CHEMICAL PROPERTIES OF *MYXOCOCCUS XANTHUS* ANTIBIOTIC TA

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Antibiotic TA was purified and crystallized from culture fluids of *Myxococcus xanthus* TA. The antibiotic ($C_{34}H_{57}O_9N$, M.W. 623.8) contained the following functional groups: ketone, lactone, secondary amide, methoxy-substituted diene (λ_{max} 239 nm), primary alcohol and three secondary alcohols, two of which were *cis*-vicinal. Mild alkaline hydrolysis opened the lactone with concomitant loss of antibiotic activity. Periodate oxidation also destroyed biological activity.

The myxobacterium *Myxococcus xanthus* strain TA (ATCC 31046) produces a wide-spectrum antibiotic when grown under nutritionally limiting conditions.[1,2] The antibiotic, referred to as TA, is bactericidal to growing cultures only. Recently, it was demonstrated that antibiotic TA inhibits incorporation of diaminopimelic acid and uridine diphosphate-*N*-acetylglucosamine into *Escherichia coli* cell walls without altering the ratio of cross-linked to uncross-linked peptidoglycan.[3] Formation of the lipid intermediate was not blocked by TA, demonstrating that TA interferes with polymerization of the lipid-disaccharide-pentapeptide.

We report here the purification and crystallization of the antibiotic, its sensitivity to acid and alkaline hydrolysis, periodate oxidation and physicochemical properties.

Analytical Methods

Antibiotic TA activity was determined by the paper disc assay method,[4] using the sensitive *Escherichia coli* ESS[5] as the test organism. Varying quantities of the antibiotic in chloroform or ethanol were applied to 5.5 mm diameter discs of Whatman 3 MM filter paper and dried. The discs were then placed on nutrient agar plates which had been overlayed with soft agar (0.7%) containing nutrient broth and 10^8 *E. coli* ESS. The diameter of the zone of inhibition was recorded after 18 hours at 37°C. The units of antibiotic activity were determined from a standard curve of zone of inhibition as a function of antibiotic concentration. One unit of antibiotic yields a diameter of inhibition of 6.5 mm (excluding the 5.5 mm disc). One unit corresponds to 0.012 μg of crystalline antibiotic (83,333 units/mg).

The ultraviolet spectra were observed with a Gilford model 240 spectrophotometer, 1-cm light path, using optical grade methanol and cyclohexane as solvents. The infrared spectrum was obtained with a Perkin-Elmer model 177 spectrophotometer. ^{1}H NMR spectra were measured at 270 MHz in $CDCl_3$ on a Bruker WH-270 spectrometer, using tetramethylsilane as internal reference. ^{13}C NMR spectra were measured at 22.63 MHz in $CDCl_3$ on a Bruker WH-90 spectrometer. Melting points were taken on an electrothermal melting point apparatus. Analytical column chromatography of the purified antibiotic was carried out on Bio-Gel P-2 (exclusion limit 2,600) on a column of 76×0.9 cm (diameter), using 25% ethanol in 0.01 M phosphate buffer, pH 7.0, for development.

TLC was performed on silica gel plates, F-254 type 60 (Merck), using acetone - benzene (1 : 1) for development. Periodate oxidation was carried out in 50% ethanol; periodate consumption was followed spectrophotometrically.[6]

Fermentation and Isolation

A late exponential phase culture of *Myxococcus xanthus* strain TA (1.5 liters) was inoculated into 500 liters of media containing 5 kg Casitone (Difco), 500 g serine, 500 g alanine and 1 kg $MgSO_4 \cdot 7H_2O$. The fermentation was carried out for 64 hours at 30°C with 10 liters/minute air and 150 rpm (no baffles) as previously described.[7] After centrifugation at 4°C to remove the cells, the clarified culture supernatant fluid was mixed with 5 kg Florisil to adsorb the antibiotic. After collecting the Florisil by centrifugation, the antibiotic was obtained from it by exhaustive extraction with acetone. The solvent was evaporated *in vacuo* leaving about 1 liter of water, which was extracted four times with 1 liter of chloroform - methanol (95: 5). The combined chloroform - methanol phases were evaporated to dryness, yielding 15.8 g dry material containing 5×10^7 units of antibiotic activity.

The dried material was dissolved in $CHCl_3$ and applied to a silica column (prepared from 700 g silicic acid in 2 liters $CHCl_3$). The column dimensions were 5 cm diameter and 72.5 cm height. After washing the column with 1.5 liters of $CHCl_3$, the activity was eluted with 2.5~3.5 liters chloroform - methanol (96: 4). The yield of column-purified antibiotic was 3.6×10^7 units with a specific activity of 1.2×10^4 units per mg. The concentrated antibiotic was then placed on prewashed TLC plates (20 × 20 cm, silica gel, 60 F-254), approximately 100 mg per plate, and developed with acetone - benzene (1: 1, v/v). The antibiotic was located on the TLC plates by its UV absorption, scratched from the plate and extracted with acetone. Acetone was evaporated and the dried antibiotic dissolved in 4 ml of ethanol. After standing overnight at 0°C, crystals, which appeared, were removed by filtration. Since these crystals contained less than 0.5% of the antibiotic activity, they were discarded. To the ethanol supernatant was added 16 ml of water and the turbid solution placed at 4°C. The white needles that formed were recrystallized three times from 20% ethanol in water and the collected on a sintered glass funnel. On drying under desiccation the needles tended to lose their crystallinity and form a gum. The final yield of antibiotic was 206 mg and 2×10^7 units. The purification scheme is summarized in Fig. 1.

Fig. 1. Isolation of TA from *Myxococcus xanthus* culture broth.

Culture broth (5.5×10^7 units, 490 liters)
↓ adsorption onto 5 kg Florisil, extraction into acetone
Acetone extract (10 liters)
↓ vacuum-concentrated and extracted with equal volume of chloroform - methanol (95: 5), evaporated dryness and residue dissolved in chloroform
Chloroform extract (5×10^7 units, 15.8 g)
↓ column chromatography on silica gel using chloroform - methanol (96: 4), active fractions combined and dried under vacuum.
Crude antibiotic TA (3.6×10^7 units, 3 g)
↓ preparative TLC on silica gel using benzene - acetone (1: 1), active band extracted with acetone and crystallized three times from 20% ethanol in water at 4°C.
Crystalline antibiotic TA (2×10^7 units, 206 mg).

Results

Freshly prepared, dried, crystalline antibiotic TA melted at 79°C (uncorrected). On exposure to air and light, the compound slowly decomposed to a biologically inactive material with concomitant loss of ultraviolet absorbance. Thus, the antibiotic was stored in a desiccator in the dark. IR: ν_{max}^{KBr} 3430 (OH); 2960, 2935, 2875 (CH); 1740, 1710, 1665 (3×CO); 1535, 1460, 1410, 1375, 1365, 1220, 1145, 1110, 970, 915, 730 cm^{-1}.

Analytical column chromatography on Bio-Gel P-2 yielded a single peak with a molecular weight

of 750±200. Elementary analysis gave C 64.82, H 9.05, N 2.21% and direct oxygen 23.39%, corresponding to an empirical formula of $C_{34.2}H_{56.8}O_{9.2}N_{1.0}$. From the molecular weight estimate (P-2 column), ^{13}C NMR data (34 carbon atoms) and elementary analysis, the molecular formula is $C_{34}H_{57}O_9N$, M.W. 623.8.

Mass Spectroscopy

The molecular formula of TA was confirmed directly by mass spectra (EI, 70 eV, 250°C) *m/z* (%): 623 (M^+, 7%), 621 (M−2H, 7), 605 (M−H_2O, 10), 603 (621−H_2O, 7), 591 (M−MeOH, 35), 589 (621−MeOH, 13), 573 (M−MeOH−H_2O, 53), 555 (M−MeOH−$2H_2O$, 30), 544 (M−79, 13), 537 (M−MeOH−$3H_2O$, 16), 512 (544−MeOH, 10) and 95.086 (C_7H_{11}, 100).

NMR Spectroscopy

The ^1H NMR data for antibiotic TA are shown in Table 1. Four methyl and one methoxy groups were indicated: 2 CH_2–CH_3 (0.830 and 0.923 ppm), –CHCH_3 (0.864 ppm), CH_3–CH–CO– (1.154 ppm), and C=C–OCH_3 (3.37 ppm). Three secondary hydroxyl (–C*H*OH, 3.56, 3.83 and 3.99 ppm) and one primary hydroxyl (–C–CH_2OH, 3.88 and 4.20 ppm) groups were located. Subsequently, it was shown (see periodate oxidation) that two of the secondary hydroxyl groups must be *cis*-vicinal. Thus, all of the oxygens and nitrogen in TA can be accounted for by the four hydroxyl groups, the *sec* amide, lactone, methoxy and ketone functions.

Fig. 2 suggests several groupings. Fragments **A** and **C** are proposed on the basis of double irradiation experiments (Table 2). The two methylene groups adjacent to the secondary alcohol shown in fragment B are suggested as a result of the quintet observed at 3.88 ppm. Fragment **D** is proposed because the single proton on the carbon atom adjacent to the lactone (5.21 ppm) was split by two protons.

The ^{13}C NMR spectrum indicated a total of 34 carbon atoms (22.63 MHz, $CDCl_3$) δ: 212.5s, 176.2s, 171.2s, 139.5d, 134.8s, 129.7d, 125.9d, 73.6d, 73.6d, 71.4d, 70.8t, 68.2d, 58.0q, 45.4, 45.1, 43.1, 42.4, 40.7, 37.2, 36.5, 35.9, 34.6, 33.9, 30.35, 30.35, 28.3, 26.4, 23.8, 22.2, 19.6, 18.2, 17.3, 13.7 and 11.8.

Table 1. ^1H NMR data for antibiotic TA in $CDCl_3$.

Shift (ppm)*	Multiplicity	No. of protons
0.830	t (J=7 Hz)	3
0.864	d (J=7 Hz)	3
0.923	t (J=7.3 Hz)	3
1.154	d (J=7 Hz)	3
1.20~2.0		23 (approx.)
2.29 and 2.36	m	5 (together)
2.59	quar (J=7 Hz)	1
3.22	m	1
3.37	s	3
3.56	m	3
3.88	quin (J=4 Hz)	1
3.88	d (J=11 Hz)	1
3.99	m	1
4.20	d (J=11 Hz)	1
5.21	dd (J=7.5 & 5 Hz)	1
5.36	dd (J=14 & 10 Hz)	1
6.05	d (J=11 Hz)	1
6.23	dd (J=14 & 11 Hz)	1
6.61	t (J=5 Hz)	1 (exchangeable)

* The position of the exchangeable alcoholic protons are not presented because they varied with temperature and concentration.

Fig. 2. Suggested chemical groupings according to ^1H NMR experiments.

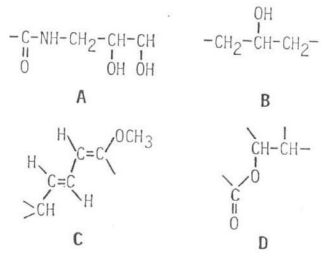


Table 2. ^{1}H NMR double irradiation experiments.

	Irradiated proton	Changed proton
Fragment **A**	6.61 t, J=5 Hz	3.58 m→dd, J=14, 4 Hz
		3.22 m→dd, J=14, 6.5 Hz
	3.99 m	3.58 m→dd, J=14, 5 Hz
		3.22 m→dd, J=14, 5 Hz
		3.54 m→dd
	3.58 m	3.22 m→dd, J=6.5, 5 Hz
		3.99 m→br s
		6.61 t→br d, J=5 Hz
	3.22 m	3.99 m→br s
		3.58 m →dd, J=4, 5 Hz
		6.61 t→br s
Fragment **C**	6.23 dd J=14, 11 Hz	6.05 d→s
		5.36 dd→d, J=10 Hz
	6.05 d J=11 Hz	6.23 dd→d, J=14 Hz
	5.36 dd J=14, 10 Hz	6.23 dd→d, J=11 Hz

UV Spectroscopy

Antibiotic TA showed an absorption maximum at 239 nm (ε_{max} 27,000) in methanol or cyclohexane. The fact there was no bathochromic shift with increasing solvent polarity indicates that the absorption was not due to an α,β-unsaturated ketone, but more likely a result of a substituted diene.[5] The relatively high extinction coefficient rules out homoannular (cisoid) dienes. The predicted absorption maximum of fragment **C**, Fig. 2, would be 217+(2×5)+6=233 nm.

Acid and Alkaline Hydrolyses

Antibiotic TA in 50% ethanol was hydrolyzed in 0.1 M KOH at 30°C (Table 3). The half-life of antibiotic activity was about 6 minutes under these conditions. By 30 minutes, over 95% of the activity was destroyed. Examination of the products formed by TLC revealed two new ultraviolet absorbing components; TA-OH-1 and TA-OH-2, with Rf of 0.16 and 0.05, respectively. Compound TA-OH-1 was extracted with $CHCl_3$, while TA-OH-2 was extracted by $CHCl_3$ only after acidification. Both compounds were prepared in larger amounts and purified on short columns of silicic acid eluted with chloroform - methanol (9: 1) for ^{1}H NMR examination. The data indicated that the lactone (Fig. 2, **D**) was cleaved yielding the free acid (TA-OH-2) and the ethyl ester (TA-OH-1). This was consistent with the subsequent finding that TA-OH-1 could be further hydrolyzed by alkali to TA-OH-2. UV-Absorption spectra for acid TA-OH-2 and ethyl ester TA-OH-1 resembled that of the parent compound. It should be added that no other hydrolysis products were observed on TLC after spraying with 10% H_2SO_4 in ethanol.

The antibiotic was considerably more stable to acid than alkali. In 0.1 M HCl at 30°C or 70°C for 2 hours, there was no detectable change in antibiotic activity, UV absorption or behavior on TLC. However, at 100°C in 0.1 M HCl, the antibiotic activity was destroyed with a half-life of about 15 minutes (Table 4). Chromatographic examination of the products formed by acid hydrolysis revealed UV-absorbing spots with R_{TA}=1.56 and 0.54 (weak). No additional products were seen following spraying with 10% H_2SO_4 in ethanol.

Table 3. Alkaline hydrolysis of antibiotic TA at 30°C*.

Time	Antibiotic activity (%)	Rf (TLC)	
		Extract 1 ($CHCl_3$)	Extract 2 ($CHCl_3$ - HCl)
0	100	0.32	0.32
5 minutes	89	0.32, 0.16	0.32, 0.16, 0.05
10 minutes	29	0.32, 0.16	0.32, 0.16, 0.05
30 minutes	1.4	0.16	0.16, 0.05
60 minutes	0	0.16 (trace)	0.16, 0.05
2~12 hours	0	—	0.16, 0.05
24 hours	0		0.05

* Antibiotic TA (0.2 mg in 0.1 ml ethanol) was added to a solution containing 0.4 ml ethanol and 0.5 ml freshly prepared 0.2 M KOH. At various times, 0.1 ml was removed and extracted first with $CHCl_3$ and water and then $CHCl_3$ - 0.01 M HCl. The chloroform extracts were assayed for antibiotic activity and chromatographed on TLC plates.

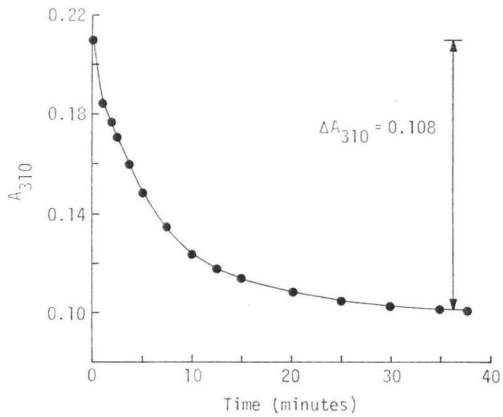
Table 4. Acid hydrolysis of antibiotic TA at 100°C*.

Time	Antibiotic activity (%)	R_{TA} (TLC)
0	100	1.00
1 minute	88	1.00
5 minutes	80	1.00, 1.56
15 minutes	49	1.00, 1.56
30 minutes	25	1.00, 1.56
45 minutes	13	1.00, 1.56
1 hour	6	1.56, 0.54
2 hours	3.3	1.56, 0.54

* The reaction was performed as described in Table 3 except that HCl was used in place of KOH.

Fig. 3. Periodate oxidation of antibiotic TA.

Antibiotic (1 mg) in 1 ml 50% ethanol was oxidized with 2.5 mM $NaIO_4$ in 0.2 M acetate buffer, pH 4.5, at room temperature. Absorbance at 310 nm due to antibiotic TA was subtracted from all values.



Periodate Oxidation

Kinetics of the periodate oxidation of antibiotic TA were examined by measuring the reduction of IO_4^- (to IO_3^-) at 310 nm (Fig. 3). Since 2.5 μmoles sodium periodate (time zero) had an A_{310} of 0.210, the observed decrease in absorbance of 0.108 corresponded to a reduction of 1.29 μmoles periodate per mg antibiotic. Considering the molecular weight of TA is 623.8 (from mass spectra), 0.8 moles periodate were consumed per mole antibiotic. Thus, the data suggest that TA contains a single 1,2-diol (Fig. 2, A). The rapid rate of oxidation of TA (50% completion in 3 minutes) indicates that the diol has a *cis*-conformation[6]. Periodate oxidation of antibiotic TA caused a complete loss of biological activity.

The products of periodate oxidation of TA were examined by extracting the final reaction mixture with chloroform, concentrating the chloroform phase and applying it to TLC plates. After development in acetone - benzene (1: 1), three new UV-absorbing components were observed, (Rf 0.58, 0.69 and 0.72); by comparison the pure antibiotic had an Rf of 0.40. Each of the products was eluted from the TLC plate and its UV-spectrum determined in methanol. Each of the new components had a spectrum (λ_{max}^{MeOH} 239 nm) identical to TA.

Discussion

Although the chemical structure of antibiotic TA has not yet been elucidated, several points have

been clarified. The molecular formula of TA, derived from elementary composition and spectroscopic analyses, is $C_{34}H_{57}O_9N$, M.W. 623.8. According to this formula there are 7 unsaturations in the molecule. Three of the unsaturations are due to the following carbonyl groups: saturated ketone (1710 cm^{-1}, 212.5 ppm), secondary amide (1660 cm^{-1}, 171 or 176 ppm) and lactone (1740 cm^{-1}, 171 or 176 ppm). Two of the unsaturations are due to a substituted diene (Fig. 2, **C**) which is also responsible for the absorption maximum at 239 nm. The remaining two unsaturations are most easily explained by proposing that TA contains 2 rings. One of the two rings is a lactone.

Mild alkaline conditions (0.1 M KOH, 30°C, 1 hour) cleaved the lactone, opening one of the rings and causing a loss of biological activity, but no change in the UV spectrum. Similarly, acid hydrolysis as well as periodate oxidation convert TA into a number of new, biologically inactive components exhibiting UV-absorbance resembling that of the parent compound while formation of small fragments could not be detected. Attempts are currently being made to prepare suitable derivatives of TA for X-ray structural determination.

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