BMY-28190, A NOVEL ANTIVIRAL ANTIBIOTIC COMPLEX

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(Received for publication February 3, 1988)

BMY-28190, an antibiotic complex active against herpes simplex virus type 1 (HSV-1) was produced by the cultured broth of *Streptoalloteichus hindustanus* sp. nov., a producing strain of tallysomycins A and B[1]. The antibiotic complex was recovered from the broth with Amberlite IRC-50 resin and separated from the coproduced tallysomycins and nebramycins by a series of chromatographies. BMY-28190 exhibited weak inhibitory activity toward Gram-positive and Gram-negative bacteria and strong inhibitory activity toward HSV-1. Structural studies disclosed that BMY-28190 is a novel complex of γ-poly-D-α,γ-diaminobutyric acids with an average MW of 5,130.

In an earlier paper[1], we reported isolation of new antitumor antibiotics, tallysomycins A and B, and the aminoglycoside antibiotic nebramycin from the cultured broth of *Streptoalloteichus hindustanus* E465-94. In our search for antiviral antibiotics among microbial metabolites, the broth of this strain showed strong activity toward herpes simplex virus type 1 (HSV-1) which was not caused by the tallysomycins or nebramycins. BMY-28190, the active principle was isolated from the broth by a weakly acidic ion exchange resin and purified by column chromatography using a similar type ion exchanger and silica gel. BMY-28190 is a novel complex of γ-homopolymers of D-α,γ-diaminobutyric acid (D-α,γ-DAB) and exhibits strong inhibition against HSV-1. This paper describes isolation, physico-chemical properties, structure and biological activity of BMY-28190.

Production and Isolation

Taxonomic studies on *S. hindustanus* E465-94 (ATCC 31158) have been reported[2]. The strain was cultivated as described[1] and the harvested broth (30 liters, pH 7.5) was centrifuged. The clear supernate was applied on a column of Amberlite IRC-50 (NH_4^+ type, 3 liters) which was developed with water, 0.01 N NH_4OH, 0.25 N NH_4OH and then with 1.0 N HCl successively. The antiviral activity and nebramycins were eluted by 0.25 N NH_4OH and tallysomycins A and B by 1.0 N HCl. The fractions containing BMY-28190 were concentrated *in vacuo*, and the residue was chromatographed on a column of Amberlite CG-50 (NH_4^+ type, 2×64 cm). After washing with water and elution of most of the nebramycins with 0.1 N NH_4OH, the antiviral activity was eluted with 0.4 N NH_4OH. Upon monitoring the eluate by TLC (SiO_2, $CHCl_3$ - MeOH - 28% NH_4OH - H_2O, 1 : 4 : 2 : 1), the fractions containing BMY-28190 were pooled and evaporated *in vacuo* to afford a semi-pure sample (870 mg). This solid was rechromatographed on a Silica gel column (Wako C-200, 1.5×35 cm) with elution by $CHCl_3$ - MeOH - 28% NH_4OH - H_2O (1 : 4 : 2 : 1) mixture first and then of the upper layer of $CHCl_3$ - MeOH - 28% NH_4OH (1 : 1 : 1). The relevant fractions were concentrated, and the concentrate was applied on a column of Sephadex G-25 (1.0×30 cm) for desalting. Elution was carried out with water, and the course of elution was followed by the TLC as described above. Evaporation of the appropriate fractions yielded a white solid of homogeneous BMY-28190 (54 mg).

Physico-chemical Properties

Physico-chemical data for BMY-28190 are summarized in Table 1. BMY-28190 is readily soluble in water and dimethyl sulfoxide, slightly soluble in methanol and ethanol and practically insoluble in other organic solvents. It gave positive response to ninhydrin, Dragendorff, Rydon-Smith reagents but negative to ferric chloride and anthrone reagents. BMY-28190 does not exhibit absorption in the UV and visible region. The IR spectrum (Fig. 1) indicated amino (3400 cm^{-1}) and amide (1640 and 1540 cm^{-1}) functionalities. BMY-28190 is a complex of several components with similar properties, as depicted in the HPLC (Fig. 2).

Structural Studies

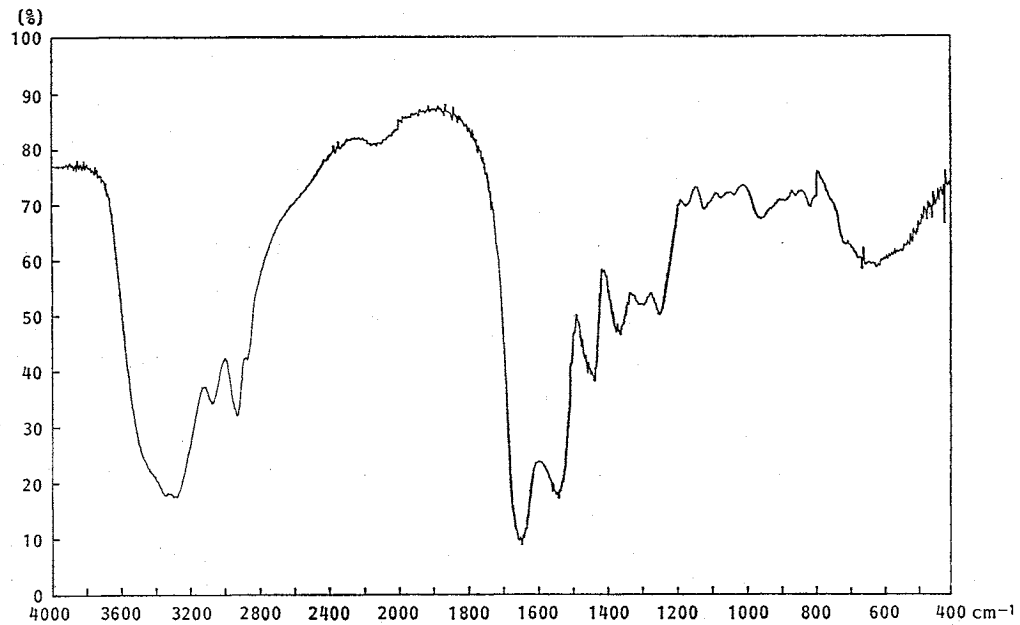
As revealed by HPLC, BMY-28190 is a complex of several components; an average MW of 5,700 was obtained by gel filtration using Sephadex G-50. BMY-28190 was hydrolyzed with 6 N HCl under reflux for 18 hours. The hydrolysate contained only one ninhydrin-positive substance whose be-

Table 1. Physico-chemical properties of BMY-28190.

Nature:	White amorphous solid
MP:	165~167°C
$[\alpha]_D^{25}$ (c 1.0, H_2O):	−19°
Microanalysis:	
Calcd for $C_4H_8N_2O \cdot \frac{1}{3}H_2CO_3$:	
	C 43.08, H 7.23, N 23.18.
Found:	C 43.80, H 7.84, N 23.00.
MW:	5,700 (Sephadex G-50 gel filtration chromatography),
	5,100~5,200 (DNP method)[7]
TLC, SiO_2*:	Rf 0.02 ($CHCl_3$ - MeOH - 28% NH_4OH - H_2O, 1:4:2:1),
	Rf 0.72 ($CHCl_3$ - MeOH - 28% NH_4OH, 1:1:1, upper layer)

* Silica gel (Kiesegel $60F_{254}$, Merck). Detection by I_2 vapour and ninhydrin reagent.

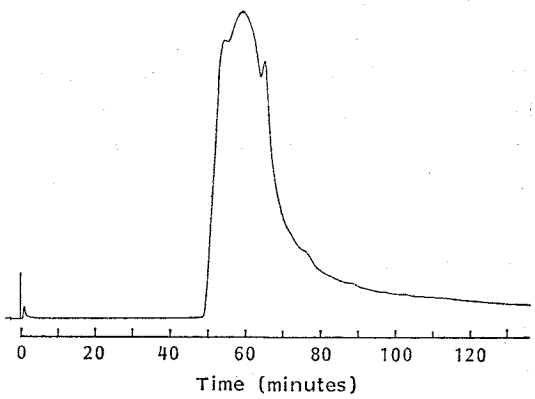
Fig. 1. IR spectrum of BMY-28190 (KBr).



havior in TLC and amino acid analysis was identical with that of α,γ-DAB. This substance was isolated from the hydrolysate by Amberlite CG-50 chromatography and obtained as colorless needles by crystallization from aqueous ethanol. Its spectral data and optical rotation ($[\alpha]_D^{26.5}$ $-20°$)[3] confirmed that it was D-α,γ-DAB.

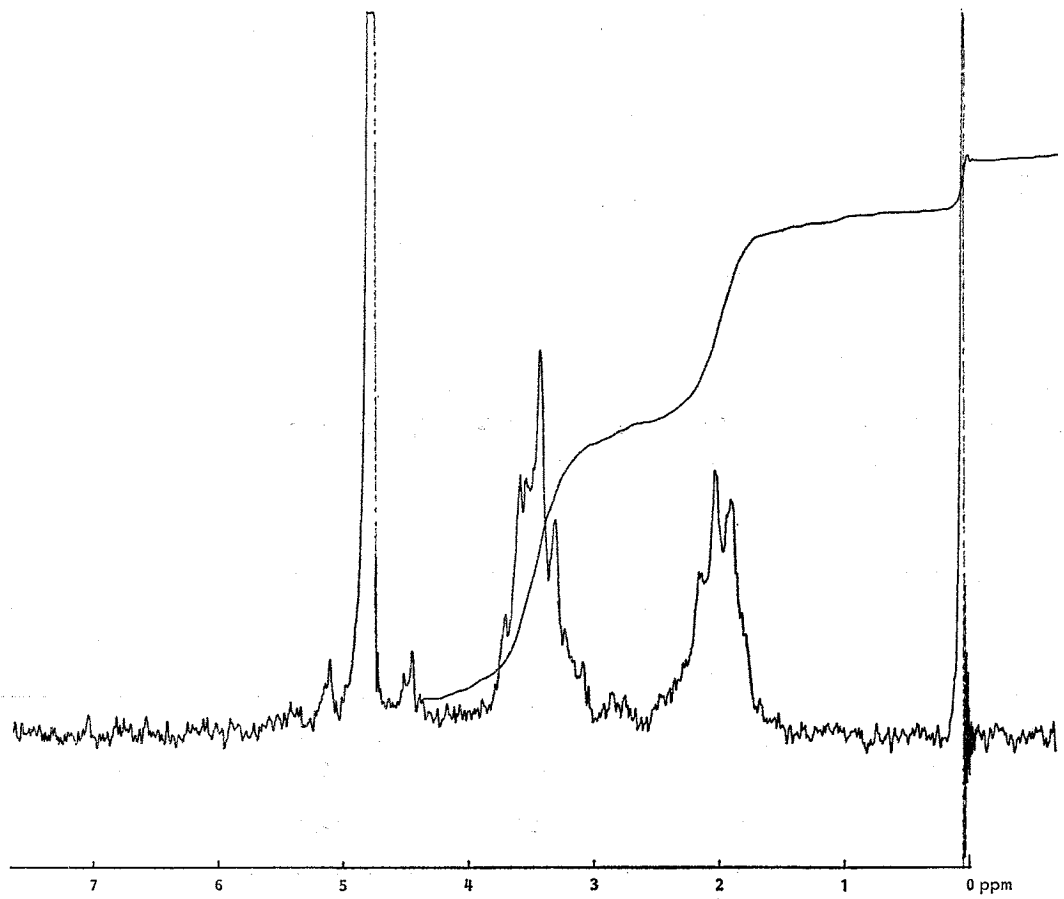
The ^{1}H NMR of BMY-28190 (Fig. 3) resembled that of DAB exhibiting only two multiplet signals centered at 2.00 (2H) and 3.43 ppm (3H). Upon acidification, one proton of the latter signal underwent down-field shift (Δ 0.48 ppm) and appeared as a triplet. In accordance with the ^{1}H NMR, the ^{13}C NMR of BMY-28190 showed four carbon signals at 34.2 (t), 36.7 (t), 53.3 (d) and 177.1 ppm (s) which corresponded well to those of DAB. When determined at pD 2.0, the higher field methylene and carbonyl carbons

Fig. 2. HPLC of BMY-28190.



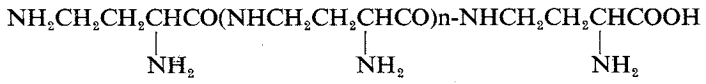
Tosoh Co. TSK Gel 3000 SW (7.5×600 mm). 1/15 M phosphate buffer, pH 7.0. 0.7 ml/minute, UV at 210 nm.

Fig. 3. ^{1}H NMR spectrum of BMY-28190 (60 MHz in D_2O).



demonstrated protonation shifts of 2.9 and 6.7 ppm, respectively. These spectral data coupled with the hydrolysis result provided convincing evidence that BMY-28190 is γ-homopolymer of D-α,γ-DAB.

For determination of the polymerization rate of DAB, BMY-28190 was reacted with 2,4-dinitrofluorobenzene in aqueous ethanol. The resulting 2,4-dinitrophenyl (DNP)-BMY-28190 was hydrolyzed with 7.1 N H_2SO_4 for 10 hours, and two DNP-DABs produced were separated and purified by preparative TLC. The major one (mp 204~205°C) and the minor one (mp 123~124°C) were identified as α-DNP-DAB[4,5] and α,γ-di-DNP-DAB[5,6], respectively, by comparison with authentic samples. α,γ-Di-DNP-DAB should have been derived from the *N*-terminal and α-DNP-DAB from the other peptide portion of BMY-28190 confirming the assigned linear γ-homopolymer structure of the antibiotic. 2,4-Dinitrophenyllysine has been reported to partly decompose during acid hydrolysis[7,8]. Under the hydrolytic condition used for DNP-BMY-28190, 26.9% of α,γ-di-DNP-DAB and 47.0% of α-DNP-DAB were found to decompose. Taking into consideration these decomposition rates, the ratio of α,γ-di-DNP-DAB and α-DNP-DAB produced in the hydrolysis of DNP-BMY-28190 was calculated as 1 : 50.3 indicating an average MW of 5,130 (51~52 homopolymer) for the antibiotic.



BMY-28190
n=49~50 (average)

Biological Activity

Antiviral Activity

Antiviral activity of BMY-28190 was assessed by the plaque reduction assay and dye-uptake assay[9] using the HSV-1-vero cell system. ε-Poly-L-lysine[10], a structurally related agent produced by *Streptomyces* No. 346 and two types of synthetic α-poly-L-lysine (MW 3,500 and 25,000) were tested comparatively as reference compounds. The results are shown in Table 2 together with their cytotoxicity against host cells. By plaque reduction assay, BMY-28190 was the most potent among the compounds tested, showing an ID_{50} of 0.84 μg/ml. ε-Poly-L-lysine and higher molecular α-poly-L-lysine were slightly less active than BMY-28190, and lower molecular α-poly-L-lysine was the least active. BMY-28190 and ε-poly-L-lysine were comparably active by the dye-uptake

Table 2. Activity against herpes simplex virus type 1.

	Plaque reduction assay		Dye-uptake assay	
	Activity vs. HSV-1 (ID_{50}: μg/ml)	Cytotoxicity vs. vero cell ($TCID_{50}$: μg/ml)	Activity vs. HSV-1 (ID_{50}: μg/ml)	Cytotoxicity vs. vero cell ($TCID_{50}$: μg/ml)
BMY-28190	0.84	170	2.8	540
ε-Poly-L-lysine	2.6	70	2.7	>100
α-Poly-L-lysine (MW 3,500)	42	>400	100	>100
α-Poly-L-lysine (MW 25,000)	1.8	25	>20	20

Cells: Vero cells. Medium: EAGLE MEM containing 5% fetal bovine serum.

Table 3. Antibacterial activity by the broth dilution method.

Test organisms	MIC (μg/ml)		
	BMY-28190	ε-Poly-L-lysine	α-Poly-L-lysine
Staphylococcus aureus Smith	6.3	6.3	12.5
S. aureus BX-1633-2	6.3	6.3	25
Streptococcus faecalis A9612	>100	50	>100
Escherichia coli Juhl	6.3	6.3	12.5
Klebsiella pneumoniae A9977	3.1	6.3	12.5
Proteus mirabilis A9554	25	6.3	50
Serratia marcescens A20019	>100	25	>100
Enterobacter cloacae A9656	100	25	>100
Haemophilus influenzae A2241	100	12.5	100
Pseudomonas aeruginosa A9930	100	12.5	>100

assay, while the two α-poly-L-lysines were nearly inactive. BMY-28190 showed relatively weak cytotoxicity and had the best selectivity in these assay systems.

Antibacterial Activity

The *in vitro* antibacterial activity of BMY-28190 was determined by the 2-fold serial broth dilution method in nutrient broth using ε-poly-L-lysine and α-poly-L-lysine (MW 3,500) as reference compounds. As shown in Table 3, these three compounds exhibited moderate inhibitory activity against Gram-positive and Gram-negative bacteria with a similar antibacterial spectrum. In terms of MICs, BMY-28190 was comparably active to ε-poly-L-lysine against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* but less active than ε-poly-L-lysine against other organisms. α-Poly-L-lysine was the least active against all test strains.

Table 4. Antiviral activity of partial hydrolysates of BMY-28190 by dye-uptake assay.

Peptide fragment No.	Number of DAB	Anti-HSV-1 activity ID_{50} (μg/ml)
D-α,γ-DAB	1	>660
1	3~4	>660
2	6~7	>660
3	8~9	>660
4	9~10	>660
5	12~13	>660
6	15~16	220
7	16~17	38
8	21~22	23
BMY-28190	51~52	2.8

Acute Toxicity

The LD_{50} of BMY-28190 was 22.6 mg/kg following intramuscular administration to male *dd*Y mice.

Partial Acid Hydrolysis of BMY-28190 and Anti-HSV-1 Activity of the Products

In order to examine the relationship between peptide chain length and antiviral activity, partial acid hydrolysis of BMY-28190 was carried out by heating with 6 N HCl at 100°C for 1 hour. The hydrolysate was chromatographed on a CM-cellulose column to yield 8 peptide fragments. Numbers of D-α,γ-DAB in each peptide were determined by the DNP method as described before, and antiviral activity against HSV-1 was assessed by dye-uptake assay (Table 4). Small peptides with less than 12 D-α,γ-DAB residues were practically inactive against the viruses, while peptide fragments 6, 7 and 8 having more than 15 amino acids showed weak antiviral activity. Potency increased with the length of the peptide chain. BMY-28190 was more active than these peptides.

Discussion

BMY-28190, a complex of novel antiviral antibiotics, was produced by *S. hindustanus* E465-94 together with tallysomycins and nebramycins. It is active against herpes simplex virus type 1 both by plaque reduction assay and dye-uptake assay and shows moderate antibacterial activity against certain Gram-positive and Gram-negative bacteria. Structural studies revealed that BMY-28190 is a mixture of linear γ-homopolymers of D-α,γ-DAB with an average MW of 5,130.

SHIMA and SAKAI reported the isolation of ε-poly-L-lysine from the fermentation broth of *Streptomyces albulus*[10], and in their recent papers, they demonstrated that the compounds had bacteriophage inactivation activity[11] and antimicrobial activity[12]. ε-Poly-L-lysine was demonstrated to show antiviral activity against HSV-1 in our test system. BMY-28190 is the second example of bioactive homopolymers of amino acids obtained from natural origins. It is worthy to note that BMY-28190 is a larger polymer (n=51~52) than ε-poly-L-lysine (n=25~30) and is composed of D-amino acid in contrast to L-amino acid polymers of the latter. In addition, it is interesting that homopolypeptides consisting of more than 15 D-α,γ-DAB residues were active against HSV-1, while smaller polymers were inactive as revealed by partial hydrolysis experiments.

Acknowledgments

We want to thank late Prof. H. SAKAI of the University of Osaka Prefecture for providing us the sample of ε-poly-L-lysine. Thanks are also due to the members of Microbiology, Fermentation and Analytical groups for their excellent technical assistance.

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