LASPARTOMYCIN, A NEW ANTI-STAPHYLOCOCCAL PEPTIDE

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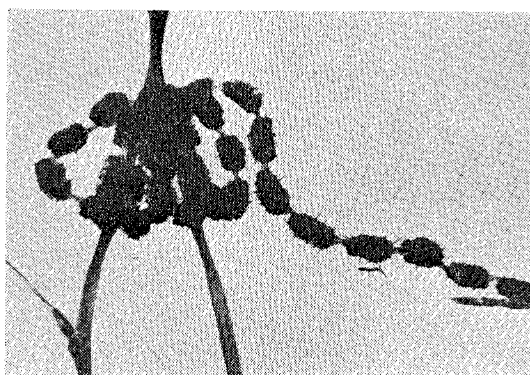
A new peptide antibiotic, laspartomycin, was isolated from a *Streptomyces* designated *Streptomyces viridochromogenes* var. *komabensis* HAMADA *et* OKAMI. The isolation, purification, physicochemical, and biological properties of the antibiotic were described. Laspartomycin was differentiated from related antibiotic such as aspartocin, amphomycin, glumamycin, zaomycin and crystallomycin by amino acid composition and behavior in paper chromatographic and thin-layer chromatographic systems.

Increase of pathogens resistant to antibiotics has been noted and the need for elimination of this problem is now urgently in recognized. Staphylococci often cause stubborn diseases and their resistance to antibiotics are frequently reported. In the authors' laboratory, a search has been conducted for new substances active against resistant staphylococci. Isolation of a new antibiotic named laspartomycin from fermented beer of a streptomyces and its characteristics are reported in this paper.

Producing Strain

A streptomycete was isolated from a soil sample collected in Tokyo City and found to produce a new antibiotic, laspartomycin. This streptomycete was designated strain No. M 307-M 5 in the authors' laboratory. It showed the following characteristics on taxonomic examination. Microscopically, strain No. M 307-M 5 branches monopodially and aerial mycelia develop with spiral structure. Whorl formation was not observed. The surface of the conidial spore has a spiny structure under electronmicroscope as seen in Plate 1.

Plate 1. Electron microscopic photograph of the strain No. M 307-M 5 (Direct magnification ×7,500)



Characteristics of the strain on various media are as follows:

1) Glycerol-CZAPEK agar (incubated at 27°C): Colorless to pale yellowish brown growth is later shaded with dusty rose (8 ie Color Harmony Manual, published by Container Corporation of America, Chicago) or with light brown. White aerial mycelium develops. Faint violet-colored or faint yellowish soluble pigment is often produced.

Reverse of the growth is yellowish brown or reddish brown.

2) KRAINSKY glucose asparagine agar (incubated at 27°C): Colorless growth changes to pale yellow and is gradually shaded with reddish to reddish brown tone (Dusty Rose 8 ie by Color Harmony Manual). In some cases, colorless growth changes to pale yellow or pale yellowish brown. Aerial mycelium is white or shaded with bluish tinge (Lt Aqua 18 ec, by Color Harmony). No or slightly yellowish soluble pigment is produced. Reverse of the growth is yellowish brown or brown.
3) Calcium malate agar (incubated at 27°C): Growth is colorless to pale yellow. Aerial mycelium is white or shaded with bluish tinge. No soluble pigment is produced. Calcium malate around the growth is solubilized. Reverse of the growth is cream-colored.
4) Peptone solution (containing 1.0 % sodium nitrate) (incubated at 27°C): Colorless growth is observed. No aerial mycelium and no soluble pigment are produced. Nitrite is not formed from nitrate.
5) Starch agar plate (incubated at 27°C): Colorless growth is later shaded with purple tinge and further becomes dark red (Old Wine 8 ng by Color Harmony Manual). In some cases, it becomes pale yellow or brownish yellow. Aerial mycelium is white or shaded with bluish tinge (Lt Aqua 18 ec by Color Harmony Manual). No or slightly yellowish soluble pigment is produced. Reverse of the growth is yellowish brown or reddish brown. Hydrolysis of starch is weak.
6) Potato plug (incubated at 27°C): Colorless growth changes to yellowish brown or dark yellowish brown with many wrinkles. In some cases, color of the growth is shaded with violet color. Aerial mycelium is thin and white. Soluble pigment is light brown.
7) Nutrient agar (incubated at 37°C): Colorless growth changes to pale brownish. No aerial mycelium is observed. Brown soluble pigment is produced.
8) Nutrient agar (incubated at 27°C): Colorless growth changes to light brown or reddish brown and is later shaded with grayish red brown color. No aerial mycelium is observed. Brown soluble pigment is produced.
9) LOEFFLER coagulated serum (incubated at 37°C): Colorless growth changes to pale brown or yellowish brown. No aerial mycelium is observed. Light brown to reddish brown soluble pigment is produced. Coagulated serum is not liquefied.
10) Gelatin stab (incubated at 20°C): Colorless to light brown growth is observed, but no aerial mycelium. Brown soluble pigment is produced. Liquefaction of gelatin occurs.
11) Milk (incubated at 37°C): Growth is colorless to yellowish brown. No aerial mycelium is observed. Yellowish brown soluble pigment is produced. Medium to strong coagulation and peptonization of milk are observed.
12) Tyrosine agar (incubated at 27°C): Growth is colorless to brownish. No aerial mycelium is observed. Soluble pigment with blackish tinge is produced. Tyrosinase reaction is positive.
13) CZAPEK solution containing cellulose as the sole carbon source (incubated at 27°C): Growth is colorless. Decomposition of cellulose is not observed.
14) Utilization of carbon sources in PRIDHAM-GOTTLIEB basal medium (incubated at 27°C): Growth with glycerol, arabinose, xylose, rhamnose, galactose, glucose, mannose, fructose, mannitol, inositol, lactose, maltose, sucrose, raffinose, salicin, dextrin, inulin and starch. No growth with sorbitol and dulcitol.

As shown by the characteristics described above, the strain M 307-M 5 belongs to the genus *Streptomyces*, and its characteristics can be summarized as follows: no whorl; closed spiral; spiny structure on surface of spore; yellowish brown to light brown (purplish tinge in some cases) growth; white aerial mycelium shaded with

bluish tinge; chromogenic type which produces brownish soluble pigment on organic media but either no or slightly yellowish pigment on synthetic media; cream-colored, yellowish brown, brown or reddish brown reverse of the growth; medium to strong proteolytic activity; weak hydrolysis of starch.

Among known species, strain No. M 307-M 5 has most many common characteristics with the group of *Streptomyces viridochromogenes* (KRAINSKY 1914) WAKSMAN *et* HENRICI, 1948 (The Actinomycetes, Vol. 2, p. 287, William and Wilkins, Baltimore). Of this group, *Streptomyces viridochromogenes* is different from strain No. M 307-M 5 in respect of greenish to blackish pigmentation of growth and its reverse. The group of *Streptomyces viridochromogenes* and related species have been described by R. E. BENNETT in J. Bacteriology, Vol. 85, p. 681 in 1963. On comparing with the species described in this references, strain No. M 307-M 5 is found to be related to *Streptomyces chartreusis* CALHOUN *et* JOHNSON, 1956 (The Actinomycetes, Vol. 2, p. 192), *S. coerulescens* GAUZE *et al.* 1957, *S. coeruleorubidus* GAUZE *et al.* 1957 and *S. coeruleofuscus* GAUZE *et al.* 1957. On comparing with these four species, strain No. M 307-M 5 is most closely related to *S. chartreusis*. However, strain No. M 307-M 5 is different from *S. chartreusis* in respects to proteolytic activity, reduction of nitrate and hydrolysis of starch. *S. coerulescens* is differentiated from the strain by the pattern of carbohydrate utilization. The comparison is shown in Table 1. Considering the result of the comparison, it is thought proper to assign strain No. M 307-M 5 to one of varieties of *S. viridochromogenes*. Thus it was named *S. viridochromogenes* var.

Table 1. Comparison of the strain M 307-M 5 with *S. viridochromogenes*, *S. chartreusis*, *S. coerulescens*, *S. coeruleorubidus* and *S. coeruleofuscus*

	M 307-M 5	*S. chartreusis*	*S. coerulescens*	*S. coeruleorubidus*	*S. coeruleofuscus*	*S. viridochromogenes*
Spiral	closed spirals	short tight spirals to loose open spirals	short spirals	long open spirals	short open spirals (some closed spirals)	open or compact spirals
Color of aerial mycelium	white to bluish-white to bluish	bluish gray to blue-green	blue to blue-green	blue to blue-green	blue to bluish gray	blue to blue-green
Color of reverse	cream color, yellowish brown, brown, reddish-brown	cinnamon, light amber	brown	reddish brown	brown	bluish green to greenish black
Surface of spore	spiny	spiny	spiny	spiny	spiny	spiny
Antibiotics produced	laspartomycin	chartreusin	coerulomycin	—	—	antifungal activity
Chromogenecity	+	+	+	+	+	+
Nitrate reduction	−	+	−	+	+	+ or −
Utilization of carbohydrate						
Sorbitol	−	−	−	−	−	−
Raffinose	+	+	−	−	+	+
Proteolysis	medium to strong	slow				+
Hydrolysis of starch	weak	good				+

komabensis HAMADA *et* OKAMI based on the name of the area, Komaba where this organism was isolated.

Fermentation

The survey of media for production of the antibiotic resulted in the choice of glycerol as the sole carbon source and digested soybean meal (Prorich, product of Ajinomoto Co.) as a nitrogen source. In a medium consisting of 2 % glycerol, 2 % soybean meal (trade name, Prorich), 0.2 % yeast extract, 0.1 % K_2HPO_4, 0.2 % NaCl and 0.1 % $MgSO_4 \cdot 7H_2O$, production of 450 mcg/ml of laspartomycin was achieved at 168-hours, when it was cultured on a reciprocating shaking machine with 8 cm amplitude, 140 strokes per minute at 27°C.

Isolation and Purification

Laspartomycin was present mainly in the liquid portion of the fermented beer but some also in the mycelium mass. Fermented broth (7.5 L) was extracted at pH 2 with *n*-butanol (5,180 ml). The antibiotic in the solvent layer was re-extracted into water at a pH greater than 10. The aqueous extract (5,100 ml) was re-extracted with *n*-butanol (1,700 ml and 1,000 ml) at pH 2, and concentrated to about 50 ml under reduced pressure. To the concentrated solution ethyl acetate (500 ml) was added to precipitate a brown crude powder (3.691 g) containing the antibiotic. A methanol solution (30 ml) of this powder was passed through a column (2×26 cm) containing Alumina Woelm Neutral (60 g), and the elution was performed with 0.1 N NH_4OH - MeOH (2 : 8 in volume). The active eluate (1,000 ml) was concentrated and dried *in vacuo* to yield 2.263 g of yellowish powder. Further purification was made by column (3×24 cm) chromatography using 70 g of silica gel (Kanto Chemical Co., 60~80 mesh) and the mixture of $CHCl_3$ - MeOH - H_2O - EtOAc (100 : 40 : 7 : 40 by volume) as the developing solvent. Active eluates were combined and evaporation *in vacuo* gave 1.377 g of powder. The powder was dissolved in *n*-butanol (50 ml), and the solution washed with water (50 ml) at pH 9 and pH 2. The butanol layer was further washed with water (50 ml) 6 times, and then dehydrated with Na_2SO_4. The butanol solution was concentrated to dryness. The residual solid (1.283 g) was dissolved in methanol (50 ml), treated with active charcoal and finally 1.265 g of pure laspartomycin was obtained. The purified laspartomycin thus obtained showed one spot on a thin layer chromatogram and is described in the next section.

Physical and Chemical Properties of Laspartomycin

Laspartomycin is obtained as a colorless powder of an acidic peptide having an isoelectric point at about pH 2.6. It shows decomposition at 199°C, weakly positive ninhydrin and biuret reactions, and negative MOLISCH and anthrone reactions. It decolorizes potassium permanganate solution. It is soluble in water (*ca.* 0.2 g/ml), methanol, ethanol and butanol, but is not or slightly soluble in ethyl acetate, butyl acetate, ethyl ether, benzene, acetone or petroleum ether. Optical rotation in 1 % methanol solution, $[\alpha]_D^{20}$ is −17.5°. As seen in Fig. 1, its spectrum has only end

absorption. Elemental analysis of its free acid is as follows: C 53.96 %, H 7.61 %, N 13.07 %, O 25.22 %, no halogen and sulfur. Infrared spectrum in a KBr tablet (Fig. 2) shows following absorption bands (cm^{-1}): 3330, 3060, 2940, 1730, 1660, 1530, 1440, 1340, 1230, 1175, 1025, 1005, 980, 925, 870 and 850.

On thin-layer chromatograms using silica gel, a single spot is detected by permanganate at Rf values of 0.14, 0.13, 0.45, 0.163, 0.58 and 0.76 with the following solvent systems: *n*-butanol saturated with water, acetic acid - chloroform (2 : 1), secondary butanol - formic acid - water (75 : 15 : 10), *n*-propanol - acetic acid - chloroform (1 : 4 : 2), *n*-butanol - acetic acid - water (4 : 1 : 2) and *n*-butanol - pyridine - water (2 : 1 : 1), respectively.

Fig. 1. Ultraviolet absorption spectrum of laspartomycin

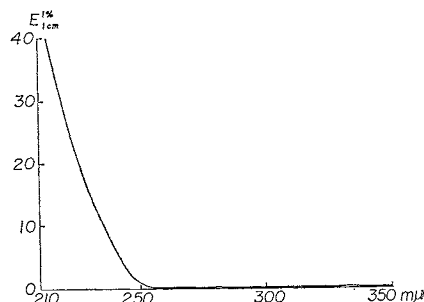
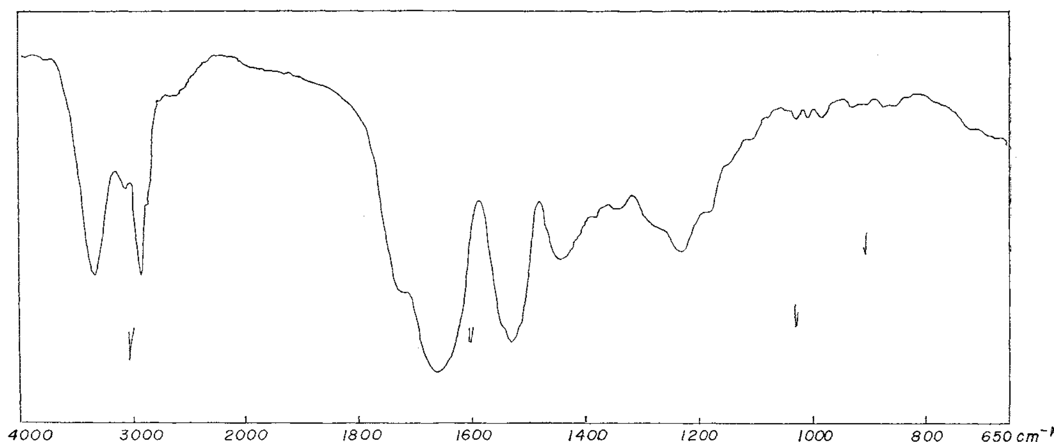


Fig. 2. Infrared absorption spectrum of laspartomycin (KBr)



With ascending paper chromatography (Toyo filter No. 51) using *n*-butanol saturated with water, 5 % NH_4Cl, *n*-butanol - acetic acid - water (4 : 1 : 2), *n*-butanol - pyridine - water (2 : 1 : 1), *n*-butanol - pyridine - acetic acid - water (20 : 5 : 5 : 10) and benzene - methanol (4 : 1), laspartomycin shows a single spot by bioautography at Rf values, 0.45, 0.23, 0.91, 0.41, 0.80 and 0.70, respectively.

The hydrolysate of laspartomycin with 6 N HCl at 105°C for 20 hours was subjected to two dimensional paper chromatographic analysis, using high voltage electrophoresis with acetic acid - formic acid - water (75 : 25 : 900) in one direction and *n*-butanol - acetic acid - water (4 : 1 : 2) in the other. Nine spots were detected by ninhydrin and isatin reactions.

Amino acid analysis of the hydrolysate gave each four moles of aspartic acid and glycine, each one mole of threonine, proline, pipecolic acid, isoleucine, and α,β-diamino-butyric acid, with proline calculated as 1 mole.

In addition to these amino acids, it gave two unknown amino acids, one of them

being located in the same position as arginine. This amino acid was not arginine, as indicated by its negative SAKAGUCHI reaction. The results described above are shown in Table 2.

Table 2. Amino acids of laspartomycin

	Found amino acid μmoles per 1 mg of sample	Molar ratio (Pro.=1.00)
Aspartic acid	1.59	3.87
Threonine	0.458	1.11
Unknown	++	—
Proline	0.411	1.00
Glycine	1.55	3.77
Pipecolic acid	++	—
Isoleucine	0.490	1.19
α,β-Diaminobutyric acid	0.251*	0.611*
Unknown	0.199**	0.484**

* Calcd. using the const. of α, γ-diaminobutyric acid
** Calcd. as arginine

Table 3. Stability of laspartomycin solutions (2 mg/ml) at 60°C for 30 minutes

Laspartomycin solution	Activity remained
pH 2. aq. sol.	75 %
pH 5. 〃	100
pH 7. 〃	105
pH 9. 〃	96
0.1 N HCl sol.	106
0.1 N NaOH sol.	100
Control aq. sol.	100

Activity was determined by a cylinder plate method using *Micrococcus flavus* as the test organism.

Table 4. Antibacterial activity of laspartomycin

Test organism	Minimal inhibitory concentration mcg/ml
Staphylococcus aureus FDA 209 P	1.56
〃 〃 R 1	1.56
〃 〃 R 2	1.56
〃 〃 R 3	1.56
〃 〃 R 4	1.56
〃 〃 R 5	3.12
〃 Smith	1.56
〃 Terajima	1.56
〃 52-34	3.12
〃 〃 R 6	3.12
〃 193	1.56
〃 〃 R 7	1.56
Micrococcus flavus 16	<0.39
Sartina lutea PCI 1001	0.78
Bacillus subtilis PCI 219	6.25
Bacillus anthracis	0.78
Mycobacterium 607	>100
Escherichia coli NIHJ	>100
Pseudomonas aeruginosa A_3	>100
Proteus vulgaris OX 19	>100
Klebsiella pneumoniae PCI 602	>100
Shigella flexneri 1 a Ew 8	>100
Serratia marcescens	>100

R 1=Kanamycin resistant, R 2=Streptothricin & streptomycin resistant, R 3=Bryamycin resistant, R 4=Novobiocin resistant, R 5=Actinomycin resistant, R 6=Erythromycin, carbomycin & tetracycline resistant, R7=Erythromycin resistant

Table 5. Antifungal activity of laspartomycin

Test organism	Minimal inhibitory concentration mcg/ml
Cladospolium spharosperum	25.0
Colletotrichum phomoides	12.5
Fusarium roseum	50.0
Fusarium lini	>100.0
Fusarium oxysporium	>100.0
Gibberella fujikuroi	12.5
Gloeosporium kaki	12.5
Glomerella laginarium	100.0
Helminthosporium sesameum	25.0
Ophiobolus miyabeanus	50.0
Pellicularia filamentosa (Sasaki)	6.25
Pyricularia grisea	12.5
Schlerotium rofsii	12.5
Alternaria kikuchiana	12.5
Xanthomonas oryzae	>100.0
Absidia spinosa	>100.0
Candida tropicalis	100.0
Candida pseudotropicalis	>100.0
Candida Yu-1200	>100.0
Candida albicans 3147	>100.0
Candida krusei	>100.0
Saccharomyces cerevisiae	>100.0
Torula utilis 4001	6.25
Botrytis bassiana	>100.0
Cryptococcus neoformans	3.12
Pseudomonas fluorescens	>100.0
Helminthosporium oryzae	25.0
Trichophyton mentagrophytes 598	50.0
Trichophyton asteroides	50.0
Aspergillus niger	>100.0
Penicillium chrysogenum 49-133	25.0
Penicillium lilacinum 8021	>100.0
Pyricularia oryzae	6.25

On the basis of the elemental analysis and the amino acid content, a tentative molecular formula is calculated to be $C_{82}H_{137\sim139}N_{17}O_{29}$ and its molecular weight is 1,825~1,827.

When laspartomycin in dilute hydrochloric acid was titrated with 1 N NaOH, its titration equivalent for neutralization was 455 and its molecular weight was estimated to be 1820.

The stability of laspartomycin is shown in Table 3.

Biological Activity of Laspartomycin

An acute toxic reaction was not shown by intravenous injection of 5 mg of laspartomycin to a mouse. When mice were infected with *Staphylococcus aureus* Smith strain and laspartomycin was administered 1.5 hours after the infection, intraperitoneal injection of over 25 mcg per mouse saved all the mice. As shown by the antimicrobial spectrum in Tables 4 and 5, laspartomycin is active against Gram-positive bacteria and some of fungi. Laspartomycin does not show any hemolysis at 800 mcg/ml in agar plate containing 10 % human blood or 10 % rabbit blood.

Discussion

Among antibiotics which are active against Gram-positive bacteria and which are acidic or amphoteric peptides, aspartocin[1], amphomycin[2], zaomycin[3], glumamycin[4], and crystallomycin[5] are most closely related to laspartomycin.

Authentic samples of amphomycin, glumamycin and aspartocin were differentiated from laspartomycin by ascending paper chromatography. Rf values of amphomycin, glumamycin, aspartocin and laspartomycin detected by bioautography are 0.15, 0.03, 0.03, 0.70 with solvent system of benzene - methanol (4 : 1), 0.07, 0.05, 0.05, 0.45 with *n*-butanol saturated with water, and 0.15, 0.10, 0.10, 0.41 with *n*-butanol - pyridine - water (2 : 1 : 1), respectively.

They are also differentiated by thin-layer chromatography on silica gel. Rf values of amphomycin, glumamycin, aspartocin and laspartomycin detected by decolorization of permanganate and bioautography are 0.03, 0.03, 0.03, 0.14 with *n*-butanol-saturated with water, 0.026, 0.020, 0.020, 0.13 with acetic acid-chloroform (2 : 1), 0.35, 0.44, 0.44, 0.58 with *n*-butanol - acetic acid - water (4 : 1 : 2), and 0.27, 0.70, 0.64, 0.76 with *n*-butanol - pyridine - water (2 : 1 : 1), respectively.

Zaomycin[3] has been described to remain at the spotted position on paper chromatography with *n*-butanol saturated with water and benzene - methanol (4 : 1) and therefore can be differentiated from laspartomycin. Crystallomycin[6] has been described to contain no isoleucine.

Thus, the antibiotic from the strain M 307-M 5 was differentiated from all known similar antibiotics and was named laspartomycin.

Acknowledgement

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