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# Bioconversion and Biosynthesis of 16-Membered Macrolide Antibiotics. X.[1] Final Steps in the Biosynthesis of Spiramycin, using Enzyme Inhibitor: Cerulenin

Satoshi Ōmura, Chiaki Kitao, Hidetaka Hamada, and Haruo Ikeda

*School of Pharmaceutical Sciences, Kitasato University*[2]

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Final steps in the biosynthesis of spiramycin was studied by using cerulenin, a specific inhibitor of fatty acid and polyketide biosynthesis. The spiramycin-related compounds were tested for transformation with *Streptomyces ambofaciens*, a spiramycin producing strain, under the condition inhibiting the biosynthesis of aglycone by cerulenin. Forocidin I (4′-demycarosyl 9-deforosaminyl-spiramycin I) was converted into forocidin III (3-propionyl forocidin I), neospiramycin I (4′-demycarosyl spiramycin I), neospiramycin III (3-propionyl neospiramycin I), and spiramycin III (3-propionyl spiramycin I). Neospiramycin I was also converted to neospiramycin III and spiramycin III. Spiramycin I was rapidly transformed into spiramycin III, while neospiramycin III was not converted to any other compounds. These results suggested that the binding of forosamine to aglycone precedes the mycaroside formation, and that the acylation of aglycone at C-3 occurs in the final step of spiramycin biosynthesis.

**Keywords**——bioconversion; biosynthesis; 16-membered macrolide antibiotics; spiramycin; cerulenin; polyketide

Cerulenin is a specific inhibitor of the condensing enzyme which catalyzes the condensation reaction of acyl CoA in fatty acid biosynthesis.[3] Cerulenin also inhibits the biosynthesis of metabolites such as leucomycin,[4] tylosin,[1] tetracycline,[5] and 6-methylsalicylic acid[6] which are presumably biosynthesized *via* polyketide intermediates, without reduction in the growth of those producing microorganisms even at a sufficiently high concentration. On the other hand cerulenin does not inhibit the synthesis of dihydrostreptomycin, alazopeptin which is a peptide antibiotic, or prumycin which is amino acid amino sugar antibiotic. None of these are expected to be synthesized *in vivo* through polyketide.[5] It can be concluded from these results that the mechanism of condensation between acetate and malonate in fatty acid synthesis and that of polyketide biosynthesis are similar to each other, and that both condensation steps are inhibited by cerulenin. In the previous investigation on the biosynthesis of macrolide antibiotic, tylosin,[1] we reported that the intermediates of tylosin biosynthesis are converted into tylosin under the condition that allows cerulenin to inhibit the formation of aglycone. This indicated that cerulenin can be satisfactorily applied to biosynthetic studies of compounds produced *via* polyketide, in place of previously employed methods involving blocked mutant or radio isotope methods.

Spiramycin is produced by *Sm. ambofaciens*[7] and it possesses two amino sugars (mycaminose and forosamine) and one neutral sugar (mycarose). According to the classification by fundamental skeletal arrangement of lactone ring, spiramycin belongs to magnamycin

1) Part IX: S. Ōmura, C. Kitao, J. Miyazawa, H. Imai, and H. Takeshima, *J. Antibiotics*, **31**, 254 (1978).
2) Location: *5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan.*
3) S. Ōmura, *Bacteriol. Rev.*, **40**, 681 (1976).
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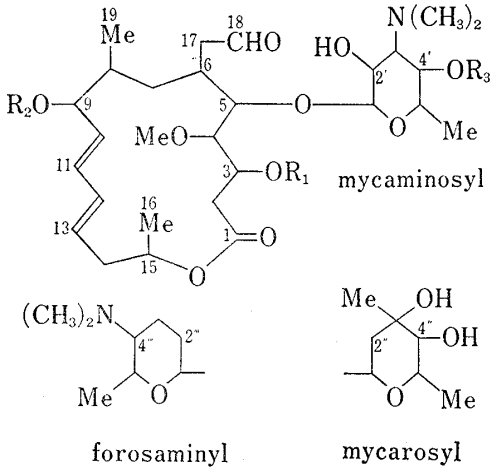
6) H. Ohno, T. Ohno, J. Awaya, and S. Ōmura, *J. Biochem.*, **78**, 1149 (1975).
7) S. Pinnert-Sindico, L. Ninet, J. Preud'hoome, and C. Cosar, *Antibioti. Ann.* **1954—1955**, 724.

group macrolide antibiotic[8] like leucomycin, platenomycin, maridomycin *etc.* Three components of spiramycins, namely spiramycin I (SP–I) which has a hydroxyl group at C-3 of aglycone, spiramycin II (SP–II) in which the hydroxyl group is acetylated, and spiramycin III (SP–III) in which the same position is propionylated are known. With regard to the acylation of hydroxyl group at C-3 of aglycone in platenomycin biosynthesis, Furumai *et al.*[9] have reported that the acylation takes place prior to linking of amino sugar (mycaminose) to aglycone. By contrast, we[10] have reported that this acylation occurs in the final step of the biosynthesis of leucomycins. On the other hand, there have been no reports in connection with acylation in spiramycin biosynthesis which stimulate much of our interests. This paper describes the order of glycosidation of sugars, forosamine and mycarose, and the acylation in the course of spiramycin biosynthesis.

## Experimental

**Preparation of Spiramycin-related Compounds**——The structures of the compounds prepared for the bioconversion are shown in Fig. 1. SP-I and III were generously provided by Kyowa Hakko Kogyo Co.,




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| forocidin I (FO-I) | H | H | H |
| forocidin III (FO-III) | $COC_2H_5$ | H | H |
| neospiramycin I (NSP-I) | H | forosaminyl | H |
| neospiramycin III (NSP-III) | $COC_2H_5$ | forosaminyl | H |
| spiramycin I (SP-I) | H | forosaminyl | mycarosyl |
| spiramycin III (SP-III) | $COC_2H_5$ | forosaminyl | mycarosyl |
| leucomycin V (LM-V) | H | H | mycarosyl |

Fig. 1. Structures of Spiramycin-related Compounds

Ltd. Neospiramycin I (NSP-I) and III (NSP-III) were obtained by the following acid hydrolysis of SP-I and III. The samples of SP-I and III were dissolved in 2 N HCl and stirred for 6 hr at 37°. The reaction mixture was then neutralized with 2 N NaOH and extracted many times with chloroform. The extract was concentrated *in vacuo* and the residue was subjected to silica gel column chromatography using a solvent system of chloroform: methanol (40: 1 and 30: 1) to obtain NSP-I and III as white powder, respectively. Similarly, forocidin I (FO-I) and III (FO-III) were obtained by the acid hydrolysis of SP-I and III with 2 N HCl for about 15 hr at 37°. FO-I was identical with demycarosyl leucomycin V obtained by the acid hydrolysis of leucomycin V (LM-V) produced by a mutant strain of *Sm. kitasatoensis*.

**Fermentation and Bioconversion**——Spiramycin producing strain *Sm. ambofaciens* KA-1028 was employed for the present studies. The stock culture on oatmeal agar was inoculated into the seed medium containing (in %) glucose 2.0, peptone 0.5, dried yeast 0.3, meat extract 0.5, NaCl 0.5, and $CaCO_3$ 0.3 (pH adjusted to 7.0 prior to sterilization), and cultured for 2 days at 27° on a reciprocal shaker. The seed culture was then transferred (2%) into 500 ml Sakaguchi flask containing 100 ml of production medium (in %: glucose 1.0, dried yeast 1.0, $NaNO_3$ 0.1, NaCl 0.5, and $CaCO_3$ 1.0, pH 7.5). It was then cultured for a desired period on a reciprocal shaker at 27°. For the bioconversion experiment, 20 mcg/ml of cerulenin was added to the culture at the beginning and every 24 hr thereafter to prevent the formation of aglycone. After 48 hr of cultivation, 100 mcg/ml of each spiramycin-related compound was added and the culture was incubated

8) S. Ōmura and H. Takeshima, *Kagaku to Seibutsu*, **15**, 381 (1977).
9) T. Furumai, K. Kaneda, and M. Suzuki, *J. Antibiotics*, **28**, 789 (1975).
10) S. Ōmura, J. Miyazawa, H. Takeshima, C. Kitao, K. Atsumi, and M. Aizawa, *J. Antibiotics*, **29**, 1131 (1976).

for an additional 24 hr. The antibiotic activity of spiramycin in the cultured broth was determined with paper disc-agar plate method using *Sarcina lutea* PCI 1001 as test microorganism.

**Isolation and Identification of Transformed Product**——After incubation cultured broth was collected, centrifuged at 3000 rpm for 15 min, and the extract was concentrated *in vacuo*. The residue was then subjected to silica gel (Kieselgel $GF_{254}$, Merck Co.,) thin-layer chromatography (TLC) developing with solvent system; chloroform: methanol: 10% aq. ammonia (2: 1: 1, bottom layer). The spiramycin-related compounds and their transformed products loaded on the silica gel plate were detected with double beam chromatogram scanner (Shimadzu Seisakusho Co., Ltd., model CS-910) at 232 nm. The transformed products were isolated and purified by preparative silica gel TLC using the same solvent system as described above. The isolated product was identified by TLC (20% $H_2SO_4$ and $I_2$ as indicator), ultraviolet (UV) spectra and mass spectra using authentic samples.

## Results

### Effect of Cerulenin on Spiramycin Production

The biosynthesis of spiramycin was completely inhibited when 20 mcg/ml of cerulenin was added into the culture every 24 hr. Figure 2 shows the time course of spiramycin production with or without cerulenin. In the ordinal culture, 100 mcg/ml of spiramycin was produced at 72 hr, and the concentration decreased gradually thereafter. However, when 20 mg/ml of cerulenin was added to the culture at the beginning and every 24 hr thereafter, the production of spiramycin was completely inhibited without affecting the appearance of mycelial growth or pH values of medium. Addition of the same amount of cerulenin during

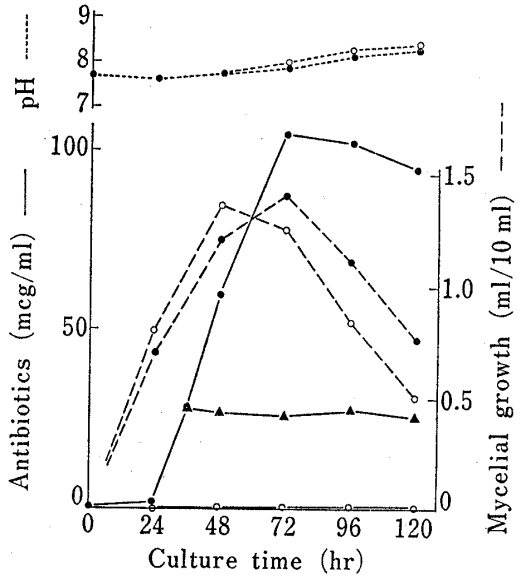



Fig. 2. Time Course of Spiramycin Production

●: CRL not added.
○: CRL added.
▲: CRL added at 36 hr.

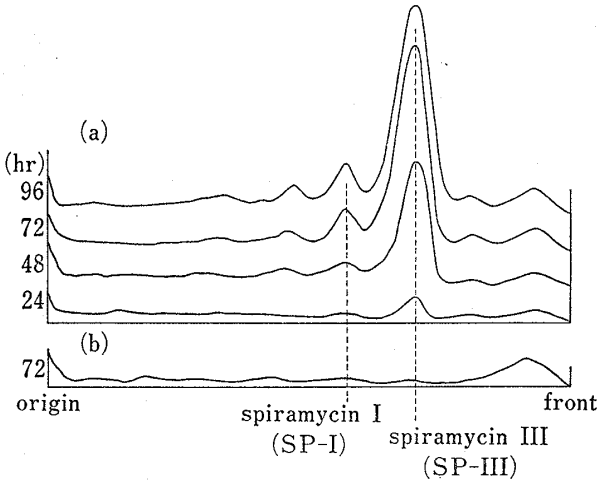



Fig. 3. Time Course of Spiramcyin Production

Silica gel TLC developed with solvent system-A, and detected by double beam chromatogram scanner at 232 nm.
a: CRL not added.
b: CRL added and extracted at 72 hr.

the course of spiramycin production, *e.g.* at 36 hr, also resulted in complete inhibition of antibiotic formation. The time course of spiramycin production was also followed by silica gel thin-layer chromatography and double beam chromatogram scanner (Fig. 3). When cerulenin was not added to the culture, two major components of spiramycin were produced; one of which with a higher *Rf* value (0.68) was identified as SP-III, and the other with lower *Rf* value (0.56) was SP-I (Fig. 3-a). It is obvious that the strain KA-1028 mainly produces SP-III and less amount of SP-I. On the other hand, when cerulenin was added to the culture, no compound having UV absorption at 232 nm could be detected even after 72 hr

(Fig. 3-b). This is why the bioconversion experiments could be carried out under these conditions.

**Bioconversion of Spiramycin-related Compounds**

The *Rf* values of spiramycin and its related compounds on silica gel thin-layer chromatography plate were examined in the following binary solvent systems. In the solvent system-A ($CHCl_3$: MeOH: 10% aq. $NH_4OH$, 2: 1: 1, bottom layer), as shown in Fig. 4-a, each compound was clearly observed separately except for LM-V and NSP-I. In the solvent system-B (benzene: acetone, 1: 2), however, only LM-V had much higher *Rf* value (0.45) than the other compounds. Thus LM-V could be distinguished from NSP-I using the solvent system-B.

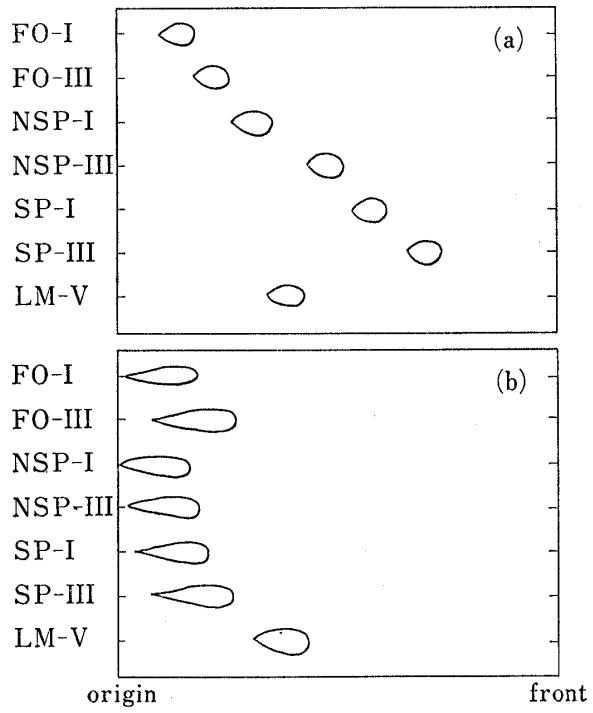



Fig. 4. TLC pattern of Spiramycin Related Compounds detected with 20% aq. $H_2SO_4$

a: solvent system-A ($CHCl_3$: MeOH: 10% aq. $NH_4OH$, 2: 1: 1, bottom layer).
b: solvent system-B (benzene: acetone, 1: 2).

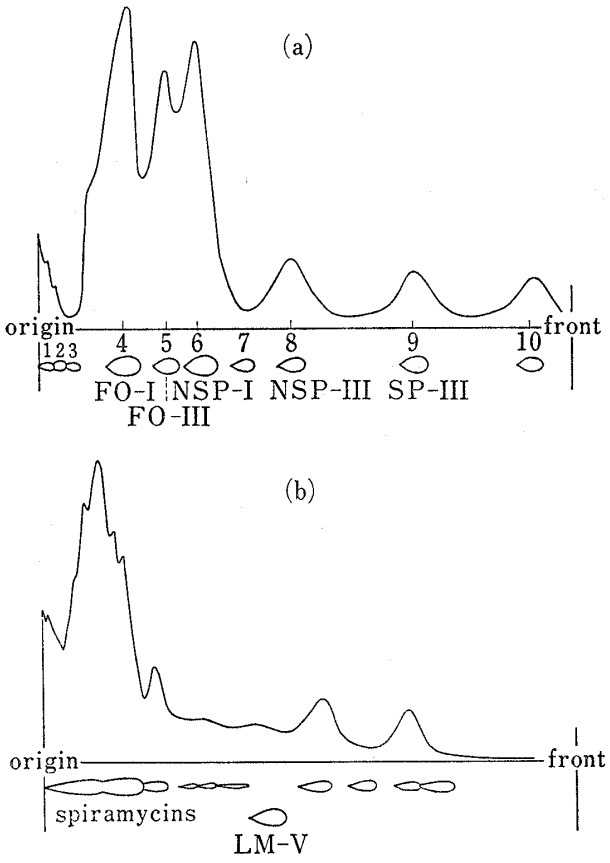



Fig. 5. Bioconversion of Forocidin I

TLC scanned at 232 nm.
a: developed with solvent system-A.
b: developed with solvent system-B.

(a) **Forocidin I**——Addition of FO-I into the cerulenin-supplemented culture resulted in transformation into four compounds. As shown in Fig. 5-a, ten spots appeared on TLC plate when it was developed with the solvent system-A. Spot 1, 2, 3, and 10 were found to be neither transformed products nor spiramycin-related compounds because these compounds always appeared in the ordinary culture. Spot 7 seemed to be a degradation product of cerulenin. Spot 4 was residual FO-I. Spot 5 was identified as FO-III by comparison of TLC and mass spectral analysis (Fig. 6-a: $M^+$ *m/e* 614, *m/e* 541 ($M^+$—$C_2H_5COO^-$), 423, 190, 174, 87). Spot 6 seemed similar to LM-V on TLC, but it was not LM-V as clearly seen on the TLC developed with solvent system-B. It was identified as NSP-I by mass spectral analysis (Fig. 6-b: $M^+$ *m/e* 699, *m/e* 525, 367, 190, 174, 158, 142, 87). Both spots 8 and 9 were also identified as NSP-III (Fig. 6-c: $M^+$ *m/e* 755, *m/e* 681 ($M^+$—$C_2H_5COOH$), 491, 349, 190, 174, 158, 142, 87) and as SP-III (Fig. 6-d:

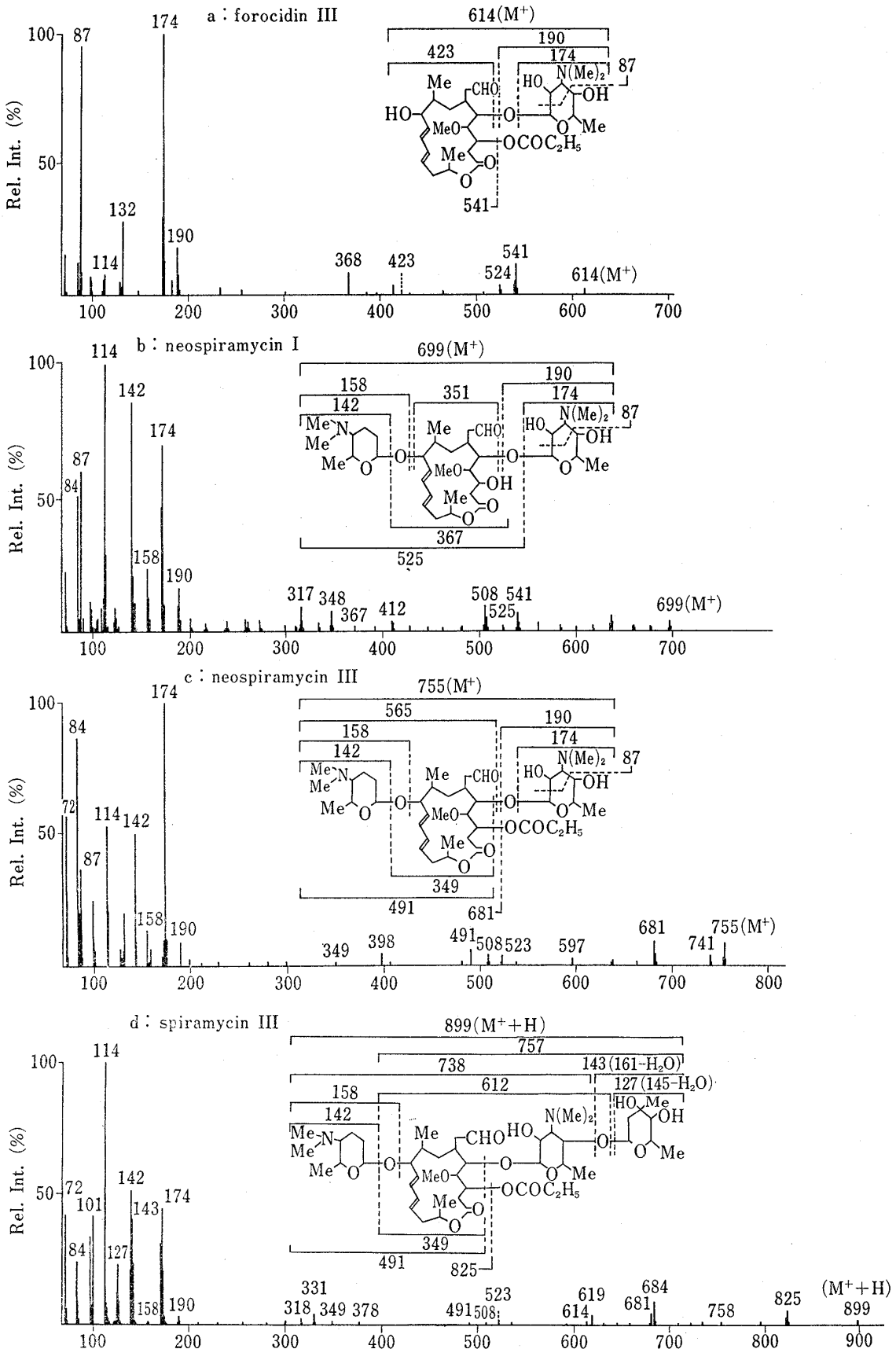



Fig. 6. Mass Spectra of Forocidin III, Neospiramycin I, Neospiramycin III, and Spiramycin III

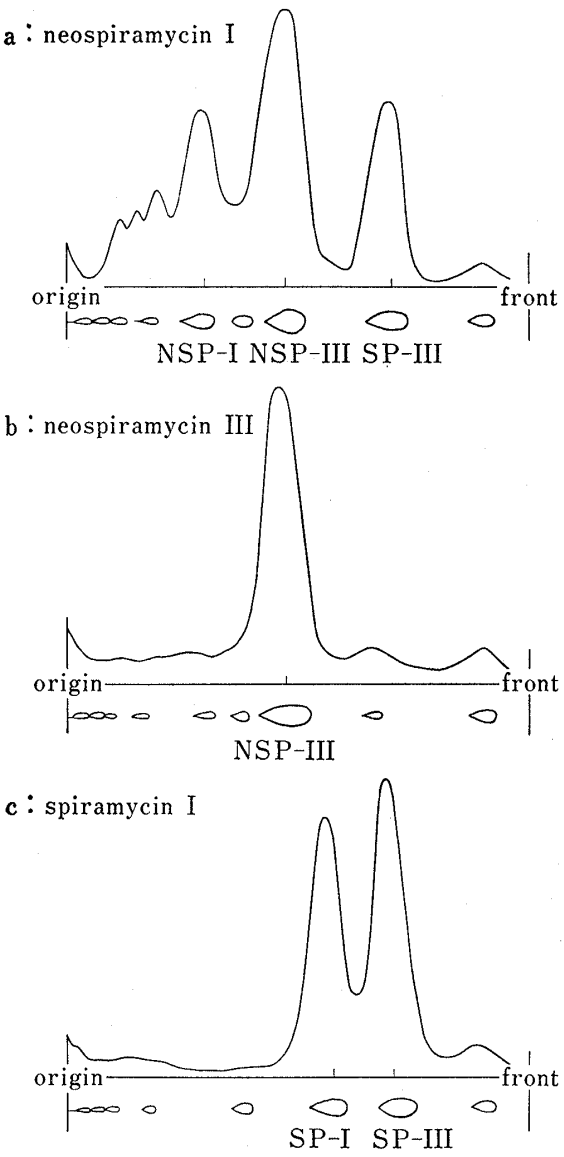



Fig. 7. Bioconversion of Neospiramycin I, Neospiramycin III, and Spiramycin I

TLC developed with solvent system-A scanned at 232 nm.

$M^+$+H *m/e* 899, *m/e* 825 ($M^+$+H−$C_2H_5$-COOH), 758, 614, 491, 349, 190, 174, 158, 143 (161−$H_2O$), 142, 127 (145−$H_2O$), respectively.

**(b) Neospiramycin I**——NSP-I was transformed into NSP-III and SP-III as shown in Fig. 7-a. Each compound was isolated by preparative TLC and identified by mass spectral analysis. SP-I was not detected in the extract since the rapid acylation at C-3 of aglycone might have occurred before or after the formation of linkage between mycarose and mycaminose.

**(c) Neospiramycin III and Spiramycin I**——NSP-III was not transformed to any of spiramycin components (Fig. 7-b), while SP-I was readily converted to SP-III (Fig. 7-c).

## Discussion

The plausible biosynthetic pathway from FO-I to SP-III is summarized in Fig. 8 considering the results described above. The formation of aglycone derived from acetate, propionate, butyrate, and unknown precursor carrying two carbons was completely inhibited by cerulenin. Under these conditions, FO-I was transformed to FO-III, NSP-I, NSP-III, and SP-III. The remarkable fact that FO-I was not converted into LM-V led us to conclude that the linking of forosamine to C-9 of aglycone is much more predominant than that of mycarose to C-4′ of mycaminose. The addition of NSP-I into the cerulenin-supplemented culture also resulted in its transformation into NSP-III and SP-III. Two processes were assumed as the biosynthetic pathway from NSP-I to SP-III: one *via* NSP-III, and the other *via* SP-I. The former could be ruled out by the fact that NSP-III was not transformed to any compounds as shown in Fig. 7-b, and the latter therefore seemed the more likely because the rapid transformation of SP-I into SP-III took place as shown in Fig. 7-c. Consequently the biosynthetic pathway from FO-I to SP-III could be most reasonably rationalized assuming that FO-I is initially transformed into NSP-I which is finally converted into SP-III *via* SP-I by the acylation at C-3 of the aglycone. The acylation took place in a variety of conversion steps. This seemed to suggest that the specificity of this acylating enzyme of *Sm. ambofaciens* KA-1028 is rather loose. Nevertheless it is noted that no acylated compounds were transformed into SP-III. Therefore it was concluded that the acyl group of aglycone was introduced in the final step in the biosynthesis of spiramycin. In the natural culture (namely, in the absence of cerulenin), the strain of KA-1028 produced spiramycin without accumulating its intermediates. This seemed to suggest that the biosynthesis rapidly exhibited by concerting whole enzyme systems. On the other hand, in the present biosyn-

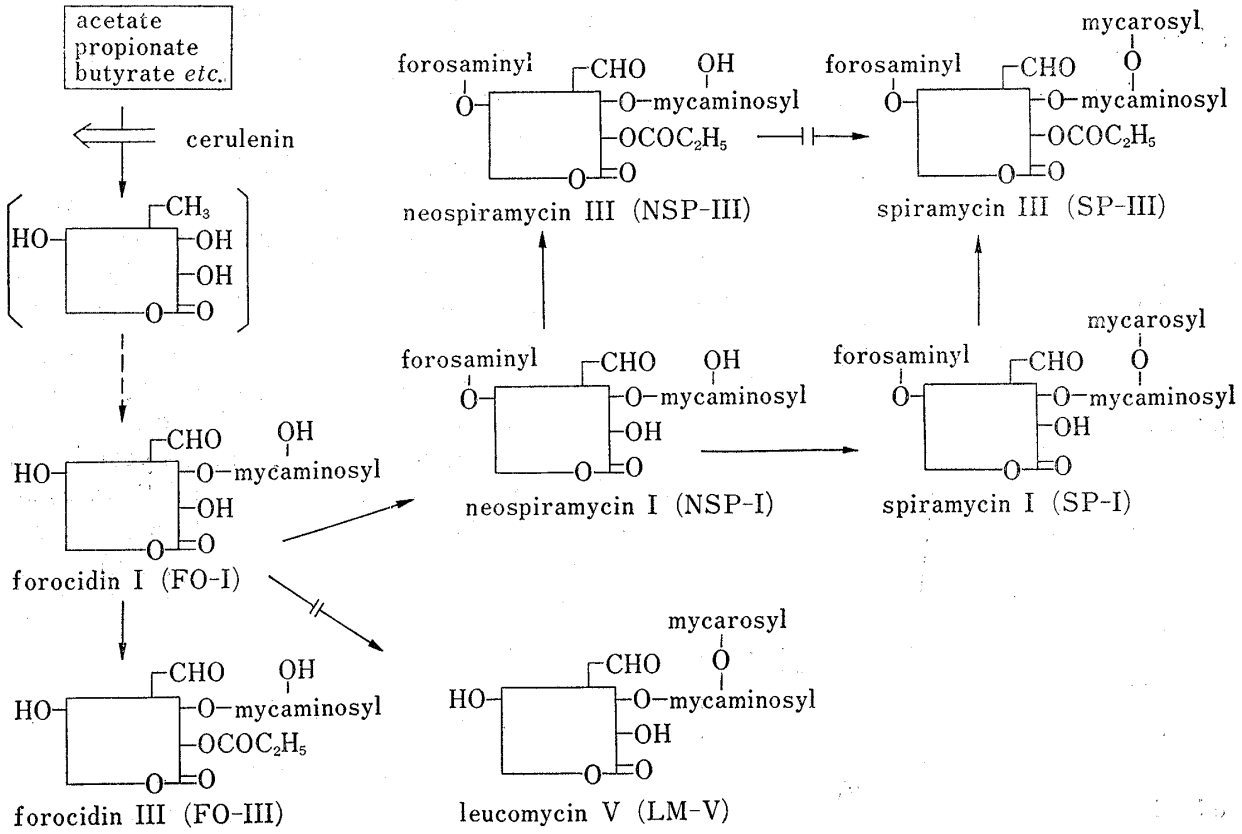



Fig. 8. Proposed Scheme for Spiramycin Biosynthesis from Forcidin I

Aglycone moiety of each compound is illustrated by box shape.

thetic study using the exogenous precursors added to the culture where the formation of aglycone was blocked by cerulenin, we could observe in some cases the accumulation of intermediate. The reason why the intermediate was accumulated is not clear yet, although it can be expected that the enzyme system leading the intermediates to the final products did not flow in good cooperation in the presence of excess amount of exogenous precursors. It should be interesting to compare these results with that of platenomycin biosynthesis reported by Furumai *et al.*[9] who concluded using blocked mutant method that the acylation of aglycone (platenolide I or II) takes place before the linking of mycaminose. Though our experimental conditions are not identical with these authors, these investigations should give us much interesting problems whether or not these differences on acylation are due to the genetic nature of the individual producing microorganisms.

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