DIFFICIDIN AND OXYDIFFICIDIN: NOVEL BROAD SPECTRUM ANTIBACTERIAL ANTIBIOTICS PRODUCED BY *BACILLUS SUBTILIS*

I. PRODUCTION, TAXONOMY AND ANTIBACTERIAL ACTIVITY†

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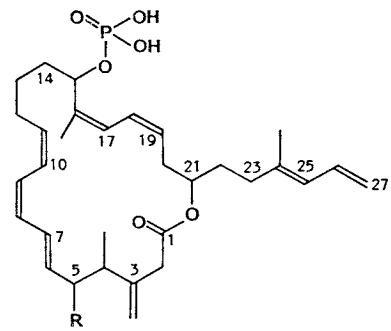
Difficidin and oxydifficidin, two novel macrocyclic polyene lactone phosphate esters were discovered in fermentation broths of each of two strains of *Bacillus subtilis*: ATCC 39320 and ATCC 39374. Difficidin and oxydifficidin each showed a broad spectrum of activity against aerobic and anaerobic bacteria. Many of the susceptible aerobes and anaerobes were human pathogens resistant to one or more antibiotics. Difficidin and oxydifficidin when administered intraperitoneally protected mice against an otherwise lethal bacteremia caused by *Klebsiella pneumoniae* (ED_{50} in mg/kg of 1.31 and 15.6 respectively). Neither difficidin nor oxydifficidin were effective when administered *via* the subcutaneous route.

In the course of screening for new antibiotics, difficidin and oxydifficidin, two novel macrocyclic polyene lactone phosphate esters[1], (Fig. 1) were discovered in fermentation broths of each of two strains of *Bacillus subtilis*: ATCC 39320 and ATCC 39374. Bacillin[2] was also isolated from fermentation broths of each strain.

Fig. 1. Structure of difficidin and oxydifficidin.



Difficidin R = H
Oxydifficidin R = OH

Difficidin and oxydifficidin demonstrated broad and potent activities *in vitro* against aerobic and anaerobic bacteria, many of which are human pathogens that are resistant to one or more antibiotics. Both antibiotics were active *in vivo* when administered *via* the intraperitoneal route, but not *via* the subcutaneous route, to mice infected with *Klebsiella pneumoniae*. Mode of action studies[3] indicate that difficidin is bactericidal and acts by inhibiting protein synthesis.

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Materials and Methods

Fermentation

"A" Stage: Culture ATCC 39320 is maintained in the lyophilized state in a 1.0-ml ampule containing 0.15 ml of a skim milk suspension of the culture.

"B" Stage: Vessel; 250-ml 3-baffled Erlenmeyer flask containing 50 ml of medium per flask. Medium; glucose 1 g/liter, soluble starch 10 g/liter, beef extract 3 g/liter, Ardamine pH 5 g/liter, NZ-Amine type E 5 g/liter, $MgSO_4 \cdot 7H_2O$ 0.05 g/liter, 1.34 M phosphate buffer (KH_2PO_4 91 g/liter, Na_2HPO_4 95 g/liter) 0.02%, $CaCO_3$ (after pH adjustment to 7.0~7.2) 0.05%, distilled water 1,000 ml. Inoculum; contents of one lyophilization tube into each "B" flask. Incubation; 24 hours at 28°C on a rotary shaker with a 5 cm-throw at 220 rpm.

"C" Stage: Vessel; 250-ml Erlenmeyer flasks containing 40 ml of medium. Medium; dextrin 40 g/liter, distillers solubles 7 g/liter, yeast extract 5 g/liter, $CoCl_2 \cdot 6H_2O$ 100 mg/liter, distilled water 1,000 ml, pH adjustment to 7.3. Inoculum; 2 ml from "B" stage. Incubation; 1~4 days at 28°C on a rotary shaker with a 5 cm-throw at 220 rpm.

Fermentation titers and chemical isolation procedures were monitored by HPLC as described by WILSON *et al.*[1] and/or by bioassay on susceptibility plates containing *Vibrio percolans* ATCC 8461 seeded into Difco Nutrient agar +0.2% Difco yeast extract.

Taxonomy

Standard taxonomic characterization was carried out based on BERGEY's Manual of Determinative Bacteriology. 8th Ed.[4] and GORDON *et al.*[5].

Antibacterial Activity *In Vitro*

Aerobic Bacteria: MICs for aerobic strains of bacteria were determined in agar dilution assays. Cultures were inoculated from stock slants prepared from cultures stored in the Merck Culture Collection (designated as MB numbers), incubated in Trypticase soy broth at 35°C for 18 hours and applied to the surface of medicated Trypticase soy agar with a Denley Multipoint Inoculator at an inoculum of 10^6 cfu/spot. The susceptibility plates were medicated with aqueous solution of antibiotic. Difficidin and oxydifficidin test samples of $\geq$90% purity were prepared as described by WILSON *et al.*[1]. Cefoxitin sodium (Mefoxin) and streptomycin sulfate were used as a control for comparing MICs. Assay plates were incubated at 35°C for 20~24 hours. Endpoints were read to $\leq$5 discrete colonies.

Anaerobic Bacteria: MICs of strains of anaerobic bacteria were determined in agar dilution assays in a manner similar to that described for aerobic bacteria, except for the following differences: (1) Wilkins-Chalgren agar was used as the assay medium; (2) assay plates were incubated for 44 hours under anaerobic conditions.

Antibacterial Activity *In Vivo*

The therapeutic efficacy of difficidin and oxydifficidin was determined in standard mouse protection tests. An experimental systemic infection was produced in CD1 female mice (Charles River Breeding Lab., Wilmington, MA) by the intraperitoneal injection of a suitably diluted broth culture of *K. pneumoniae* (MB 4005 in the Merck Culture Collection). The test antibacterial compounds and the control drug (streptomycin sulfate) were administered parenterally (intraperitoneally or subcutaneously) immediately after infection and again 6 hours later. At least 4-fold dilutions of each agent were tested. There were 5 mice at each dose level. All mice were observed for a period of 7 days after which the median effective dose (ED_{50}) was calculated by the method of KNUDSEN and CURTIS[6]. Under the conditions of the test, all infected untreated mice died within 48 hours.

Results

Fermentation

Fermentation yields were 270 μg/ml for difficidin and 160 μg/ml for oxydifficidin. Purification

procedures[1] resulted in isolation of material of ≥90% purity for use in all antibiotic testing.

Taxonomy

Morphological and Physiological Characteristics of *B. subtilis* ATCC 39320

The morphological and physiological properties of ATCC 39320 are as follows.

Morphology: Gram-positive, non-vacuolated vegetative rods with rounded ends; average size 0.9×2.3~3.6 μm; occurring singly. Rods are motile. Spores are produced under aerobic conditions. Spores are 0.5×1.0 μm (average size), oval to cylindrical, predominantly central, sporangia not swollen.

Colonial Appearance: Flat, round with irregular edge, surface dull, edge becoming opaque as colony ages. Dull, wrinkled entire pellicle on surface of broth. No pigmentation on Trypticase soy agar. Growth at 28°C, 37°C, no growth at 60°C.

Positive Reactions: Catalase, Voges-Proskauer, gelatin, nitrate reduction, utilization of citrate, acid from glucose, arabinose, mannitol, xylose, sorbitol and sucrose, hydrolysis of starch.

Negative Reactions: Urease, indole, utilization of propionate, arginine dihydrolase, acid from rhamnose and melibiose, no growth in anaerobic agar (stabs or plates incubated in anaerobic jars), no growth in glucose broth or nitrate broth under anaerobic conditions.

Comparison with culture descriptions in BERGEY's Manual of Determinative Bacteriology. 8th Ed.[4] and GORDON *et al.*[5] indicate that ATCC 39320 (MB 4488) is a strain of known species *B. subtilis*.

Morphological and Physiological Characteristics of *B. subtilis* ATCC 39374

The morphological and physiological properties of ATCC 39374 are the same as those indicated above for ATCC 39320, except with respect to the appearance of the colonies of the microorganism, which are as follows.

Colonial Appearance: At 24 hours, raised, round, mucoid. As colony ages, edge becomes dry, opaque and irregular. Central mucoid area continues to dry, becoming opaque and wrinkled. Dull, wrinkled entire pellicle on surface of broth. No pigmentation on Trypticase soy agar. Growth at 28°C, 37°C, no growth at 60°C.

Antibacterial Activity vs. Aerobes

Table 1 demonstrates that difficidin has a broad spectrum of activity against aerobic bacteria, with MICs of 0.5~16 μg/ml except for *Streptococcus faecalis* (>128 μg/ml). Difficidin MICs were generally 2~8× lower for a majority of the strains than those of oxydifficidin. Difficidin was superior to cefoxitin vs. *Proteus* sp., one strain of methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Serratia marcescens*. Difficidin was superior to streptomycin vs. *S. aureus*, *Proteus* sp., *Salmonella typhimurium*, *P. aeruginosa* and *S. marcescens*.

Antibacterial Activity vs. Anaerobic bacteria

Against anaerobes (Table 2), difficidin and oxydifficidin each demonstrated a broad spectrum, particularly against strains of *Clostridium difficile*, *Bacteroides* sp. and *Fusobacterium mortiferum*.

Antibacterial Activity *In Vivo*

The data in Table 3 show that when administered intraperitoneally, difficidin and oxydifficidin were effective in protecting mice against an otherwise lethal infection of *K. pneumoniae* MB 4005

Table 1. Antibacterial spectrum of difficidin and oxydifficidin vs. aerobic bacteria.

Organism	MB No.	MIC (μg/ml)[a]			
		Difficidin	Oxydifficidin	Cefoxitin	Streptomycin
Staphylococcus aureus Gm^R $Meth^R$	4310	8.0	32.0	>128	>128
S. aureus	2868	16.0	>128.0	2	>128
S. aureus	2865	8.0	32.0	2	16
Streptococcus faecalis	2864	>128.0	>128.0	>128	>128
Escherichia coli TEM 2+	4351	8.0	16.0	4	1
E. coli TEM 2+ DC2	4352	8.0	8.0	1	2
E. coli DC2	4353	8.0	4.0	1	2
E. coli	2891	8.0	4.0	128	16
Salmonella typhimurium	3860	8.0	32.0	8	>128
Enterobacter cloacae P99+	2646	16.0	32.0	>128	4
E. cloacae P99−	2647	8.0	64.0	16	4
E. aerogenes	2828	8.0	32.0	>128	8
Klebsiella pneumoniae K1+	4354	8.0	8.0	4	8
K. pneumoniae	4005	8.0	8.0	4	2
Morganella morganii Sm^R	2833	2.0	2.0	16	>128
Proteus vulgaris	2829	0.5	8.0	16	4
P. mirabilis Gm^R	2830	8.0	32.0	32	32
Pseudomonas aeruginosa R PL 11+	3350	8.0	16.0	>128	>128
P. aeruginosa	2835	8.0	32.0	>128	>128
P. aeruginosa	4279	8.0	32.0	>128	64
Serratia marcescens	2840	4.0	16.0	64	>128

[a] Agar dilution assay, inoculum 10^6 cfu/spot.
Gm: Gentamicin, Meth: methicillin, Sm: streptomycin, ^{R}: resistance.

Table 2. Antibacterial spectrum of difficidin and oxydifficidin vs. anaerobic bacteria.

Organism	MB No.	MIC (μg/ml)[a]		
		Difficidin	Oxydifficidin	Cefoxitin
Actinomyces naeslundii	4053	>128	64	0.015
Eubacterium limosum	3344	>128	>128	0.25
Propionibacterium acnes	2249	1	8	≤0.008
Peptostreptococcus anaerobius	3282	0.06	0.06	≤0.008
Clostridium perfringens (NCCLS control strain)	4418	>128	>128	0.5
C. ramosum	4272	2	2	4
C. difficile $Clind^R$, Fox^R	4273	0.125	0.06	64
C. difficile Fox^R	4380	0.06	0.06	128
Bifidobacterium dentium Fox^R	4427	32	>128	32
Bacteroides fragilis $Metr^R$, Pen^R	4324	0.125	0.25	2
B. fragilis Pen^R	3214	0.125	0.125	8
B. fragilis $Clind^R$	4360	0.25	0.25	32
B. fragilis NCCLS	4419	0.06	0.25	4
B. distasonis Pen^R	4361	0.06	0.06	16
B. distasonis Pen^R, Fox^R	3445	>128	>128	64
B. ovatus Pen^R	3248	0.125	0.25	8
B. thetaiotaomicron $Clind^R$	4362	0.25	0.25	16
B. thetaiotaomicron $Clind^R$ (NCCLS control strain)	4420	0.06	0.25	16
Veillonella alcalescens	1952	0.25	0.06	0.5

[a] Agar dilution assay, inoculum 10^6 cfu/spot.
Clind: Clindamycin, Fox: cefoxitin, Metr: metronidazol, Pen: benzylpenicillin, ^{R}: resistance.

Table 3. Therapeutic efficacy against an experimental *Klebsiella pneumoniae* bacteremia in mice[a].

Antibiotic[b]	ED_{50}[c] (mg/kg×2 ip doses, 95% confidence limits)	TD_{50}[d] (mg/kg×2 ip doses)	MIC (μg/ml)
Difficidin	1.31 (1.04~1.65)	>50	8
Oxydifficidin	15.62 (10.83~22.54)	>84.5	8
Streptomycin	0.26 (0.16~0.45)	NT	2

[a] Infection was established in CD1 female mice by intraperitoneal injection of an appropriate broth dilution of the pathogen. The challenge dose contained at least $100 \times LD_{50}$, and all infected, untreated mice died within 48 hours.

[b] Antibiotics were administered at 0 and 6 hours after infection.

[c] ED_{50}: Median effective dose (mg/kg) that would protect 50% of infected mice. Values are geometric means of 5 tests (3 tests for oxydifficidin).

[d] Median toxic dose (mg/kg) that would kill 50% of treated infected mice[1].

NT: Not tested.

(ED_{50} of 1.31 and 15.6 mg/kg respectively). Neither antibiotic was effective when administered *via* the subcutaneous route. The drugs were not toxic at the levels tested.

Discussion

Difficidin and oxydifficidin are novel broad spectrum antibacterial antibiotics which demonstrated excellent *in vitro* activity against both aerobic and anaerobic strains of bacteria, many of which are pathogenic to humans. Both antibiotics when administered intraperitoneally protected mice against an experimental *K. pneumoniae* bacteremia. The inability to demonstrate *in vivo* activity *via* the subcutaneous route suggests that difficidin and oxydifficidin are metabolized and/or are not reaching the site of infection in time to prevent it.

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