TIACUMICINS, A NOVEL COMPLEX OF 18-MEMBERED MACROLIDE ANTIBIOTICS

I. TAXONOMY, FERMENTATION AND ANTIBACTERIAL ACTIVITY

ROBERT J. THERIAULT, JAMES P. KARWOWSKI, MARIANNA JACKSON, ROLAND L. GIROLAMI, GABRIELA N. SUNGA, CHARLENE M. VOJTKO and LYLE J. COEN

Department, 47P, Anti-Infective Research, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

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A complex of 18-membered macrolide antibiotics has been discovered in the fermentation broth of strain AB718C-41. The producing culture, isolated from a soil sample collected in Hamden, Connecticut, was identified as a strain of *Dactylosporangium aurantiacum* and was designated *D. aurantiacum* subsp. *hamdenensis* subsp. nov. The antibiotic complex was produced in a New Brunswick 150-liter fermentor using a medium consisting of glucose, soybean oil, soybean flour, beef extract and inorganic salts. Several of the antibiotics were active against sensitive and multiple antibiotic-resistant strains of pathogenic Gram-positive bacteria.

In the course of screening for new antibiotics, fermentation broths of a soil isolate designated AB718C-41 were shown to inhibit antibiotic-resistant strains of *Staphylococcus aureus* and *Streptococcus pyogenes*. The producing culture was identified as *Dactylosporangium aurantiacum* subsp. *hamdenensis* subsp. nov. strain AB718C-41. Although active fermentation broths were initially produced in shake flasks and 14-liter fermentors, fermentation yields were higher and more reproducible in a New Brunswick 150-liter fermentor. Purification studies of the antibiotic activity resulted in the isolation of six novel 18-membered macrolide antibiotics. Members of the antibiotic complex have been named tiacumicins A, B, C, D, E and F. This paper reports the taxonomy and fermentation of the producing culture and the antibacterial activity of the tiacumicin antibiotic complex. The isolation and elucidation of the structures of the tiacumicins are described in an accompanying paper[1].

Materials and Methods

Microorganisms

Strain AB718C-41 was isolated in our laboratory from a soil sample collected in Hamden, Connecticut, U.S.A. A subculture was deposited at the ARS Patent Collection of the Northern Regional Research Center, United States Department of Agriculture, Peoria, Illinois, U.S.A. and assigned accession number NRRL 18085. For comparative purposes we obtained *D. aurantiacum* ATCC 23491 from the American Type Culture Collection (ATCC). The bacterial strains used to evaluate the tiacumicins were from the stock culture collection in our laboratory and from the ATCC.

Taxonomic Studies

Most morphological and physiological characteristics were determined using the methods and media described by the International Streptomyces Project (ISP)[2] and WAKSMAN[3]. ATCC medium 172[4] was added for morphological studies; NaCl tolerance was examined on nutrient and LUEDEMANN[5]

agars; and starch, tyrosine and casein hydrolyses were determined by the method of GORDON *et al.*[6]. Observations were made after incubation at 28°C for 14 days. Color names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Science Color Council - National Bureau of Standards (ISCC-NBS) Centroid-Color Charts[7]. Whole-cell sugars were identified by the procedure of LECHEVALIER[8], and the diaminopimelic acid isomer was determined by the method of BECKER *et al.*[9].

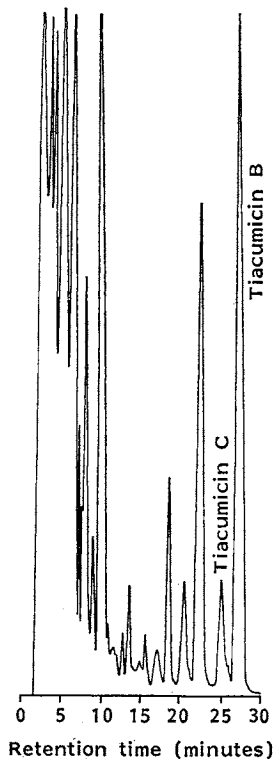
Fermentation Studies

Strain AB718C-41 was grown on agar slants of ATCC medium 172[4]. Slants were incubated at 30°C for 10 to 14 days and then stored at 4°C until needed.

Agar slant cultures were used to inoculate 25×150 mm seed tubes closed with stainless steel caps and containing 10 ml of seed medium consisting of glucose 0.1%, starch 2.4%, yeast extract (Difco) 0.5%, Tryptone (Difco) 0.5%, beef extract (Scott) 0.3% and $CaCO_3$ 0.4%. The medium was prepared in distilled water and adjusted to pH 7.3 prior to sterilization. Seed tubes were incubated at 30°C for 96 hours on a rotary shaker at 250 rpm (3.2 cm stroke). Five percent vegetative inoculum was then used to inoculate second passage 2-liter Erlenmeyer seed flasks closed with rayon plugs and containing 600 ml of the same seed medium. Flasks were also incubated on a rotary shaker at 30°C for 72 hours.

Five percent vegetative inoculum from the second passage seed flasks was used to inoculate a New Brunswick 150-liter fermentor charged to a volume of 80 liters with a medium consisting of glucose monohydrate (added after sterilization) 2%, soybean flour 1%, beef extract (Scott) 0.3%, soybean oil 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03%, $CaCO_3$ 0.3% and antifoam XFO-371 (Ivanhoe Chem. Co.) 0.01%. The medium was prepared in distilled water and adjusted to 7.0 prior to sterilization at 121°C and 1.05 kg/cm² for 1 hour. The fermentation was carried out for 162 hours with an agitation rate of 200 rpm, an aeration rate of 0.7 vol/vol/minute and a head pressure of 0.35 kg/cm² at 30°C.

Fig. 1. HPLC separation of a fermentation broth extract containing tiacumicins B and C.



Packed cell volumes were determined by centrifugation at 600×*g* for 30 minutes in 15 ml conical tubes. Glucose concentrations were determined by the method of HOFFMAN[10].

HPLC

Fermentation broths for HPLC analysis of levels of tiacumicins B and C were adjusted to pH 7 and extracted twice with 1/2 vol of EtOAc. EtOAc extracts were combined, solvent was removed under reduced pressure and the residue was reconstituted in MeOH to a concentration 100 times that of the original fermentation broth. Analyses were performed using a Waters model 6000A solvent delivery system and a U6K injector. Tiacumicins B and C were chromatographed on a 25 cm 7 μm C-18 Adsorbosphere HS column (Alltech. Assoc.) with a mobile phase consisting of acetonitrile - 0.1% H_3PO_4 (50:50) at a flow rate of 1 ml/minute. Tiacumicins B and C were detected at 266 nm using a Kratos SF 770 UV detector. Quantitative analyses were conducted using external standards of tiacumicins B and C with retention times of 27 and 25 minutes, respectively. An HPLC scan of a fermentation broth extract is shown in Fig. 1.

In Vitro Potency Determination

MICs of the tiacumicins were determined using the standard agar dilution procedure[11] on brain heart infusion agar.

Results and Discussion

Taxonomy

Morphological Characteristics

The vegetative mycelium of isolate AB718C-41 is compact, fine (0.3~0.5 μm in diameter), and irregularly branched. Fragmentation of hyphae did not occur when grown on either agar or submerged in liquid culture. Aerial mycelia were not observed. Masses of finger-shaped sporangia emerging directly from the vegetative mycelium were formed on only a few of the media studied. The sporangia typically measure 0.8 μm×2.3~3.5 μm as determined by scanning electron microscopy. Each contains a single, straight row of 2 to 4 spores (Fig. 2). The spores were released and became motile about 45 minutes after the sporangia were flooded with water. Globose bodies, as described by THIEMANN *et al.*[12] and SHARPLES and WILLIAMS[13], were formed on some media (Fig. 3).

Cultural and Physiological Characteristics

The cultural and physiological characteristics of strain AB718C-41 are detailed in Tables 1 and 2, respectively. The carbon source utilization pattern is given in Table 3. Growth was moderate on most media with sporangium formation restricted to inorganic salts - starch (ISP No. 4), CZAPEK sucrose and calcium malate agars. Diffusible pigment was not produced in any of the media shown in Table 1, but a strong brown (55)[7] color was elaborated in nutrient agar in which tyrosine was suspended according to the method of GORDON *et al.*[6]. Strain AB718C-41 tolerates 1.5% NaCl on both nutrient and LUEDEMANN agars but cannot grow in the presence of 3.0% NaCl. Growth occurs at 15°C, a feature that is shared with only *Dactylosporangium matsuzakiense*[14] of the described *Dactylosporangium* species.

Whole-cell Chemical Analysis

Analysis of the whole-cell hydrolysates of strain AB718C-41 and *D. aurantiacum* ATCC 23491

Fig. 2. Scanning electron micrograph of sporangia of strain AB718C-41 from a 14-day old culture grown on calcium malate agar (bar=5 μm).

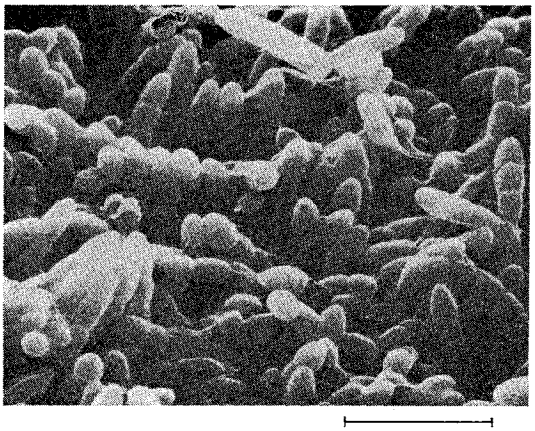


Fig. 3. Scanning electron micrograph of the globose bodies of strain AB718C-41 from a 14-day old culture grown on ISP No. 5 agar (bar=5 μm).

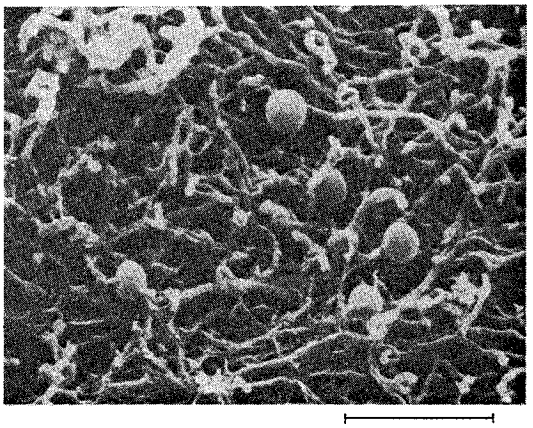


Table 1. Cultural characteristics of strain AB718C-41.

Culture medium	Color of vegetative mycelium	Growth	Sporangia	Globose bodies
ISP No. 2 (yeast extract - malt extract agar)	Moderate orange (53)	Abundant	Absent	Absent
ISP No. 3 (oatmeal agar)	Pale orange yellow (73) with strong orange (50) spots	Moderate	Absent	Absent
ISP No. 4 (inorganic salts - starch agar)	Light orange yellow (70)	Moderate	Poor	Present
ISP No. 5 (glycerol - asparagine agar)	Light orange yellow (70)	Moderate	Absent	Present
ISP No. 6 (peptone - yeast extract - iron agar)	Moderate orange yellow (71)	Moderate	Absent	Absent
ISP No. 7 (tyrosine agar)	Light orange (52)	Moderate	Absent	Absent
CZAPEK sucrose agar	Pale orange yellow (73) with strong orange (50) spots	Poor	Abundant	Present
Nutrient agar	Light orange yellow (70)	Moderate	Absent	Absent
Calcium malate agar	Yellow white (92)	Moderate	Abundant	Present
ATCC No. 172	Light orange (52) with strong brown (55) spots	Abundant	Absent	Absent
Glucose asparagine agar	Pale orange yellow (73)	Moderate	Absent	Absent
Hickey-Tresner agar	Light orange (52)	Moderate	Absent	Absent
Bennett agar	Light orange (52)	Moderate	Absent	Absent

Table 2. Physiological characteristics of strain AB718C-41.

Test	Reaction
H_2S production	+
Gelatin liquefaction	−
Casein hydrolysis	+
Starch hydrolysis	+
Tyrosine hydrolysis	+
Nitrate reduction	+
Milk peptonization	+
Milk coagulation	−
Calcium malate hydrolysis	−
Melanin formation[a]	+
Growth temperature range[b]	15~37°C
NaCl tolerance	1.5%

[a] Dark soluble pigment is produced on nutrient agar containing tyrosine but not on peptone - yeast extract - iron agar (ISP No. 6) or tyrosine agar (ISP No. 7).
[b] Hickey-Tresner agar.

Table 3. Utilization of carbon sources by strain AB718C-41.

Compound	Growth	Compound	Growth
Glucose	++	Cellulose	−
L-Arabinose	++	Dulcitol	−
D-Xylose	++	Glycerol	−
Inositol	−	Sorbitol	−
D-Fructose	++	Lactose	+
Sucrose	+	Maltose	++
Rhamnose	++	Mannose	++
Raffinose	++	Sorbose	−
Mannitol	++	Ribose	−
D-Galactose	++	None	−
Salicin	+		

++ Good growth, + moderate growth, − no growth.

demonstrated the presence of hydroxydiaminopimelic acid in both. Neither LL- nor *meso*-diaminopimelic acid was observed in either strain. Xylose, arabinose and galactose were also detected in both cultures although these sugars were present in somewhat different ratios. The presence of major amounts of xylose and arabinose indicates that strain AB718C-41 can be assigned to whole-cell sugar pattern D in the classification scheme of LECHEVALIER and LECHEVALIER[15]. Although most *Dactylosporangium* species contain xylose and arabinose[14,16~18], it is not unusual to also find galactose in cells of this genus and other Actinoplanaceae[14,19,20].

Species Determination

The morphological characteristics of isolate AB718C-41 placed it in the species *Dactylosporangium* which was first described by THIEMANN *et al.*[12]. Published descriptions of *Dactylosporangium* species suggested that the culture bore a very close resemblance to *D. aurantiacum*. A laboratory comparison of the two cultures showed that, although they were quite similar, they were not identical. The differences are shown in Tables 4 and 5. The latter culture grows over a temperature range of 22~37°C[12] whereas AB718C-41 can grow from 15~37°C. In our laboratory, AB718C-41 hydrolyzed tyrosine suspended in nutrient agar and formed a dark soluble pigment on this medium, but ATCC 23491 did neither. ATCC 23491 is reported to form 3~4 spores per sporangium[12]; AB718C-41 forms 2~4 spores per sporangium. The ratios of whole-cell sugars also vary somewhat between cultures. Lastly, it is interesting to note that *D. aurantiacum* ATCC 23491 was reported to grow in the presence of 3.0% NaCl[16], but in our laboratory it could only tolerate 1.5% NaCl. AB718C-41 showed the same NaCl tolerance.

Because of its close similarity to *D. aurantiacum* ATCC 23491 we consider strain AB718C-41 a subspecies of this culture. We have designated it *Dactylosporangium aurantiacum* subsp. *hamdenensis* subsp. nov. by virtue of its place of origin.

Fermentation

The time course of the tiacumicins fermentation is shown in Fig. 4. Glucose was depleted by 138 hours while the packed cell volume increased up to 162 hours. The maximum yield of tiacumicin B (18.8 mg/liter) was obtained by 114 hours. Tiacumicin C was produced at a significantly lower concentration but was still accumulating by 162 hours. Other tiacumicins were not detected by HPLC in 100-fold concentrated ethyl acetate extracts of the 150-liter fermentor fermentation broth but were isolated from a 6,000-liter fermentation.

Table 4. Whole-cell sugars of strain AB718C-41 and *Dactylosporangium aurantiacum* ATCC 23491.

	Xylose	Arabinose	Galactose
AB718C-41	+++	++	Trace
ATCC 23491	+++	+	++

Table 5. Differential characteristics of *Dactylosporangium aurantiacum* subsp. *hamdenensis* strain AB718C-41 and *D. aurantiacum* ATCC 23491.

Characteristic	*D. aurantiacum* subsp. *hamdenensis*	*D. aurantiacum* ATCC 23491
Growth at 15°C	+	−
Tyrosine hydrolysis with soluble pigment	+	−
Spores in a sporangium	2~4	3~4

Fig. 4. Time course of fermentation in a New Brunswick 150-liter fermentor.
■ Packed cell volume, ● tiacumicin B, □ tiacumicin C, △ residual glucose, ▲ pH.

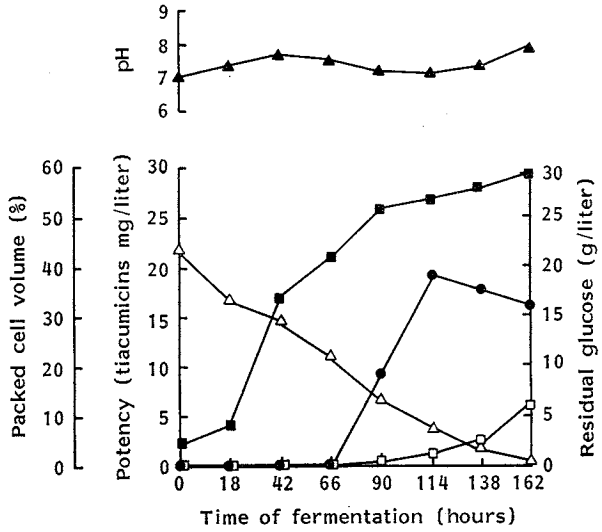


Table 6. Potency of tiacumicins A, B, C, D, E and F against a variety of aerobic bacteria.

Organism	MIC (μg/ml)					
	A	B	C	D	E	F
Staphylococcus aureus ATCC 6538P	100	6.2	50	25	25	12.5
S. aureus CMX 686B	100	12.5	50	>25	25	12.5
S. aureus A5177[a]		6.2	50	>25	25	12.5
S. aureus 45		12.5	100	>25	25	12.5
S. aureus RAR2		6.2	25	>25	12.5	6.2
S. epidermidis 3519		12.5	25	>25	12.5	12.5
Micrococcus luteus 4678		≤0.78	≤0.39	0.2	0.1	≤0.05
Enterococcus faecium ATCC 8043	100	6.2	100	>25	12.5	6.2
Streptococcus bovis A5169		50	100	>25	100	100
S. agalactiae CMX 508		50	100	>25	100	100
S. pyogenes EES 61		12.5	25	>25	25	6.2
S. pyogenes 930	100	3.1	25	>25	50	12.5
Escherichia coli Juhl		>100	>100	>25	>100	>100
E. coli DC2		>100	>100	>25	>100	>100
Enterobacter aerogenes ATCC 13048		>100	>100	>25	>100	>100
Klebsiella pneumoniae ATCC 8045		>100	>100	>25	>100	>100
Providencia stuartii CMX 640		>100	>100	>25	>100	>100
Pseudomonas aeruginosa BMH10		>100	>100	>25	>100	>100
P. aeruginosa A5007		>100	>100	>25	>100	>100
P. cepacia 2961		>100	>100	>25	>100	>100
Acinetobacter sp. CMX 669		>100	>100	>25	>100	>100

[a] Resistant to ampicillin, methicillin, tetracycline and erythromycin.

Minimal inhibitory concentrations (MICs) of tiacumicins were determined using the standard agar dilution procedure on brain heart infusion agar.

Table 7. Potency of tiacumicin B against a variety of anaerobic bacteria.

Organism	Tiacumicin B MIC (μg/ml)	Organism	Tiacumicin B MIC (μg/ml)
Bacteroides fragilis ATCC 25285	>100	*Fusobacterium nucleatum* ATCC 25586	100
B. fragilis 784	>100	*Fusobacterium* sp. GS-10	100
B. fragilis UC-2	>100	*Veillonella parvula* ATCC 10790	100
B. fragilis SFM 2906A	>100	*Clostridium perfringens* ATCC 13124	100
B. fragilis SFM 2975-7	>100	*C. perfringens* SFBC 2026	≤0.05
B. fragilis SFM 2929-1	>100	*C. perfringens* 788	≤0.05
Bacteroides sp. SFM 2975-2	>100	*C. difficile* ATCC 17857	100
B. vulgatus 792	>100	*C. ramosum* 7	100
B. vulgatus SFBC 2375	100	*Propionibacterium acnes* 132	6.25
B. disiens ATCC 29426	>100	*Peptococcus asaccharolyticus* ATCC 14963	>100
B. thetaiotaomicron ATCC 29742	>100	*P. magnus* ATCC 29328	0.4
B. thetaiotaomicron 106	>100	*Peptostreptococcus* sp. TB-11	25
B. thetaiotaomicron ATCC 29741	>100	*P. micros* ATCC 33270	100
B. melaninogenicus ATCC 25845	100	*P. anaerobius* ATCC 27337	≤0.05
B. loescheii ATCC 15930	>100		
B. bivius B6140	100		

Minimal inhibitory concentrations (MICs) of tiacumicin B were determined using the standard agar dilution procedure on Wilkins-Chalgren agar.

Antimicrobial Activity

As shown in Table 6, tiacumicins B and F showed moderate activity against pathogenic strains of *Staphylococcus aureus*, including a multiple antibiotic-resistant strain, *Streptococcus pyogenes* and *Enterococcus faecium*. They had nearly equal activity and were more potent than tiacumicins A, C, D and E. Tiacumicin B showed very limited but potent activity against a few anaerobic bacteria (Table 7).

Acknowledgments

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