Antibiotics A21459 A and B, New Inhibitors of Bacterial Protein Synthesis

I. Taxonomy, Isolation and Characterization

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Novel cyclic peptide antibiotics A21459 A and B are produced by a member of the genus *Actinoplanes* sp. These antibiotics inhibit bacterial protein synthesis and have selective antimicrobial activity against clostridia, mycoplasma and some Gram-negative bacteria.

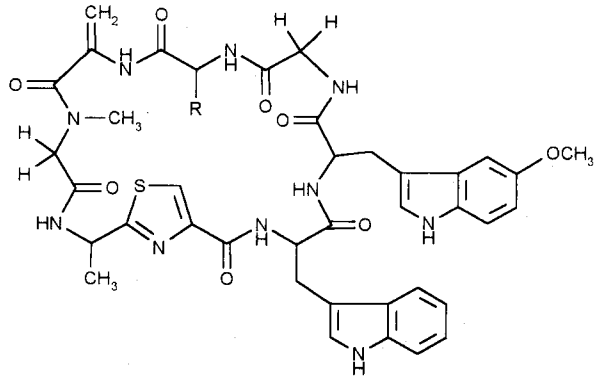
In the course of a screening program for new antibacterial agents produced by rare actinomycetes, we discovered the novel antibiotics A21459 A and B. These antibiotics have cyclic peptide structures[1] which are shown in Fig. 1. We describe the taxonomy and fermentation of the producer strain, isolation of the antibiotics and their biological activity.

Materials and Methods

Taxonomy of Producing Strain

The enantiomeric form of diaminopimelic acid was determined by paper chromatography of whole-cell hydrolysate[2]. Whole-cell sugars were determined by gas chromatography[3]. Fatty acids and menaquinones were analyzed according to SADDLER *et al.*[4] and O'DONNELL *et al.*[5], respectively.

Fig. 1. Structure of A21459 A and B.



R=$-CH_2-CH_3$ A21459 A
R=$-CH_3$ A21459 B

Fermentation

The strain was grown on a seed medium consisting of starch noredux 2.4%, glucose 0.1%, yeast extract 0.5%, soybean meal 0.5%, tryptone 0.5%, beef extract 0.4% and $CaCO_3$ 0.5%. A 72-hour culture in Erlenmeyer flasks was inoculated (5%) into 4 liters and, after 48 hours incubation, into 30 liters of seed medium. A 48-hour culture was then inoculated into 200 liters of production medium consisting of glucose 2.5%, soybean meal 1%, yeast extract 0.1%, peptone 0.4%, beef extract 0.4% and $CaCO_3$ 0.5%. The 4, 30 and 200-liter fermenters were run at 28°C with 1 v/v/minute aeration and 900, 500 and 250 rpm stirring, respectively.

Agar Diffusion Assay

Production was monitored with agar diffusion assay using *Neisseria caviae* ATCC 14659 grown overnight at 37°C in Todd Hewitt agar (Difco).

Isolation

Harvested broth was filtered and mycelia were extracted with methanol-acetone 1:2 (v/v). The extract was then concentrated to water residue and was extracted at pH 7 with ethyl acetate. A crude preparation of A21459 complex was obtained upon addition of petroleum ether to the concentrated organic phase. The complex and individual factors were then purified by subsequent rounds of flash chromatography on silica gel eluted with mixtures of ethyl acetate and methanol.

HPLC Analysis

The purification process was monitored by HPLC on a 250 × 4.6 mm Bakerbond Octyl (5 m) column eluted at 1.8 ml/minute flow rate with a 20 minutes linear gradient from 10% to 50% of phase B. Phase A and B were 40 mM $HCOONH_4$-CH_3CN-tetrahydrofuran 80:10:10 and

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20 : 40 : 40 (v/v), respectively. Factor A and B eluted with 12.3 and 11.6 minutes retention times, respectively. UV detection was at 230 nm.

Antimicrobial Activity

MICs for *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus* spp., *Salmonella typhimurium*, *Shigella dysenteriae* and *Pseudomonas aeruginosa* were determined by broth microdilution in Iso-Sensitest broth (Oxoid). Other organisms tested by broth microdilution included streptococci (Difco Todd Hewitt broth), *Bacillus subtilis* (Difco Penassay broth), *Neisseria gonorrhoeae* (Difco GC Base broth +1% BBL IsoVitaleX, incubation in 5% CO_2), *Clostridium perfringens* (Difco AC broth, anaerobic incubation), *Neisseria caviae* (Difco Brain Heart Infusion broth) and *Candida albicans* (Difco Yeast Nitrogen Base broth). For the following organisms, MICs were determined by agar dilution: *Haemophilus influenzae* (Difco GC Base agar +1% IsoVitalex and 0.001% hemin, incubation in 5% CO_2); *Clostridium difficile*, *Bacteroides fragilis* and *Propionibacterium acnes* (Difco Wilkins-Chalgren agar, anaerobic incubation). MIC for *Mycoplasma gallisepticum* was determined by broth macrodilution using Difco PPLO broth +1% glucose and 10% horse serum. Inoculum for *M. gallisepticum* was 1% (v/v) of a 48 hours broth culture; inocula for other organisms were $10^4 \sim 10^5$ colony-forming units (CFU) per ml or per spot. *C. albicans* was incubated at 30°C, all other organisms at 37°C. *N. gonorrhoeae*, *P. acnes*, *C. difficile*, *B. fragilis* and *M. gallisepticum* were incubated for 48 hours; all other organisms were incubated for 24 hours. MBCs were determined using a broth macrodilution method (media as above) and inocula of approximately 10^6 CFU/ml. MBC was taken as the lowest concentration that killed 99.9% of inoculum in 24 hours.

Mechanism of Action

Hyperpermeable mutant of *S. typhimurium*, TA1538[6] was grown in M9 broth[6], supplemented with glucose 40 g, L-histidine 100 mg and biotin 1.2 mg in 1 liter of medium. Cultures in logarithmic growth were utilized when cell density reached approximately 10^7 CFU/ml. With the exception of protein synthesis (TCA-precipitated samples heated for 15 minutes at 75°C), macromolecular syntheses were followed as cold 5% TCA-precipitable material retained by Sartorius SM glass fiber filters. Instafluor II (Packard) was used as the scintillation liquid. For DNA synthesis, Me-[^{3}H]-thymidine 2 μCi and deoxyadenosine 50 μg were added in 1 ml of medium; for RNA synthesis, [^{3}H]-uracil 1 μCi, 5 μg; for protein synthesis, [^{3}H]-phenylalanine 1 μCi, 1 μg; for cell wall peptidoglycan, [^{3}H]-N-acetylglucosamine 4 μCi, 7 μg in 1 ml of medium.

Mutation to Resistance

An overnight culture of *P. vulgaris* ATCC 881 was diluted to approximately 10^2 CFU/ml in Oxoid No. 2 Nutrient broth and 0.5 ml aliquots were distributed into 22 tubes. After incubation at 37°C for 18 hours, two tubes were pooled for determination of total cell count and the contents of each of the remaining tubes were plated by agar inclusion onto plates containing 50 μg/ml of A21459. Colonies were counted after 18 hours incubation at 37°C. The rate of mutation to resistance was calculated by the LURIA-DELBRÜCK method[7].

Results

Taxonomy of the Producing Strain

The strain A21459 contained *meso*-diaminopimelic acid as the sole peptidoglycan diamino acid. Sugar analysis showed xylose and a complex mixture of ribose, arabinose, mannose, galactose and glucose. Fatty acid profile consisted of *iso*- and *anteiso*-branched chain and straight chain fully saturated acids. 13-methyltetradecanoic acid, 14-methylpentadecanoic acid and *n*-octadecanoic acid, were the prominent constituents. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and monoacylphosphatidylinositol dimannoside were the major polar lipids found. The only isoprenoid quinone found was a tetrahydrogenated menaquinone with nine isoprene units. Microscopic examination of vegetative mycelium revealed the presence of branched hyphae bearing globular sporangia (Fig. 2) with irregular shape. The strain A21459 was classified as *Actinoplanes* sp. in accordance with VOBIS[8].

Fermentation

Maximal production was obtained after 72 ~ 96 hours of incubation. The strain produced 11.5 mg/liter of a complex of A21459 A and B present in equal amounts.

Isolation and Physico-chemical Properties

A21459 A and B, once isolated, had very low solubility in water and showed no ionizable functions either in water or in non-aqueous solvents. The antibiotics gave

Fig. 2. Light micrograph of sporangia of strain A21459 grown on soil agar medium for 7 days at 28°C.

Bar represents 10 μm.

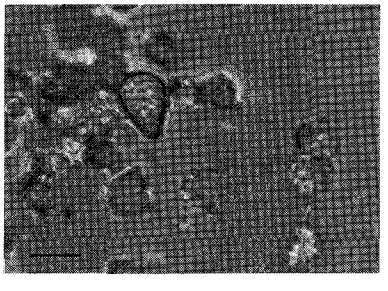


Table 1. Physico-chemical properties.

	Molecular formula	MW	UV maxima*
A21459 A	$C_{41}H_{46}N_{10}O_8S$	838	280, 290 nm
A21459 B	$C_{40}H_{44}N_{10}O_8S$	824	280, 290 nm

* in 0.1 N NaOH and 0.1 N HCl.

positive colour reaction upon heating with 4-methoxybenzaldehyde-H_2SO_4-ethanol 1:1:1 (v/v). Table 1 contains their molecular formulas, molecular weights and UV maxima. Structure determination is described in the accompanying paper[1].

Biological Activity

Because no differences in activity were seen between A21459 A and B, we report the antimicrobial activity of the complex. As shown on Table 2, A21459 was most active against a Gram-positive anaerobe (*Clostridium difficile*) and a fastidious Gram-negative organism (*Neisseria caviae*), with MICs of 0.03~0.13 μg/ml, but it was significantly less active against *Clostridium perfringens* and other fastidious Gram-negative species (*Neisseria gonorrhoeae*, *Haemophilus influenzae*), with MICs ranging from 8~64 μg/ml. Among other Gram-positive bacteria, A21459 was active against *Mycoplasma gallisepticum* and had slight activity against *Staphylococcus aureus*, but was inactive against streptococci, *Enterococcus faecalis*, *Bacillus subtilis*, *Propionibacterium acnes* and *Mycobacterium tuberculosis*. Among other Gram-negative organisms, *Pseudomonas aeruginosa* was moderately susceptible, as were various species of *Proteus*, although other members of the Enterobacteriaceae were uniformly resistant, with the exception of hyperpermeable mutants. The antibiotic was bacteriostatic, as judged by the fourfold or greater difference in the MICs and MBCs against isolates of *Ps. aeruginosa* and *P. vulgaris* (Table 2). This was confirmed in *Ps. aeruginosa* with a time-kill experiment (data not shown).

Mechanism of action was determined in a hyperpermeable mutant of *Salmonella typhimurium*. As shown in Fig. 3, RNA and DNA synthesis were unaffected, over the course of the experiment, by 50 μg/ml of A21459, while protein synthesis was immediately and completely blocked upon addition of the antibiotic. Peptidoglycan synthesis was partially inhibited, starting at about 5~10 minutes after antibiotic addition.

Rate of mutation to resistance in *P. vulgaris* was approximately 1×10^{-8}/cell/generation. Four independently arising mutants (*i.e.*, from different tubes) were purified and tested for their level of resistance by streaking on agar plates containing different concentrations of A21459. They all grew at 200 μg/ml, which is the solubility limit of the antibiotic. When tested for cross-resistance to other antibiotics, the mutants were all as susceptible as the parental strain to rifampicin, chloramphenicol, tetracyclines, ampicillin and gentamicin, suggesting that resistance was due to a change in the target of the antibiotic, rather than to a change in permeability. This was confirmed by means of a modified protein synthesis cell-free system[9] showing IC_{50} at 2 mg/liter for the parental strain and no inhibition up to 50 mg/liter for one of the resistant mutants.

Table 2. Antimicrobial activity of A21459 complex.

Microorganism	MIC (μg/ml)
Staphylococcus aureus (2 isolates)	32~64
Streptococcus pyogenes L49	>128
S. pneumoniae L44	>128
S. dysgalactiae ATCC 9926	>128
Enterococcus faecalis ATCC 7080	>128
Bacillus subtilis ATCC 6633	>128
Propionibacterium acnes (3 isolates)	>128
Clostridium perfringens L290	8
C. difficile (3 isolates)	0.03
Mycoplasma gallisepticum L431	2
Neisseria caviae ATCC 14659	0.13
Neisseria gonorrhoeae (4 isolates)	64
Haemophilus influenzae (3 isolates)	16~64
Escherichia coli (4 isolates)	>128
Enterobacter cloacae L1349	>128
Klebsiella pneumoniae L142	>128
Proteus mirabilis (2 isolates)	16
Proteus rettgeri L1503	32
Proteus vulgaris ATCC 881*	4
Salmonella typhimurium L389	>128
S. typhimurium TA1538 (hyperpermeable)	8
Shigella dysenteriae L87**	>128
Pseudomonas aeruginosa (4 isolates)	4~32
Bacteroides fragilis (3 isolates)	>128
Candida albicans L145	>128

* MBC>128.
** MBC>128 (Performed on a single isolate for which the MIC was 4 μg/ml).

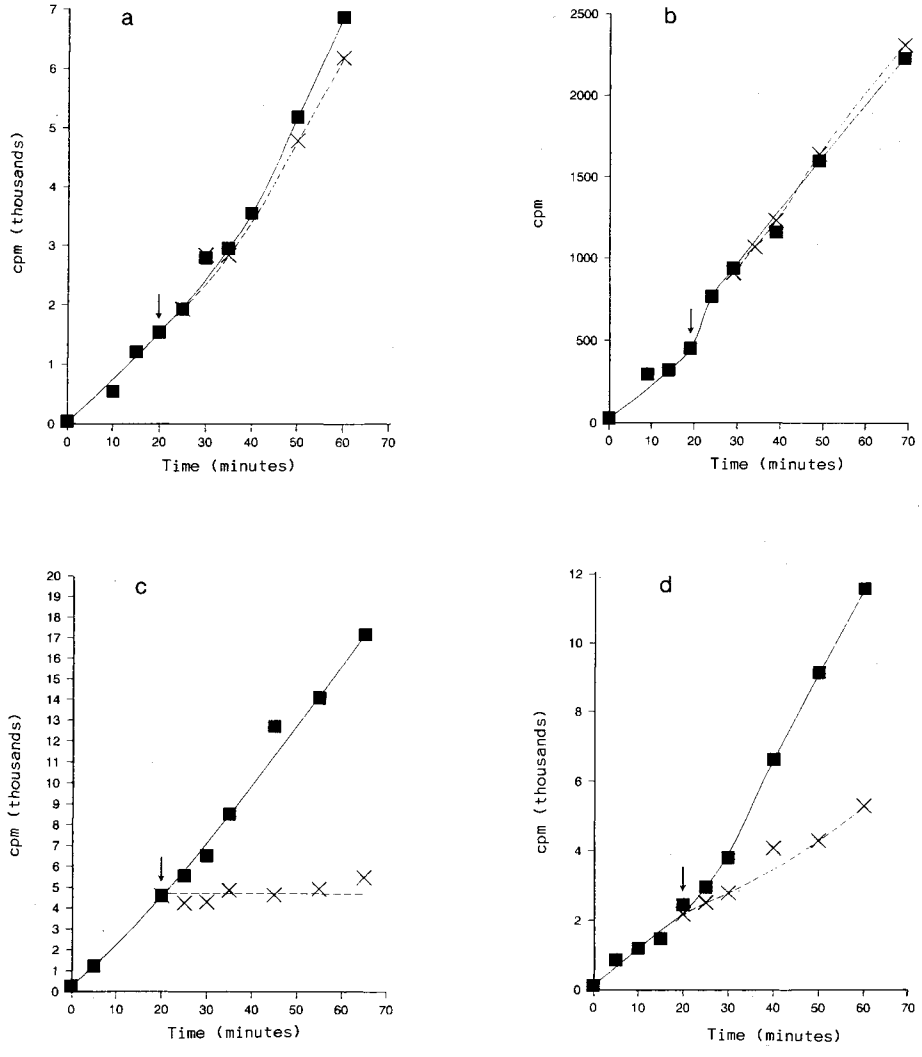
Discussion

A21459 has a rather unusual spectrum of antibacterial activity. The antibiotic is very active against *C. difficile* and mycoplasma and shows activity specifically against some Gram-negative bacteria. Intrinsic resistance appears due in some cases to lack of permeability (Table 2) although mutation to resistance in susceptible species appears to be mediated by the protein synthesis apparatus which is the target of the antibiotic.

A21459 A and B have novel structures. They are cyclic peptides constituted of α-amino acids and one thiazolyl

Fig. 3. Inhibition of macromolecular syntheses by A21459.

a. DNA synthesis, b. RNA synthesis, c. protein synthesis, d. peptidoglycan synthesis; ■, control culture; ×, 50 μg/ml of A21459. The cultures were divided and the antibiotic was added at the times indicated by the arrows.



amino acid. Biosynthesis of thiazoles is typically from cysteine and carboxylic group of one adjacent amino acid[10]. The peptidic macrocycle of A21459 appears thus originated from eight α-amino acids. Among microbial products, a peptidic macrocycle formed by eight α-amino acids is reported only for fungisporin[11,12]. Fungisporin is an antibiotic isolated from spores of several species of *Penicillium* and *Aspergillus* and is distinct from A21459 in structure and biological activity.

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