TEICHOMYCINS, NEW ANTIBIOTICS FROM *ACTINOPLANES TEICHOMYCETICUS* NOV. SP.

II. EXTRACTION AND CHEMICAL CHARACTERIZATION

M. R. BARDONE, M. PATERNOSTER and C. CORONELLI

Research Laboratories, Gruppo Lepetit S.p.A.
Via Durando, 38 20158 Milano, Italy

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The teichomycins are new antibiotics produced by *Actinoplanes teichomyceticus* nov. sp. Teichomycin A_1 is a phosphorus-containing antibiotic, active *in vitro* and *in vivo* against gram-positive bacteria, and active only *in vitro* against gram-negative bacteria. Teichomycin A_2 is a chlorine-containing antibiotic, active *in vitro* and *in vivo* against gram-positive bacteria. Isolation, purification and physical and chemical properties of the two antibiotics are reported. Teichomycin A_1 is a member of the phosphoglycolipid class of antibiotics while teichomycin A_2 is related to the group of the glycopeptide antibiotics.

The teichomycins are a complex of antibiotics produced by *Actinoplanes teichomyceticus* nov. sp. ATCC N° 31121[1]. In this report the isolation and preliminary characterization of the two major components of the antibiotic mixture are described. The two products are referred to as teichomycin A_1 and teichomycin A_2.

Teichomycin A_1 is active *in vitro* against gram-positive and gram-negative bacteria and teichomycin A_2 is active *in vitro* only against gram-positive bacteria[1]. Both antibiotics inhibit bacterial growth by interfering with cell-wall synthesis[2]. They are very effective in protecting mice which have been infected with medically important pathogens such as: *Staphylococci, Streptococci,* and *Diplococci.* They show low acute toxicity in mice[1].

Isolation

Teichomycins produced during the fermentation of *Actinoplanes teichomyceticus*[1] are present both in the filtered medium and in the mycelial cake and can be extracted from the fermentation broth by the procedure described in Fig. 1. The crude antibiotic preparation obtained from the mycelial cake and from filtrates were combined and purified by column chromatography on Sephadex LH-20, using *n*-propanol - ethyl acetate - 0.2 N NH_4OH (10: 7: 7) as eluent.

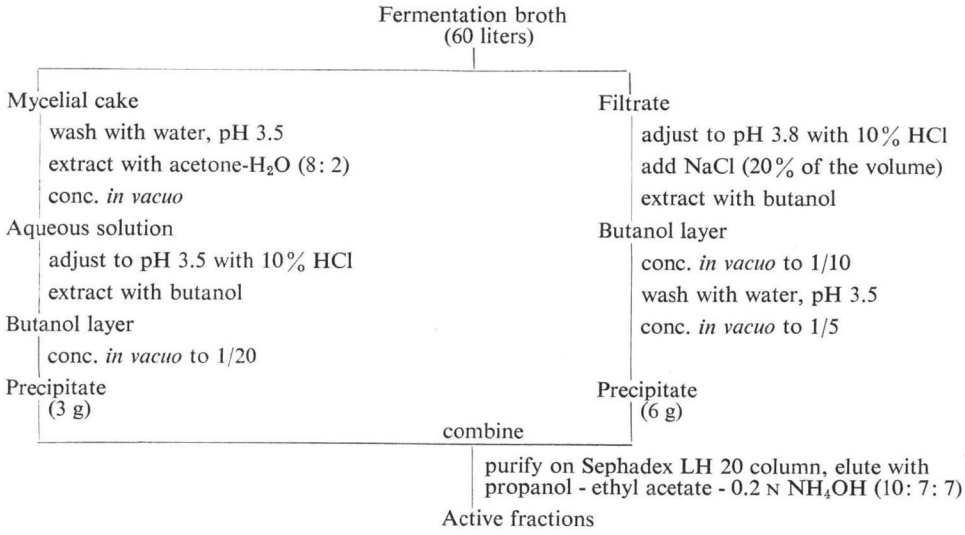
The fractions were analyzed by cellulose TLC using *n*-propanol - petroleum ether - conc.NH_4OH (4: 1: 4) as the solvent system with bioautography by *Staphylococcus aureus.* Teichomycin A_1 has an Rf 0.85 and teichomycin A_2 an Rf 0.6. The fractions corresponding to the two active products were collected and the antibiotics obtained as whitish amorphous powders.

Physical and Chemical Properties of Teichomycin A_1

Teichomycin A_1 was purified further by column chromatography on silica gel - Celite (1: 1) (v/v), using as eluent *n*-butanol - CH_3COOH - H_2O (8: 2: 2).

The antibiotic, which melts with decomposition at 220°C, is soluble in water at pH 7.0, in dimethylformamide and dimethylsulfoxide. It is partially soluble in methanol and ethanol, insoluble in

Fig. 1. Isolation and purification of teichomycins



diluted mineral acids and in non-polar organic solvents.

Teichomycin A_1 exhibits no characteristic UV-absorption between 220~360 nm. It gives positive FEHLING, TOLLENS, $KMnO_4$, anthrone and MOLISCH reactions and negative GRIESS and SCHIFF reactions. The IR spectrum is shown in Fig. 2. The elemental analysis, given as the average of four different analyses, is as follows: C 52.9, H 7.6, N 5.25, ash 3.26. Analyses of the ash revealed phosphorus. The presence of phosphorus in the molecule was confirmed by detection of radioactivity in a highly purified preparation of teichomycin A_1 produced in the presence of ^{32}P-phosphate. The percentage of P present in teichomycin A_1, as determined by chemical method, is 0.96.

The minimal molecular formula, tentatively determined for one atom of P in the molecule, is: $C_{142}H_{245}N_{12}O_{69}P$, with a calculated molecular weight of 3,255. Two different systems were used to determine the molecular weight of the antibiotic: analytical ultracentrifugation and gel filtration on Sephadex G-75. The molecular weight was established by ultracentrifugation on a Spinco Model E analytical ultracentrifuge and calculated with the SVEDBERG equation[3], after the sedimentation and diffusion coefficients had been determined by the ultracentrifuge. The gel filtration on Sephadex G-75 was performed on a column 1.6×114 cm, with Blue Dextran and vitamin B_{12} as internal markers.

The results are the following:

Solvent system	Ultracentrifuge	Sephadex G-75
Veronal buffer pH 8.5	17,700 daltons	
SÖRENSEN's phosphate buffer pH 7.38		20,000 daltons
MCILVAIN citrate buffer pH 4.4		30,000 daltons
9 M Formamide	57,400 daltons	

The antibiotic gave different molecular weights depending on the polarity and the pH of the solvent system used for the determination, suggesting that teichomycin A_1 aggregates in aqueous solutions. The size of the aggregates varied with the polarity and the pH of the solvent systems used for the determination. The lowest molecular weight value probably is that of a multimer, as the higher values are not regular multiples of the lowest one.

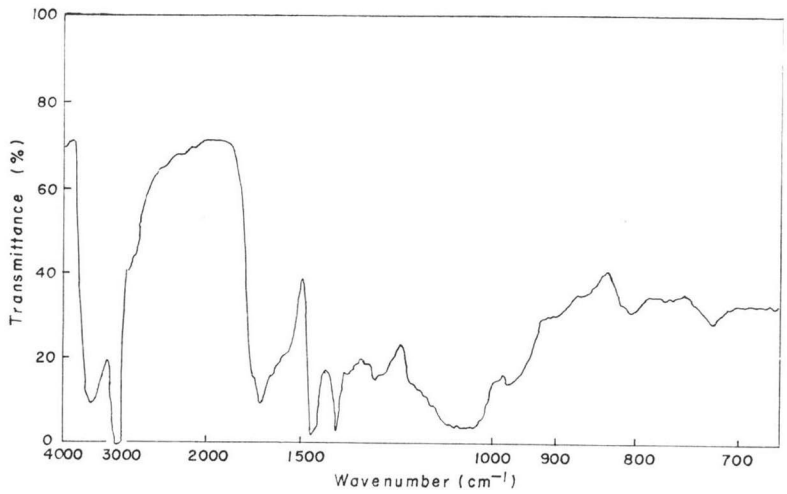
The behaviour of teichomycin A_1 in paper and thin-layer chromatography is shown in Table 1.

Table 1. Chromatographic behaviour of teichomycin A_1 and teichomycin A_2

Elution systems	Rf values	
	Teichomycin A_1	Teichomycin A_2
°1) *n*-Butanol saturated with M/15 phosphate buffer, pH 6.0	0.0	0.0
°2) *n*-Butanol saturated with water containing 2% of *p*-toluenesulfonic acid	0.05	0.13
°3) *n*-Butanol saturated with water containing 2% ammonium hydroxide	0.0	0.0
°4) M/15 Phosphate buffer, pH 6.0, saturated with *n*-butanol	0.2	0.25
°5) 20% Aqueous solution of NH_4Cl	0.0	0.0
°6) *n*-Butanol - methanol - water (40: 10: 20) with 0.75% of methyl orange	0.42	0.37
°7) *n*-Butanol - methanol - water (40: 10: 20)	0.46	0.41
°8) Ethyl acetate saturated with water	0.0	0.0
°9) *n*-Propanol - *n*-butanol - 10 N NH_4OH (2: 3: 4)	0.55	0.43
*10) *n*-Propanol - ethyl acetate - conc. NH_4OH (2: 1: 2)	0.48	0.10
**11) *n*-Propanol - petroleum ether 35/60 - conc. NH_4OH (4: 1: 4)	0.85	0.6

° Paper chromatography on Whatman No. 1
* Thin-layer chromatography on silica gel.
** Thin-layer chromatography on cellulose.
The spots were visualized by microbiological assay with *Staphylococcus aureus*.

Fig. 2. Infrared spectrum of teichomycin A_1



Hydrolysis of the antibiotic in 2 N H_2SO_4 in a sealed tube at 100°C for 2 hours gave a mixture of at least three TOLLENS-positive products, two of which were also ninhydrin-positive. These reactions and the absorptions at 3300 and 1180 cm^{-1} in the IR spectrum (Fig. 2) are indicative of the presence of carbohydrate residues in the molecule.

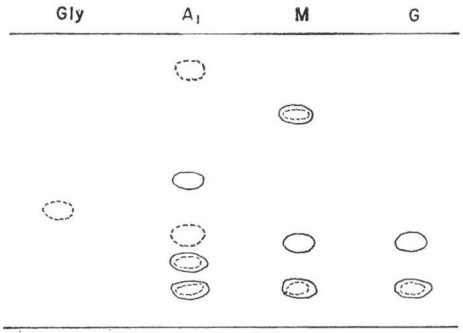
Furthermore, two other ninhydrin-positive products were found on the chromatographic analysis of the hydrolyzate of teichomycin A_1 (Fig. 3).

Physical and Chemical Properties of Teichomycin A_2

Teichomycin A_2 was dissolved in methanol - water (1: 1) and treated with acidic ion-exchange resin

(IR 120 or Dowex 50) up to pH 4.01 to remove the excess of base. After quick removal of the resin the antibiotic precipitated from the solution cooled at 4°C. Teichomycin A_2 is an amorphous powder which melts with decomposition at 260°C. The antibiotic is soluble in water at pH >7.0 and in methanol-water mixtures. It is partially soluble in methanol and in ethanol, insoluble in non-polar organic solvents. The antibiotic gives positive reactions with FEHLING, TOLLENS, $KMnO_4$, $FeCl_3$ and FOLIN-CIOCALTEU reagents and negative reactions with GRIESS, anthrone and SCHIFF reagents. It gives a dark violet color with conc. H_2SO_4. The IR spectrum is shown in Fig. 4. The elemental analysis, given as the average of three different analyses is as follows: C 54.20, H 5.70, N 6.80, Cl 3.30. The minimum molecular formula, calculated for one atom of Cl in the molecule, is : $C_{48}H_{61}N_5ClO_{20}$, which corresponds to a minimum molecular weight of 1,062.

Fig. 3. Chromatographic pattern of teichomycin A_1 and moenomycin after acid hydrolysis



A_1 : Teichomycin A_1 after hydrolysis in 2 N HCl
Gly: Glycine
M : Moenomycin after hydrolysis in 2 N HCl
G : Glucose+Glucosamine
Paper chromatography on Whatman No. 1
Elution system: *n*-butanol - acetic acid - water (2: 1: 1)
Visualization systems: ninhydrin ------
TOLLENS ——

The UV spectrum shows an absorption maximum at 278 nm ($E^{1\%}_{1cm}$ 55) in acidic and neutral solutions and an absorption maximum at 297 nm ($E^{1\%}_{1cm}$ 74) in alkaline solution (Fig. 5). The behaviour of teichomycin A_2 at different pH's, with bathochromic shift from acidic to basic conditions, indicates the presence of ionizable functions related to the chromophore.

Potentiometric titrations with 0.1 N NaOH in methylcellosolve - H_2O (4: 1) (v/v) gave a value of pK 7.4. The same titration made in the presence of 4% formaldehyde gave a pK 5.66. These values indicate the presence of a primary or secondary amino group which interacts with formaldehyde to

Fig. 4. Infrared spectrum of teichomycin A_2

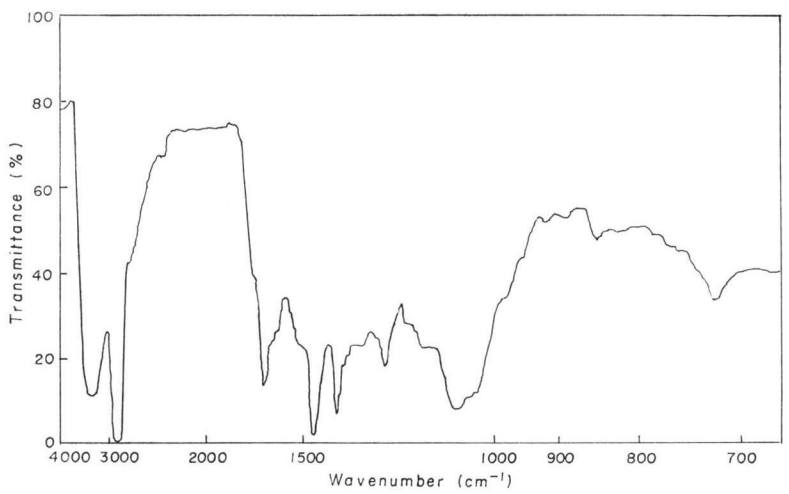
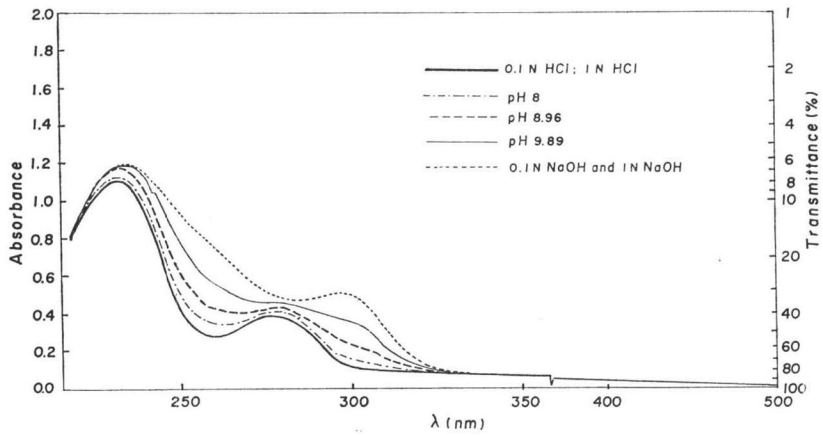


Fig. 5. Ultraviolet spectrum of teichomycin A_2



give a lower pK value. This basic function, titrated with NaOH, is present as a cation. Potentiometric titration with $HClO_4$ in dimethylsulfoxide solution indicated only one basic function. Potentiometric titrations of teichomycin A_2 with 0.1 N HCl in methylcellosolve - H_2O (4: 1) (v/v) gave a value of pK 4.95. This value must be related to an acidic group which, since it is titrated with acid, must be present as a salt.

The amphoteric character of the antibiotic has been confirmed by electrofocusing. The value p_I 6.5 was determined by thin-layer electrofocusing on polyacrylamide gel and microbiological assay with *S. aureus* as detecting system. The behaviour of teichomycin A_2 in paper and thin-layer chromatography is reported in Table 1.

Hydrolysis of the antibiotic in 2 N H_2SO_4 in a sealed tube at 100°C for 2 hours gave a mixture of two TOLLENS-positive products, one of which was also ninhydrin-positive. These reactions indicate the presence of two reducing sugars, including an aminosugar. These sugars have been isolated from the hydrolyzed mixture by means of ion-exchange resins. The acidic products obtained from hydrolysis were removed from the solution by adsorption on a basic ion-exchange resin (Amberlite IR 904). The neutral and basic products were adsorbed on an acidic ion-exchange resin (Dowex CG-50) and eluted with water and 0.1 N HCl. The TOLLENS-positive product was present in the neutral eluates and identified as D-mannose by comparison of its physico-chemical properties (Rf, $[\alpha]_D$, IR, GC, MS) with the standard sample. The TOLLENS and ninhydrin-positive product was present in the acidic eluates and identified as D-glucosamine by comparison of its physico-chemical properties (Rf, $[\alpha]_D$, IR, GC, MS) with the standard sample.

The acidic products adsorbed on the anionic resin were eluted with 0.1 N HCl; among them a substance was found which still showed some biological activity. It no longer contains sugar, indicating that loss of the D-mannose and D-glucosamine does not completely inactivate the teichomycin A_2 molecule. Hydrolysis in 6 N HCl sealed tube for 18 hours at 100°C gave a mixture of at least three ninhydrin-positive products, indicating the presence of aminoacids. The amide bands at 1660 cm^{-1} ($C{=}O_{st}$) and at 1040~970 cm^{-1} ($C{-}O_{st}$) in the IR spectrum of teichomycin A_2 are in accord with the presence of a peptide moiety.

Table 2. Chemical data on the glycolipid phosphorus-containing antibiotics

	P%	Formula	Glucose	Glucosamine	$2NH_2$-2, 6-dideoxy glucose	Other neutral carbohydrate	Glycine
Moenomycins	1.7~2	$C_{70}H_{121}N_5O_{40}P$	+	+	+	+*	n.r.
Diumycins	1.89~1.91	$C_{68\sim72}H_{93\sim107}N_5O_{38\sim40}Na_3P$	+	+	—	n.r.	—
Prasinomycins	2.43~2.28	$C_{62\sim74}H_{96\sim130}N_{5\sim6}O_{32\sim40}P$	n.r.	+	+	n.r.	+
Macarbomycins	2.12	$C_{68\sim79}H_{123\sim144}N_{6\sim7}O_{41\sim48}P$	+	+	—	n.r.	+
RP 11837	2.25	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
RP 8036	2.2	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
RP 19402	2.4	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Teichomycin A_1	0.96	$C_{142}H_{245}N_{12}O_{69}P$	—	+	—	+	—

* This pattern is related to an hydrolysis after 3~5 hours in 2 N HCl 100°C.
n.r. not reported

Discussion

The two antibiotics produced by *A. teichomyceticus* have different biological and chemical properties.

Teichomycin A_1 is characterized by the presence of phosphorus and the ability to form aggregates in solution, with the size depending on the pH and the polarity of the solvent. In contrast teichomycin A_2 contains no phosphorus, but does contain chlorine which is absent in teichomycin A_1. The properties described lead us to conclude that the two products are members of two different classes of antibiotics. The mechanism of action of teichomycin A_1 which inhibits cell-wall synthesis, the presence of phosphorus in the molecule and the property of aggregating in aqueous solvents suggest its similarity to the phosphoglycolipid antibiotics including the moenomycins[4], diumycins[5], prasinomycins[6], macarbomycins[7], RP 11837[8], RP 8036[9] and RP 19402[10]. These antibiotics contain different neutral and basic sugars. The presence of neutral and basic carbohydrate residues was also established for teichomycin A_1. The chromatographic pattern of an acid hydrolyzate of teichomycin A_1 differed from the pattern for hydrolyzed moenomycin as shown in Fig. 3. Further studies are in progress in order to identify the sugar moieties of teichomycin A_1. The data on teichomycin A_1 ,compared with those on the other phosphoglycolipid antibiotics are presented in Table 2. The percentage of phosphorus and the absence of glucose and of 6-deoxyglucosamine distinguish teichomycin A_1 from the other antibiotics of the class.

The mechanism of action of teichomycin A_2, its biological properties and the ability to give active products after hydrolysis suggest a similarity between this product and the glycopeptide class of antibiotics, such as vancomycin[11], the ristomycins[12], the ristocetins[13], the actinoidins[14], LL-AV 290[15] and the mannopeptins[16].

Teichomycin A_2 differs from the known antibiotics of this class in that its net charge, is almost neutral (Pi=6.5) while it is basic for the others. Furthermore, teichomycin A_2 differs from the ristomycins, ristocetins, and mannopeptins in that teichomycin A_2 contains chlorine. Teichomycin A_2 can be distinguished from vancomycin by TLC analysis; teichomycin A_2, has an Rf 0.10 while vancomycin has an Rf 0.21 (solvent system 10 of Table 1).

Furthermore, teichomycin A_2 differs from the other glycopeptide antibiotics in carbohydrate composition as shown in Table 3. Teichomycin A_2 is the first antibiotic of this class which contains the basic carbohydrate D-glucosamine. In the other antibiotics, the basic carbohydrate is usually 2,5-dideoxy-2-amino-glucose or -arabinose. Mannose is present in 6 of 8 antibiotics, while glucose is

Table 3. Carbohydrate composition of the glycopeptide antibiotics

Antibiotic	Neutral carbohydrate	Basic carbohydrate
Vancomycin[18]	Glucose	Vancosamine
Actinoidins[19]	Glucose-mannose	Acosamine-actinosamine
Ristomycin A[20]	D-Glucose, D-mannose, L-Rhamnose, D-arabinose	Ristosamine
Ristomycin B[20]	D-Glucose, D-mannose, L-rhamnose	Ristosamine
Ristocetins[21]	Glucose, mannose, rhamnose, D-arabinose	Acosamine or ristosamine
LL-AV 290[15,22]	Glucose, rhamnose	Acosamine or ristosamine
Mannopeptins[16]	D-Mannose	No amino sugar
Teichomycin A_2	D-Mannose	D-Glucosamine

present in every antibiotic except the mannopeptins and teichomycin A_2. Consequently we conclude that teichomycin A_2 is a new member of the glycopeptide class.

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