TEICHOMYCINS, NEW ANTIBIOTICS FROM *ACTINOPLANES TEICHOMYCETICUS* NOV. SP. IV. SEPARATION AND CHARACTERIZATION OF THE COMPONENTS OF TEICHOMYCIN (TEICOPLANIN)

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Teichomycin (teicoplanin) was found to be a mixture of five closely related components of similar polarity, designated T-A2-1, 2, 3, 4 and 5 and of one more polar component, designated T-A3. The separation of the single components was achieved by reverse phase partition chromatography and their physico-chemical and biological properties were compared. The results show that 1) the five major components have the same molecular size of about 1,900; 2) they contain the same ionizable functions, *i.e.*, a carboxyl group and an amino group which form a zwitterion, and four phenolic groups; 3) they differ in a side aliphatic chain. The component T-A3 is not described.

Teichomycin** is a glycopeptide antibiotic produced by *Actinoplanes teichomyceticus* nov. sp. ATCC 31121[1,2]. It is active against aerobic and anaerobic Gram-positive bacteria and it is under evaluation for use in man[3]. The major characteristics differentiating teichomycin from the other members of the glycopeptide antibiotics[4] is the sugar composition, *i.e.*, the presence of D-mannose and D-glucosamine[2], while the chlorinated product obtained by oxidation seems to indicate similarities to vancomycin[5]. It is worthwhile to recall that the antibiotic produced by fermentation was initially recognized as essentially two major components, *i.e.*, A_1 and A_2 and that only the component A_2 was considered worthy of further development.

During fermentation and purification studies a teichomycin product has been obtained which contains major amounts of teichomycin A_2 (T-A2) and a minor amount of another component called teichomycin A_3 (T-A3). While the paper and thin-layer chromatography procedures (usually employed for the identification of new antibiotics) showed that T-A2 was a homogeneous product, the present reverse phase high performance liquid chromatography (HPLC) has led to the identification of five major components of very similar polarity, designated T-A2-1, 2, 3, 4 and 5 corresponding to T-A2 and the more polar component, corresponding to T-A3 (Table 1 and Fig. 1).

The five major components have been chemically characterized by elemental analysis, UV, IR and ^{1}H NMR spectroscopies, and acid-base titration studies and biologically evaluated by *in vitro* and *in vivo* activity, and by acute toxicity in mice. The minor component T-A3 will be described in a succeeding paper[3].

Isolation

The single components of T-A2 were obtained in gram quantities by means of a reverse phase partition chromatography at normal pressure on a silanised Silica gel column eluted with a linear

** The recommended INN name of teichomycin is teicoplanin.

Table 1. Chromatographic conditions for the separation of teicoplanin components.

Component	Retention time (minutes)
T-A3	8.9
T-A2-1	16.9
T-A2-2	18.0
T-A2-3	18.6
T-A2-4	20.5
T-A2-5	20.9
3,5-Dihydroxytoluene (internal reference)	6.3

Column: 5 μm Zorbax ODS (Du Pont) 4.6× 150 mm.

Mobile phase: Linear gradient from 0% B to 50% B in A in 30 minutes.

A) 25 mM NaH_2PO_4 - acetonitrile, 9: 1 buffered at pH 6.0 with 1 N NaOH.

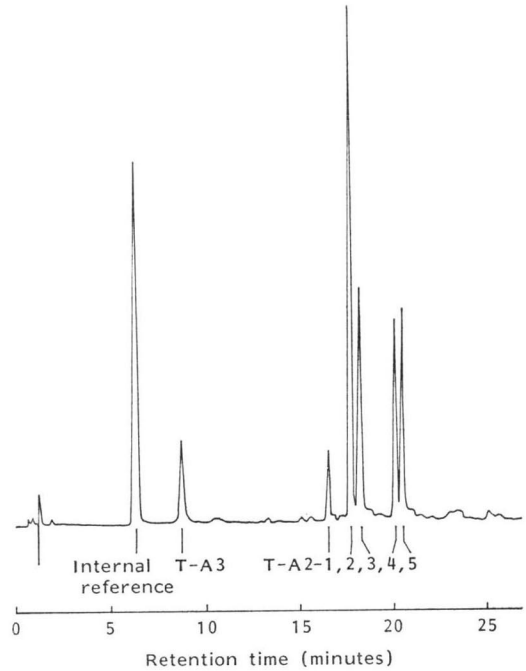
B) 25 mM NaH_2PO_4 - acetonitrile, 3: 7 buffered at pH 6.0 with 1 N NaOH.

Flow: 2 ml/minute.

Injection: 20 μl of a solution at 2 mg/ml.

Detector: UV photometer at 254 nm.

Fig. 1. HPLC gradient chromatogram of teicoplanin and 3,5-dihydroxytoluene (internal reference) under the conditions described in Table 1.



gradient from 10% to 20% acetonitrile in 0.2% aqueous ammonium formate mixture. Components T-A2-1 and T-A2-2 were obtained in pure form. Pure components T-A2-3, 4 and 5 were obtained from semipreparative HPLC on a Whatman Partisil ODS M-9 column eluted with 0.2% aqueous ammonium formate - acetonitrile, 76: 24 mixture.

Physico-chemical Characteristics

T-A2 has been described as a glycopeptide antibiotic with an approximate molecular weight of 1,900, containing D-mannose and D-glucosamine as sugars and a dichlorotriphenyl ether moiety and other aromatic moieties as amino acids[2,5].

The elemental analyses of the pure components compared with those of T-A2 (Table 2) confirm the presence of two chlorine atoms in each component, while the minor differences observed allow us to exclude significant differences in their molecular size.

UV spectra recorded at different pH values (Table 3) do not reveal differences among the components indicating that the chromophore and the ionizable functions related to it are the same.

The IR spectra of the components are also identical.

Acid-base ionization properties were studied in Methyl Cellosolve (MCS) - H_2O mixtures, in dimethylformamide (DMF) - H_2O mixtures, and in non-aqueous media. The apparent ionization constant values (pK) and equivalent weight values (eqW) are essentially the same for T-A2 and for each component. In Fig. 2 a representative titration curve of T-A2 is shown, while in Table 4 the values obtained for T-A2 and for the major component T-A2-2 are reported. The data indicate that the five functions with pK-MCS 5.0 and pK-MCS in the range of 9 to 12.5 have an acidic character because there is a negative shift of all these pK values by increasing the water content and that the function with pK-

Table 2. Elemental analysis* of teicoplanin components.

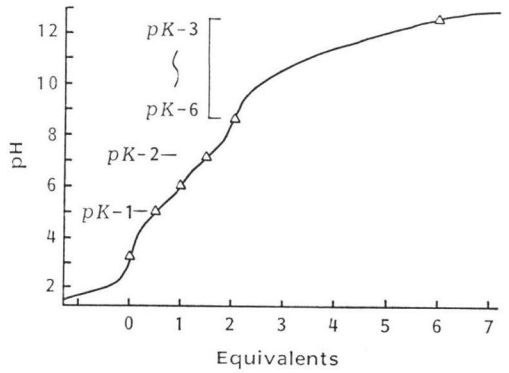
Component	C (%)	H (%)	N (%)	Cl (%)
Teicoplanin	55.3	5.52	6.56	3.68
T-A2-1	56.7	4.90	6.65	3.80
T-A2-2	56.15	5.15	6.30	3.90
T-A2-3	56.26	5.20	6.69	3.95
T-A2-4	56.50	5.10	6.50	3.80
T-A2-5	56.60	5.05	6.63	3.85

* Data determined after the samples have been dried at about 140°C under inert atmosphere to eliminate residual solvents.

Table 3. UV bands (λ_{max}, nm) and absorptivities (A) of teicoplanin components in H_2O solution at different pH values.

Component	Solvent		
	0.1 N HCl	Buffer pH 7.4	0.1 N NaOH
T-A2-1	278 (4.95)	278 (5.00)	297 (7.21)
T-A2-2	278 (4.80)	278 (4.90)	297 (7.00)
T-A2-3	278 (4.92)	278 (5.08)	297 (7.27)
T-A2-4	278 (5.25)	278 (5.25)	297 (7.55)
T-A2-5	278 (4.96)	278 (5.18)	297 (7.88)
Teicoplanin	278 (5.95)	278 (5.93)	297 (8.98)

Fig. 2. Titration of teicoplanin dissolved in MCS - H_2O, 4: 1 with 0.1 N NaOH, after having added an excess of 2.3 equivalents of 0.1 N HCl.



MCS 7.1 has a basic character, as confirmed by exclusion by the titration in non-aqueous media which shows one basic function. Consequently, the functions with *pK*-MCS 5.0 and *pK*-MCS 7.1 give rise to a zwitterion. The following assignments are then possible: *pK*-1 is a terminal carboxyl group, *pK*-2 is a terminal amino group, both confirming previous assignments[2], while the four *pK*'s (3 ~ 6) are phenolic groups.

The ^1H NMR spectra in dimethyl sulfoxide-d_6 solution were recorded at 270 MHz. Even if the spectra appear quite complex some useful information can be derived and some differences among the individual components revealed. The spectrum of the major component, T-A2-2, is reported in Fig. 3. The number of aliphatic (28 H), oxygen and nitrogen-bonded aliphatic and alcoholic (40 H), aromatic

Table 4. Ionization data of teicoplanin.

Solvent	*pK*-1	eqW	*pK*-2	eqW	*pK*-3	eqW	*pK*-4 ~ 6
MCS - H_2O, 4: 1	5.0	1,930	7.1	1,750	Continuous titration slope from pH 9 to 12.5 without a well defined end-point, but corresponding to 4 functions		
MCS - H_2O, 1: 1	4.3	1,900	7.1	1,770	Continuous titration slope from pH 8.5 to 12.0 without a well defined end-point, but corresponding to 4 functions		
DMF - H_2O, 9: 1	Single titration slope (pH 1/2 = 7.4) eqW = 880 (880 × 2 = 1,760)				10.5	1,880	Single titration slope (pH 1/2 = 12.7), eqW = 570 (570 × 3 = 1,710)
CH_3COOH	—	—	—	1,880	—	—	—
MCS - H_2O, 4: 1	4.8	1,850	7.0	1,750	Continuous titration slope from pH 9 to 12.5 without a well defined end-point, but corresponding to 4 functions		
DMF - H_2O, 9: 1	Single titration slope (pH 1/2 = 7.5) eqW = 960 (960 × 2 = 1,920)				10.8	1,870	Single titration slope (pH 1/2 = 13.2), eqW = 640 (640 × 3 = 1,920)

Fig. 3. ^{1}H NMR at 270 MHz of teicoplanin solution in DMSO-d_6 ($\sim 3 \times 10^{-2}$ mol).

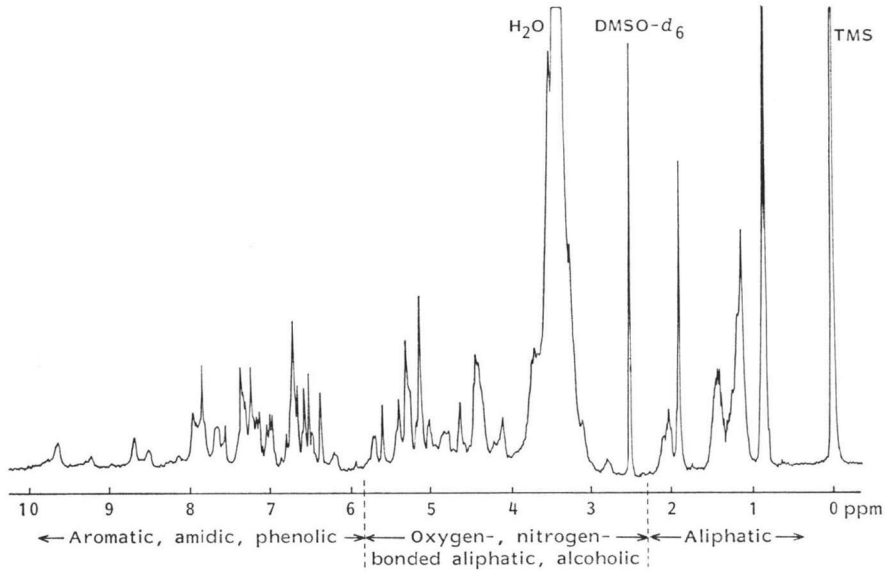
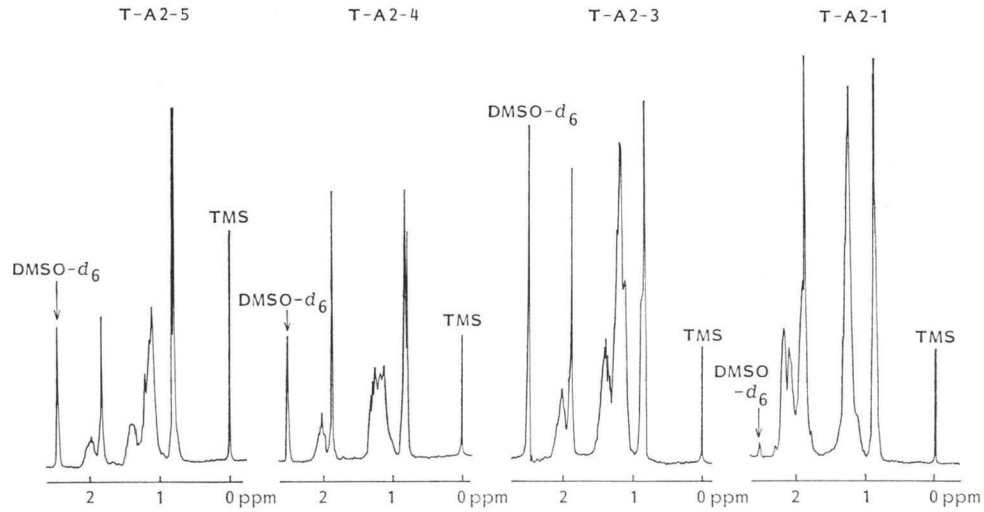


Fig. 4. ^{1}H NMR at 270 MHz of teicoplanin components in DMSO-d_6 solution in the region 0~3 ppm.



(19 H), amidic and phenolic (15 H) hydrogens total 102 H and are substantially the same for the individual components. The total number of protons is almost identical and very close to the values obtained by elemental analysis: the only significant and interesting differences are localized in the region of the spectrum arising from aliphatic saturated protons as shown in Fig. 4.

The definition of these differences as well as the exact molecular weight of each component will be published elsewhere[7]. The structure of the common aromatic portion is described in a concurrent paper[8].

Biological Properties

The *in vitro* activity against Gram-positive clinical isolates of staphylococci and streptococci (Table

Table 5. *In vitro* antibacterial activity of teicoplanin components.

Microorganisms	No. of tested strains	MIC (μg/ml)				
		T-A2-1	T-A2-2	T-A2-3	T-A2-4	T-A2-5
Staphylococcus aureus	5	0.8~1.6	0.8~1.6	0.4~0.8	0.2~0.8	0.2~0.8
S. epidermidis	4	0.2~1.6	0.1~1.6	0.2~0.8	0.2~0.8	0.2~0.8
Streptococcus pyogenes	7	0.05~0.1	0.025~0.1	0.025~0.05	0.006~0.05	0.006~0.05
S. pneumoniae	6	0.1~0.2	0.05~0.1	0.05~0.1	0.05~0.1	0.05~0.1
S. faecalis	5	0.2~0.4	0.1~0.4	0.1~0.2	0.1~0.4	0.1~0.4
S. mitis	1	0.025	0.025	0.0125	0.025	0.025
S. salivarius	1	0.2	0.2	0.1	0.05	0.05
S. sanguis	1	0.1	0.1	0.1	0.05	0.05
S. bovis	1	0.4	0.4	0.2	0.4	0.4
S. agalactiae	1	0.1	0.1	0.05	0.1	0.1

Table 6. *In vivo* antibacterial activity of teicoplanin components.

Component	ED_{50} (mg/kg/day) sc	
	S. pneumoniae L 44	*S. pyogenes* L 49
T-A2-1	0.47	0.31
T-A2-2	0.28	0.15
T-A2-3	0.27	0.13
T-A2-4	0.12	0.098
T-A2-5	0.13	0.10

Table 7. Acute toxicity in mice (ip) of teicoplanin components.

Component	Approximate LD_{50} (mg/kg)
T-A2-1	1,500 ~ 2,000
T-A2-2	1,500 ~ 2,000
T-A2-3	1,000 ~ 1,500
T-A2-4	500 ~ 1,000
T-A2-5	500 ~ 1,000

5)[3], the *in vivo* activity in mice with septicemic infections (Table 6)[3] and the acute toxicity in mice (Table 7) confirm the close similarity of the components inferred by the physico-chemical data.

Experimental

Separation of T-A2 Components 1, 2, 3, 4 and 5

Ten g of T-A2 were dissolved in 1 liter of 0.2% ammonium formate - acetonitrile (9: 1) mixture and adjusted to pH 7.5 with 1 N NaOH. This solution was passed through a column containing 500 g of silanised Silica gel 60 (Merck). The column was then eluted with a linear gradient from 10% to 20% acetonitrile in a 0.2% ammonium formate solution in a total volume of 10 liters. Fractions of 20 ml were collected and checked by means of HPLC.

In Table 1 the retention time for T-A2 components 1, 2, 3, 4 and 5 from a representative HPLC separation are reported (the operating conditions are indicated in the Table). Fractions with similar HPLC profile were combined and the organic solvent was evaporated under reduced pressure. The residual aqueous solutions were passed through a column containing 10 g of silanised Silica gel 60. The column was washed with distilled water in order to eliminate the ammonium formate and then eluted with 50% aqueous acetonitrile. The eluate was concentrated to a small volume, after adding butanol to facilitate the evaporation of the water and then precipitated with 1: 1 acetone - ethyl ether mixture. Pure T-A2-1 (410 mg) and T-A2-2 (770 mg) were obtained by these procedures.

T-A2-3, obtained as a 1: 1 mixture with T-A2-2, was further purified by HPLC on a semipreparative column under the following operating conditions:

Column: Whatman Partisil ODS M 9 10/50. Mobile Phase: 0.2% aqueous ammonium formate - acetonitrile (76: 24). Flow: 4.5 ml/minute. Detector: UV photometer at 254 nm. Load: 20 mg dissolved in 1 ml of the mobile phase.

Also in this case purification was monitored by checking each fraction by HPLC. Fractions containing pure T-A2-2 as well as fractions containing pure T-A2-3 were combined, desalted and precipitated as previously described (yield: 510 mg of T-A2-2 and 520 mg of T-A2-3). Fractions containing T-A2-4 and T-A2-5 in 1: 1 proportion (about 1 g) obtained from the first column, were separated by semipreparative HPLC using the same operating conditions described above for the purification of T-A2-3, yielding 350 mg of pure T-A2-4 and 300 mg of pure T-A2-5.

In vitro Activity

The *in vitro* activity of the single factors was determined against Gram-positive clinical isolates of staphylococci and streptococci using the two-fold dilution method in microtiter system. Penassay broth (Difco) and Todd-Hewitt broth (Difco) were used for staphylococci and streptococci respectively, the final inoculum was about 10^3 colony-forming units per ml. Minimal inhibitory concentration was read as the lowest concentration which showed no visible growth after 18~24 hours incubation at 37°C.

Experimental Infections

Groups of five mice were infected intraperitoneally. Inocula were adjusted so that untreated animals died of septicemia within 48 hours. Animals were treated subcutaneously once a day for three days starting immediately after infection. On the 10th day the value for the ED_{50} in mg/kg/day was calculated by the method of SPEARMAN and KARBER[9], on the basis of the percentage of surviving animal at each dose.

Instruments

UV spectra were run on a Unicam SP 800 spectrophotometer. IR spectra were recorded on a Perkin-Elmer Model 580 spectrometer. ^1H NMR spectra were recorded on a Bruker WH-270 cryospectrometer using tetramethylsilane (TMS) as internal reference (δ 0.00 ppm).

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