Inactivation of Fusidic Acid by Pathogenic *Nocardia*

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While *Nocardia* species produce many antibiotics such as SF2475[1], brasiliquinones[2], brasilidine A[3], nocardicyclins[4], brasilinolide A[5] and brasilicandine A[6], some *Nocardia* can cause nocardiosis. During studies on the susceptibility of pathogenic *Nocardia* towards various antibiotics, we have found that pathogenic *Nocardia* sp. showed species-specific drug resistance patterns[7]. Our studies also that revealed that most of the resistance in pathogenic *Nocardia* is due to the inactivation of antibiotics by glycosylation or phosphorylation. Indeed, rifampicin and macrolide antibiotics are inactivated by several *Nocardia* sp.[8~11].

Fusidic acid (FA, Fig. 1), is a useful antibiotic in the treatment of drug-resistant staphylococcal infections and is produced by fermentation of a strain of the fungus *Fusidium coccineum*[12]. Chemically, it belongs to a group of tetracyclic triterpenoic acids whose stereochemistry of rings A, B and C is *trans-syn-trans*, which forces ring B into a boat conformation. As will be described later, although much research on microbiological transformations of FA has been conducted, we have found a new inactivation product of FA. In the present report we describe the inactivation of FA by a *Nocardia brasiliensis* to give three products designated as FAIP-1, -2 and -3, two of which (FAIP-1 and -2) were successfully isolated by chromatography.

The inactivation of FA was carried out and the inactivation products were extracted with ethyl acetate in acidic conditions. Since FA and related compounds were easily detected with a spray reagent composed of vanillin-phosphoric acid, it was possible to differentiate fermentation broth components of *N. brasiliensis* IFM 0329 from FA and related compounds. In a TLC chromatogram two prominent spots (FAIP-1 and 2) were observed together with a faint spot (FAIP-3) and that of FA. The HPLC chromatogram of the same reaction product showed three peaks, FAIP-1 and -2 and FA, but FAIP-3 was not detected. Sephadex LH-20 chromatography is very effective for separation of the FA related compounds from the fermentation broth components of *N. brasiliensis*.

The structures of FAIP-1 and -2 were spectroscopically determined by the comparison with FA. The FAB mass spectrum of FAIP-1 shows a protonated molecule $(M+H)^+$ at *m/z* 457, indicating that FAIP-1 has a molecular weight of 456. This value corresponds to the loss of acetic acid from FA. The ^{13}C and ^{1}H NMR chemical shifts of FAIP-1 are summarized in Table 1 and are consistent with those of a reported inactive lactone derivative[13]. Additionally, it was confirmed that

Fig. 1. Structure of fusidic acid (FA).

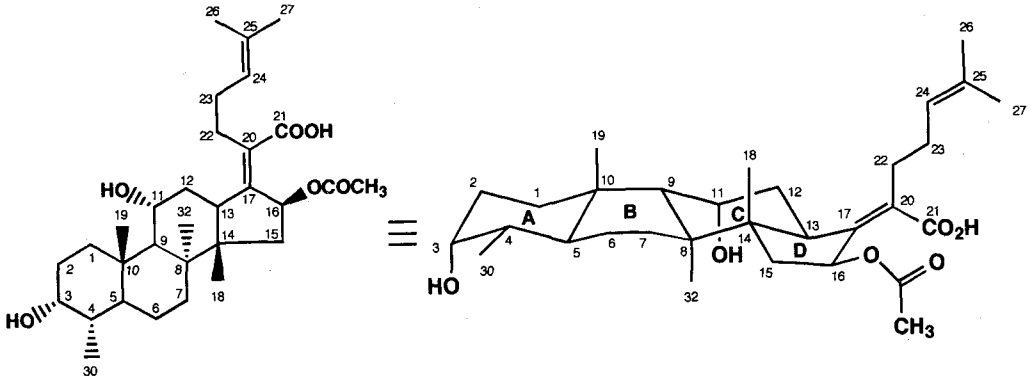

Table 1. ^{13}C and ^{1}H NMR chemical shifts of FA, FAIP-1 and FAIP-2.

	FA	FAIP-1	FAIP-2		FA	FAIP-1	FAIP-2
carbon	δ_C	δ_C	δ_C	proton	δ_H	δ_H	δ_H
C-1	~30	29.3	29.0	1-H	1.35~1.60, 2.05~2.25	1.28 t, 2.38 dd	1.23 t, 2.43 dd
C-2	~30	30.1	30.0	2-H	1.35~1.60	1.50 m, 1.64 m	1.49 m, 1.61 t
C-3	69.3	69.2	68.9	3-H	3.52	3.52 s	3.47 s
C-4	36.2	36.7	37.2	4-H	1.35~1.55	1.38 m	1.31 m
C-5	35.2	34.9	35.1	5-H	2.10	2.09 m	2.17 m
C-6	20.7	21.0	34.1	6-H	0.95~1.05, 1.35~1.55	1.05 m, 1.55 m	1.33 m, 1.41 m
C-7	30.2	30.9	69.1	7-H	0.95~1.05, 1.35~1.55	1.05 m, 1.55 m	3.62 m
C-8	39.9	40.3	45.4	-	-	-	-
C-9	48.6	49.8	50.6	9-H	1.33	1.33 s	1.23 s
C-10	36.3	36.4	36.1	-	-	-	-
C-11	66.1	65.6	65.7	11-H	4.13	4.19 s	4.19 s
C-12	36.4	31.8	31.8	12-H	1.60~1.70, 2.05~2.25	1.72 dt, 1.92 dt	1.68 dt, 1.91 dt
C-13	41.9	37.7	37.9	13-H	2.83	3.46 d	3.46 m
C-14	48.2	54.8	54.4	14-H	-	-	-
C-15	38.9	33.6	37.0	15-H	1.09 ,1.86	1.05 m, 2.23 m	1.18 dd, 2.37 m
C-16	74.0	81.3	81.9	16-H	5.75	5.12 dd	5.03 dd
C-17	139.2	171.3	171.5	-	-	-	-
C-18	17.4	19.7	18.6	18-H	0.83	0.72 s	0.75 s
C-19	22.7	23.3	24.0	19-H	0.89	0.88 s	0.83 s
C-20	135.5	121.8	121.8	-	-	-	-
C-21	174.9	175.7	175.9	-	-	-	-
C-22	~30	27.0	23.4	22-H	2.10~2.30	2.11 m, 2.22 m	2.11 m, 2.20 dt
C-23	~30	23.4	27.1	23-H	2.10~2.30	2.11 m	2.12 m
C-24	125.0	123.2	123.3	24-H	5.10	5.09 t	5.09 t
C-25	129.9	131.9	131.9	-	-	-	-
C-26	17.5	17.5	17.5	26-H	1.57	1.56 s	1.54 s
C-27	25.5	25.4	25.4	27-H	1.64	1.65 s	1.63 s
C-30	16.2	16.2	16.1	30-H	0.79	0.81 d	0.78 d
C-32	23.5	22.3	15.1	32-H	1.35	1.43 s	1.26 s
CH_3	20.7	-	-	CH_3	1.85	-	-
C=O	170.4	-	-				

FAIP-1 is identical with the lactone derivative derived from FA under basic conditions.

The molecular weight of 472 for FAIP-2 was obtained from the FAB mass spectrum, in which the $(M+H)^+$ ion appears at m/z 473 and the composition $C_{29}H_{45}O_5$ was determined by HRFAB. This molecular weight corresponds to an additional oxygen atom relative to the lactone derivative mentioned above, derivative and FAIP-2 was considered to be a hydroxylated or epoxidised form of FAIP-1 in consideration of the thin layer chromatographic behavior. The ^{13}C and ^{1}H chemical shifts of FAIP-2 are compared with those of FA and FAIP-1 (Table 1), and were assigned using COSY, HMQC and HMBC techniques (Fig. 2 (A)). It was found that the C-7 signal of FAIP-2 is shifted to δ 69.1 and the H-7 signal is observed at δ 3.62 in FAIP-2, indicating that FAIP-2 is 7-hydroxylated FAIP-1. In order to determine the absolute configuration of C-7, the NOESY spectrum was measured. Fig. 2 (B) shows the NOE correlations of FAIP-2 and the NOE correlations are clearly observed between H-7β and H-9 and CH_3-19. This indicates that the hydroxy group is equatorially oriented at C-7, and FAIP-2 is 7α-hydroxylated FAIP-1.

Because the molecular weight of FAIP-3 could be deduced to be 488 by the FAB mass spectral analysis, it may be a dihydroxylated derivative of FAIP-1. However, it was not possible to determine its structure due to limited amounts of the isolated compound.

FA is active against the Gram-positive bacteria, *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus*

Fig. 2. (A) Structure of FAIP-2 and COSY, HMBC and (B) NOE correlation in FAIP-2.

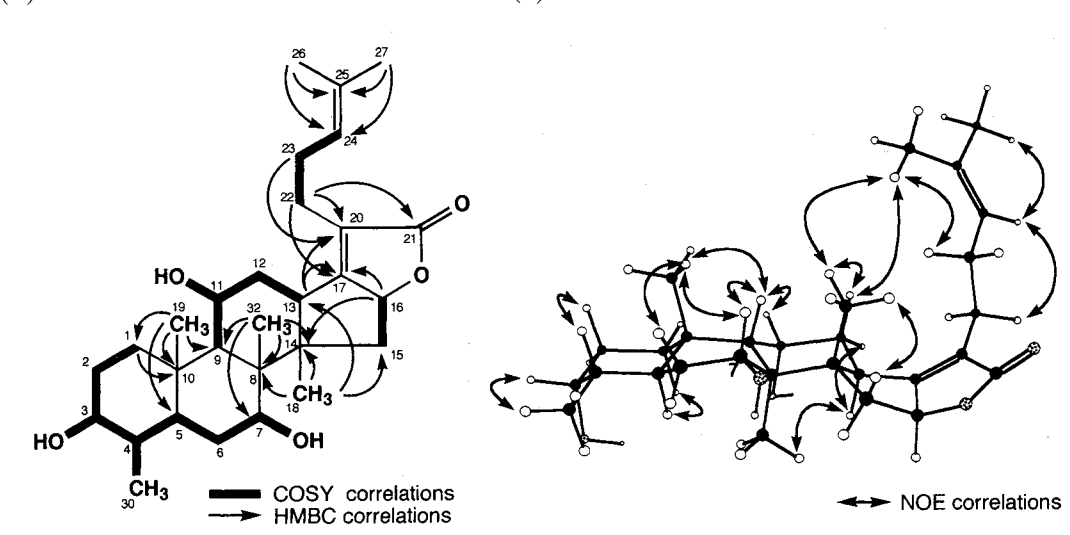

Table 2. Antimicrobial activity of FA, FAIP-1 and FAIP-2.

organism	MIC (μg/ml)		
	FA	FAIP-1	FAIP-2
Micrococcus luteus IFM 2066	≦ 0.6	5.0	660
Bacillus subtilis PCI 189	≦ 0.6	2.5	> 660

subtilis[14]. The antimicrobial activity of FAIP-1 and -2 was examined together with FA using *M. luteus* and *Bacillus subtilis* as the test organisms (Table 2). As expected, FA showed activity against these two test strains, whereas FAIP-1 showed less activity and FAIP-2 completely lost activity.

FA was discovered in 1962 and since then much research on the inactivation of FA has been conducted to determine with structure-activity relationships. Modifications of the structure and antibacterial properties of FA were attempted by microbiological processes. A total of 2254 isolates, mainly fungi, streptomycetes and bacteria, were examined and 2.9% of the organisms were found to inactivate FA[15]. Also, a variety of microorganisms including *Nocardia* sp. which are capable of transforming steroid substrates by means of biooxidative and bioreductive processes were incubated with FA and several conversion products such as 3-keto derivative, 3-epifusidic acid and 16-deacetylfusidic acid were obtained. DAEHNE *et al.*[16] demonstrated that the fungus *Acrocylindrium oryzae* introduces oxygen functions into positions 6 and 7 of FA and these products showed markedly reduced antibacterial activity. VON DER HAAR *et al.*[13] have demonstrated that several *Streptomyces* strains are resistant to FA and this resistance is mediated by structural modification of the antibiotic. They obtained two transformed derivatives (having Rf 0.61 and 0.20) of FA from the culture broth, and the structure of one (Rf 0.61) was determined to be a lactone derivative. We have obtained the same compound (FAIP-1) from the culture broth of FA with *N. brasiliensis* IFM 0329, suggesting that FAIP-2 may correspond to the compound with Rf 0.20. Subsequently, the same authors isolated an extracellular enzyme from *Streptomyces lividans*,

which catalyzes the hydrolysis of the acetoxy group at C-16, so that FAIP-1 is formed after spontaneous lactonization[17].

We have demonstrated that rifampicin and macrolide antibiotics become inactive against pathogenic *Nocardia* through the modification of the structures by phosphorylation and/or glycosylation. However, FA becomes inactive through a completely different inactivation process by *N. brasiliensis* IFM 0329, which stereospecifically oxidizes at C-7. Because FAIP-3 may be a further oxidized product of FAIP-2, oxidation is considered to be a key process for the inactivation of FA by *N. brasiliensis* IFM 0329. Although the present study is preliminary, we observed this inactivation of FA with all tested strains of *N. brasiliensis*. This type of inactivation, however, was not observed with other species of pathogenic *Nocardia*, *e.g.*, *N. asteroides*, *N. farcinica*, *N. nova* and *N. otitidiscaviarum*. Therefore, the inactivation of FA with oxidation may be a species-specific phenomenon in *N. brasiliensis*.

Experimental

MIC Determination

Antimicrobial activities were determined by a microbroth dilution method using brain heart infusion broth (BHI broth, Difco).

Inactivation of Fusidic Acid

N. brasiliensis IFM 0329 (type strain) was inoculated into a 10 ml Erlenmeyer shake flask containing 5 ml of a seed medium (2% glucose supplemented BHI medium). The inoculated flasks were shaken at 250 rpm at 32°C for 3 days. The mature seed cultures were inoculated at a 2% ratio into 100 ml Erlenmeyer flasks containing 50 ml of a medium (pH 7.2) composed of 1% glucose, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.4% sodium glutamate and the culture was incubated at 32°C for 1 day. Methanol-sterilized fusidic acid was added at a final concentration of 50 μg/ml. The culture was incubated for three more days in the same conditions. The culture was filtered and the pH of the filtrate was adjusted to 2.0 with 6 M HCl and the filtrate was extracted with ethyl acetate. After concentration of the extract *in vacuo* to dryness, the resulting residue was used for the subsequent separation.

Chromatography

TLC was performed using the following conditions: silica gel plate, Kieselgel $60F_{254}$ (E. Merck, Darmstadt, Germany); mobile phase, benzene : acetone : acetic acid (70 : 30 : 1); detection, spraying with vanillin-phosphoric acid, followed by heating at 120°C for 10~20 minutes. HPLC was carried out using the following conditions: pump, JASCO PU 980 (Tokyo, Japan); detector, JASCO UV-970; column, Cosmosil 5C18-AR (150 × 4.6 mm, Nacalai Tesque, Kyoto, Japan); mobile phase, methanol : 0.05% TFA (67 : 33); detector, UV absorbance at 215 nm; flow rate, 1 ml/minute. Silica gel and Sephadex LH-20 for chromatography were obtained from Fuji silysia (Tokyo, Japan) and Pharmacia (Uppsala, Sweden), respectively.

Purification of Inactivation Products

The inactivated products mentioned above were subjected to silica gel chromatography using chloroform : methanol (95 : 5→50 : 50) as the mobile phase to separate FAIP-1 and FAIP-2 fractions. The FAIP-1 fraction was repeatedly chromatographed on silica gel using benzene : acetone : acetic acid (90 : 10 : 1→70 : 30 : 1) as the mobile phase and the desired fraction was finally purified by Sephadex LH-20 column chromatography using methanol as the mobile phase to give 24.8 mg of FAIP-1. FAIP-1 has the following properties: colorless amorphous powder, $[\alpha]_D$ 51.4° (*c* 0.58, MeOH), positive FABMS *m/z* 457 $(M+H)^+$. The FAIP-2 fraction was repeatedly chromatographed on silica gel using chloroform : methanol (95 : 5→50 : 50) and benzene : acetone : acetic acid (80 : 20 : 1) as the mobile phases and the desired fraction was finally purified by Sephadex LH-20 column chromatography using methanol as the mobile phase to give 3.3 mg of FAIP-2. FAIP-2 has the following properties: colorless amorphous powder, $[\alpha]_D$ 13.6° (*c* 0.25, MeOH), positive FABMS *m/z* 473 $(M+H)^+$, positive HRFAB-MS *m/z* 473.3260 $(M+H)^+$ calcd. for $C_{29}H_{45}O_5$, Δ + 1.3 ppm.

Structure Analysis

^1H and ^{13}C NMR spectra were measured in DMSO-d_6 on JOEL JNM A-600 and JOEL JNM A-400 spectrometers. Chemical shifts of ^1H and ^{13}C were recorded in δ units relative to the solvent peaks (^1H: δ 2.49 and ^{13}C: δ 39.5 in DMSO-d_6). FAB mass spectra and HRFAB mass spectra were measured on a JEOL JMX-HX110 spectrometer. Glycerol was used as the matrix.

Conversion of FA to FAIP-1

Sodium fusidate (50 mg) was dissolved in methanol. The solution was adjusted to pH 11 with 5% KOH (aq)

and heated under reflux for 7 hours. The reaction mixture was acidified with 20% HCl and the solution was evaporated under reduced pressure. The residual aqueous solution was extracted three times with ethyl acetate. The extract was dried over anhydrous K_2CO_3 and evaporated to dryness. The residue was chromatographed on silica gel with benzene : acetone : acetic acid (90 : 10 : 1) as the mobile phase to give pure lactone derivative (27.1 mg) as an amorphous powder.

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