DIRECTED BIOSYNTHESIS OF 5″-FLUOROPACTAMYCIN IN *Streptomyces pactum*

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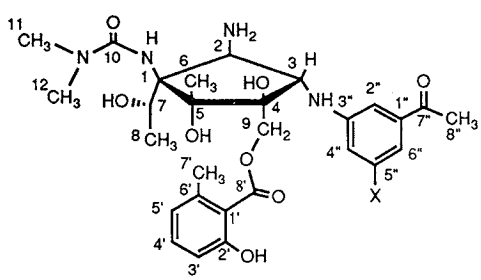
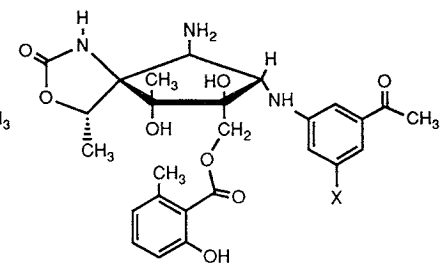
A new pactamycin analogue, 5″-fluoropactamycin, was prepared by directed biosynthesis. Supplementation of the fermentation medium of *Streptomyces pactum*, var. *pactum* with 3-amino-5-fluorobenzoic acid, an analogue of 3-aminobenzoic acid, an advanced precursor in pactamycin biosynthesis, resulted in co-production of pactamycin and the new pactamycin analogue. A similar feeding experiment with 3-amino-5-methylbenzoic acid did not result in formation of the corresponding methylated pactamycin analogue, but only in inhibition of pactamycin production. Comparison of antimicrobial and cytotoxic activities of pactamycin and 5″-fluoropactamycin showed no significant differences.

The *m*-aminoacetophenone unit of pactamycin (**1**), an antitumor antibiotic produced by *Streptomyces pactum*, var. *pactum*, represents one of the simplest examples of the "*m*-C_7N" unit found in a wide variety of antibiotics. Previous work in our laboratory demonstrated that this unit is derived from *m*-aminobenzoic acid (*m*-ABA), which is most likely formed *via* a variant on the shikimate pathway.[1] Knowledge of this advanced precursor provides the opportunity for modification of the *m*-C_7N unit of **1** by directed biosynthesis,[2] in hopes that a new analogue would retain the antimicrobial activity, but not the cytotoxicity, of pactamycin. This technique involves feeding non-physiologic amounts of an analogue of the identified advanced precursor to the wild-type organism, resulting in co-production of a new antibiotic analogue with the naturally-occurring antibiotic.

The success of directed biosynthesis relies on sufficient similarity between the advanced precursor and the synthetic precursor analog to allow enzymatic manipulation of the analogue in the identical fashion which would normally occur with the true precursor. In the case of actamycin biosynthesis, the 4-chloro, 6-chloro, *N*-methyl and *O*-methyl analogues of the precursor 3-amino-5-hydroxybenzoic acid could not be incorporated into new actamycin analogues.[3] This contrasts with the commercial production of penicillins, where administration of phenylacetic acid or phenoxyacetic acid to cultures of *Penicillium chrysogenum* results in the formation of penicillins G and V, respectively.[4]



1: X = H
2: X = F



3: X = H
4: X = F

Results

Synthesis of Precursor Analogues

In the absence of knowledge about the steric requirements of the relevant enzyme active sites in pactamycin biosynthesis, we chose *m*-ABA analogs with small substituents at the C-5 position. Thus, 3-amino-5-fluorobenzoic acid (**10**) and 3-amino-5-methylbenzoic acid (**16**) were prepared by the route shown in Scheme 1 in 31% and 20% overall yields, respectively. This general route has been used previously to prepare **14**.[5]

Feeding Experiments with the Precursor Analogues

The *m*-ABA analogues **10** and **16** were administered to resting cultures of *S. pactum*, var. *pactum* in a complex medium containing glucose, peptone, tryptone, yeast extract, molasses and oatmeal. Precursors were administered in varying concentrations, and crude pactamycin bases, consisting of pactamycin and at least five pactamycin analogues, were isolated by previously-described methods.[6] Analysis of the crude bases from each feeding experiment by low-resolution fast-atom-bombardment mass spectrometry (LRFAB-MS) provided ratios of peak heights between the signal corresponding to pactamycin (M+H *m/z* 559) and that expected for the fluorinated or methylated analogue (M+H *m/z* 577 or 573, respectively).

When **10** was administered, LRFAB-MS showed an ion at *m/z* 577 following feeding experiments with precursor concentrations of between 0.179 and 0.716 mmol/liter. Precursor concentrations of 1.25 mmol/liter and higher gave diminished or absent pactamycin production and no ion at *m/z* 577 in the LRFAB-MS spectra. When **16** was fed, LRFAB-MS did not show an ion at *m/z* 573, which would be characteristic of the desired methylated analogue, at any concentration of **16**. Higher concentrations of **16** also caused diminished pactamycin production.

Isolation and Characterization of 5″-Fluoropactamycin

Incorporation of **10** into the new pactamycin analogue 5″-fluoropactamycin (**2**) was maximal when administered at a concentration of 0.358 mmol/liter. A larger scale fermentation (1 liter) using this concentration of precursor afforded 43 mg of crude pactamycin bases. Flash chromatography provided a mixture of **1** and **2** (4:3 ratio of peak heights by LRFAB-MS) in the early fractions, what were apparently 7-deoxypactamycin and 7-deoxy-5″-fluoropactamycin (*m/z* 543 and 561, respectively, 5:2 ratio of peak heights), as well as pactamyçate (**3**) and 5″-fluoropactamyçate (**4**) (*m/z* 514 and 532, respectively,

Scheme 1.

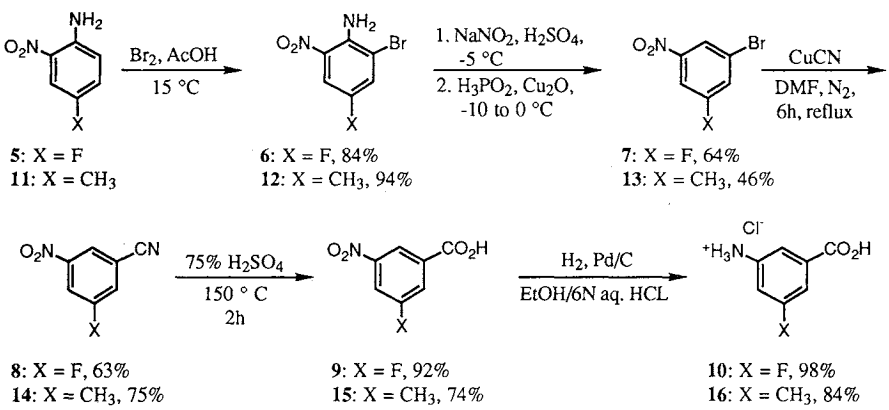
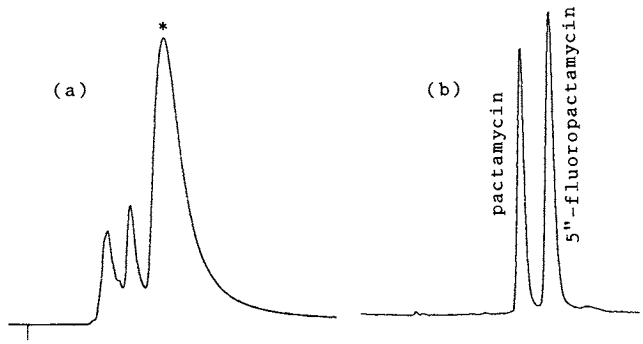


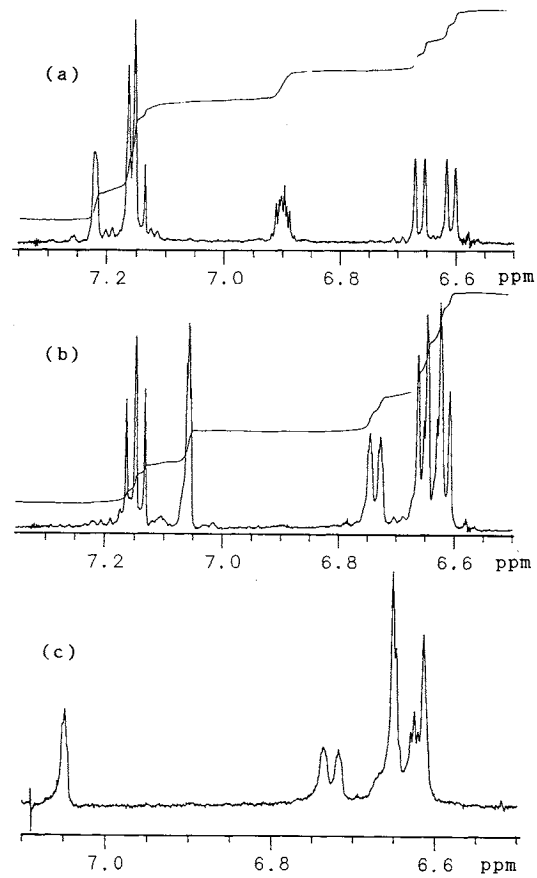
Fig. 1. (a) UV-detector tracing from HPLC of a mixture of **1** and **2** on a silica column. (b) UV-detector tracing from HPLC on an amino column of the eluant (*) from the silica column.



2:5 ratio of peak heights) in intermediate fractions, and 7-deoxyhydroxypactamycin and 7-deoxy-5″-fluorohydroxypactamycin (*m/z* 559 and 577, respectively) in late fractions. The position of the extra hydroxyl group in the last two compounds is unclear. High-resolution FAB-MS of the compounds from early and intermediate fractions having *m/z* values of 577 and 561 confirmed their formulae as those of **2** and 7-deoxy-5″-fluoropactamycin, respectively.

Further purification of the sample containing **1** and **2** by HPLC on silica and amino columns allowed the separation of these two components. Figs. 1a and 1b show, respectively, UV-detector tracings from HPLC on silica and amino columns. The peak marked with an asterisk in Fig. 1a contains both **1** and **2**; the LRFAB mass spectrum of the sample corresponding to this peak showed no change in the ratio of signal heights for these two compounds when compared to the spectrum of the sample recorded before silica HPLC. However, chromatographic conditions which were shown to give optimal resolution for **1** on an amino column were adequate to provide baseline resolution between **1** and **2** (Fig. 1b). Thus, 0.7 mg of **1** and 0.6 mg of **2** were obtained in pure form.

Fig. 2. (a) Aromatic region of the 500 MHz ^{1}H NMR spectrum of **1**. (b) Aromatic region of the 500 MHz ^{1}H NMR spectrum of **2**. (c) Aromatic region of the ^{1}H NMR spectrum of **2**, with irradiation of the triplet at 7.15 ppm.



Figs. 2a and 2b show, respectively, expansions of the aromatic regions of the 500-MHz ^{1}H NMR spectra of **1** and **2**. In the ^{1}H NMR spectrum of **1**, seven protons are apparent by integration. The multiplet centered around 7.15 ppm integrates for three protons and contains a triplet from H-4′ coupled to two doublets at 6.61 and 6.66 ppm (H-3′ and H-5′,

Table 1. Assignments of ions appearing in the FAB-MS/CID/MS spectra of pactamycin and 5″-fluoropactamycin.

Pactamycin		5″-Fluoropactamycin	
m/z[a]	Assignment	m/z[b]	Assignment
559	M+H	577	M+H
514	Loss of dimethylamine	532	Loss of dimethylamine
425	Loss of $C_8H_6O_2$ from M+H[c]	443	Loss of $C_8H_6O_2$ from M+H[c]
380	Loss of dimethylamine from m/z 425	398	Loss of dimethylamine from m/z 443
362	Loss of water from m/z 380	380	Loss of water from m/z 398
216	$C_{13}H_{14}NO_2$ by HRFAB-MS	234	$C_{13}H_{13}NO_2F$

[a] From FAB-MS/CID/MS spectrum of pactamycin; assignments made by HRFAB-MS.
[b] From FAB-MS/CID/MS spectrum of **2**; assignments made by comparison with FAB-MS/CID/MS spectrum of **1**.
[c] This corresponds to loss of 6-methylsalicylic acid minus H.

Table 2. Bioactivity assay results for **1** and **2**.

Compound	L1210 cytotoxicity assay		Antimicrobial activity *vs. B. subtilis* (size of zone of inhibition[b])					
			Amount applied to disk (μg)					
	LD_{50}[a]	LD_{90}[a]	2.0	1.0	0.20	0.10	0.050	0.025
1	2 ng/ml	12 ng/ml	2.6 cm	2.4 cm	2.1 cm	1.7 cm	1.3 cm	0.9 cm
2	2 ng/ml	12 ng/ml	2.6 cm	2.3 cm	2.3 cm	1.8 cm	1.5 cm	1.0 cm

[a] Concentration of compound required to decrease L1210 cell growth by 50% and 90%, respectively.
[b] Each value is the average of two determinations.

respectively). In the ^{1}H NMR spectrum of 7-deoxypactamycin,[7] doublets at 6.68 and 6.60 ppm were assigned to H-3′ and H-5′, respectively, but calculated values suggest that the H-3′ resonance should be the more upfield of the two.[8]

The *m*-aminoacetophenone unit of **1** gives a broad singlet (presumably H-2″) at 7.22 ppm, two apparently coincident doublets at 7.16 ppm (H-4″ and H-6″ overlapping the 6-methylsalicylate triplet above), and a multiplet (presumably H-5″) at 6.90 ppm.

The ^{1}H NMR spectrum of **2** contains the absorptions assigned for **1** to the 6-methylsalicylate unit. The 3-amino-5-fluoroacetophenone unit gives a multiplet at 7.06 ppm (H-2″), a broad doublet at 6.74 ppm (H-6″), and a doublet of triplets (revealed by irradiation at 7.16 ppm, Fig. 2c) at *ca.* 6.64 ppm (H-4″).

Comparison of the fast-atom-bombardment tandem mass spectrum (FAB-MS/CID/MS) of **2** with that of **1** provides further proof of its structure. Through the use of high-resolution FAB-MS, the molecular formulae of many ions in the FAB-MS/CID/MS spectrum of **1** were determined. Table 1 shows comparisons of analogous ions in the FAB-MS/CID/MS spectra of **1** and **2**.

Biological Activities of Pactamycin and 5″-Fluoropactamycin

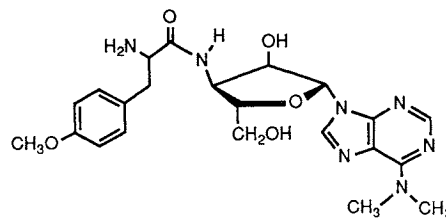
Disc-diffusion assays versus *Bacillus subtilis* and murine leukemia L1210 cell assays were performed in parallel on samples of pactamycin and 5″-fluoropactamycin to determine, respectively, their relative antimicrobial activities and cytotoxicities. The results of both assays were virtually identical for the two compounds.

Discussion

The continuing increase in resistance of Gram-positive bacteria, especially *Staphylococcus aureus*, to

currently used antimicrobials underscores the importance of finding new compounds which are effective against such organisms. Despite its cytotoxicity, pactamycin is interesting in this regard because of its activity versus Gram-positive bacteria (MIC 0.2 μg/ml *vs. Staphylococcus aureus*[9]). Modifications to its C_7N unit might be expected to be most promising for enhancing its selectivity for prokaryotic ribosomes for the following reason: Similarities between the protein synthesis inhibiting properties of pactamycin and puromycin have been described previously,[10] but there are also structural similarities; these are more noticeable when puromycin is drawn as in Scheme 2. The C_7N unit of pactamycin is seen to be analogous to the purine portion of puromycin, an area of the molecule quite likely important for its recognition by the ribosome as an aminoacyl-tRNA analogue. According to the results of the L1210 murine leukemia cell assay, fluorine substitution at the 5″ position of the C_7N unit of pactamycin is insufficient to improve its clinical properties. However, *in vivo* testing should be pursued, as the results would be clinically relevant.

Scheme 2.



Comparisons of the van der Waals radii of hydrogen, fluorine and a methyl group (1.20, 1.35 and 2.0 Angstroms, respectively[11]) offer an explanation for the ability of 3-amino-5-fluorobenzoic acid (**10**), and the failure of 3-amino-5-methylbenzoic acid (**16**) to substitute for *m*-ABA in pactamycin biosynthesis. That is, hydrogen and fluorine have essentially the same atomic radii, whereas a methyl group is significantly larger than hydrogen. Despite the fact that the methyl analogue was not incorporated, it is apparent that, at higher concentrations, as with the fluoro analogue, this compound behaves as an inhibitor of pactamycin biosynthesis.

Experimental

General

Low resolution fast atom bombardment (FAB) mass spectra were obtained on a VG Analytical ZAB-SE spectrometer using dithiothreitol-dithioerythritol matrix (magic bullet), an 8-kV accelerating potential, and bombardment of the sample with xenon atoms of 8000 eV. High-resolution and tandem FAB mass spectra were obtained on a VG Analytical 70SE-4F spectrometer, also using magic bullet matrix, an 8-kV accelerating potential and xenon bombardment. Electron-ionization (EI) mass spectra were obtained on a Finnigan-MAT CH5 mass spectrometer at 70 eV. NMR spectra were obtained on General Electric QE-300 and GN-500 spectrometers for all synthetic compounds and for **1** and **2**. Chemical shifts are reported in ppm downfield, using the solvent as internal standard. Elemental analyses were performed by the Microanalytical Laboratory, University of Illinois at Urbana-Champaign. Melting points are uncorrected and were obtained on a Thomas Hoover Uni-Melt apparatus.

HPLC utilized Alltech Econosil 10 μm silica 250 × 10 mm and Econosil 10 μm amino 250 × 10 mm columns. UV-detection for HPLC using the amino column employed a Waters 990 diode array detector, which was also used to record the UV spectra of **1** and **2**. Amino TLC utilized Merck 0.2 mm HPTLC plates with UV_{254} indicator.

Culture Conditions

Liquid media were sterilized by autoclaving for 20 minutes at 120°C in 500-ml wide-mouthed Erlenmeyer flasks (100 ml media/flask) with cotton plugs. All incubations with liquid media were conducted in a New Brunswick rotary incubator at 200 ~ 250 rpm and 32°C. Manipulations of bacterial cultures were performed using autoclaved or flame-sterilized equipment. *Streptomyces pactum*, var. *pactum*, originally obtained from ALMA DIETZ, The Upjohn Company, Kalamazoo, Michigan, was maintained in soil stocks and kept at 4°C. Sporulating colonies of *S. pactum* were raised on Hickey and Tresner Agar (Baxter Scientific) after transfer from soil stock by sterile wire loop and incubation at 32°C for 3 to 6 days. Seed cultures were then established by transferring an agar plug containing a sporulating colony from the agar surface into TY medium (5 g tryptone, 3 g yeast extract, both from Difco, in 1 liter distilled water), then

incubating for 2 days. The production medium consisted of 40 g glucose, 10 g Plantation blackstrap molasses, 5 g Gerber oatmeal, 2.5 g peptone, 2.5 g tryptone, and 2.5 g yeast extract (the latter three from Difco) in 1 liter of distilled water. Production media were inoculated by transferring 5 ml of seed medium, containing well-developed mycelia, by sterile pipet. Precursors were added to production media as aqueous solutions through a Nalgene 0.2 μm sterile syringe filter.

Bioassays

Disk-diffusion assays were conducted by the paper-disc assay method on *B. subtilis*,[12] using 10 μl of a methanol solution of the test compound. Cytotoxicity assays utilized a modification of the L1210 murine leukemia cell assay developed by R. G. HUGHES, Jr. (Roswell Park Memorial Institute, Buffalo, NY, personal communication). The samples were dissolved in methanol and applied to dry wells in 24-well tissue culture plates (Falcon). After evaporation of the methanol, 1 ml of minimum essential medium (MEM) containing 1,000 cells was added to each well. The tissue culture plates were incubated at 37°C until the control wells contained 8,000 cells. Inhibition of growth was calculated as the ratio of living cells in sample wells to those in control wells. Cells were determined to be alive if they demonstrated morphologic changes.

Synthesis of precursors

6-Bromo-4-fluoro-2-nitroaniline (6)

4-Fluoro-2-nitroaniline (**5**, 20.0 g, 128 mmol, tech. grade) was treated with 24.6 g (154 mmol, 1.2 eq.) of bromine in 75 ml of glacial acetic acid at 15°C. The mixture was stirred an additional hour at room temperature, then poured into 500 ml of water, stored overnight in the freezer, and filtered. The residue was dissolved in ethanol, treated with charcoal and recrystallized from ethanol-water, giving 25.2 g of **6** in one crop (84%), mp 74.0~74.8°C; ^{1}H NMR (CD_3OD) δ 7.74 (dd, J_{H-H}=2.8 Hz, J_{H-F}=7.3 Hz, 1H), 7.91 (dd, J_{H-H}=2.9 Hz, J_{H-F}=9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 111.6 (d, J_{C-F}=26 Hz), 112.1 (d, J_{C-F}=9 Hz), 127.6 (d, J_{C-F}=26 Hz), 131.5 (d, J_{C-F}=10 Hz), 139.4 (s), 151.8 (d, J_{C-F}=242 Hz); EI-MS *m/z* 236, 234, 190, 188, 178, 176, 163, 161, 108.

Anal Calcd for $C_6H_4BrFN_2O_2$: C 30.67, H 1.72, N 11.92, F 8.08, Br 34.00.
Found: C 30.86, H 1.74, N 11.87, F 8.05, Br 34.03.

3-Bromo-5-fluoronitrobenzene (7)

6-Bromo-4-fluoro-2-nitroaniline (**6**, 10.0 g, 42.6 mmol) was placed in a 3-necked 500-ml round-bottomed flask equipped with a mechanical stirrer, a thermometer and a dropping funnel. The flask was cooled to −5°C with a brine/ice bath, and 100 ml of concd sulfuric acid was added with vigorous stirring. Sodium nitrite (3.23 g, 46.8 mmol, 1.1 eq.) in 10 ml of water was added to the sulfuric acid mixture at −2 to −3°C during 90 minutes, then stirred for another 15 minutes, cooled to −5°C, and 50% aqueous hypophosphorous acid (30.9 ml, 292 mmol, 7 eq.) and cuprous oxide (6.70 g, 46.8 mmol, 1.1 eq.) were added in small portions with vigorous stirring during 90 minutes. After the reaction mixture was stirred an additional 10 minutes at 0°C, it was poured into 300 g of crushed ice and stored in the freezer for 1 hour, then the precipitate was filtered and washed with ice-cold water.

The residue on the filter was eluted through the filter with 400 ml of chloroform, which was washed with water. The aqueous layer was back-extracted with two 50-ml portions of chloroform and the chloroform layers were combined, dried over $MgSO_4$ and evaporated *in vacuo* to yield a dark solid. This was purified by flash chromatography (silica, 97:3 hexane-ethyl acetate, 30×8 cm column), which upon removal of solvent crystallized, affording 6.03 g (64%) of **7** as white crystals, mp 27.5~28.3°C; ^{1}H NMR ($CDCl_3$) δ 7.61 (dt, J_{H-H}=2.0, J_{H-F}=7.3, 1H), 7.92 (dt, J_{H-H}=2.1 Hz, J_{H-F}=8.1 Hz, 1H), 8.22 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 110.3 (d, J_{C-F}=26 Hz), 122.8 (d, J_{C-F}=3 Hz), 123.1 (d, J_{C-F}=9 Hz), 125.2 (d, J_{C-F}=24 Hz), 149.3 (d, J_{C-F}=7 Hz), 162.0 (d, J_{C-F}=256 Hz); EI-MS *m/z* 221, 219, 205, 203, 201, 199, 175, 173, 165, 163, 94, 74. This material was unstable at room temperature and was stored at −4°C;

Anal Calcd for $C_6H_3BrFNO_2$: C 32.76, H 1.37, N 6.37, F 8.64, Br 36.32.
Found: C 32.61, H 1.40, N 6.37, F 8.65, Br 36.37.

3-Nitro-5-fluorobenzonitrile (**8**)

According to the method used by STALEY,[13] cuprous cyanide (0.546 g, 6.1 mmol) was stored *in vacuo* overnight in a 50-ml round-bottomed flask. The reaction vessel was then placed under an anhydrous nitrogen atmosphere, and 3-bromo-5-fluoronitrobenzene (**7**, 1.32 g, 6.0 mmol) in 10 ml of freshly-distilled DMF was transferred into the flask by a gas-tight syringe. The contents were heated at reflux for 5 hours, then diluted to 100 ml with 5 ml of 6 N hydrochloric acid and water and extracted with three 50-ml portions of diethyl ether. The combined organic layers were washed with 30 ml each of water, 5% aqueous sodium bicarbonate, and water and dried over Na_2SO. The solvent was removed *in vacuo*, and the residue was purified by flash chromatography (silica, 8 : 2 hexane - ethyl acetate for first 100 ml, then 7 : 3 hexane - ethyl acetate, 3.5 × 20 cm column), then crystallized from ethyl acetate - hexane, providing 0.632 g of **8** in two crops (63%), mp 49.7 ~ 50.3°C; ^{1}H NMR ($CDCl_3$) δ 7.73 (dq, J_{H-H}=2.0 and 2.5 Hz, J_{H-F}=7.1 Hz, 1H), 8.21 (dt, J_{H-H}=2.2 Hz, J_{H-F}=7.6 Hz, 1H), 8.36 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 115.2 (d, J_{C-F}=25 Hz), 115.3 (d, J_{C-F}=17 Hz), 116.0 (d, J_{C-F}=26 Hz), 123.1 (d, J_{C-F}=4 Hz), 125.1 (d, J_{C-F}=25 Hz), 149.4 (d, J_{C-F}=9 Hz), 162.0 (d, J_{C-F}=256 Hz); EI-MS *m/z* 166, 120, 108, 100, 93.

Anal Calcd for $C_7H_3FN_2O_2$: C 50.62, H 1.82, N 16.87, F 11.44.
Found: C 50.65, H 1.80, N 16.85, F 11.36.

3-Nitro-5-fluorobenzoic Acid (**9**)

Aqueous sulfuric acid (6 ml, 75%) was added to 5-fluoro-3-nitrobenzonitrile (**8**, 0.514 g, 3.10 mmol), and the mixture was stirred at 150°C for 2 hours. The reaction mixture was poured into ice water, and sodium bicarbonate was added until gas evolution ceased. The solution was washed with diethyl ether (*ca.* 40 ml), then acidified to pH 1 and extracted with two 75-ml portions of diethyl ether. After drying ($MgSO_4$) and removal of the ether *in vacuo*, recrystallization from chloroform - petroleum ether gave 0.515 g (92%) of **9**, mp 124.0 ~ 125.0°C; ^{1}H NMR (CD_3OD) δ 8.11 (dq, J_{H-H}=1.9 and 2.8 Hz, J_{H-F}=8.4 Hz, 1H), 8.25 (dt, J_{H-H}=2.4, J_{H-F}=8.1 Hz, 1H), 8.62 (br s, 1H); ^{13}C NMR (CD_3OD) δ 115.9 (d, J_{C-F}=27 Hz), 121.1 (d, J_{C-F}=3 Hz), 123.3 (d, J_{C-F}=23 Hz), 135.8 (d, J_{C-F}=7 Hz), 150.4 (d, J_{C-F}=8 Hz, 161.8 (s), 165.6 (d, J_{C-F}=61 Hz); EI-MS *m/z* 185, 166, 139, 120, 83. 75.

Anal Calcd for $C_7H_4FNO_4$: C 45.42, H 2.18, N 7.57, F 10.26.
Found: C 45.50, H 2.21, N 7.55, F 10.22.

3-Amino-5-fluorobenzoic Acid Hydrochloride (**10**)

In a Parr hydrogenation flask, 5-fluoro-3-nitrobenzoic acid (**9**, 0.451 g, 2.44 mmol) was combined with 26 ml of ethanol, 4 ml of 6 N hydrochloric acid, and 30 mg of 5% Pd/C (Engelhard). The flask was flushed twice with hydrogen, then pressurized with hydrogen to 24 ~ 28 psi. After 12 minutes of agitation, the reaction mixture was filtered through acid-washed Celite and evaporated to dryness *in vacuo*. Recrystallization from 6 N hydrochloric acid afforded 0.457 g (98%) of **10** in one crop, mp 244°C (d.); ^{1}H NMR (D_2O) δ 7.24 (dd, J_{H-H}=1.8 Hz, J_{H-F}=8.6 Hz, 1H), 7.61 (br d or dd, 2H); ^{13}C NMR (CD_3OD - D_2O) δ 116.3 (d, J_{C-F}=26 Hz), 117.8 (d, J_{C-F}=23 Hz), 120.8 (d, J_{C-F}=4 Hz), 133.7 (d, J_{C-F}=10 Hz), 134.8 (d, J_{C-F}=8 Hz), 161.9 (s), 166.7 (d, J_{C-F}=235 Hz); EI-MS *m/z* 155, 138, 110, 83.

Anal Calcd for $C_7H_7ClFNO_2$: C 43.88, H 3.68, N 7.31, F 9.92, Cl 18.51.
Found: C 43.84, H 3.79, N 7.20, F 9.80, Cl 18.46.

4-Amino-3-nitro-5-bromotoluene (**12**)

4-Amino-3-nitrotoluene (**11**, 20.0 g, 0.131 mol) was brominated in the same manner as for the synthesis of **6**, except that the bromine addition required 30 minutes, and decolorizing charcoal was not used. Recrystallization from ethanol - water gave 28.56 g (94%) of **12** as bright orange needles, mp 63.7 ~ 66.7°C. Further recrystallization from ethanol - water provided a sample with mp 65.7 ~ 66.5°C (lit.[14] 65.5 ~ 66.5°C); ^{1}H NMR ($CDCl_3$) δ 2.28 (s, 3H), 7.56 (d, J=19 Hz, 1H), 7.94 (d, J=0.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 19.7, 111.7, 125.2, 126.2, 132.3, 139.8, 140.0; EI-MS *m/z* 232, 230, 202, 200, 186, 184, 174, 172, 159, 157, 104.

5-Bromo-3-nitrotoluene (**13**)

4-Amino-3-nitro-5-bromotoluene (**12**, 10.0 g, 43.3 mmol) was diazotized in the same manner as for the synthesis of **7** except that 150 ml of concd sulfuric acid, and 1.2 eq. of sodium nitrite were used and

the latter added during 2 hours. After the addition of hypophosphorous acid, the reaction mixture was poured into 600 g of crushed ice and stored 48 hours in the freezer, after which a flocculant precipitate was present. The mixture was extracted with three equal volumes of diethyl ether, which were dried over $MgSO_4$ and evaporated *in vacuo*. Purification by flash chromatography (silica, 95 : 5 hexane - ethyl acetate, 30 × 8 cm column), treatment with charcoal in methanol, and crystallization from methanol - water provided 4.26 g (46%) of **13** as light-orange crystals, mp 81.5~82.0°C (lit.[5] 83~84°C); ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 3H), 7.65 (s, 1H), 7.98 (s, 1H), 8.18 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.0, 122.3, 122.6, 123.7, 138.0, 141.5, 148.5; EI-MS *m/z* 217, 215, 171, 169, 90.

5-Methyl-3-nitrobenzonitrile (**14**)

5-Bromo-3-nitrotoluene (**13**, 2.59 g, 12.0 mmol) was treated with cuprous cyanide in the same manner as for the synthesis of **8**, except on twice the scale. The starting material was also added to the reaction flask in crystalline form under nitrogen, and the reaction required 6 hours. Purification by flash chromatography (silica, 55 : 45 hexane - ethyl acetate, 4.5 × 28 cm column) and recrystallization from ethyl acetate - hexane afforded 1.15 g (59%) of **14**, mp 102.0~103.0°C (lit.[5] 104~105°C); ^{1}H NMR ($CDCl_3$) δ 2.55 (s, 3H), 7.79 (s, 1H), 8.28 (s, 1H), 8.33 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.0, 113.5, 116.6, 124.2, 127.9, 138.0, 141.7, 148.0; EI-MS *m/z* 162, 116, 104, 89, 77, 63.

3-Nitro-5-methylbenzoic Acid (**15**)

As in the synthesis of **9** above, 5-methyl-3-nitrobenzonitrile (**14**, 0.800 g, 4.94 mmol) was treated with 10 ml of 75% aqueous sulfuric acid at 150°C for 1 hour. The reaction mixture was then poured into 20 ml of ice water and extracted with three equal volumes of diethyl ether. The combined ether layers were extracted with three 40-ml volumes of 5% aqueous sodium bicarbonate, which were combined, acidified to pH 1 with concd hydrochloric acid, and extracted with three 50-ml volumes of ether. The combined ether extracts were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was recrystallized from chloroform - petroleum ether, giving 0.433 g (75%) of **15** in two crops, mp 170.0~171.2°C; ^{1}H NMR ($CDCl_3$) δ 2.57 (s, 3H), 8.25 (s, 1H), 8.30 (s, 1H), 8.76 (s, 1H); ^{13}C NMR (CD_3OD) δ 21.1, 122.4, 128.4, 133.4, 136.8, 142.0, 149.4, 167.3; EI-MS *m/z* 181, 164, 151, 135, 107, 89, 77, 63.

Anal Calcd for $C_8H_7NO_2$: C 53.04, H 3.90, N 7.73.
Found: C 52.95, H 3.88, N 7.82.

3-Amino-5-methylbenzoic Acid Hydrochloride (**16**)

Hydrogenation of 5-methyl-3-nitrobenzoic acid (**15**, 0.324 g, 1.79 mmol) was accomplished in the same manner as for the synthesis of **10**. Recrystallization from 6 N hydrochloric acid gave 0.271 g (84%) of **16**, mp 240°C (d.); ^{1}H NMR (D_2O) δ 2.23 (s, 3H), 7.26 (s, 1H), 7.56 (s, 1H), 7.73 (s, 1H); ^{13}C NMR (D_2O - CD_3OD) δ 21.0, 121.7, 128.9, 131.3, 131.6, 132.5, 142.2, 169.3; EI-MS *m/z* 151, 134, 106, 77, 63.

Anal Calcd for $C_8H_{10}ClNO_2$: C 51.21, H 5.37, N 7.47, Cl 18.90.
Found: C 51.30, H 5.40, N 7.44, Cl 18.86.

Supplementation Experiments

Ten 100-ml flasks of production medium were inoculated from TY seed medium, and **10** was added as an aqueous solution to five of these flasks 36 hours after inoculation in amounts of 3.4, 6.9, 13.7, 24.0 and 34.3 mg; the remaining flasks served as controls. After another 3.5 days of incubation at 32°C, the pH of the medium in each flask was measured, and crude pactamycin bases were obtained separately from each flask to which precursor had been added. The control flasks were combined for work-up. Samples from the control fermentation corresponding to each concentration of **10** used were then analyzed by LRFAB-MS.

For the feeding experiments involving **16**, only one 100-ml flask of production medium was used as a control, and precursor was administered in amounts of 3.4, 6.7, 10.0, 16.8 and 26.9 mg. The two feeding experiments were otherwise identical.

Purification of 5″-Fluoropactamycin (**2**) and Pactamycin (**1**)

Ten 100-ml flasks of production medium were inoculated with *S. pactum* from seed culture, and 6.9 mg

of **10** was added to each flask 40 hours after inoculation. Crude pactamycin bases were obtained after another 3.5 days, and LRFAB-MS of this sample showed a 4:3 ratio of peak heights for the signals at *m/z* 577 and 559 (M+H for **2** and **1**, respectively). Flash chromatography (silica, 95:5 to 75:25 chloroform-methanol gradient, 19×2 cm column) gave fractions which were shown by LRFAB-MS to contain, in order of elution, *m/z* 559 and 577, 543 and 561, 514 and 532, and 559 and 577, corresponding to the molecular weights, respectively, of **1** and **2**, 7-deoxypactamycin and 7-deoxy-5″-fluoropactamycin, **3** and **4**, and 7-deoxyhydroxypactamycin and 7-deoxy-5″-fluorohydroxypactamycin.

The fractions containing **1** and **2** were again subjected to flash chromatography (silica, 99:1 to 97:3 chloroform-methanol gradient, 0.8×15 cm column), giving 6.8 mg of a sample which showed a 4:3 ratio of peak heights for the signals at *m/z* 577 and 559 in its LRFAB mass spectrum. HPLC (silica, 98:2 chloroform-methanol, 4 ml/minute) showed three peaks, the largest of which had a retention time of 8.2 minutes and was shown by LRFAB-MS to consist of 4.3 mg of a mixture of pactamycin and 5″-fluoropactamycin in the same ratio as before silica HPLC.

HPLC utilizing an amino semi-preparative column (68% hexane, 32% of a solution of 85:15 dichloromethane-methanol; *i.e.*, 68:27.2:4.8 hexane-CH_2Cl_2-CH_3OH, 4 ml/minute) provided separation of **1** and **2**, with retention times of 10.5 and 12.4 minutes, respectively.

2: Yield 0.7 mg; ^{1}H NMR (CD_3OD) δ 1.12 (d, J=6.5 Hz, 3H), 1.49 (s, 3H), 2.27 (s, 3H), 2.45 (s, 3H), 2.99 (s, 6H), 3.77 (d, J=2.8 Hz, 1H), 4.24 (m, 1H), 4.42 (d, J=12 Hz, 1H), 4.85 (d, J=11.9 Hz, *ca.* 2H-overlaps with solvent), 6.62 (d, J=8.4 Hz, 1H), 6.64 (d, J=11.2 Hz, 1H), 6.65 (d, J=7.35, 1H), 6.74 (dt, J=8.7, 1.6 Hz, 1H), 7.06 (t, J=1.7 Hz, 1H), 7.15 (t, J=7.9 Hz, 1H); UV (68:27.2:2.8 hexane-CH_2Cl_2-CH_3OH) λ_{max} 238; FAB-MS/CID/MS *m/z* 577→532, 514, 443, 425, 398, 290, 277, 234, 135.

Anal Calcd for $C_{28}H_{37}FN_4O_8$: *m/z* 577.2674 (M+H).
Found: *m/z* 577.2682 (HRFAB-MS).

1: Yield 0.7 mg; ^{1}H NMR (CD_3OD) δ 1.08 (d, J=6.5 Hz, 3H), 1.49 (s, 3H), 2.26 (s, 3H), 2.48 (s, 3H), 2.99 (s, 6H), 3.82 (br s, 1H), 4.15 (m, 1H), 4.44 (d, J=12.0 Hz, 1H), 4.82, 2.86 (s, overlap with solvent peak), 6.61 (d, J=7.3 Hz, 1H), 6.66 (d, J=8.4 Hz, 1H), 6.90 (m, 1H), 7.15 (m, 3H), 7.22 (br s, 1H); LRFAB$^+$, *m/z* 559.3, 514.3, 496.3, 425.3, 380.2, 362.2; HRFAB$^+$ of fragment ions from *m/z* 577, Calcd for $C_{26}H_{32}N_3O_8$: *m/z* 514.2189, found: *m/z* 514.2171; Calcd for $C_{20}H_{33}N_4O_6$: *m/z* 425.2400, found: *m/z* 425.2395; Calcd for $C_{18}H_{26}N_3O_6$: *m/z* 380.1822, found: *m/z* 380.1834; Calcd for $C_{18}H_{24}N_3O_5$: *m/z* 362.1716, found: *m/z* 362.1722; Calcd for $C_{13}H_{14}NO_2$: *m/z* 216.1025, found: *m/z* 216.1023; FAB-MS/CID/MS *m/z* 559→514, 496, 425, 380, 362, 290, 216, 135; *m/z* 425→380, 362, 290, 216; *m/z* 380→362, 216; *m/z* 362→317, 256, 214; UV (68:27.2:2.8 hexane-CH_2Cl_2-CH_3OH) λ_{max} 240 (lit.[15] (ethanol) 238).

Anal Calcd for $C_{28}H_{39}N_4O_8$: *m/z* 559.2768
Found: *m/z* 559.2777 (HRFAB-MS).

Bioactivities of Pactamycin and 5″-Fluoropactamycin

Bioassay results are shown in Table 2.

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