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The effect of cerulenin on the production of esperamicin A_1 by *Actinomadura verrucosospora*

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SUMMARY

Addition of cerulenin (0.25–1.0 mM) to cultures of *Actinomadura verrucosospora* before the onset of esperamicin synthesis inhibited the production of esperamicin A_1 by the microorganism. This result indicates that esperamicin A_1 is biosynthesized in part by the polyketide pathway. Addition of cerulenin to the cultures during the active production phase led to a net decrease in esperamicin A_1 production. The ^{14}C-acetate labeling pattern of esperamicin A_1 in the cultures with or without addition of cerulenin at the active production phase also demonstrated the instability of esperamicin A_1 in the fermentation. This suggests that esperamicin A_1 is unstable and degradation occurs during the active production phase. Addition of the neutral resin Diaion HP-20 (1%) to the fermentation enhanced the production of esperamicin A_1 by 53%.

INTRODUCTION

Esperamicin A_1 (esp), one of the most potent antitumor agents [20], was isolated from cultures of *Actinomadura verrucosospora* ATCC 39334 [11,12]. The isolation and elucidation of the structure of esp has been reported (Fig. 1) [2–6,11]. Phase I clinical trials of esp has just been completed and the start of Phase II clinical trials is imminent [1]. The mechanism of action of esp [13,22] and several related compounds, calicheamicins [25,26], neocarzinostatin [9,17] and dynemicins [21] has been reported. It appears that these compounds are capable of cleaving DNA via direct carbon radical abstraction of deoxyribose hydrogen atoms. The presence of enediyne function in these compounds is a prerequisite to their potent activities. Therefore we are interested in elucidating the biosynthetic origin of the enediyne moiety of esp. The related C_{14} dienediyne moiety of the chromophore from neocarzinostatin was reported to be derived from head-to-tail condensation of acetate units [7]. Recently, Tokiwa et al. [24] demonstrated that the enediyne portion of dynemicin A is derived from 7 head-to-tail coupled acetate units. It is therefore reasonable to assume that the formation of the enediyne moiety of esp is derived from the polyketide pathway.

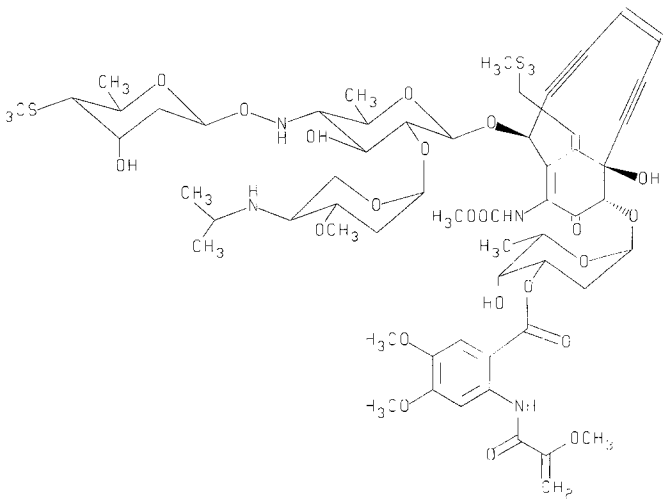

Fig. 1. Structure of esperamicin A_1.

Cerulenin is a 12 carbon fatty acid amide produced by the fungus *Cephalosporium caerulens*. It specifically inhibits the condensing enzymes involved in fatty acid and polyketide biosynthesis [10,15,18,19,23]. Therefore, we decided to examine the effect of cerulenin on the production of esp. If the enediyne ring system of esp is biosynthesized by the polyketide pathway, cerulenin would inhibit the formation of the enediyne ring and hence the production of esp. In this communication, we investigate the effect of cerulenin on the synthesis of esp by *A. verrucosospora* and conditions for improving the production of esp in the fermentation by adding the neutral resin Diaion HP-20 to the culture medium.

MATERIALS AND METHODS

Materials

Cerulenin was obtained from Sigma Chemical Company (St Louis). Sodium [1-^{14}C]acetate (58 mCi mmol^{-1}) was purchased from Du-Pont New England Nuclear Company

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(Boston). Diaion HP-20 was obtained from Mitsubishi Kasei Corporation (Tokyo).

Microorganism

The esp-producing microorganism was *Actinomadura verrucosospora* ATCC 39334. Frozen vegetative preparations were maintained in 10% glycerol–5% sucrose solution stored at −80 °C for use as working stocks.

Media

The seed medium used was medium MFSM (corn starch 2%, cerelose 0.5%, Pharmamedia 1%, Debittered Brewer's Yeast 1% and $CaCO_3$ 0.2%). The production medium used was medium H946 (Cane molasses 6%, corn starch 2%, fishmeal 2%, $CuSO_4 \cdot 5H_2O$ 0.01%, $CaCO_3$ 0.2% and NaI 0.00005%).

Fermentation conditions

To prepare an inoculum for the production phase, 4 ml of the frozen vegetative stock was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium MFSM. This seed culture was incubated at 28 °C for 3 days on a rotary shaker set at 250 rpm. 8 ml of the seed culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium H946. The fermentation was carried out at 28 °C on a rotary shaker at 250 rpm for 6–10 days. Growth of the organism was determined by the packed cell volume measured after centrifugation of 10 ml of the culture broth at 3000 rpm for 15 min on a Beckman centrifuge. Cerulenin was dissolved in ethanol at 100 times the desired final concentration before addition to the culture.

Extraction and analytical methods

The production of esp in the fermentation was monitored by HPLC using a C-18 reversed-phase column (Novapak, 3.9 × 150 mm, Waters Associates) and UV absorption at 254 nm. The solvent system was 0.05 M ammonium acetate (pH 4.5)/CH_3OH/CH_3CN (1:1:1) with a flow rate of 1 ml min^{-1}. The fermentation extracts were processed by extracting the culture broths with an equal volume of ethyl acetate. The ethyl acetate fractions were concentrated 10-fold and 25–50 μl of the concentrated extracts were used for HPLC analysis.

To determine the amount of ^{14}C-acetate incorporated into esp, the concentrated extracts (8.6–47.6 μg esp) were injected into HPLC and the fractions containing esp were collected and counted for radioactivity. A Lichrosorb RP-18 column (10 × 250 mm, Merck) was used for this study with a flow rate of 4 ml min^{-1} and the same solvent system described above.

RESULTS AND DISCUSSION

Effect of cerulenin on the production of esp when added before production phase

The effect of cerulenin on the production of esp by strain ATCC 39334 is shown in Table 1. Varying levels of cerulenin were added to the culture at day 3 of the fermentation, just before the onset of esp synthesis [12]. At all concentrations tested, cerulenin did not affect the growth nor the pH of the cultures. The growth of the organism was determined by the packed cell volume. The packed cell volume of the above fermentation ranged from 25 to 27% and the pH of the fermentation was between 7.6 and 7.7. At a cerulenin concentration of 0.5 mM, a 73% inhibition of esp production was observed. A 100% inhibition of esp synthesis was achieved in culture supplemented with a 1.0 mM cerulenin. The inhibitory effect of cerulenin on esp production by strain ATCC 39334 is similar to that of the polyketide-derived antitumor antibiotic daunorubicin produced by *Streptomyces peucetius* [16]. The addition of 0.1–0.5 mM cerulenin inhibited the production of daunorubicin by 90%. Since cerulenin inhibits the production of esp without affecting the growth of the microorganism, this indicates that esp is biosynthesized in part by the polyketide pathway.

TABLE 1

Effect of cerulenin on esperamicin A_1 production in medium H946

[Cerulenin][1] (mM)	[Esperamicin A_1][2] (μg ml^{-1})	% Inhibition
0	5.2 ± 0.4	—
0.10	4.2 ± 0.6	19.2
0.25	2.0 ± 0.3	61.5
0.50	1.4 ± 0.1	73.1
1.0	0	100

[1] Cerulenin was added to the culture at day 3 of the fermentation.

[2] The titers of esperamicin A_1 were determined at day 10 of the fermentation.

Effect of cerulenin on the production of esp when added during production phase

The effect of cerulenin on esp production when added to the culture during active production phase (day 6) is shown in Fig. 2. Without the addition of cerulenin, active esp production occurred at day 5 to day 7 and reached a maximum of 4.8 μg ml^{-1} at day 7. The esp titer was constant through day 10. Addition of cerulenin to the culture during the active production phase, at day 6, not only inhibited further production of esp but led to a drop in the titer of esp. The rate of degradation was dependent on the concentration of cerulenin added to the culture. The higher the concentration of cerulenin added, the higher the rate of degradation of esp. In the culture supplemented with 0.25 mM cerulenin, the production of esp dropped gradually from 3.5 μg ml^{-1} at day 6, to 2.8 μg ml^{-1} at day 7, and to 1.8 μg ml^{-1} at day 8. The rate of drop in titer was about 0.7–1.0 μg ml^{-1} day^{-1}. In the cultures supplemented with high concentrations (0.5 and 1.0 mM) of cerulenin, there was a more significant drop in esp titers, 1.9–2.2 μg ml^{-1} day^{-1}.

Since we determined that cerulenin did not degrade esp (data not shown), the above data indicate that esp is unstable

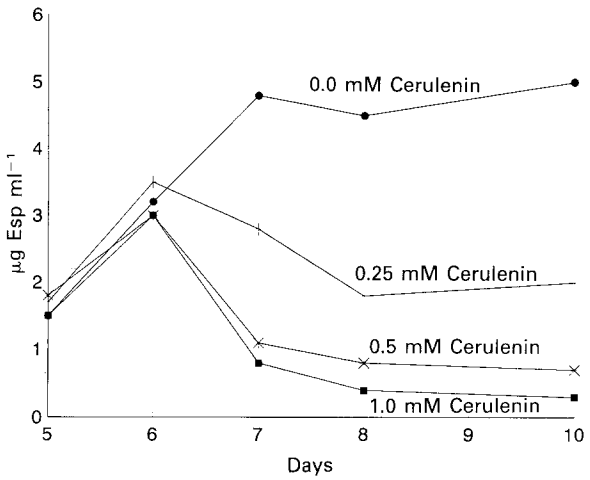

Fig. 2. Effect of cerulenin on esperamicin A_1 production by *A. verrucosospora* ATCC 39334. Various concentrations of cerulenin were added to the cultures at day 6 of the fermentation.

and a continuous degradation of esp may occur during the active production phase. The increase in titer from day 5 to day 7 in the control fermentation (no cerulenin) may reflect the balance between synthesis and degradation of esp. The rate of synthesis of esp was higher than the rate of degradation. In the culture supplemented with 0.25 mM cerulenin, the rate of degradation of esp was higher than the rate of synthesis. This was due to the inhibitory effect of cerulenin on esp production, which led to a gradual drop in esp titer. When 1.0 mM cerulenin was added to the culture at the active production phase, it completely inhibited further production of esp. This led to a sharp drop in esp titer because of the degradation of the existing esp in the culture.

^{14}C-labeling pattern of esp in cerulenin-supplemented cultures

The above hypothesis was tested by the following experiment, and the results are summarized in Table 2. Sodium [1-^{14}C]acetate was added to the culture of strain ATCC 39334 at day 4 to yield a final concentration of 2.5 μCi ml^{-1}. At day 6, cerulenin (0.25 and 1.0 mM) together with unlabeled sodium acetate (0.1%) were added to the cultures. The purpose of adding cold sodium acetate to the cultures was to dilute the concentration of ^{14}C-acetate and lower the rate of incorporation of ^{14}C-acetate into esp. Only esp produced from day 4 to day 6 would be labeled by ^{14}C-acetate. The rate of incorporation of ^{14}C-acetate into esp that was produced from day 6 to day 8 would be negligible, and any new synthesis of esp during this period would lower the specific activity of ^{14}C-esp in the fermentation.

With no added cerulenin, the specific activity of ^{14}C-esp was about 1644 dpm/10 μg at day 6. When unlabeled sodium acetate (0.1%) was added at day 6, the specific activity of ^{14}C-esp dropped significantly to 495 dpm/10 μg at day 7. The increase of esp production from day 6 to day 7 was 0.9 μg ml^{-1}, or 32%. The decrease in specific activity was about 70%, more than double the amount of the dilution factor accounted for by the increase in esp production. The same trend was observed at day 8 in the control culture. There was a 5% increase in esp titer, but the specific activity of ^{14}C-esp further dropped by 40%. The above data indicate that there is more de novo synthesis of esp than the net increase in titer and therefore some preformed esp (^{14}C-labeled) must be degraded.

When cerulenin (0.25 mM) and sodium acetate (0.1%) were added to the culture at day 6, the specific activity of ^{14}C-esp dropped by 44% at day 7, with a further drop of 18% at day 8, even though there was a decrease in esp titer in the fermentation. The above data further support the previous finding that cerulenin, at a concentration of 0.25 mM, did not completely inhibit the production of esp. New esp was synthesized from day 6 to day 8, thereby lowering the specific activity of ^{14}C-esp. The drop in titer in this case indicated that the rate of degradation of esp was higher than the rate of synthesis. For the culture supplemented with 1 mM cerulenin and 0.1% sodium acetate at day 6, there was no significant difference in the specific activity of ^{14}C-esp from day 6 to day 8. Cerulenin, at a concentration of 1.0 mM, completely inhibited further synthesis of esp in the fermentation and hence no dilution of specific activity of ^{14}C-esp. All the above data support the finding that degradation of esp occurs during the active production phase.

Effect of neutral resin on the production of esp

Jarvis et al. [8] demonstrated the use of resin to improve the production of trichothecenes from *Mycothecium verrucaria*. Marshall et al. [14] also reported that the addition of neutral

TABLE 2

Incorporation of sodium [1-^{14}C]acetate into esperamicin A_1

Cerulenin (mM)	Day 6		Day 7		Day 8	
	(μg ml^{-1})	dpm/10 μg	(μg ml^{-1})	dpm/10 μg	(μg ml^{-1})	dpm/10 μg
0	2.8 ± 0.3	1644 ± 91	3.7 ± 0.4	495 ± 40	3.9 ± 0.4	280 ± 23
0.25	3.3 ± 0.6	1830 ± 33	2.6 ± 0.4	1004 ± 68	1.4 ± 0.6	686 ± 69
1.0	2.7 ± 0.4	1689 ± 48	0.7 ± 0.1	1757 ± 83	0.3 ± 0.1	1833 ± 72

Sodium [1-^{14}C]acetate was added to the cultures to yield a final concentration of 2.5 μCi ml^{-1} at day 4 of the fermentation. Cerulenin, together with sodium acetate (final concentration of 0.1%), were added to the cultures at day 6 of the fermentation.

resins to the fermentation enhanced the production of rubradirin by *Streptomyces achromogenes v. rubradiris*. We examined the effect of Diaion HP-20, a nonionic highly porous resin, on the production of esp. The resin was added to the production culture at the time of inoculation to yield a final concentration of 1%. The production of esp in the control (no resin) and resin-supplemented culture was 5.3 and 8.1 $\mu g\ ml^{-1}$, respectively. The production of esp increased by 53% with the addition of resin. The resin may bind esp and prevent it from further metabolism and degradation.

The esp-producing organism, *A. verrucosospora*, is sensitive to esp in the fermentation. In the agar culture, no growth of *A. verrucosospora* was observed when the agar was supplemented with 20 $\mu g\ ml^{-1}$ of esp. Binding esp to the resin may reduce the contact of esp to its producing organism and may lead to higher production of esp. Optimization of the time of resin addition, the amount of resin used, and the comparison of various kinds of resin in enhancing the production of esp by *A. verrucosospora* is in progress.

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