NEW STREPTOTHRICIN-GROUP ANTIBIOTICS, AN-201 I AND II

SCREENING, FERMENTATION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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Two streptothricin-group antibiotics, AN-201 I and II, were newly discovered and isolated from the culture broth of *Streptomyces nojiriensis* C-13. These antibiotics were purified by IRC-50 (H^+) and CM-Sephadex C-50 chromatography, and paper electrophoresis. Structural analysis of AN-201 I and II showed that they were N^{β}-acetylated derivatives of streptothricin E and D, respectively. They had antibacterial activities against several strains of *Escherichia coli*, *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*, and showed a strong selective cytotoxic effect on 3T3 cells transformed with SV-40 as compared with their normal cells in a test system *in vitro* as well as *in vivo*.

UDAKA and MIYASHIRO described a novel microbial test system[1] that was applicable to the screening of macromolecular antitumor antibiotics having molecular weights of greater than 700. On application of this new screening program, they found many peptide antibiotics with molecular weights of about 10,000~13,000 amongst microbial products and characterized some of them[2~5].

This paper describes new antibiotics with molecular weights of about 700 which were also obtained by applying this new screening system.

Materials and Methods

Microorganisms

Streptomyces nojiriensis C-13 was employed in this study for the production of AN-201 I and II. *Escherichia coli* W3876 and its macromolecule permeable mutants, MP1 and MP2 were used to estimate the molecular sizes of these antibiotics[1]. Strain MP2 and its DNA repair mutants were used for determining activities of antibiotics[1].

Antitumor Activity *In Vitro*

Normal 3T3 cells and SV-40 transformed 3T3 cells, SV3T3, were cultured in Eagle minimal essential medium supplemented with 10% calf serum, 5 μg/ml of cefazolin and 100 μg/ml of streptomycin. The cell cultures were performed on 2.8 ml tissue culture plates (Falcon 3047, growth area 2.0 cm^2) at 37°C in a 7% CO_2 incubator. The initial cell density was 4×10^3 cells/ml for 3T3 and 2×10^3 cells/ml for SV-3T3.

After 1-day culture, the used medium was replaced by some newly prepared with various concentrations of antibiotics, and the cultures were continued for a further 2 days. The ED_{50} values were determined by measuring the viable cell densities. The N/T ratio equals the ED_{50} of 3T3 divided by the ED_{50} of SV3T3.

Results

Screening of New Antibiotics

Culture fluids of actinomycetes were subjected to the macromolecular antibiotic detecting system in which macromolecule permeable *E. coli* strains, MP1 and MP2, were employed. Of 636 samples tested, 12 samples showed stronger growth inhibitory effects on MP1 or MP2 than on their parent strain, but the effect on MP1 and MP2 was similar. Since antibiotics with molecular weights of less than about 1,200 and 700 are permeable to MP1 and the parental strain, respectively, the molecular weight of these antibiotics seemed to be between 700 and 1,200[1].

By subjecting them to both DNA damage assay and mutagenicity tests[1] the 12 new isolates were classified into two groups according to their activities in the test systems. Seven samples including the one named AN-201 did not exhibit increased toxicity toward a *uvr*A and *rec*A defective strain, UR3. Therefore, these antibiotics were considered not to interact directly with DNA. The other five samples of culture fluids showed increased toxicity both toward REC9 (a *rec*A mutant) and UR3, but not toward UV28 (a *uvr*A mutant), indicating the DNA-degrading property of these antibiotics. Mutagenic activity was not detected for the antibiotics of these two groups.

AN-201 type antibiotics were studied further because they showed greater cytotoxic activity toward 3T3 cancer cells than the parental cells. Strain C-13 of *S. nojiriensis* was chosen as an AN-201-producing organism.

Fermentation

Fig. 1 shows fermentation kinetics of AN-201 production. A slant culture of *S. nojiriensis* C-13 was inoculated into 100 ml of seed medium (Table 1) in a 500-ml flask, and the flask was incubated for 72 hours at 27°C with shaking. The pre-seed culture (3 liters) thus obtained was inoculated into 20 liters of the seed medium which was incubated for 24 hours at 30°C. The whole seed culture was transferred to 280 liters of the production medium (Table 1). The fermentation was carried out for 28.5 hours at 30°C at an agitation speed of 350 rpm and with air flow rate of 1/2 v/v/minute. Antibacterial activity of the fermentation broth was 450 units/ml as determined by the method described in the previous paper[2].

Fig. 1. Time course of antibiotic production by *S. nojiriensis* C-13.

The fermentation was carried out in a 300-liter jar-fermentor. The pH was controlled with NaOH. (●), Antibacterial activity; (○), growth (DCW stands for dry cell weight); (△), glucose; (▲), pH.

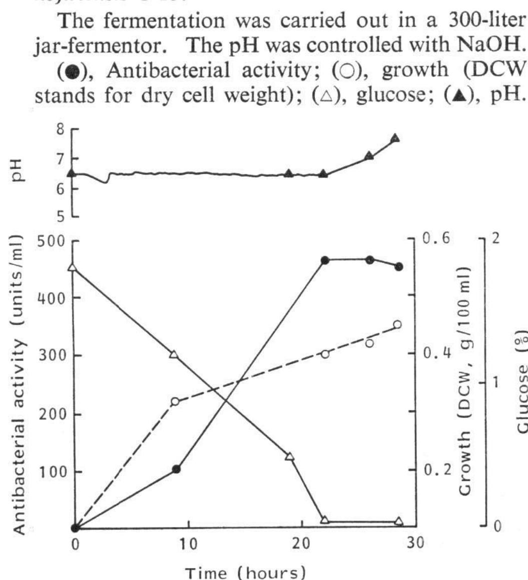


Table 1. Composition of fermentation media.

Component	Seed medium (%)	Production medium (%)
Glucose	1.0	2.0
Starch	1.0	
Polypeptone	0.5	0.5
Meat extract	0.5	1.0
Dried yeast		0.5
NaCl	0.3	0.3
K_2HPO_4	0.1	0.1
$CuSO_4 \cdot 5H_2O$	0.001	
$FeSO_4 \cdot 7H_2O$	0.001	
$MnCl_2 \cdot 4H_2O$	0.001	
$ZnSO_4 \cdot 7H_2O$	0.0002	
$MgSO_4 \cdot 7H_2O$	0.1	
pH	7.0	7.2

Isolation

Mycelia were removed by centrifugation of the culture liquid, and the pH of the supernatant was adjusted to 7.3 with HCl. The supernatant (280 liters) was directly applied to a column (volume: 10 liters) of IRC-50 (H^+). The column was washed with 20 liters of H_2O. Then elution was made with 0.2 N HCl. After harvesting of about 43 liters of the eluate, its pH was adjusted to 5.0 with NaOH. It was concentrated to 3 liters by evaporation and lyophilized to give 450 g of a dry powder. The powder was dissolved in 2 liters of 50% methanol and insoluble materials were removed by centrifugation. After the soluble fraction was dried with a rotary evaporator, the dry matter was dissolved in 500 ml of methanol. To the methanol solution, 2.5 liters of acetone was added to obtain 10.2 g of a precipitate containing antibiotics. The precipitate was dissolved in 100 ml of 0.5 M pyridine - acetate - H_2O buffer (pH 4.8) and the solution was passed through a column of 400 ml of CM-Sephadex C-50 (Pharmacia). The column was washed with 400 ml of the same buffer. The elution was made with 6.8 liters of 1 M pyridine - acetate - H_2O, followed by 6.4 liters of 2 M pyridine - acetate - H_2O buffer and 500 ml of 4 M pyridine - acetate - H_2O buffer. Substances A′ and B′ were eluted with the 1 M buffer in fraction No. 132~170 and No. 172~340, respectively, and substances C′, A and B with the 2 M buffer in the fraction No. 342~360, No. 372~410 and No. 482~522, respectively. Substance C was obtained with 4 M buffer in fraction No. 572~620. All these samples were lyophilized and subjected to further purification by paper electrophoresis.

The paper electrophoresis was carried out for 1.5 hours at 30 V/cm employing Toyo filter paper No. 51, using pyridine - acetate - H_2O (10: 100: 890) as the electrophoresis buffer. All these samples moved toward the cathode, and the relative mobilities of A′, B′, C′, B and C were 0.71, 0.79, 0.89, 1.03 and 1.08, respectively, as compared to 1.00 of A. The six separated components of antibiotics were eluted with water, and lyophilized to give about 65 mg of A′, 200 mg of B′, 220 mg of C′, 400 mg of A, 420 mg of B and 140 mg of C in the form of the acetate salt.

Chemical Structure

Chemical structures of these antibiotics were determined as shown in Fig. 2. The antibiotics, A, B and C, were identified as streptothricin F, E and D, respectively. They all had a β-lysine moiety, 1 mol in streptothricin F, 2 mol in streptothricin E, and 3 mol in streptothricin D. The structures of A′, B′ and C′ were the same as those of A, B and C, respectively, except that they had 1 mol of *N*-acetyl group.

Of them, streptothricin F, E and D had been already found in a microbial culture fluids,[6~9] and N^{β}-acetylstreptothricin F had never been found in a microbial culture fluid but was chemically synthesized from streptothricin F[10]. N^{β}-Acetylated derivatives of streptothricins E and D, named AN-201 I and II, were not known before.

Details concerning the determination of the chemical structures of these antibiotics will be described in a subsequent paper.

Biological Properties

Antibacterial Activity

These antibiotics belonging to the streptothricin group were compared with each other particularly in connection with the influence of β-lysine residues and their N^{β}-acetylation on the activity as shown in Table 2. The results were not so clear cut, but could be interpreted as follows: 1) as the number of β-lysine residues increases, the antibacterial activity of antibiotics becomes more potent, and 2) N^{β}-acetylation of the β-lysine moiety markedly weakens the activity.

Table 2. Antibacterial spectra of streptothricin group antibiotics.

Microorganism	MIC (μg/ml)					
	A -L*	B -L-L	C -L-L-L	A′ -AL	B′ -AL-L	C′ -AL-L-L
E. coli W3876	1.2	1.2	1.2	>200	80	35
〃 MP1	1.0	1.0	0.3	>200	21	5.0
〃 MP2	1.0	1.0	0.3	>200	20	4.0
〃 UR3	1.2	1.3	0.4	>200	23	6.0
Pseudomonas aeruginosa ATCC 10145	100	100	100	>200	>200	>200
Bacillus subtilis ATCC 6633	0.2	0.1	0.1	>200	2.7	1.5
Micrococcus luteus ATCC 9341	2.0	1.5	1.5	>200	5.0	5.0
Staphylococcus aureus FDA 209P	0.2	0.2	0.1	>200	2.0	2.0

A: Streptothricin F, B: streptothricin E, C: streptothricin D,
A′: N^{β}-acetylstreptothricin F, B′: AN-201 I, N^{β}-acetylstreptothricin E,
C′: AN-201 II, N^{β}-acetylstreptothricin D.
* Underneath each streptothricin, the side chain is abbreviated as follows: L, β-lysine; AL, N^{β}-acetyl-β-lysine.

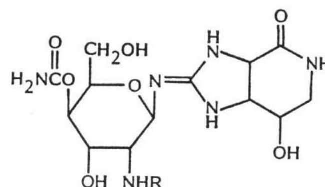
Antibiotics of the streptothricin group, including the newly found AN-201 I and II, had only a very weak activity against *Pseudomonas aeruginosa*.

Effect on Tumor Cells

As in the case of antibacterial activity, an increase in β-lysine residues in the molecule enhanced the cytotoxicity toward both 3T3 and SV3T3. N^{β}-Acetylation of β-lysines brought about a decrease in the cytotoxic effect of streptothricins, although the extent was far less than that for the antibacterial activity. Every antibiotic showed a selective cytotoxicity with an N/T ratio ranging between 1.3 and 4.5 (Table 3, Figs. 3 and 4).

The size of transplanted Lewis lung carcinoma cells was reduced by about 60% by injecting intraperitoneally 1~3 mg/kg of AN-201 II or streptothricin E into a mouse daily for 10 days. Nine mg/kg of AN-201 II gave a 74% reduction of tumor cells under similar conditions. Streptothricin F, D, N^{β}-acetylstreptothricin F and AN-201 I were much less effective.

Fig. 2. Chemical structures of streptothricin group antibiotics.
(A): Streptothricin F,
(A′): N^{β}-acetylstreptothricin F,
(B): Streptothricin E,
(B′): AN-201 I (N^{β}-acetylstreptothricin E),
(C): Streptothricin D,
(C′): AN-201 II (N^{β}-acetylstreptothricin D).



R = ←β-lys (-L) (A)
←β-lys-β-lys (-L-L) (B)
←β-lys-β-lys-β-lys (-L-L-L) (C)
←β-Aclys (-AL) (A′)
←β-Aclys-β-lys (-AL-L-L) (B′)
←β-Aclys-β-lys-β-lys (-AL-L-L) (C′)

Table 3. Selective cytotoxic effect of streptothricin group antibiotics on 3T3 cells.

Antibiotic*	ED_{50} (μg/ml)		N/T ratio
	3T3(N)	SV3T3(T)	
A -L	60	40	1.5
B -L-L	45	10	4.5
C -L-L-L	20	5	4.0
A′ -AL	65	45	1.4
B′ -AL-L	55	25	2.2
C′ -AL-L-L	50	15	3.3

* See Table 2 for explanation of symbols.

Discussion

Streptothricin was one of the earliest antibiotics with a broad antimicrobial spectrum found in

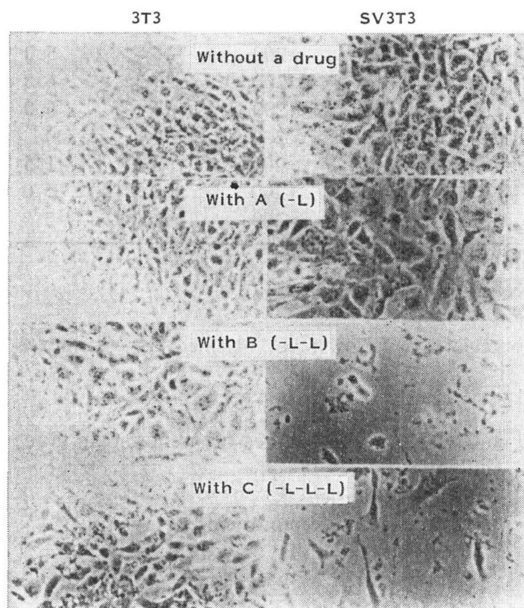


Fig. 3. Cytotoxic effect of streptothricin F, E and D on 3T3 and SV3T3 cells.

Experimental conditions are given in Materials and Methods. The concentration of antibiotics was 10 μg/ml.

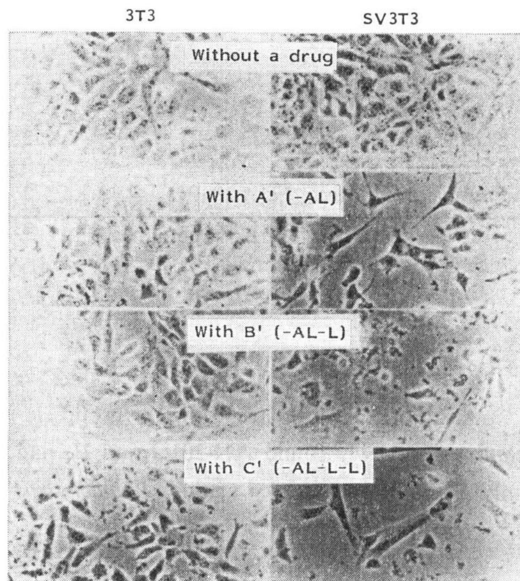


Fig. 4. Cytotoxic effect of N^{β}-acetylstreptothricin F, E (AN-201 I) and D (AN-201 II) on 3T3 and SV3T3 cells.

Experimental conditions are given in Materials and Methods. The concentration of antibiotics was 40 μg/ml.

actinomycetes and had been studied extensively[6,7]. Streptothricins are known to contain a heterocyclic β-amino acid (streptolidine), an aminosugar (*d*-gulosamine), and several β-lysines in their side chains[9].

We found six kinds of antibiotics in the culture broth of *S. nojiriensis* C-13. Two of them, N^{β}-monoacetylated streptothricin E and D, are new antibiotics, although N^{β}-monoacetylated streptothricin F was prepared from streptothricin F chemically[10,11].

The results described in this paper seem to be of great value for elucidating the structure-activity relationship of the streptothricin-group antibiotics (Table 2). The amino group of the β-lysine residue was essential for the antibacterial activity of the antibiotics, because antibacterial activity of streptothricin F drastically diminished with the acetylation of the amino group of the β-lysine moiety. Antibacterial activity of streptothricin E and D also decreased markedly with the same acetylation. However, these antibiotics retained some antibacterial activity probably owing to their other β-lysine moiety, whose amino group was not acetylated. Therefore, β-lysine seemed to have a critical role in manifestation of the antibacterial activity of this group of antibiotics. For unknown reasons, addition of a β-lysine moiety to streptothricin E or its acetylated compound increased more profoundly their antibacterial activities toward *E. coli* than toward other bacteria tested.

The cytotoxic effect of the *N*-acetylated form of the streptothricins exhibited a striking contrast to their antibacterial effect. For instance, streptothricin F and its *N*-acetylated compound showed a cytotoxic effect on both 3T3 and SV3T3 cells, to the same extent, but that was not the case for the antibacterial activity. It is noteworthy that deacetylation probably proceeds more rapidly in SV3T3 cells than in bacterial cells to the active form of streptothricins. Thereby, the transformed cells might be more sensitive to AN-201 (I or II) compared to 3T3 cells.

The *in vitro* results that AN-201 I and II had selective toxicity toward cancer cells were roughly reproduced with the *in vivo* system of Lewis lung carcinoma but not that of L1210 cells. Since AN-201 II showed a potent *in vivo* antitumor activity, it may have some practical value.

Acknowledgments

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