THE HAZIMICINS, A NEW CLASS OF ANTIBIOTICS

TAXONOMY, FERMENTATION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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The hazimicins, a new class of broad spectrum antibiotics with at least 2 active components (5 and 6), were isolated from the fermentation of *Micromonospora echinospora* var *challisensis* SCC 1411.

The complex was separated from the broth by a solvent extraction procedure, and the individual components were separated by column chromatography. The two primary active components are isomers, with unique structures shown to be di-tyrosine analogs containing two isonitrile groups. The antibiotic has *in vitro* and *in vivo* activity against Gram-positive and Gram-negative bacteria, and *in vitro* activity against yeasts and dermatophytes.

In our continuing efforts to discover novel antibiotic structures, a strain of Micromonospora has been found which produces a new class of antibiotics, called the hazimicins. The organism produces a six component complex of which components 5 and 6 have been isolated and purified. These display unusual activity on agar against various Gram-positive and Gram-negative bacteria and yeast, as evidenced by hazy zones of inhibition. In addition, a hazimicin mixture of components 5 and 6 was active in MICs against a large number of microorganisms and protected mice against *Staphylococcus* sp., and to a lesser degree *Escherichia* sp., infections.

This paper details the taxonomy of the producing organism, the fermentation and isolation of the antibiotic complex as well as components 5 and 6, and purification, identification, characterization, ^{14}C-labelling and biological activity of the components.

Details of the structure determination of hazimicins 5 and 6 appear elsewhere[1].

Taxonomy

The organism producing hazimicin was isolated from a soil sample collected in a field of bunch grass and sage in Challis, Idaho. The culture was deposited in the Schering Culture Collection under the accession number SCC 1411.

Macroscopic determinations, including growth characteristics, color of the vegetative mycelium, and diffusible pigment formation, were made after 14 to 21 days at 30°C on media defined by SHIRLING and GOTTLIEB[2], and WAKSMAN[3]. Each color designation assigned to the vegetative mycelial pigments consisted of a color name[4] and color chip number[5]. Carbohydrate utilization was determined by the procedure of LUEDEMANN and BRODSKY[6] and decomposition of organic compounds followed the procedures of GORDON *et al.*[7], and GOODFELLOW[8]. Antibiotic susceptibility testing was done as described by HORAN and BRODSKY[9]. Morphologic observations were based on the growth of the strain in broth

Table 1. Growth characteristics of SCC 1411 on various descriptive media.

Medium		
Bennett agar	G:	Good
	S:	Raised, convoluted
	AM:	Absent
	DFP:	Absent
	C:	g5nl, chocolate
Czapek - sucrose agar	G:	Fair
	S:	Raised, granular
	AM:	Absent
	DFP:	Faint yellow brown
	C:	g4ne, luggage tan
Glycerol - asparagine agar (ISP No. 5)	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4gc, rose beige
Nutrient agar	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4ie, cork tan
Potato - dextrose agar	G:	Moderate
	S:	Raised, granular
	AM:	Present; white-rose bloom
	DFP:	Absent
	C:	g5li, dark rose taupe
Emerson agar	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4ec, light rose beige
NZ-Amine glucose agar	G:	Good
	S:	Raised, deeply folded, waxy
	AM:	Absent
	DFP:	Absent
	C:	g4ne, luggage tan
Yeast extract - glucose agar	G:	Good
	S:	Flat, granular to folded
	AM:	Absent
	DFP:	Slight gray brown
	C:	g5nl, chocolate
Tomato paste - oatmeal agar	G:	Good
	S:	Raised, plicate
	AM:	Absent
	DFP:	Absent
	C:	g7ni, rose brown
Yeast extract - malt extract agar (ISP No. 2)	G:	Good
	S:	Raised, folded
	AM:	Absent
	DFP:	Absent
	C:	g3ie, camel
Oatmeal agar (ISP No. 3)	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4ic, suntan
Inorganic salts - starch agar (ISP No. 4)	G:	Moderate to fair
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	Center, PO, black; periphery, g5ig, rose taupe
Starch agar (Waksman No. 21)	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4ie, cork tan
Gelatin agar	G:	Moderate to fair
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g2le, mustard
Casein agar	G:	Fair to poor
	S:	Flat, granular
	AM:	Absent
	DFP:	Absent
	C:	g4ge, rose beige
Tyrosine agar (modified)	G:	Moderate
	S:	Raised, slightly folded
	AM:	Absent
	DFP:	Faint brown
	C:	g5ng, brick red
Starch agar (modified)	G:	Good
	S:	Raised, plicate
	AM:	Absent
	DFP:	Slight rose
	C:	g4lc, dusty orange

G=Growth; S=surface characteristics; AM=aerial mycelium; DFP=diffusible pigment; and C=color of the growth.

for 7 days as described by LUEDEMANN and BRODSKY[6].

Test media were inoculated with a washed mycelial suspension from a broth culture shaken at 30°C for 72 hours in a liquid medium (ATCC medium 172)[10].

Table 2. Carbohydrate utilization of SCC 1411.

Utilization of	Result	Utilization of	Result
D-Arabinose	±	Lactose	±
L-Arabinose	+++	Maltose	+++
Cellibiose	++	Mannitol	±
Dextrin	++	Mannose	+++
Dulcitol	±	Melibiose	±
Erythritol	±	Melizitose	±
Fructose	+	Raffinose	∓
L-Fucose	±	Rhamnose	±
Galactose	∓	Ribose	±
Glucose	+++	Sucrose	+++
α-m-d-Glucoside	±	Trehalose	+++
Inositol	±	D-Xylose	+++
Inulin	+		

+++ Good, ++ moderate, + fair, ∓ fair to poor, ± poor.

Table 3. Physiologic characteristics of SCC 1411.

Test		Result
Growth in the presence of (50 μg/ml)	Gentamicin	+
	Sisomicin	+
	Kanamycin	+
	Erythromycin	−
	Everninomicin	−
	Cycloserine	−
	Tetracycline	−
	Gentamicin A	+
NaCl (%)	1.0	+++, good
	2.0	+, fair to poor
	3.0	±, poor
Hydrolysis of	Adenine	−
	Hypoxanthine	−
	Tyrosine	+
	Xanthine	−
	Chitin	+
	Casein	+
	Starch	+
	DNA	+
	Gelatin	+
Growth at	27°C	++, moderate
	35°C	+++, good
	40°C	+++, good
	45°C	±, poor
	50°C/8 hours	±, poor
Grown on	Potato+$CaCO_3$	+++, good
	Potato−$CaCO_3$	±, poor

Morphologic Observations

Abundant rough walled spores (1.0~1.5 μm in diameter) were formed along the length of fine (0.5~0.8 μm in diameter) slightly branched mycelial strands. Spores occurred singly or in clusters and were sessile or on short to long sporophores. Electron microscopic observation of spore whole mounts showed protrubances or warts on the surface of both the spores and sporophores. Cell wall analysis indicated the presence of *meso*-diaminopimelic acid, while whole cells characteristically contained arabinose and xylose.

Appearance on Various Media

The macroscopic appearance of SCC 1411 on various descriptive media is presented in Table 1. The vegetative mycelial pigments ranged from tan-orange to light rose. A slight white to pink bloom was formed on potato - dextrose agar. Diffusible pigments when formed were gray-brown to rose. Good growth occurred on most media; poor growth occurred on glucose - asparagine, peptone - glucose, peptone - iron, and calcium malate agars.

Physiologic Characteristics

Tables 2 and 3 present the results of the physiologic tests applied to SCC 1411. The following characteristics were used to define this strain: good growth on L-arabinose, glucose, maltose, mannose, sucrose, trehalose, and D-xylose; slight growth on galactose; poor growth on lactose, raffinose, rhamnose and ribose; growth in the presence of gentamicin, sisomicin and kanamycin; growth at 40°C; inability to grow in the presence of 3% NaCl, on acid potato, or at 45°C.

Identification and Classification

Based on morphologic and cell wall analysis, SCC 1411 was identified as a strain belonging to the genus Micromonospora. The formation of warty spores, vegetative mycelial pigmentation, the occurrence of a bloom on some media, growth in the presence of aminoglycoside antibiotics, inability to utilize

Table 4. Comparison of distinguishing properties of *Micromonospora* sp. SCC 1411, *M. echinospora* NRRL 2985, and *M. purpurea* NRRL 2953.

Test		*Micromonospora* sp. SCC 1411	*M. echinospora* NRRL 2985	*M. purpurea* NRRL 2953
Carbohydrate utilization	D-Arabinose	±, poor	±, poor	±, poor
	L-Arabinose	+++, good	+++, good	+++, good
	Fructose	+, fair	±, poor	±, poor
	Galactose	+, fair	±, poor	±, poor
	α-m-d-Glucoside	±, poor	±, poor	±, poor
	Raffinose	±, fair to poor	±, poor	±, poor
	Rhamnose	±, poor	+++, good	±, poor
	Ribose	±, poor	±, poor	+, fair to poor
	D-Xylose	+++, good	+++, good	+++, good
Growth in the presence of: (50 μg/ml)	Gentamicin	+	+	+
	Sisomicin	+	+	+
	Kanamycin	−	−	−
	Erythromycin	−	−	−
	Everninomicin	−	−	−
NaCl (%)	2.0	+, fair	+, fair	+, fair
	3.0	±, poor	±, poor	±, poor
Growth at	40°C	+++, good	+++, good	+++, good
	45°C	±, poor	±, poor	±, poor
Growth on	Potato +$CaCO_3$	+++, good	+++, good	+++, good
	Potato −$CaCO_3$	±, poor	±, poor	±, poor
Formation of	Warty spores	+	+	−

lactose, and poor growth on galactose, all suggested that this culture was most closely related to the *M. echinospora* – *M. purpurea* group.

Table 4 compares some key characteristics of SCC 1411 to *M. echinospora* NRRL 2985 and *M. purpurea* NRRL 2953. SCC 1411 differs from *M. purpurea* in the formation of spores, from *M. echinospora* in the lack of utilization of rhamnose, and from both in the production of hazimicin. Based on these finding, SCC 1411 was considered to be a variety of *M. echinospora* designated *M. echinospora* var *challisensis* SCC 1411.

Production of Antibiotic Complex

The inoculum for antibiotic production was prepared in a medium which contained 0.3% beef extract, 0.5% tryptone, 0.5% yeast extract, 0.1% dextrose, 2.4% potato starch and 0.2% calcium carbonate. The medium was adjusted to pH 7.5 before sterilization. A 300-ml Erlenmeyer flask containing 70 ml of the above medium was inoculated with 4 to 5 ml of a stock suspension of the antibiotic producing culture maintained at −20°C. The flask was incubated at 30°C on a rotary shaker for 48 hours.

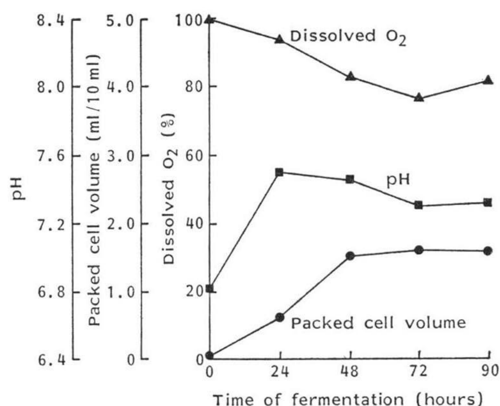
Twenty-five ml of the seed culture was transferred to a 2-liter Erlenmeyer flask containing 500 ml of the same medium and incubated as above. This second stage seed culture was used as the inoculum to initiate the fermentation in 14-liter New Brunswick Scientific laboratory fermentors batched with 10 liters of a medium containing: 0 5% yeast extract, 1.0% dextrose, 2.0% soluble potato starch, 0.5% casein hydrolysate, 0.4% calcium carbonate and 0.024% cobalt chloride. The fermentation was carried out at 30°C with 3.5 liters of air per minute and agitation at 350 rpm. Total antibiotic activity was monitored at regular intervals by bioassay against *Micrococcus luteus* and *Escherichia coli*. The pH, dissolved oxygen levels, and growth profile of the organism during fermentation was also monitored (Fig. 1). The

amounts of different components of the antibiotic complex were assessed by thin-layer chromatography on Whatman LK6DF plates in a solvent system consisting of chloroform - methanol (8: 2) followed by bioautography against *M. luteus*.

Extraction and Isolation

A 100-liter fermentation was extracted twice with equal volumes of ethyl acetate. After separation, the solvent phases were combined and concentrated to dryness. The dried residue was dissolved in acetone, separated from biologically inactive oils, added to a mixture of ethyl ether - hexane (6: 4), and the resulting precipitate (12 g) was filtered and dried over vacuum.

Fig. 1. Time course study of *M. echinospora* var *challisinsis*.



Bioautography (against *M. luteus*) of hazimicin complex on silica gel thin-layer plates following chromatography in a solvent system consisting of chloroform - methanol (9: 1), demonstrated that the complex consisted of at least seven biologically active components. Two components which represented the major portion of the activity have been designated hazimicin component 5 and hazimicin component 6 (based on decreasing Rf values).

Isolation of both components was achieved by chromatography of 12 g of the complex on Sephadex LH-20 and elution with ethanol. The desired fractions, determined by chromatography, were combined and evaporated to dryness. Two grams of a purified mixture was obtained in this manner.

The 2 g mixture of components 5 and 6 was further separated by chromatography on a silica gel column (height 76.2 cm, width 7.6 cm; eluted with chloroform - methanol (9: 1); rate 1 ml/minute). The column was monitored by determining the activity of each fraction against *M. luteus* using a disc diffusion assay. The active fractions were bioautographed as described above and the desired fractions containing either component 5 or 6 combined. Precipitation of the individual components yielded 118 mg of hazimicin component 5 and 150 mg of component 6.

Physico-chemical Characteristics of Hazimicin Components 5 and 6

Hazimicin components 5 and 6 obtained as off-white amorphous powders were found to have similar physico-chemical properties. Both components were soluble in ethanol, methanol and acetone but insoluble in ether and petroleum ether. The UV absorption spectra in methanol of both components were identical (Fig. 2) and showed a peak at 290 nm with an $E^{1\%}_{1cm}$ of 148 and an inflection point at 240 nm.

Elemental analysis showed that both compounds contained 60.86% C, 5.17% H and 13.81% N which is consistent with the molecular formula $C_{20}H_{18}N_4O_4$. Neither compound gave a molecular ion in its mass spectrum. Their infrared spectra were identical (Fig. 3) and revealed characteristic absorption bands at 2160 cm^{-1}, 1680 cm^{-1} and 1612 cm^{-1}.

The chromatographic pattern of hazimicins 5 and 6 were found to be identical in all but one of the systems tried. The Rf values of both components on Whatman #1 paper and on Analtech silica gel GF thin-layer plates are given in Table 5.

Both components were stable from pH 6~8 at room temperature and at pH 6~7 up to 100°C for 15 minutes in aqueous solutions. They both lost activity immediately at room temperature, at pH 2~4,

Fig. 2. UV absorption spectra of hazimicin in methanol.

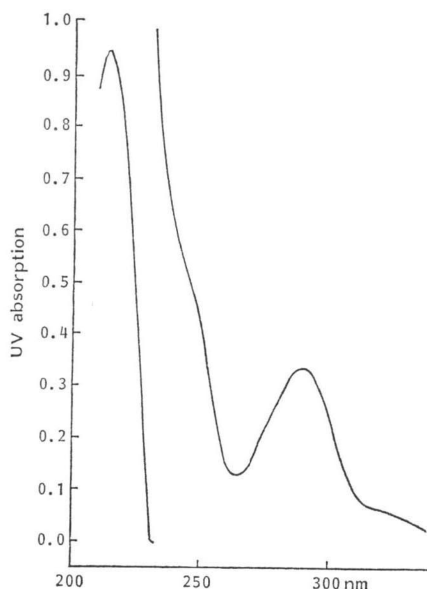


Table 5. Chromatographic migration of hazimicin components 5 and 6.

System	Rf**	
	5	6
Paper chromatography*		
1-Butanol - water - acetic acid (4: 5: 1) upper phase ascending	0.76	0.76
Propanol - water - acetic acid (10: 8: 1) ascending	0.76	0.76
Propanol - pyridine - acetic acid - water (15: 10: 3: 12) ascending	0.60	0.60
80% Methanol plus 3% NaCl descending***	0.86	0.86
Toluene - petroleum ether - acetone (3: 2: 3) descending	0.50	0.50
Chloroform - methanol - 17% ammonium hydroxide	0.00	0.00
Thin-layer chromatography****		
Acetone - toluene (3: 2)	0.43	0.43
Chloroform	0.00	0.00
Methanol	0.80	0.80
Toluene - methanol (22: 3)	0.15	0.15
Toluene - methanol (94: 6)	0.05	0.05
Chloroform - methanol (9: 1)	0.26	0.14

* Whatman No. 1 paper.
** Detection: bioautography *vs* *M. luteus*.
*** Paper buffered with 0.95 M Na_2SO_4 plus 0.05 M $NaHSO_4$.
**** Analtech silica gel GF TLC plates.

and pH 10. Hazimicin components 5 and 6 were interconvertible in the presence of water. This reaction was accelerated in the presence of ammonium hydroxide.

The total structure and stereochemistry of hazimicin components 5 and 6 have been reported separately by WRIGHT *et al.*[1]. The structures, elucidated by spectroscopic and X-ray crystallographic studies, have been shown to be di-tyrosine analogs containing two isonitrile groups.

From the above data it was concluded that hazimicin components 5 and 6 are novel members of a small but growing group of isonitrile-containing antibiotics[1].

Fig. 3. Infrared spectrum of hazimicin (Nujol).

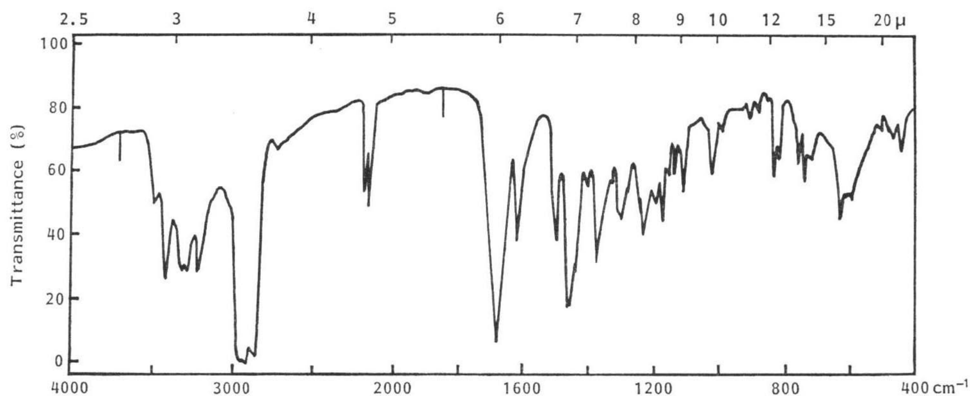


Table 6. *In vitro* activity of hazimicin complex and components 5 and 6 against various bacteria and Candida.

Organisms	Agar diffusion zone size (hazy) (mm) at 2 concentrations (μg/disc), 24 hours					
	Hazimicin complex		Component 5		Component 6	
	40	4	40	4	40	4
Candida albicans 406[a]	11	0	13	9	11	0
E. coli A10536[b]	25	9	27	12	25	11
M. luteus A9341[b]	25	11	26	13	25	11
S. aureus A6538P[b]	25	11	30	14	25	12

[a] Sabouraud dextrose agar pH 5.6.
[b] Nutrient agar pH 6.8.

Table 7. *In vitro* activity of hazimicin mixture against various bacteria and fungi.

Organisms	No. of strains	Average MICs (μg/ml, 24 hours incubation)
Bacillus sp.	2	24
Mycobacterium fortuitum	1	17.5
Nocardia asteroides	1	17.5
Staphylococcus sp.	14	8
Streptococcus sp.	8	23
Streptomyces griseus	1	17.5
Enterobacter sp.	2	>128
Escherichia sp.	4	128
Klebsiella sp.	3	>128
Providencia sp.	2	>128
Pseudomonas sp.	4	>128
Salmonella sp.	2	>128
Serratia sp.	2	>128
Shigella dysenteriae	1	>128
Actinomyces israelii[a]	1	17.5
Bacteroides fragilis[a]	1	>50
Clostridium novyi[a]	1	>50
Propionibacterium acnes[a]	1	>50
Candida albicans[b]	1	17.5
Saccharomyces cerevisiae[b]	1	17.5
Epidermophyton floccosum[b]	1	17.5
Trichophyton mentagrophytes[b]	1	17.5

[a] 48 hours incubation.
[b] 72 hours incubation.

Table 8. *In vivo* activity of hazimicin mixture against a Staphylococcus or Escherichia infection in mice.

Compound	Route	Organism	Inoculum/mouse	PD_{50}* (mg/kg)
Hazimicin	SC	*S. aureus*	3.6×10^{7}	3
〃	PO	〃	〃	5
Cephalothin	SC	〃	〃	<0.8
〃	PO	〃	〃	4
Hazimicin	SC	*E. coli*	1.2×10^{6}	100
〃	PO	〃	〃	>100
Cephalothin	SC	〃	〃	60
〃	PO	〃	〃	100

* 72 hours after infection

^{14}C-Labelling of Hazimicin Components 5 and 6

Shake-flask fermentations were performed to study incorporation of radiolabelled precursors into hazimicin. Ten μCi of DL-[3-^{14}C]tyrosine or L-[*methyl*-^{14}C]methionine was added to 50 ml of the fermentation medium in 250 ml flasks at 48 hours, and the organism was exposed to the radiolabelled precursors by shaking the flask for an additional 48 hours.

The results of these studies showed that 9% of DL-[3-^{14}C]tyrosine was incorporated into hazimicin component 5 and 5% into hazimicin component 6; and 0.2% of L-[*methyl*-^{14}C]methionine was incorporated into component 5 and 0.3% into component 6.

Biological Properties

In vitro agar diffusion tests to determine the activity of hazimicin complex and components 5 and 6, were done on nutrient agar at pH 6.8 and Sabouraud dextrose agar at pH 5.6 using 6 mm paper discs. The results, shown in Table 6, indicate that all 3 preparations have broad spectrum activity at 40.0 μg/

disc but the zones were all hazy. At 4.0 μg/disc the activity was significantly lower and at 0.4 μg/disc no activity was seen. Since components 5 and 6 are interconvertible in the presence of water, all subsequent testing was performed on a mixture of the two components.

In vitro dilution tests to determine minimum inhibitory concentrations (MICs) were done using Mueller-Hinton broth pH 7.4 for bacteria, thioglycolate broth pH 7.1 for anaerobes, and Sabouraud dextrose broth pH 5.7 for fungi. The results, shown in Table 7, indicate that hazimicin mixture had good activity (MICs 8 μg/ml) against the 14 strains of *Staphylococcus* sp. studied. It had weak activity (MICs 8～24 μg/ml) against other Gram-positive bacteria, yeasts, dermatophytes, Actinomyces and some strains of *Escherichia* sp. The antibiotic was inactive (MICs>128 μg/ml) against all other Gram-negative bacteria.

The *in vivo* subcutaneous and oral activity of hazimicin mixture was determined in experimental infections (PD_{50}s) in mice against *S. aureus* and *E. coli* and the results are shown in Table 8. It can be seen that hazimicin mixture was active against *S. aureus* (PD_{50} 3 and 5 mg/kg), but only slightly active against the *E. coli* (PD_{50} 100 mg/kg or greater). The subcutaneous LD_{50} of the antibiotic in mice was greater than 200 mg/kg.

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