TEICOPLANIN, ANTIBIOTICS FROM *ACTINOPLANES TEICHOMYCETICUS* NOV. SP.

VI. CHEMICAL DEGRADATION: PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF ACID HYDROLYSIS PRODUCTS

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Teicoplanin is an antibiotic complex consisting of five closely related factors, T-A2-1, 2, 3, 4 and 5 and a more polar factor, T-A3-1. By controlled acid hydrolysis the complex is transformed into pseudoaglycones and finally into a single aglycone with consecutive removal of three sugar units.

Quantitative determination of sugars obtained by degradative reactions and NMR/LC-MS studies on suitable derivatives confirmed that all the components carry one *N*-acyl-D-glucosamine and that at least two of them are characterized by *N*-decanoyl and *N*-undecanoyl chains on the D-glucosamine unit. The hydrolysis products still possess *in vitro* and *in vivo* activity.

Teicoplanin*, an antibiotic complex produced by fermenting a strain of *Actinoplanes teichomyceticus* ATCC 31121[1,2], is a glycopeptide type active against Gram-positive bacteria[3] by specifically inhibiting the bacterial cell wall synthesis[4].

Degradative studies[2,5,6] of the complex led to the identification of three aromatic amino acidic moieties carrying hydroxyl groups: a chlorine bearing triphenyl ether, a diphenyl ether and a diphenyl, constituting a polypeptide skeleton. Two sugars, D-mannose and D-glucosamine were also detected in all the components of the complex[7]. All these data confirmed the preliminary indication[2] that teicoplanin belonged to the vancomycin family antibiotics, with particular similarity to ristocetin[8], A35512B[8], actaplanin[9], A47934[10] and A41030.[10] In this paper we describe some reactions that yielded biologically active products and some preliminary structural information.

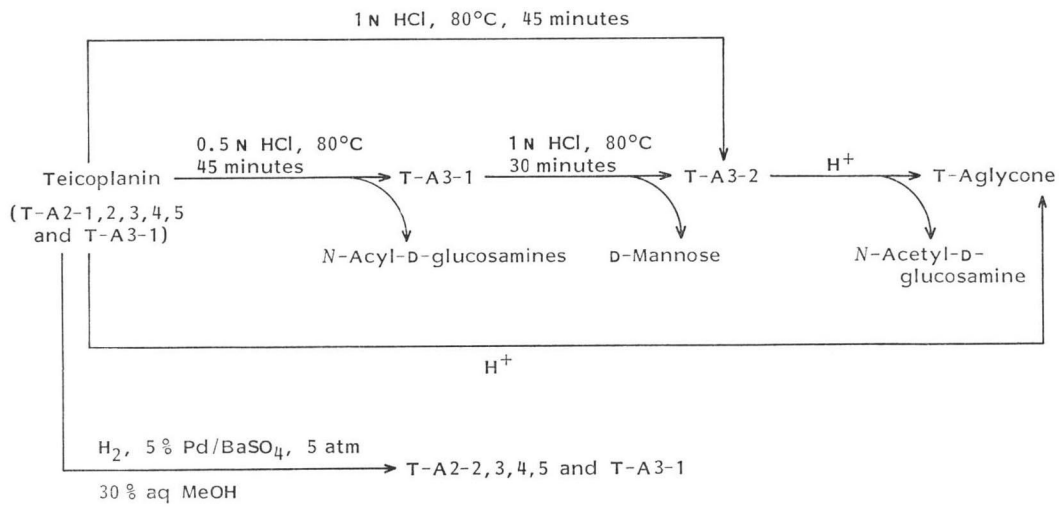
Chemistry

Reverse-phase thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) showed[7] that teicoplanin is a mixture of five closely related factors (formerly called teichomycin A_2)[2] designated T-A2-1, 2, 3, 4 and 5, corresponding to the most of the substance, and a more polar factor designated T-A3-1. Water of crystallization and an inorganic residue were detected by thermogravimetric and elemental analysis.

Potentiometric titrations of the teicoplanin complex gave an equivalent weight (eqW) of about 1,900 (Table 2) and revealed the presence of one acidic and one basic function attributable to a COOH and an NH_2 group, and of four weak acidic groups identified as phenolic hydroxyls belonging to the polyphenyl aromatic units mentioned above[6].

* Teicoplanin is the recommended INN of teichomycin.

Chart 1.



Hydrogenation

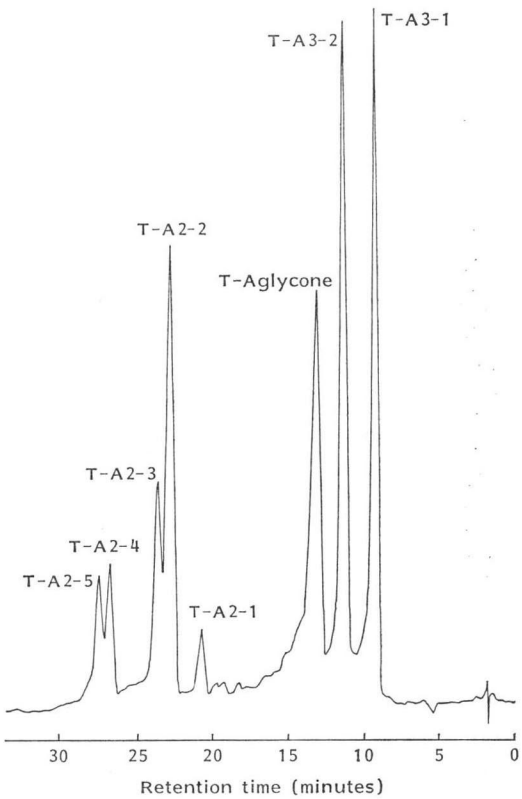
When the teicoplanin complex was hydrogenated under mild conditions (30% aqueous MeOH, 5% Pd on $BaSO_4$, room temperature, 5 atm) the only observable modification in its HPLC profile was the complete transformation of component T-A2-1 into component T-A2-3.

Acid Hydrolysis

Mild hydrolysis (0.5 N HCl, 80°C) led to the transformation of the teicoplanin complex into the single (HPLC) antibiotic T-A3-1. By sugar analysis (see later) T-A3-1 lost one glucosamine unit and still contains one mannose and one glucosamine unit. By stronger hydrolytic treatment (1 N HCl, 80°C) T-A3-1 produced a new compound designated T-A3-2 which had lost the mannose unit (Chart 1). Further acid treatment of T-A3-2 displaced the remaining sugar and a product was isolated which was shown to be the aglycone (T-aglycone)[11].

Selected reaction conditions transformed teicoplanin directly into T-A3-2 or T-aglycone. All these hydrolysis products are less lipophilic than teicoplanin (Fig. 1).

Fig. 1. HPLC gradient profile of a mixture of teicoplanin and hydrolysis products under the conditions described in the Experimental Section (values of retention time are listed in Table 1).



The physico-chemical properties of these hydrolysis products are listed in Tables 1 and 2 in comparison with those of the parent teicoplanin. By interpretation of the elemental analytical data and

Table 1.

Compound	Elemental analysis (%)[a]				Formula	MW (Calcd)	$[\alpha]_D^{20}$ (c 1, DMF)	HPLC[b] (K)
	C	H	N	Cl				
Teicoplanin	55.30	5.52	6.56	3.68	$C_{89}H_{106}N_9O_{35}Cl_2$	1,933	−29°	1.00
T-A3-1	55.46	4.50	7.20	4.67	$C_{72}H_{69}N_8O_{27}Cl_2$	1,549	−34°	0.41
T-A3-2	56.74	4.27	7.99	5.11	$C_{66}H_{59}N_8O_{23}Cl_2$	1,403	−44°	0.50
T-Aglycone	58.27	3.73	7.86	6.04	$C_{58}H_{45}N_7O_{18}Cl_2$	1,198	nd	0.54

a) Values corrected for weight loss (measured by Thermal Gravimetric Analysis) and for inorganic residues (determined after heating the samples at 900°C in oxygen atmosphere).

b) See Experimental Section. K is the ratio of the retention time of the compounds to the retention time of component T-A2-2 (22.4 minutes), Fig. 1.

nd=not determined.

eqW values given by potentiometric titration the formulas and molecular weights (MW) reported were obtained. In particular, potentiometric titrations showed that the three hydrolysis products still possess one terminal COOH and one primary NH_2 group with *pKa* values identical to those titrated in teicoplanin, forming an internal salt. Furthermore, all the hydrolysis products show one phenol group in addition to the four present in teicoplanin.

Thus, it was concluded that in all the products the acidic and basic functions most likely belong to the aglycone moiety, and that the *N*-acyl-D-glucosamines are attached to one phenolic oxygen.

Taking into account that a single product (T-A3-1) was obtained from teicoplanin it was confirmed that the components of teicoplanin are closely related and that the markers of the factors are on one glucosamine unit.

Our data on the difference between the respective MW's and on the NH_2 functions, as well as the ^1H NMR investigation at Cambridge University[12], led to the hypothesis that the markers mentioned above are aliphatic residues bonded to glucosamine as amides.

Sugar Analysis

The similarity of teicoplanin to vancomycin type antibiotics suggested that the sugars might be bonded to the aglycone moiety (or between themselves) through acetalic linkages. In fact, teicoplanin was unaffected by treatment with $NaBH_4$. The sugar determination was made by high-performance thin-layer chromatography (HPTLC). A mixture of standard *N*-acetyl-D-glucosamine, D-glucosamine and D-mannose was treated with 2 N H_2SO_4 under various conditions in order to determine their stability (Table 3). *N*-Acetyl-D-glucosamine was used as one of the standards because its presence had been demonstrated in previous experiments[12]. The *N*-acetyl-D-glucosamine was partially or totally degraded while the D-mannose was only slightly affected (20%) by the acid treatment.

Samples of teicoplanin and of the hydrolysis compounds T-A3-1 and T-A3-2 were analyzed and calculations were based on the MW's determined by potentiometric titrations. After 15 minutes of hydrolysis of teicoplanin 0.2 mol of *N*-acetyl-D-glucosamine, about 0.5 mol of D-glucosamine and about 0.6 mol of D-mannose were found. A longer heating (1 hour) gave values in agreement with those from blank experiments showing no *N*-acetyl-D-glucosamine; the D-glucosamine was about 1.2 mol and D-mannose 0.7 mol. Therefore, it could be deduced that teicoplanin contains one mol of D-mannose and two mol of D-glucosamine (one being *N*-acetylated) per mol of aglycone.

Hydrolysis of T-A3-1 (15 minutes) gave 0.3 mol of *N*-acetyl-D-glucosamine, 0.2 mol of D-glucos-

Table 2.

Compound	Potentiometric titrations[a]						UV λ_{max}, nm ($E^{1\%}_{1cm}$)[a]	IR (cm^{-1}, Nujol)[a]
	Solvent	pK-1	eqW	pK-2	eqW[b]	pK-3~pK-7 (DMF - H_2O, 9: 1)		
Teicoplanin	MCS - H_2O, 4: 1	5.0	1,930	7.1	1,750	pK-3 10.5, eqW 1,880	279 (59.6) in 0.1 N HCl,	3250 (br, ν_{NH}, ν_{OH}),
	CH_3COOH	—	—	—	1,880	pK-4~6, single titration slope,	298 (89.8) in 0.1 N NaOH,	1645 (amide I),
	DMF - H_2O, 9: 1	pH 1/2 7.4, eqW 880×2=1,760				pH 1/2 12.7, eqW 570×3= 1,710	279 (59.3) in pH 7.38 buffer	1610 (ν_{COO^-}), 1590 ($\delta_{NH_3^+}$), 1510 (amide II)
T-A3-1	MCS - H_2O, 4: 1	5.0	1,517	7.0	1,541	Single titration slope,	279 (76.4) in 0.1 N HCl,	3300 (ν_{NH}, ν_{OH}),
	CH_3COOH	—	—	—	1,490	pH 1/2 12.6,	298 (149.8) in 0.1 N NaOH,	1660 (amide I),
	DMF - H_2O, 9: 1	pH 1/2 7.5, eqW 740×2=1,480				eqW 290×5=1,450	279 (88.7) in pH 7.38 buffer	1610 (ν_{COO^-}), 1595 ($\delta_{NH_3^+}$), 1515 (amide II)
T-A3-2	MCS - H_2O, 4: 1	5.0	1,370	7.1	1,307	Single titration slope,	279 (81.3) in 0.1 N HCl,	3310 (ν_{NH}, ν_{OH}),
	CH_3COOH	nd				pH 1/2 12.8,	298 (150.4) in 0.1 N NaOH,	1655 (amide I),
	DMF - H_2O, 9: 1	pH 1/2 7.5, eqW 681×2=1,362				eqW 268×5=1,340	279 (90.9) in pH 7.38 buffer	1610 (ν_{COO^-}), 1595 ($\delta_{NH_3^+}$), 1515 (amide II)
T-Aglycone	MCS - H_2O, 4: 1	4.78	1,247	6.90	—	Single titration slope,	279 (82.9) in 0.1 N HCl,	3250 (ν_{NH}, ν_{OH}),
	CH_3COOH	—	—	—	—	pH 1/2 13.7,	297 (155.6) in 0.1 N NaOH	1645 (amide I),
	DMF - H_2O, 9: 1	pH 1/2 7.1, eqW 626×2=1,252				eqW 252×5=1,260		1610 (ν_{COO^-}), 1595 ($\delta_{NH_3^+}$), 1520 (amide II)

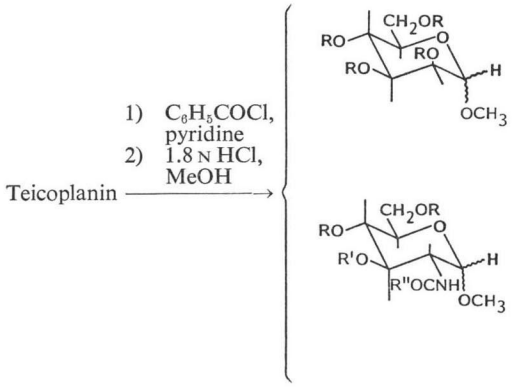
[a] See the Experimental Section. [b] eqW: Equivalent molecular weight. nd=not determined.

Table 3. Quantitative sugar determination.

Compound	Time of hydrolysis	*N*-Acetyl-D-glucosamine (mol)		D-Glucosamine (mol)		D-Mannose (mol)	
		Calcd	Found	Calcd	Found	Calcd	Found
Mixture of standards	0	1	0.94	1	0.89	1	0.97
〃	15	1	0.47	1	1.03	1	0.80
〃	60	1	0.00	1	1.33	1	0.90
Teicoplanin	15	1	0.20	1	0.52	1	0.65
〃	60	1	nd	1	1.23	1	0.73
T-A3-1	15	1	0.30	—	0.20	1	0.60
〃	60	1	nd	—	0.40	1	0.78
T-A3-2	60	1	nd	—	0.20	—	0.00

nd=not detectable

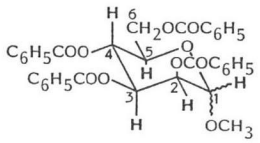
Chart 2.



Compound	OCH_3	R
Ia	α	COC_6H_5
b	β	COC_6H_5

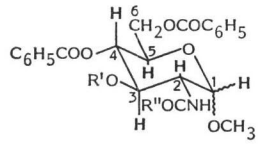
Compound	OCH_3	R	R′	R″
IIa	α	COC_6H_5	COC_6H_5	C_9H_{19}
b	β	COC_6H_5	COC_6H_5	C_9H_{19}
IIIa	α	COC_6H_5	H	C_9H_{19}
b	β	COC_6H_5	H	C_9H_{19}
IVb	β	COC_6H_5	H	$C_{10}H_{21}$
V	—	COC_6H_5	COC_6H_5	$C_{10}H_{21}$

Table 4. ^1H NMR data for methyl D-mannopyranoside tetrabenzoates at 270 MHz in $CDCl_3$ solution (δ in ppm, J in Hz, internal reference TMS).



Proton	Compound **Ia** (α)			Compound **Ib** (β)		
	δ	Multiplicity	J (Hz)	δ	Multiplicity	J (Hz)
H-1	5.00	d	1	4.89	d	0.5
H-2	5.73	dd	1, 3	5.98	dd	0.5, 3
H-3	5.95	dd	3, 10	5.64	dd	3, 10
H-4	6.15	dd	10, 10	6.06	dd	10, 10
H-5	4.46	ddd	10, 2.5, 4.5	4.15	ddd	10, 5, 3
CH_2-6	4.53 and 4.74	2dd	2.5, 12.5 4.5, 12.5	4.55 and 4.77	2dd	5, 12.5 3, 12.5
four C_6H_5	7.8~8.2	m	—	7.2~8.2	m	—
OCH_3	3.54	s	—	3.58	s	—

Table 5. ^{1}H NMR data for 2-acylamino-2-deoxy-3,4,6-tri- and 4,6-di-*O*-benzoyl-D-methylglucopyranosides at 270 MHz in $CDCl_3$ solution. (δ in ppm, J in Hz, internal reference TMS).



	IIa (α, R′=COC_6H_5, R″=C_9H_{19})			IIb (β, R′=COC_6H_5, R″=C_9H_{19})		
H-1	4.89	d	J=3.5	4.78	d	J=8.5
H-2	4.66	ddd	J=3.5, 9, 9	4.23	ddd	J=8.5, 9, 9.5
H-3	5.72	m	—	5.64	dd	J=9.5, 9.5
H-4		m		5.78	dd	J=9.5, 10
H-5	4.33	ddd	J=9, 2.5, 4.5	4.11	ddd	J=10, 5, 2.5
CH_2-6	4.46 and	2dd	J=4.5, 12	4.47 and	2dd	J=5, 12
	4.63		J=2.5, 12	4.63		J=2.5, 12
CONH	5.98	d	J=4	5.73	d	J=9
R′	0.7~2.2	m	—	0.7~2.2	m	—
OCH_3	3.52	s	—	3.52	s	—
C_6H_5	7.2~8.2	m	—	7.2~8.1	m	—
R″	7.2~8.2	m	—	7.2~8.1	m	—
	IIIa (α, R′=H, R″=C_9H_{19})			**IIIb** (β, R′=H, R″=C_9H_{19})		
H-1	4.77	d	J=3.5	4.67	d	J=8
H-2	4.48	ddd	J=3.5, 9, 9	4.18	ddd	J=8, 9, 9
H-3	3.84	ddd	J=9, 9, 4	3.84	ddd	J=9, 9, 4.5
H-4	5.23	dd	J=9, 9	5.21	dd	J=8.5, 9
H-5	4.00	ddd	J=9, 2.5, 4	3.76	ddd	J=8.5, 5.5, 3
CH_2-6	4.69 and	2dd	J=12, 2.5	4.63 and	2dd	J=12, 5.5
	4.81		J=12, 4	4.82		J=12, 3
CONH	5.77	d	J=9	5.63	d	J=9
R′	3.07	d	J=4	3.25	d	J=4.5
OCH_3	3.46	s	—	3.52	s	—
C_6H_5	7.3~8.1	m	—	7.3~8.1	m	—
R″	0.7~2.2	m	—	0.7~2.2	m	—

	IVb (β, R′=H, R″=$C_{10}H_{21}$)					
H-1	4.56	d	J=8	CONH	5.80	d	J=5
H-2	4.24	ddd	J=8, 9, 5	R′	2.24 and	t and	J=7.5
H-3	3.55	ddd	J=9, 9, 5		0.8~1.7	m	
H-4	5.36	dd	J=9, 11	OCH_3	3.54	s	—
H-5	4.02	ddd	J=11, 4.5, 2.5	C_6H_5	7.3~8.1	m	—
CH_2-6	4.63 and	2dd	J=2.5, 12.5	R″	4.57	d	J=5
	4.77		J=4.5, 12.5				

amine, likely deriving from the portion of *N*-acetyl-D-glucosamine that was hydrolyzed, and 0.6 mol of D-mannose. After heating one hour no *N*-acetyl-D-glucosamine was revealed, but 0.4 mol of D-glucosamine and about 0.8 mol of D-mannose were found.

The same treatment of T-A3-2 gave only 0.2 mol of D-glucosamine and no D-mannose. The small amount of D-glucosamine formed from *N*-acetyl-D-glucosamine in comparison with the values obtained from blank experiments (about 0.4 mol) may be explained as a variation of the hydrolysis reaction which has only been qualitatively interpreted.

The T-aglycone showed no sugars.

These data confirm that one molecule of D-glucosamine carrying the markers of the factors was removed from teicoplanin by hydrolysis giving the pseudo-aglycone T-A3-1 which still contains one mol each of *N*-acetyl-D-glucosamine and D-mannose. Stronger hydrolysis removed the D-mannose giving T-A3-2 which carries the *N*-acetyl-D-glucosamine.

Some information about the connections of the sugar units were obtained by degradative hydrolysis of teicoplanin after perbenzoylation and methanolysis (Chart 2). Treatment of teicoplanin with benzoyl chloride in pyridine yielded crude perbenzoylteicoplanin which was methanolyzed. The mixture of several compounds was separated by silical gel column chromatography followed by preparative TLC. The relevant fractions were analyzed by ^{1}H NMR at 270 MHz in $CDCl_3$ (the chemical shifts and coupling constants are reported in Tables 4 and 5) and by liquid chromatography-mass spectrometry (LC-MS). Quasi-molecular ions $(M+H)^+$, $(M-H)^-$ and M^-, as base peaks, and some fragment ions are shown in Tables 6 and 7.

Table 6. Negative ion CI mass spectra (base peaks are underlined).

Compound	F 9-10/1		F 11/2	
	I	II	III	IV
$(M-H)^-$	609	<u>658</u>		
↓ −phCOOH				
h		536		
↓ −phCOO				
i		415		
↓ −phCOOH				
l		293		
M^-			<u>555</u>	<u>569</u>
↓ −phCOO				
m			434	448

The integrated use of the two techniques led to elucidation of the structures even if the compounds were not isolated in a pure state.

The following sugars were identified: α-D-methylmannopyranoside tetrabenzoate (**Ia**) and its β-

Table 7. Positive ion CI mass spectra (base peaks are underlined).

Compound	F 9-10/1		F 11/2		F 11/3
	I	II	III	IV	V
$(M+H)^+$	611	<u>660</u>	<u>556</u>	<u>570</u>	<u>674</u>
↓ −phCOOH					
a	489	538	434	448	552
↓ −phCOOH					
b	367	416	312	326	
↓ −phCOO					
c	246				
$(M+H)^+$					
↓ $-CH_3OH$					
d	<u>579</u>	628	524	538	642
↓ −phCOOH					
e	457	506	402	416	520
↓ −phCOOH					
f	335	384	280	294	398
↓ −phCOOH					
g		262			276

Table 8. *In vitro* antibacterial activity.

Organism	MIC (μg/ml)			
	Teicoplanin	T-A3-1	T-A3-2	T-Aglycone
Staphylococcus aureus ATCC 6538	0.1	0.4	0.2	0.25
S. aureus Tour	0.4	0.4	0.2	0.05
S. aureus Tour[a)]	0.8	1.6	0.8	0.2
S. aureus Tour[b)]	0.8	0.8	0.4	0.4
S. epidermidis ATCC 12228	0.4	0.4	0.05	0.0125
Streptococcus pyogenes C203	0.05	1.6	1.6	0.05
S. pneumoniae UC41	0.05	1.6	1.6	0.05
S. faecalis ATCC 7080	0.2	1.6	0.8	0.1
Escherichia coli SKF 12140	>800	>800	>800	25
Proteus vulgaris X 19 H ATCC 881	>100	>100	>100	50
Pseudomonas aeruginosa ATCC 10145	>100	>100	>100	>100

a) Inoculum 10^4 cfu/ml.
b) Determined in the presence of 30% bovine serum.

anomer (**Ib**)[13], confirmed also by comparison with authentic samples; 2-decanoylamino-2-deoxy-3,4,6-tri-*O*-benzoyl-α-D-methylglucopyranoside (**IIa**) and its β-anomer (**IIb**); 2-decanoylamino-2-deoxy-4,6-di-*O*-benzoyl-α-D-methylglucopyranoside (**IIIa**) and its β-anomer (**IIIb**); 2-undecanoylamino-2-deoxy-4,6-di-*O*-benzoyl-β-D-methylglucopyranoside (**IVb**); 2-undecanoylamino-2-deoxy-3,4,6-tri-*O*-benzoyl-methylglucopyranoside (**V**).

The identification of a reasonable quantity of D-mannopyranoside tetrabenzoates was indicative of a terminal D-mannose. The low yield derives from extensive debenzoylation that was shown to occur for a standard sample under the same methanolysis conditions.

The presence of 2-decanoyl- and 2-undecanoyl-amino-2-deoxyglucopyranose, demonstrated by isolation of partially and totally benzoylated methylglucopyranosides and independently demonstrated in Cambridge[12], confirms the hypothesis, derived from preliminary NMR studies on teicoplanin, that at least two factors are characterized by these aliphatic chains on the glucosamine moiety.

Antibacterial Activity

In Table 8 we compare the MIC of T-A3-1, T-A3-2 and T-aglycone with those of teicoplanin, for several bacterial species. Against staphylococci, T-aglycone was somewhat more active than the other three compounds, in particular against *Staphylococcus epidermidis*. Against streptococci, T-aglycone was as active as teicoplanin while T-A3-1 and T-A3-2 were much less active. Only T-aglycone showed some activity against *Escherichia coli* and *Proteus vulgaris*. As T-aglycone was the most active compound, it was tested against several clinically isolated methicillin-resistant staphylococci; the results (MIC 0.05~0.2 μg/ml) confirmed the activity observed with laboratory strains. As in the case of teicoplanin, the MIC of the other three compounds were only slightly affected by addition of bovine serum (*Staphylococcus aureus* Tour, Table 8). In mice with septicemia resulting from intraperitoneal infection with *S. pyogenes* C203, T-A3-1 and T-A3-2 were less active than teicoplanin (sc ED_{50}: T-A3-1 2.64 mg/kg, T-A3-2 2.46 mg/kg, teicoplanin 0.11 mg/kg), as expected from their *in vitro* activities against this organism. T-Aglycone was quite effective in curing this infection (sc ED_{50} 0.95 mg/kg), although less so than teicoplanin and less than might have been expected from its MIC.

Discussion

Teicoplanin is a glycopeptide antibiotic complex possessing a core heptapeptide similar to ristocetin[8], A35512B[8], actaplanin[9], A47934[10] and A41030[10]. Teicoplanin consists of five closely related factor (T-A2-1, 2, 3, 4 and 5) each one containing one mol of *N*-acyl-D-glucosamine, one mol of *N*-acetyl-D-glucosamine and one mol of D-mannose, and of a pseudo-aglycone (T-A3-1) deriving from T-A2 factors by loss of the *N*-acyl-D-glucosamines.

The teicoplanin complex is transformed into the single pseudo-aglycone T-A3-1 by hydrolysis. By suitable hydrolytic treatment D-mannose is removed from T-A3-1 giving a second pseudo-aglycone (T-A3-2) still carrying *N*-acetyl-D-glucosamine. Finally, stronger hydrolysis leads to the T-aglycone.

The *N*-acyl chains on a terminal D-glucosamine are the differentiating factors of the components of the complex, two of them being characterized as decanoyl and undecanoyl residues. The sugars are attached to the aglycone at three separate sites and the *N*-acyl-D-glucosamines are attached to one phenolic oxygen.

The hydrolysis compounds maintain *in vitro* and *in vivo* antibacterial activity.

Experimental

Evaporation was carried out with a rotary evaporator at 45°C (bath temperature) *in vacuo*.

Column chromatographies were done on silica gel 60, 0.06~0.2 mm, or silanized silica gel 60, 0.06~0.2 mm (Merck).

TLC were done on silanized silica gel F_{254} plates (Merck), unless otherwise indicated.

UV spectra were run on a Unicam SP 800 spectrophotometer.

IR spectra were recorded on a Perkin-Elmer Model 580 spectrometer.

^{1}H NMR spectra were recorded with a Bruker WH-270 cryospectrometer using tetramethylsilane (TMS) as internal reference (δ=0.00 ppm).

The optical rotations were measured with a Perkin-Elmer Model 241 polarimeter in a 0.1-cm cell.

HPLC were run with a Varian Model 5000LC pump equipped with a 20 μl loop injector Rheodyne Model 7125 and a Perkin-Elmer LC15 detector at 254 nm. Column: pre-column (5 cm) packed with Perisorb RP-8 (30 μm) Merck followed by a column Hibar RT 250-4 Merck prepacked with LiChrosorb RP-8 (10 μm). Eluent: CH_3CN - 0.2% aq $HCOONH_4$, linear step-gradient ranging from 10% CH_3CN to 30%. Injection: 20 μl. Flow rate: 2 ml/minute. The reactions were monitored by injecting samples of the solutions diluted enough to obtain a final concentration of 1 mg/ml. Final products were checked by injecting solutions of 10 mg each of the hydrolysis derivatives or 30 mg of teicoplanin in 10 ml of a CH_3CN - 0.2% aq $HCOONH_4$, 1: 1 mixture.

HPTLC determinations were made on HPTLC silica gel 60 F_{254} plates 10×20×0.1 cm (Merck) with a Camag Linomat III sample applicator (100 μl Hamilton Syringe Model) equipped with a Camag TLC Monochromator Version, a HP 3357 Data System and a Perkin- Elmer Model 561 recorder. The densitometric conditions selected were: wavelength 366 nm (mercury lamp); sensitivity 2; span 10; slit length 5 mm×0.1 mm; scan speed 1 mm/second.

LC-MS analyses were made with an apparatus consisting of a Constametric model III pump equipped with a Perkin-Elmer LC15 UV 254 nm detector and a Hewlett-Packard 5985B mass spectrometer equipped with LC-MS interface, positioned downstream from the UV detector. The samples were dissolved in 1 ml of CH_2Cl_2 and 20 μl were injected. Separation was achieved in a 10-cm Brownlee Labs RP-8 (10 μm) column connected to a 3-cm RP-8 (10 μl) pre-column by isocratic elution with CH_3CN - H_2O, 75: 25 as mobile phase, at a flow rate of 0.8 ml/minute. Spectra were recorded in positive and negative ion chemical ionization.

Potentiometric titrations were carried out under three different conditions: a) the compounds were dissolved in methylcellosolve (MCS) - H_2O, 4: 1, an excess of 0.01 N HCl in the same solvent mixture was added and the resulting solution was titrated with 0.01 N NaOH; b) to confirm the presence of the basic functions, the compounds were also titrated in non-aqueous medium (glacial acetic acid) with 0.01 N $HClO_4$; c) weak acidic functions attributable to phenolic groups were demonstrated by titration

with 0.1 N KOH of solutions of the compounds in DMF - H_2O, 9: 1 after the addition of an excess of 0.1 N HCl in the same solvent mixture.

Hydrogenation of Teicoplanin

A solution of 100 mg of teicoplanin (composition: T-A2-1 13.1%, T-A2-2 38.6%, T-A2-3 19.3%, T-A2-4 9.2%, T-A2-5 9.5%, T-A3-1 10.4%) in 10 ml of 30% aq MeOH was hydrogenated at 5 atm and room temperature in the presence of 100 mg of 5% Pd on $BaSO_4$. After 6 hours of stirring, 100 mg of the catalyst was added and the reaction mixture was stirred under hydrogen for another 2 hours. The reaction mixture was then filtered, and analyzed by HPLC. The composition was: T-A2-1 1.8%, T-A2-2 37.8%, T-A2-3 31.6%, T-A2-4 9.5%, T-A2-5 9.2%, T-A3-1 10.0%.

Preparation of T-A3-1 from Teicoplanin

a) Teicoplanin (5 g) was added under vigorous stirring at one time to 60 ml of a solution of 0.5 N HCl preheated to 80°C. The stirring and the temperature were maintained for 45 minutes, then the cloudy reaction mixture was rapidly filtered. The solution was cooled to 0~5°C and 10 ml of 6 N HCl was added. A suspension formed, which was stirred for 15 minutes at 0~5°C. The precipitate was collected, washed with 20 ml of cold 1 N HCl then with Et_2O, and dried at room temperature on KOH pellets obtaining 4.5 g of crude material which contained 74% of T-A3-1 and 16% of T-A3-2 (HPLC).

The product (3 g) was suspended in 150 ml of a mixture of 0.2% aq $HCOONH_4$ - CH_3CN, 95: 5. The pH was brought to 7.5 with 1 N NaOH and the product dissolved. The resulting solution was applied to a column containing 150 g of silanized silica gel prepared in the same solvent mixture. The column was developed with a linear gradient 5 to 21% CH_3CN in 0.2% aq $HCOONH_4$ elution, collecting 150 of 20-ml fractions which were checked by HPLC. Fractions 70 to 96 containing T-A3-1 were combined and the CH_3CN was removed. The residual aqueous solution was applied to a column of 10 g of silanized silica gel in distilled water. After washing with distilled water until the salts were completely eliminated the product was eluted with a 1: 1, CH_3CN - H_2O mixture. The solution collected was concentrated to a small volume, acidified to pH 3.5 with 1 N HCl and the antibiotic was precipitated by adding acetone. After drying at room temperature 0.9 g of pure T-A3-1 was obtained.

Preparation of T-A3-2

From Teicoplanin: Teicoplanin (10 g) was added while stirring to 150 ml of 1 N HCl preheated to 80°C. After 45 minutes the reaction mixture was cooled to 0~5°C and 30 ml of 37% HCl was added. Stirring was maintained for 10 minutes at 0~5°C then the solid was filtered off, washed with 20 ml of 2 N HCl then with 200 ml of Et_2O and finally dried at room temperature over KOH overnight obtaining 8.3 g of crude T-A3-2. This material (6.2 g) was dissolved in 500 ml of 80% aq MeOH and 30 g of silica gel was added. After adding 200 ml of 1-BuOH the solvents were evaporated, 30 ml of MeOH was added and the mixture was evaporated to dryness. The residue was put at the top of a column containing 300 g of silica gel in CH_3CN. The column was developed using 300 ml each of the following solvent mixtures: CH_3CN; CH_3CN - H_2O, 95: 5; CN_3CN - H_2O, 90: 10; CH_3CN - H_2O, 85: 15. The eluates were discarded, then the column was developed with a linear gradient obtained by mixing 3.5 liters each of the solvent mixtures CH_3CN - H_2O, 83: 17 and CH_3CN - H_2O, 70: 30 at a rate of 375 ml/hour.

Fractions of 25 ml were collected and monitored by HPLC. Fractions 170 to 200 were combined, 400 ml of 1-BuOH was added and the solution was concentrated to a small volume. Acetone was added to the cloudy solution. After standing at 10°C for 1 hour the precipitate was filtered, washed with 300 ml of acetone then with 200 ml of Et_2O and dried at room temperature for 4 days obtaining 1.9 g of pure T-A3-2.

From T-A3-1: T-A3-1 (0.1 g) was added to 1.5 ml of 1 N HCl preheated to 80°C. After 30 minutes the reaction mixture was cooled and checked by HPLC. The peak corresponding to the starting compound disappeared and the peak of T-A3-2 became visible. The yield in T-A3-2 calculated by comparison with standard samples, was 0.05 g.

Quantitative Determination of Carbohydrates

N-Acetyl-D-glucosamine, D-glucosamine and D-mannose were determined by HPTLC with post-elution derivatization against an external calibration curve. Accurately weighed samples (standards about 10 mg each; teicoplanin, T-A3-1 and T-A3-2 about 40 mg) were added to 2 ml of 2 N H_2SO_4 and heated at 120°C (bath temperature) for the established times. After cooling, 2 ml of water was added and the pH was brought between 6 and 7 by adding a saturated solution of $Ba(OH)_2$ under stirring. The suspension was centrifuged for 15 minutes, decanted and the residue was carefully washed with water. The filtrate was evaporated to dryness at 60°C. Water was added (10 ml for standards, 4 ml for teicoplanin and the hydrolysis derivatives), the suspension was sonicated and filtered on Swinnex HA 02500, then 1 ml of the filtered solution was diluted to 5 ml with MeOH.

HPTLC silica gel plates washed overnight with MeOH, dried under warm air flow (1 minute), dipped in 100 ml of 30% methanolic buffer pH 8, then dried in oven for 1 hour were used. Standard solutions (3, 4, 5, 7 and 9 μl) and 6 μl of each sample were alternatively applied on a 10-mm band at a speed of 50 mm/μl and 5 seconds/μl. Elution was done with an EtOH - H_2O - conc NH_4OH, 90: 30: 1 mixture in presaturated chamber at room temperature for 6 cm. The plates were dried in oven at 95°C for 5 minutes, then dipped in 1 liter of a derivatization agent (prepared by dissolving 20 g of diphenylamine and 20 ml of freshly distilled aniline in 150 ml of 85% phosphoric acid, then diluting with 1 liter of acetone) and dried at 120°C for 15 minutes. The Rf values were: D-glucosamine 0.4, D-mannose 0.7, *N*-acetyl-D-glucosamine 0.9. After the densitometric scanning of the plates a basic computer program was run for the quantitative evaluation of the spots by reference to the five applied standards. This program calculated the best fitting calibration curve from among seven different linearizing equations by chosing it on the basis of the higher correlation coefficient. Then this program used the chosen calibration curve for calculating the amount of the sample spots.

Analysis of Carbohydrates

Benzoyl chloride (10 ml) was added dropwise to a stirred suspension of 5 g of teicoplanin in 25 ml of dry pyridine at 0°C. The reaction was left at room temperature overnight and then stirred for 8 hours. MeOH was added (50 ml) to decompose the excess of benzoyl chloride and the reaction mixture was kept at room temperature overnight. After concentration to dryness, the oily residue was washed repeatedly with Et_2O and then dissolved in 50 ml of 1.8 N methanolic HCl. The solution was refluxed for 2 hours, left overnight at room temperature and evaporated to dryness. The oily residue was redissolved in 50 ml of MeOH and re-evaporated many times. Finally, it was dissolved in EtOAc (200 ml) and the solution was extracted with water acidified with 10% HCl. The organic phase was separated, washed with water (100 ml), then with 5% aq $NaHCO_3$, dried over anhydrous Na_2SO_4 and concentrated to a 5-ml volume. Et_2O was added (200 ml) then the precipitate was filtered off and the solution was evaporated to dryness obtaining 1.2 g of a yellow oil that was dissolved in 5 ml of Et_2O and applied to a column containing 50 mg of silica gel slurried with *n*-hexane. Elution was carried out using 100 ml of *n*-hexane, then by a stepwise gradient of 10 to 90% Et_2O in *n*-hexane, followed by 200 ml of Et_2O and 200 ml of EtOAc. Fractions of 50 ml each were collected. Fractions 2 to 8 containing methyl benzoate were discarded.

Fractions 9~10, 11, 12, 13~14 were evaporated to dryness, redissolved in 2 ml of CH_2Cl_2 and separately chromatographed by preparative TLC on silica gel PLC 60 F_{254} plates (Merck) in the system *n*-hexane - Et_2O, 2: 8. Four zones that were revealed by UV were scraped off and extracted with MeOH. The extracts were evaporated giving four oily residues designated F 9-10/1, F 11/2, F 11/3, and F 13-14 that were checked by TLC using a *n*-hexane - Et_2O, 3: 7 mixture as mobile phase.

Standard samples of methyl-α- and β-D-mannopyranoside tetrabenzoate (**Ia** and **Ib**) were prepared from D-mannose by the procedure described above. After chromatography on a silica gel column eluted with *n*-hexane then with *n*-hexane - Et_2O, 1: 1 fractions containing **Ia** and **Ib** were collected and evaporated. The two compounds were recrystallized from MeOH. TLC (*n*-hexane - Et_2O, 3: 7): **Ia**, Rf 0.70; **Ib**, Rf 0.45.

Determination of Antibacterial Activity

Minimum inhibitory concentration (MIC) was determined using the two-fold dilution method in

microtiter system. The media used were: Todd-Hewitt broth (Difco) for streptococci; Iso-Sensitest broth (Oxoid) for staphylococci and Gram-negative bacteria. The final inoculum was about 10^4 cfu/ml. MIC was read as the lowest concentration which showed no visible growth after 18~24 hours incubation at 37°C.

Experimental Infection: Groups of five mice have been infected intraperitoneally with *S. pyogenes* C203. Inocula have been adjusted so that untreated animals died of septicemia within 48 hours. Animals have been 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[14], on the base of the percentage of surviving animals at each dose.

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