TETRONOMYCIN, A NOVEL POLYETHER OF UNUSUAL STRUCTURE

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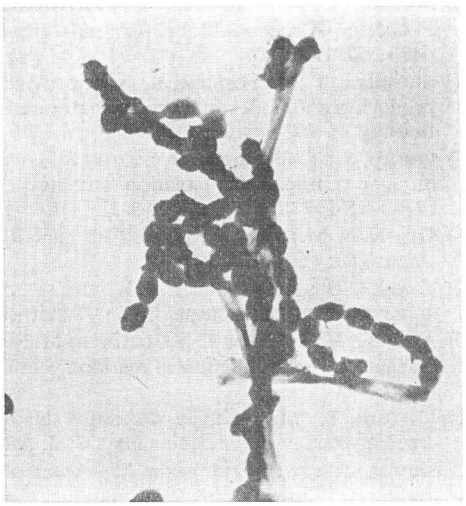
Tetronomycin, $C_{34}H_{50}O_8$, isolated from a strain of *Streptomyces* sp. nov. represents a novel polycyclic ionophore polyether. The crystal structure and absolute configuration were established by X-ray analysis of the mono-*O*-acetyltetronomycin silver salt. Tetronomycin is the first metabolic polyether which contains a tetronic acid moiety instead of the essential carboxylic acid function. A trisubstituted cyclohexane ring and an interesting molecular conformation of the silver salt represent additional unique structural features. Extensive NMR-studies enabled the assignment of chemical shifts and the correlation of the proton and carbon signals. Tetronomycin exhibits activity against Gram-positive bacteria.

Tetronomycin was isolated in the course of our screening program for novel antibiotics from a culture of a new strain *Streptomyces* sp. nov. (S 53161/A). The metabolite, which is effective against Gram-positive bacteria, represents a new member of the ionophorous polyether group and is distinguished by several unusual structural features. This paper describes the taxonomy of the producing organism and presents the isolation, characterization, biological activities and the chemical structure of tetronomycin.

Taxonomy

The tetronomycin-producing strain (NRRL 11266) was isolated from a soil sample collected in Berkeley California in 1974. The microorganism was identified as a *Streptomyces* species (nov.) having Type I cell-wall analysis and whose main characteristics, white aerial mycelia, sporophores with hooks and spirals, melanin production and spiny spores, is similar to *Streptomyces longisporus*,[1] with the exception that the spores are not long but round to oval, 0.5~0.6 nm by 0.7~0.9 nm, and the culture does not utilize sucrose (Fig. 1).

Fig. 1. Electron micrograph of spores of *Streptomyces* sp. strain NRRL 11266 (oatmeal agar ×19,000).



Fermentation

Fermentation was performed on a 500-liter scale in stainless steel fermentors. Two hundred milliliters of a dense spore suspension of the

culture NRRL 11266 were used to inoculate 50 liters of the vegetative growth medium, consisting of 1.0% dextrin, 1.0% glucose, 0.5% pepton (Cadoley Labs, Omaha, Nebraska), 0.5% yeast extract (NG & SF Delft, Holland) and 0.1% $CaCO_3$ with a pH 7.2. Incubation of the inoculated vegetative medium was made at 27°C, stirring at 200 rpm and aerating with 1 liter air/minute/liter medium for 4 days. A second stage for the vegetative culture was made by inoculating the previous vegetative cultures to 150 liters of a medium, consisting of 0.3% beef-extract, 0.5% Trypton, 0.1% glucose, 2.4% soluble starch, 0.5% yeast extract and 0.2% $CaCO_3$ with a pH 7.2. Incubating at 27°C and stirring at 150 rpm with the same aeration rate as before was done for 3 days. The vegetative culture was then inoculated to 500 liters of the fermentation medium containing 1.0% glucose, 1.0% soluble starch, 0.5% corn steep liquor (Nurnpan Inc., Düsseldorf, Germany), 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.5% $CaCO_3$ with a pH 7.0. Media were sterilized at 120°C for 20 minutes. The inoculated fermentation tank was incubated for 4 days at 27°C, stirring at 120 rpm and aerating with 1 liter air/minute/liter of medium.

Isolation of Tetronomycin

The fermentation broth (420 liters) adjusted to pH 7.0 was separated by centrifugation yielding 20 kg mycelial cake. The broth filtrate was extracted three times with 400 liters of ethyl acetate, the combined organic layers were washed with water (100 liters) and evaporated yielding 204 g of crude extract. The mycelium was homogenized with 70 liters of methanol, and then with 70 liters of methanol - water (9: 1). The filtered extracts were combined, and the methanol was removed by concentrating under addition of water. The aqueous concentrate (50 liters) was extracted several times with 50 liters of ethyl acetate. After washing with water the combined organic layers were evaporated yielding 309 g residue. A methanolic solution of the pooled extracts from broth filtrate and mycelium (513 g) was placed on a column

Table 1. Physico-chemical properties of tetronomycin.

	Tetronomycin (Na salt)	Tetronomycin (free acid)
Nature	White amorphous powder	Light yellow oil
mp (°C)	107~110	—
$[\alpha]_D^{20}$	+125.5° (*c* 0.8, MeOH)	+141.3° (*c* 0.7, $CHCl_3$)
pKa	2.52 in methylcellosolve - H_2O (8: 2)	—
MW (MS)	608	—
Formula	$C_{34}H_{49}NaO_8$ (608.74)	$C_{34}H_{50}O_8$ (586.76)
Anal. Found	C 67.0, H 8.4, Na 3.2, O 20.2	C 69.4, H 8.8, O 21.6%
Calcd.	C 67.1, H 8.1, Na 3.8, O 21.0	C 69.6, H 8.6, O 21.8%
UV (MeOH)	λ_{max} 252 nm (log ε 4.25)	λ_{max} 256 nm (log ε 3.97)
	301 nm (log ε 3.95)	290 nm (log ε 4.02)
IR (CH_2Cl_2)	3380 cm^{-1} OH	3500 cm^{-1} OH
	1740 cm^{-1} CO	1778 cm^{-1} CO
	968 cm^{-1} –CH=CH–(*trans*)	976 cm^{-1} –CH=CH– (*trans*)
TLC* (Kieselgel Merck 60, 0.25 mm)	Rf 0.22 (hexane - acetone, 8: 2)	
	Rf 0.32 (diethylether)	
	Rf 0.33 (toluene - diethylether, 1: 1)	
	Rf 0.36 (dichloromethane - acetone, 9: 1)	

* Detection was performed by spraying with a solution of ceric-sulfate (0.2%) in 50% H_2SO_4 followed by heating at 130°C thus producing brown spots.

prepared with 3 kg of Sephadex LH-20. Elution with methanol yielded 61.4 g of crude fractions exhibiting activity against *Staphylococcus aureus*. This material was dissolved in chloroform - acetone (95: 5) and rechromatographed using 1 kg silica gel (Merck, 0.063~0.2 mm). Elution with chloroform - acetone (95: 5~6: 4) produced 14.7 g active solid which was further purified on 1.1 kg Sephadex LH-20 using dichloromethane - methanol (1: 1) as solvent mixture. Final purification of the main fractions (2.6 g) was achieved on a silica gel column (Merck, 0.063~0.2 mm) starting with dichloromethane - acetone - triethylamine (89: 10: 1) and then switching to dichloromethane - acetone - triethylamine (66: 33: 1). The tetronomycin, isolated as a mixture of the sodium and calcium salts, represents an amorphous, colorless powder (mp 103~108°C) with a yield 1.78 g.

To prepare the free acid a solution of the salt mixture in acetone was acidified with 10% H_3PO_4 in ethanol. After the usual work up procedure tetronomycin free acid was obtained as a light yellow oil.

The sodium salt of tetronomycin, gained from the free acid by treating a dichloromethane solution with 1 N $NaHCO_3$, was obtained as an amorphous powder with mp 107~110°C. Rf values on thin-layer chromatography and physico-chemical properties are listed in Table 1.

The Structure of Tetronomycin

Tetronomycin sodium salt dissolves readily in dichloromethane, chloroform, ethyl acetate, acetone and methanol but is only poorly soluble in water. The molecular formula ($C_{34}H_{49}NaO_8$) was established by elemental analysis, ^{13}C NMR and mass spectra (MW 608). UV and IR spectra (Figs. 2 and 3) were rather ambiguous, however the presence of a hydroxyl group, an α,β-unsaturated carbonyl (lactone), several ether functions and a disubstituted *trans* olefin could be recognized. These structural elements

Fig. 2. IR Spectrum of tetronomycin (Na-salt in CH_2Cl_2).

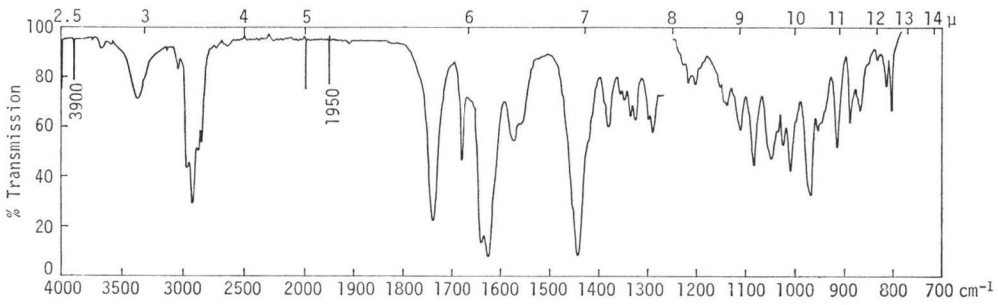


Fig. 3. IR Spectrum of tetronomycin (free acid in CH_2Cl_2).

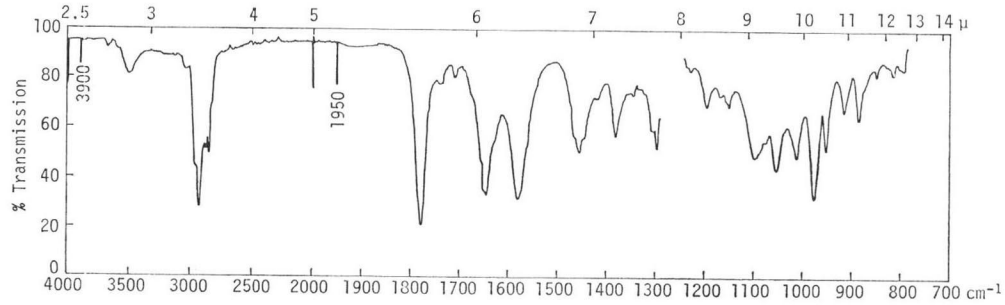


Fig. 4. ^{1}H NMR Spectrum of tetronomycin (Na-salt in $CDCl_3$).

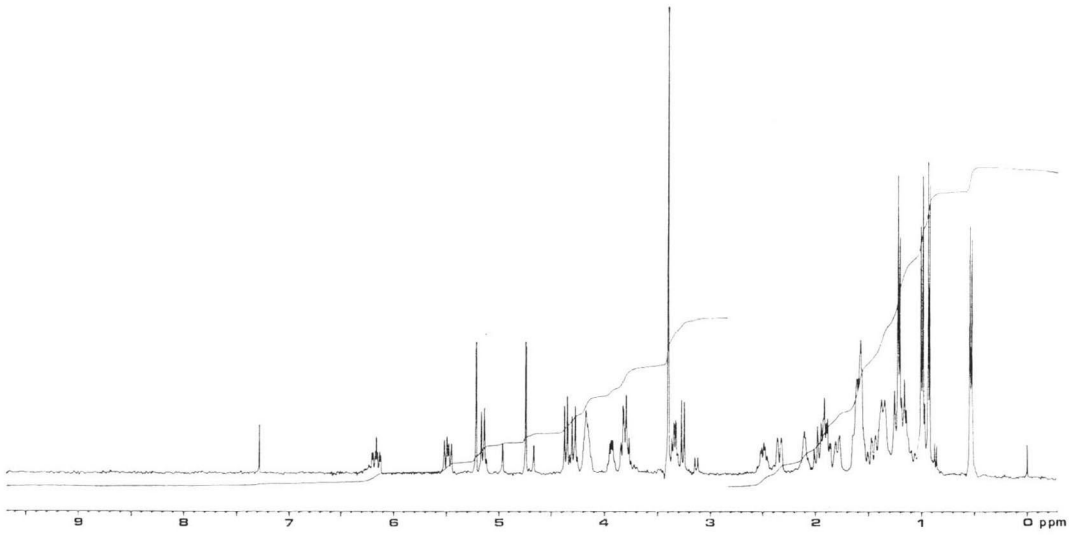
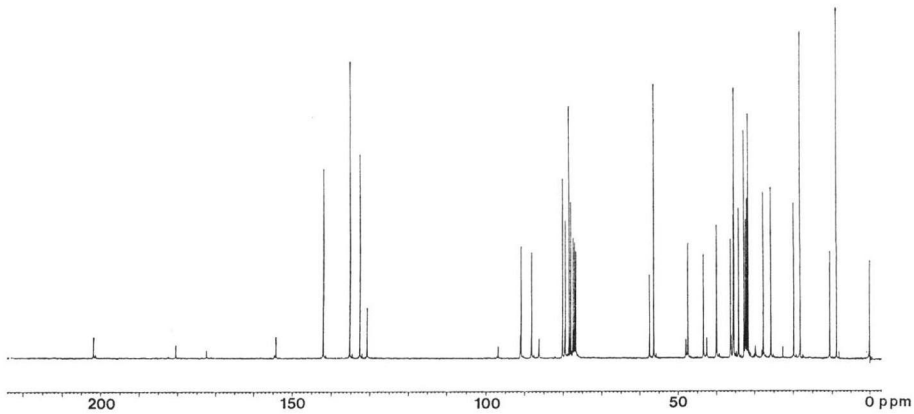


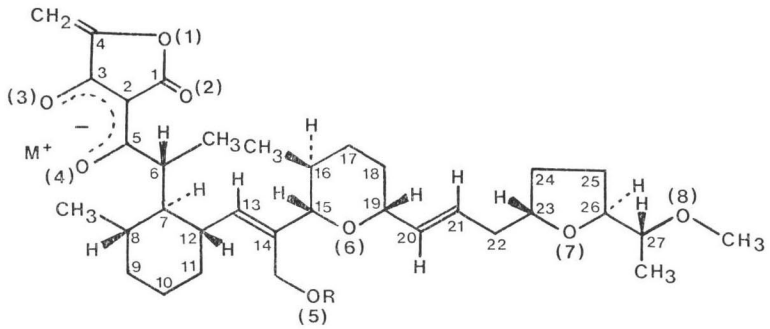
Fig. 5. ^{13}C NMR Spectrum of tetronomycin (Na-salt in $CDCl_3$).



were supported by corresponding signals in the proton NMR spectrum (in $CDCl_3$, 360 MHz; Fig. 4): δ 4.36 (d) for OH, δ 5.48 (dxd) and 6.15 (dxt) for >CH–CH=CH–CH_2–. Moreover the spectrum exhibited the presence of a terminal methylene [δ 4.73 (d) and δ 5.20 (d)] and a trisubstituted double bond [δ 5.12 (d)]. Methyl signals in the range of δ 0.5~1.2 (4 d) revealed four CH–CH_3 groups and the signal at δ 3.40 (s) reflected an O–CH_3 group. In the ^{13}C NMR spectrum the signals of all 34 carbon atoms were resolved and assigned to five methyls, ten methylenes, 13 methin groups (included five CH–O–) and six tetra-substituted carbon atoms (among them three carbonyl groups) (Table 3). Although tetronomycin contains obviously an acidic group (*pKa* 2.52) the IR spectrum of the sodium salt did not show a characteristic absorption band for a carboxylate ion. Another striking observation concerned the strong shift of a carbonyl frequency in the IR accompanying the transformation of the sodium salt (1740 cm^{-1}) to the free acid (1778 cm^{-1}).

In spite of these anomalies, the physico-chemical data of the new metabolite, the formation of metal complexes soluble in nonpolar solvents and the biological properties suggested that tetronomycin

Fig. 6. Structural formula of tetronomycin.



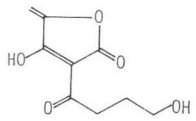
M=Na, R=H Tetronomycin (sodium salt)
M=Ag, R=Ac *O*-Acetyltetronomycin (silver salt)

belongs to the class of ionophoric polyethers.[2,3] Other antibiotics of this class *e.g.* septamycin[4], mutalomycin[5,7] and the noboritomycins A and B[6,7] have been isolated earlier in our laboratories.

Acetylation of tetronomycin with Ac_2O - pyridine furnished a mono-*O*-acetyl derivative which in the form of the sodium salt easily crystallized from ether. Elemental analysis and MS (*m/z* 650) confirmed the assumed formula $C_{36}H_{51}NaO_9$. The IR spectrum lacked an OH-absorption band and the proton NMR spectrum exhibited a new signal at δ 2.07 consistent with the CH_3CO-group. On treatment with $AgNO_3$ the acetyl derivative afforded well-formed prisms of the silver salt suitable for X-ray crystallographic analysis. The structure and absolute configuration of tetronomycin revealed by this analysis is given in Fig. 6.

Tetronomycin is distinguished from all other polyether antibiotics by several unique structural features: It is the first compound of this class in which the usual carboxylic acid group at one end of the molecule is replaced by an acyl-ylidenetetronic acid moiety. Acyl- or ylidenetetronic acids as structural elements have been encountered in a number of natural products especially in some fungal metabolites.[8,9] The same partial structure of an acyl-ylidenetetronic acid occurs for instance in dehydrocarolic acid (Fig. 7) isolated from *Penicillium cinerascens* Biourge.[10,11]

Fig. 7. Dehydrocarolic acid (hydrated form).

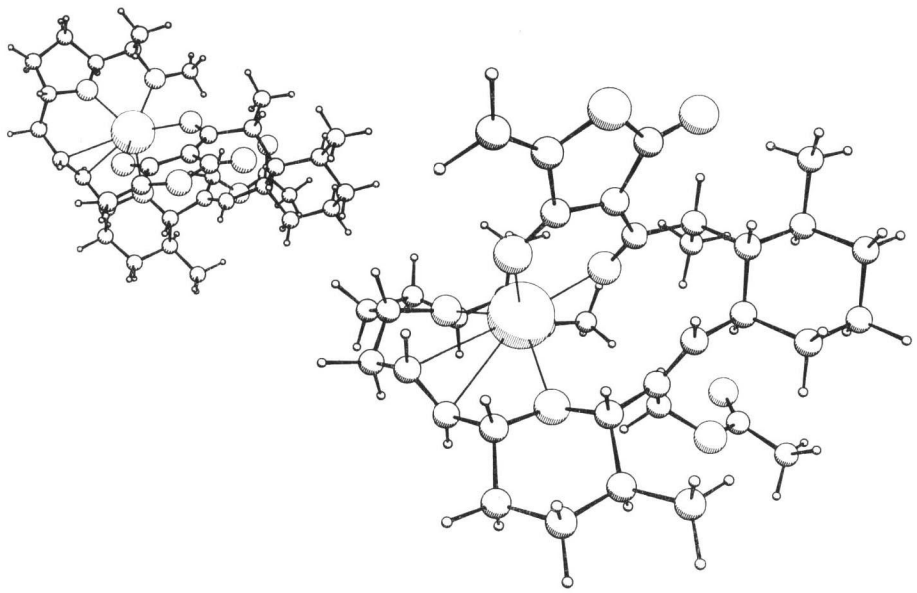


The combination of a tetronic acid with a polyether side chain as in tetronomycin is of considerable interest from the point of view of biosynthesis. It can be assumed that the tetronic acid moiety arises either by oxidative cleavage of polyketide derived aromatic intermediates or by condensation of a C_4 dicarboxylic acid (from KREBS' cycle) with the α-methylene group of a β-keto acid of the polyketide chain.[8,9]

Another prominent structural detail consists in the trisubstituted cyclohexane ring representing to our knowledge the first example of a homocyclic aliphatic ring in a polyether metabolite.

A third interesting observation concerns the molecular conformation of the mono-*O*-acetyltetronomycin silver salt. X-Ray analysis (see below) showed that the silver ion is complexed by the acidic diketone, by the tetrahydropyran and tetrahydrofuran oxygen atoms, by the terminal ether oxygen, and interestingly, by the C-20 double bond.

Fig. 8. Crystal structure of the mono-*O*-acetyltetronomycin silver salt.
The upper inset view shows the silver coordination more clearly.



X-Ray Crystallography

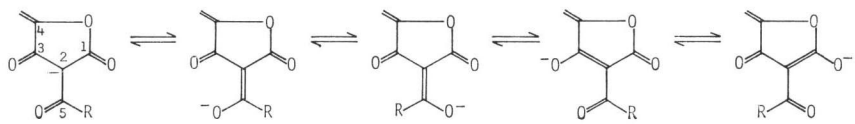
Crystal data: Mono-*O*-acetyltetronomycin silver salt: $C_{38}H_{51}AgO_9$
MW=735, orthorhombic, a=11.474 (3), b=16.160 (16), c=19.234 (7) Å.
U =3566 Å^3, space group $P\,2_12_12_1$ (D_2^4, No. 19)
D_c =1.37, Z=4, CuK_α radiation, graphite monochromator, λ=1.54178 Å, μ(CuK_α)=52.8 cm^{-1}.

Intensity data were measured at room temperature on an Enraf-Nonius CAD4F diffractometer, yielding 2688 significant reflections from a total of 4022 measurements. The structure was solved by means of a combination of MULTAN and heavy-atom techniques and was refined by block-diagonal least-squares to a final R-factor of 0.065. The absolute configuration was determined by application of the HAMILTON R-factor ratio test, which yielded a result significant at much better than the 0.005 level. Estimated standard deviations are $\leq$0.02 Å for bond lengths and $\leq$1.6° for bond and torsion angles. The structure of the silver salt in the crystal is shown in Fig. 8; full crystallographic details will be published elsewhere.[12]

NMR Spectra of Tetronomycin

A total assignment of the ^{1}H NMR spectrum of tetronomycin sodium salt (Fig. 4 and Table 2) was obtained by use of a number of homo-decoupling experiments.[13a] The spectrum in $CDCl_3$ exhibits signals from two isomers occurring in the ratio 85:15, which may be attributed to hindered rotation about the C_2–C_5 bond, caused by electron delocalization of the tetronic acid moiety (Fig. 9).[11,14]

Fig. 9. Tautomeric forms of tetronomycin*.



* The presence of the ketonic form can be inferred by the carbonyl frequency at 1778 cm^{-1} in the IR spectrum of the free acid in solvents of low polarity (CH_2Cl_2).[15,16]

Table 2. Assignments of the ^1H NMR spectra for the sodium salt of tetronomycin in $CDCl_3$ by 360 MHz with TMS=0 ppm.

Hydrogen	δ (ppm)	Hydrogen	δ (ppm)
4-CH_2	5.22 4.74	16-CH_3	0.52
H-C 6	3.93	H-C 17 eq	1.79
6-CH_3	0.99	H-C 17 ax	1.1 ~1.3
H-C 7	1.89	H-C 18 eq	1.52~1.67
H-C 8	1.32~1.42	H-C 18 ax	1.45
8-CH_3	1.21	H-C 19	3.82
H-C 9 eq	1.52~1.67	H-C 20	5.48
H-C 9 ax	1.1 ~1.3	H-C 21	6.16
H-C 10 eq	1.52~1.67	H-C 22	2.33 1.96
H-C 10 ax	1.1 ~1.3	H-C 23	4.1 ~4.2
H-C 11 eq	1.32~1.42	H-C 24	2.11 1.52~1.67
H-C 11 ax	1.0	H-C 25	1.85~1.95 1.52~1.67
H-C 12 ax	2.49	H-C 26	4.1 ~4.2
H-C 13	5.15	H-C 27	3.33
14-CH_2	4.29 3.78	27-CH_3	0.92
14-OH	4.36	27-OCH_3	3.40
H-C 15	3.26		
H-C 16	1.32~1.42		

Table 3. Assignments of the ^{13}C NMR spectra for the sodium salt of tetronomycin in $CDCl_3$ by 90.5 MHz with TMS=0 ppm.

Carbon	δ (ppm)	Carbon	δ (ppm)
C-1	172.59	14-CH_2	56.29
C-2	96.82	C-15	90.86
C-3	180.45	C-16	34.07 (b)
C-4	154.22	16-CH_3	18.16
4-CH_2	88.21	C-17	35.27 (a)
C-5	201.95	C-18	31.58 (a)
C-6	43.31	C-19	79.45
6-CH_3	8.76	C-20	132.62
C-7	47.32	C-21	135.23
C-8	32.64 (b)	C-22	39.87
8-CH_3	19.84	C-23	78.05
C-9	35.40 (a)	C-24	31.93 (a)
C-10	25.76	C-25	27.73
C-11	32.20 (a)	C-26	80.17
C-12	36.18	C-27	78.46
C-13	141.94	27-CH_3	10.43
C-14	130.72	27-OCH_3	57.42

a), b) Signals could be interchanged.

At higher temperatures (~55°C) the signals of the minor component coalesce with those of the major component. Only the signals of the predominant isomer were interpreted.

The ^{13}C NMR spectrum of tetronomycin sodium salt (Fig. 5) was assigned as shown in Table 3 by comparison of the ^{1}H undecoupled spectrum with all possible single-frequency proton decoupled spectra.[13b] If, for example, a given proton resonance can be identified in a ^{1}H NMR spectrum it is then possible to completely irradiate only that proton at low rf-power. This results in changes in the ^{13}C spectrum such that the signal from the carbon attached to that proton collapses to a singlet while other carbon resonances retain some C–H coupling. It was thus possible to correlate the ^{1}H and ^{13}C spectral lines of tetronomycin.

Table 4.

Organism	MIC (μg/ml)
Staphylococcus aureus (res. tetracycline and streptomycin)	0.1
Staphylococcus aureus	0.03
Staphylococcus aureus (res. penicillin)	0.1
Streptococcus faecalis	0.1
Micrococcus lysodeikticus	0.03
Bacillus subtilis	0.1
Micrococcus luteus (res. macrolides)	0.3
Clostridium pasteurianum	0.01
Neisseria pharyngis	0.3
Mycoplasma laidlawii	0.1

Biological Properties

Tetronomycin sodium salt shows a broad antibiotic activity against all Gram-positive bacteria tested and is also active against several *Mycoplasma* and *Neisseria* species. Cross resistance is not observed with common classes of antibiotics. MIC values of a few representative strains, obtained by broth dilution assay using brain heart infusion broth, are given in Table 4.

Activity against other Gram-negative bacteria is lacking as well as an inhibition of yeasts and filamentous fungi. This spectrum of activity is consistent with that of a polyether compound, and so is the toxicity of tetronomycin. LD_{50} was estimated to be lower than 10 mg/kg when tested in mice intraperitoneally.

Experimental

NMR spectra were run on a Bruker HX-90-E or WH-360 using TMS=0 ppm as internal standard.

Tetronomycin

A solution of 300 mg mixed Na and Ca salts in 20 ml of acetone was acidified with 2 ml of 10% H_3PO_4 in ethanol under vigorous stirring for 10 minutes. After dilution with 10 ml of water and concentration *in vacuo* the remaining aqueous solution was extracted with 100 ml of dichloromethane. The organic phase was washed with water and evaporated to yield 276 mg tetronomycin free acid as slightly yellow oil (Table 1).

Tetronomycin Sodium Salt

250 mg tetronomycin in 50 ml dichloromethane were shaken with 5 ml 1 N $NaHCO_3$. The organic layer was washed twice with 2.5 ml of water, dried over Na_2SO_4 and evaporated *in vacuo*. The pure sodium salt was obtained as an amorphous powder with mp 107~110°C (physico-chemical data see Table 1).

Mono-*O*-acetyltetronomycin Sodium Salt

A mixture of 200 mg tetronomycin sodium salt in 4 ml of acetic anhydride - pyridine (1: 1) was stirred at 20°C for 90 minutes. The residue obtained after evaporation *in vacuo* was taken up in dichloromethane, washed with 4 ml of water and then shaken with 1 ml 1 N $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated, yielding 204 mg mono-*O*-acetyltetronomycin sodium salt; white crystals from ether, mp 189~194°C (M.W. 650.79).

Anal. Calcd. for $C_{36}H_{51}NaO_9$: C 66.4, H 7.9, Na 3.5, O 22.1
Found: C 66.0, H 7.9, Na 3.1, O 22.0

Mono-*O*-acetyltetronomycin Silver Salt

A solution of 200 mg mono-*O*-acetyltetronomycin sodium salt in 25 ml of dichloromethane was shaken with 2 ml of 1 N HCl. The organic layer was washed several times with 5 ml of water and then shaken with 5 ml of 30% $AgNO_3$ for 5 minutes. After separation and filtration, the organic phase was evaporated *in vacuo*. The residue (199 mg) crystallized from ether in prisms with mp 164~168°C (M.W. 735.66).

Anal. Calcd. for $C_{38}H_{51}AgO_9$: C 58.8, H 7.0, Ag 14.7, O 19.6
Found: C 58.8, H 7.1, Ag 13.9, O 19.4

Note added in proof

In a recent publication (D. H. DAVIES, *et al.*, J.C.S. Chem. Comm. 1981, 1073) the X-ray analysis of an antibiotic, M139603, has been reported, having a very similar structure to tetronomycin. The reported antibiotic differs structurally in the substitution of methyl groups on C(22) and C(24) (see Fig. 6), in the absence of a methylene on C(4), and, somewhat surprisingly, in the absolute configuration of all comparable chiral centers.

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