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Isolation, structure elucidation and physicochemical properties of novel antibiotic polypeptide, ε-(*L*-β-lysine) polypeptide from *Streptomyces* sp. DWGS2

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Abstract *:* During the screening of material which has the antimicrobial activity against aminoglycoside-resistant bacteria, A new material ε-(L-β-lysine) polypeptide from a culture medium of *Streptomyces* sp.(DWGS2) was isolated, and the structure and the physicochemical properties of the new material were elucidated. The new material was separated by column chromatography of the culture medium using Dowex1×2, Silica gel, and Sephadex LH20 etc. The chemical structure and molecular weight were determined with the data of various NMR experiments, MALDI mass, and ESI mass experiments. The antimicrobial activity of ε -(L-β-lysine) polypeptide is not only better than equal to the activity of known aminoglycoside type of antibiotics(MIC=3.125 - 6.25ug/mL) but also effective against aminoglycoside-resistant bacteria and fungi. If the mechanism of antimicrobial activity against aminoglycoside- resistant bacteria is figured out, the ε -(L-β-lysine) polypeptide can be utilized for the treatment of diseases caused by aminoglycoside-resistant bacteria.

INTRODUCTION

Antitumor antibiotics, including bleomycin and tallysomycin are clinically used against certain malignant lymphomas, squamous cell carcinomas, and testicular carcinoma. This glycopeptide has a metal-binding moiety that cleaves the specific DNA sequence, sugar rings(glucose, mannose, tallose) and tail regions(bithiazole, sulfonium, lysine) that recognize and bind noncovalently to specific DNA base sequences[1]. Screening of the culture broth of soil-derived microorganisms has been continued to provide an important

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source of novel natural products possessing potentially useful biological activities[2]. During the course of a screening program to discover new fermentation-derived anti-microbial compounds, especially having the activity against aminoglycoside-resistant bacteria[3], the new material, ε-(L-β-lysine) polypeptide (m.w. 2300) was isolated from a culture medium of *Streptomyces* sp.(DWGS2). This report describes the complete structure elucidation by NMR. Physicochemical properties and isolating procedure of ε-(L-β-lysine) polypeptide are also described in detail.

EXPERIMENTAL

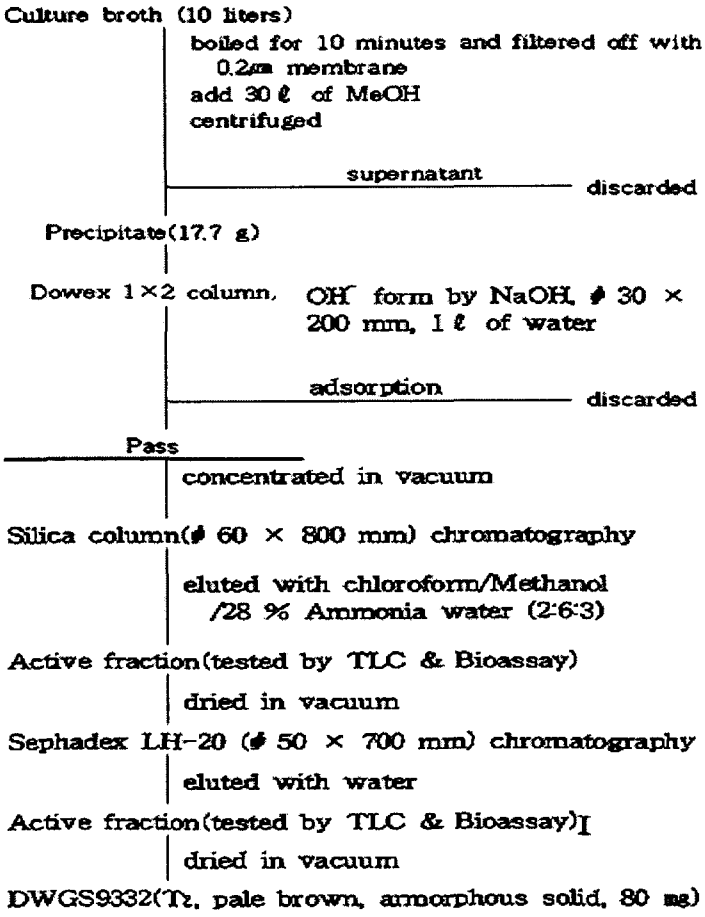
Materials

Culture media for fermentation and bio-assay were purchased from Difco and all kinds of eluting reagent for column chromatography were used with analytical reagent grade. Precoated kieselgel 60 F254(No. 5715) for TLC plate and silica gel for column chromatography were purchased from Merck and Dowex 1×2(No. 44290, Cl^- form strong basic; 50-100 mesh) for ion exchange resin from Fluka and Sephadex LH-20(No. 17-0090) for Gel filtration from Pharmacia..

Isolation and Purification

The isolation procedure of the active compound from the culture broth of *streptomyces sp.* DWGS2 is schematically shown in scheme 1. Culture broth(10 L) was boiled in order to be sterilized for 10 min. and filtered with 0.2 um membrane filter. Three times(30L) of methanol in volume was added in this clear filtered broth and then stood alone overnight at 4°C and centrifuged to give the active precipitate. This precipitate was dissolved in 1 L of water and applied to Dowex-1×2 column(40 × 200 mm) which is anion exchange(^-OH form) to remove coloring and anionic impurities and subsequently washed with 1L of water. The eluate gathered was concentrated in vacuum to give active residue, which was applied to silica gel column(Silica gel 60, MERK 70 ~ 230 mesh, ASTM 60 × 800 mm) and eluted with a solution which was composed with chloroform/methanol/28% ammonia water (2:6:3). Eluted fraction was collected by fraction collector(EYELA DC 12000) and for each fraction TLC(Rf : 0.35) and bioassay was performed simultaneously to find out the active fraction. Silica plate was developed with chloroform/methanol/28% ammonia water (2:6:3) and sprayed by dragendorff reagent[4,5] (purple color, for determining of nitrogen containing compounds, basic bismuth nitrate 0.64 mg, potassium iodide 1.6 g, acetic acid 20 mL dissolved in 80 mL of water.). Bioassay was performed by cylinder plate method. 20 uL of test sample was loaded on the medium made of nutrient agar containing 1.0%(w/v) peptone, 0.3%(w/v) beef extract, 0.2%(w/v) sodium chloride, which was inoculated with *Bacillus*

subtilis ATCC6633(incubated at 37°C for 12hrs), *Serratia marcescens* Sma 62(incubated at 30°C for 12hrs) and *S. marcescens* AG4410(incubated at 30°C for 12hrs). Antibacterial activity was revealed as a clear zone of inhibition of growth.



Scheme 1. Isolation and purification procedure of ε-(L-β-lysine) polypeptide

The active fraction was concentrated with vacuum freezer dryer. The aqueous residue applied to Sephadex LH-20 column and eluted with water and for each fraction TLC and bioassay was performed again to find out the active fraction. The active fraction was dried in vacuum to obtain the active compound(pale brown, amorphous solid, 80 ㎎).

Biological Activity

Biological activity was tested at Central Research Laboratories of Dong-Wha pharmaceutical company. The antimicrobial activity of ε-(L-β-lysine) polypeptide is not only better than equal to the activity of known aminoglycoside type of antibiotics (MIC=3.125 - 6.25ug/mL) but also effective against aminoglycoside-resistant bacteria and fungi. The data are summarized in Table 1 and Table 2, respectively.

Table 1. Minimum inhibition concentration(MIC, ug/mL) of ε-(L-β-lysine)polypeptide

Strains	ε-(L-β-lysine)poly peptide	Reference	
		Tm*	Sm
1. *Acinetobacter calcoaceticus* 19606	3.125	6.25	50
2. *Citrobacter freudii* 8090	3.125	6.25	50
3. *Enterobacter cloacae* 23355	3.125	6.25	25
4. *Escherichia coli* 25922	3.125	6.25	25
5. *Hamemophillus influenzae* 35056	3.125	6.25	25
6. *Klebsiella pneumoniae* 13883	6.250	12.50	50
7. *Proteus vulgaris* 13315	3.12	3.125	25
8. *Pseudomonas aeroginosa* 27853	3.125	6.25	50
9. *Salmonella typhimurium* 14028	3.125	6.25	25
10. *Shigella flexneri* 12022	3.125	3.125	25
11. *Serratia marcescens* 8100	3.125	3.125	6.25
12. *Staphylococcus aureus* 25923	3.125	3.125	12.5
13. *Staphylococcus epidermidis* 12228	3.125	6.25	12.5
14. *Streptococcus faecalis* 19433	3.125	6.25	25
15. *Streptococcus pneumoniae* 6303	3.125	3.125	12.5
16. *Bacillus subtilis* 6633	3.125	3.125	12.5

* Tm ; tobramycin , Sm ; Streptomycin

Table 2. MIC of ε-(L-β-lysine)polypeptide against fungi

strains	ε-(L-β-lysine)polypeptide	reference
		ketoconazole
C. albicans *	0.25mg/mL	0.25 mg/mL
S. schenckii	0.25mg/mL	-

* *C. albicans* ; *Candida albicans* ATCC 28366(separated clinically),
S. schenckii ; *Sporothrix schenckii* ATCC 58251(separated clinically)
- no activity

Spectroscopic and Thermal Properties

IR Spectrum was obtained by UNICAM Mattson 1000. IR spectrum for the active compound has absorptions at 3423, 3276 cm^{-1}, which exhibit that the active compound has N-H_2 bond and at 3084, 2938, 1561, 1453, 623 cm^{-1}, which exhibit -NH_3^+, N-H, aliphatic C-H, -CH_2, C-N respectively and at 1653, 1107 cm^{-1}, which exhibit carbonyl function of amide and CO-N-C respectively. IR spectrum for hydrolyzed product has absorptions at 3210, 2970, 1560, 1440, 1220, 808, 660 cm^{-1}, which exhibit N-H, aliphatic C-H, carbonyl, methylene, C-N for primary amine and two peaks of –NH_2 , respectively. The Experiment of difference scanning colorimeter was performed with Perkin-Elmer DSC 7. The DSC pattern shows that the active compound decompose at 295.6°C and the decomposition starts at around 270°C

NMR Experiments

All NMR spectra were measured on Varian unity 300 (300 MHz), JEOL Lambda 400 (400 MHz). ^1H NMR and ^{13}C NMR chemical shifts are reported in ppm with the chemical shift of the residual protons of the solvent used as internal standard. NMR experiments for the active compound were carried out under the 10% D_2O solvent (90% H_2O) at 22°C. ^1H NMR, ^{13}C NMR, DEPT experiment were performed and COSY, HMQC and HMBC experiments were performed in order to determine the network of the structure. All NMR signal assignments and J-coupling are summarized in Table 3 and 4.

MALDI-TOF-MS and ESI-MS Experiments

Mass spectra were measured on Jeol JMS SX102A and HX110/110A for EI and FAB and VG Quattro ESI-MS for ESI-ms, Kratos Kompact MALDI 2 for MALDI(Matrix Assisted Laser Desorption Ionization) spectrum. Any mass data was not able to be obtained with EI and FAB mass but the meaningful data for the active compound was obtained with MALDI-MS using 2,5-dihydroxybenzoic acid(DHB)[6] as a matrix. A part of the active compound was dissolved in 50 part in weight of 50% acetonitrile solution made of DHB saturated water and put on polished stainless steel probe and let be dried. MALDI-MS spectrum of the active compound shows the peaks, m/z of 1819.8, 1946.2, 2074.1, 2202.2, 2329.8, 2457.4, 2585.2, 2712.1, 2842.9, 2966.8, 3100.6. For ESI-MS, a part of the active compound and hydrolyzed product was dissolved in around 500 part in weight of water respectively and injected into ESI-MS(positive).

RESULTS AND DISCUSSION

In the isolating process we found that nitrogen was contained in the compound because the active compound was colored to purple by dragendorff reagent and the hydrolyzed product was colored to violet by Ninhydrine reagent. MALDI-MS spectrum of the active compound shows the peaks in the fig. 1, 1819.8(n=13), 1946.2(n=14), 2074.1(n=15), 2202.2(n=16), 2329.8(n=17), 2457.4(n=18), 2585.2(n=19), 2712.1(n=20), 2842.9(n=21), 2966.8(n=22), 3100.6(n=23) and these peaks were explained follow formula.

$$\text{Molecular ion} = [\text{DHB}(154) - H_2O(18) + M(128 \times n + 18) + H]^+$$

These peaks tell us that active compound is a polymer composed of the monomer with molecular weight 128. The mass spectrum of hydrolyzed product shows that the molecular weight of the monomer is 146 and from NMR data of the monomer it is determined that the monomer is L-β-lysine which is a dextro isomer having an optical rotation, $[\alpha]^{24}_D = +50.6$ (c=1%,H_2O). H.E. Carter,[9] and D. Keirs[10] repoted that the specific rotation of L-β-lysine was +24 and +18 respectively. Our the specific rotation value is bigger than that of H.E. Carter, and D. Keirs, which is thought like that our L-β-lysine is purer than these results.

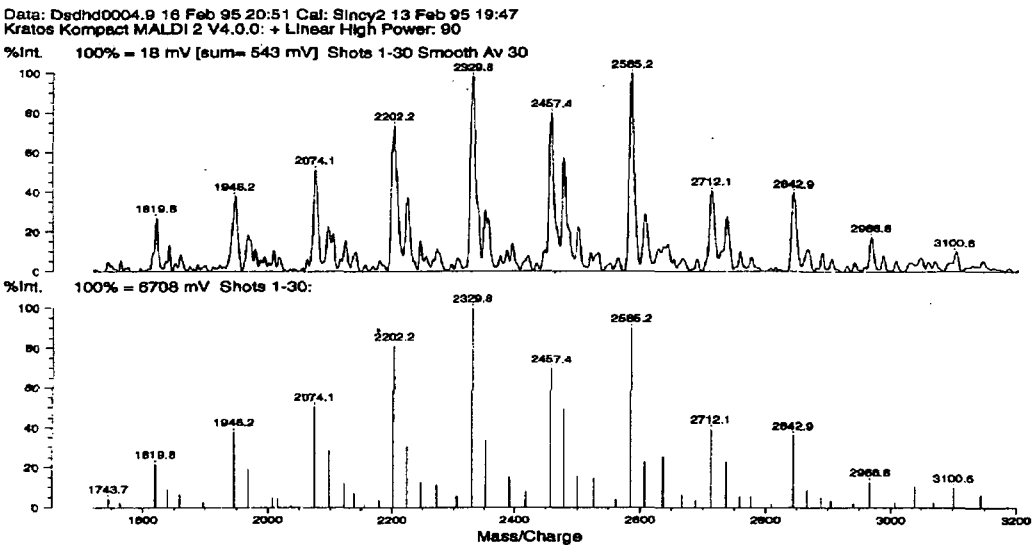


Fig. 1. MALDI-mass spectrum of ε-(L-β-lysine) polypeptide

Table 3. ^{1}H and ^{13}C-NMR signal assignments for ε-(L-β-lysine) polypeptide

No	^{13}C		DEPT		^{1}H (multiplicity)	
	ε-(L-β-lysine) polypeptide	L-β-lysine	ε-(L-β-lysine) polypeptide	L-β-lysine	ε-(L-β-lysine) polypeptide	L-β-lysine
C-1	174.371	176.566	C	C		
C-2	39.606	37.437	CH2	CH2	2.522(dd) 2.592(dd)	2.385 2.610
C-3	51.353	48.675	CH	CH	3.526(m)	3.440
C-4	32.031	29.095	CH2	CH2	1.624(m)	1.614
C-5	26.729	22.949	CH2	CH2	1.536(m)	1.591
C-6	41.371	39.165	CH2	CH2	3.134(m)	2.906
N-7					8.160(t)(NH)	
N-8						

- not detected

Table 4. Scalar NMR connectivities used to assign ^{1}H and ^{13}C signals in ε-(L-β-lysine) polypeptide

proton	HMBC		COSY	
	ε-(L-β-lysine) polypeptide	L-β-lysine	ε-(L-β-lysine) polypeptide	L-β-lysine
H-2a	C-1, C-3, C-4	C-1, C-3, C-4	H-2b, H-3	H-3
H-2b	C-1, C-3, C-4	C-1, C-3, C-4	H-2a, H-3	H-3
H-3	C-1, C-2, C-4, C-5	C-1, C-4, C-5	H-2a, H-2b, H-4	H-2a, H-2b, H-4
H-4	C-2, C-3, C-5, C-6	C-3, C-5, C-6	H-3, H-5	H-3, H-4, H-5
H-5	C-3, C-4, C-6	C-4, C-6	H-4, H-6	H5, H6
H-6	C-1, C-4, C-5	C-4, C-5	H-5, H-7	H5
H-7	C-1, C-6		H-6	

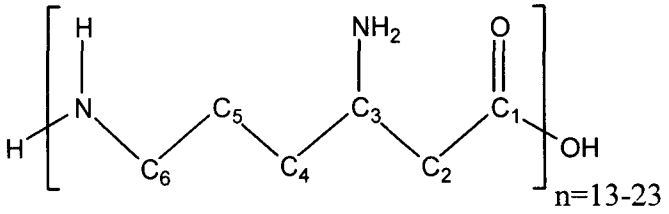


Fig. 2. Molecular structure of ε-(L-β-lysine) polypeptide

The active compound was determined to be ε-(L-β-lysine) polypeptide. In the ESI-MS spectrum we were not able to assign all peaks because the spectrum is very complicated, but assigned some peaks to 2194.29(128 × 17 + 18), 2451.72(128 × 19 + 18), 2577.69(128 × 20 + 18). These data also support that the active compound is ε-(L-β-lysine) polypeptide. NMR signal assignments and heteronuclear correlation experiments were enable to accomplish of the molecular skeleton of ε-(L-β-lysine) polypeptide as shown in Fig. 2. As described in introduction, ε-(L-β-lysine) is a tail region of tallysomycin-A which known to be recognizing the certain DNA sequence. The product of hydrolysis of the polypeptide can be used as further DNA-durg binding studies.

The antimicrobial activity of ε-(L-β-lysine) polypeptide is not only better than equal to the activity of known aminoglycoside type of antibiotics(MIC=3.125 - 6.25ug/mL) but also effective against aminoglycoside-resistant bacteria and fungi. ε-(L-β-lysine) polypeptide can be utilized for the treatment of diseases caused by aminoglycoside-resistant bacteria if the mechanism of antimicrobial activity against aminoglycoside-resistant bacteria is figured out.

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