Macquarimicins, Microbial Metabolites from *Micromonospora*

I. Discovery, Taxonomy, Fermentation and Biological Properties

MARIANNA JACKSON, JAMES P. KARWOWSKI, ROBERT J. THERIAULT, RONALD R. RASMUSSEN, DENA M. HENSEY, PATRICK E. HUMPHREY, SUSAN J. SWANSON, GRANT J. BARLOW, USHA PREMACHANDRAN and JAMES B. MCALPINE

New Lead Discovery, Abbott Laboratories,
Abbott Park, Illinois 60064, U.S.A.

(Received for publication February 27, 1995)

A novel series of microbial metabolites were discovered in fermentation broths of two soil isolates. Both cultures were identified as strains of *Micromonospora chalcea*. Production of the metabolites, named macquarimicins, was monitored by an HPLC assay. A seven-day fermentation yielded 27 mg/liter of macquarimicin A. With MICs of 50 to 100 μg/ml, macquarimicin A has only very low activity against strains of *Bacteroides* and other anaerobes. Macquarimicin B has inhibitory activity against the leukemia cell line P-388.

The *Bacteroides fragilis* group of Gram-negative anaerobic bacteria, which constitute the predominant portion of normal human colonic flora, are frequently recovered from clinical specimens. *B. fragilis* and *B. thetaiotamicron* are found in most abdominal infections and may be seen in infections at other sites. The *Bacteroides* group is more resistant than most other anaerobes to available antimicrobial agents[1]. We used *B. fragilis* as the primary target organism in screening microbial metabolites for agents which inhibit anaerobic bacteria. From this screen we found two microorganisms producing a family of novel compounds. This paper will describe the production of the agents by fermentation, the identification of the producing microorganisms and the biological activity of the macquarimicins. A companion paper will describe the isolation of the active components and the elucidation of their chemical structures[2].

Materials and Methods

Microorganisms

Micromonospora strain AB 965S-73 was isolated from a soil sample collected on a construction site at Macquarie University in Sydney, Australia. The second producer, *Micromonospora* sp. AB 969J-62, was from soil collected in Henrico County, Virginia, U.S.A. The *Micromonospora* neotype strain, *M. chalcea* ATCC 12452, was obtained from the American Type Culture Collection. The bacteria used to characterize the activity of macquarimicin A were from the ATCC and from our laboratory collection.

Taxonomic Studies

The producers of macquarimicin were compared with the *Micromonospora* neotype strain on media described by SHIRLING and GOTTLIEB[3] and by WAKSMAN[4] and on ATCC medium 172 with incubation at 28°C for 22 days. Color names were assigned to the mycelial growth and to the spore masses on the basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts[5]. Carbon source utilization was determined as described by SHIRLING and GOTTLIEB[3]. The diaminopimelic acid isomer was determined by the method of BECKER *et al.*[6]. Whole-cell sugars were identified by the procedure of LECHEVALIER[7]. Lysozyme sensitivity was tested in glycerol broth as suggested by GORDON *et al.*[8] Menaquinones were extracted as described by ATHALYE *et al.*[9] and analyzed by mass spectroscopy.

Fermentation

Macquarimicin was produced in a 150-liter New Brunswick fermentor. Inoculum was grown in a medium consisting of glucose monohydrate 0.1%, soluble starch 2.4%, yeast extract (Difco) 0.5%, tryptone (Difco) 0.5%, beef extract (Scott) 0.3% and $CaCO_3$ 0.4%. Distilled water was used, and the pH was adjusted to 7. Seed preparation was a sequence of two steps, the first in 10 ml of medium in 25 × 150 mm culture tubes, the second in 600 ml of medium in 2-liter Erlenmeyer flasks. Both the tubes and the flasks were incubated at 30°C on a rotary shaker at 250 rpm, the tubes for 96 hours and the second-stage flasks for 72 hours. The fermentation medium consisted of sucrose 2%, cottonseed flour (Proflo, Traders) 1%, yeast extract 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $ZnSO_4 \cdot 7H_2O$ 0.0005%, $CoCl_2 \cdot 6H_2O$ 0.0001% and $CaCO_3$ 0.1%. The medium

was prepared in distilled water, and the pH was adjusted to 7.3. The fermentor was charged with 70 liters of medium and sterilized in place for 1 hour at 121°C and 1.05 kg/cm^2. Agitation was 200 rpm, and aeration was 0.7 vol/vol/minute. A head pressure of 0.35 kg/cm^2 was maintained. Antifoam, XFO-371 (Ivanhoe Chemical), was added initially at 0.01% and then was available on demand. The fermentation was harvested on the 7th day.

Analysis of Fermentation Samples

Macquarimicin production was analyzed by HPLC. A 4.6 × 250 mm column packed with Spherisorb 5 C-8 (Phenomenex) was eluted with 0.1% H_3PO_4-CH_3CN (70:30) at pH 3. The flow rate was 1 ml/minute. Fermentation broth samples were prepared for assay by extraction with ethyl acetate. The solvent was removed *in vacuo*, and methanol was added to give a 100-fold concentration with respect to the broth. The methanol sample was filtered through an HPLC syringe filter before injection. Detection was at 240 nm. Growth was evaluated as packed cell volume by centrifuging the fermentation broth in a graduated conical tube at 600 × *g* for 20 minutes. Residual carbohydrate was determined by the phenol-sulfuric acid method of DUBOIS *et al.*[10].

Evaluation of Biological Activity

Minimal inhibitory concentrations (MICs) of macquarimicin were determined by the agar dilution method. Anaerobes were grown on Wilkins Chalgren agar incubated in a BBL Gas-Pak anaerobic chamber at 37°C. Aerobes were grown on Mueller-Hinton agar. Inoculum concentration was 1×10^4 cells per inoculation. Plates were incubated for 24 hours. The MIC was determined as the lowest concentration of macquarimicin with not more than 4 colonies per inoculation spot. For assessment of potential antitumor activity, P-388 leukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The antiproliferative effect of the agents against P-388 cells was determined using an MTT colorimetric method[11].

Results and Discussion

Strains AB 965S-73 and AB 969J-62 form well-developed, branched mycelia. Aerial hyphae were absent. Non-motile spores are borne singly on monopodial sporophores (see Figs. 1 and 2). The vegetative mycelium is orange on most media, and mature sporulation is brown to brownish black in color. The diaminopimelic acid found in whole-cell hydrolysates was the *meso* isomer. The diagnostic sugars present were xylose and arabinose indicating cell wall type IID[12]. The morphology and cell wall type identify these strains as members of the genus *Micromonospora*[13]. The macquarimicin-producing strains were compared with the *Micromonospora* neotype strain, *M. chalcea* ATCC 12452. The cultural characteristics of

Fig. 1. Scanning electron micrograph of *Micromonospora chalcea* AB 965S-73.

Bar represents 1.0 μm.

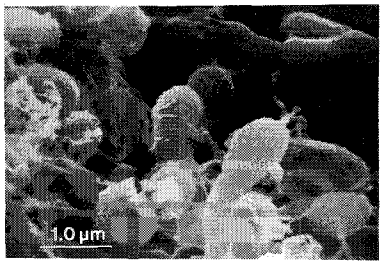


Fig. 2. Scanning electron micrograph of *Micromonospora chalcea* AB 969J-62.

Bar represents 2.0 μm.

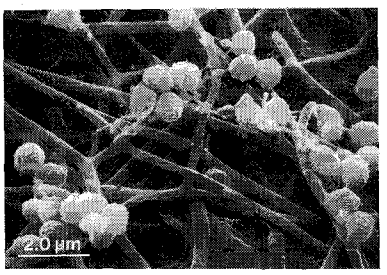
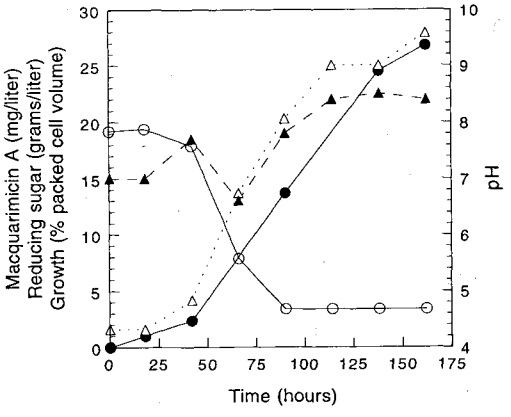


Fig. 3. Time course of the macquarimicin fermentation in a 150-liter fermentor.

● Macquarimicin A, ○ reducing sugar, △ growth, ▲ pH.



the three cultures are given in Table 1. The comparison of carbon source utilization is shown in Table 2. The macquarimicin-producing strains were found to be sensitive to lysozyme as was *M. chalcea*. Menaquinone MK-10(H_6) was found in extracts of AB 969J-62 while both MK-10(H_4) and MK-10(H_6) were in AB 965S-73 (see Table 3). These are the two most common menaquinones found among the micromonosporae. While the strains are not identical they have considerable phenotypic homology with each other and with *M. chalcea*. We consider both producing cultures to be

Table 1. Cultural characteristics of the macquarimicin-producing organisms, comparison with *Micromonospora chalcea* ATCC 12452.

Medium		*M. chalcea*	AB 965S-73	AB 969J-62
Yeast extract - malt extract agar	G*:	Abundant	Abundant	Abundant
ISP-2	V:	Strong brown (55)	Strong brown (55)	Moderate yellow pink (29)
	S:	Brown black (65)	Brown black (65)	Dark gray yellow brown (81)
Oatmeal agar	G:	Abundant	Abundant	Moderate
ISP-3	V:	Deep orange (51)	Strong orange (50)	Pale orange yellow (73)
	S:	Slight	Brown black (65)	Brown black (65)
Inorganic salts - starch agar	G:	Abundant	Abundant	Moderate
ISP-4	V:	Vivid orange (48)	Strong orange (50)	Moderate orange (53)
	S:	Absent	Brown black (65)	Dark gray yellow brown (81)
Glycerol - asparagine agar	G:	Moderate	Poor	Poor
ISP-5	V:	Dark orange yellow (72)	Pale orange yellow (73)	Pale orange yellow (73)
	S:	Moderate brown (58)	Dark yellow brown (78)	Brown black (65)
Peptone - yeast extract iron agar	G:	Abundant	Moderate	Moderate
ISP-6	V:	Vivid orange (48)	Strong yellow brown (74)	Moderate orange (53)
	S:	Absent	Moderate olive brown (95)	Dark gray yellow brown (81)
Tyrosine agar	G:	Abundant	Moderate	Moderate
ISP-7	V:	Deep yellow brown (75)	Moderate orange yellow (96)	Light orange (52)
	S:		Dark olive brown (96)	Brown black (65)
Nutrient agar	G:	Abundant	Abundant	Abundant
	V:	Deep orange (51)	Moderate orange yellow (71)	Light orange (52)
	S:	Absent	Dark olive brown (96)	Dark yellow brown (78)
Czapek Dox agar	G:	Abundant	Abundant	Abundant
	V:	Strong yellow brown (74)	Moderate orange yellow (71)	Light orange (52)
	S:	Moderate orange yellow (71)	Absent	Absent
Calcium malate agar	G:	Poor	Absent	Poor
	V:	Light orange (52)		
	S:	Moderate olive brown (95)		Dark gray yellow brown (81)
ATCC #172 agar	G:	Abundant	Abundant	Abundant
	V:	Strong orange (50)	Moderate orange (53)	Vivid orange (48)
	S:	Brown black (65)	Deep brown (56)	Dark gray yellow brown (81)

* G=growth; V=color of vegetative mycelium; S=color of mature sporulation. Observations after 22 days incubation at 28°C. No soluble pigments were observed.

Table 2. Utilization of various compounds as the sole source of carbon.

Carbon source	*M. chalcea* ATCC 12452	AB 965S-73	AB 969J-62	Carbon source	*M. chalcea* ATCC 12452	AB 965S-73	AB 969J-62
None	−	−	−	Mannitol	−	−	−
Glucose	++	++	−	Melibiose	++	++	+
Adonitol	−	−	−	Raffinose	++	++	+
Arabinose	++	++	++	Rhamnose	−	−	−
Cellulose	−	−	−	Salicin	+	+	+
Dulcitol	−	−	−	Sorbitol	−	−	−
Fructose	++	+	+	Starch	−	−	−
Galactose	++	++	+	Sucrose	++	++	++
Inositol	−	−	−	Trehalose	++	++	+
Lactose	++	−	++	Xylose	++	++	+

++ Strong utilization; + moderate utilization; − not utilized. Basal medium was ISP-9 (Difco). Incubation was at 28°C for 30 days.

Table 3. Additional comparison of macquarimicin producers with *M. chalcea* ATCC 12452.

Characteristic	*M. chalcea*	AB 965S-73	AB 969J-62
Lysozyme sensitivity	Sensitive	Sensitive	Sensitive
Menaquinone	MK-10 (H_4)	MK-10 (H_4) and MK-10 (H_6)	MK-10 (H_6)
Diaminopimelic acid	*Meso-*	*Meso-*	*Meso-*
Diagnostic sugars	Xylose & arabinose	Xylose & arabinose	Xylose & arabinose

Table 4. *In vitro* activity of macquarimicin A against a panel of anaerobic and aerobic bacteria.

Microorganism	MIC (μg/ml)
Anaerobes	
Bacteroides bivius B 6140	50
B. disiens ATCC 29426	100
B. fragilis ATCC 25285	50
B. fragilis 784	50
B. fragilis SFM 2906A	100
B. fragilis SFM 2975-7	50
B. loescheii ATCC 15930	100
B. melaninogenicus ATCC 25845	50
B. thetaiotaomicron ATCC 29741	100
B. thetaiotaomicron ATCC 29742	100
B. vulgatus 792	200
B. vulgatus.SFBC 2375	100
Clostridium difficile ATCC 9689	100
C. difficile ATCC 17857	>200
C. perfringens ATCC 13124	100
C. perfringens SFBC 2026	50
Fusobacterium nucleatum ATCC 25586	50
Peptococcus asaccharolyticus ATCC 14963	100
P. magnus ATCC 29328	200
Peptostreptococcus anaerobius ATCC 27337	50
P. micros ATCC 33270	100
Propionibacterium acnes 132	100
Veillonella parvula ATCC 10790	>200
Aerobes	
Staphylococcus aureus (5 strains)	>100
S. epidermidis (2 strains)	>100
Streptococcus (5 strains)	>100
Escherichia coli (5 strains)	>100
Klebsiella pneumoniae ATCC 8045	>100
Pseudomonas aeruginosa (4 strains)	>100

Table 5. Inhibitory activity of macquarimicins B and C against P-388.

Macquarimicin	IC_{50} (μg/ml)
B	0.3
C	30.0

strains of *M. chalcea*. A time course of the fermentation is shown in Fig. 3. The HPLC assay was effective in quantitating macquarimicin A in crude fermentation samples. A yield of 27 mg/liter macquarimicin A was obtained in a seven day fermentation. In addition to macquarimicin A, related compounds were observed in fermentation broths of strain AB 969J-62.

Purified macquarimicin A, evaluated with a panel of anaerobic bacteria, gave disappointing results (Table 4). MICs ranged from 50 to 100 μg/ml against *B. fragilis* and *B. thetaiotamicron* and from 50 to >200 μg/ml against other anaerobes. Macquarimicin had MICs> 100 μg/ml against 22 Gram-positive and Gram-negative bacteria grown aerobically. Macquarimicins B and C were found to inhibit the leukemia cell line P-388 (Table 5) with macquarimicin B more potent than C.

Conclusion

Macquarimicin was discovered as an antianaerobic agent, albeit of low potency, and preliminarily reported as the first of a novel chemical class in 1986[14]. Another family of the same structural type, the cochleamycins, have been discovered as antitumor agents from a streptomycete[15]. Re-examination of the macquarimicin fermentation yielded two new members of the family, B and C, which, like the cochleamycins, show activity against leukemia cell lines.

Acknowledgments

The authors wish to thank A. M. BUKO for mass spectral measurements and M. F. MILLER and F. A. SEILER for electron microscopy.

References

1) JOUSIMIES-SOMER, H. R. & S. M. FINEGOLD: Anaerobic gram-negative bacilli and cocci. *In* Manual of Clinical Microbiology, 5th Ed. *Eds.*, A. BALOWS *et al.* American Society for Microbiology, 1991
2) HOCHLOWSKI, J. E.; M. M. MULLALLY, R. HENRY, D. M. WHITTERN & J. B. MCALPINE: Macquarimicins, microbial metabolites from *Micromonospora*. II. Isolation and structural elucidation. J. Antibiotics 48: 467~470, 1995
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. II. Classification, Identification and Descriptions of Genera and Species. Williams & Wilkins, 1961
5) KELLY, K. L. & D. B. JUDD: ISCC-NBS Color-name Charts Illustrated with Centroid Colors. U. S. Dept. of Comm. Suppl. to Cir. No. 553, 1976
6) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Micrbiol. 12: 421~423, 1964
7) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
8) GORDON, R. E.; D. A. BARNETT, J. E. HANDERHAN & C. H.-N. PANG: *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. Int. J. Syst. Bacteriol. 24: 54~63, 1974
9) ATHALYE, M.; M. GOODFELLOW & D. E. MINNIKIN: Menaquinone composition in the classification of *Actinomadura* and related taxa. J. Gen. Microbiol. 130: 817~823, 1984
10) DUBOIS, M.; K. A. GILLES, J. K. HAMILTON, P. A. REBERS & F. SMITH: Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350~356, 1956
11) CARMICHAEL, J.; W. G. DEGRAFF, A. F. GAZDAR, J. D. MINNA & J. B. MITCHELL: Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res. 47: 936~942, 1987

12) LECHEVALIER, M. P. & H. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
13) KAWAMOTO, I.: Genus *Micromonospora* Ørskov. *In* Bergey's Manual of Systematic Bacteriology, Vol. 4. *Ed.*, S. T. WILLIAMS, pp. 2442~2450, Williams & Wilkins, 1989
14) JACKSON, M.; J. P. KARWOWSKI, R. J. THERIAULT, D. M. HENSEY, P. E. HUMPHREY & G. J. BARLOW: Macquarimicin, the first member of a new class of antibiotics. I. Discovery, taxonomy, fermentation and biological properties. Abstracts of Papers of the 26th Intersci. Conf. on Antimicrob. Agents Chemother., No. 912, New Orleans, 1986
15) SHINDO, K. & H. KAWAI: Novel antibiotics, cochleamycins A and B. J. Antibiotics 45: 292~295, 1992