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Mechanism of Action and Selective Toxicity of Ascamycin, a Nucleoside Antibiotic

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An unidentified *Streptomyces* sp. produces two nucleoside antibiotics, ascamycin and its dealanyl derivative. In contrast to the broad antibacterial activity of dealanylascamycin against various gram-negative and gram-positive bacteria, ascamycin showed selective toxicity against *Xanthomonas citri* and *X. oryzae*. Both ascamycin and dealanylascamycin inhibited the protein synthesis of *X. citri*, but only dealanylascamycin inhibited that of *Escherichia coli*. In cell-free systems from *E. coli* and *X. citri*, both antibiotics, at ca. 0.04 μg/ml, inhibited the polyuridylate-directed synthesis of polyphenylalanine by ca. 50%. These data suggest that ascamycin cannot permeate the bacterial membrane. The dealanylating activity toward ascamycin was found only on the cell surface of bacteria susceptible to ascamycin. Dealanylascamycin must then have been transported into cytoplasm, where it inhibited protein synthesis.

Recently, we have reported the isolation and the structure of a new nucleoside antibiotic, ascamycin (Fig. 1A), from the culture filtrate of a *Streptomyces* sp. (3). The same strain also produces the dealanyl derivative (Fig. 1B) which is indistinguishable from the antibiotic AT-265 (9). In contrast to the broad antibacterial activity of dealanylascamycin against various gram-positive and gram-negative bacteria, ascamycin showed selective antibacterial activity against only *Xanthomonas citri* and *X. oryzae* among the microorganisms tested.

To elucidate the remarkable biological difference between ascamycin and dealanylascamycin, we studied the mechanisms of action of both compounds and the dealanylating activity toward ascamycin on the cell envelope of bacteria susceptible to ascamycin. The results showed that ascamycin could not permeate the cell membrane. After dealanylation, however, the dealanylascamycin became permeable and showed antibacterial activity.

MATERIALS AND METHODS

Pure crystalline ascamycin and dealanylascamycin were prepared as previously described (3) and used for experiments on the basis of weight. [*methyl*-^{3}H]thymidine (670 mCi/mmol), [2-^{14}C]uridine (59 mCi/mmol), and L-[*U*-^{14}C]phenylalanine (504 mCi/mmol) were purchased from New England Nuclear Corp. and used for experiments without dilution. Polyuridylic acid was obtained from Yamasa Shoyu Co., Ltd. *Escherichia coli* tRNA was a product of Sigma Chemical Co.

Bacterial strains. *Staphylococcus aureus* IFO12732 and *Pseudomonas aeruginosa* IFO13130, which were purchased from the Institute for Fermentation, Osaka, Japan, were cultured at 37°C. Plant pathogenic bacteria, *X. citri* IFO3781 and *X. oryzae* IFO3312 (from the Institute for Fermentation) were cultured at 28°C. *Bacillus subtilis* ATCC 6051 was obtained from the American Type Culture Collection, Rockville, Md. *E. coli* BE1186 (*thr leu pro his arg thi lac gal ara xyl mtl tsx str sup uvrA ruv tolC*) (6) was a gift from Dr. Kawamata (Osaka University). *Salmonella typhimurium* TV119 (*metA trpB fra str rfb-430*) (10) was given by Dr. Izaki (Tohoku University).

Antibacterial activity. The susceptibility of bacteria to ascamycin and dealanylascamycin was determined by the conventional paper disk-agar plate method. Paper disks contained 4 μg of the antibiotic.

Macromolecular synthesis of bacterial cells. *E. coli* BE1186 and *X. citri* IFO3781 were grown aerobically at 30°C to an optical density at 550 nm (OD_{550}) of 0.6 in an L-shaped Monod tube containing 5 ml of the complete medium. Cells were washed with saline and transferred into 5 ml of the supplemented minimal salt medium containing 4 g of glycerol, 8.8 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, 1 g of

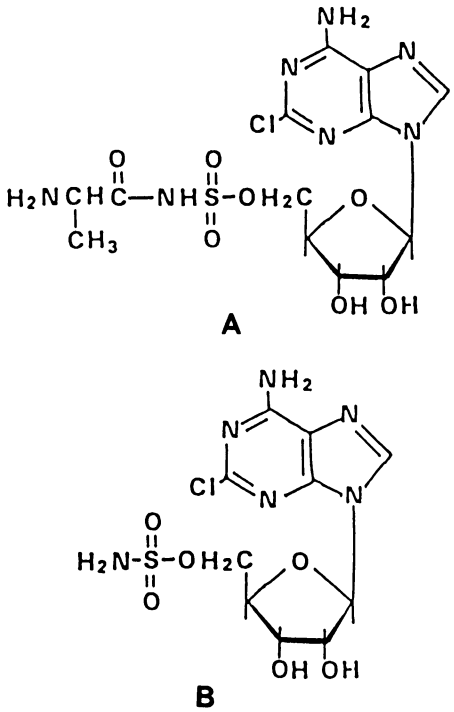

FIG. 1. Structures of ascamycin (A) and dealanylascamycin (B).

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TABLE 1. Antimicrobial spectra of ascamycin and dealanylascamycin[a]

Organism	Diam of inhibition zone (mm) treated with:	
	Ascamycin	Dealanyl-ascamycin
Staphylococcus aureus IFO12732	–[b]	12
Bacillus subtilis ATCC 6051	–	11
Escherichia coli BE1186	–	22
Salmonella typhimurium TV119	–	21
Pseudomonas aeruginosa IFO1313	–	30
Xanthomonas citri IFO3781	37	39
Xanthomonas oryzae IFO3312	29	33

[a] Paper disks (diameter, 8 mm) were used containing 4 μg of the antibiotic.
[b] –, No effect.

NH_4Cl, and 20 mg of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water.

[^{3}H]thymidine, [^{14}C]uridine, or [^{14}C]phenylalanine (1 μCi) was added to 2 ml of the culture. Antibiotics were added 5 min before addition of the radioactive compounds. After the addition of labeled precursors, 0.1-ml samples of the culture were added to 5 ml of 10% ice-cold trichloroacetic acid at appropriate intervals. The acid-insoluble fractions were collected on glass filters (GF/C; diameter, 25-mm; Whatman, Inc.), which were then washed and dried. The filters were counted in a toluene-Triton X-100 scintillator.

Preparation of S-30 fractions of *X. citri* and *E. coli* and polyuridylate-directed polyphenylalanine synthesis in vitro. The S-30 fraction was prepared as described by Nirenberg and Matthaei (5). Polyuridylate-directed polyphenylalanine synthesis was assayed as follows. Each 500-μl portion of the reaction mixture contained 50 mM Tris-hydrochloride (pH 7.5), 60 mM NH_4Cl, 15 mM magnesium acetate, 1 mM ATP, 50 μM GTP, 5 mM phosphoenolpyruvate (pH 7, adjusted by KOH), 20 μg of pyruvate kinase, 200 μg of *E. coli* tRNA, 30 μg of polyuridylate, 1 μCi of [^{14}C]phenylalanine, 5 OD_{280} units of S-30 fraction, and various amounts of inhibitors. Thereaction mixture was incubated at 30°C and then stopped by 25% trichloroacetic acid solution containing 2% casein. The radioactivity of the trichloroacetic acid-insoluble fraction in a 50-μl sample was measured by a liquid scintillation counter.

Assay for dealanylation of ascamycin. *X. citri* IFO3781, *E. coli* BE1186, and *B. subtilis* ATCC 6051 were grown in nutrient broth containing 0.2% yeast extract. Cells were collected at the late-logarithmic-growth phase and washed with buffer A (10 mM Tris-hydrochloride [pH 7.6], 10 mM $MgCl_2$, 100 mM KCl, 2 mM 2-mercaptoethanol).

Packed cells were disrupted by a sonicator (UR-200P, Tomy Seiko Co., Ltd.). After unbroken cells were removed, the extract was centrifuged at 100,000 × *g* for 1 h. The supernatant and the precipitate were regarded as cytoplasmic and envelope fractions, respectively. Each fraction was suspended in 10 mM Tris-hydrochloride (pH 7.6) at a concentration of 10 OD_{280} units per ml (ca. 10 mg/ml) and assayed for ascamycin-dealanylating activity. The reaction mixture contained 0.1 OD_{280} units of the subcellular fraction and 2 μg of ascamycin in buffer A. It was incubated at 37°C for 90 min and examined by cellulose thin-layer chromatography (solvent system, isopropanol–1 N ammonia [7:3, vol/vol]). The conversion to dealanylascamycin was detected by the alteration of the R_f value (from ascamycin [0.57] to dealanylascamycin [0.50]) and the appearance of the antibacterial activity against *E. coli* BE1186. As *E. coli* BE1186 is resistant to ascamycin and susceptible to dealanylascamycin, the conversion from ascamycin to dealanylascamycin can be determined by the appearance of antibacterial activity against *E. coli* BE1186.

RESULTS

Antimicrobial activity of ascamycin and dealanylascamycin on solid media. Ascamycin (4 μg per disk) had detectable activity against only *X. citri* and *X. oryzae* among the strains used. In contrast to ascamycin, dealanylascamycin showed

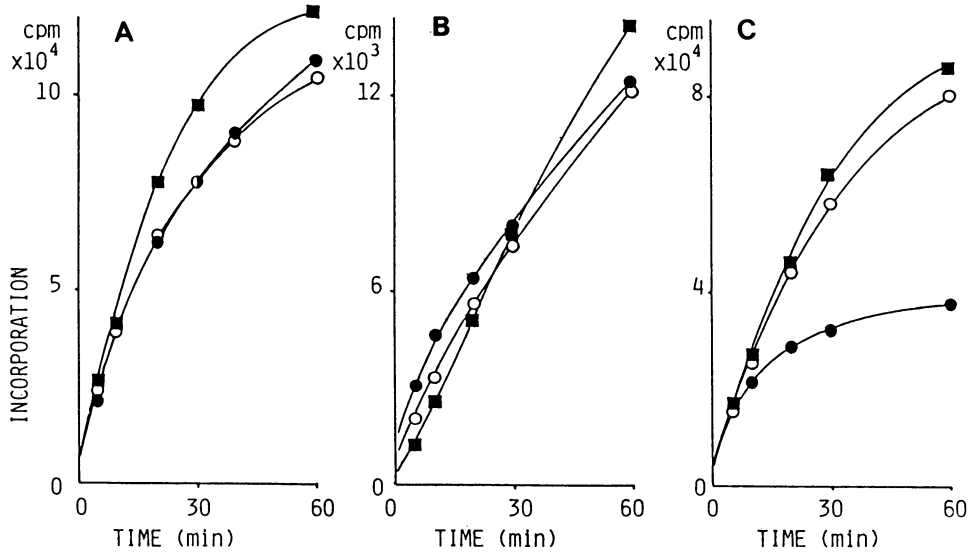

FIG. 2. Time course of macromolecular synthesis of *E. coli* BE1186 growing in minimal medium. The following labeled compounds (0.5 μCi/ml) were added to the medium: (A) thymidine, (B) uridine, and (C) phenylalanine. Symbols: ■, control; ○, 2 μg of ascamycin per ml; ●, 2 μg of dealanylascamycin per ml.

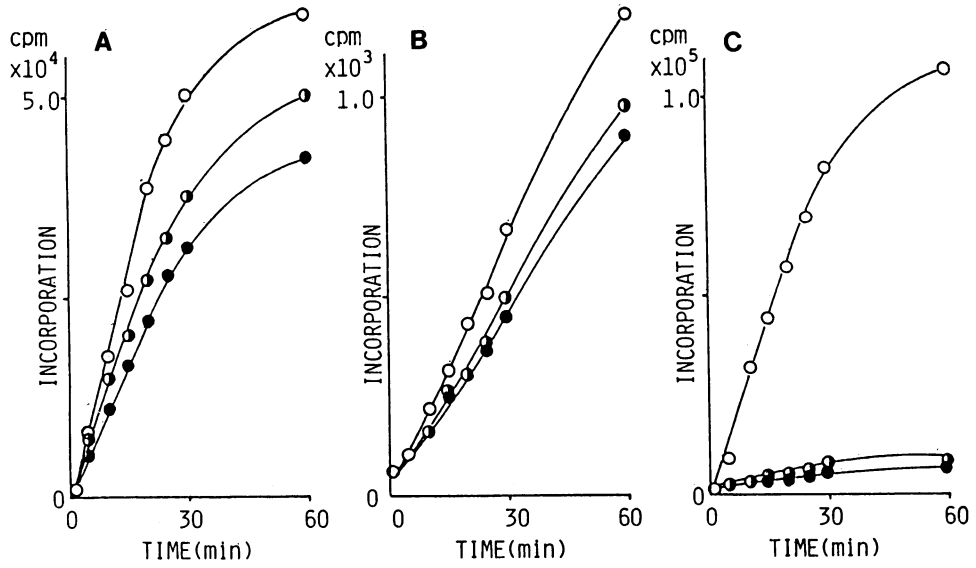

FIG. 3. Effect of ascamycin on macromolecular synthesis of *X. citri* IFO3781 intact cells growing in minimal medium. The following labeled compounds (0.5 μCi/ml) were added to the medium: (A) thymidine, (B) uridine, and (C) phenylalanine. Symbols: ○, control; ◑, 0.5 μg of ascamycin per ml; ●, 5 μg of ascamycin per ml.

strong activity against various bacteria as shown in Table 1. Dealanylascamycin was more effective against gram-negative than against gram-positive bacteria, because the effects of dealanylascamycin against the *S. aureus* and *B. subtilis* strains were minimal.

Effects of ascamycin and dealanylascamycin on macromolecular synthesis. The effects of ascamycin and dealanylascamycin on macromolecular synthesis in *E. coli* were measured by the incorporation of radioactive thymidine, uridine, and phenylalanine into acid-insoluble fractions. When dealanylascamycin at 2 μg/ml (5.3×10^{-6} M) was added to a growing culture of *E. coli*, inhibition of protein biosynthesis occurred. However, no effect on DNA and RNA biosynthesis was observed. Ascamycin had no influence on the incorporation of labeled precursors into *E. coli* (Fig. 2). In contrast to *E. coli*, incorporation of phenylalanine into *X. citri* was strongly inhibited by both ascamycin and dealanylascamycin (Fig. 3).

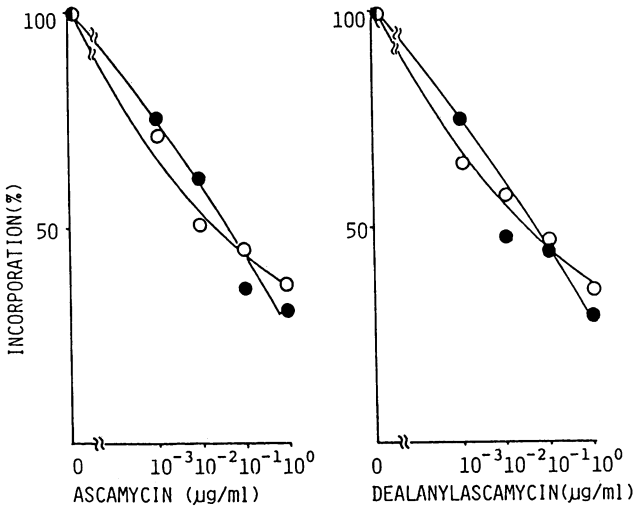

FIG. 4. Polyuridylate-directed polyphenylalanine synthesis by cell-free systems from *E. coli* BE1186 and *X. citri* IFO3781. Symbols: ○, S-30 fraction from *E. coli* BE1186; ●, S-30 fraction from *X. citri* IFO3781. Reaction mixtures were incubated at 30°C for 30 min, and the radioactivity in the trichloroacetic acid-insoluble fraction was measured. The incorporation ratio of the ascamycin-treated fraction to the nontreated fraction (control) was plotted against the concentration of ascamycin.

Inhibition of polyuridylate-directed polyphenylalanine synthesis. The data with intact cells indicated that the primary action of both ascamycin and dealanylascamycin was at the level of protein synthesis. To investigate why growth and protein synthesis were inhibited by ascamycin in *X. citri* but not *E. coli*, we examined the effects of ascamycin and dealanylascamycin on polyuridylate-directed polyphenylalanine synthesis in cell-free extracts from both organisms (Fig. 4). Both compounds had an equivalent inhibitory effect on cell-free protein synthesis in both extracts; at ca. 1.0×10^{-7} M (0.03 to 0.05 μg of either ascamycin or dealanylascamycin per ml), incorporation of phenylalanine was reduced by ca. 50%. These data suggest that ascamycin penetrated *X. citri* but not *E. coli*. Presumably, dealanylascamycin penetrates both species and inhibits protein synthesis and growth of intact cells.

Enzymic conversion of ascamycin to dealanylascamycin. The dealanylation of ascamycin was examined by using subcellular fractions of *B. subtilis* and *E. coli*, which are resistant to ascamycin, and of *X. citri*, which is susceptible to ascamycin. Ascamycin (final concentration, 100 μg/ml) was added to each subcellular fraction (0.1 OD_{280} units). After 90 min of incubation, the reaction mixture was applied for cellulose thin-layer chromatography. Dealanylation was detected by the decrease of the R_f value and the appearance of activity against *E. coli* BE1186.

Only the envelope fraction of *X. citri* had activity which converted ascamycin to dealanylascamycin (Fig. 5). The envelope fractions from the ascamycin-resistant organisms had no such activity. The cytoplasmic fractions from both

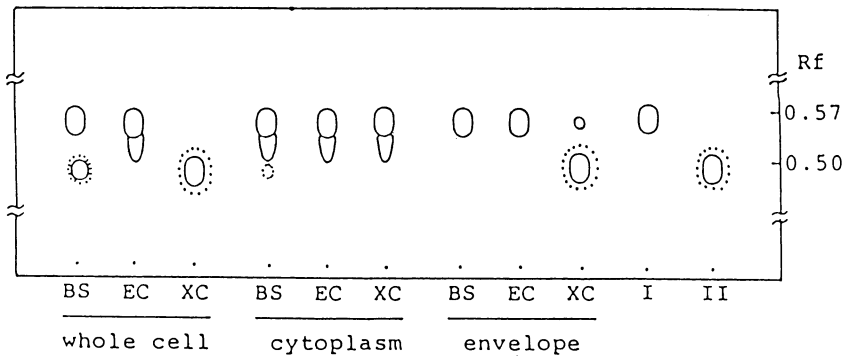

FIG. 5. Dealanylation of ascamycin by cell fractions as examined by cellulose thin-layer chromatography (solvent system, isopropanol–1 N ammonia [7:3, vol/vol]). Abbreviations: BS, *B. subtilis*; EC, *E. coli*; XC, *X. citri*; I, ascamycin; II, dealanylascamycin. Symbols: ○, detected by UV; ⁘, detected by antibacterial activity, with *E. coli* BE1186 as an indicator strain.

ascamycin-susceptible and -resistant organisms showed weak dealanylating activity. The membrane-bound dealanylating enzyme from *X. citri* is presumed to be novel by preliminary characterization. The dealanylation of ascamycin was not observed with a commercially available leucine aminopeptidase (data not shown).

DISCUSSION

Prodrugs such as bacilysin and alaphosphin include an antibacterial moiety whose uptake into the bacterial cell is facilitated by incorporation with another moiety to form a more readily transported molecule (7). For example, bacilysin, a dipeptide antibiotic consisting of alanine and anticapsin (8), is effectively transported into cells. Bacilysin is then hydrolyzed to alanine and the inhibitory agent anticapsin (4). Similarly, alaphosphin (L-alanyl-L-1-aminoethylphosphonic acid) is a synthetic antibacterial agent which mimics a peptide (2). Its activity depends on membrane transport of the dipeptide and on intracellular metabolism to release an active moiety, L-1-aminoethylphosphonic acid (1). In both cases, alanine facilitates penetration of the active agent through the membrane.

The presence of alanine in ascamycin appears to inhibit, rather than facilitate, the penetration of the dealanyl derivative into *E. coli*. Although dealanylascamycin is presumably transported by a nucleoside transport system, alanylation of the sulfamoyl nucleoside apparently interferes with such transport.

The demonstration of dealanylating activity in the cell envelope of *X. citri*, which is susceptible to ascamycin, but not in the cell envelopes from ascamycin-resistant organisms suggests that the difference in susceptibility lies not in the ability to transport ascamycin, but rather in the ability to transform ascamycin into a transportable moiety. The dealanylating enzyme from the cell envelope of *X. citri* is currently undergoing purification for further study.

The observation that alanine blocks uptake and that some organisms can remove it and thereby stimulate uptake raises the possibility of selectively targeting toxic agents to some organisms, but not others, by alanylating the active compound. Of course, the effectiveness of this tactic will depend on the characteristics of the transport and dealanylating mechanisms of the organisms under consideration.

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