PREPARATION OF FLUORINATED ANTIBIOTICS FOLLOWED BY ¹⁹F NMR SPECTROSCOPY

II. FLUORINATED ACTINOMYCINS

Sir:

In the previous paper[1], we reported the preparation of fluorovulgamycins obtained by supplementation of fluorinated precursors to the fermentation broth of a vulgamycin-producing organism. As a result, it turned out that *para*-fluorovulgamycin exhibited higher activity against *Micrococcus luteus* than the mother compound, vulgamycin. During these experiments we established the methodology to prepare fluorinated antibiotics by the combined use of biosynthetic pathway and ¹⁹F NMR spectroscopy. As a next step, we have tried to introduce the fluorine atom into the sites of antibiotics which are believed to play an important role for expressing the biological activities.

Actinomycin known to exhibit antitumor activities against P1514 leukemia and DBRB mammary adenocarcinoma[2] consists of two peptide chains and the phenoxazine ring called actinocin[3]. The antibiotic shows its antitumor activities by binding with double stranded DNA at the actinocin moiety to inhibit DNA-dependent RNA polymerase[4,5]. Thus, it is very interesting to replace hydrogen in actinocin with fluorine; this modification would result in the change of the electric distribution pattern in actinocin and therefore affect the biological activities of actinomycins. The actinocin moiety of actinomycin has been proved by radioactive isotope experiments[6,7] to be biosynthesized from the phenyl ring of tryptophan with the 5-position of tryptophan corresponding to the 7-position of actinocin. With these considerations in mind we investigated the fluorination of actinocin by feeding 5-fluorotryptophan to the fermentation broth of *Streptomyces parvullus* KCC S-0601 which produced actinomycin D.

The strain was cultured in 500-ml Erlenmeyer flasks each containing 100 ml of a medium consists of starch 2.5%, soybean meal 1.5%, dry yeast 0.2% and CaCO₃ 0.4% (pH 6.2 before autoclaving). After 48 hours of cultivation, 5-fluorotryptophan was added to the medium at a concentration of 300 μg/ml and the cultivation was conducted for 4 days at 27°C on a rotary shaker. The active substance in the fermenta-

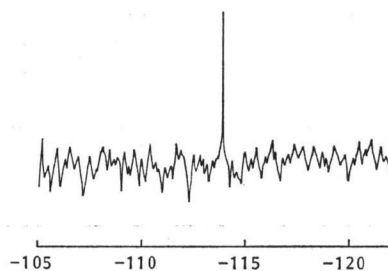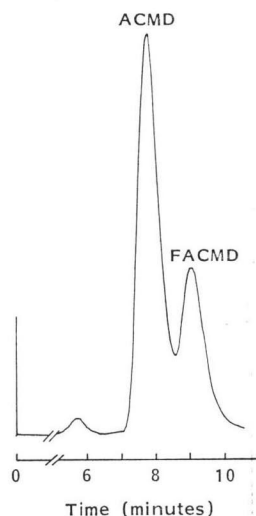Fig. 1. ¹⁹F NMR spectrum of the EtOAc extract of the cultured broth supplemented with 5-fluorotryptophan.

Fig. 2. Separation of fluoroactinomycin D and actinomycin D by HPLC.

Column condition: Column, Radial Pak C₁₈; solvent, MeCN - H₂O, 1:4; flow rate, 3 ml/minute; detector, UV₂₅₄.

tion broth (1 liter) was extracted with EtOAc. A fluorine signal (δ_F −114.0) derived from the metabolite of the precursor was observed in ¹⁹F NMR spectrum of the EtOAc extract (Fig. 1). Fluoroactinomycin D was purified by monitoring this fluorine signal. The organic layer was dried under reduced pressure to give a red powder. This material was dissolved in MeOH and applied to a Toyopearl HW40F column which was developed with MeOH. The appearance of the fluorine signal in the fractions containing actinomycin D suggested the presence of fluoroactinomycin D. The combined fraction containing actinomycin D (ACMD) and fluoroactinomycin D (FACMD) was concen-

Table 1. Physico-chemical properties of fluoroactinomycin D and fluoroactinomycin X_2.

	FACMD*	ACMD	FACMX$_2$	ACMX$_2$
MP (°C)	227.0~229.0	245.0~247.0	239.0~241.5	248.0~253.0
UV λ_{max}^{MeOH} nm (ε)	240 (29,500),	238 (27,200),	240 (23,700),	236 (26,800),
	425 (15,800),	425 (18,800),	426 (14,600),	426 (18,000),
	443 (16,400)	443 (20,000)	445 (15,300)	444 (19,900)
FD-MS m/z	1,273 (M+H)	1,255 (M+H)	1,287 (M+H)	1,269 (M+H)
Molecular formula	$C_{62}H_{85}N_{12}O_{16}F$	$C_{62}H_{86}N_{12}O_{16}$	$C_{62}H_{83}N_{12}O_{17}F$	$C_{62}H_{84}N_{12}O_{17}$
Rf value	0.58	0.58	0.67	0.67
(CHCl$_3$ - MeOH, 9:1)				

* Abbreviations: FACMD=Fluoroactinomycin D, ACMD=actinomycin D, FACMX$_2$=fluoroactino-mycin X$_2$, ACMX$_2$=actinomycin X$_2$.

Fig. 3. The ^{1}H NMR spectrum of fluoroactinomycin D.
Inserts show the relevant portions of the ^{1}H NMR spectrum of ACMD.
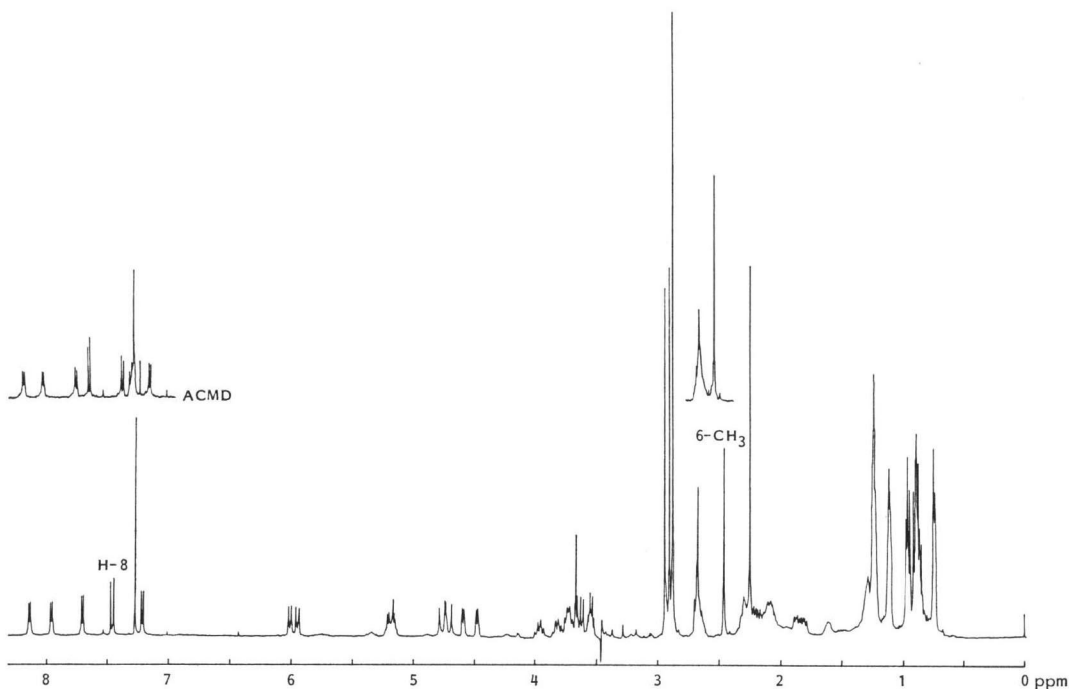
trated *in vacuo* to give a red powder which was then subjected to HPLC to separate FACMD from ACMD. A typical HPLC elution pattern is shown in Fig. 2. FACMD was isolated as red crystals (8 mg).

In a similar way, fluoroactinomycin X$_2$ (FACMX$_2$) was obtained by cultivation of an actinomycin X$_2$ (ACMX$_2$) producing strain, *Streptomyces* sp. H-63 isolated in our laboratory, followed by the purification procedures as described above.

Physico-chemical properties of FACMD and

FACMX$_2$ are compared with those of ACMD and ACMX$_2$ (Table 1). Rf values of fluorinated actinomycins were identical with those of the corresponding nonfluorinated actinomycins.

The structure determination of FACMD was performed by the comparison of the ^{1}H NMR and ^{13}C NMR spectra between FACMD and ACMD. Although the signals derived from the peptide chains of FACMD were almost identical with those of ACMD (data not shown), the changes of chemical shifts and splitting patterns in the actinocin moiety were brought about by

Table 2. ^{13}C NMR assignments of the actinocin moiety in fluoroactinomycin D.

Carbon	δ_C (J_{C-F} Hz)	$\Delta\delta_C$*
C5a	141.2 (br)	+1.0
C6	114.7 (20)	−12.6
C7	159.9 (247)	+34.2
C8	113.5 (26)	−16.5
C9	133.0 (br)	+4.1
C9a	125.7	−6.8
6-CH₃	7.8	−7.5

* Changes of the chemical shift values between fluoroactinomycin D and actinomycin D.

Fig. 4. Structures of fluoroactinomycin D and fluoroactinomycin X₂.
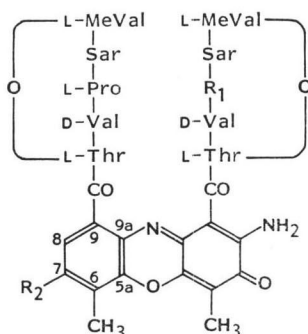
	R_1	R_2
ACMD	L-Pro	H
FACMD	L-Pro	F
ACMX₂	L-oxoPro	H
FACMX₂	L-oxoPro	F

Table 3. Antitumor activities of FACMD, FACMX₂, ACMD and ACMX₂.

Drug	Dose (mg/kg/day)	T/C (%) of MST*** P388 leukemia	T/C (%) of MST*** B16 melanoma
FACMD	0.05	NT	158
	0.1	203	182
	0.2	221	172
	0.4	236*	NT
ACMD	0.05	NT	184
	0.1	239	188
	0.2	218*	39
	0.4	58**	NT
FACMX₂	0.00625	154	172
	0.0125	162	199
	0.025	165	51
ACMX₂	0.00625	152	165
	0.0125	155	184
	0.025	172	181

NT: Not tested.

For abbreviations of the drugs, see Table 1.

P388 leukemia cells (1.0×10^6) were implanted intraperitoneally on day 0 into CDF₁ mice. B16 melanoma cells (10% homogenate: 0.5 ml) were given into BDF₁ mice in the same manner.

*,** The antibiotics were administered intraperitoneally on day 1~5, day 1~4(*) or day 1~3(**).

*** Ratio of median survival time of test and control mice.

the introduction of fluorine (Fig. 3). When a fluorine atom was introduced into the 7-position of actinocin of ACMD, the H-8 *ortho*-proton showed an upfield shift by 0.17 ppm with a coupling constant of 10 Hz in the 400 MHz ¹H NMR spectrum (Fig. 3). Furthermore, the methyl signal (6-CH₃) showed an upfield shift by 0.09 ppm and coupling with the fluorine atom (1 Hz).

In the 100 MHz ¹³C NMR spectra of FACMD, the carbon directly attached to the fluorine showed a downfield shift by 34.2 ppm (Table 2) and coupling with the fluorine atom (J_{C-F} = 247 Hz). The neighboring carbons (C6 and C8) exhibited an upfield shift of 12.6~16.5 ppm and the magnitude of C-F coupling was in the range of 20~26 Hz. C5a and C9 were shifted downfield by 1.0~4.1 ppm and C9a and 6-CH₃

exhibited an upfield shift of 6.8~7.5 ppm. These NMR spectral changes agree well with the literature values[8,9]. From all the results described above, the structure of FACMD was determined to be depicted in Fig. 4.

Analogous NMR spectral changes observed between FACMX₂ and ACMX₂ can be reasonably explained by the introduction of a fluorine atom into the 7-position of the actinocin moiety in ACMX₂. Since no change of the NMR spectral data was observed except for the actinocin moiety (data not shown), the structure of FACMX₂ was elucidated as shown in Fig. 4.

Antitumor activities of these fluoroactinomycins against murine P388 leukemia and B16 melanoma were compared with those of actinomycins (Table 3). FACMX₂ and ACMX₂ were active against both tumors at the same degree but the former exhibited toxicity at the highest dose (25 μg/kg) against B16 melanoma. Although FACMD and ACMD showed nearly equal antitumor activities on tested tumors at

$0.05 \sim 0.1$ mg/kg, FACMD prolonged the survival time as compared with ACMD at higher doses.

The LD_{50} (mg/kg) values of actinomycins and their fluorinated derivatives were as follows (male ICR mice, ip); FACMD 0.66 mg/kg, ACMD 1.74 mg/kg, $FACMX_2$ 0.14 mg/kg, $ACMX_2$ 0.30 mg/kg. From these results it became apparent that fluorination of ACMD and $ACMX_2$ increased the acute toxicities.

FACMD gave prolongation of the life span (Table 3) in spite of the increased acute toxicity. These very interesting results indicated the different responses between successive injections of the drug into the tumor bearing mice and a single administration into normal mice. For this reason it is suggested that the introduction of a fluorine atom into ACMD results in the change of dose-response curve and/or pharmacokinetics of the drug. The *in vivo* behavior of FACMD will be elucidated in detail by further experiments.

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