DIRECT SELECTION OF A SPECIFICALLY BLOCKED MUTANT OF *ACTINOMADURA BRUNNEA*

ISOLATION OF A THIRD 8-METHOXY SUBSTITUTED CHLORTETRACYCLINE

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Actinomadura brunnea produces 2′-*N*-methyl-8-methoxychlortetracycline. A derivative of this strain has been isolated that is specifically blocked in methylation of the 2′-amino position. This isolate was detected by screening approximately 30,000 colonies of a mutagenized population of *Actinomadura brunnea* using a direct soft agar overlay with an *Escherichia coli* indicator. The antibiotic produced by the blocked mutant has been identified as 8-methoxychlortetracycline (Sch 36969) based upon its biological activity, relative mobility on TLC and HPLC, and spectroscopic data.

The two preceding papers[1,2] have described the isolation and characterization of two novel tetracycline antibiotics: 2′-*N*-Methyl-8-methoxychlortetracycline (2′-NCH_3-8MCTC, Sch 33256) and 4a-hydroxy-8-methoxychlortetracycline (4a-OH-8MCTC, Sch 34164). The former compound has relatively weak activity against Gram-negative organisms which is expected as a result of methylation of the 2′-NH_2 position[3]. In order to improve the antimicrobial spectrum of this compound and to obtain a third 8-methoxy substituted chlortetracycline, we have isolated a mutant of *Actinomadura brunnea* that is specifically blocked in methylation of the 2′-amino position. The overlay method used to identify this mutant directly, as well as the characterization of the antibiotic produced (8-methoxychlortetracycline, 8MCTC, Sch 36969) are the subjects of this report.

Materials and Methods

Media

MFM9-1 Contains (per liter): Beef extract 3 g, Tryptone 5 g, yeast extract 5 g, glucose 1 g, soluble starch 24 g, calcium carbonate 2 g and Dow Corning Antifoam B 1 ml. The medium is adjusted to pH 7.5 prior to sterilization.

MFM11 Contains (per liter): Yeast extract 5 g, NZ-Amine-A 5 g, glucose 10 g, soluble starch 20 g, calcium carbonate 4 g, 1 mM $CoCl_2$ 1 ml and antifoam 1 ml.

TM26 Contains (per liter): Soluble starch 10 g, yeast extract 5 g, agar 15 g and antifoam 2 ml. The pH is adjusted to 7.1 prior to autoclaving.

SSS Medium Contains (per liter): Peptone 4 g, soluble starch (Difco) 20 g, yeast extract 4 g, glucose 10 g and $MgSO_4 \cdot 7H_2O$ 0.5 g. The pH is adjusted to 7.5 prior to autoclaving. After sterilization, TES buffer at pH 7.2 is added to a final concentration of 25 mM.

Mutagenesis

A frozen whole broth of a spontaneous high level streptomycin-resistant mutant of *A. brunnea*

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ATCC 53108, was used to inoculate (5%) 10 ml of SSS medium in 25 mm tubes containing glass rods (5 mm by 75 mm). The cultures were grown for 27 hours at 30°C at 300 rpm (New Brunswick G-52 shaker). A 25-ml aliquot of the pooled cultures was fragmented for 1 minute (on ice) at 75 watts using a Branson Model 350 sonicator and then transferred to a 250-ml Erlenmeyer flask containing 35 ml of SSS medium (total volume=60 ml). The culture was incubated at 30°C in an Aquatherm water bath shaker at 260 rpm throughout mutagenesis sampling periods. After 15 minutes incubation, a 10-ml sample was taken for the unexposed population control, then *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to final concentration of 500 μg/ml. Ten ml samples were taken at 90, 120, 150, 180 and 210 minutes. Each individual sample was treated as follows: Cells were collected by centrifugation (Beckman J2-21 at 5,000 rpm for 10 minutes) and the pellet was resuspended in 10 ml of SSS medium and incubated at 30°C, 300 rpm for 16 hours. They were then sonicated for 20 seconds at 75 watts and frozen at −20°C. A pre-determined viable count (cfu) was used to determine the appropriate dilution that would yield approximately fifty colonies on each 100 mm diameter TM26 plate for the overlay step.

Overlay Procedure

The mutagenized population used (210 minutes NTG exposure) was plated onto TM26 and the colonies were allowed to grow until they reached a diameter of 2 to 3 mm. They were then directly overlaid with 10 ml of Difco nutrient agar (containing 80 μg/ml of 2,3,5-triphenyltetrazolium chloride) which had been inoculated with a fresh, saturated culture of *Escherichia coli* OLA290R5 (1 ml per 125 ml agar). The overlaid plates were incubated at 35°C overnight. *A. brunnea* colonies that were surrounded by a clear zone of inhibition were then transferred to MFM9-1 agar plates containing 100 μg/ml of streptomycin.

Fermentation

Cultures were grown either in 25 mm test tubes with 10 ml of medium or in 2-liter flasks with 500 ml of medium at 30°C and 300 rpm (New Brunswick G-52 shaker). Ten ml MFM9-1 inocula were seeded with 0.5 ml of a frozen whole broth and grown for 2 days. The inocula were then transferred (5%) to MFM11 fermentation medium and grown for 4 days at 30°C, 300 rpm.

Isolation

Fermentation whole broths were adjusted to pH 2 with 6 M sulfuric acid and the mycelia removed by centrifugation (Beckman J2-21 centrifuge, 5,000 rpm for 30 minutes). This acidified supernatant was used directly for some analyses. The active components were extracted from the acidified supernatant with an equal volume of water - saturated butanol. The solvent was removed by evaporation and the residue resuspended in water - methanol (1 : 1) for HPLC analysis or chloroform - methanol (1 : 1) for TLC analysis.

TLC

TLC analysis was performed on silica gel plates (Whatman LK6DF) which were developed in chloroform - methanol - 0.2 M acetate buffer (pH 3.5) (2 : 2 : 1, lower phase) at room temp until the solvent front was within 1 cm of the top of the plate.

Bioautography

Dried TLC plates were placed face down on neomycin assay agar (pH 8.0) seeded with either *Staphylococcus aureus* 209P as the representative Gram-positive organism or *E. coli* OLA290R5 as the Gram-negative indicator strain. After 30 minutes the TLC plate was removed and the agar plate incubated at 35°C overnight.

HPLC

The aqueous samples were filtered through a 0.2-μm membrane filter and were then applied to a Waters μBondapak C-18 column (3.9 mm×30 cm). The mobile phase consisted of a 15-minute linear gradient from 100% buffer A (methanol - water - 0.2 M phosphate buffer (pH 2.5) in the ratio of 30 : 60 : 10) to 100% buffer B (methanol - acetonitrile - water - 0.2 M phosphate buffer (pH 2.5) in the ratio of 50 : 20 : 20 : 10) and run at a rate of 1 ml/minute.

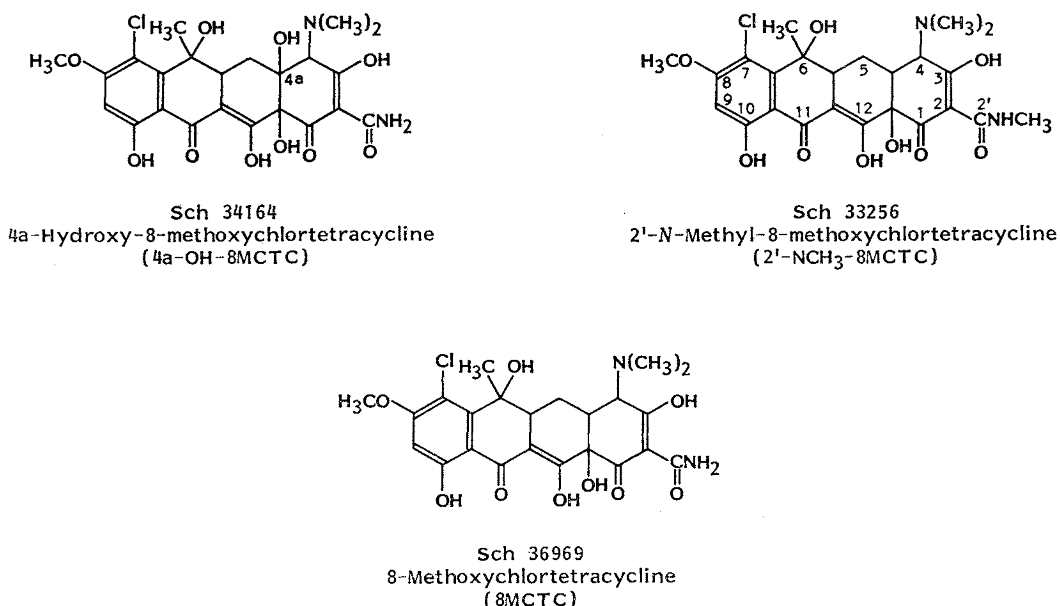
Results and Discussion

In order to obtain the desired antibiotic structure (8-methoxychlortetracycline) we assumed that a blocked mutant of *A. brunnea,* unable to methylate the 2′-amino position of 2′-*N*-methyl-8-methoxychlortetracycline (Sch 33256, Fig. 1), could be isolated and would produce the desired compound. The basis for detecting this specific mutant is the observation that loss of the methyl group on the 2′-amino moiety should result in a marked increase in activity against Gram-negative organisms[3]. Because this specific mutation would be a rare event, the screening procedure would have to be as efficient as possible. The scheme that we devised is outlined in Fig. 2.

The indicator strain chosen from our in-house strain collection, *E. coli* OLA290R5 had an MIC of 2 μg/ml for chlortetracycline as compared to an MIC of 16~32 μg/ml for 2′-*N*-methyl-8-methoxychlortetracycline (2′-NCH_3-8MCTC). A spontaneous streptomycin-resistant derivative of *A. brunnea* (ATCC 53108) was mutagenized, allowed to grow out overnight, diluted and plated so that each plate would receive approximately 50 cfu. When this population had grown sufficiently, all 600 plates were directly overlaid with the *E. coli* OLA290R5 indicator strain. Of the nominally 30,000 *A. brunnea* cfu plated, two discrete colonies were surrounded by sharp, clear zones of growth inhibition of the *E. coli* indicator. The *A. brunnea* mutants were picked directly onto MFM9-1 agar plates containing 100 μg/ml of streptomycin to purify the putative block mutants and kill any of the residual streptomycin-sensitive *E. coli* OLA290R5.

The antibiotic elaborated by these mutants was analyzed by TLC followed by bioautography against Gram-positive (Fig. 3A) and Gram-negative (Fig. 3B) organisms. Despite the resolution of this system, it appears that the antibiotic activity produced by the putative blocked mutant, has a mobility greater than that of 4a-hydroxy-8-methoxychlortetracycline (4a-OH-8MCTC) but less than that of the parental compound, 2′-NCH_3-8MCTC. Evidence for this difference is seen more clearly when the bioautography against the *E. coli* indicator is examined. Here the putative blocked mutant

Fig. 1. Structures of tetracyclines.



exhibits Gram-negative activity that is not only absent from the parental 2′-NCH_3-8MCTC but it also has a different mobility compared to a 4a-OH-8MCTC standard.

The antibiotic produced by the putative blocked mutant was isolated by extraction of acidified fermentation supernatants with butanol and analyzed by HPLC[4]. This material exhibited a retention time of 11.8 minutes which is intermediate between that of chlortetracycline (9.2 minutes) and 2′-NCH_3-8MCTC (14.0 minutes) (Fig. 4) and is consistent with the absence of 2′-*N*-methylation in the *A. brunnea* mutant isolated.

An improved producer was derived from the blocked mutant by selecting for resistance to tetracycline (100 μg/ml in MFM9-1 plates). As

Fig. 2. Flow chart outlining selection procedure for the blocked mutant and improved-producing isolate (Tet 7).

Isolation of a mutant blocked in 2′-*N*-methylation.

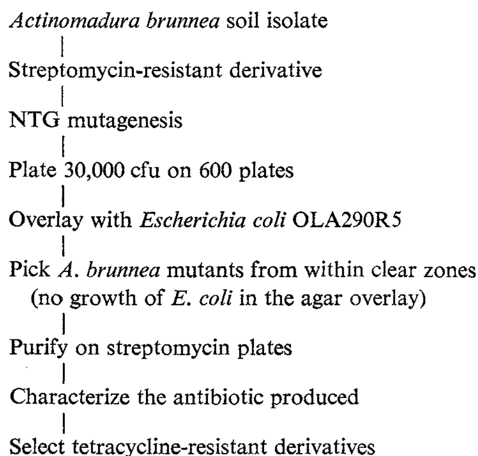


Fig. 3. Bioautography analysis of fermentation products vs. *Staphylococcus aureus* 209P (Panel A) and *Escherichia coli* OLA290R5 (Panel B).

Lane 1; blocked mutant, lane 2; streptomycin-resistant isolate, lane 3; soil isolate, lane 4; 2′-NCH_3-8MCTC standard, lane 5; 4a-OH-8MCTC standard.

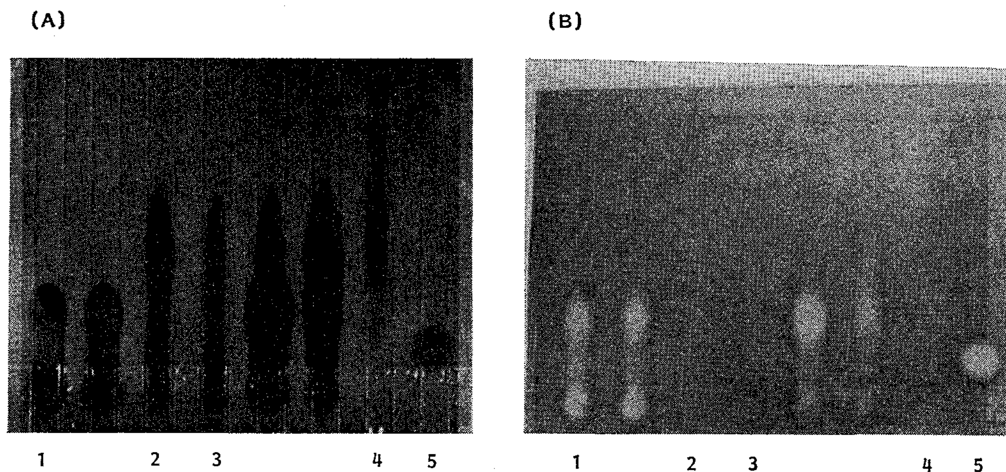


Table 1. Physico-chemical properties of 8-methoxychlortetracycline (Sch 36969).

UV λ_{max}^{MeOH} nm (ε)	204 (19,140), 235 (17,250), 250 (16,440), 274 (13,140), 373 (19,115)
IR (KBr) cm^{-1}	3530, 1650, 1610, 1580, 1380, 1240
FAB-high resolution MS $(M+H)^+$ (m/z)	Measured: 509.1284 Calcd for $C_{23}H_{26}N_2O_9Cl$: 509.1324
^1H NMR ($(CD_3)_2SO$) δ	1.16 (CH_3), 1.50 (CH), 2.25 (CH), 2.84 ($N(CH_3)_2$), 2.95 (CH), 3.2 (CHN), 3.94 (OCH_3), 6.7 (=CH), 5.4, 7.0, 9.10, 9.5 (exchangeable NH_2 and OH protons)
$[\alpha]_D^{26}$	−116.4 (c 0.5, MeOH)

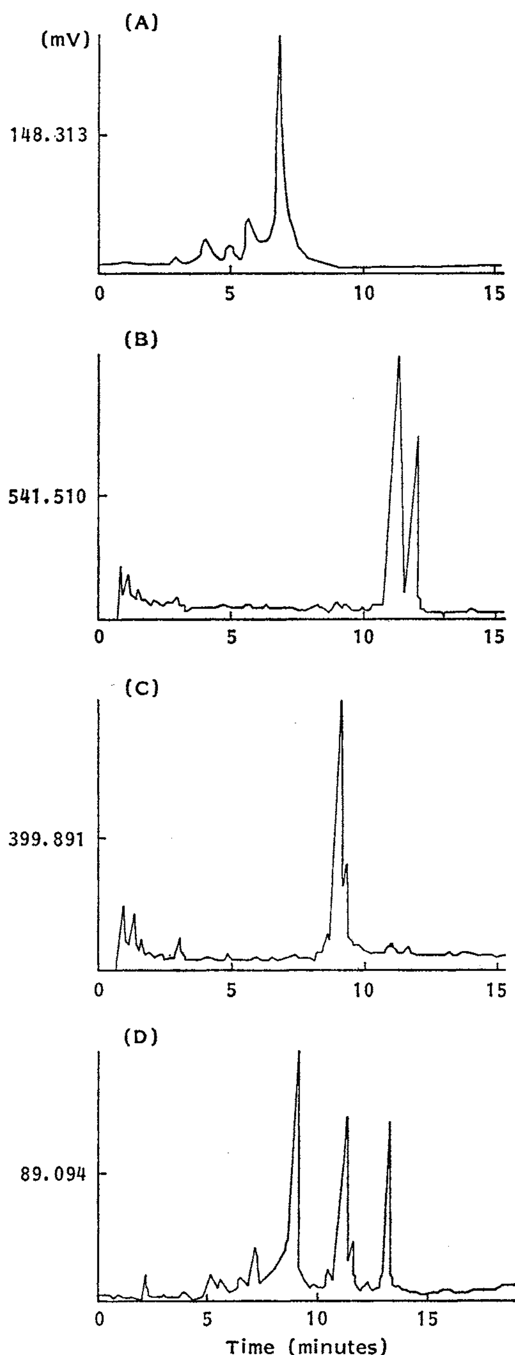


Fig. 4. HPLC analysis of fermentation products.

Panel A; chlortetracycline standard, panel B; crude preparation from soil isolate, panel C; crude preparation from blocked mutant, panel D; coinjection of chlortetracycline standard and purified preparations from the soil isolate and blocked mutant.

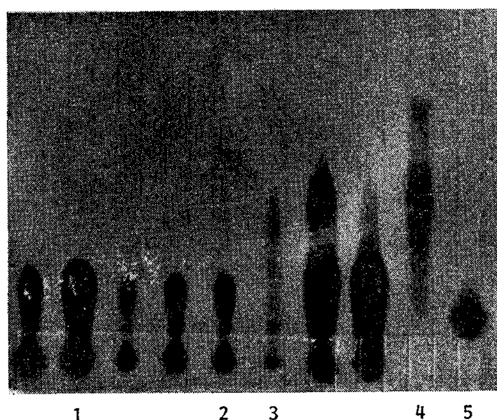


Fig. 5. Bioautography analysis of fermentation products vs. *Staphylococcus aureus* 209P.

Lane 1; tetracycline-resistant improved isolate (Tet 7), lane 2; blocked mutant, lane 3; streptomycin-resistant isolate, lane 4; 2′-NCH_3-8MCTC standard, lane 5; 4a-OH-8MCTC standard.

Table 2. ^{13}C NMR spectral data of Sch 36969 and Sch 33256.

Position	^{13}C NMR ($(CD_3)_2SO$)	
	Sch 36969	Sch 33256
C-1	194.0	193.1
C-2	95.3	96.5
CONHR	171.9(R=H)	169.9(R=CH_3)
C-3	187.3	186.3
C-4	68.3	68.0
$N(CH_3)_2$	41.5[a]	41.5[a]
C-4a	34.4	34.8
C-5	27.0	26.9
C-5a	42.4[a]	42.3[a]
C-6	73.4	73.3
CCH_3	20.5	20.4
C-6a	148.5	148.5
C-7	108.7	108.6
C-8	163.3	163.2
C-9	100.1	100.0
C-10	161.9	161.8
C-10a	111.6	111.6
C-11	190.6	190.6
C-11a	105.4	105.4
C-12	174.3	174.1
C-12a	73.6	73.6
OCH_3	56.9	56.9
NCH_3	—	26.5

[a] Indicates peaks under DMSO peak, observed when spectrum was run in D_2O - dioxane.

expected from previous work[1] a significant increase in titer was observed (Fig. 5). The tetracycline-resistant derivative (designated Tet 7) was fermented and the antibiotic produced was purified by HPLC. This material exhibited a fast atom bombardment mass spectrum (FAB-MS) molecular ion $(M+H)^+$ of *m/z* 509 which is 14 mass units less than that of 2′-NCH_3-8MCTC, consistent with the loss of a methyl group.

The biological activity of this antibiotic, combined with its relative mobility on HPLC and TLC and its FAB-MS molecular weight, all indicate that it is 8-methoxychlortetracycline (8MCTC) and, therefore, the mutant isolated is specifically blocked in 2′-*N*-methylation.

These conclusions were confirmed by further spectroscopic analyses. The physico-chemical properties of 8MCTC are closely related to 2′-NCH_3-8MCTC and chlortetracycline, as shown in Table 1. The absence of *N*-methylation at the C-2′ position was revealed by the fragmentation pattern of the right-hand part of the molecule in the electron impact mass spectrum (EI-MS)[5]. Further evidence was provided by NMR studies. The ^1H NMR showed the absence of NCH_3 peak at δ 3.12 as observed in 2′-NCH_3-8MCTC. The ^{13}C NMR spectrum is identical to 2′-NCH_3-8MCTC except for the absence of a peak due to a methyl carbon at 26.5 ppm as shown in Table 2. Therefore, based on the spectral data, the structure is 8MCTC as shown in Fig. 1.

We have used an overlay procedure, that was both rapid and accurate to find a mutant of *A. brunnea* blocked in a specific antibiotic biosynthetic step. This was facilitated by the availability of a suitable indicator strain that essentially would yield an all-or-none response. This overlay technique has also been used with a high level producer of *A. brunnea* that elaborated a mixture of 2′-NCH_3-8MCTC and 8MCTC. The overlay screen allowed the isolation of a mutant in which the ratio of 2′-NCH_3-8MCTC to 8MCTC had been shifted from approximately 80:20 to 60:40. Therefore, an overlay procedure like the one described here should permit the efficient screening of large populations of mutagenized organisms in situations where a specific activity is desired regardless of whether the organism is producing a single compound or a mixture of components.

The biological activity of the three 8MCTCs described in this and the two preceding papers[1,2] is presented in the accompanying manuscript[6].

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