A NOVEL TETRACYCLINE FROM *ACTINOMADURA BRUNNEA*

FERMENTATION, ISOLATION AND STRUCTURE ELUCIDATION

Mahesh Patel, Vincent P. Gullo, Vinod R. Hegde, Ann C. Horan, Frank Gentile, Joseph A. Marquez, George H. Miller, Mohindar S. Puar and J. Allan Waitz

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

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A novel tetracycline antibiotic, Sch 33256, was isolated from a culture broth of a new species of *Actinomadura*. The antibiotic was isolated by solvent extraction, Sephadex G-25 column chromatography and crystallization. The structure was determined by comparison of the spectra with that of chlortetracycline. Spectroscopic analysis of the compound yielded 2′-*N*-methyl-8-methoxychlortetracycline as the proposed structure.

During the course of searching for new microbial fermentation products, a novel solvent extractable antibiotic complex has been obtained from an organism isolated from a soil sample collected near Phoenix, Arizona. The producing culture, SCC 1676, was found to have the macroscopic, microscopic and whole-cell hydrolysis properties of the genus *Actinomadura*.

In this paper, we describe the fermentation, isolation and structure elucidation of the major antibiotic component Sch 33256[1]. Detailed taxonomy and biological properties will be reported elsewhere.

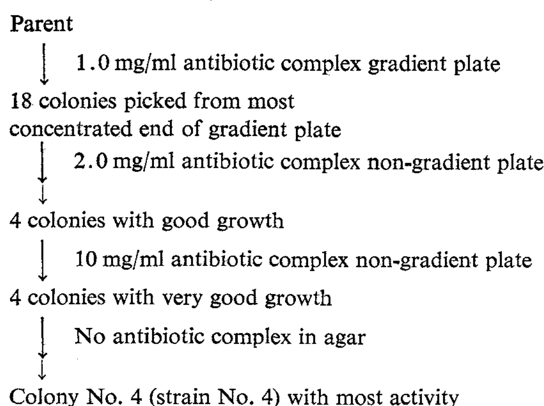
Fermentation

Fermentation studies were carried out in shake flasks and 14-liter fermentors. The inoculum for antibiotic production was prepared in a medium containing beef extract 0.3%, Tryptone 0.5%, yeast extract 0.5%, Cerelose 0.1%, potato starch 2.4% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.0 prior to sterilization. A 300-ml Erlenmeyer flask containing 70 ml of this medium was inoculated with 3 ml of a stock suspension of the producing strain which had been maintained at −20°C. The flask was incubated at 30°C on a rotary shaker at 300 rpm, for 48 hours. Twenty-five ml of the resulting seed culture was transferred to a 2-liter Erlenmeyer flask containing 500 ml of the above medium and incubated as above. The entire contents were used to inoculate a 14-liter fermentor containing 10 liters of the production medium consisting of yeast extract 0.5%, NZ-Amine 0.5%, Cerelose 1%, soluble starch 2%, $CaCO_3$ 0.4% and 1 mM $CoCl_2$ 10 ml. The pH of the medium was adjusted to 7 before the addition of $CaCO_3$.

The fermentation was carried out at 30°C with aeration of 3.5 liters/minute and agitation at 350 rpm. The antibiotic production was monitored at regular intervals by bioassay against *Staphylococcus aureus* 209P and *Escherichia coli* A-10536. The results from the 14-liter fermentation indicated poor production and a long fermentation time (96~120 hours). To obtain a better producing isolate, strain improvement was performed using an antibiotic gradient with Sch 33256 in agar as a selective pressure as described in Fig. 1.

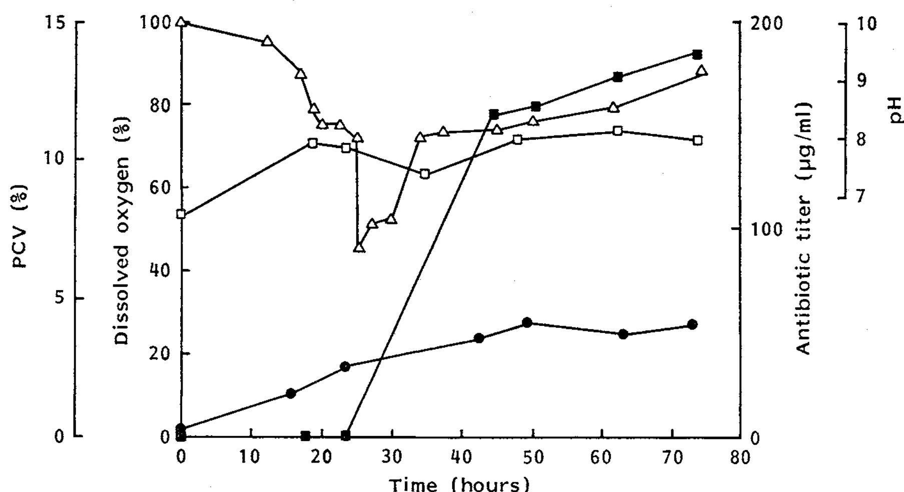
The parent culture was plated on agar containing a gradient of 0.0 to 1.0 mg/ml of the antibiotic complex. Eighteen colonies were picked from the 1.0 mg/ml end of the plate. These colonies were

Fig. 1. Strain selection.



Vessel	Strain	Sch 33256 (μg/ml)	Improvement (%)
250-ml flask	Parent	92	
	Strain No. 4	264	286
14-liter fermentor	Parent	124	
	Strain No. 4	184	148

Fig. 2. Fermentation profile of strain No. 4.
● Packed cell volume (PCV), △ dissolved oxygen, ■ antibiotic titer, □ pH.



replated on a plate containing 2.0 mg/ml of antibiotic. Four colonies with good growth were picked and replated on a 10-mg/ml plate. All four colonies survived, and from these colonies one showed the most antibiotic production, as shown in Fig. 1. Antibiotic titers were measured by bioassay against *S. aureus* 209P using chlortetracycline as a standard. Strain No. 4 showed a 286%-increase in antibiotic production in flasks and a 148%-increase in 14-liter tanks. The time course study for a typical 14-liter fermentation of strain No. 4 is shown in Fig. 2. As shown, peak antibiotic titer levels were achieved in 60~70 hours as compared to 96~120 hours for the parent. The pH, dissolved oxygen, growth profile of the organism and antibiotic production were monitored.

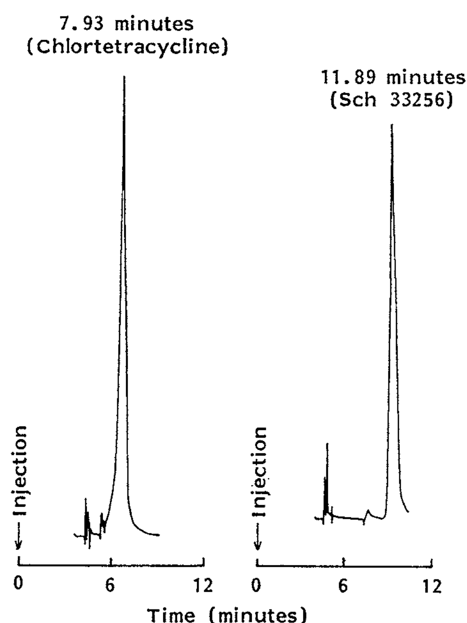
Isolation

The whole broth (10 liters) was adjusted to pH 2 and filtered. The filtrate was adjusted to pH 8.5 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 precipitated with a mixture of ether - hexane (1 : 4). The resulting yellow antibiotic complex was treated with EDTA in water at pH 1.5 and extracted with methylene chloride at pH 6.5. Final purification of Sch 33256 was achieved by Sephadex G-25 column chromatography using 0.02 N HCl as the eluting solvent[2]. The fractions were monitored by HPLC. The desired fractions were pooled and lyophilized. The 10 liters fermentation yielded 150 mg of pure Sch 33256.

Purity of Sch 33256 was determined by HPLC[3,4] using chlortetracycline as a standard shown in Fig. 3. The purity of Sch 33256 was comparable to a USP chlortetracycline standard.

Fig. 3. HPLC comparison of Sch 33256 with chlortetracycline.

Column: Chromegabond C-2 5 μm (ES Ind.) 4.6 mm × 15 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.



Physico-chemical Properties

Sch 33256 was differentiated from most other known antibiotic by paper and TLC. After development of TLC plates with a chloroform - methanol - water mixture (2 : 2 : 1, lower phase) Sch 33256 gave a bright yellow fluorescence, enhanced by ammonia vapors indicative of the tetracycline family of antibiotics.

Physico-chemical data of Sch 33256 are listed in Table 1. Sch 33256 is a yellow crystal. 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

Table 1. Physico-chemical properties of Sch 33256.

MP (°C, dec)	180~185
UV λ_{max}^{MeOH} nm (ε)	234 (17,400), 280 (15,700), 378 (19,500)
IR (KBr) cm^{-1}	3420 (br), 1660, 1608, 1574, 1432, 1414, 1380, 1240, 1210
CI-MS (NH_3) $(M+1)^+$ (m/z)	523
HREI-MS (m/z)	Found: 522.1414
	Calcd for $C_{24}H_{27}N_2O_9Cl$: 522.1405
EI-MS (m/z)	522, 479, 429, 393, 252, 227, 184
^1H NMR ($CD_3OD+(CD_3)_2CO$) δ	1.26 (CH_3), 3.09 (NCH_3), 3.22 ($N(CH_3)_2$), 4.02 (OCH_3), 6.62 (=CH), 9.72 (NH)
$[\alpha]_D^{26}$	−105.5° (c 0.5, MeOH)
$[\theta]_\lambda \times 10^{-4}$	$[\theta]_{259}=-9.7$, $[\theta]_{297}=+7.1$, $[\theta]_{328}=-3.0$

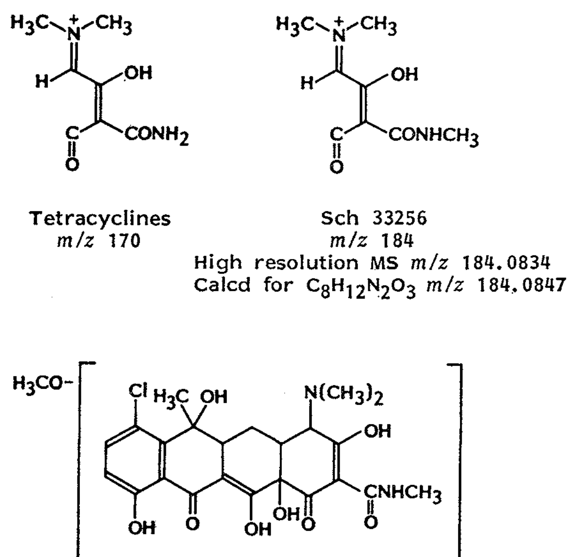
CI-MS: Chemical ionization mass spectrum.

Table 2. ^{13}C NMR spectral data of Sch 33256 and chlortetracycline.

Position	^{13}C NMR ($(CD_3)_2SO$)	
	Sch 33256	Chlortetra-cycline
C-1	193.1	193.4
C-2	96.5	95.6
CONHR	169.9 ($R=CH_3$)	172.1 (R=H)
C-3	186.3	187.3
C-4	68.0	68.1
$N(CH_3)_2$	41.5[a]	41.0[a]
C-4a	34.8	34.9
C-5	26.9	27.1
C-5a	42.3[a]	42.0[a]
C-6	73.3	70.4
CCH_3	20.4	25.0
C-6a	148.5	143.6
C-7	108.6	121.2
C-8	163.2	139.7
C-9	100.0	118.9
C-10	161.8	160.7
C-10a	111.6	117.0
C-11	190.6	193.4
C-11a	105.4	106.1
C-12	174.1	175.7
C-12a	73.6	73.2
OCH_3	56.9	—
NCH_3	26.5	—

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

Fig. 4. Mass spectral fragments and partial structure of Sch 33256.



amide at 1660 and 1608 cm^{-1}. The electron impact mass spectrum (EI-MS) gave an $(M+H)^+$ of m/z 523 and suggested the presence of chlorine. The high resolution (HR) EI-MS of Sch 33256 is in agreement with a molecular formula of $C_{24}H_{27}N_2O_9Cl$.

The ^1H NMR (200 MHz) of Sch 33256 was found to be similar to that of chlortetracycline except for the presence of two additional methyl groups at 3.09 and 4.02 ppm.

The assignment of all the carbons in the ^{13}C NMR spectrum (50.3 MHz) of Sch 33256 and chlortetracycline is shown in the Table 2[5]. The chemical shifts of most of the carbons are similar to chlortetracycline. The major differences are in the C-7, C-8, C-9 and the two methyl groups at 56.9 and 26.5 ppm.

Structure Elucidation

The physico-chemical characteristics of Sch 33256 are closely related to chlortetracycline except for the presence of NCH_3 and OCH_3 substitution. Methylation at the C-2′ position was confirmed by the fragmentation pattern of the right hand part of the molecule in the EI-MS[6] as shown in Fig. 4 and by the additional NCH_3 in the ^1H NMR (at 3.09 ppm) and ^{13}C NMR (at 26.5 ppm).

Based on ^1H and ^{13}C NMR, the additional methoxy substituent can only be located on the aromatic ring at the C-8 or C-9 position. The exact location was confirmed by the chemical shifts in the ^{13}C NMR of the C-7, C-8 and C-9 carbons when compared to chlortetracycline (Table 2). Further confirmation for a methoxy substituent at the C-8 position was based on the UV spectrum as compared to chlortetracycline in Fig. 5. Chlortetracycline has an absorbance at 355 nm and Sch 33256 has an absorbance at 378 nm, which is consistent with the methoxy substituent at the C-8 position.

Fig. 5. UV spectra of Sch 33256 and chlortetracycline.

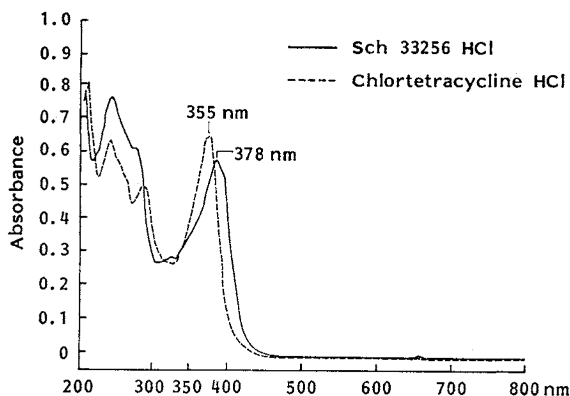
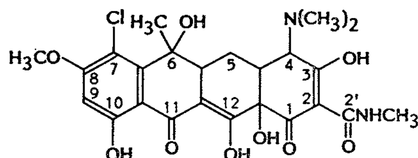


Fig. 6. Structure of Sch 33256.



2'-*N*-Methyl-8-methoxychlortetracycline
(2'-NCH3-8MCTC)

Table 3. *In vitro* activity of Sch 33256.

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

Based on the evidence presented the structure of Sch 33256 is shown in Fig. 6.

Biological Properties

The *in vitro* antibacterial activity of Sch 33256 is shown in Table 3. Evaluation of the *in vitro* data showed that Sch 33256 was primarily active against Gram-positive bacteria (MICs 0.125 to 2 μg/ml) with significantly less activity against Gram-negative organism (16~64 μg/ml). The lack of Gram-negative activity was consistent with the known structure activity relationship for 2'-*N*-methyl substituted tetracyclines[7].

In summary, we have reported a novel chlortetracycline from a new species of *Actinomadura*, differing from other known tetracyclines by 8-methoxy and 2'-*N*-metyl substitution. Extensive structural modification has been made on tetracyclines including the carboxamido moiety and the C-9 position but to date no tetracycline analogs have been reported with a substituent at the C-8 position[8,9].

References

1) Patel, M.; V. Gullo, V. Hegde, A. Horan, F. Gentile, J. A. Marquez, G. Miller, M. Puar & J. A. Waitz: A novel tetracycline produced by *Actinomadura brunnea*. Program and Abstracts of the 25th Intersci. Conf. on Antimicrob. Agents Chemother., No. 231, 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 tetra-

cycline hydrochloride and related antibiotics. J. Am. Chem. Soc. 97: 6248, 1975
7) HOFFMAN, D. R.: The mass spectra of tetracyclines. J. Org. Chem. 31: 792~796, 1966
8) VALCARI, U.: Tetracyclines, chemical aspects and some structure activity relationship. Symp. Giovanni Lorenzini Found. Vol. 10. *In* New Trends in Antibiotics: Research and Therapy. *Eds.*, G. G. GRASSI & L. D. SABATH, pp. 3~25, Elsevier/North-Holland Biomedical Press, Milano, 1981
9) MITSCHER, L. A.: The chemistry of tetracycline antibiotics. *In* Medicinal Research Series. Vol. 9. *Ed.*, G. L. GRUNEWALD, pp. 95~99, Marcel Dekker, New York, 1978