PREPARATION OF FLUORINATED ANTIBIOTICS FOLLOWED BY ^{19}F NMR SPECTROSCOPY

I. FLUORINATED VULGAMYCINS

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Attempts to prepare fluorinated vulgamycins have been followed by ^{19}F NMR spectroscopy. Isolation, structural elucidation and biological activities of fluorovulgamycins are reported.

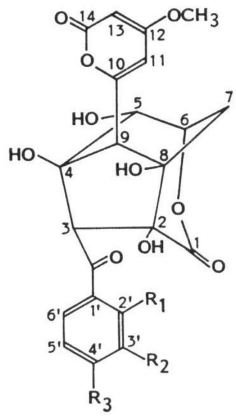
In 1973 ISONO *et al.* described the biosynthetic preparation of 5-fluoropolyoxin L and 5-fluoropolyoxin M[1], by supplementation of 5-fluorouracil to the fermentation broth of *Streptomyces cacaoi* var. *asoensis*. It is worth noting that these fluoro derivatives of polyoxins exhibited an inhibitory effect to some Gram-positive bacteria, while natural polyoxins are only active against some kinds of fungi.

More recently, TOSCANO *et al.*[2] obtained fluorinated erythromycins by a mutasynthetic experiment in which a new semisynthetic aglycone, fluoroerythronolide, was added to the fermentation broth of the strain *S. erythreus* ATCC 31772, which is a blocked mutant unable to biosynthesize erythronolide. The transformation products, (8*S*)-8-fluoroerythromycins A and B, are as active against Gram-positive bacteria as the natural erythromycins. However, the serum level of (8*S*)-8-fluoroerythromycin A in rats was reported to be higher than that of erythromycin A. These findings together with the well-known antitumor activity of 5-fluorouracil suggest that replacement of hydrogen by fluorine in a given molecule may result in modification of the biological activities of the parent compound. Although hydrogen and fluorine are nearly isosteric, the fluorine atom has the strongest electronegativity among all the elements and the C-F bond energy is higher than that of the C-H bond[3]. Therefore, one can expect to find new biological activities in fluorinated derivatives. With this in mind, we attempted to prepare fluorinated derivatives of known antibiotics by the use of the biosynthetic method combined with ^{19}F NMR spectroscopy.

^{19}F NMR spectroscopy is a very effective method to detect the incorporation of fluorinated precursors into the metabolites of concern, since its sensitivity is very high and therefore fluorinated metabolites in the fermentation broth can be detected after very simple purification procedures, if any are required, even at concentrations as low as 20 μg/ml. Furthermore, this methodology does not require obtaining mutants which are blocked at specific biosynthetic steps[2].

As the first target of our investigation we selected vulgamycin (VM), an antibiotic active against Gram-negative bacteria which was isolated from *S. hygroscopicus* No. A-5294[4]. Its structure was determined independently by a biosynthetic method[5] and by X-ray analysis[6] as shown in Fig. 1.

Fig. 1. Structures of vulgamycin and fluorovulgamycins.



	R_1	R_2	R_3
VM	H	H	H
OFVM	F	H	H
MFVM	H	F	H
PFVM	H	H	F
3′,4′-DiFVM	H	F	F

For abbreviations, see Table 3.

It has been proved by stable isotope experiments[5] that the pyrone ring and nonaromatic moiety of vulgamycin are derived from acetic acid units with the aromatic part being derived from benzoic acid. Therefore we have undertaken to introduce fluorine into the benzoyl unit by using fluorobenzoic acids as precursors.

Experimental

Instrument

^{1}H NMR and ^{13}C NMR spectra were recorded on a Jeol GX-400 spectrometer. Mass spectral determinations were carried out on a Shimadzu LKB 9000 GC/MS spectrometer. UV spectra were recorded on a Hitachi-32 spectrophotometer.

^{19}F NMR spectra were recorded on a Jeol GX-400 spectrometer under the following conditions; resonance frequency 376 MHz, data points 16K, spectral width 50 KHz, acquisition time 0.164 second, and pulse delay 1.0 second. Potassium fluoride was used as an external standard.

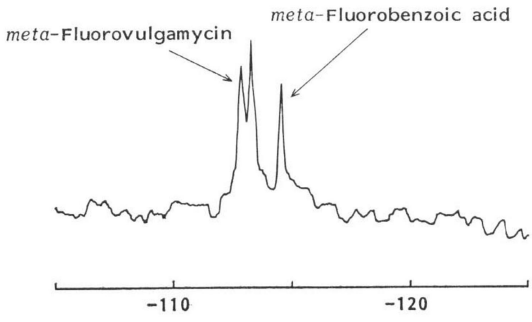
Results

Fermentation

S. hygroscopicus No. A-5294 was cultivated at 27°C in 500-ml Erlenmeyer flasks, each containing 100 ml of a medium consisting of glucose 5.0%, soy bean meal 1.0%, Ebios 0.4%, meat extract 0.2%, NaCl 0.2%, KCl 0.2%, and $CaCO_3$ 0.02% (pH 7.2). After 36 hours of cultivation, a fluorinated precursor such as *ortho*-, *meta*-, *para*- or 3′,4′-difluorobenzoic acid was added to the cultured broth at a concentration of 100 μg/ml and the cultivation was continued for a further 120 hours.

Fig. 2. ^{19}F NMR spectrum of the cultured broth supplemented with *meta*-fluorobenzoic acid.

The ^{19}F chemical shifts are expressed in ppm relative to external potassium fluoride. The peak at −113.2 ppm is due to an unknown metabolite unrelated to vulgamycin.



The fluorinated metabolites in the fermenta-

Table 1. ^{19}F NMR spectral data.

	δ_F
ortho-Fluorobenzoic acid	−111.4
ortho-Fluorovulgamycin	−112.4
meta-Fluorobenzoic acid	−114.2
meta-Fluorovulgamycin	−113.7
para-Fluorobenzoic acid	−107.7
para-Fluorovulgamycin	−106.7
3′,4′-Difluorobenzoic acid	−134.5, −140.8
3′,4′-Difluorovulgamycin	−133.8, −140.0

Spectra were taken in MeOH.

Fig. 3. Flow diagram of the isolation procedures of fluorovulgamycin.

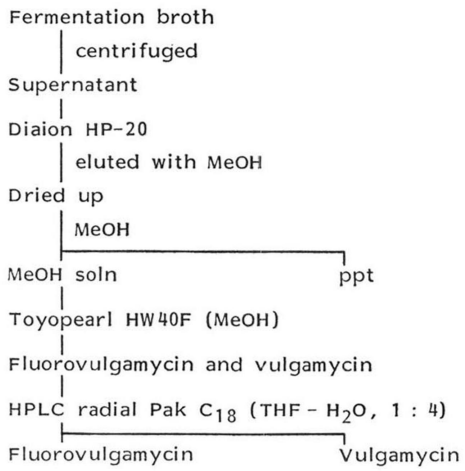


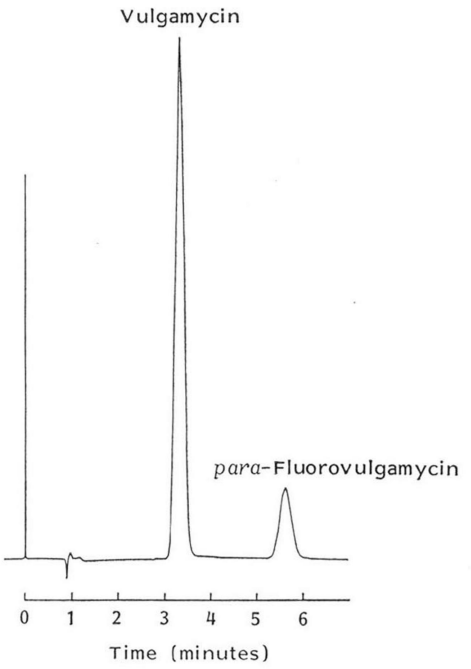
Table 2. Retention times of vulgamycin and fluorovulgamycins.

Antibiotic	Retention time (minutes)
Vulgamycin	3.3
ortho-Fluorovulgamycin	2.9
meta-Fluorovulgamycin	5.7
para-Fluorovulgamycin	5.6
3′,4′-Difluorovulgamycin	7.1

HPLC conditions are shown in Fig. 4.

Fig. 4. HPLC of vulgamycin and *para*-fluorovulgamycin.

Column conditions: column, Radial Pak C_{18}; solvent, THF - H_2O (1: 4); flow rate, 3 ml/minute; detector, UV_{254}.



tion broth could be easily recognized by the use of ^{19}F NMR spectroscopy. For example, as shown in Fig. 2, two signals due to the precursor and fluorinated vulgamycin were observed in the ^{19}F NMR spectrum of the cultured broth supplemented with *meta*-fluorobenzoic acid as a precursor. By judging from the peak height of the signals, it was suggested that about 70% of the added precursor was metabolized. The ^{19}F NMR spectral data of fluorovulgamycins and fluorobenzoic acids are given in Table 1.

In addition to these two resonances, another ^{19}F-signal due to an unknown metabolite is observed in Fig. 2. Similar phenomena were observed when *ortho*-, *para*- or 3′,4′-difluorobenzoic acid

Table 3. Physico-chemical properties of vulgamycin and fluorovulgamycins.

	VM	OFVM	MFVM	PFVM	3′,4′-DiFVM
Nature	White crystals	White crystals	White crystals	White crystals	White powder
MP (°C)	162.5~165.0	187.0~189.0	156.0~158.5	157.0~159.5	167.5~170.0
Molecular formula	$C_{22}H_{20}O_{10}$	$C_{22}H_{19}O_{10}F$	$C_{22}H_{19}O_{10}F$	$C_{22}H_{19}O_{10}F$	$C_{22}H_{18}O_{10}F_2$
EI-MS, *m/z*	444	462	462	462	480
λ_{max}^{MeOH} nm (ε)	249 (11,200), 283 (7,300)	242 (10,900), 284 (8,600)	243 (11,000), 283 (8,300)	252 (11,900), 280 (8,400)	247 (9,800), 283 (7,500)

Abbreviations: VM vulgamycin, OFVM *ortho*-fluorovulgamycin, MFVM *meta*-fluorovulgamycin, PFVM *para*-fluorovulgamycin, 3′,4′-DiFVM 3′,4′-difluorovulgamycin.

was employed as a precursor. Isolation of these compounds is under investigation.

The purification of fluorovulgamycins was accomplished by monitoring ^{19}F NMR signals throughout this work.

Isolation

The isolation procedure is depicted in Fig. 3. The filtered broth (1 liter) was adsorbed on a Diaion HP-20 column and eluted with 100% methanol. The eluate was evaporated to dryness under reduced pressure and the residue was dissolved in methanol. After removal of insoluble materials by centrifugation, the supernatant was concentrated and applied to a Toyopearl HW40F column which was developed with methanol. The unchanged precursors were removed by this procedure. Combined fractions containing vulgamycin and fluorovulgamycin were concentrated *in vacuo* to give a solid residue which was subjected to HPLC to separate the fluorinated vulgamycin from vulgamycin. A typical HPLC elution pattern and retention times are shown in Fig. 4 and Table 2, respectively. Thus, fluorovulgamycins, *ortho*-fluorovulgamycin (OFVM, 6 mg), *meta*-fluorovulgamycin (MFVM, 8 mg), *para*-fluorovulgamycin (PFVM, 8 mg), and 3′,4′-difluorovulgamycin (3′,4′-DiFVM, 9 mg), were isolated in pure forms.

Physico-chemical Properties

As shown in Table 3, fluorovulgamycins were obtained as white crystals or powder. They showed the same Rf values (0.56, silica gel TLC, chloroform - acetone (1 : 1)), and could not be distinguished from vulgamycin. The UV spectral data are also summarized in Table 3.

Structure Determination

The structures of these four fluorovulgamycins were determined based on ^{1}H and ^{13}C NMR spectral analysis. The 400 MHz ^{1}H NMR spectrum of each fluorovulgamycin showed in common the presence of two *meta*-coupled protons due to the pyrone ring (H-11, H-13), a methylene (H-7), a methoxy, four methines (H-3, H-5, H-6, H-9) and di- or tri- substituted phenyl protons. The assignments of these signals are summarized in Table 4. The ^{1}H NMR spectra of these fluorovulgamycins are in good agreement with that of vulgamycin except for the aromatic region.

When a fluorine atom was introduced into the benzoyl moiety, *ortho* and *para* protons showed upfield shifts of about 0.2~0.3 ppm but *meta* protons were little affected[7]. In addition, H-F spin coupling was observed, with a magnitude of 8~12 Hz for *ortho* protons and 6~10 Hz for *meta* protons[1]. The chemical shifts and splitting patterns of these fluorovulgamycins were almost identical with those of the corresponding fluorobenzoic acids. Based on these spectral data, the structures of fluorovulgamycins have been determined as shown in Fig. 1.

The 100 MHz ^{13}C NMR spectra of fluorovulgamycins (Table 5) also revealed a pyrone ring (C-10 to C-14), a methoxy carbon and a benzoyl function. In the aromatic carbon region, changes of the chemical shifts were observed by the introduction of fluorine. The aromatic carbon directly attached to fluorine showed a downfield shift of 30~35 ppm[8] and it was coupled with the halogen atom with a coupling constant of *ca.* 250 Hz[8]. The vicinal carbon exhibited an upfield shift of *ca.* 14 ppm, with the coupling constant being 10~20 Hz, while the carbons *meta* to fluorine showed a downfield shift of about 0~3 ppm, with the coupling constant being 10 Hz. These ^{13}C NMR data, shown in Table 5,

Table 4. ^{1}H NMR spectra of vulgamycin and fluorovulgamycins.

	VM	OFVM	MFVM	PFVM	3′,4′-DiFVM
H-3	4.68 (1H, s)	4.67 (1H, s)	4.62 (1H, s)	4.64 (1H, s)	4.57 (1H, s)
H-5	4.64 (1H, d, 4.4)	4.61 (1H, d, 4.4)	4.64 (1H, d, 4.4)	4.65 (1H, d, 4.4)	4.64 (1H, d, 4.5)
H-6	4.79 (1H, dt, 4.4, 2.9)	4.73 (1H, m)	4.89 (1H, dt, 4.4, 2.9)	4.79 (1H, dt, 4.4, 2.9)	4.78 (1H, dt, 4.5, 3.0)
H-7	1.86 (1H, dt, 14.6, 2.9)	1.82 (1H, dt, 14.7, 2.9)	1.86 (1H, dt, 14.6, 2.9)	1.87 (1H, dt, 14.6, 2.9)	1.85 (1H, dt, 15.0, 3.0)
	2.63 (1H, dd, 14.6, 2.9)	2.60 (1H, dd, 14.7, 2.9)	2.62 (1H, dd, 14.6, 2.9)	2.62 (1H, dd, 14.6, 2.9)	2.62 (1H, dd, 15.0, 3.0)
H-9	4.72 (1H, d, 2.9)	4.75 (1H, d, 2.9)	4.68 (1H, d, 2.9)	4.68 (1H, d, 2.9)	4.64 (1H, d, 3.0)
H-11	6.39 (1H, d, 2.0)	6.38 (1H, d, 2.4)	6.38 (1H, d, 2.4)	6.39 (1H, d, 2.4)	6.38 (1H, d, 2.2)
H-13	5.64 (1H, d, 2.0)	5.63 (1H, d, 2.4)	5.64 (1H, d, 2.4)	5.65 (1H, d, 2.4)	5.64 (1H, d, 2.2)
OCH_3	3.88 (3H, s)	3.89 (3H, s)	3.89 (3H, s)	3.89 (3H, s)	3.89 (3H, s)
H-2′	7.92 (1H, dd, 8.3, 1.5)		7.62 (1H, dt, 9.8, 2.0)	8.00 (1H, dd, 10.0, 7.4)	7.82 (1H, ddd, 11.0, 9.5, 2.8)
H-3′	7.46 (1H, dd, 8.3, 7.3)	7.17 (1H, ddd, 12.0, 8.5, 2.0)		7.20 (1H, t, 10.0)	
H-4′	7.57 (1H, dt, 7.3, 1.2)	7.57 (1H, m)	7.33 (1H, dt, 8.0, 2.0)		
H-5′	7.46 (1H, dd, 8.3, 7.3)	7.25 (1H, dt, 9.0, 2.0)	7.50 (1H, dt, 8.0, 5.9)	7.20 (1H, t, 10.0)	7.38 (1H, dt, 11.0, 9.5)
H-6′	7.92 (1H, dd, 8.3, 1.5)	7.79 (1H, dt, 9.0, 3.0)	7.74 (1H, dd, 8.0, 2.0)	8.00 (1H, dd, 10.0, 7.4)	7.77 (1H, m)

For abbreviations, see Table 3.

Spectra were taken in CD_3OD at 400 MHz with TMS as internal standard. Values in parentheses represent coupling constants in Hz.

Table 5. ^{13}C NMR spectra of vulgamycin and fluorovulgamycins.

	VM	OFVM	MFVM	PFVM	3′,4′-DiFVM
C-1	175.6	173.3	175.5	175.5	174.8
C-2	80.7	80.1	80.7	80.7	80.5
C-3	54.6	55.0	54.9	54.6	54.6
C-4	77.5	76.6	77.5	77.5	77.2
C-5	71.2	69.3	71.1	71.1	71.0
C-6	77.4	75.6	77.4	77.4	77.2
C-7	36.6	35.9	36.6	36.6	36.6
C-8	79.9	78.7	79.9	79.9	79.7
C-9	56.6	56.6	56.9	56.5	56.4
C-10	162.3	161.0	162.1	162.2	161.5
C-11	107.2	104.9	107.2	107.2	106.9
C-12	173.5	170.6	173.5	173.5	172.8
C-13	89.0	87.9	89.0	88.9	88.8
C-14	167.0	163.6	167.1	167.1	166.5
OCH_3	56.9	56.8	57.0	56.9	56.9
Benzoyl carbonyl	197.8	193.1	196.4	196.1	194.4
C-1′	140.8	128.8 (11)	142.9 (9)	137.4	137.7
C-2′	129.6	160.1 (253)	115.8 (23)	132.4 (12)	118.1 (br)
C-3′	129.5	116.7 (21)	164.0 (247)	116.3 (24)	150.8 (250)
C-4′	134.1	134.5 (9)	120.8 (24)	167.1 (252)	154.2 (255)
C-5′	129.6	124.4	131.4 (9)	116.3 (24)	118.1 (br)
C-6′	129.5	130.1	125.5	132.4 (12)	126.7

For abbreviations, see Table 3.

Expressed in ppm from internal TMS. Values in parentheses are coupling in Hz with fluorine. Spectra were taken in CD_3OD at 100 MHz except for *ortho*-fluorovulgamycin which was dissolved in $(CD_3)_2SO$.

Table 6. Antimicrobial activities of vulgamycin and fluorovulgamycins (MIC, μg/ml).

Organisms	VM	OFVM	MFVM	PFVM	3′,4′-DiFVM
Staphylococcus aureus IFO 12732	>100	>100	>100	>100	>100
Bacillus subtilis IFO 3134	>100	>100	>100	>100	>100
Micrococcus luteus ATCC 9341	12.5~50	100	50~100	6.25~12.5	25~50
Pseudomonas aeruginosa IFO 12582	>100	>100	>100	>100	>100
Salmonella typhimurium IID 971	>100	>100	>100	>100	>100
Escherichia coli IFO 12734	>100	>100	>100	>100	>100
Saccharomyces cerevisiae ATCC 9763	>100	>100	>100	>100	>100
Candida albicans No. Yu 1200	>100	>100	>100	>100	>100
Penicillium chrysogenum ATCC 10002	>100	>100	>100	>100	>100
Trichophyton mentagrophytes	>100	>100	>100	>100	>100

For abbreviations, see Table 3.

corroborated the structures of the fluorovulgamycins.

Antimicrobial Activities

Antimicrobial activities of fluorovulgamycins and vulgamycin are shown in Table 6. They were not effective against the test organisms except for *Micrococcus luteus* ATCC 9341. *para*-Fluorovulgamycin showed stronger activity than vulgamycin against this organism. On the whole, the antimicrobial activities of vulgamycin were little affected by the introduction of fluorine atoms into the

benzoyl moiety. Therefore, it may be assumed that this aromatic part is distant from the active site of vulgamycin. As described herein, the technique using ^{19}F NMR as a detection method for incorporation of fluorine is useful in preparing fluorinated substances and its applications to other antibiotics are now in progress.

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