INACTIVATION OF FUSIDIC ACID BY RESISTANT *Streptomyces* STRAINS

BEATE VON DER HAAR, DOUWE ROSENBERG†, WERNER DITTRICH and HILDGUND SCHREMPF*

Fachbereich Biologie/Chemie, University Osnabrück,
Germany
†Schering Aktiengesellschaft,
Berlin, Germany

(Received for publication January 21, 1991)

Streptomyces lividans and several other *Streptomyces* species are resistant to the steroid-like antibiotic fusidic acid. This resistance is mediated by structural modification of the antibiotic. Using TLC, CD, UV, IR, NMR and mass spectroscopy the structure of one of the resulting inactive compounds was determined. It is derived from fusidic acid by the loss of an acetyl group and the formation of a lactone ring between C-21 and C-16. In addition, helvolic acid, a compound closely related to fusidic acid, has been shown to be modified.

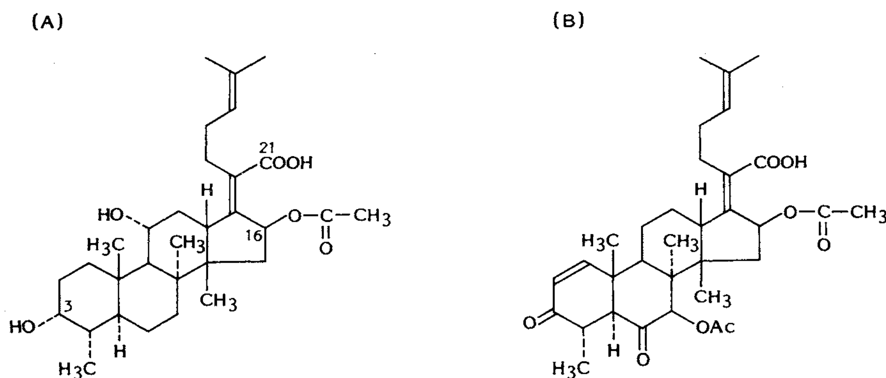
Streptomycetes are Gram-positive bacteria which produce a wide range of different antibiotics and exhibit numerous types of antibiotic resistance[1].

In addition to unstable chloramphenicol and tetracycline-resistance determinants we recently noticed that *Streptomyces lividans* also has unstable fusidic acid-resistance[2]. Since fusidic acid-resistance was not known to occur within *Streptomyces* strains it was of interest to analyze its nature.

Fusidic acid is a steroid-like antibiotic which is synthesized by the fungus *Fusidium coccineum*[3], and is clinically used to inhibit growth of *Staphylococcus aureus* following infection. Fusidic acid inhibits protein synthesis of both prokaryotic and eukaryotic organisms. It has been demonstrated that it binds to the prokaryotic elongation factor G (EFG) and thus prevents the translocation step[4]. Spontaneous fusidic acid-resistant strains of *Escherichia coli* and *Staphylococcus aureus* have a modified EFG[5]. In addition, certain *S. aureus* strains possess plasmid encoded resistance which prevents drug uptake[6].

In this paper we demonstrate that *S. lividans* and several other *Streptomyces* strains inactivate fusidic acid (Fig. 1A) and we have determined the structure of one of the resulting compounds. Helvolic acid (Fig. 1B) which is related to fusidic acid has also been shown to be modified.

Fig. 1. Structures of fusidic acid (A) and helvolic acid (B).



Materials and Methods

Strains

S. lividans 1326 and *Streptomyces coelicolor* A3(2) (His^-, Ura^-, $SCP1^-$ derivative: M110) and *Streptomyces reticuli* were kindly donated by D. A. HOPWOOD (John Innes Institute, Norwich) and H. ZÄHNER (University Tübingen), respectively. Fusidic acid-sensitive derivatives from *S. lividans* 1326 were identified in our laboratory. All other strains were obtained from the German Culture Collection, DSM, Braunschweig.

Chemicals

Chemicals and silica TLC plates were obtained from Merck. Fusidic acid and helvolic acid were purchased from Sigma.

Media

YEME-medium[7] was used for cultivation of *Streptomyces* strains, L broth (LB)-medium[7] for cultivation of *Micrococcus luteus* and minimal medium[7] for identification of modified fusidic acid.

Cultivation

Strains were propagated on YEME-agar. To analyse the inactivation of fusidic acid or helvolic acid 5 ml minimal medium were inoculated with spores or with mycelium (if non-sporulating variants were used). After incubation at 30°C for 36 hours, the cultures were diluted 1 : 100 with minimal medium and 5 μg/ml fusidic acid or helvolic acid was added. The cultures were shaken at 30°C for various lengths of time. The culture supernatant (10 ml) was extracted twice with 2 ml ethyl acetate. The organic phase was evaporated completely and the solid residue was dissolved in 50 μl ethanol. Aliquots of 1 ~ 5 μl were spotted on filter discs (for biological tests) or on TLC plates for further analysis.

Antibiotic Disc Assay

M. luteus was grown to the logarithmic phase in LB-medium. LB-agar plates were overlaid with soft LB-agar (0.75%) which was seeded with *M. luteus* (0.2 ml culture/20 ml). After drying, filter discs containing various amounts of fusidic acid or helvolic acid were placed on the plates. After incubation at 37°C, zones of inhibition were analyzed.

TLC

Aliquots of 1 ~ 5 μl were spotted on TLC plates (20 cm × 20 cm). After drying the plates were placed in a glass chamber which contained chloroform - methanol (9 : 1). After the organic phase had reached the end of the plate, it was dried, sprayed with 50% H_2SO_4 in methanol, incubated for 10 minutes at 110°C and the Rf's were calculated.

Large Scale Isolation of Modified Fusidic Acid

The 10-ml liquid minimal-medium were inoculated with 10^7 spores of *S. lividans* 1326 and incubated 48 hours at 30°C. The culture was diluted with 140 ml fresh minimal medium and incubation was continued for another 48 hours. Each of 10-Erlenmeyer flasks containing 500 ml minimal medium with 50 μg/ml fusidic acid was inoculated with 10 ml preculture. Incubation was continued over a period of 7 days at 30°C. After removal of the mycelia by centrifugation, 250 ml portions of the supernatant were each extracted three times with 30 ml ethyl acetate. After drying with Na_2SO_4, the combined organic phases were evaporated and the residue was redissolved in 10 ml methanol. The material was adsorbed to 20 g Silica gel 60H and placed on a preparative Silica gel 60 column (20 cm × 6 cm). Flash-chromatography was performed at 0.5 bar using chloroform - methanol (95 : 5). Aliquots of 3 ml were collected; 5 μl of each fraction were spotted on TLC plates. After analysis on TLC plates using chloroform - methanol (9 : 1), fractions containing substances of identical Rf were combined and the organic solvents evaporated. The compounds of Rf 0.61 and Rf 0.20 were used for structure determination.

Structure Determination

The structure of the modified fusidic acid was analyzed using the following methods and conditions: UV-absorption spectra, solvent methanol; circular dichroism spectra, solvent methanol; IR spectra, KBr/water; NMR spectra, solvent DMSO, TMS as internal reference, ^{1}H NMR at 500 MHz, ^{13}C NMR at 125 MHz, homo- and hetero correlation; mass spectra (MS), EI ionization at 70 eV, chemical ionization with NH_3 gas.

Results

Biological Tests and TLC

The wild-type strain, *S. lividans* 1326 is inhibited by 15 μg/ml fusidic acid, while spontaneously derived variants are inhibited at lower concentrations (2.5~7.5 μg/ml). The variant 90-2 was chosen for further comparative analyses since its growth was prevented in the presence of 2.5 μg/ml fusidic acid. *M. luteus* is sensitive to fusidic acid and could be used to determine fusidic acid concentrations as low as 0.4 μg/ml in antibiotic disc assay.

Comparative analyses of *S. lividans* wild-type and its variant 90-2 revealed that after 20 hours of

Table 1. Antimicrobial activities of culture extracts of *Streptomyces lividans* 1326 and the variant 90-2 after growth in the presence or absence of fusidic acid (Fus) and the Rf values of identified compounds.

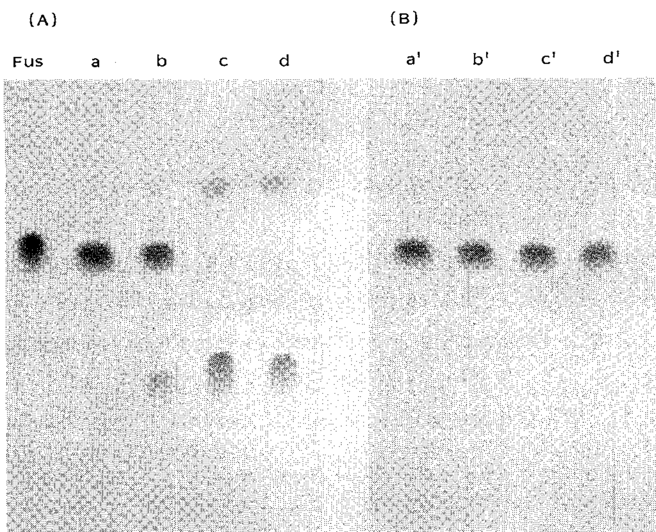
Extract	Time	Zones of inhibition		Rf of identified compounds		
		−Fus	+Fus	+Fus		
1326	0	0	39		0.45	
	4	0	38	0.20	0.45	0.61
	8	0	25	0.20	0.45	0.61
	12	0	21	0.20	0.45	0.61
	16	0	19	0.20	0.45	0.61
	20	0	0	0.20		0.61
90-2	0	0	40		0.45	
	4	0	40		0.45	
	8	0	40		0.45	
	12	0	40		0.45	
	16	0	40		0.45	
	20	0	40		0.45	

Table 2. Antimicrobial activity and Rf of compounds present in culture extracts after growth in the presence of helvolic acid (Hel).

Extract	Time	Zones of inhibition		Rf of identified components		
		−Hel	+Hel	+Hel		
1326	0	0	21		0.61	
	4	0	20		0.61	
	8	0	14	0.44	0.61	
	12	0	11	0.44	0.61	
	16	0	9	0.44	0.61	
	20	0	0	0.44	0.61	0.76
90-2	0	0	20		0.61	
	4	0	20		0.61	
	8	0	20		0.61	
	12	0	20		0.61	
	16	0	20		0.61	
	20	0	20		0.61	

Fig. 2. Modification of fusidic acid (Fus) by *Streptomyces lividans* 1326 (wild-type) and the sensitive derivative *S. lividans* 90-2.

(A) *S. lividans* 1326. (B) *S. lividans* 90-2.



S. lividans 1326 (A) and *S. lividans* 90-2 (B) were incubated with 5 μg/ml fusidic acid (Fus) for 0 hour (a and a'), 4 hours (b and b'), 16 hours (c and c') and 20 hours (d and d'); the extracted compounds were analyzed by TLC with chloroform - methanol (9 : 1) and visualized with 50% H_2SO_4 in methanol. Purified Fus was used as reference.

cultivation no biologically active compound could be extracted with ethyl acetate from the culture medium of the wild-type. This is in contrast to the variant *S. lividans* 90-2 (Table 1).

Helvolic acid (Fig. 1B) is another steroid-like antibiotic and is closely related to fusidic acid (Fig. 1A). It could be shown that helvolic acid was also converted, after 20 hours of growth, to two inactive compounds by the wild-type strain, but not by the variant 90-2 (Table 2).

Table 3. Optical and MS data of modified fusidic acid.

UV (nm)	λ_{max}^{MeOH} 224	$E_{1cm}^{1\%}$ 277
CD	218 nm	ε +27.8
	250 nm	ε − 9.72
IR (cm^{-1})		1690, 1730～1760
EI-MS (*m/z*)		456 (M, $C_{29}H_{44}O_4$), 438 ($M-H_2O$), 420 ($438-H_2O$), 405 ($420-CH_3$), 69 (C_5H_9)
CI-MS (*m/z*)		457 (M+H)

In order to analyze the modified products of both fusidic acid and helvolic acid, concentrated ethyl acetate culture extracts were analyzed by using TLC (Fig. 2). Over a period of 20 hours *S. lividans* wild-type converted fusidic acid (Rf 0.45) quantitatively to two compounds (Rf 0.61 and Rf 0.20; Table 1) and helvolic acid (Rf 0.61) to two products (Rf 0.44 and Rf 0.76; Table 2). *S. lividans* 90-2 was sensitive to helvolic acid and did not modify this substance (Table 2).

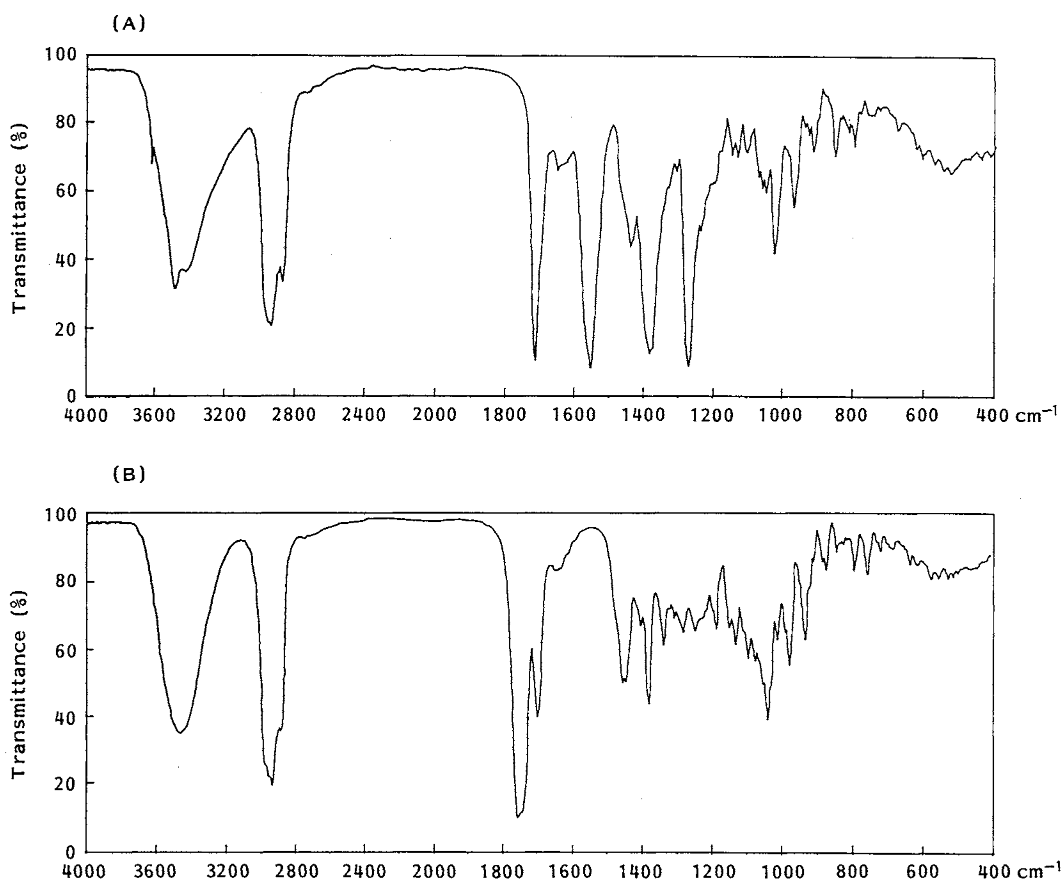
Structure Elucidation

About 100 mg of the modified products were isolated and structure elucidation of one modified compound (Rf value 0.61) was performed using UV, IR, MS, NMR and CD as described under Materials and Methods.

Using EI ionization conditions it could be demonstrated (Table 3) that the highest mass of the fusidic acid derivative corresponded to *m/z* 456. In addition *m/z* 438 ($M-H_2O$), *m/z* 420 ($M-2H_2O$) and

Fig. 3. IR spectra of fusidic acid and of its derivative.

(A) Fusidic acid. (B) Fusidic acid derivative.



m/z 405 (*m/z* 420 $-CH_3$) could be identified. The signal *m/z* 69 corresponded to C_5H_9. The CI spectrum has a signal equivalent to *m/z* 457. Fusidic acid has a MW of 516; the reduction in MW of 60 of the derivative could be explained by the loss of the acetyl group.

Using IR analysis the acetyl group of fusidic acid can be identified as a signal at 1710 cm^{-1} (Fig. 3A). The altered product has a new intense band at 1750 cm^{-1} and an additional weaker signal at 1690 cm^{-1} (Fig. 3B and Table 3). These data indicate that the free acetyl group is absent from the derivative.

Using ^1H and ^{13}C NMR spectroscopy most of the carbons and hydrogens in fusidic acid and the modified product (Rf 0.61) were identified by cross correlation (Table 4). Within the modified product only 6 methyl groups are present. The H-atoms 16-H and 24-H of fusidic acid are located at 5.75 and 5.10 ppm, respectively, whereas the corresponding atoms of the derivative are both found at 5.10 ppm. From these data it is clear that the modified compound (Rf 0.61) has lost the acetyl group of fusidic acid at C-16 and a condensation between the newly formed hydroxyl group and the carboxyl group has occurred.

The second modified product of Rf 0.20 had very low MW. It is very likely to be a degradation product and has not been analyzed.

Occurrence of Fusidic Acid-resistance

Several other *Streptomyces* strains are also resistant to fusidic acid. *S. coelicolor* A3(2) (derivative

Table 4. ^{1}H and ^{13}C NMR data of fusidic acid and modified fusidic acid.

Atom	Fusidic acid	Modified fusidic acid	Atom	Fusidic acid	Modified fusidic acid
	δ_C	δ_C		δ_H	δ_H
C-1	~30	29.3	1-H	1.35~1.60, 2.05~2.25	1.30 (1β), 2.38 (1α)
C-2	~30	30.1	2-H	1.35~1.60	1.50, 1.64
C-3	69.3	69.2	3-H	3.52	3.52
C-4	36.2	36.8	4-H	1.35~1.55	1.38
C-5	35.2	34.9	5-H	2.10	2.09
C-6	20.7	21.0*	6-H	0.95~1.05, 1.35~1.55	1.05, 1.55
C-7	30.2	30.9*	7-H	0.95~1.05, 1.35~1.55	1.05, 1.55
C-8	39.9	40.3	—	—	—
C-9	48.6	49.8	9-H	1.33	1.33
C-10	36.3	36.4	—	—	—
C-11	66.1	65.6	11-H	4.13	4.19
C-12	36.4	31.8	12-H	1.60~1.70, 2.05~2.25	1.72, 1.92
C-13	41.9	37.7	13-H	2.83	3.51
C-14	48.2	54.8	14-H	—	—
C-15	38.9	33.6	15-H	1.09, 1.86	1.05, 2.23
C-16	74.0	81.3	16-H	5.75	5.10
C-17	135.5*	171.2	—	—	—
C-18	17.4	19.6	18-H	0.83	0.72
C-19	22.7	23.3	19-H	0.89	0.88
C-20	139.2*	121.8	—	—	—
C-21	174.9	175.7	—	—	—
C-22	~30	27.0*	22-H	2.10~2.30	2.10~2.25
C-23	~30	23.4*	23-H	2.10~2.30	2.10~2.25
C-24	125.0	123.2	24-H	5.10	5.10
C-25	129.9	131.9	—	—	—
C-26	17.5*	17.5*	26-H	1.57	1.56
C-27	25.5*	25.4*	27-H	1.64	1.65
C-30	16.2	16.2	30-H	0.79	0.81
C-32	23.5	22.3	32-H	1.35	1.43
CH_3	20.7		CH_3	1.85	
C=O	170.4			—	

* Assignment could be interchanged.

M110), *Streptomyces citreofluorescens* and *S. lividans* 40434 convert fusidic acid quantitatively to the same compounds (Rf 0.61 and Rf 0.20) as *S. lividans* 1326. *Streptomyces canescens*, *Streptomyces hirsutus* and *Streptoverticillium flavopersicum* which are resistant to lower levels of fusidic acid, inactivate it slowly but not quantitatively. *S. reticuli* is resistant to fusidic acid but forms two products (Rf 0.67 and Rf 0.14), which differ from those formed by *S. lividans*. *Streptomyces prasinopilosus* and *Streptomyces platensis*—which are sensitive to fusidic acid—are incapable of modifying the drug.

Discussion

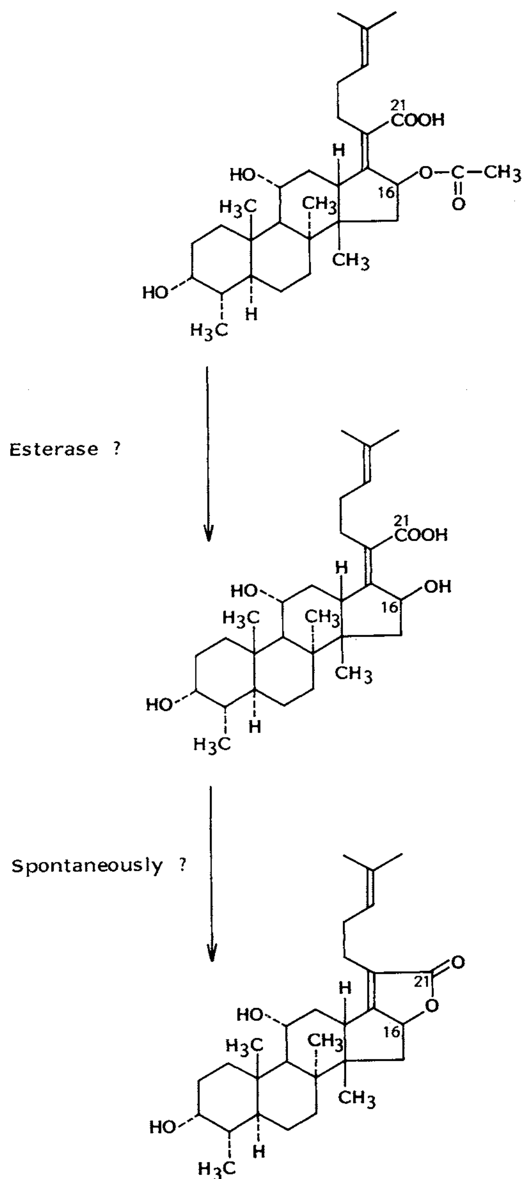
It has been demonstrated for the first time that several *Streptomyces* species are resistant to the steroid-like antibiotic fusidic acid and that they generate two products from fusidic acid. The structure of one of the substances (having a Rf 0.61) is derived from fusidic acid by the loss of the acetyl group, an event which is probably catalyzed by an esterase. The formation of the lactone ring between C-21 and C-16 may occur spontaneously (Fig. 4).

The Rf of the compound formed by *S. reticuli* is slightly different; it could be explained by a modification which might occur alone or in addition to the condensation. The compound with the Rf 0.22 has a

MW considerably lower than fusidic acid; it has not been further investigated since it probably represents a degradation product. Interestingly, fusidic acid-resistant strains exhibit helvolic acid-resistance and are also able to inactivate it.

GODTFREDSEN *et al.*[8] had analyzed different chemically synthesized derivatives of fusidic acid. One of them was also a lactone-derivative, the activity of which was 10^{-4} lower against *S. aureus* than that of fusidic acid. Another derivative, 3-oxofusidic acid, has been synthesized[8] and shown to be produced by *Corynebacterium simplex*[9]; its activity is about 10^{-3} lower than that of fusidic acid. Recently, it was demonstrated that *Rhodococcus erythropolis*[10] inactivates fusidic acid to a so far uncharacterized product. Another interesting type of resistance to fusidic acid is found on transposons related to Tn9. In this case, type I chloramphenicol acetyltransferase (type I CAT) binds fusidic acid with high affinity but does not acetylate the drug[11]. We exclude an involvement of a corresponding enzyme since we could show that resistance to chloramphenicol within *S. lividans* is not conferred by type I CAT[2]. Also the examined fusidic acid-sensitive variants still show resistance to chloramphenicol.

Fig. 4. Proposed two step-mechanism for modification of fusidic acid.



Since fusidic acid-sensitive variants are derived frequently from tetracyline-sensitive variants of *S. lividans*[2], further studies should allow us to clone a gene encoding the putative esterase enzyme and enable us to determine possible deletion events within the genomes of the variants as have been found for other unstable traits within streptomycetes[12].

Mycelia of *S. lividans* 90-2 cultivated in the absence of fusidic acid or helvolic acid can continue to grow if added to fresh media containing these antibiotics. Since the TLC analyses had proven the lack of inactivation by the variant and also no revertants had been found, one or several additional resistance mechanism(s)—*i.e.* efficient efflux, no uptake or a modified EFG-factor—is (are) likely to occur within *Streptomyces* strains.

Acknowledgments

We are very grateful to Dr. ROSENMEYER, FB Biologie/Chemie, University Osnabrück, for generous help in performing flash-chromatography and expert advice during the isolation and initial characterization studies. We thank L. SCHMIEDING for expert typing and correction of the manuscript and B. GEILHORN for analysing some *Streptomyces* strains during a graduate course.

The work was supported by the Deutsche Forschungsgemeinschaft, SFB 145/B7.

References

1) CUNDLIFFE, E.: Self defence in antibiotic-producing organisms. Br. Med. Bull. 40: 61～67, 1983

2) SCHREMPF, H.; A. KESSLER, V. BRÖNNEKE, W. DITTRICH & M. BETZLER: Genetic instability in streptomycetes. *In* Genetics and Molecular Biology of Industrial Microorganisms. *Ed.*, C. H. HERSHBERGER *et al.*, pp. 133~140, American Society for Microbiology, 1989
3) GODTFREDSEN, W. O.; S. JAHNSSEN, H. LORCK, K. ROHOLT & L. TYBRING: Fusidic acid: a new antibiotic. Nature 193: 987, 1962
4) YAMAKI, H.: Inhibition of protein synthesis by fusidic and helvolinic acids, steroidal antibiotics. J. Antibiotics, Ser. A 18: 228~232, 1965
5) TANAKA, N.; T. KINOSHITA & H. MASUKAWA: Mechanism of protein synthesis inhibition by fusidic acid and related antibiotics. Biochem. Biophys. Res. Commun. 30: 278~283, 1968
6) CHOPRA, I.: Mechanisms of resistance to fusidic acid in *Staphylococcus aureus*. J. Gen. Microbiol. 96: 229~238, 1976
7) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic Manipulation of Streptomyces: A Laboratory Manual. John Innes Foundation, 1985
8) GODTFREDSEN, W. O.; W. VON DAEHNE, L. TYBRING & S. VANGEDAL: Fusidic acid derivatives. I. Relationship between structure and antibacterial activity. J. Med. Chem. 9: 15~22, 1966
9) DVONCH, W. & G. GREENSPAN: Microbiological oxidation of fusidic acid. Experiencia 15: 517, 1966
10) DABBS, E. R: Fusidic acid resistance in *Rhodococcus erythropolis* due to an inducible extracellular inactivating enzyme. FEMS Microbiol. Lett. 40: 135~138, 1987
11) PROCTOR, G. N.; J. MCKELL & R. H. ROWND: Chloramphenicol acetyltransferase may confer resistance to fusidic acid by sequestering the drug. J. Bacteriol. 155: 937~939, 1983
12) SCHREMPF, H.: Genetic instability: amplification, deletion, and rearrangements within *Streptomyces* DNA. *In* Microbiology—1985. *Eds.*, L. LEIVE *et al.*, pp. 436~440, American Society for Microbiology, 1985