ISOLATION AND IDENTIFICATION OF N-FORMYLFORTIMICIN A

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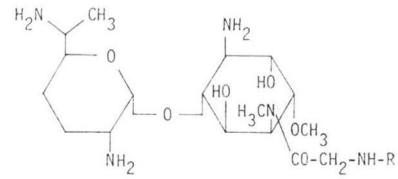
(Received for publication March 11, 1980)

Since the discovery of fortimicin A (**I**) and B in 1977[1], a number of derivatives and analogues were reported as by-products of *Micromonospora* sp. These include fortimicin C (**II**), D, KE[2] and E[3]. A *Micromonospora* strain with code number SF-1854 was isolated from a soil sample collected at the lakeside of Inawashiro, Fukushima Prefecture, Japan, and was found to produce several antibiotics. Among them, substance SF-1854 was isolated and characterized as a new derivative of **I**, N-formylfortimicin A (**III**). The present paper describes the isolation, identification and biological property of substance SF-1854.



I: R=H
II: R=$CONH_2$
III: R=CHO
IV: R=CH=NH

Materials and Methods

Microorganism

The isolate was designated as *Micromonospora olivoasterospora* SF-1854 from a taxonomic study, and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession number of FERM-P 3141.

Fermentation

The medium for the seed culture was 0.5% glucose, 0.5% starch and 3.0% soybean meal. The first seed culture was prepared by shaking 3 spoonful spores of strain SF-1854 in 50 ml tubes at 28°C for 3 days. This was transferred to the culture in 100-ml flasks, and the last seed culture was fermented in a 30-liter jar at 28°C for 48 hours.

The production medium was composed of 3.0% starch, 3.5% soybean meal, 1.5% wheat embryo, 0.2% soybean oil, 0.25% NaCl and pH 7.0 before sterilization. Fermentation was carried out at 28°C for 80 hours under aeration in a 300-liter jar fermenter containing 200 liters of the production medium and 5% seed culture. The antibiotic titer was assayed using *Bacillus subtilis* ATCC 6633 and the paper disc method.

Physico-chemical Measurements

IR spectrum was recorded on a Hitachi model 215 IR spectrometer in nujol mull. PMR spectrum was determined in D_2O using a Varian XL-100 spectrometer with DOH as an external standard. EI-MASS spectra were obtained with a JMS-01SG double-focussing mass spectrometer at 75 eV. N-Salicylidene-O-TMS derivatives were prepared by the procedure already reported[4]. TLC was performed on silica gel TLC plates (E.Merck F_{254}) or cellulose TLC plates (E.Merck) with the following solvent systems: (a) the upper layer of $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1), (b) 10%NH_4OAc - MeOH (2: 1). PPC was conducted ascendingly with the lower phase of $CHCl_3$ - MeOH - 17%NH_4OH (2: 1: 1).

Results and Discussion

Isolation of Substance SF-1854

The culture broth fermented for 80 hours was adjusted to pH 4 by addition of hydrochloric acid, and filtered. The filtrate was adjusted to pH 7.5 with sodium hydroxide, and the resulting precipitate was removed by filtration. The solution was passed through a column of Amberlite IRC-50 (Na^+, 15 liters), which was washed with water, and eluted with 0.5 N hydrochloric acid. The first eluate (45 liters) was discarded, and the next eluate (30 liters) was collected and treated with Amberlite IR-45 (OH^-). The resulting alkaline solution of pH 9 was passed through a column of active carbon (1 liter), and the antibiotic adsorbed was eluted with an equal mixture of 0.1 N hydrochloric acid and acetone. The active fractions were neutralized with Amberlite IR-45 (OH^-), and concentrated to 1.5 liters,

which were charged on a column of Amberlite CG-50 (NH_4^+, 500 ml). After washing with water, elution was effected with 0.1 N ammonium hydroxide. Concentration and neutralization with sulfuric acid gave a crude powder of SF-1854 (920 mg, 25% purity). Fortimicin A was recovered from a column by elution with 0.3 N ammonium hydroxide.

A part of the crude powder (300 mg) was dissolved in water, and applied on a short column (20 ml) of Amberlite CG-50 (NH_4^+). This was washed with H_2O, and eluted with 0.075 N ammonium hydroxide. The active fractions were collected, and concentrated to 4 ml, to which was added excess of salicylaldehyde dissolved in ethanol. The resulting yellow precipitate was collected by filtration, and distributed counter-currentwise between chloroform and water. The bioactive organic layer was taken up, and evaporated to dryness to give the salicylidene SCHIFF base of SF-1854 (99 mg). This was again distributed counter-currentwise between acidic water (pH 2.0) and chloroform, and the active aqueous fractions were combined, and neutralized with Amberlite IR-45 (OH^-). Concentration and lyophilization gave a white amorphous powder of SF-1854 sulfate (31 mg). Conversion of the sulfate into free base was accomplished by passing over Amberlite CG-50 (NH_4^+) followed by elution with 0.1 N ammonium hydroxide and lyophilization.

Physico-chemical Properties

Substance SF-1854 sulfate is a white powder, and melted above 220°C with decomposition. It is soluble in water, but almost insoluble in common organic solvents. The antibiotic showed positive color reactions to ninhydrin, LEMIEUX and GREIG-LEABACK reagents, but negative SAKAGUCHI and silver nitrate reactions. It is fairly stable in acidic and neutral but unstable in alkaline solution.

SF-1854 was optically active, $[\alpha]_D^{25}+67.0°$ (c 1.0, H_2O), and no UV maximum was observed in the aqueous solution. IR and PMR spectra are shown in Figs. 1 and 2. The molecular weight of 433 was determined by the mass spectrometry of the free base. Elemental analysis: Found: C 31.04, H 6.58, N 10.49. Calcd. for $C_{18}H_{35}N_5O_7 \cdot 1.5H_2SO_4 \cdot 6H_2O$: C 31.39, H 7.32, N 10.17. The antibiotic showed a distinguishable Rf value (0.30) from fortimicin A (0.25) and B (0.86) on PPC. Rf values on silica gel TLC was 0.19 (solvent a), and those on cellulose TLC 0.09 (solvent a) and 0.31 (solvent b).

Identification of SF-1854

The gross structure of SF-1854 was obtained first from the PMR spectrum shown in Fig. 2. It was very similar to that of fortimicin A (**I**) except for a signal at 8.34. In particular, a OCH_3 signal at 3.64, NCH_3 at 3.31, $C-CH_3$ at 1.48, $-CH_2-$ at 2.15, and an anomeric proton at 5.43 in **III** were seen as such in **I**. An extra signal of 8.34 could be assigned to a formyl or formimino proton, but the lack of an IR band around 1720 cm^{-1} denied the formimino group[6]. Further evidence on the presence and location of a formyl group was obtained from the mass analysis of the free base (**III**) and the N-salicylidene-O-TMS derivative (**V**). As shown in Chart 1, besides molecular ions and related ones, both compounds showed characteristic aminosugar ions at m/e 143 and 495. The same fragment ions were observed

Fig. 1. IR spectrum of substance SF-1854 sulfate in nujol mull.

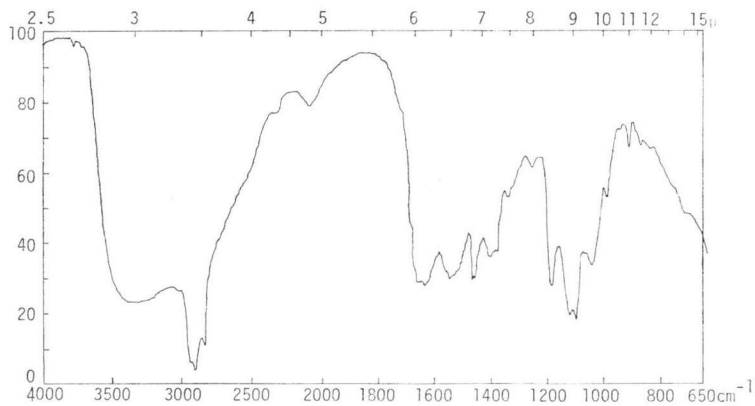
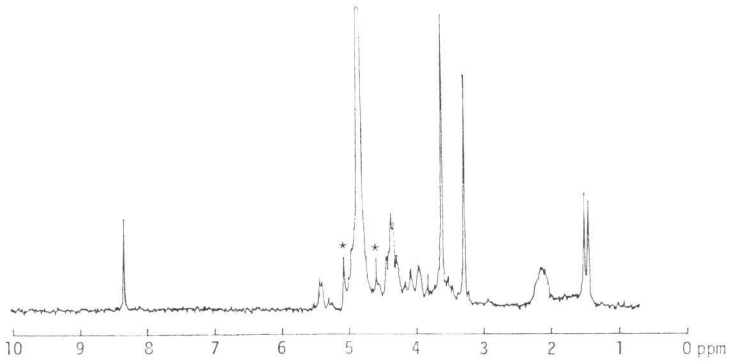
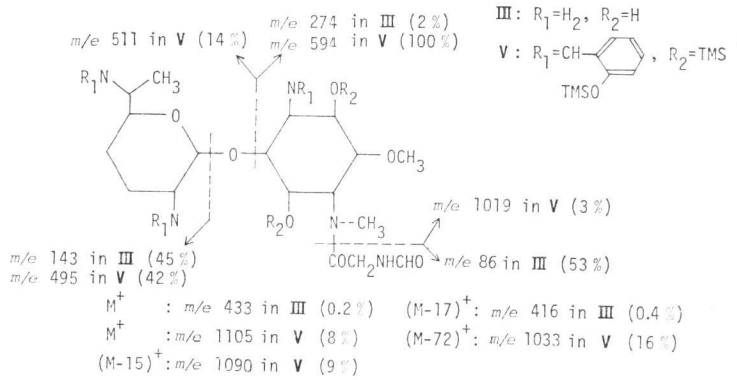


Fig. 2. 100 MHz PMR spectrum of substance SF-1854 sulfate in D_2O.



*: side band.

Chart 1. Mass fragmentation of SF-1854 (III) and N-salicylidene-O-TMS derivative (V)*.



* Figures in parentheses indicate relative abundance against base peaks at *m/e* 44 in III and *m/e* 594 in V.

also in the mass spectra of fortimicin A (I) and its N-salicylidene-O-TMS derivative (VI).

Aminocyclitol ions of III and V appeared at *m/e* 274 and 594 with strong intensity, which were absent in the spectra of I and VI. Instead, the latter substances showed the corresponding ions at *m/e* 246 and 742. The differences in *m/e* value 28 between I and III, and *m/e* 247 between V and VI could be reasonably explained by a formyl group substituted in an amino function. Furthermore, the ions arising from the cleavage of the N-methylamide linkage were observed at *m/e* 86 in III and *m/e* 1019 in V, suggesting that a formyl group was located in the glycine moiety. The presence of glycine was indicated chemically by the drastic acid hydrolysis. Treatment of III with 5 N hydrochloric acid at 100°C for 2 hours gave glycine, which was identified by TLC and amino acid analyzer.

Final confirmation of III as N-formylfortimicin A was made by comparing the physicochemical properties of III and a degradation product of dactimicin (IV)*. Alkaline hydrolysis of IV gave N-formylfortimicin A as one of products[6], which gave values indistinguishable in IR and PMR spectra, Rf and biological activity from those of SF-1854.

Biological Activity

The antibacterial spectrum of SF-1854 is shown in Table 1. It exhibited 8~16 times weaker activity against Gram-positive and -negative bacteria than fortimicin A. However, the activity against *Salmonella*, *Pseudomonas* and *Streptococcus faecalis* were relatively close to fortimicin A.

* Dactimicin was previously reported as substance SF-2052[5].

Table 1. Antibacterial spectrum of SF-1854.

Test organism	MIC (mcg/ml)*		Test organism	MIC (mcg/ml)*	
	SF-1854	Fortimicin A		SF-1854	Fortimicin A
Staphylococcus aureus Rosenbach FDA 209-P JC-1	6.25	0.39	*Salmonella typhimurium* LT-2	12.5	3.13
Staphylococcus aureus Smith S-424	12.5	1.56	*Salmonella enteritidis* No.11(Tōkai)	12.5	3.13
Staphylococcus aureus No. 26	50	1.56	*Salmonella* species D-0006	12.5	3.13
Staphylococcus aureus ApO-1	50	1.56	*Shigella dysenteriae* Shigae	25	3.13
Staphylococcus epidermidis ATCC 14990	6.25	0.39	*Klebsiella pneumoniae* PCI-602	25	6.25
Streptococcus faecalis ATCC 8043	50	50	*Klebsiella pneumoniae* 22 #3038	50	6.25
Bacillus subtilis ATCC 6633	12.5	0.78	*Proteus morganii* Kōno	50	6.25
Bacillus anthracis No. 119	6.25	0.39	*Proteus vulgaris* OX_{19}	50	3.13
Escherichia coli (M) Cast. & Chalm. NIHJ JC-2	25	6.25	*Proteus rettgeri* J-0026	>50	12.5
Escherichia coli No. 29	50	12.5	*Proteus mirabilis* J-0010	50	6.25
Escherichia coli W 677 (A-20684)	12.5	1.56	*Serratia marcescens* No. 1	12.5	1.56
Escherichia coli A-0001	50	12.5	*Serratia marcescens* No. 2	25	1.56
			Pseudomonas aeruginosa E-2	>50	100
			Pseudomonas aeruginosa IAM-1007	50	25
			Vibrio parahaemolyticus K-5	50	12.5

* The MICs were determined on MUELLER-HINTON agar with inoculum size of 10^8 cell/ml.

Acknowledgements

The authors wish to thank Miss S. MIKI for mass spectra, and Mr. K. MIYAUCHI for antibacterial spectra.

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