DACTYLOCYCLINES, NOVEL TETRACYCLINE DERIVATIVES PRODUCED BY A *Dactylosporangium* sp.

II. STRUCTURE ELUCIDATION

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Fermentation of *Dactylosporangium* sp. (ATCC 53693) produces a mixture of tetracycline derivatives from which several related tetracycline glycosides, the dactylocyclines, were isolated and their structures determined. The most abundant glycoside in initial fermentations was found to be dactylocycline A. Each glycoside proved to be acid sensitive and readily hydrolyzed to a common aglycone, dactylocyclinone. While the aglycone was cross resistant with tetracycline, the dactylocyclines proved active against certain tetracycline-resistant organisms.

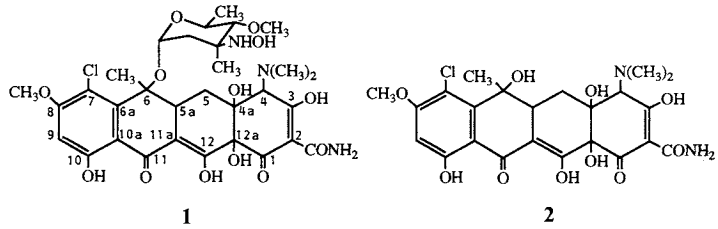
Since the initial isolation of chlortetracycline, tetracycline derivatives have played an important role in antiinfective therapy. In contrast to previously described fermentation products, the dactylocyclines are the first naturally occurring tetracycline derivatives which retain a C-2 amide but lack cross resistance with tetracycline. The producing organism, fermentation and biological properties of the antibiotic are reported in the previous paper.[1] In this publication, we describe the structural elucidation of the dactylocyclines. Studies defining the absolute stereochemistry and conformation of these unusual tetracycline derivatives are presented in the following paper.[2]

Isolation and Identification of Antibiotics

Tetracyclines are potent chelators and, as such, are notoriously difficult to chromatograph. Acidic conditions can be used to prevent chelation or disassociate already formed complexes, thereby allowing recovery of free tetracyclines. The dactylocyclines, however, proved to be chelators that were unstable under conditions typically used to purify tetracyclines. Furthermore, *Dactylosporangium* sp. strain No. SC14051 produced a complex mixture of tetracycline derivatives whose exact composition varied, from fermentation to fermentation, during the course of our studies. In combination, these factors complicated the isolation of pure products from fermentation broth.

Since the dactylocyclines lack cross resistance with tetracycline while the major decomposition product, dactylocyclinone (Fig. 1), is cross resistant with tetracycline, we relied on a tetracycline-resistance assay to guide isolation studies. Samples were tested by disc diffusion on two strains of *Staphylococcus aureus*, one resistant (SC2400) and the other sensitive (FDA 209P) to tetracycline. Isolation of the dactylocyclines was achieved through a combination of partition, ion exchange and reversed phase chromatographies. Scheme 1, detailing the optimized isolation of dactylocycline A (**1**), is typical of the general strategy for purifying dactylocyclines. The use of concentrated crude samples (as opposed to dried fractions) in the

Fig. 1. Structures of dactylocycline A (**1**) and dactylocyclinone (**2**).



1 **2**

Scheme 1. Isolation of pentostatin.

Dactylosporangium sp. SC14051
190 liters broth supernatant

↓ pH 5, EtOAc extraction

Concentrated crude extract

↓ Bio·Rad AG MP-50 resin (pyridinium$^+$ form)
CH_3CN-H_2O wash
Pyridine-CH_3CN-H_2O elution

Concentrated cation exchange eluate

↓ Bio·Rad AG MP-50 resin (Na^+ form)
CH_3CN-H_2O wash
NaCl-CH_3CN-H_2O gradient elution
desalting of active fractions

Crude dactylocyclines

↓ Bio·Rad AG MP-1 resin (Cl^- form) sorption at pH 4.5
CH_3CN-H_2O elution

Enriched dactylocyclines

↓ MCI gel CHP20P resin
CH_3CN-H_2O gradient elution

Dactylocycline A
(48 mg)

Table 1. Isolated yields (mg) of dactylocyclines.

Producer	SC14051	SC14051	SC14051 subclone
Fermentation	Shake flasks	A 250-liter tank	Shake flasks
Fermentation volume (liter)	190	84	130
Dactylocyclinone	91	107	166
Dactylocycline A	30	—[a]	—
Dactylocycline B	46	11	91
Dactylocycline C	11	—	14
Dactylocycline D	18	14	16
Dactylocycline E[b] Dactylocycline F[b]	16	—	9

[a] None isolated.
[b] Isolated as a mixture.

first three steps of the isolation was especially critical for maximal recovery of purified glycosides. In all cases, however, a substantial amount of dactylocyclinone (**2**) was separated during the course of glycoside isolation.

Dactylocycline A (**1**) was the major glycosylated product isolated from initial fermentations although several closely related glycosides were also obtained (Table 1). Some attempts were made to increase production of dactylocyclines. Scaled up fermentations in tanks, requiring antifoam, resulted in decreased dactylocycline production and lower recoveries after chromatography. A subclone of SC14051, selected for its apparently high production of antibiotics active against the tetracycline-resistant strain, gave no substantial improvement in overall yield of dactylocyclines. We found that dactylocycline B was the major tetracycline glycoside isolated from fermentations of the subclone (Table 1).

Although some rapid chromatographic methods for distinguishing the dactylocyclines (without significant decomposition to the aglycone) were developed, mass spectrometry provided the most convincing evidence for identifying components. Both desorption chemical ionization (DCI) and fast atom bombardment (FAB) were used for initial characterization of the antibiotics. Typically, a protonated molecular ion and the fragment corresponding to dactylocyclinone were observed in DCI mass spectra. Ionization by FAB provided mass spectra with the protonated molecular ion predominating (Fig. 2).

Fig. 2. Positive ion FAB mass spectrum of dactylocycline D.

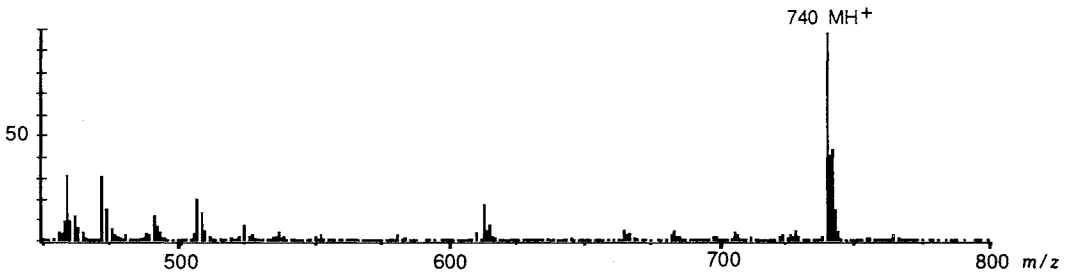


Fig. 3. Daughter ion mass spectrum of dactylocycline D.

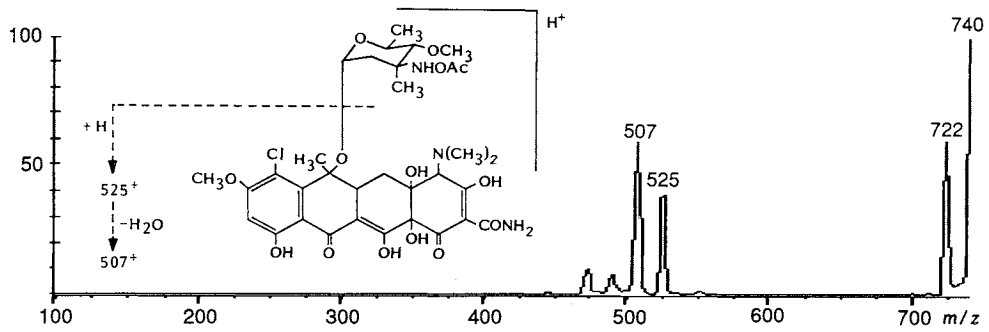


Table 2. High resolution mass spectral data for the dactylocyclines.

Component	Exact mass[a]	Predicted formula (Δamu)
Dactylocyclinone	524.1191	$C_{23}H_{25}N_2O_{10}Cl$ (−0.0007)
Dactylocycline A	697.2236	$C_{31}H_{40}N_3O_{13}Cl$ (−0.0014)
Dactylocycline B	711.2001	$C_{31}H_{38}N_3O_{14}Cl$ (−0.0042)
Dactylocycline C	727.1990	$C_{31}H_{38}N_3O_{15}Cl$ (−0.0002)
Dactylocycline D	739.2357	$C_{33}H_{42}N_3O_{14}Cl$ (+0.0001)
Dactylocycline E	682.2097	$C_{31}H_{39}N_2O_{13}Cl$ (−0.0044)
Dactylocycline F	777.2498	$C_{36}H_{44}N_3O_{14}Cl$ (−0.0014)

[a] Exact mass of neutral species derived from measurement of MH^+ ion.

However, the characteristic fragmentation to the aglycone ion could be confirmed for each dactylocycline through MS/MS daughter ion experiments (Fig. 3). This common mass spectral fragmentation, along with diagnostic UV absorption spectra and visible fluorescence, provided early evidence that the dactylocyclines were a series of tetracycline derivatives based on the dactylocyclinone nucleus. Table 2 summarizes high resolution FAB-MS data obtained for the dactylocyclines which allowed prediction of molecular formulae.

Structure Elucidation

Structure elucidation studies began with dactylocyclinone (**2**), the tetracycline nucleus common to all dactylocyclines. In addition to obtaining **2** during the course of isolation studies, it could also be generated by mild acid hydrolysis (pH <4, room temperature) of any dactylocycline. Regardless of the source, dactylocyclinone was always found to be a mixture of epimers at C-4. Comparison of ^1H NMR, ^{13}C NMR (Table 3) and HPLC coinjection data[3] allowed identification of aglycone **2** as Sch 34164.[4]

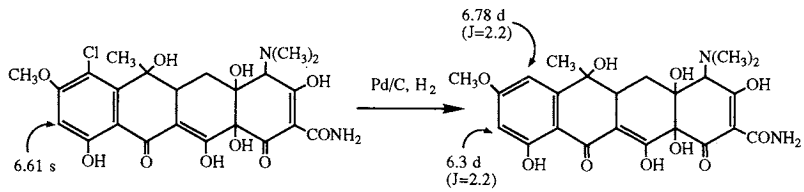
Although Sch 34164 had been described previously, several aspects of the structure determination for dactylocyclinone required more rigorous analysis. First, the aromatic ring substitution pattern (8-methoxy vs. 9-methoxy) could not be determined unambiguously by simple inspection of NMR data obtained for

Table 3. ^{13}C NMR chemical shifts (ppm) for dactylocyclinone and related tetracyclines (DMSO-d_6).

Position	Chlortetracycline·HCl[4]	Sch 34164[4]	Dactylocyclinone[a] Major epimer	Dactylocyclinone[a] Minor epimer	Position	Chlortetracycline·HCl[4]	Sch 34164[4]	Dactylocyclinone[a] Major epimer	Dactylocyclinone[a] Minor epimer
1	193.4	193.1	193.1	192.6	6a	143.6	148.6	149.2	149.0
2	95.6	96.2	101.0	99.2	7	121.2	108.6	109.5	—
$CONH_2$	172.1	172.7	172.2	171.8	8	139.7	163.1	163.6	—
3	187.3	186.4	191.7	—	8-OCH_3	—	56.9	56.9	—
4	68.1	70.0	69.7	sh	9	118.9	100.0	100.5	—
$N(CH_3)_2$	41.0	40.7	45.6	—	10	160.7	161.6	162.0	—
4a	34.9	76.9	78.5	80.3	10a	117.0	111.6	112.1	—
5	27.1	31.4	26.4	32.6	11	193.4	189.7	190.1	—
5a	42.0	42.3	40.8	—	11a	106.1	104.3	104.4	—
6	70.4	73.0	74.5	75.3	12	175.7	173.6	176.4	176.0
6-CH_3	25.0	20.1	20.6	20.5	12a	73.2	73.5	74.3	—

[a] Free base.

Scheme 2. Hydrogenolysis of dactylocyclinone to deschlorodactylocyclinone.



Dactylocyclinone Deschlorodactylocyclinone

dactylocyclinone. While the placement of OCH_3 at C-8 and Cl at C-7 is biosynthetically reasonable,[5] spectroscopic proof was desirable. Clear evidence for assigning a C-8 methoxy group was obtained from ^1H NMR data for the hydrogenolysis product of **2**, deschlorodactylocyclinone (Scheme 2). Secondly, the bridgehead hydroxyl assignment to C-4a (as opposed to C-5a) was not obvious since critical couplings and NOE's to the remaining bridgehead proton were not observed. The position of hydroxylation was firmly established by long range heteronuclear correlations observed for dactylocyclinone as shown in Table 4. Further verification was obtained by recording the ^{13}C NMR spectrum of **2** after acidification with DCl, where characteristic upfield shifts for C-3 and C-4a (β carbons relative to the dimethylamino bearing carbon) could be seen. Finally, the assignment of doubled signals to resonances in the A ring (Tables 3 and 4) provides evidence that dactylocyclinone is epimeric at C-4. This is consistent with both the isolation conditions and the well documented epimerization of tetracyclines in acetate

Table 4. Long range correlations assigning C-4a-OH in dactylocyclinone[a] (C_5D_5N).

Proton assignment	^1H δ	Carbon assignment	^{13}C δ
6-C*H*$_3$	1.7 d	C-6	75 d
		C-6a	150 d
		C-5a	42 d
5a-*H*	4.1 d	C-6	75 d
		C-6a	150 d
		CH_3	23 d
		C-5, C-5′	28, 35
4-*H*	4.8	$N(CH_3)_2$	45
		C-5	28
		C-4a	77
4-*H*′	4.0	C-5′	35
		C-4a′	75

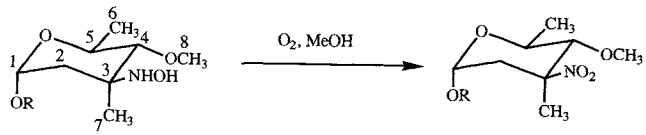
[a] Mixture of C-4 epimers.

Table 5. Exchangeable proton chemical shifts observed for dactylocycline A and related tetracyclines (DMSO-d_6).

Position	Tetracycline hydrochloride[6]	Tetracycline methyliodide[7]	Dactylocyclinone (2)	Dactylocycline A (1)
$CONH_2$	9.5, 9.1	9.4, 9.5	9.0, 8.8	9.1
C-3-OH	—[a]	—	—	—
C-6-OH	—	5.0	5.1	—
C-10-OH	11.7	11.7	12.5	12.7
C-12-OH	15.0	15.2	14.7	14.8
C-12a-OH	broad	7.7	broad	7
Other			4.3	4.3, 11, 18

[a] Not observed.

Scheme 3. Identification of sugar substituents in dactylocyclines A and B.

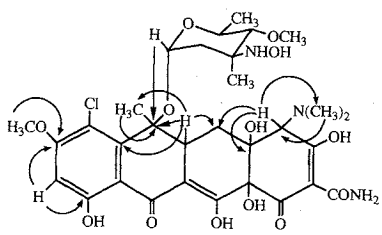


Dactylocycline A (**1**) Dactylocycline B (**3**)

1-Heq: No ax/ax coupling with 2-Hα or 2-Hβ (4 Hz)
4-Hax: $J_{4\text{-H}/5\text{-H}} = 10$ Hz, NOE to 2-Hα and C6-CH_3
5-Hax: $J_{4\text{-H}/5\text{-H}} = 10$ Hz
C-7ax: Strong NOE to 5-H

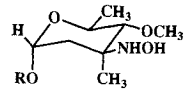
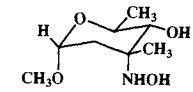
Fig. 4. Identification of glycosylation site in dactylocycline A.

The arrows indicate critical multiple bond ^1H-^{13}C correlations (C_5D_5N).



buffers.[6] It is interesting to note that, in contrast, the glycosides appeared much less susceptible to epimerization at C-4.

Table 6. ^{13}C NMR chemical shifts observed for hydroxylamino sugar derivatives.

Position	Dactylocycline A[a] 	Sugar from reduced viriplanin[b] 
	1	**4**
C-1	95.4[c]	97.8
C-1-OCH_3	—	55.0
C-2	38.9	38.4
C-3	60.3	57.4
C-3-CH_3	19.1	23.3
C-4	82.5[c]	80.0
C-4-OCH_3	61.2[c]	—
C-5	67.9[c]	65.2
C-5-CH_3	18.4[c]	18.2

[a] C_5D_5N.
[b] $CDCl_3$, see Ref. 12.
[c] Assignments obtained from HETCOR data.

Chemical and spectroscopic studies on the dactylocyclines confirmed them to be simple elaborations of the dactylocyclinone nucleus. It could already be deduced from MS/MS fragmentation and hydrolysis studies that each dactylocycline contained a single and distinct acid labile functionality. Due to sample size limitations, full spectroscopic studies were performed only on dactylocycline A. An accounting of exchangeable protons in **1** (Table 5) revealed that a substituent could be accomodated at C-6-OR. Identification of the substituent in **1** as a hydroxylamino pyranoside followed interpretation of NMR chemical shift, coupling and NOE data (Scheme 3). Finally,

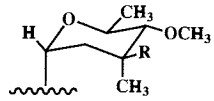
Table 7. ^1H NMR chemical shifts and coupling constants observed for hydroxylamino sugar derivatives.

Position	**1** (DMSO-d_6)	**1** (CD_3OD)	**1** (C_5D_5N)	**3** ($CDCl_3$)[11]
1-H	4.72 d (4.3)	4.93 d (4)	5.26 d (4)	4.64 d (4)
C-1-OCH_3	—	—	—	3.36 s
2-Hax	1.80 dd (14, 4)	2.03 m	2.5 m	1.65 dd (14, 4)
2-Heq	1.95 br d (14)	2.50 d (13.2)	2.6 d (14)	1.89 d (14)
C-3-CH_3	1.07 s	1.59 s	1.68 s	1.39 s
4-H	3.01 d (9.8)	3.24 d (8.4)	3.65 m	3.21 d (10)
C-4-OCH_3	3.42 s	3.55 s	3.71 s	—
5-H	3.8 m	4.1 m	4.35 dq (10, 6)	3.81 dq (10, 6)
C-5-CH_3	1.22 d (6)	1.42 d (6)	1.68 d (6)	1.34 d (6)

the glycosidic linkage at C-6 of dactylocycline A was proven by heteronuclear multiple bond correlation spectroscopy (Fig. 4).

During prolonged NMR experiments (in CD_3OD at room temperature), dactylocycline A oxidized to a nitro sugar-containing glycoside **3**, later found to be identical with dactylocycline B (Scheme 3). Similar nitro sugar residues have been described for the antibiotics everninomycin[9~11] and viriplanin[11] although the corresponding hydroxylamino sugar derivatives were not observed as fermentation products. A summary of relevant NMR data for the sugar subunit of dactylocycline A and closely related sugars is provided in Tables 6 and 7. While the presence of a hydroxylamine group in **1** could be verified by a positive Griess test (pyrolysis to HNO_2), the nitro group in dactylocycline B could only be inferred from the predicted molecular formula, chemical shift measurements and derivatization studies. The assigned sugar structures, however, are consistent with the greater basicity observed during low voltage electrophoresis of **1** compared to **2** and the formation of a monoacetyl derivative of **1** (and not of **2**) after treatment with neat acetic anhydride.

Table 8. Summary of dactylocycline structures and bioactivities.



Dactylocycline	R= (from MW)	Antibiotic activity (mm zone, 50 μg)	
		S. aureus (tet^s)[a]	*S. aureus* (tet^r)[b]
A	NHOH	19	17
B	NO_2	13.5	13
C	NO_2+O	18	17
D	NHOAc	19	19
E	OH	20[c]	17[c]
F	Unknown	[c]	[c]
Dactylocyclinone	No C-6 substituent	22.5[d]	0

[a] Tetracycline-sensitive *Staphylococcus aureus*.
[b] Tetracycline-resistant *Staphylococcus aureus*.
[c] Tested as a mixture of dactylocyclines E and F.
[d] Hazy zone of inhibition.

The chemical shifts for protons in the tetracyclic nucleus of all dactylocyclines are highly conserved. Thus, the dactylocyclines are a series of C-6-glycosidic tetracyclines containing different nitrogenous sugars. Table 8 lists the sugar subunits identified for dactylocyclines isolated to date and their relative antibiotic potencies. In each dactylocycline, the novel glycosidation at C-6 appears to impart activity against tetracycline-resistant strains. While dactylocyclinone is a weakly active, Gram-positive selective tetracycline, many other potent broad spectrum tetracyclines are known. However, a strategy for overcoming tetracycline-resistance mechanisms for these antibiotics is needed. The structural features of the dactylocyclines may provide new insights into the development of tetracycline analogs with activity against tetracycline-resistant organisms.

Experimental

^1H and ^{13}C NMR spectra were recorded in CD_3OD, C_5D_5N or DMSO-d_6 using JEOL GX-270, JEOL GX-400 or Bruker AM 500 spectrometers. Chemical shift values are given in ppm downfield of internal TMS or from the central solvent lines of the deuterated solvents used for ^{13}C NMR spectra. Long range correlations were observed by proton-detected heteronuclear multiple bond correlation spectroscopy.[13] FAB mass spectra (8 keV Xe) were obtained using a VG-ZAB-2F instrument (Vacuum Generators, Ltd.). Exact mass measurements were performed at a resolution of 8000 (m/Δm) using polyethylene glycol as an internal standard. MS/MS daughter ion experiments were carried out *via* mass analyzed ion kinetic energy spectroscopy (MIKES), using nitrogen as the collision gas at a pressure sufficient to attenuate the main beam by ~50%. Desorption chemical ionization mass spectra were obtained using a Finnigan TSQ46 instrument with ammonia as the reagent gas. Thin layer chromatographic analyses were performed on Macherey-Nagel RPC18 plates, developing with 0.5 M oxalic acid - methanol - acetonitrile (6:2:2). HPLC comparisons were performed on an HP 1090 liquid chromatograph with a Waters microbondapak phenyl column (0.45 × 30 cm), eluting with 1 ml/minute 30% DMF in buffer (0.5 mM Na_2EDTA, 15 mM citric acid, 20 mM sodium citrate and 50 mM KNO_3 at pH 4.2) and detecting at 280 nm. Low voltage paper electrophoresis was performed at 250 V, 12 V/cm for 1 hour with 50 mM buffers of varying pH.

Acid Hydrolysis of Dactylocycline A (**1**) to Dactylocyclinone (**2**)

Treatment of **1** with 1 N HCl at room temperature for 40 minutes provides 75% hydrolysis to **2**, as judged by TLC. Complete hydrolysis of **1** is achieved with 1 N HCl at 50°C for 40 minutes. FAB-MS positive ion, *m/z* 525 $(M+H)^+$; negative ion *m/z* 523 $(M-H)^-$.

Hydrogenolysis of Dactylocyclinone (**2**) to Deschlorodactylocyclinone

Dactylocyclinone (7.3 mg) was dissolved in 10 ml methanol to which 10 μl triethylamine was added. A solution of 1 mg 10% palladium on charcoal in 10 ml methanol was prepared, and the dactylocyclinone solution was added to it after flushing with nitrogen gas. The mixture was stirred at room temperature for 48 hours under a hydrogen atmosphere. Products recovered after filtering through Celite showed a 70% conversion to deschlorodactylocyclinone by ^1H NMR integration. FAB-MS positive ion, *m/z* 491 $(M+H)^+$.

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