BIOSYNTHESIS OF FLUOROTHREONINE AND FLUOROACETIC ACID BY THE THIENAMYCIN PRODUCER, *STREPTOMYCES CATTLEYA*

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An antimetabolite, THX, was isolated from fermentation broths of the thienamycin producer, *Streptomyces cattleya*, when the organism was grown in the presence of a fluorine-containing substrate. THX was subsequently identified as one of the four possible stereoisomers of 4-fluorothreonine. Inorganic fluoride or any one of a number of organofluorine compounds can be used as precursors of 4-fluorothreonine. In addition, ^{19}F NMR has provided evidence that the organism synthesizes fluoroacetate under the same fermentation conditions. The *in vitro* antibacterial spectrum of 4-fluorothreonine is also presented.

In the course of studies to improve the production of thienamycin[1] by fermentation, it was discovered that under certain fermentation conditions *Streptomyces cattleya* synthesizes an antimetabolite which was initially designated, THX. This paper describes the isolation and identification of THX as a stereoisomer of 4-fluorothreonine. Studies on the biosynthesis of 4-fluorothreonine by *S. cattleya* are also presented. There are very few reports in the literature on the identification of fluorine-containing natural products. The biosynthesis of fluoroacetic acid and compounds derived from it, such as fluorocitrate, ω-fluoropalmitate, and ω-fluorooleate, by higher plants have been reported[2~6]. In addition, nucleocidin, an anti-trypanosomal antibiotic produced by *Streptomyces calvus*, has been found to contain a fluororibosyl group[7]. As far as the authors are aware, the present communication represents the first report on the ability of a microorganism to produce a fluoroamino acid from inorganic fluoride.

Materials and Methods

Chemicals

DL-Fluorocitrate was obtained from Calbiochem; 4-fluoroglutamate from Fairfield Chemical Co.; sodium fluoroacetate from Pfaltz and Bauer, Inc.; *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Regis Co.; 2-(*N*-morpholino)ethane sulfonic acid (MES) from Sigma Chemical Company. All other chemicals were of reagent grade.

Cultures

S. cattleya (NRRL 8057) and mutants derived from it (MA 5176, MA 5617 and THC-3807E) were used for these studies.

Fermentation Procedures

Tank Fermentations: Seed cultures of *S. cattleya* (THC-3807E) were prepared in a medium con-

taining the following (concentrations in g/liter, distilled water): sucrose (30), distiller's solubles (15), yeast autolysate (5), corn gluten meal (5). Presterile pH was 7.5. Seed developed in this medium was inoculated into 130 liters (200-liter fermentor) of production medium which consisted of glycerol 20 g, distiller's solubles (Morinaga Nyugyo Co.) 22.5 g, corn steep liquor (Ohji Cornstarch Co.) 22.5 g, Pharmamedia 7.5 g, soy casein (Katayama Chemical Co.) 5 g, sodium succinate 1.0 g, $CaHPO_4 \cdot 2H_2O$ 2.5 g, $CoCl_2 \cdot 6H_2O$ 0.01 g and antifoam (polypropylene glycol-2000, P-2000; Dow Chemical Co.) 0.5 g per liter of tap water. Presterile pH was 7.5. The fermentation was carried out at 28°C for 5 days with aeration at 100 liters/minute and agitation at 110 rpm.

Shake Flask Fermentations: For complex medium studies, the inoculum was developed in the seed medium (40 ml medium/250-ml 3-baffle flask) described previously. The complex flask production medium consisted of glycerol 30 g, distiller's solubles 10 g, corn steep liquor 23 g, Pharmamedia 7.5 g, dry yeast 4.5 g, sodium succinate 1.0 g, glycine 1.5 g, $CaHPO_4 \cdot 2H_2O$ 0.5 g, $CoCl_2 \cdot 6H_2O$ 0.02 g and $MgSO_4 \cdot 7H_2O$ 0.01 g in 1 liter of tap water. Presterile pH was 7.5. Fluorine-containing substrates were added to the medium at concentrations indicated elsewhere in the text. Shake flasks (20 ml medium/250-ml flask) were incubated at 27~28°C on a rotary shaker at 220 rpm (5.1-cm throw) for both the seed and production stages. For the preparation of resting cells, a synthetic medium was used for the growth of the cells. The seed medium (40 ml medium/250-ml 3-baffle flask) for these studies consisted of monosodium L-glutamate monohydrate 5.0 g, NH_4Cl 1.5 g, K_2HPO_4 2.0 g, inositol 0.4 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.025 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, *p*-aminobenzoic acid 0.0001 g, $CoCl_2 \cdot 6H_2O$ 0.01 g, $CaCO_3$ 0.25 g and glycerol 10.0 g in 1 liter of distilled water. Presterile pH was 7.0. The synthetic production medium consisted of monosodium L-glutamate monohydrate 3.75 g, L-isoleucine 2.4 g, NH_4Cl 0.75 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CoCl_2$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.025 g, glycerol 10 g and MES 16.0 g in 1 liter of distilled water. When required, KF was added at a concentration of 2.0 g/liter. Each shake flask (250-ml) contained 20 ml synthetic medium.

Preparation of Resting Cells

Resting cells were prepared by growing *S. cattleya* for 72 hours in the synthetic medium as described above. The cells were isolated by centrifugation and washed three times with 50 ml of 50 mM sodium MES buffer, pH 6.5. The washed cells were resuspended in the same buffer in a final volume equivalent to one-fourth of the starting volume. Incubation mixtures for resting cells were made up in culture tubes (25×150 mm) and contained washed cells (2.0 ml; 26 mg/ml dry weight cells) and 50 mM MES buffer or fluorine-containing compound in MES buffer (2.0 ml). The tubes were incubated at 220 rpm and 27°C. After incubation, the cells were removed by centrifugation and the supernatant solutions analyzed as described elsewhere in the text.

Bioassay

Quantitative determination of 4-fluorothreonine was performed by the paper disk agar diffusion method with *Pseudomonas aeruginosa* MB 2835 as test organism on Davis minimal medium. Prior to assay, any carbapenems present in the broth supernatant were destroyed by heat treatment (30 minutes at 50°C or 15 minutes at 70°C, pH 3). Other secondary metabolites produced by *S. cattleya*, such as cephamycin C and penicillin N[1], are not bioactive against this organism. None of the fluorine-containing compounds tested as possible precursors of 4-fluorothreonine was bioactive on the test organism at the concentrations used.

Isolation and Identification of THX

Isolation: Cells were removed from the fermentation broth (total volume of 125 liters) by filtration. The filtrate was loaded onto a column of Amberlite IRA-4015 (OH^- form; 15 liters bed volume). The column was washed with water and the absorbed activity eluted with 1.0 M HCl. The fractions were assayed by bioassay as described previously. Fractions containing activity were combined and loaded onto a column of Amberlite IR-120 (H^+ form; 16 liters bed volume). The column was washed with water and the activity eluted with 2.0 M NH_4OH. The active fractions were combined and concentrated *in vacuo* to a volume of about 1 liter. The concentrated fraction was applied to a column of activated charcoal and eluted with water. The active fractions were concentrated *in vacuo* to yield an oily material (54 g). The oil was dissolved in water and applied to a column of Amberlite CG-50 (NH_4^+ form; type

I) and eluted with water. The active fractions were combined and concentrated as before. Further purification was carried out by column chromatography on silica gel (2 liters bed volume; Wako gel W-200, Wako Pure Chemicals, Japan). The solvent used was acetonitrile - acetic acid - water (9 : 1 : 1). The active fractions were concentrated to dryness affording 8.02 g of a pale yellow powder. This powder was dissolved in a small amount of water and ethanol was added to induce crystallization. The material was recrystallized from ethanol - water to give colorless needles. Yield 1.6 g; mp 181~182°C (dec); $[\alpha]_D^{20}$ −18° (*c* 1, H_2O); analysis Found C 35.19, H 5.79, N 10.08.

Table 1. NMR parameters of THX (D_2O).

δ	Assignment
3.87 d, 4.7	NCHC=O
4.36 d q, 25.0, 4.5	FCH_2CH
4.68 ddd, 47, 10.7[a], 3.8[a]	FCH_2

[a] Splittings indicate a slight geminal non-equivalence and second order effects resulting from the ABX system but are represented as doublets for purpose of simplification.

Identification: Combined mass spectral and NMR analysis of the antimetabolite, THX, purified from fermentation broth of *S. cattleya* led to its identification as 4-fluorothreonine. NMR analysis was carried out with a Varian SC 300 MHz spectrometer equipped with a Fourier transform accessory at ambient temperature (20~24°C). The compound was dissolved in D_2O; sodium 3-trimethylsilylpropionate-(2,2,3,3-d_4) (TSP) was used as an internal standard. The NMR spectrum was composed of five discrete spin-coupled signals between 3.87 and 4.77 (Table 1) which initially suggested the presence of three methylenes and two methines based on relative area and multiplicity considerations. This appraisal, however, proved to be incompatible with the results from double irradiation experiments. The suspicion that the interpretational difficulties arose from spin-interaction by an unseen nucleus was verified by the demonstration that the separations between the multiplets at 4.33 and 4.41 as well as those between the signals at 4.61 and 4.96 were independent of the magnetic field strength and hence represented coupling constants of 25 Hz and 47 Hz, respectively. Coupling constants of this magnitude strongly suggested an FCH_2CH-grouping and were a major factor in leading to the fluorothreonine proposal. Mass spectral analysis was carried out with an LKB 9000 Mass Spectrometer. The mass spectrum showed peaks of *m/z* 104, 92, 74, 57, and 44. Silylation and deuterosilylation gave M353 (d_q=M380) which suggested the addition of three silyl groups and an original molecular weight of 137. The presence of an intense *m/z* 218 (d_q=*m/z* 236) indicated an α-amino acid. Measured exact mass was 137.0499; $C_4H_8NO_3F$ requires 137.04897.

There are four possible stereoisomers of fluorothreonine. The configuration of the natural product was established by comparison with chemically synthesized 4-fluorothreonines of known stereochemistry. These results will be described in a separate communication (J. KOLLONITSCH, L. M. PERKINS and B. H. ARISON, manuscript in preparation).

Gas Chromatography-Mass Spectrometry (GC-MS)

The mass spectrometer used was a MAT-212 in electron impact mode (90 eV). Samples were prepared for analysis as follows: 1 ml of cell-free broth was lyophilized, then further dried *in vacuo* for 15 minutes. To the residue was added a 1 : 1 mixture of BSTFA in pyridine (400 μl) and the solution heated at 50°C in a closed tube for 30 minutes. Separations were performed on a 10-meter SE-30 capillary column at 120°C.

^{19}F NMR

Pulsed Fourier transform ^{19}F NMR spectra were obtained at 94.1 MHz with a Varian XL-100 Spectrometer at ambient temperature (20~24°C). The solvent used was D_2O. The internal standard used was C_6F_6 (ϕ=163.0). The following are the chemical shifts of the compounds observed: fluorocitrate [ϕ=190.3 ppm; J=48.5 Hz (d)]; fluoroacetate [ϕ=216.9 ppm; J=48.3 Hz (t)]; 4-fluoroglutamate [multiplet; ϕ=180.8 ppm; full width $\sum J_{MF}$=117.8 Hz] and 4-fluorothreonine [multiplet; ϕ=231.6 ppm; J=25.0 Hz (d); J=46.9 Hz (t)]. The resonance of 4-fluorothreonine was shifted 1~2 ppm downfield in some of the broth samples. The exact cause of this shift is unknown but may reflect the presence of trace metal ions in these samples.

Results

Production of 4-Fluorothreonine in Complex Media

Since the antimetabolite activity of 4-fluorothreonine was first detected in a medium containing soy bean casein, it was initially thought that the casein was the source of the fluoroorganic compound. However, further experimentation showed that *S. cattleya* produced 4-fluorothreonine whenever the medium was supplemented with either inorganic or organic fluorine compounds. The effects of halide compounds and soy bean casein from various sources on the production of 4-fluorothreonine in shake flasks containing complex fermentation medium are shown in Table 2. 4-Fluorothreonine was produced when the basal medium was supplemented with potassium fluoride, sodium fluoride, fluoroacetic acid, *m*- or *p*-fluorophenylalanine or soy bean casein from two of the three sources tested. Elemental analysis of the soy bean caseins that gave positive results revealed the presence of about 0.7% fluorine as inorganic fluoride, an amount sufficient to support the 4-fluorothreonine synthesis observed. The addition of chloro-, bromo-, or iodo-compounds failed to result in production of other haloorganic derivatives.

Table 3 summarizes the results of a shake flask experiment in which three different strains of *S. cattleya* were grown in complex fermentation medium and the 4-fluorothreonine produced determined either by bioassay or GC-MS. The original soil isolate of *S. cattleya* (NRRL 8057) produces 4-fluorothreonine. One of the mutant strains tested, MA 5176, an improved thienamycin producer, makes 4-fluorothreonine; the other mutant strain tested, MA 5617, also improved for thienamycin production, does not synthesize 4-fluorothreonine. The addition of NaF to the fermentation medium had no effect on either the rate or extent of produc-

Table 2. Effect of halide compounds on 4-fluorothreonine production by *S. cattleya* (THC-3807E) in complex fermentation medium.

Addition (mM)	4-Fluorothreonine* (μg/ml)
None	0
KF (2)	168
KF (10)	134
NaF (2)	144
NaCl (2)	0
NaBr (2)	0
KI (2)	0
Soy bean casein (0.3 % w/v)	
from Kishida Chemical Co.	150
from Katayama Chemical Co.	144
from Wako Chemical Co.	0
m-Fluoro-DL-phenylalanine (2)	160
p-Fluoro-DL-phenylalanine (2)	12
Fluoroacetic acid (2)	24

* Determined by bioassay.

Table 3. Production of 4-fluorothreonine by various strains of *S. cattleya* in complex medium.

Strain	4-Fluorothreonine (μg/ml)	
	Bioassay	GC-MS
NRRL 8057	85	70
MA 5176	170	120
MA 5617	<5	<1~5

Fig. 1. Production of 4-fluorothreonine by resting cells of *S. cattleya* (MA 5176).

△; 4-Fluoroglutamate (12 mM), ▲; DL-fluorocitrate (2 mM), ○; sodium fluoroacetate (20 mM), ●; KF (2 mM).

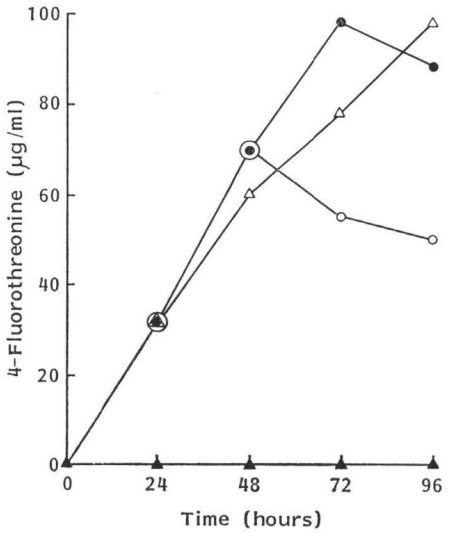

Table 4. Identification of fluorine compounds by ^{19}F NMR in resting cell reaction mixtures (MA 5176).

Fluorinated substrate	Organofluorine compound identified by ^{19}F NMR			
	F-Acetate	F-Citrate	F-Glutamate	F-Threonine
KF	F-Acetate	None	None	F-Threonine
Fluoroacetate	F-Acetate	None	None	F-Threonine
Fluorocitrate	None	F-Citrate	None	None
4-Fluoroglutamate	F-Acetate	None	F-Glutamate	F-Threonine
4-Fluorothreonine	F-Acetate	None	None	F-Threonine

Table 5. *In vitro* antimicrobial activity of 4-fluorothreonine.

Organism*	MIC (μg/ml)	
	Nutrient agar	Davis medium**
Escherichia coli NIHJ	>100	<0.2
Klebsiella pneumoniae D-11	>100	<0.2
Serratia marcescens IAM-1223	>100	3.12
Enterobacter aerogenes IAM-12348	>100	0.78
Salmonella paratyphi OB-1001	>100	<0.2
Pseudomonas aeruginosa D-15	>100	<0.2
P. aeruginosa OB-1002	>100	<0.2
Acinetobacter calcoaceticus IAM-12087	>100	<0.2
Proteus vulgaris OB-1003	50	ND***
P. mirabilis OB-1004	>100	ND
Morganella morganii OB-1005	>100	ND
Alcaligenes faecalis ATCC-8750	>100	<0.2
Staphylococcus aureus 209-P	>100	ND
S. epidermidis IAM-12012	>100	ND
Corynebacterium exerosis 53-K-1	>100	6.25
Micrococcus luteus PCI-1001	>100	0.39
M. flavus D-12	50	ND
Bacillus anthracis IID-115	50	ND
B. mycoides "0"	50	ND
B. subtilis PCI-219	>100	<0.2

* Inoculum size, 10^6; culture time, 48 hours; temperature, 27~29°C.

** Component (g/liter): K_2HPO_4(7), KH_2PO_4(2), $MgSO_4$(0.1), $(NH_4)_2SO_4$(1), sodium citrate (0.5), glucose (2), agar (15).

*** Not determined.

tion of thienamycin by any of the cultures tested (data not shown).

Production of Fluoroacetic Acid in Complex Media

The fermentation broths from the experiment of Table 3 were also examined by ^{19}F NMR in order to determine if any other fluoroorganic compounds besides 4-fluorothreonine were made. Two fluorine-containing compounds were observed by ^{19}F NMR: (1) 4-fluorothreonine made by both NRRL 8057 and MA 5176 and; (2) fluoroacetic acid made by NRRL 8057, MA 5176, and MA 5617. The amount of fluoroacetic acid excreted by the three cultures was estimated by comparison with standards at 2~3 mM.

Resting Cells Studies

Resting cells of *S. cattleya* are known to produce thenamycin[8]. The data presented in Fig. 1 show that resting cells of *S. cattleya* also produced 4-fluorothreonine when incubated with a fluorine-

containing substrate. Growth of the cells in the presence of KF was not required to produce resting cells competent to synthesize 4-fluorothreonine. 4-Fluoroglutamate, fluoroacetate and KF were all used as a source of fluorine for the synthesis of 4-fluorothreonine by MA 5176. Fluorocitrate was not used as a precursor. The diminution of 4-fluorothreonine titer observed after 48 hours in the presence of fluoroacetate was reproducible but its cause is unknown. The supernatant solutions of each of the reaction mixtures (Fig. 1) were examined for the presence of other organofluorine compounds by ^{19}F NMR after 96 hours of incubation; in addition, a similar reaction mixture to which 4-fluorothreonine (10 mM) had been added as substrate was also examined. The results are summarized in Table 4. Both fluoroglutamate and fluorothreonine were converted to fluoroacetate by resting cells of MA 5176. Fluorocitrate appeared to be inert to metabolism by *S. cattleya*. ^{19}F NMR confirmed the synthesis of 4-fluorothreonine by the resting cells.

Biological Properties of 4-Fluorothreonine

The *in vitro* antimicrobial activity of 4-fluorothreonine was examined against a variety of microorganisms on both Davis minimal medium and nutrient agar. The results are shown in Table 5. 4-Fluorothreonine exhibited potent activity against the test organisms on the minimal medium but only poor activity on the complex medium. Since 4-fluorothreonine is an analog of threonine, reversal of its growth inhibition of *Pseudomonas aeruginosa* MB 2835 was examined. The antimicrobial activity of 4-fluorothreonine against *P. aeruginosa* MB 2835 was readily reversed by L-serine or L-threonine while L-alanine, L-cysteine and glycine were partially effective.

4-Fluorothreonine showed a weak PD_{50} (77 mg/kg) in mice infected with *Escherichia coli* Juhl when administered intramuscularly. It was ineffective in mice infected with *Staphylococcus aureus* Smith. The LD_{50} of 4-fluorothreonine in mice was 320 mg/kg when administered intravenously.

Discussion

It has been demonstrated that 4-fluorothreonine is a secondary metabolite of *S. cattleya* when the culture is provided with an exogenous source of fluorine. ^{19}F NMR analysis of the fermentation broths has provided evidence that fluoroacetic acid is excreted by the microorganism under the same conditions. Evidence has also been presented to show that not all strains of *S. cattleya* that can produce thienamycin produce 4-fluorothreonine.

One possible route for the biosynthesis of 4-fluorothreonine is the following:

$$F^- + ? \rightleftharpoons \text{fluoroacetate} \rightleftharpoons \text{fluoroacetaldehyde} \overset{\pm\text{glycine}}{\rightleftharpoons} \text{4-fluorothreonine}$$

If this pathway is correct, MA 5617 is blocked either in the conversion of fluoroacetate to fluoroacetaldehyde or in the condensation reaction with glycine. Additional work will be required, however, to confirm this hypothesis. The ability of resting cells of *S. cattleya* to synthesize 4-fluorothreonine provides a valuable tool for the study of this interesting biosynthetic pathway.

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