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

II. ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION

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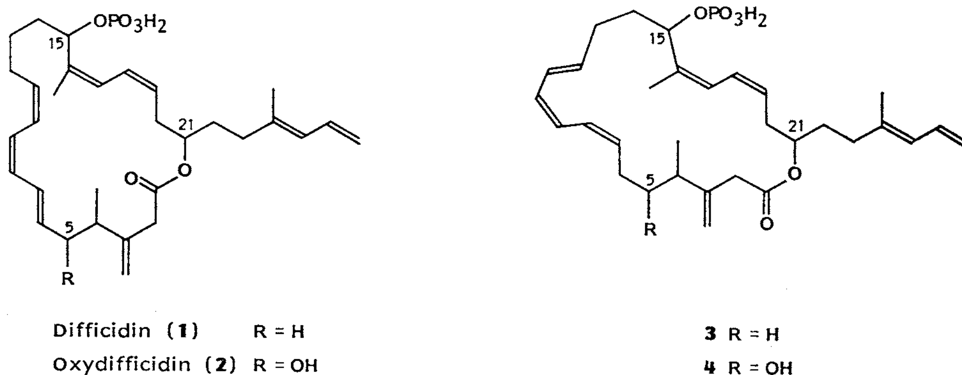
The isolation of difficidin (1) and oxydifficidin (2) from fermentation broth of *Bacillus subtilis* ATCC 39320 and the physico-chemical characterization of these labile antibiotics are described. The structures of the compounds represent a new class of antibiotics, characterized as highly unsaturated 22-membered macrolide phosphates. Difficidin and oxydifficidin undergo reversible thermal isomerization to **3** and **4** respectively. Biological evaluation of the isomers is presented.

The preceding paper[1] describes the discovery of two novel antibiotics, difficidin (**1**) and oxydifficidin (**2**), as products of the fermentation of *Bacillus subtilis* strains ATCC 39320 and ATCC 39374. The compounds exhibit good antibacterial activity against both aerobic and anaerobic organisms. The mode of action of difficidin is addressed in the following the paper[2]. During isolation work on difficidin and oxydifficidin, minor antibacterial components **3** and **4** were also isolated from fermentation broth. This paper presents the isolation and physico-chemical properties of compounds **1**~**4**. Experimental data show that **3** and **4** arise from thermal isomerization of difficidin and oxydifficidin respectively. The *in vitro* and *in vivo* antibacterial potencies of **3** and **4** are compared to those of difficidin and oxydifficidin.

Scheme 1.



Results

Isolation

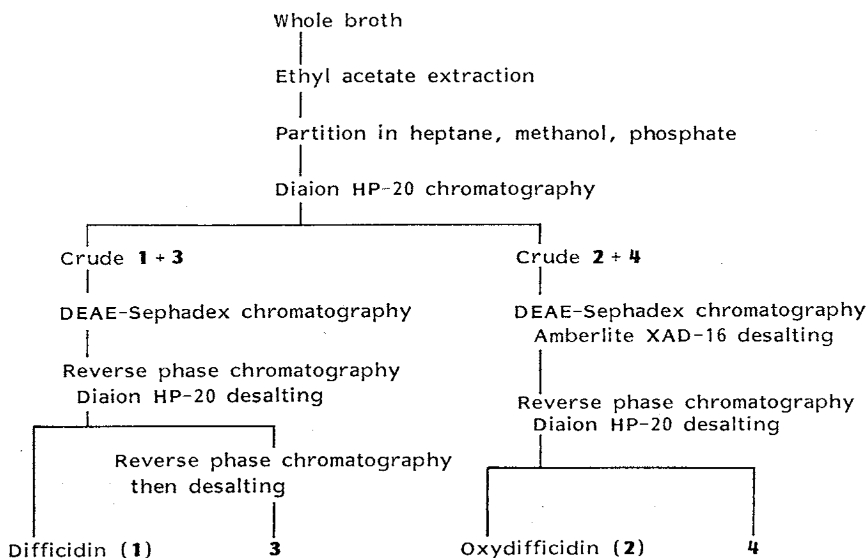
Difficidin, oxydifficidin and isomers **3** and **4** were isolated from fermentation of *Bacillus subtilis* ATCC 39320. Fermentation conditions are described in an accompanying paper[1]. The isolation scheme is summarized in Fig. 1. Antibiotics **1**~**4** were extracted from acidified whole broth into ethyl acetate. The extract was heavily contaminated with polyglycol 2000, used as defoamer during fermentation. The bulk of the defoamer was removed by partitioning the extract concentrate between heptane and methanol - aqueous phosphate. Adsorption of the aqueous layer on Diaion HP-20 followed by selective elution afforded crude mixtures of difficidin/**3** and of oxydifficidin/**4**. The difficidin containing fraction was further purified on DEAE-Sephadex A25 (Cl^-). Subsequent chromatography on LiChroprep RP-18 resin separated difficidin and its isomer **3**. Desalting afforded pure difficidin. Antibiotic **3** required repeat reverse phase chromatography to reach homogeneity. In a similar fashion oxydifficidin and **4** were separated and purified. All antibiotics were isolated and stored as the potassium salts.

Physico-chemical Properties

Structure Characterization

Oxydifficidin forms a tris(trimethylsilyl) derivative **5** that exhibits a molecular ion by electron impact mass spectrometry (EI-MS) at *m/z* 776.4100, corresponding to the formula $C_{40}H_{69}O_7PSi_3$ (calcd 776.4089). Accordingly, the molecular formula of oxydifficidin was assigned as $C_{31}H_{45}O_7P$ (MW 560). In support of this assignment, negative ion fast atom bombardment mass spectrometry (FAB-MS) of oxydifficidin gave a base ion at *m/z* 559 $(M-H)^-$. Elemental analysis was also in agreement with the proposed molecular formula of oxydifficidin (see Experimental). Fragment ions in the EI-MS of oxydifficidin and of its silyl derivative **5** at *m/z* 462.3095 ($C_{31}H_{42}O_3$, calcd 462.3134, $M-H_3PO_4$) and at *m/z* 534.3528 ($C_{34}H_{50}O_3Si$, calcd 534.3529, $M-HOPO(O\text{-trimethylsilyl})_2$) respectively

Fig. 1. Isolation of difficidin, oxydifficidin and their thermal isomers.



identify the phosphorous to be present as a phosphate monoester. This is confirmed by the rapid dephosphorylation of oxydifficidin by alkaline phosphatase. The UV spectrum of oxydifficidin exhibits an absorption maximum at 273 nm (ε 30,200), characteristic of a triene moiety. In addition, an absorption maximum is present at 235 nm (ε 59,600), suggesting the presence of two diene chromophores. Extensive NMR and MS studies established ultimately the structure of oxydifficidin to be **2**. Oxydifficidin is therefore a macrolide antibiotic containing a highly unsaturated 22-membered lactone with a hydroxyl substituent at C-5 and a phosphate group at C-15. The stereochemistry of the double bonds is as indicated in **2**.

Difficidin forms a bis(trimethylsilyl) derivative that affords a molecular ion by EI-MS at *m/z* 688.3741 ($C_{37}H_{61}O_6PSi_2$, calcd 688.3744). Therefore difficidin has a molecular formula $C_{31}H_{45}O_6P$ (MW 544), differing from oxydifficidin by one oxygen atom. Negative ion FAB-MS of difficidin shows a strong $(M-H)^-$ peak at *m/z* 543, consistent with the assigned molecular formula. Subsequent NMR work showed the structure of the antibiotic to be **1**. The structures of isomers **3** and **4** were determined primarily by extensive NMR studies.

^1H NMR assignments for difficidin and oxydifficidin pertinent to discussions in this paper are summarized in Table 1. The full description of the structural elucidation of compounds **1**~**4** will be published separately.

Stability

Difficidin and oxydifficidin are sensitive to pH, temperature and oxygen. Effects of solution pH are summarized in Table 2. Oxydifficidin is significantly less stable than difficidin under alkaline conditions. At pH 11 the macrolide ring of oxydifficidin (**2**) is cleaved, leading to a mixture of seco-acid salt **7** and δ-lactone **8**. It seems most likely that there is initial translactonization of **2** to **9**, which then undergoes competitive hydrolysis to **7** or isomerization to **8**. Both antibiotics exhibit instability at pH 3.5. In addition to pH sensi-

Table 1. ^1H NMR assignments for difficidin and oxydifficidin.

Proton	Chemical shift (δ) and multiplicity[a]	
	Oxydifficidin	Difficidin
2-H_A	2.98 br d (16)	2.92 d (15)
2-H_B	3.25 br d (16)	3.01 d (15)
3-CH_A	4.95 br s	4.84 br s
3-CH_B	5.02 br s	4.92 br s
4-H	~2.52 obsc	2.38 m
4-CH_3	1.18 d (6.5)	1.08 d (6.5)
5-H_A	4.30 br t (4)	2.22 m
5-H_B	—	2.22 m
6-H	5.79 dd (5, 15)	5.66 ddd (5.5, 9, 15)
7-H	6.78 br dd (11.5, 15)	6.52 dd (11, 15)
15-H	~5.21 m obsc	~5.17 m obsc
21-H	~4.91 br m	~4.92 m

[a] Spectra were recorded at 300 MHz in CD_3OD - $CDCl_3$ (1:4) at 25°C using TMS as internal standard. Multiplicity is abbreviated as follows: Singlet (s), doublet (d), triplet (t), multiplet (m), broad (br), obscured (obsc). Coupling constants (*J*=Hz) are reported in parentheses.

Table 2. pH Stability of difficidin and oxydifficidin at 25°C.

	t_{90} (hours)[a]		
	pH 3.5	pH 9	pH 11
Difficidin	2	—	17
Oxydifficidin	1.3	2	0.02

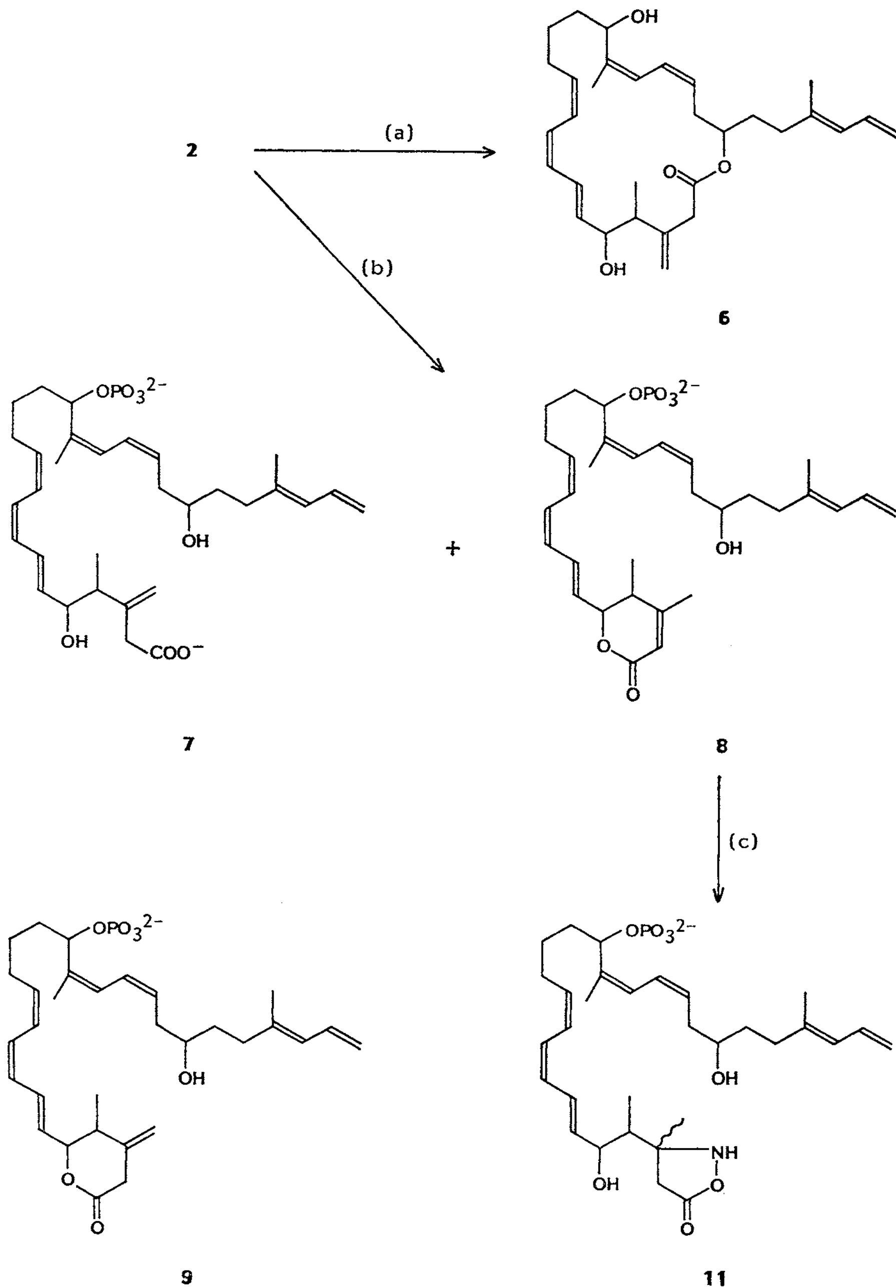
[a] Time at which 90% of the antibiotic remains.

Table 3. Thermal isomerization of compounds **1**~**4** at 60°C.

Isomer pair	Equilibrium distribution[a]
Difficidin - **3**	63: 37
Oxydifficidin - **4**	29: 71

[a] Compounds were tested as solutions in methanol - aqueous citrate (1:1) pH 5.5. Final product distributions were reached after *ca.* 20 hours, independent of the starting compound.

Scheme 2.



(a) Alkaline phosphatase, (b) ≥pH 8, (c) NH_2OH.

tivity, difficidin and oxydifficidin undergo temperature dependent isomerization to **3** and **4** respectively. The process is reversible. Equilibrium ratios of the two isomer pairs at 60°C are shown in Table 3. Finally, the antibiotics are sensitive to air oxidation, particularly when stored as solids. Elemental analysis showed that a lyophilized sample of oxydifficidin potassium salt picked up approximately 4.6 oxygen atoms per molecule after 1 day storage in air at 23°C.

Table 4. *In vitro* antibacterial spectrum of **3** and **4** against aerobic human pathogens.

Organism	MB No.	MIC (μg/ml)[a]			
		Difficidin	**3**	Oxydifficidin	**4**
Staphylococcus aureus	2865	4	128	16	128
Streptococcus faecalis	4710	1	4	4	—
Escherichia coli	2891	8	8	16	32
Enterobacter cloacae P99+	2646	4	8	16	32
E. aerogenes	2828	4	16	16	64
Klebsiella pneumoniae	4005	2	4	8	>128
Morganella morganii Sm^R	2833	2	2	4	—
Proteus vulgaris	2829	1	2	4	32
P. mirabilis Gm^R	2830	2	4	4	32
Pseudomonas aeruginosa	2835	4	16	16	128
Serratia marcescens	2840	2	8	8	64

[a] Agar dilution test with 10^4 cfu/spot.
Sm: Streptomycin, Gm: gentamicin, ^{R}: resistance.
—: Not tested.

Antibacterial Properties

The antibacterial properties of difficidin and oxydifficidin have been reported[1]. Isomers **3** and **4** are also antibacterial. *In vitro* potencies are summarized in Table 4. In both cases the thermal isomer exhibits an antibacterial spectrum similar to that of the parent antibiotic but is less potent. Lower potency is also reflected in *in vivo* results against a *Klebsiella pneumoniae* infection in mice (Table 5). Protection was observed when compounds **1**~**4** were administered intraperitoneally but not when dosed subcutaneously.

Table 5. *In vivo* efficacy of **3** and **4** against *Klebsiella pneumoniae* MB 4005 in mice.

Compound	ED_{50} (mg/kg)	
	ip Therapy	sc Therapy
Difficidin	1.4	>100
3	3.8	>100
Oxydifficidin	21	>100
4	95	>100
Streptomycin	0.31	1.9

Discussion

The susceptibility of difficidin and oxydifficidin to isomerization and oxidative processes has been described above. In order to minimize oxidation, storage of crude and purified samples of the antibiotics as lyophilized or precipitated solids was avoided whenever possible. Instead samples were stored as solutions, preferably in aqueous methanol. Thermal isomerization to **3** and **4** could be halted by storing the solutions at −80°C. During the isolation, solutions were stored at −80°C when volumes permitted, otherwise at −15°C. Despite these precautions, significant amounts of **3** and **4** were produced during the isolation steps. For instance, at harvest the ratio of **4** to oxydifficidin in broth was 5 : 95. After carrying out the steps outlined in Fig. 1 leading to the column feed for reverse phase chromatography, the ratio had increased to 35 : 65. Difficidin isomerizes less rapidly. The ratio of **3** to difficidin prior to the reverse phase chromatography step was 10 : 90.

Careful control of pH was also important during isolation. Translactonization of oxydifficidin and of **4** proceeds under mildly alkaline conditions (see Table 2). The resulting δ-lactone **8** and the corresponding δ-lactone (**10**) derived from **4** have chromatographic properties very similar to those of oxydifficidin and could only be removed effectively from the latter on a milligram scale. On occasion initial isolation steps produced material, in which the combined percentage of δ-lactones **8** and **10** relative to oxydifficidin was considered high (>10%). In these cases the δ-lactones in the crude oxydifficidin fraction were selectively converted with hydroxylamine to a pair of more polar com-

pounds that were readily removed from oxydifficidin during reverse phase chromatography. The structures of these polar derivatives are likely 5-oxo-1,2-oxazolidine adducts **11** and **12**, derived from δ-lactones **8** and **10** respectively. This is based on IR and NMR data on the isolated reaction product of **8** with hydroxylamine.

Difficidin is considered to be a new antibiotic since of the few reported triene macrolides[3], none has physico-chemical properties matching those of difficidin. The known triene proticin[4,5] has biological and physico-chemical properties similar to those of oxydifficidin or its isomer **4**. Thermal isomers **3** and **4** exhibit antibacterial spectra similar to those of difficidin and oxydifficidin but are somewhat less potent than the parent compounds.

Experimental

NMR spectra were recorded on a Varian XL-300 spectrometer in CD_3OD - $CDCl_3$ (1 : 4) as solvent with TMS as internal standard unless otherwise stated. FAB-MS data were collected using a Varian MAT 731 spectrometer, ions being produced by bombardment with xenon atoms of about 7 kV energy. High resolution EI-MS data were collected at 3 kV energy on a Finnigan MAT 212 spectrometer with PFK as reference standard. IR and UV spectra were determined on a Nicolet Model 7199 fourier transformation (FT)-IR spectrometer and a Beckman Model 5260 spectrophotometer respectively. UV extinction coefficients are calculated assuming samples to be the mono-potassium salts for **1**~**4**, **8** and **11** and to be the di-potassium salt for **7**.

During isolation work compounds **1**~**4** were monitored and quantitated by HPLC. An HPLC system employing a Dupont Zorbax ODS column (4.6 mm i.d. × 25 cm) operated at 40°C was used for most work. The flow rate was 1.2 ml/minute and the UV absorbance of column effluent was monitored at 273 nm. Mobile phases consisting of a mixture of methanol and 0.01 M potassium phosphate pH 7 were routinely used for assays of difficidin (**1**), oxydifficidin (**2**) and thermal isomers **3** and **4**. With an eluant composition of methanol - buffer (75 : 25), the retention times of **1** and **3** were 374 seconds and 570 seconds respectively. With an eluting mixture of methanol - buffer (67 : 33), the retention times of **2** and **4** were 422 seconds and 737 seconds respectively. HPLC assays of δ-lactones **8** and **10** and hydroxylamine adducts **11** and **12** were performed with a mobile phase of methanol - 0.02 M sodium citrate (70 : 30) pH 4.8. The retention times of the various compounds were: **11** (260 seconds), **12** (279 seconds), **8** (344 seconds), **10** (361 seconds), **2** (390 seconds), **4** (586 seconds). A second HPLC system using a Whatman Partisil 5 PAC column (4.6 mm × 25 cm) at 40°C was developed to permit convenient, simultaneous quantitation of difficidin and oxydifficidin. This system was used to analyze fractions from the Diaion HP-20 chromatography step of Fig. 1. With a mobile phase of methanol - 0.067 M phosphoric acid (85 : 15) and a flow rate of 2.1 ml/minute, the retention times of difficidin and oxydifficidin were 450 seconds and 504 seconds respectively. Quantitation of thermal isomers **3** and **4** was not possible with this system.

Desalting of Antibiotic Solutions

Aqueous methanol solutions of an antibiotic containing 35% or less methanol were desalted by adsorbing the antibiotic on Amberlite XAD-16 or Diaion HP-20, washing the resin with methanol - water (3 : 7) and eluting the antibiotic with methanol. Initial aqueous methanol solutions containing more than 35% methanol were first diluted with water to reduce the methanol content to 35% before adsorption on resin.

Antibacterial Evaluation

The *in vitro* and *in vivo* results for compounds **1**~**4** summarized in Tables 4 and 5 respectively, were obtained following procedures described in an accompanying paper[1].

Primary Isolation of Crude Difficidin/Oxydifficidin

Whole broth (1,250 liters, pH 7.4)[1], containing 345 g of difficidin and 202 g of oxydifficidin, was acidified to pH 4.8 with sulfuric acid. To the broth was added 50 kg of sodium chloride and 190 liters of methanol. The mixture was extracted with ethyl acetate. The extract was concentrated to 100 liters and diluted with 500 liters of methanol and 1,000 liters of heptane. The mixture was ex-

Table 6. Crude antibiotic composition in Diaion HP-20 rich cuts.

	Volume (liters)	Difficidin (g)	Oxydifficidin (g)	Total solids (g)
Crude difficidin conc	9	170	15	610
Crude oxydifficidin conc	18	0	110	730

tracted at pH 7.5 with 500 liters of 0.02 M potassium phosphate. The resulting 950 liters aqueous methanol layer was decanted and assayed to contain 247 g of difficidin and 132 g of oxydifficidin. The solution was diluted with 640 liters of distilled water and adsorbed onto a 380-liter column of Diaion HP-20 resin. After washing the resin with 1,100 liters of methanol - water (3 : 7), oxydifficidin and difficidin were eluted from the resin with methanol - water (7 : 3) and with methanol respectively. The 300-liter oxydifficidin rich cut and the 900-liter difficidin rich cut were separately concentrated. The concentrates were stored at −80°C and had compositions shown in Table 6.

Difficidin (**1**)

A 1.3-liter aliquot of crude difficidin concentrate (Table 6), containing 25 g of difficidin and 2.1 g of difficidin thermal isomer **3**, was diluted with sufficient water to increase the water content of the sample to 70%. The pH 6.1 solution was adsorbed on a 1-liter column of DEAE-Sephadex A25 (Cl^-) resin. After washing the resin with 1 liter of methanol - water (3 : 7) and then 8 liters of methanol - water (9 : 1) to remove neutral lipophilic impurities including residual polyglycol 2000 defoamer, the antibiotic was eluted with 3% ammonium chloride in methanol - water (9 : 1). The 2.4-liter difficidin rich cut was diluted with sufficient 0.05 M potassium phosphate pH 7 to reduce the methanol content to 35%. The solution was charged on a 1-liter column (13 cm i.d. × 7.5 cm) of LiChroprep RP-18 resin (25 ~ 40 μm) at 100 ml/minute. The column was washed with 4 liters of methanol - 0.05 M potassium phosphate (35 : 65) pH 7 followed by 4 liters of methanol - buffer (45 : 55). The column was then eluted with 4 liters each of methanol - buffer (55 : 45, 65 : 35 and 75 : 25). Eluate was collected in a series of forty-eight, 200-ml fractions. The difficidin rich cut, fractions 12 ~ 30, was desalted on 1 liter of Diaion HP-20 resin. The methanolic Diaion HP-20 eluate was concentrated to 400 ml, containing 15.9 g of difficidin, potassium salt contaminated with 2.8% by weight of the corresponding salt of difficidin thermal isomer **3**. Negative ion FAB-MS *m/z* 543 $(M-H)^-$; EI-MS of bis(trimethylsilyl) derivative *m/z* 688.3741 (M^+, $C_{37}H_{61}O_6PSi_2$, calcd 688.3744); IR (KBr) cm^{-1} 1728 (s, C=O), 990 (s, C-O-P); UV λ_{max}^{MeOH} nm (log ε) 235 (4.78), 263 (4.35), 273 (4.48), 284 (4.37); ^1H NMR (see Table 1).

Anal Calcd for $C_{31}H_{43.5}O_6PK_{1.5}\cdot H_2O$: C 60.1, H 7.4, P 5.0.
Found: C 59.4, H 7.2, P 4.9.

Difficidin Thermal Isomer (**3**)

Fractions 36 ~ 45 from the LiChroprep RP-18 step described above were combined and desalted on 200 ml of Diaion HP-20 resin. The resulting methanol eluate, containing 1.0 g of difficidin and 1.2 g of **3**, was concentrated to 30 ml and buffered with 2 ml of 1 M potassium phosphate pH 7. The solution was chromatographed in three portions on a Whatman ODS-3 M/20 Partisil column (2.1 cm i.d. × 25 cm). The desired compound was eluted with a linear gradient from 6 : 4 to 8 : 2 methanol - 0.03 M potassium phosphate pH 7. The rich cuts from the three runs were combined, desalted on 35 ml of Diaion HP-20 resin, and re-chromatographed in a single portion on a Whatman ODS-3 M/20 column as described above. The resulting rich cut was desalted on 35 ml of Diaion HP-20 resin to afford 0.48 g of **3** as the potassium salt, contaminated with 1% by weight of difficidin salt. EI-MS of bis(trimethylsilyl) derivative *m/z* 688.3744 (M^+, calcd for $C_{37}H_{61}O_6PSi_2$ 688.3744); negative ion FAB-MS *m/z* 543 $(M-H)^-$; IR (KBr) cm^{-1} 1740 (s, C=O); UV λ_{max}^{MeOH} nm (log ε) 235 (4.72), 239 (sh, 4.70), 264 (sh, 4.22), 273 (4.34), 284 (4.24); ^1H NMR (300 MHz, CD_3OD - $CDCl_3$, 1 : 4) δ 1.01 (d, *J*=7 Hz, 4-CH_3), 3.04 (d, *J*=15 Hz, 2-H_A), 3.17 (d, *J*=15 Hz, 2-H_B), 4.82 (m, 21-H), 4.91 (br s, 3-CH_A), 4.93 (br s, 3-CH_B).

Oxydifficidin (**2**)

A 4.3-liter aliquot of crude oxydifficidin concentrate (Table 6), containing 26.3 g of oxydifficidin and 11.3 g of oxydifficidin thermal isomer, was diluted with sufficient water to increase the water con-

tent of the sample to 80%. The pH 6.2 solution was adsorbed onto a 1.2-liter column of DEAE-Sephadex A25 (Cl^-) resin. The resin was washed with 4 liters of methanol - water (2:8) followed by 9 liters of methanol - water (9:1). The antibiotic was eluted with 3% ammonium chloride in methanol - water (9:1). The 860-ml rich cut was diluted with 2.4 liters of water and adsorbed at pH 7 onto 1.3 liters of Amberlite XAD-16 resin. The resin was washed successively with 2 liters of water, 2 liters of 0.05 M potassium phosphate pH 7 and 2 liters of methanol - water (2:8). Elution of the resin with methanol afforded a 1.4-liter rich cut containing 25.3 g of oxydifficidin, 11 g of **4** and 40 g of total solids. The rich cut was concentrated to 470 ml. A 204-ml aliquot, containing 11 g of oxydifficidin and 4.8 g of **4**, was diluted with an equal volume of 0.05 M potassium phosphate pH 7 and applied to a 2.2-liter column (7 cm i.d. × 57 cm) of LiChroprep RP-18 resin (25~40 μm). After washing the resin with 8 liters of methanol - 0.05 M potassium phosphate (50:50) pH 7, oxydifficidin was eluted from the column with methanol - buffer (55:45). Subsequent elution with methanol - buffer (60:40) afforded oxydifficidin isomer **4**. The oxydifficidin rich cut was desalted on 500 ml of Amberlite XAD-16 resin. The resulting methanol rich cut was concentrated to a final volume of 150 ml, containing 7.4 g of oxydifficidin, potassium salt contaminated with 4% by weight of the corresponding salt of **4**. Negative ion FAB-MS *m/z* 559 $(M-H)^-$; EI-MS of tris(trimethylsilyl) derivative *m/z* 776.4100 (M^+, $C_{40}H_{69}O_7PSi_3$, calcd 776.4089); IR (KBr) cm^{-1} 1727 (s, C=O), 1025 (s, P-O-C); UV λ_{max}^{MeOH} nm (log ε) 235 (4.78), 265 (sh, 4.37), 273 (4.48), 284 (4.37); ^1H NMR (see Table 1).

Anal Calcd for $C_{31}H_{43.5}O_7PK_{1.5}\cdot H_2O$: C 58.6, H 7.2, P 4.9.
Found: C 58.2, H 7.3, P 4.7.

Oxydifficidin Thermal Isomer (**4**)

Appropriate fractions from the 2.2-liter LiChroprep RP-18 chromatography of crude oxydifficidin described above were combined and desalted on 500 ml of Amberlite XAD-16. The resulting methanol rich cut was concentrated to 52 ml. The final concentrate contained 2.7 g of oxydifficidin thermal isomer **4** potassium salt, contaminated with 5% by weight of the potassium salt of oxydifficidin. EI-MS of tris(trimethylsilyl) derivative *m/z* 776.4086 (M^+, calcd for $C_{40}H_{69}O_7PSi_3$ 776.4089), 534, 315, 299, 243; negative ion FAB-MS *m/z* 559 $(M-H)^-$; IR (KBr) cm^{-1} 1740 (s, C=O), 990 (C-O-P); UV λ_{max}^{MeOH} nm (log ε) 235 (4.79), 239 (sh, 4.78), 265 (4.32), 273 (4.44), 284 (4.34); ^1H NMR (300 MHz, CD_3OD - $CDCl_3$, 1:4) δ 0.99 (d, J=7 Hz, 4-CH_3), 3.10 (d, J=15 Hz, 2-H_A), 3.24 (d, J=15 Hz, 2-H_B), 3.50 (m, 5-H), 4.86 (m, 21-H), 5.10 (br s, 3-CH_A), 5.12 (br s, 3-CH_B).

Stability Studies on Difficidin and Oxydifficidin

Effect of pH: Acid stability of difficidin and oxydifficidin was examined using 50% aqueous methanol solutions containing 100 μg/ml of antibiotic and 20 mM sodium citrate. Final solutions had an apparent pH of 3.5. For alkaline stability studies, aqueous solutions containing 100 μg/ml of antibiotic were buffered with either 20 mM TAPS-HCl pH 9.0 or 20 mM sodium carbonate pH 11.0. All samples were held at 23±1°C and assayed by HPLC. Results are summarized in Table 2.

Effect of Temperature: The thermal stabilities of difficidin, compound **3**, oxydifficidin and compound **4** were examined under identical conditions. A 100-μg/ml solution of the antibiotic in methanol - 20 mM sodium citrate (1:1) pH 5.5 was heated under argon at 60°C. Aliquots were periodically removed and assayed by HPLC. Results are summarized in Table 3.

Oxydifficidin δ-Lactone (**8**) and Seco-acid (**7**)

A 210-mg sample of oxydifficidin in 45 ml of water was adjusted to pH 11. After 2 hours at room temp, the solution was acidified to pH 7.5. HPLC analysis showed a product ratio of 69% **8**/31% **7**. The solution was concentrated to 2 ml and chromatographed on a 90-ml Dupont Zorbax ODS column (21.2 mm i.d. × 25 cm) using a linear gradient from 58:42 to 65:35 methanol - 0.02 M potassium phosphate pH 7. Fractions of the earlier eluting seco-acid salt were combined, concentrated and desalted on 6 ml of LiChroprep RP-18. The final product contained 48 mg of **7** and was stored as an aqueous solution at pH 9. Negative ion FAB-MS *m/z* 577 $(M-H)^-$; IR (KBr) cm^{-1} 1585 (s, COO^-); UV λ_{max}^{MeOH} nm (log ε) 239 (4.73), 262 (sh, 4.59), 272 (4.61), 280 (sh, 4.48); ^1H NMR (300 MHz, CD_3OD - $CDCl_3$, 1:4) δ 2.98 (d, J=16 Hz, 2-H_A), 3.00 (d, J=16 Hz, 2-H_B), 3.69 (quintet,

J=5 Hz, 21-H), 3.99 (dd, J=7.6 and 9.1 Hz, 5-H), 4.96 (d, J=2 Hz, 3-CH_A), ~5.04 (obscured, 3-CH_B).

Fractions containing δ-lactone **8** were similarly concentrated and desalted on 10 ml of LiChroprep RP-18 to afford 114 mg of **8** which was stored as an aqueous solution at pH 6.6. EI-MS of tris(trimethylsilyl) derivative m/z 776.4086 (M^+, $C_{40}H_{69}O_7PSi_3$, calcd 776.4089); IR (KBr) cm^{-1} 1708 (s, C=O); UV λ_{max}^{MeOH} nm (log ε) 236 (4.79), 266 (sh, 4.53), 273 (4.58), 281 (sh, 4.49); ^1H NMR (300 MHz, CD_3OD - $CDCl_3$, 1:4) δ 1.22 (d, J=7.1 Hz, 4-CH_3), 1.99 (dd, J=1.2 and 1.5 Hz, 3-CH_3), 2.41 (br, quintet J=~6.5 Hz, 4-H), 3.67 (br quintet, J=~5.5 Hz, 21-H), 4.72 (ddd, J=1, 5.7 and 7.1 Hz, 5-H), 5.73 (dd, J=7.1 and 15 Hz, 6-H), 5.79 (quintet, J=1.2 Hz, 2-H).

Enzymatic Dephosphorylation of Oxydifficidin to **6**

To a solution of 43 mg of oxydifficidin in 8 ml of 0.025 M MOPS-HCl buffer, pH 7 at 30°C was added 460 units of alkaline phosphatase (calf intestinal mucosa, P-L Biochemicals). After 60 minutes incubation at 30°C, the milkly white suspension was extracted twice with ether. The combined extract was washed successively with 0.025 M Tris-HCl buffer pH 8, water and brine. The solution was dried over Na_2SO_4, concentrated to an oil and chromatographed on 2 ml of Silica gel G_{60} in ethyl acetate - hexane (25:75) to afford 33 mg of **6**. EI-MS of bis(trimethylsilyl) derivative m/z 624 (M^+, $C_{37}H_{60}O_4Si_2$); UV λ_{max}^{MeOH} nm (log ε) 235 (4.60), 263 (sh, 4.18), 273 (4.29), 283 (4.18); ^1H NMR ($CDCl_3$ spiked with CD_3OD) δ 1.16 (d, J=6.5 Hz, 4-CH_3), 3.03 (d, J=15.5 Hz, 2-H_A), 3.22 (d, J=15.5 Hz, 2-H_B), 4.23 (br t, J=5.0 Hz, 5-H), 4.74 (br dd, J=4 and 9 Hz, 15-H), 4.93 (quintet, 21-H), 5.84 (br dd, J=5 and 15.5 Hz, 6-H).

Hydroxylamine Treatment of Oxydifficidin δ-lactone (**8**)

A solution of 12 mg of oxydifficidin δ-lactone (**8**) in 3 ml of methanol - water (1:1) was treated with 0.8 ml of 2 N aqueous hydroxylamine, pH 7. The solution was stirred at pH 7 and room temp for 18 hours, after which the solution contained about 5 mg of **11**. Several products more polar than **11** were also present and appear to be derived from **11**. To remove hydroxylamine, the reaction mixture was diluted with 9 ml of water and adsorbed on 3 ml of LiChroprep RP-18 resin. The resin was washed successively with water, 20 mM potassium phosphate pH 7 and again with water. Crude **11** was eluted from the resin with methanol and chromatographed on a 90 ml Dupont Zorbax ODS column (21.2 mm i.d.×25 cm) using a solvent gradient from 58:42 to 65:35 methanol - 20 mM potassium phosphate pH 7. Further decomposition of **11** took place during the chromatography. The recovery of **11** after desalting on 1.5 ml of LiChroprep RP-18 was 1.1 mg (HPLC: 89% pure by UV at 273 nm): IR (KBr) cm^{-1} 1782 (s), 1722; UV λ_{max}^{MeOH} nm (log ε) 240 (4.74), 263 (sh, 4.54), 273 (4.58, assumed value based on **8**), 283 (sh, 4.46); ^1H NMR (300 MHz, CD_3OD - $CDCl_3$, 1:4), 2:1 mixture of diastereomers **a** and **b**, δ 0.84 (d, J=7 Hz, isomer **a** 4-CH_3), 1.01 (d, J=7 Hz, isomer **b** 4-CH_3), 1.14 (s, isomer **b** 3-CH_3), 1.38 (s, isomer **a** 3-CH_3), 2.37 (d, J=15 Hz, isomer **b** 2-H_A), 2.53 (d, J=15 Hz, isomer **a** 2-H_A), 2.78 (d, J=15 Hz, isomer **a** 2-H_B), 2.94 (d, J=15 Hz, isomer **b** 2-H_B), 3.64 (m, 21-H), 4.05 (partially obscured by solvent, 5-H), 5.65 (dd, J=8 and 15 Hz, 6-H).

Removal of δ-Lactones **8** and **10** from Crude Oxydifficidin

Crude antibiotic (130 g total solids) contained 20 g of oxydifficidin, 10 g of thermal isomer **4** and 3.5 g of combined δ-lactones **8** and **10**. The material in 3 liters of methanol - water (2:1) was treated with 1.5 liters of 2 N aqueous hydroxylamine pH 7. The solution was stirred 20 hours at room temp, after which 0.2 g of **8**+**10** remained. The solution was diluted with water to 14 liters and processed through DEAE-Sephadex A25 and XAD-16 to afford a mixture (45 g total solids) containing 17 g of oxydifficidin, 7 g of **4** and 0.2 g of **8**+**10**.

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