A NEW TETRACYCLINE ANTIBIOTIC FROM A *DACTYLOSPORANGIUM* SPECIES

FERMENTATION, ISOLATION AND STRUCTURE ELUCIDATION

MAHESH PATEL, VINCENT P. GULLO, VINOD R. HEGDE, ANN C. HORAN, JOSEPH A. MARQUEZ, RICHARD VAUGHAN, MOHINDAR S. PUAR and GEORGE H. MILLER

Department of Microbial Products, Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003, U.S.A.

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An actinomycete identified as a *Dactylosporangium* sp. produces a new tetracycline, 4a-hydroxy-8-methoxychlortetracycline (Sch 34164). The addition of magnesium ions to complex fermentation media increased the antibiotic titers. Sch 34164 was isolated by solvent extraction and Sephadex G-25 column chromatography. The novel structure was proposed based on spectroscopic analysis. The shift of C-4a (35 to 77 ppm) and C-8 (140 to 163 ppm) in the ^{13}C NMR as compared to chlortetracycline was indicative of the novel hydroxyl and methoxy substituents, respectively.

An actinomycete, designated SCC 1695, isolated from a sample collected in the Kasie Valley of Zambia was found to produce in fermentation a new tetracycline antibiotic. The antibiotic was isolated from fermentation broth by solvent extraction and purified by Sephadex G-25 column chromatography. The producing strain was characterized and found to have the macroscopic, microscopic and whole-cell hydrolysis properties of the genus *Dactylosporangium*.

This paper describes the production, isolation, physico-chemical properties and structure elucidation of Sch 34164[1]. Taxonomy and biological properties[2] will be reported elsewhere.

Fermentation

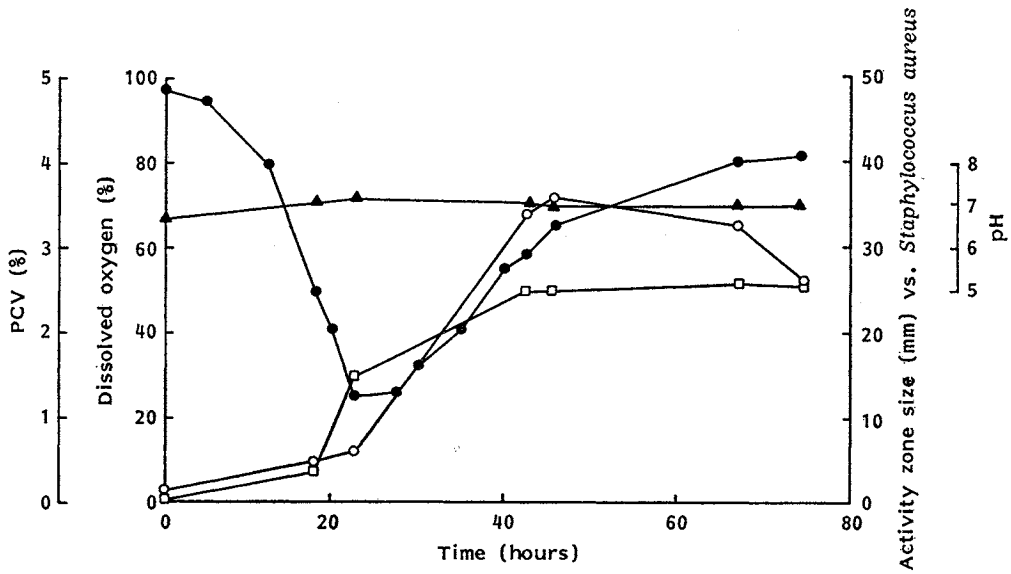
The fermentation studies were carried out in shake flasks and 14-liter fermentors. An aliquot of the frozen whole broth was used to inoculate a germination medium composed of Cerelose 0.1%, potato starch 2.4%, beef extract 0.3%, yeast extract 0.5%, Tryptone 0.5% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.0 prior to sterilization. The seed culture was incubated at 30°C on a rotary shaker at 300 rpm for 48 hours. Twenty five ml of the resulting seed culture were transferred to a 2-liter Erlenmeyer flask containing 500 ml of the above medium and incubated for an additional 48 hours at 30°C. The entire content was used to inoculate a 14-liter fermentor containing 10 liters of the production medium consisting of Cerelose 1.0%, soluble starch 2%, yeast extract 0.5%, NZ-Amine 0.5%, $CaCO_3$ 0.4% and 1 mM $CoCl_2$ 1 ml. The fermentation was carried out at 30°C, 300 rpm and aeration of 3.5 liters per minute for 96 hours.

Antibiotic production in shake flasks and tanks was monitored at 72 and 96 hours by whole broth disc-agar diffusion assay against *Staphylococcus aureus* and *Escherichia coli*. Since the initial titers were extremely low, experiments in shake flasks focusing on the production medium were initiated to increase antibiotic titers. The fermentation medium discussed previously was supplemented with various ions at the concentration shown in Table 1. All ionic solutions were added post steriliza-

Table 1. Effect of ions on the production of Sch 34164.

Ion	Added (μmol)	Whole broth agar diffusion zone size (6.35 mm discs)			
		72 hours		96 hours	
		S. aureus 209P (mm)	*E. coli* A-10536 (mm)	*S. aureus* 209P (mm)	*E. coli* A-10536 (mm)
None		9	0	8	0
Fe	100	0	0	9	0
	200	9	0	9	0
Zn	25	10	0	9	0
	50	11	0	11	0
Co	50	7	0	9	0
	100	7	0	9	0
Cu	5	0	0	9	0
	10	7	0	7	0
Mn	25	0	0	7	0
	50	7	0	8	0
Ca	100	9	0	9	0
	200	0	0	7	0
Mg	1,250	21	17	20	19
	2,500	22.5	20	21.5	21

Fig. 1. Standard fermentation parameters.
○ Packed cell volume (PCV), ● dissolved oxygen, □ activity zone size, ▲ pH.



tion. Magnesium at the optimum concentration, significantly increased the activity of the fermentation broth against both *S. aureus* and *E. coli*. A typical time course study in 14-liter fermentors with magnesium supplementation is shown in Fig. 1. The pH, dissolved oxygen, growth profile of the organism and antibiotic production were monitored.

Isolation

The whole broth (10 liters) was adjusted to pH 4 and extracted two times with 10 liters of ethyl

acetate. The extracts were pooled and concentrated to dryness. The active residue was dissolved in acetone and the antibiotic complex was precipitated with a mixture of diethyl ether - hexane (1 : 4). The resulting precipitate was treated with EDTA in water at pH 1.5 and extracted with methylenechloride at pH 6.5. Final purification of Sch 34164 was achieved by Sephadex G-25 column chromatography using 0.02 N HCl as the eluting solvent[3]. The fractions were monitored by HPLC and the desired fractions pooled and lyophilized. A 10-liter fermentation gave 60 mg of pure Sch 34164.

The purity of Sch 34164 was determined by HPLC[4,5] using chlortetracycline as a standard (Fig. 2). The purity of Sch 34164 was comparable to USP chlortetracycline standard.

Physico-chemical Properties

Sch 34164 was differentiated from most other known antibiotics by TLC using a mixture of chloroform - methanol - water (2 : 2 : 1, lower phase). The compound gave a bright yellow fluorescence on a thin layer plate, enhanced by ammonia vapors which is indicative of the tetracycline family of antibiotics.

Physico-chemical data of Sch 34164 are listed in Table 2. Sch 34164 is a yellow powder. The hydrochloride salt is freely soluble in water. The IR spectrum in KBr showed the presence of NH, OH stretching at 3600~3200 and an amide at 1650 and 1611 cm^{-1}. High resolution fast atom bombardment mass spectrum (FAB-MS) gave an $(M+1)^+$ of m/z 525.1273 which calculated for the molecular formula $C_{23}H_{26}N_2O_{10}Cl$.

The ^1H NMR of Sch 34164 was found to be similar to that of chlortetracycline except for the presence of one additional methyl group at 4.02. The assignment of all the carbons in the ^{13}C NMR spectrum of Sch 34164 and chlortetracycline are shown in the Table 3[5]. The chemical shifts of most of the carbons are similar to chlortetracycline. The major differences are in C-4a, C-7, and C-8, C-9 and the presence of an additional methyl group at 56.9 ppm.

Structure Elucidation

The physico-chemical characteristics of Sch

Fig. 2. HPLC comparison of Sch 34164 with chlortetracycline.

Column: Chromegabond C-2 5 μm (ES Ind.) 4.6 mm×5 cm. Mobile phase: Buffer - DMF (80 : 20) [buffer 1; 5 mM EDTA, buffer 2; 15 mM citric acid, buffer 3; 20 mM sodium citrate, buffer 4; 50 mM potassium nitrate]. UV: 280 nm (0.05 aufs). Flow rate: 1 ml/minute.

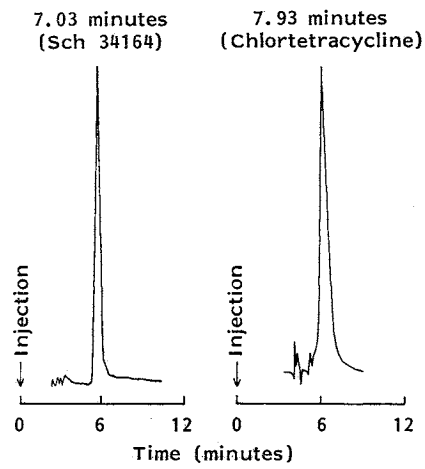


Table 2. Physico-chemical properties of Sch 34164.

UV λ_{max}^{MeOH} nm (ε)	234 (16,750), 280 (15,500), 377 (18,000)
IR (KBr) cm^{-1}	3400 (br), 1611, 1570, 1558, 1461, 1423, 1414, 1380, 1309, 1240, 1209
FAB-MS $(M+1)^+$ (m/z)	Found: 525.1273 Calcd for $C_{23}H_{26}N_2O_{10}Cl$: 525.1267
^1H NMR ($CD_3OD+(CD_3)_2CO$) δ	1.26 (CH_3), 3.3 ($N(CH_3)_2$), 4.02 (OCH_3), 6.62 (=CH), 9.72 (NH_2)
$[\theta]_\lambda \times 10^{-4}$	$[\theta]_{258}=-11.8$, $[\theta]_{293}=+2.2$, $[\theta]_{328}=-2.1$

34164 are closely related to chlortetracycline except for the presence of an additional hydroxyl and a methoxyl[6] substitution in the molecule. Based on ^{1}H and ^{13}C NMR, the additional methoxy substituent could only be located on an aromatic ring at C-8 or C-9 position. The exact location was confirmed by the chemical shift in the ^{13}C NMR spectra of the C-7, C-8 and C-9 carbons when compared to chlortetracycline (Table 3). Further confirmation for the methoxy substituent at C-8 position was based on the bathochromic shift in the UV spectrum as compared to chlortetracycline. Chlortetracycline has an absorbance at 355 nm while Sch 34164 has an absorbance at 377 nm, which is consistent with the methoxy substituent at the C-8 position. The additional hydroxyl function was assigned to the C-4a position based on the downfield shift of this carbon as compared to chlortetracycline (Table 3).

Table 3. ^{13}C NMR data of Sch 34164 and chlortetracycline.

Position	^{13}C NMR ($(CD_3)_2SO$)	
	Sch 34164	Chlortetracycline
C-1	193.1	193.4
C-2	96.2	95.6
CONHR	172.7	172.1
C-3	186.4	187.3
C-4	70.0	68.1
$N(CH_3)_2$	40.7	41.0[a]
C-4a-R	76.9	34.9
C-5	31.4	27.1
C-5a	42.3	42.0[a]
C-6	73.0	70.4
CCH_3	20.1	25.0
C-6a	148.6	143.6
C-7	108.6	121.2
C-8	163.1	139.7
C-9	100.0	118.9
C-10	161.6	160.7
C-10a	111.6	117.0
C-11	189.7	193.4
C-11a	104.3	106.1
C-12	173.6	175.7
C-12a	73.5	73.2
OCH_3	56.9	—
NCH_3	—	—

[a] Indicates peaks under $(CD_3)_2SO$ peak, observed when spectrum was run in D_2O - dioxane.

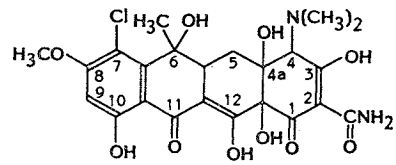
Based on the data presented the structure of Sch 34164 is shown in Fig. 3.

Biological Properties

The *in vitro* antibacterial activity of Sch 34164 is shown in Table 4. The *in vitro* evaluation showed that Sch 34164 was active against both Gram-positive and Gram-negative organism.

In summary, we have reported on an additional novel 8-methoxychlortetracycline[7] from a new species of *Dactylosporangium* having a novel substituent at the C-4a position.

Fig. 3. Structure of Sch 34164.



4a-Hydroxy-8-methoxychlortetracycline
(4a-OH-8MCTC)

Table 4. *In vitro* activity of Sch 34164.

Organisms (No. of strains)	Geometric mean MICs (μg/ml)		
	Sch 34164	Sch 33256	Tetracycline
Gram-negative aerobic bacteria (23)[a]	7.8	33.0	2.3
Staphylococcus (63)[b]	3.1	0.52	0.52
Streptococcus (25)[c]	0.45	0.15	0.36

[a] Includes *Escherichia coli* (9), *Klebsiella* sp. (8), *Enterobacter* sp. (4) and *Salmonella-Shigella* sp. (2).

[b] Includes methicillin-resistant (9) and -susceptible (54) strains.

[c] Includes *Streptococcus pneumoniae* (5), *Streptococcus viridans* (2), *Streptococcus faecalis* (2), *Streptococcus faecium* (4), Group A (5), Group B (1), Group C (3) and Group G (3) Streptococci.

References

1) PATEL, M.; V. GULLO, V. HEGDE, A. HORAN, J. A. MARQUEZ, R. VAUGHAN, M. PUAR & G. MILLER: A novel tetracycline produced by *Micromonospora vesca*: Taxonomy, fermentation, isolation and structure. Program and Abstracts of the 25th Intersci. Conf. on Antimicrob. Agents Chemother., No. 230, p. 132, Minneapolis, Sept. 29~Oct. 2, 1985
2) CACCIAPUOTI, A.; E. L. MOSS, Jr., F. MENZEL, Jr., C. A. CRAMER, W. WEISS, D. LOEBENBERG, R. S. HARE & G. H. MILLER: *In vitro* and *in vivo* characterization of novel 8-methoxy derivatives of chlortetracycline. J. Antibiotics 40: 1426~1430, 1987
3) GRIFFITHS, B. W.: Gel chromatography of tetracyclines and derivatives of tetracyclines. J. Chromatogr. 38: 41~46, 1968
4) KNOX, J. H. & J. JURAND: Mechanism of reverse phase separation of tetracyclines by high-performance liquid chromatography. J. Chromatogr. 186: 763~782, 1979
5) ASZALOS, A.: Fast determination of tetracycline antibiotics in different media by high performance liquid chromatography. Chromatographic 20: 313~321, 1985
6) GULBIS, J. & G. W. EVRETT, Jr.: Carbon 13, nuclear magnetic resonance. Spectral analysis of tetracycline hydrochloride and related antibiotics. J. Am. Chem. Soc. 97: 6248, 1975
7) PATEL, M.; V. P. GULLO, V. R. HEGDE, A. C. HORAN, F. GENTILE, J. A. MARQUEZ, G. H. MILLER, M. S. PUAR & J. A. WAITZ: A novel tetracycline from *Actinomadura brunnea*. Fermentation, isolation and structure elucidation. J. Antibiotics 40: 1408~1413, 1987