PYRROLOMYCINS F_1, F_{2a}, F_{2b} AND F_3, NEW METABOLITES PRODUCED BY THE ADDITION OF BROMIDE TO THE FERMENTATION

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New antibiotics, pyrrolomycins F_1, F_{2a}, F_{2b} and F_3 were produced by *Actinosporangium vitaminophilum* sp. nov. when bromide ion was added to the fermentation medium. Addition of other halide ions such as chloride, iodide and fluoride ion showed no effect on pyrrolomycin production, affording polychlorinated pyrrolomycins A, B, C, D and E, but no F components. All four new antibiotics contain 2~4 mol of bromine, and their substitutional position was determined by X-ray analysis and synthesis, supported by spectroscopic analysis. They are strongly active against Gram-positive bacteria and fungi.

Pyrrolomycins (PM) A, B, C, D and E[1~5] are polychlorinated pyrrole antibiotics produced by *Actinosporangium vitaminophilum* sp. nov. Replacement of chlorine with other halogens in chlorine-containing antibiotics has been successfully achieved in cases of chlorotetracycline[6] and pyrrolnitrin[7]. Accordingly, an attempt was made to modify the biosynthetic pathway of PM by the addition of halide ion other than chloride. Among fluoride, bromide and iodide examined, only bromide was effective, and new members of the PM group that contained bromine in the molecule were obtained.

This paper describes the fermentation, isolation, physico-chemical properties, structural elucidation and biological properties of new bromine-containing PM-F_1, F_{2a}, F_{2b} and F_3. This also includes the synthesis of PM F_1.

Effect of Halide Ions on the Production of PM Components

PM antibiotics produced in the fermentation broth were analyzed by means of HPLC. Fig. 1A illustrates a chromatographic pattern of the cultured broth of *Actinosporangium vitaminophilum* sp. nov. without addition of halide ions. PM-A, E, B, D and C are eluted in that order with C predominating. When 0.1% of NaBr was added in the culture medium, none of these PMs were detected in HPLC. Instead, peaks due to PM-F_1, F_{2a}, F_{2b} and F_3 appeared (Fig. 1B). The relative ratio of each PM-F components depended on the amount of NaBr added. As the amount of NaBr was increased from 0.05% to 0.1% and 0.2%, the relative ratio of PM-F_1, substituted with four bromine atoms, was markedly increased, while PM-F_3, substituted with two atoms of bromine and two atoms of chlorine was decreased. PM-F_{2a} and PM-F_{2b} which contained three atoms of bromine and one atom of chlorine were less sensitive to the added NaBr. In contrast, when other halogen ions (Cl, I or F) were added, the HPLC pattern was little changed, and was similar to that of the control fermentation (Fig. 1A).

Fig. 1. Effect of NaBr on the production of PM-Fs.

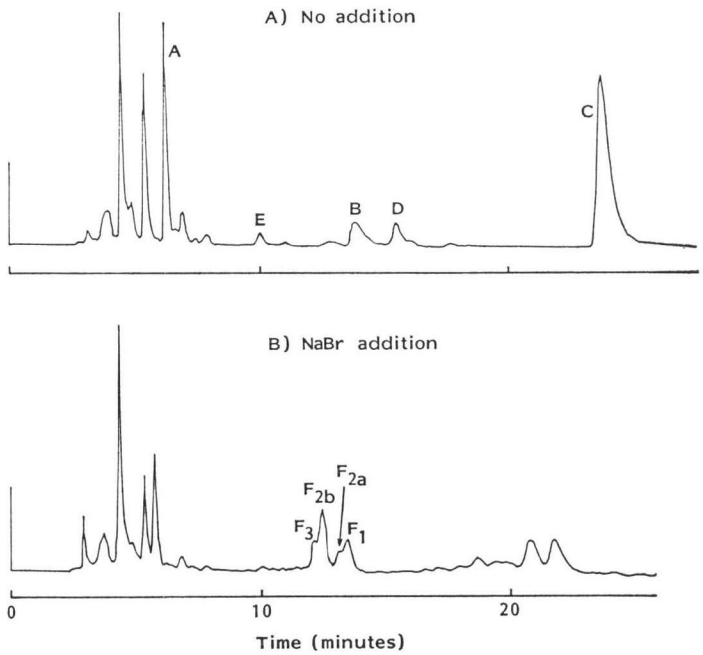


Table 1. Physico-chemical properties of PM-F components.

	PM-F_1	PM-F_{2a}	PM-F_{2b}	PM-F_3
Appearance	Yellow needles	Yellow needles	Yellow needles	Yellow needles
mp (°C)	188~190	182	206~208	192~193
Mol. wt. (MS)	499	455	455	411
Mol. formula	$C_{11}H_5NO_2Br_4$	$C_{11}H_5NO_2Br_3Cl$	$C_{11}H_5NO_2Br_3Cl$	$C_{11}H_5NO_2Br_2Cl_2$
Rf on TLC[1]	0.30	0.30	0.30	0.30
Rt on HPLC[2] (minutes)	13.9	13.7	12.8	12.4
UV(λ_{max}^{MeOH} nm)	268, 316	267, 315	267, 315	268, 314
IR (KBr, cm^{-1})	3250, 1620	3270, 1620	3270, 1620	3270, 1620
^1H NMR(Me_2CO-d_6, δ, ppm)	6.91 (d), 7.57 (dd), 7.73 (d)	6.93 (d), 7.54 (dd), 7.78 (d)	6.93 (d), 7.53 (dd), 7.66 (d)	6.98 (d), 7.61 (dd), 7.73 (d)

1) SiO_2; hexane - EtOAc - AcOH (100: 20: 1)
2) TSK-GEL LS-410

Physico-chemical Properties of Pyrrolomycin F Components

Table 1 summarizes physico-chemical properties of PM-F_1, F_{2a}, F_{2b} and F_3, isolated by the procedures described in the Experimental section. All of PM-F components are yellow needles and have the same molecular formulae namely $C_{11}H_5NO_2X_4$(X expresses halogen), as well as the same Rf values on TLC. All of them showed similar solubility and UV spectra. IR absorption bands due to NH of pyrrole ring and hydrogen bonded carbonyl group were observed. High halogen contents of these antibiotics amounting to 56~64% is a distinctive feature.

Structural Elucidation

IR and mass spectra as well as molecular formulae of PM-F_1, F_{2a}, F_{2b} and F_3 closely resembled those of PM-C or D. Therefore, the structure of the four compounds were supposed to resemble PM-C or D,

Chart 1. Chemical structures of PMs.

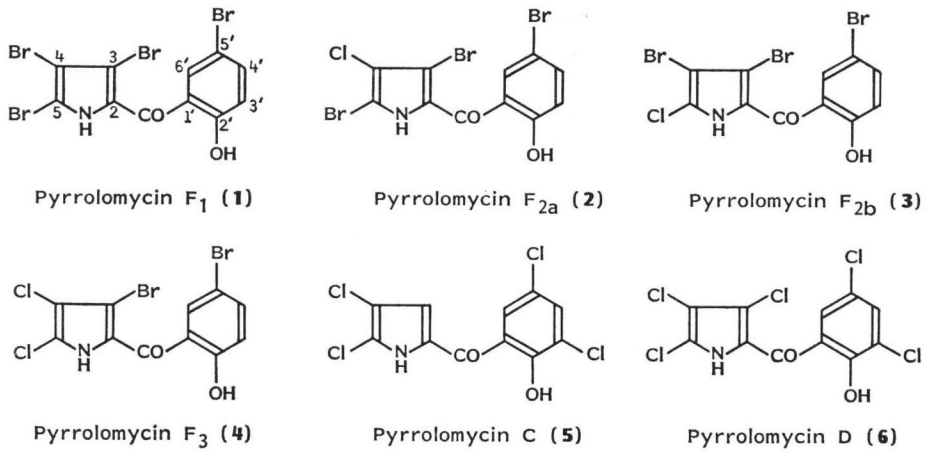
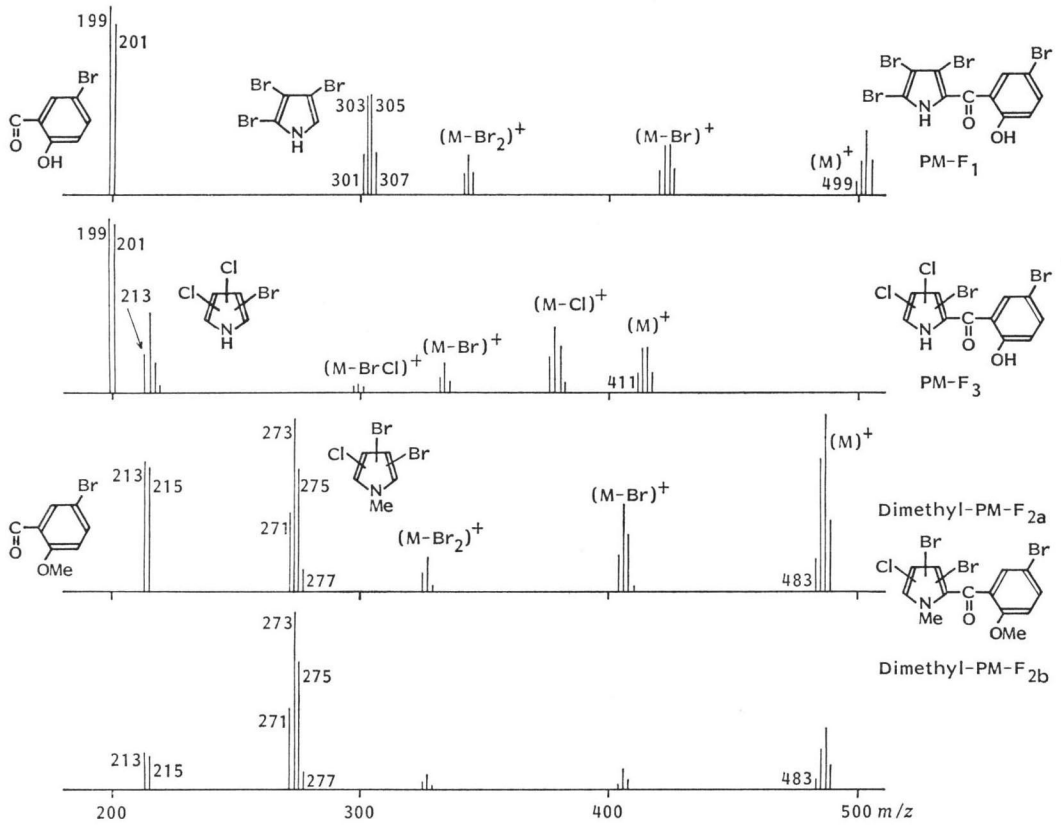
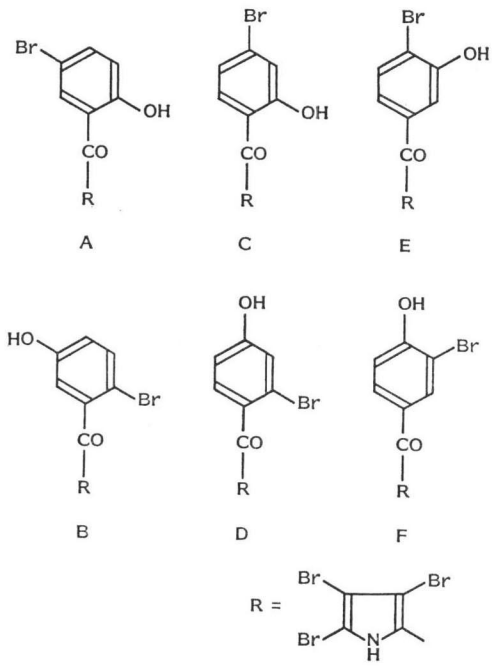


Fig. 2. Mass spectra of PM-Fs.



which were determined as **5** and **6** in Chart 1, respectively. Accordingly, structural elucidation of PM-F components were conducted by the following procedure, being compared with PM-C or D. The four halogens are bromine only in PM-F_1, while in the other components, they are a mixture of bromine and chlorine. Therefore, structural investigation was carried out first on PM-F_1, in which no differentia-

Chart 2. Possible structures for PM-F_1.



tion of bromine and chlorine was required. The mass spectrum of PM-F_1 is shown in Fig. 2. Fragment ion peaks observed at m/z 199 and 201 were assigned to the benzoyl moiety substituted with monobromo and monohydroxyl groups, and ions at m/z 301, 303, 305 and 307 to the pyrrole moiety substituted with three bromine atoms. This was confirmed by ^{1}H NMR. According to ^{1}H NMR, three signals observed at δ 6.91 (d, J=8.8 Hz), 7.57 (dd, J=8.8 and 2.5 Hz) and 7.73 ppm (d, J =2.5 Hz) suggested 1, 2, 4 trisubstituted benzene ring linked by a carbonyl-carbon. The substituted position of a benzoyl group on the pyrrole ring had probably an α position, because of the spectral similarity to PM-C or D with an α substitution. Among six possible structures for PM-F_1 in Chart 2, structure A was confirmed to be the structure of PM-F_1 which was synthesized from pyrrole following the synthesis of PM-C and D[5] shown in Chart 3.

The mass and ^{1}H NMR spectra shown in Fig. 2 and Table 1 indicated that the benzoyl moiety of PM-F_{2a}, F_{2b} and F_3 were identical with PM-F_1. Therefore, the difference of the three components must be in the position of the halogen ions on the pyrrole ring. In PM-F_1, the pyrrole ring was substituted with three bromine atoms, but in PM-F_{2a} and F_{2b} it was substituted with two bromine and one chlorine atoms and in PM-F_3 with one bromine and two chlorine atoms. The difference between PM-F_{2a} and F_{2b} is the position of the halogen atoms in the pyrrole ring. The structural elucidation of these three antibiotics by spectral data or synthetic method was found to be very difficult. Therefore, the

Chart 3. Synthesis of PM-F_1.

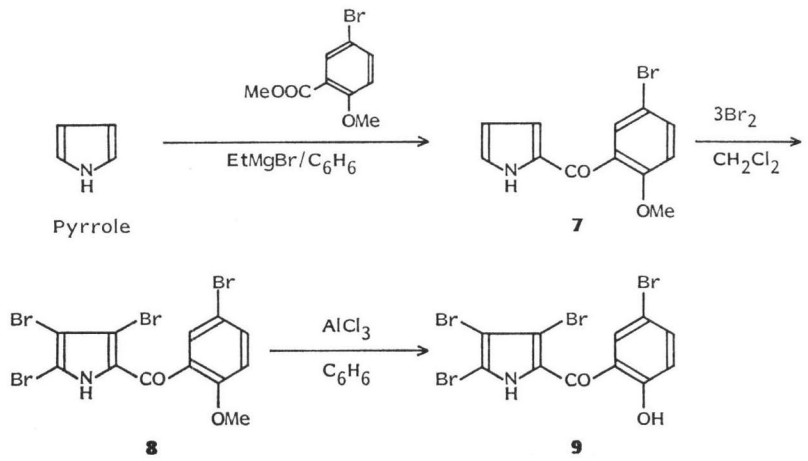
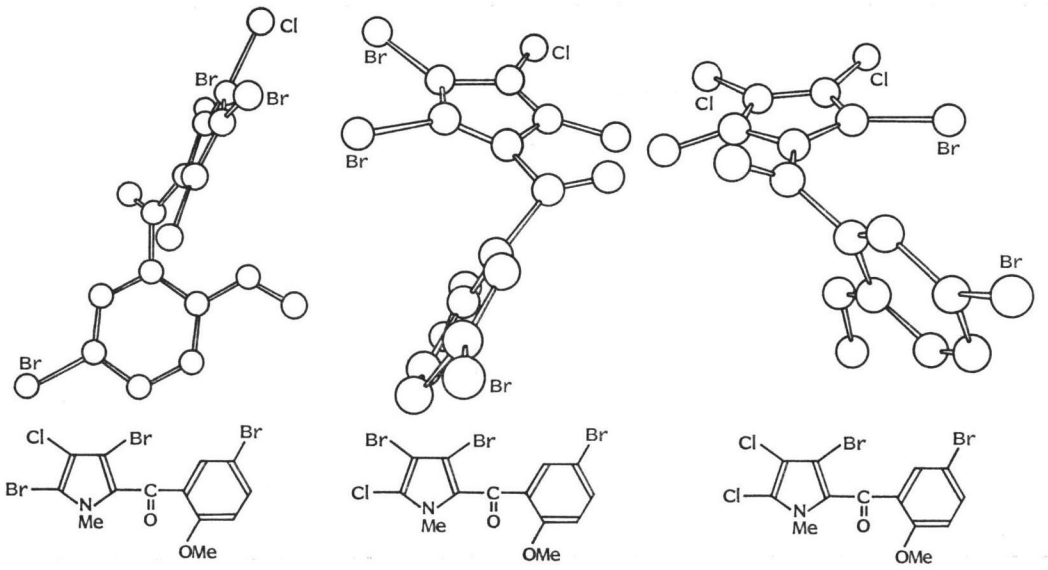


Fig. 3. X-Ray analysis of dimethylpyrrolomycins F_{2a}, F_{2b} and F_3.



Crystal system; Triclinic	Triclinic	Triclinic
Space group; $P\bar{1}$	$P\bar{1}$	$P\bar{1}$
Lattice constants		
a 8.527, *b* 11.676, *c* 8.395	*a* 8.267, *b* 12.098, *c* 8.260	*a* 12.008, *b* 8.242, *c* 8.209
α 75.2, β 110.0, γ 95.8	α 108.3, β 92.0, γ 103.3	α 91.8, β 108.2, γ 102.8
R=7%	R=10%	R=9%
Dimethyl-PM-F_{2a}	Dimethyl-PM-F_{2b}	Dimethyl-PM-F_3

structures of PM-F_{2a}, F_{2b} and F_3 were determined by X-ray crystallographic analysis as **2**, **3** and **4** in Chart 1, respectively. X-Ray analysis was performed using crystals of *N*, *O*-dimethyl derivatives of PM-F_{2a}, F_{2b} and F_3 prepared by treatment with diazomethane, because single crystals of PM-F components appropriate for X-ray analysis were not available. The perspective views and crystal data of three compounds are given in Fig. 3, in which the hydrogen atoms are not drawn. As shown in Table 2, the shift of ^{13}C-chemical shift going from PM-F_1 to F_{2a}, F_{2b} and F_3 was reasonably explained, indicating a rational assignment.

Table 2. ^{13}C Chemical shifts[a] of PM-F components.

Carbon	Chemical shift (multiplicity)			
	PM-F_1	PM-F_{2a}	PM-F_{2b}	PM-F_3
2	131.0(s)	130.8(s)	129.5(s)	128.0(s)
3	106.9(s)	103.9(s)	106.6(s)	104.5(s)
4	105.8(s)	116.9(s)	101.6(s)	114.1(s)
5	109.6(s)	107.7(s)	123.0(s)	120.3(s)
1′	124.8(s)	125.5(s)	126.4(s)	125.0(s)
2′	158.7(s)	158.7(s)	158.0(s)	158.5(s)
3′	120.0(d)	120.3(d)	120.0(d)	120.0(d)
4′	137.6(d)	137.3(d)	136.7(d)	137.6(d)
5′	111.4(s)	111.1(s)	111.0(s)	111.4(s)
6′	134.4(d)	134.5(d)	133.8(d)	134.3(d)
C=O	184.6(s)	184.2(s)	183.8(s)	184.5(s)

[a] Chemical shifts are reported in ppm down field from internal TMS.

Biological Properties

The *in vitro* activities of PM-F components against various bacteria and fungi are shown in Table 3. All PM-F components possess strong activities against Gram-positive bacteria and certain fungi, and moderate activities against Gram-negative bacteria. The LD_{50} value of PM-F_1 was approximately 50 mg/kg, tested by intraperitoneal injection to mice. It was found that the bioactivity of the bromine-

Table 3. Antimicrobial activities of PM-F components.

Test organisms	MIC (μg/ml)			
	PM-F_1	PM-F_{2a}	PM-F_{2b}	PM-F_3
Staphylococcus aureus 209P JC-1	<0.05	≤0.025	≤0.025	<0.05
Streptococcus faecalis ATCC 8043	<0.05	0.10	0.05	<0.05
Bacillus anthracis No. 119	<0.05	≤0.025	0.05	<0.05
Escherichia coli NIHJ JC-2	6.25	12.5	>50	6.25
Citrobacter freundii GN 346	6.25	12.5	>50	12.5
Salmonella typhi O-901-W	6.25	12.5	>50	12.5
Shigella sonnei EW 33 Type I	6.25	25	>50	12.5
Klebsiella pneumoniae PCI 602	6.25	12.5	>50	6.25
Proteus vulgaris OX-19	3.13	12.5	12.5	3.13
Serratia marcescens MB-3848	3.13	3.13	3.13	3.13
Pseudomonas aeruginosa MB-3829	12.5	100	>50	25
Candida albicans C-A-24	100	>100	>100	100
Cryptococcus neoformans Cr-1	0.78	0.78	3.12	0.78
Trichophyton mentagrophytes No. 1	0.78	0.78	1.56	0.78
Trichophyton interdigitale No. 2	0.78	0.78	12.5	3.13
Aspergillus fumigatus Saito	>100	>100	>100	>100

containing PMs were greatly enhanced as compared to the chlorine-containing PMs, while acute toxicity was not significantly different.

Discussion

Replacement of chlorine with bromine in the biosynthetic pathway of the chlorine-containing antibiotics has been achieved in cases of *Streptomyces aureofaciens* that produced chlorotetracycline[6] and *Pseudomonas* strains that produced pyrrolnitrin[7]. These organisms produced the respective bromine-containing antibiotics, bromotetracycline and bromonitrins, when the medium was supplemented with bromide ion. In the former case, halogen-exchange was the only reaction that occurred. In the latter, three bromonitrins were produced and one of them was halogen-exchanged pyrrolnitrin. It was noted, however, that all compounds obtained by the addition of bromine ion in *Actinosporangium vitaminophilum* sp., nov., were not only halogen-exchanged but also were changed in the substituted positions. Thus, the benzene ring was substituted with bromine only at C-5′ position, while it was substituted with two chlorine atoms at the C-3′ and C-5′ positions, as seen in PM-C and D. Further noteworthy was the competitive reaction of chlorine and bromine in the pyrrole moiety whereas bromination was predominant in the benzene ring. Therefore, it will be of interest to determine whether the enzyme systems involved in bromination is single or multiple.

A second feature of PM-F components is their strong antimicrobial activity. The activity of pyrrolnitrin was reduced to about one tenth when there was bromine exchange[7]. However, PM-F components possessed equal or more potent activity than the parent antibiotics even more than PM-D, which is the most active component among PM-A to E. Many bromine-containing metabolites were produced by marine bacteria, but most of them show weak activity. An exception is pentabromopseudilin[9], that is composed of tribromopyrrole and dibromobenzene and which exhibits strong activity against Gram-positive bacteria, but is inactive against *Candida*. PM-F components are that the first bromo-pyrrole antibiotics showing activity against fungi.

Experimental

General Methods

Melting points were determined on a Yamato MP-21 and uncorrected. IR spectra were recorded

on a Model 260–10 Hitachi infrared spectrophotometer. ^1H NMR spectra were obtained on a Varian T-60 NMR spectrometer. ^{13}C NMR spectra were recorded on a Jeol FX-200 NMR spectrometer. HPLC were made on a liquid chromatograph apparatus (Waters assosiates) under the following condition, column; TSK-GEL LS-410 (4×300 mm, Toyo Soda), eluent; CH_3CN - H_2O - 0.2 M CH_3COONH_4 (13: 7: 1) (pH 6.4 by Et_3N), flow rate; 0.7 ml/minute, detection; UV 313 nm. The lattice constants and intensity data on X-ray analysis were obtained on a Philips four-circle X-ray diffractometer using graphite-monochromated CuKα radiation. MIC values were determined by agar dilution method according to the standard methods recommended by the Japan Society of Chemotherapy.

Fermentation and Isolation of PM-F Components

Actinosporangium vitaminophilum sp. nov. was grown in 20 ml of a seed medium (Table 4) in a 100-ml flask at 28°C on a rotary shaker for 5 days. The seed culture (4 ml) was inoculated into 80 ml of the same medium in a 500-ml flask and cultured at 28°C for 2 days. The 2nd seed (50 ml) was transferred into 1 liter of the same medium in a 5-liter flask and cultured at 28°C for 2 days. One liter of the 3rd seed was inoculated into a 50-liter jar fermentor containing 35 liters of the production medium (Table 4). The fermentation was performed at 28°C for 5 days. Fermentation titer was determined by the disc diffusion assay using *Bacillus subtilis* ATCC 6633 as the test organism.

Table 4. Fermentation medium of PM-F components.

Seed medium		Production medium	
Glucose	2%	Maltose syrup	2%
Peptone	0.5	Soybean oil	0.15
Meat extract	0.2	Soybean meal	1
Yeast extract	0.3	Distiller's solubles	0.25
Soybean meal	0.2	Pharmamedia	0.5
$CaCO_3$	0.1	$FeSO_4 \cdot 7H_2O$	0.0005
NaBr	0.1	$NiCl_2 \cdot 6H_2O$	0.00005
		$CoCl_2 \cdot 6H_2O$	0.00005
		$CaCO_3$	0.1
		NaBr	0.1

The whole broth (25 liters) obtained was adjusted to pH 2 and extracted with EtOAc (20 liters). The solvent layer was washed with water and evaporated to a black oil. This was chromatographed on an alumina column (200 g) developing with EtOAc - conc. HCl (500: 7). The active fractions were concentrated, and an oily material (4.4 g) was chromatographed on a silica gel column (200 g), developing with benzene - EtOAc (10: 1). Evaporation of solvents furnished a yellow powder (0.98 g), a part of which (150 mg) was subjected to a column chromatography over Sephadex LH-20 (650 ml) developing with MeOH - water (3 : 2). Evaporation of active fractions gave 34 mg of PM-F_1, 62 mg of PM-F_2 mixture and 18 mg of PM-F_3. A solution of 1 mg of PM-F_2 mixture in MeOH was injected into the HPLC column packed with TSK-GEL LS-410. Chromatographic separation under the condition employed for analysis of broth, afforded PM-F_{2a} and F_{2b}. Twenty repetition of the process resulted in a yield of 6 mg of PM-F_{2a} and 7 mg of F_{2b}, respectively.

2-(5′-Bromo-2′-methoxybenzoyl)pyrrole (**7**)

To a solution of pyrrole (1.46 g, 0.02 mol) in anhydrous benzene (100 ml) 10 ml of EtMgBr (3 mol in ether) was added under cooling and the reaction mixture was stirred at 20°C for 30 minutes. To the solution, 6 ml of methyl 5-bromo-2-methoxybenzoate was added and the reaction mixture was left overnight at room temperature. The mixture was poured into 100 ml of ice water and acidified with 10 ml of conc. HCl. The organic layer was separated, dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in 50 ml of 80% methanol containing 4.0 g of sodium hydroxide and the solution was refluxed for 10 minutes. The solution was evaporated under reduced pressure and the residue was extracted with 100 ml of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated. After the recrystallization from hexane - ethyl acetate, 0.61 g of 2-(5′-bromo-2′-methoxybenzoyl)pyrrole (**7**) was obtained as colorless crystals (yield 11%), mp 86~87°C.

Anal. Calcd. for $C_{12}H_{10}NO_2Br$: C 51.45, H 3.60, N 5.00.
Found: C 51.25, H 3.45, N 4.89.

2-(5′-Bromo-2′-hydroxybenzoyl)-3,4,5-tribromopyrrole (**9**) (PM-F_1)

To a solution of **7** (280 mg, 0.001 mol) in anhydrous methylen chloride (10 ml) bromine (0.16 ml,

0.003 mol) was added in one portion and the reaction mixture was left for 1 hour at room temperature. The reaction mixture was evaporated under reduced pressure and the residue was dissolved in 50 ml of benzene. To the solution 500 mg of anhydrous aluminium chloride was added and the mixture was stirred at room temperature for 2 hours. The mixture was washed with dil.HCl and water, then dried over anhydrous sodium sulfate and evaporated under reduced pressure. Recrystallization of the residue from benzene - hexane gave 143 mg (28%) of pure 2-(5′-bromo-2′-hydroxybenzoyl)-3,4,5-tribromopyrrole (**9**) as the yellow needles. Compound **9** was identical with natural PM-F_1 in melting point (188~190°C) and other physico-chemical properties.

N,O-Dimethyl-PM-F_3

PM-F_3 (20 mg) was dissolved in 2 ml of acetone and to the solution excess diazomethane solution in ether was added. The reaction mixture was left for 2 hours and evaporation of solvents under reduced pressure gave 20 mg of *N,O*-dimethyl-PM-F_3. Recrystallization from methanol - hexane afforded pure *N,O*-dimethyl-PM-F_3, mp 142°C.

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