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

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITY

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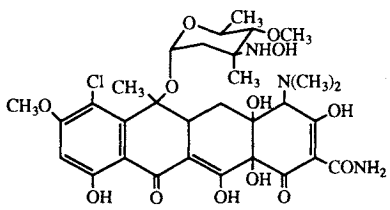
A screen for antibiotics with activity against tetracycline-resistant microorganisms has led to the isolation of *Dactylosporangium* sp. (ATCC 53693), a producer of several novel tetracycline derivatives. The major fermentation products, dactylocyclines A and B, were purified and MIC values determined against tetracycline-resistant and tetracycline-sensitive Gram-positive bacteria. The dactylocyclines represent the first naturally occuring tetracycline C2 amides which lack cross resistance with tetracycline.

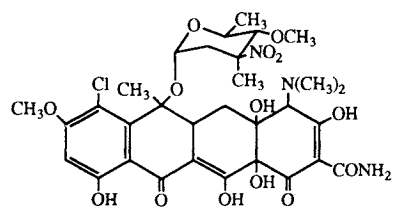
Tetracycline-resistance is widespread among bacteria and there is a need for agents with activity against resistant clinical isolates. With the goal of identifying such an agent, we employed a screen which was extremely sensitive to tetracycline and tetracycline-like compounds. The screen was based on the observation that inhibition of *Escherichia coli* SC12623, (which carries the tetracycline-resistance determinant Tn10) by cetotetrine was reversed in the presence of tetracycline.[1] This phenomenon occurs since cetotetrine (or chelocardin), a tetracycline-related compound,[2] is able to inhibit *E. coli* SC12623 without inducing its resistance mechanism. Tetracycline, at concentrations well below its MIC, can effectively induce the bacterial resistance mechanism and thus rescue the microorganism from an otherwise inhibiting concentration of cetotetrine. During the course of the screen, a *Dactylosporangium* sp. was found which produced a mixture of tetracycline derivatives that lacked cross resistance with tetracycline. The two major components have been named dactylocyclines A and B (Fig. 1).

This paper describes the producing culture, fermentation, isolation, physico-chemical characterization and biological properties of the dactylocyclines. Subsequent manuscripts describe the structure elucidation,[3] stereochemistry and conformational studies.[4]

Fig. 1. Structures of dactylocyclines A and B.



Dactylocycline A



Dactylocycline B

Materials and Methods

Taxonomic Studies

Colony morphology studies were carried out on International Streptomyces Project (ISP) medium No. 4[5] and calcium malate agar.[6] Color codes were assigned to the reverse pigments according to the Color Harmony Manual (4th Ed.).[7] Cell wall analysis was determined by thin layer chromatography.[8]

Primary Screen

E. coli SC12623 was used as the assay organism. The organism was grown overnight in Antibiotic Assay Broth (Baltimore Biological Laboratory (BBL), Cockeysville, MD) at 37°C on a rotary shaker (Model G333, New Brunswick Scientific Co., New Brunswick, NJ). A 1% inoculum was used to inoculate an agar medium containing: BBL seed agar 30.5 g and NaCl 5 g per liter of distilled water. Triphenyl tetrazolium chloride (0.06 mg/ml) and cetotetrine (0.75 μg/ml) were added at time of inoculation. The agar plates were prepared in subdued light to protect cetotetrine from inactivation. Incubation was overnight in the dark at 37°C. Active compounds were detected by the presence of red zones of growth around the discs or wells containing the fermentation material.

Protein Biosynthesis Assay

Test samples, either as liquids (250 μl) or air dried on discs, were added to 2 ml of Antibiotic Assay Broth (BBL) in sterile tubes. An overnight culture of *Bacillus licheniformis* SC9262 (0.5 ml) and 100 μl of a benzylpenicillin solution (100 μg/ml) were added to each tube and allowed to incubate for 2.5 hours at 37°C. During the incubation period, benzylpenicillin served to induce the production of β-lactamase by *B. licheniformis* through *de novo* protein biosynthesis. Following incubation, a solution (500 μg/ml) of a chromogenic cephalosporin such as PADAC (Hoechst AG)[9] was added to test for the presence of β-lactamase. Inhibition of a rapid color change was observed in the presence of protein synthesis inhibitors such as tetracyclines.

Physico-chemical Measurements

^{1}H NMR spectra were recorded in CD_3OD with internal TMS using a JEOL GX-400 spectrometer. FAB mass spectra were obtained using a VG-ZAB 2F instrument. IR spectra were obtained on micro KBr pellets with a Mattson Sirius 100 FT-IR spectrometer and UV spectra were obtained on solutions in 1 cm cells with a Shimadzu UV-260 UV-visible recording spectrophotometer.

Results

Taxonomy of the Producing Strain

Dactylosporangium sp. SC14051 was isolated from a sample of leaf litter in marsh water collected in New Jersey. The strain was deposited in the American Type Culture Collection, where it has been assigned the accession number ATCC 53693.

Morphological Properties

Growth of *Dactylosporangium* sp. SC14051 on ISP No. 4 agar was moderate with no evidence of aerial mycelium. Sporangia were scant but globose bodies were present in large numbers throughout the vegetative mycelium. Reverse color was in shades of light apricot to varying shades of orange. No soluble pigment was evident on the medium. On calcium malate agar, growth was moderate with abundant production of sporangia but globose bodies were scarce. Reverse color ranged from colorless to shades of orange. No soluble pigment was produced. On media favoring the production of true sporangia, globose bodies were scarce. They were prevalent in shaken culture, however.[10]

This organism was characterized by the production of short finger-like sporangia arising directly from the vegetative mycelium on the surface of the agar. Each sporangium contained a straight row of spores,

usually 3~4. Flooding the agar surface of a sporangial culture with sterile water caused the spores to be released by rupture of the upper part of the sporangium, probably due to internal pressure at the base of the sporangium. The spores remained immobile for approximately thirty minutes after which they exhibited the characteristic motility of zoospores. The other distinctive microscopic feature of the organism was the production of globose bodies borne laterally on the vegetative mycelium. But for their smaller size they could be mistaken for spherical sporangia common in some members of the *Actinoplanaceae*. On microscopic examination they did not contain spores of any kind, but rather masses of amorphous material some of which eventually lyse leaving empty cells. These cells appear highly refractile.

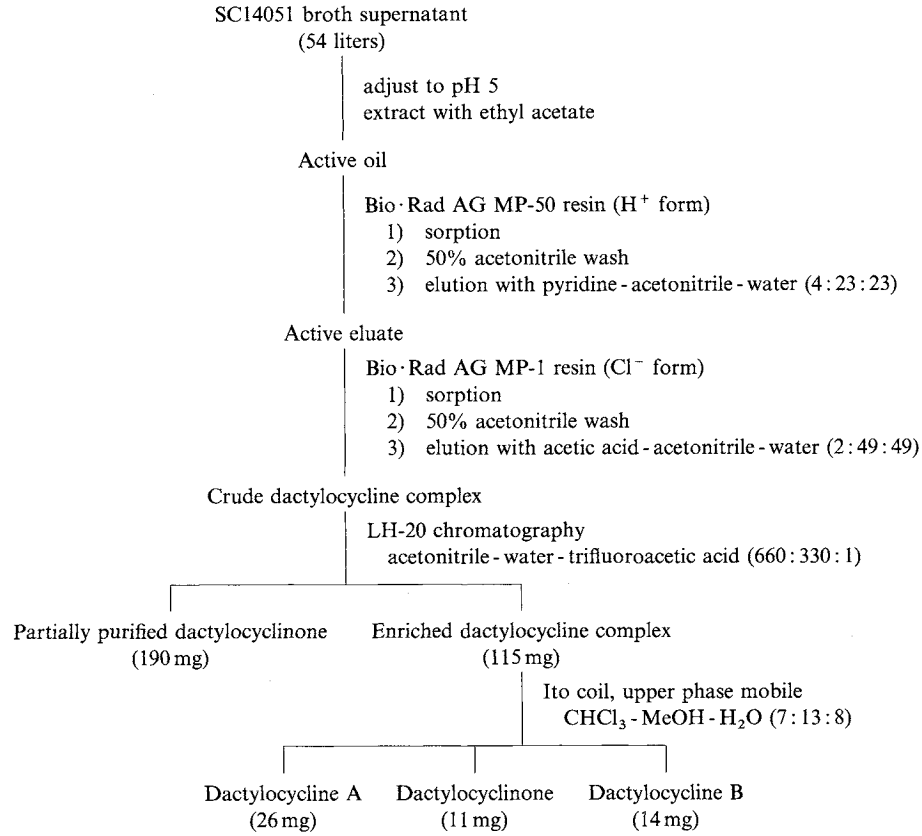
Cell Wall Analysis

Acid hydrolysates of whole cells contained *meso*-diaminopimelic acid and glycine in the cell wall, with xylose and arabinose as the prevailing sugar moieties. This is characteristic of a Type II cell wall.[11]

Basis for Genetic Identification

The absence of aerial mycelia and the morphological characteristics, *i.e.* production of finger-like sporangia containing motile spores and sterile globose bodies coupled to a Type II cell wall, place this organism in the genus *Dactylosporangium*, a member of the family *Actinoplanaceae* in accordance with the description of the genus by THIEMANN *et al.*[10] and SHARPLES and WILLIAMS.[12]

Scheme 1. Isolation of dactylocyclines A and B.



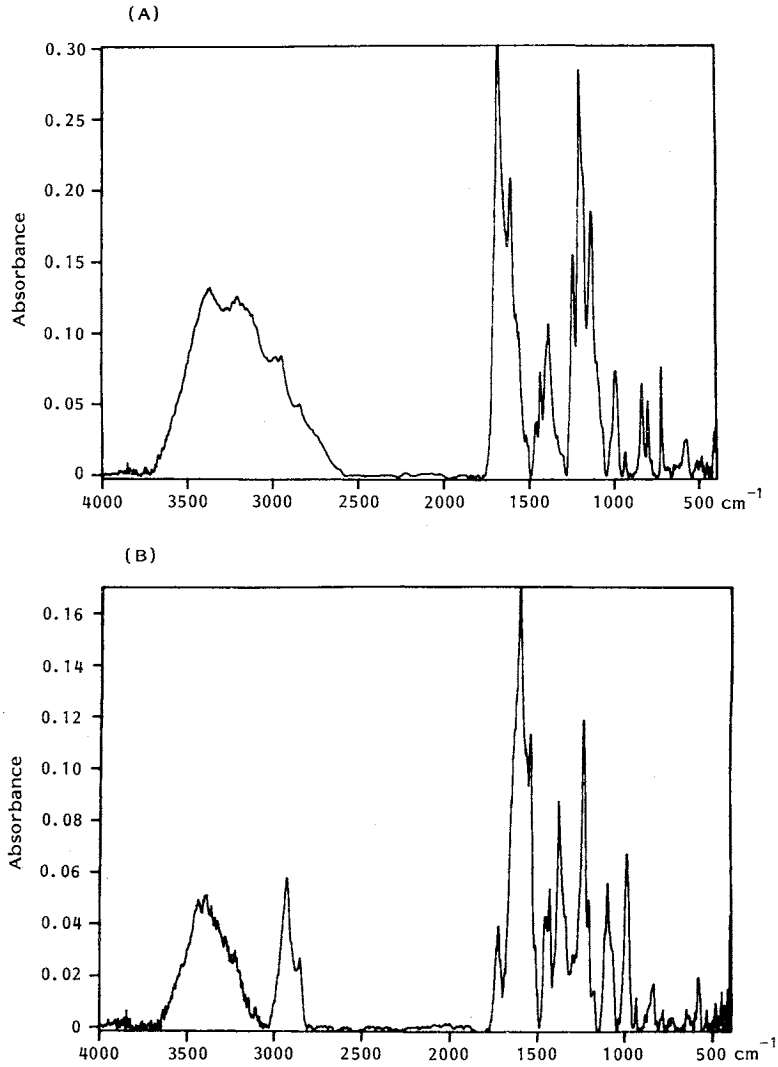
Fermentation

Dactylosporangium sp. SC14051 was cultured and maintained on agar slants composed of oatmeal 2% and tomato paste 2% in tap water. Inoculum was prepared in Erlenmeyer flasks (500 ml) containing 100 ml

Table 1. Physico-chemical properties of dactylocyclines A and B.

Property	Conditions	Dactylocycline A	Dactylocycline B
FAB-MS molecular ion (*m/z*)	+Ion mode	698	712
	−Ion mode	696	710
UV λ_{max} nm ($E^{1\%}$)	MeOH	369 (160),	373 (190),
		261 (170),	262 (200),
		238 (200)	238 (250)
	MeOH+NaOH	386 (150),	385 (180),
		279 (170),	281 (200),
		243 (210)	243 (260)

Fig. 2. IR spectra (KBr) of dactylocyclines A (A) and B (B).



of a seed medium consisting of glucose 1 g, soluble starch 24 g, beef extract 3 g, Tryptone 5 g, yeast extract 5 g, and $CaCO_3$ 4 g (pH 7.0) per liter of water. The inoculated seed flasks were incubated at 28°C on a rotary shaker with a 5-cm throw at 300 rpm for 96 hours.

Fermentation was carried out in 500-ml Erlenmeyer flasks containing 100 ml portions of the same medium used for the seed medium. The flasks were inoculated with a 1% inoculum and incubated at 28°C for 144 hours on a rotary shaker at the same speed used for the seed culture. The production of activity was measured by the protein biosynthesis assay described above.

Isolation

The first isolation of dactylocyclines was achieved through a combination of ion exchange and liquid-liquid partition chromatographies. The method shown in Scheme 1 allowed identification of dactylocyclines as novel tetracycline derivatives with activity against tetracycline-resistant strains of Gram-positive microorganisms. The aglycone of the dactylocyclines, dactylocyclinone, was also recovered by this process and found to be identical to Sch 34164.[13]

Fig. 3. ^1H NMR spectra (in CD_3OD) of dactylocyclines A (A) and B (B).

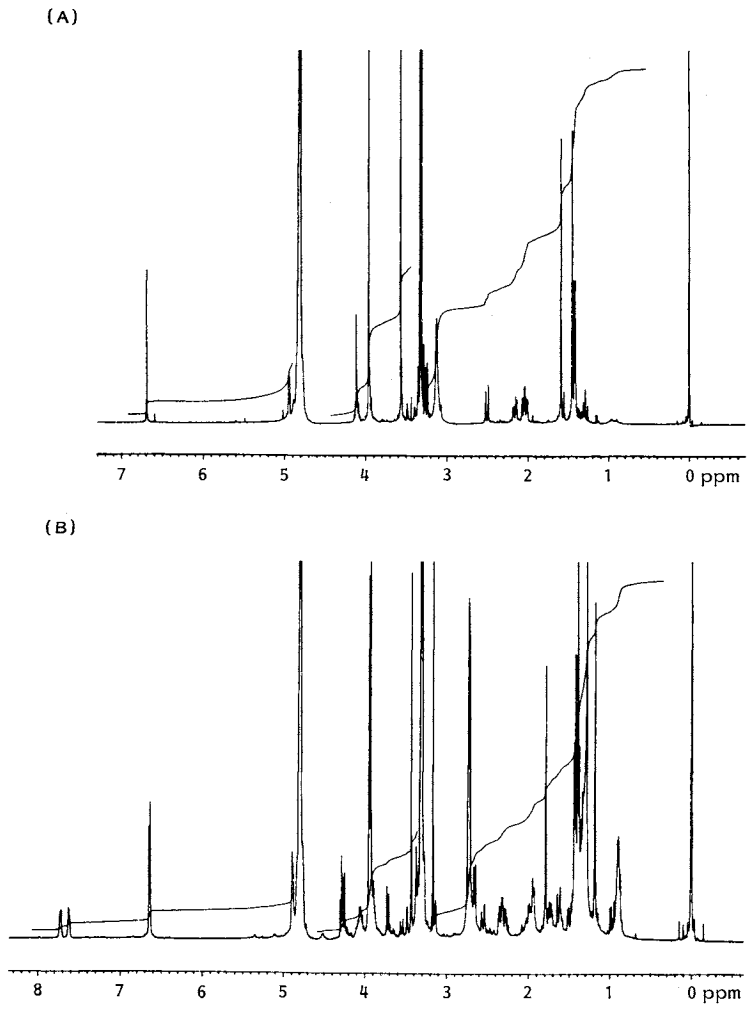


Table 2. Antimicrobial activities of several tetracyclines.

Organism[a] (10^4 cfu inoculum level)	MIC (μg/ml)			
	Tetracycline	Dactylocycline A	Dactylocycline B	Dactylocyclinone
Staphylococcus aureus SC2399[b]	0.4	1.6	6.3	6.3
S. aureus SC10016[b]	100	6.3	3.1	>100
S. aureus SGB 42[b]	0.2	1.6	3.1	3.1
S. aureus SGB 45[b]	100	3.1	3.1	>100
S. epidermidis SC9052[b]	0.8	6.3	3.1	12.5
S. epidermidis SC9087[b]	50	3.1	3.1	>100
Streptococcus faecalis SC9011[b]	1.6	25	6.3	25
S. faecalis SC9776[b]	>100	25	6.3	>100
Enterobacter cloacae SC12364[c]	3.1	>100	>100	100
E. cloacae SC12548[c]	>100	>100	>100	>100
Klebsiella pneumoniae SC12333[c]	0.8	>100	>100	12.5
K. pneumoniae SC12532[c]	>100	>100	>100	>100
Escherichia coli SC12302[c]	1.6	6.3	25	3.1
E. coli SC12549[c]	>100	>100	>100	>100
E. coli SC12196[c]	1.6	>100	>100	25
E. coli SC12623[c]	100	>100	>100	>100

[a] Pairs of organisms were used in which the first one listed is tetracycline-sensitive and the second one is resistant to tetracycline.
[b] Medium composition: Beef extract 1.5 g, yeast extract 3 g, peptone 6 g, glucose 1 g, agar 15 g, in 1 liter distilled water.
[c] Medium: DST agar.

Characterization

Dactylocyclines A and B were identified as closely related functionalized tetracycline derivatives by their characteristic UV spectra, displaying bathochromic shifts in basic solutions, and mass spectra (Table 1). The IR (Fig. 2) and ^{1}H NMR (Fig. 3) spectra, obtained for the initially isolated antibiotics, were also consistent with functionalized tetracyclines. Detailed spectroscopic characterization of the dactylocyclines is presented in the following manuscripts.

Antimicrobial Activity of Dactylocyclines A and B

The biological activity of dactylocyclines and dactylocyclinone was assessed by agar dilution assay (Table 2). The dactylocyclines are active against Gram-positive bacteria, but not active, or weakly active, against Gram-negatives. When tested against tetracycline-resistant and -sensitive pairs of bacteria, the dactylocyclines are not cross resistant with tetracycline. In contrast, dactylocyclinone is cross resistant with tetracycline and is also more active than the dactylocyclines against Gram-negative bacteria. We infer from these results that elaboration at C6, as found in the dactylocyclines, confers activity against tetracycline-resistant organisms.

Acknowledgments

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