BIOCONVERSION AND BIOSYNTHESIS OF NANAOMYCINS USING CERULENIN, A SPECIFIC INHIBITOR OF FATTY ACID AND POLYKETIDE BIOSYNTHESES

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The biosynthetic relationship of the nanaomycins produced by *Streptomyces rosa* var. *notoensis* OS-3966 was studied by means of a bioconversion method using the antibiotic cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses. Nanaomycin D was considered to be the first component produced from the hypothetical intermediate "polyketide". It is proposed that the biosynthetic sequence for the nanaomycin is: nanaomycin D→nanaomycin A→nanaomycin E→nanaomycin B. Nanaomycin B can be converted to nanaomycin A by non-enzymatic dehydration; however, nanaomycin A is rapidly bioconverted to nanaomycin E, which is the major component synthesized by the nanaomycin-producing strain.

Nanaomycins (NNMs) are antifungal and antimycoplasmal antibiotics that possess a benzoisochromane-quinone skeleton (Fig. 1). Five components, NNM-A, B, C, D and E, have been isolated by ŌMURA *et al.*[1~6] from the producing organism, *Streptomyces rosa* var. *notoensis* OS-3966. A sixth component, 4a-*epi*-NNM-B, was obtained by treatment of NNM-E with a reducing agent such as sodium thiosulfate in an acidic solution[6]. NNM-B and 4a-*epi*-NNM-B are converted chemically to NNM-A by dehydration under alkaline conditions[2,6]. NNM-D can also be prepared chemically by oxidation of NNM-A[5].

The biosynthetic origin of NNM was studied by TANAKA *et al.*[3] using ^{13}C-labeled compounds and ^{13}C-NMR spectroscopy. It was established that the carbon skeleton of NNM was derived from eight acetate units. However, the biosynthetic relationship of the NNM components has not been studied to date. Some interesting reactions appear to be involved in the biosynthesis of each of the NNM components. As a consequence the relationship of the NNM-components to one another was examined with *S. rosa* var. *notoensis* using cerulenin[7], a novel tool employed for studies of the biosyntheses of fatty acids and the natural compounds (derived from polyketide intermediates) such as macrolide antibiotics[8~12], tetracycline[8], 6-methylsalicylic acid[13], and flavanone[14].

Materials and Methods

Cultivation and Bioconversion

Streptomyces rosa var. *notoensis* OS-3966 was used throughout the investigation. A stock culture of strain OS-3966 grown on glucose-asparagine agar slants was inoculated into a 500-ml SAKAGUCHI flask containing 50 ml of a medium composed of 2.0% glucose, 1.0% meat extract, 0.5% NaCl, and 0.3% $CaCO_3$ (the pH was adjusted to 5.0 with 2 N HCl before autoclaving). The flask was incubated for 48 hours at 37°C on a reciprocal shaker to provide a seed culture. The bioconversion of NNM was carried out as follows. Two milliliters of the seed culture were transferred to a 500-ml SAKAGUCHI flask containing 50 ml of the above medium supplemented with 40 μg of cerulenin per ml and 100 μg

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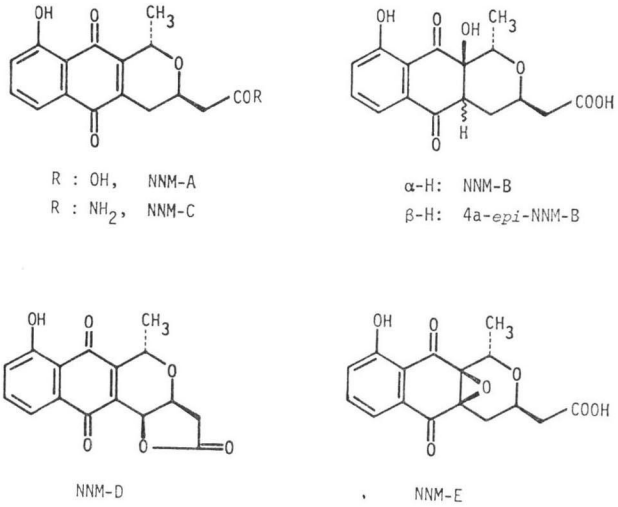
Fig. 1. Structures of nanaomycins.



of NNM per ml. The flasks were incubated at 37°C for the times indicated. NNM and cerulenin were added in a small volume of ethanol. The bioconversion of NNM-A, D and E was then examined during further incubation. As cerulenin is an unstable compound, an equivalent amount of the antibiotic was added to the culture every 24 hours.

Extraction and Analysis of NNMs

After incubation the culture broth was adjusted to pH 2.0 with 0.5 N HCl and centrifuged at 3,000 rpm for 10 minutes. NNMs, present in the supernatant fluid, were assayed by a conventional paper disc diffusion method using *Bacillus subtilis* PCI 219 as test organism and NNM-E as standard. Mycelial growth was measured as the packed mycelial volume in 10 ml of the broth after centrifugation at 3,000 rpm for 10 minutes.

A conversion ratio (%) of an NNM component to the other components was determined as follows. The supernatant fluid was extracted twice with an equal volume of ethyl acetate and the combined extracts were evaporated *in vacuo*. The residue was dissolved in a small volume of methanol and applied to silica gel thin-layer chromatographic plates (Kieselgel 60 F_{254}, Merck) using chloroform - methanol (5: 1) as solvent. The Rf values of NNM-A, B, C, D and E were 0.45, 0.22, 0.51, 0.71 and 0.40, respectively. The amounts of NNMs, separated on a silica gel plate, were determined with a dual wavelength chromatogram scanner (Model CS-910, Shimadzu Seisakusho Co., Ltd.) at 274 nm for NNM-A, C and D, and at 232 nm for NNM-B and E. The amounts of NNM components formed were expressed as molar ratios (conversion ratio, %) to the NNM component added to a culture as substrate.

Results and Discussion

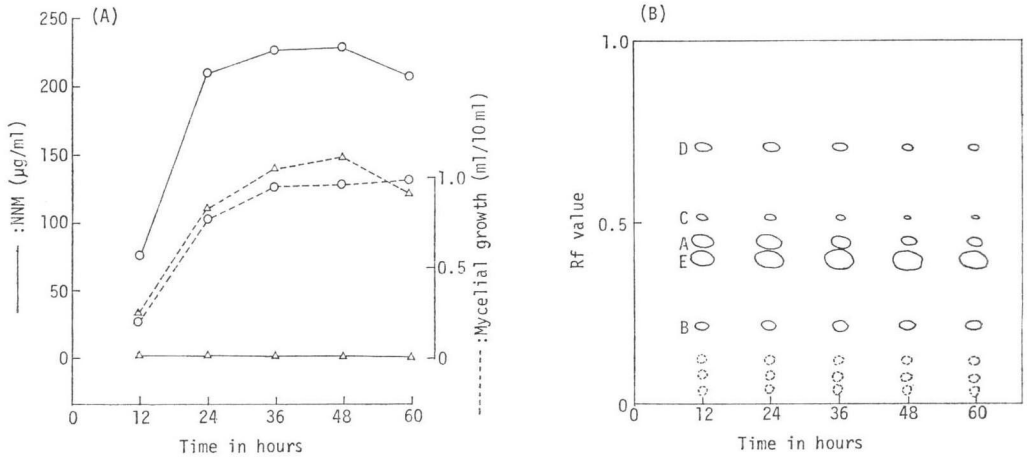
Time Course of NNM-Production in the Absence of Cerulenin

The time course of NNM production in relation to mycelial growth is shown in Fig. 2-A. Strain OS-3966 produced about 230 μg of NNM per ml (determined by bioassay as NNM-E). Fig. 2-B reveals the formation of the NNM components as a function of time. The major components synthesized in the early stage of cultivation were NNM-A and NNM-E whereas NNM-E accumulated predominantly in the late stages of the fermentation. From this experiment, however, it was difficult to delineate the biosynthetic relationships of the NNM components to each other. As a consequence a study was carried out to examine the bioconversion of the NNMs in the presence of cerulenin.

Fig. 2. Time course of NNM-production by *Streptomyces rosa* var. *notoensis*.

(A) Effect of cerulenin on NNM-production and mycelial growth.
○: Cerulenin not added
△: Cerulenin (40 μg/ml) added

(B) Analysis of NNM-components in the culture filtrate by TLC.
Culture filtrate was extracted with ethyl acetate and the extract was quantitatively applied to silica gel TLC which was developed with $CHCl_3$-MeOH=5: 1.



Effect of Cerulenin on NNM-Production and Mycelial Growth

It has been reported that the addition of 20~40 μg of cerulenin per ml to the culture of a polyketide-producing actinomycete such as *Streptomyces kitasatoensis* (leucomycin), *S. ambofaciens* (spiramycin) or *S. fradiae* (tylosin) inhibited antibiotic production without a concomitant effect on mycelial growth[10~12]. In the case of the NNM-producing strain, OS-3966, addition of 40 μg of cerulenin per ml at the onset and at 24 hour intervals completely inhibited NNM-production without inhibiting mycelial growth (Fig. 2-A). Even at 80 μg of cerulenin per ml normal growth occurred; by contrast 100 μg of cerulenin per ml affected growth considerably (data not shown). As a result, the bioconversion experiments were carried out adding cerulenin (40 μg/ml) at 24 hour intervals: cerulenin is considered to inhibit only the polyketide synthesis in NNM biosynthesis.

Fig. 3. Bioconversion of NNM-D in the cerulenin-supplemented culture.

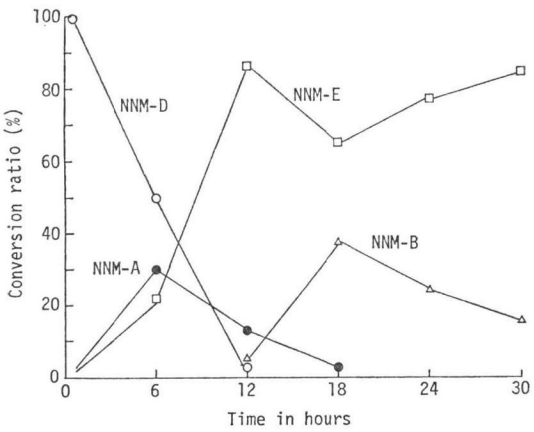
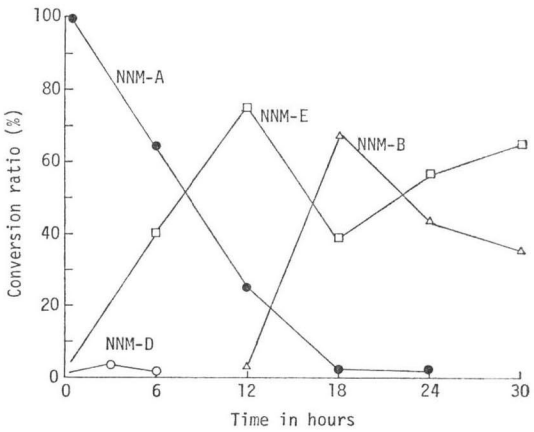


Fig. 4. Bioconversion of NNM-A in the cerulenin-supplemented culture.



Bioconversion of NNMs

Fig. 3 shows the time course of the conversion of NNM-D to the other NNM components. NNM-D was utilized rapidly disappearing within 12 hours. The first component evident in the culture medium was NNM-A followed by NNM-E. The NNM-A produced initially disappeared also, while the level of NNM-E increased rapidly reaching a maximum at 12 hour. In addition, NNM-B formation began after 12 hours, reached a maximum at 18 hour, and then slowly disappeared from the medium. When NNM-A was added to the cerulenin-treated culture, it was converted predominantly to NNM-E (Fig. 4). It was also evident that the appearance of NNM-B after 12 hours coincided with a decrease in the amount of NNM-E present. As shown in Fig. 5, the addition of NNM-E resulted in the formation of NNM-B during the first 12 hours; however, this was followed by an increase in NNM-E and a concomitant decrease of NNM-B. It was also noted that a small amount of NNM-A appeared after 6 hours although this component did not accumulate to any significant extent.

Fig. 5. Bioconversion of NNM-E in the cerulenin-supplemented culture.

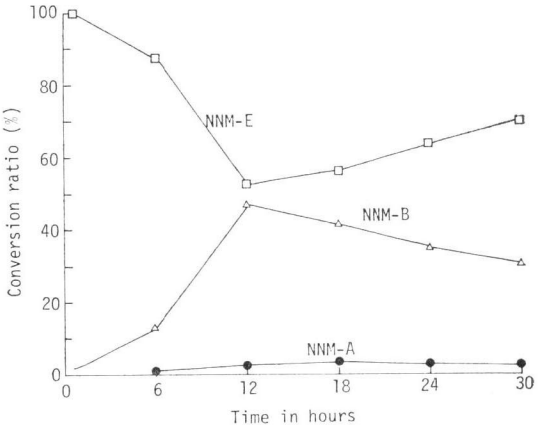
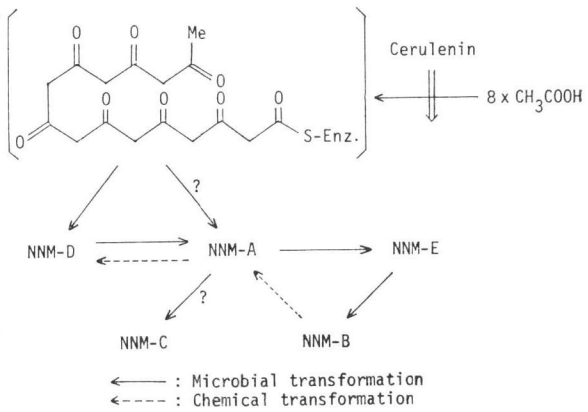


Fig. 6. Biosynthetic route of nanaomycins.



ŌMURA *et al.*[2,5,6] have reported that these compounds can undergo chemical interconversions (*e.g.*, NNM-A→NNM-D, NNM-E→NNM-B, and NNM-B→NNM-A). As a result, the non-enzymatic conversion of NNM components was examined in the NNM-production medium under the same incubation condition (37°C/30 hours) as was used in the bioconversion experiments (see Materials and Methods). The study revealed that there was a slight conversion of NNM-A to NNM-D and a rapid conversion of NNM-B to NNM-A (data not shown). Therefore, the small amount of NNM-D formed during the incubation of NNM-A with the culture (Fig. 4) probably arose by a non-enzymatic reaction. In fact, when a crude enzyme extract from strain OS-3966 was incubated with NNM-D as substrate, the latter compound was rapidly converted to NNM-A; by contrast, NNM-D was not synthesized from NNM-A under the same conditions. In the experiments shown Figs. 3, 4 and 5, the level of NNM-E decreased to same extent during the incubation presumably as a result of bioconversion to NNM-B. However, further synthesis of NNM-E occurred during the late stage of incubation. By contrast, it was observed that NNM-B first accumulated in the medium, then it gradually disappeared with a small amount of NNM-A appearing late in the incubation. From the evidence obtained, it was concluded that (1) the NNM-B which accumulated was converted to NNM-A by a non-enzymatic reaction, (2) NNM-A was converted to NNM-E by an enzymatic reaction, and (3)

NNM-E was the major product formed since the bioconversion of NNM-A to NNM-E took place more rapidly than that of NNM-E to NNM-B. The formation of a trace amount of NNM-C was observed only when an excess amount of NNM-A was present in the culture medium (data not shown). From the above results, it is proposed that the biosynthesis of the NNMs occurs in the following sequence: NNM-D→NNM-A→NNM-E→NNM-B. The biosynthetic pathway and the chemical transformation of NNMs are summarized in Fig. 6.

Many NNM-related compounds such as kalafungin[16], frenolicins[17], griseusins[18], granaticin[19], and medermycin[20] have been described to date. SNIPES *et al.*[19] reported that granaticin is derived from eight acetate units and one glucose unit, and that the five-membered lactone ring moiety of granaticin is derived enzymatically by oxidation of the reduced component, dihydrogranaticin. By contrast, in the case of the conversion of NNM-A (dihydro-NNM-D) to NNM-D, only a chemical reaction took place, and microbial transformation was not observed. Therefore, it is suggested that the first NNM-component derived from the polyketide intermediate is NNM-D. Alternatively, it is possible that NNM-A is the first component derived from the polyketide intermediate. Conceivably, NNM-D may be derived from NNM-A by chemical oxidation. A study of the biosynthetic relationship of NNM-A to NNM-D using a cell-free system is now in progress. These findings will be reported in a separate paper.

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