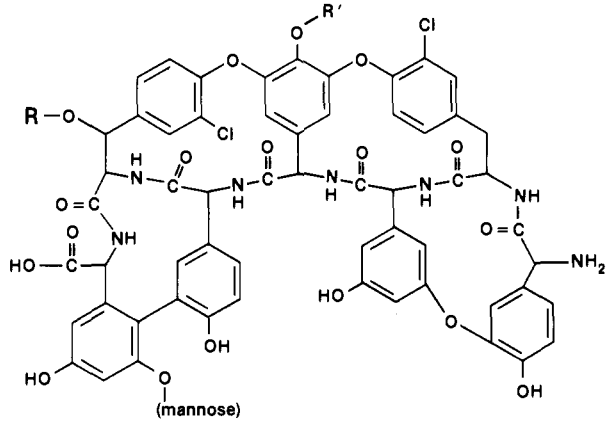
for ortho, ~0.2 ppm for para) when a sugar or an acyl group is linked to the peptide core through the particular phenolic group.[8,10] When the NMR spectra of **1** and the teicoplanin complex are compared, the aromatic resonances for rings E, F, and G show little change in position, indicating that phenols on those rings are not sugar attachment sites in teicoplanin. The ring D resonances, however, are both shifted downfield substantially; D-2 occurs at 6.52 ppm and D-4 at 6.71 ppm in the intact teicoplanin. Part of the chemical shift change for the D-2 proton can be attributed to the influence of the adjacent peptide carboxy terminal, which is $COOCH_3$ in **1** but COOH in the parent teicoplanin. The influence of such a change can be evaluated from Table I. Three of the compounds listed in Table I have methyl esters attached to D-1′: **1**, actaplanin ψ-aglycon, and A35512B ψ-aglycon. The average chemical shifts for the ring protons for the three materials are 6.07 ppm for D-2 and 6.42 ppm for D-4. Two antibiotics in Table I have free carboxyl groups attached to D-1′, A41030B and vancomycin; the average ring D chemical shifts for these two are 6.28 ppm for D-2 and 6.41 ppm for D-4. Comparison of the two sets of averages indicates that hydrolysis of a carboxy-terminal ester attached to D-1′ should cause a downfield shift of ~0.2 ppm for the D-2 resonance but have little or no effect at position D-4. The observed changes on going from **1** to intact teicoplanin are shifts downfield of 0.43 ppm (D-2) and 0.31 ppm (D-4), and these large differences indicate the presence of a sugar on ring D in teicoplanin. Such a linkage is confirmed by the observation of mutual NOEs between the D-4 proton (6.71 ppm) and an anomeric singlet (mannose) at 5.24 ppm; no NOE is observed between 5.24 ppm and proton D-2 (6.52 ppm). These results suggest that the mannose is linked through the teicoplanin D-5 phenol, ortho to proton D-4 (shift ~0.3 ppm for sugar linkage) and para to proton D-2 (shift ~0.2 ppm for sugar linkage plus ~0.2 ppm for absence of the methyl ester); the D-5 phenol is a mannose attachment site for many other glycopeptides as well (ristocetin,[9] actaplanin,[8] and probably A35512B[10]). The ring B phenol has no ortho or para protons, but this is the only site remaining for attachment of the *N*-acetylglucosamine to the peptide core. The teicoplanin factors have differing acyl chains, and they are reported to be converted to a common degradation product at low pH (loss of ~300 units in molecular weight).[6] Sequential hydrolysis studies on the actaplanin glycopeptides have shown that the first major hydrolysis step at low pH is loss of the sugar moiety attached to the B-4 phenol of the aglycon.[8] If the hydrolysis path is the same for the teicoplanins, they should each lose acylglucosamine from position B-4 and be converted to a common product, as is reported (loss of 315 mass units would be expected for the major teicoplanin factor).

The attachment of mannose to phenol D-5 and of acylglucosamine to phenol B-4 of the teicoplanin aglycon leads to **11**



11

as the common structure of the teicoplanin glycopeptides. The resonances of the acyl-chain protons are overlapped to a considerable extent, but decoupling experiments suggest that the $C_{10}H_{19}O$ group on the major teicoplanin factor is an isodecanoyl chain, **12**. The chemical shifts (in ppm) are listed below on the structure.

$$-CO-\underset{2.02}{CH_2}-\underset{1.43}{CH_2}-\underset{1.14-1.26}{(CH_2)_4}-\underset{1.44}{CH}-\underset{0.83}{(CH_3)_2}$$

12

Conclusions

The teicoplanins are new members of the glycopeptide class of antibiotics; they have the general structure **11**. They are unique among the glycopeptides in containing, in addition to sugars and a complex peptide core, a long acyl chain. The factors differ only in the identity of their acyl groups; for the major factor the group is a branched $-C_{10}H_{19}O$ (isodecanoyl). The common teicoplanin aglycon is identical with antibiotic A41030B; attached to this core are three substituents: *N*-acetylglucosamine at the A-1′ benzylic hydroxyl group, *N*-acylglucosamine at the B-4 phenol, and mannose at the D-5 phenol.

Acknowledgment. We thank Lina Coronelli of Gruppo Lepetit for the sample of teicoplanin (teichomycin A_2 complex). Most of the NMR spectra described in ths report were recorded by T. K. Elzey, and Gerry Wells has been especially helpful in the preparation of the manuscript.

Registry No. **1**, 91032-25-6; **11** (acyl = $-CO(CH_2)_6CH(CH_3)_2$), 91032-26-7; teicoplanin complex, 61036-64-4.

Structure Elucidation of the Teicoplanin Antibiotics

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Abstract: By a combination of chemical, ^1H and ^{13}C NMR, and mass spectrometric studies, the structures of the five major components of the antibiotic teicoplanin, produced by *Actinoplanes teichomyceticus*, have been elucidated. The components all have structures closely related to those of the glycopeptide antibiotics of the vancomycin group. The teicoplanin components are differentiated from one another by the presence of various *N*-acylglucosamine moieties, where the acyl group is a C_{10} or C_{11} fatty acid. Such groups are not present in any other known member of the vancomycin group.

The teichomycins are antibiotics produced by a recently discovered species of actinomycete, *Actinoplanes teichomyceticus*.[1] They have been separated into two major components, teichomycin A_1 and teicoplanin (the major component of which was formerly

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named teichomycin A_2).[2] Teichomycin A_1 appears to be a phosphoglycolipid antibiotic, while teicoplanin was already thought, when the present work was started, to be a member of the vancomycin group of glycopeptide antibiotics. Preliminary evidence for the latter hypothesis was obtained from studies of the biological properties of the antibiotic (i.e., activity against Gram-positive bacteria by inhibition of cell wall synthesis) and by chemical studies which showed that teicoplanin contained peptide and sugar moieties.[2]

Teicoplanin is of particular interest because in vitro studies show it to be more active than vancomycin (minimum inhibitory concentrations are 2–8-fold smaller)[3] against many Gram-positive bacteria and in vivo studies show that effective doses for mice may be more than an order of magnitude lower than those of vancomycin; moreover, teicoplanin is of low toxicity.[1]

Recent work has shown that teicoplanin's major component (T-A2) consists of five major factors, designated T-A2-1 to T-A2-5, and small amounts of other compounds.[4] Our studies have been confined to determining the structures of the above five major factors of the T-A2 complex. In practice this has involved determining also the structures of simpler compounds, obtained by partial hydrolysis of the T-A2 complex. We have used a combination of ^1H and ^{13}C NMR, fast atom bombardment mass spectrometry (FAB MS), chemical degradations, and gas chromatography-mass spectrometry (GCMS) to elucidate the structures.

Experimental Section

Samples of teicoplanin, its separate factors, and partial hydrolysis products were supplied by Gruppo Lepetit S.p.A., Milan, Italy.

FAB mass spectra were recorded on a Kratos MS-50 instrument fitted with a standard FAB source and a high-field magnet. The sample (~10 nmol) was dispersed in a few microliters of α-thioglycerol: diglycerol (1:1, v/v) matrix and bombarded with a 6-9 keV beam of Xe atoms. Good negative ion FAB mass spectra of the antibiotics and derivatives were recorded also when α-aminoglycerol was used as the matrix.

Electron impact (EI) mass spectra were recorded on an A.E.I. MS 902 instrument.

^1H NMR spectra were usually obtained by using a modified Bruker WH-400 spectrometer operating in the Fourier transform mode. Solutions were approximately 14 mM in $[^2H_6]Me_2SO$, prepared from samples previously dried in vacuo over P_2O_5, sometimes after lyophilization from D_2O. Spectra were obtained over the temperature range ambient to ~70 °C. Typical accumulations were made using a spectral width of 4000-5200 Hz, acquisition in 8K data points, and quadrature detection and phase alternation. For the measurement of chemical shifts and coupling constants, appropriate Gaussian multiplication of the FID was used. NOE and decoupling difference spectra were acquired by using standard techniques; no line broadening was required. ^{13}C NMR spectra were obtained at 100.6 MHz and also at 62.9 MHz on a Bruker WM-250 instrument. Spectra of antibiotics were measured at ambient temperature by using $[2H_3]MeCN/H_2O$, 1:1, v/v, as solvent and sample concentrations of 20–60 mM (10-mm o.d. tube, 62.9 MHz) or down to 10 mM (5-mm o.d. tube, 100.6 MHz). Accumulations were made with spectral widths of 13 000–14 000 Hz (62.9 MHz) or 20 000 Hz (100.6 MHz) and acquisition in 8K or 16K data points. For DEPT[5] spectra and spectra of only quaternary carbons[6] (QONLY), the following applied. At 62.9 MHz the 90° pulse for both ^{13}C and ^1H was ~25 μs. At 100.6 MHz the 90° pulses were, for ^{13}C, 13 μs and, for ^1H, 19 μs with the 5-mm probe and, for ^{13}C, 23.5 μs and, for ^1H, 34 μs with the 10-mm probe. The delay, $1/(2J)$, was set at 3.7 ms for DEPT and 3.3 ms for QONLY sequences. ^{13}C chemical shifts are quoted relative to CD_3CN = 1.2 ppm. ^{13}C NMR spectra of fatty acids were measured from samples in $CDCl_3$. HPLC was performed on a Spherisorb 10 ODS reversed-phase column on a Varian 5000 machine.

GLC was performed using a column of 3% OV17 on Supelcoport 80–100M m (sugar derivatives) or 10% DEGS on Unisorb AW 80–100M (fatty acid derivatives) on a Perkin-Elmer Sigma 4B machine or using a capillary column of carbowax 20M (decenoic acids) on a Carlo Erba 6130 machine.

GCMS was performed using a column of SE-54 and a programmed temperature rise (100–280 °C) on a Finnigan 1020 instrument. Mass spectra were automatically taken at 1-s intervals using both EI (70 eV) and chemical ionization (CI, methane) conditions.

Preparative TLC was carried out on Merck plates coated with silica GF_{254}. The solvent was $CHCl_3$ or 10% MeOH in $CHCl_3$, and visualization was by UV light.

Solutions of diazomethane in ether were prepared from Diazald/KOH.

Hydrolysis of T-A3-2. T-A3-2 (10 mg) was dissolved in aqueous HCl (6 M, 2 mL), and the solution was heated at 105 °C in a sealed tube for 10 min. After cooling, the unsealed contents were freeze-dried. HPLC was performed using 5–75% CH_3CN in 5% aqueous AcOH. Three fractions were collected, which gave compounds having molecular weights of 1400 (starting material), 1197 (aglycon), and 1214 (unidentified), as determined by FAB MS. The hydrolysate also yielded a product, which was identified as glucosamine by TLC in four solvent systems using authentic glucosamine as standard. The glucosamine was further characterized by subjecting a similar hydrolysate to acetylation/silylation.[7] GLC showed peaks at essentially the same retention times (4.2, 10.3 min) as those from similarly derivatized commercial glucosamine (4.1, 10.2 min).

Acetylation, Methylation, and Hydrolysis of T-A2-2 (T-A3-1). T-A2-2 (100 mg) was treated with Ac_2O/H_2O (1:1, 12 mL) for 2 h (until no ninhydrin positive species remained) to give a 10:1 mixture (by HPLC) of mono- and diacetylated T-A2-2, $(M + Na)^+$, 1942 and 1984. The product was dissolved in CH_3CN (30 mL), CH_3OH (30 mL),and H_2O (6 mL) and treated with excess ethereal diazomethane for 14 h [until the product gave no bathochromic shift in its UV spectrum (λ_{max} in H_2O = 265 nm) on addition of NaOH]. The largest peak in the FAB spectrum was $(M + Na)^+$, 2012 (corresponding to addition of five methyl groups, i.e., one to a carboxyl and four to phenolic positions).

The protected T-A2-2 was hydrolyzed in aqueous HCl (6 M, 50 mL) for 8 h at 105 °C under N_2. After centrifugation, the supernatant yielded a product giving only $(M - H)^-$, 375 (corresponding, in T-A2-2, to having one phenolic position blocked on the diphenyl unit and/or none blocked on the diphenyl ether).

Further acetylation with Ac_2O (5 mL), H_2O (5 mL) and $(C_2H_5)_3N$ (0.1 mL) overnight and methylation with methanolic HCl (0.1 M, 50 mL) overnight was followed by preparative TLC. This gave two products (R_f 0.72 and 0.68) which, in their EI mass spectra, both had molecular ion peaks at m/z 488. These ions correspond to the derivatives anticipated from a diphenyl ether fragment with both its phenolic groups originally unblocked and from a biphenyl fragment with one of its phenolic groups blocked.

Treatment of T-A3-1 in a similar way showed that it contained five free phenolic groups but from the molecular ions seen after hydrolysis, $(M - H)^-$, 375 and 361, and after further protection, m/z 488, 502, and 474, it appeared that some sugar was on the diphenyl ether.

Oxidative Degradation of T-A2-2 (T-A2). Anhydrous K_2CO_3 (0.2 g) was added to a suspension of T-A2-2 in CH_3OH (10 mL) and CD_3I (2 mL). The mixture was heated under reflux at 80 °C for 6 h. The solvent was evaporated and the solid residue was suspended in H_2O (2 mL), collected by filtration, washed with H_2O (2 × 1 mL), and dried in vacuo over P_2O_5.

The perdeuteriomethylated T-A2-2 was added to 2 M HCl/CH_3CN (1:1, v/v, 2 mL) preheated to 80 °C and was heated for 45 min. The reaction mixture was cooled to 0–5 °C and freeze-dried, and remethylation was performed with CH_3I in place of CD_3I using the conditions described immediately above.

The remethylated product was suspended in H_2O (5 mL), and a solution of $KMnO_4$ (0.6 g) in H_2O (11 mL) and 2 M NH_3 (1.75 mL) was added. The mixture was stirred at 70–75 °C for 6 h, filtered, acidified (HCl), and extracted with EtOAc (3 × 20 mL). The extract was dried ($MgSO_4$) and evaporated. The product was dissolved in CH_3OH (1 mL), cooled in ice, and treated with an excess of ethereal diazomethane for 10 min. Solvents were evaporated and preparative TLC was performed to give fractions having R_f 0.4 and 0.34. These yielded, respectively, compounds having EI mass spectra showing m/z 534, 536, 538 (9:6:1) and m/z 227. The same ions were found for T-A2 complex. The entire procedure was repeated for both T-A2-2 and T-A2 complex with reversal of the order of use of CD_3I and CH_3I. This gave rise to compounds having m/z 537, 539, 541 (9:6:1) and m/z 224. The compounds are Ia

(1) Parenti, F.; Beretta, G.; Berti, M.; Arioli, V. *J. Antibiot.* **1978**, *31*, 276–283.
(2) Bardone, M. R.; Paternoster, M.; Coronelli, C. *J. Antibiot.* **1978**, *31*, 170–177.
(3) Varaldo, P. E.; Debbia, E.; Schito, G. C. *Antimicrob. Agents Chemother.* **1983**, *23*, 402–406.
(4) Borghi, A.; Coronelli, C.; Faniuolo, L.; Allievi, G.; Pallanza, R.; Gallo, G. G. *J. Antibiot.*, submitted for publication.
(5) Doddrell, D. M.; Pegg, D. T.; Bendall, M. R. *J. Magn. Reson.* **1982**, *48*, 323–327.
(6) Bendall, M. R.; Pegg, D. T.; Doddrell, D. M.; Johns, S. R.; Willing, R. I. *J. Chem. Soc., Chem. Commun.* **1982**, 1138–1140.
(7) Hara, S.; Matsushima, Y. *J. Biochem.* **1972**, *71*, 907–909.

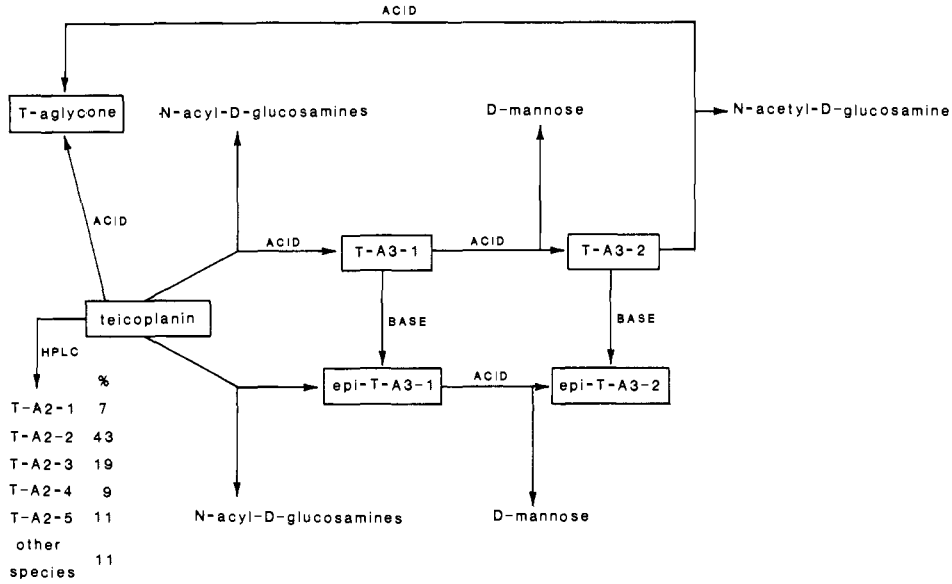


Figure 1. Interrelationship of teicoplanin, T-A2 factors,[4] and hydrolysis products.[16,17]

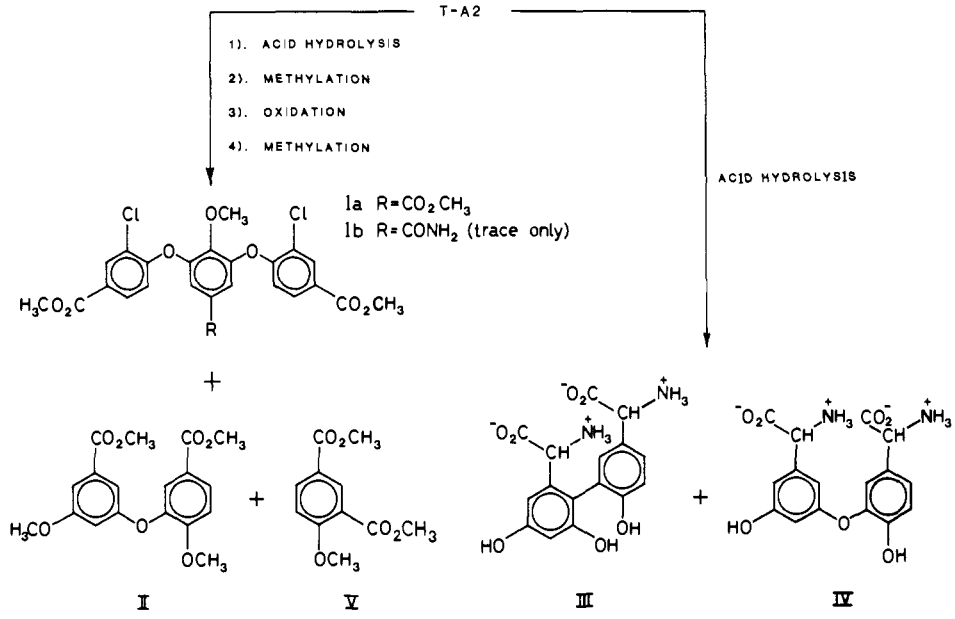


Figure 2. Fragments obtained by chemical degradations[18] of T-A2.

and V (Figure 2). For Ia, the ratio of molecular ion abundances reflects the chlorine substitution. If higher molecular weight ions were obtained when CD_3I was used first, this indicates that the fragment carried no sugar in the intact antibiotic and vice versa.

Identification of Fatty Acids.[8] **Isolation of Acids and Conversion to Methyl Esters.** T-A2 complex (or isolated factor), 5–10 mg, was hydrolyzed in aqueous HCl (2 M, 10 mL) under *n*-hexane (7 mL) and N_2 at 80 °C overnight. The cooled mixture was separated, and the hexane layer was washed with H_2O (5 mL), dried, and evaporated to give fatty acid, which was esterified with ethereal diazomethane to give methyl esters for GLC and GCMS analysis.

***N*-Pyrrolidides.**[9] A sample of methyl ester (10–50 μg) was heated in pyrrolidine/AcOH (10:1, 0.5 mL) for 40 min at 110 °C in a sealed vessel. The cooled mixture was extracted with CH_2Cl_2 (2 × 5 mL), and the combined extracts were washed with dilute HCl (2 mL) and H_2O (2 mL), dried, and evaporated to give *N*-pyrrolidides for GLC or GCMS analysis.

Standard Acids. *N*-Decanoic acid was commercially available. The other saturated acids were prepared by modified literature procedures using oxidation[10] of appropriately substituted cyclohexanols or cyclopentanols, followed by reduction using the Huang–Minlon modification of the Wolff–Kishner reaction. Thus, 8-methylnonanoic acid was prepared from 1-(isopentyl)cyclopentanol, 9-methylnonanoic acid from 1-(isopentyl)cyclohexanol, and 8-methyldecanoic acid from 1-(3-methylpentyl)cyclopentanol.

(*E*)-4-Decenoic acid was prepared by a literature procedure.[11] (*Z*)-4-Decenoic acid was prepared using the *Z* alkenyl cuprate[12,13] derived from di-*n*-pentyl cuprate and acetylene, which was quenched with ethylacrylate[14] in a one-pot preparation of the ethyl ester. This ester was then hydrolyzed to the free acid.

Ozonolysis: T-A2-1. Ozonolysis of the acid from T-A2-1 in the presence of BF_3/MeOH[15] gave dimethyl succinate, which was compared by GLC with both authentic dimethyl succinate and with the product of ozonolysis of prepared (*E*)-4-decenoic acid. All three samples had the same retention times.

Discussion and Results

It is helpful in the first instance to consider the scheme shown in Figure 1, which indicates the interrelationship of the species

(8) A more detailed account will be published elsewhere.
(9) Andersson, B. A.; Holman, R. T. *Lipids* **1974**, *9*, 185–190.
(10) Fieser, L. F.; Szmuszkovicz, J. *J. Am. Chem. Soc.* **1948**, *70*, 3352–3355.
(11) Ireland, R. E.; Mueller, R. H.; Willard, A. K. *J. Am. Chem. Soc.* **1976**, *98*, 2868–2877.
(12) Normant, J. F.; Alexakis, A. *Synthesis* **1981**, 841–870.
(13) Fujisawa, T.; Sato, T.; Kawara, T.; Naruse, K. *Chem. Lett.* **1980**, 1123–1124.
(14) Näf, F.; Degen, P. *Helv. Chim. Acta* **1971**, *54*, 1939–1949.
(15) Ackman, R. G. *Lipids* **1976**, *12*, 293–296.

Table I. Molecular Weights of T-A2 Factors and Acid Hydrolysis Products of Teicoplanin

species	M_r	species	M_r
T-A2-1	1875	T-A2-5	1891
T-A2-2	1877	T-A3-1	1562
T-A2-3	1877	T-A3-2	1400
T-A2-4	1891	T-aglycon	1197

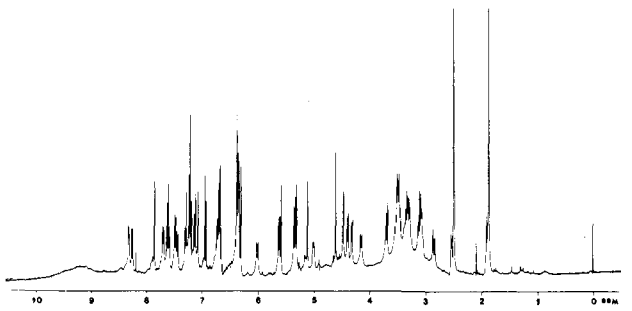


Figure 3. 400-MHz ^1H NMR spectrum of T-A3-2 in $[^2H_6]Me_2SO$ at ~60 °C.

we have studied. The transformations using basic conditions were carried out relatively recently and will be described in more detail elsewhere.[16] Hence most of the structural elucidation was carried out using the samples that were available earlier. The first samples studied were the acid hydrolysis products T-A3-1 and T-A3-2,[17] together with the separated factors T-A2-1 to T-A2-5.[4]

At the time we initiated our investigations, T-A3-2 was thought to be an aglycon as it was known to be formed with concomitant loss of D-glucosamine and D-mannose and no other sugars had been detected in a total hydrolysis. As the sugar aglycon ratio was not determined quantitatively in early work,[2] it was thus assumed there were only two sugar moieties altogether.

Most of the other important information available to us about the chemical nature of T-A2 is summarized in Figure 2. The fragments that have been obtained[18] account for seven different aromatic rings. Compounds Ia,b have also been obtained from degradations of vancomycin,[19] while compound III has been found for all known members of the vancomycin group. Compounds II and IV have identical oxygen substitution patterns with those found for analogous fragments from ristocetin,[20] actaplanin (A4696),[21] and antibiotic A35512B.[22] They lack the methyl group found for ristocetin or the chlorine atom found for A35512B.

The last useful piece of chemical evidence available was that T-A2 has one strongly acidic and one basic function;[2] i.e., the antibiotic factors each carry one free carboxyl and one free amino group only.

Structure of the Aglycon. It was appropriate to establish first that the aglycon was indeed similar to the aglycons of the other members of the vancomycin group. The molecular weights of T-A3-2, T-A3-1, and the T-A2 factors were first determined by FAB MS (Table I).

It was immediately apparent that, contrary to expectation, T-A3-2 could not be an aglycon as the "addition" of the fragments Ib, III, and IV (Figure 2), to give a vancomycin/ristocetin-type structure, would give a molecular weight of about 1200, not 1400 as found. At the same time, ^1H and ^{13}C NMR clearly indicated the presence of a sugar moiety; thus T-A3-2 was found to be a pseudoaglycon (ψ-aglycon). Most work on the structure of the aglycon has nevertheless been carried out on T-A3-2 because the true aglycon (T-aglycon) has only recently been prepared in useful amounts.[17]

The ^1H NMR spectrum of T-A3-2 is reproduced in Figure 3. Assignment of the aglycon portion was achieved by using decoupling difference, nOe difference, and variable-temperature studies, an approach used previously for vancomycin[23] and ristocetin.[24] The data are given in Tables II and III and the derived

Table II. Chemical Shifts and Coupling Constants of the Carbon-Bound Protons of the Aglycon Portion of T-A3-2[a] Compared with Analogous Data for Ristocetin[24] and Vancomycin[23]

	T-A3-2		ristocetin A		vancomycin	
proton[b]	δ[c]	J[c]	δ	J	δ	J
x_1	4.6	s[d]	4.83	s	nc	nc
x_2	4.97	ddd	5.09	dd	4.86	dd
x_3	5.34	10.4	5.25	10	nc	nc
x_4	5.60	8.2	5.65	8.2	5.71	8
x_5	4.30	5	4.73	6.5	4.50	5
x_6	4.13	12	4.38	12	4.22	12
x_7	4.46	6.1	4.55	5	4.50	5
z_2	3.31	dd	nc	nc	nc	nc
z_2'	2.84	13.5	nc	nc	nc	nc
z_6	5.30	s	5.17	s	5.13	s
1b	6.67	s	6.59	s		
1e	7.11	8.3	7.18	~8, ~2		
1f	6.92	8.3	7.03	~8		
2b	7.26	s	7.12	nc	7.42	s
2e	7.18	~8	7.25	8	7.20	8
2f	7.60	0	7.86	~8	7.57	8
3b[e]	6.33	s	6.45	s		
3d	6.39	s				
3f	6.29	s	6.42	s		
4b	5.57	s	5.38	s	5.63	s
4f	5.10	s	5.33	s	5.21	s
5b	7.05	s	7.26	2	7.19	s
5e	6.65	8.4	6.77	8	6.73	8
5f	6.70	8.4	6.84	8, ~2	6.78	8
6b	7.84	s	7.55	nc	7.87	s
6e	7.21	8.4	7.20	8	7.28	8
6f	7.27	8.1	7.41	8.2	7.48	8
4d	6.36	s	6.85	2	6.30	2
7f	6.29	s	6.32	2	6.44	2

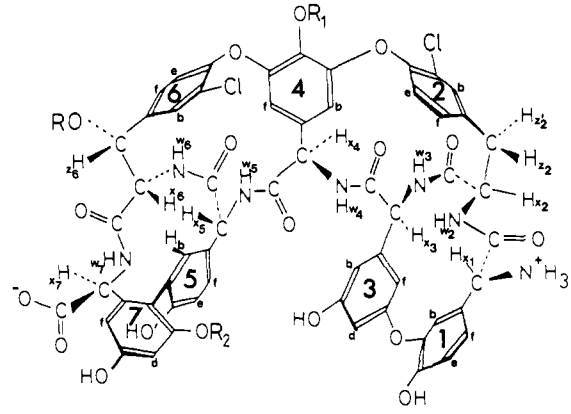
[a] Reported values measured at 60 °C, 400 MHz. [b] The nomenclature used here represents a new proposal for systematic nomenclature that can be used for both ^1H and ^{13}C data for vancomycin group antibiotics. Residues are numbered starting at the N-terminus; CO groups are designated y_n; lettering of aromatic carbons/protons is such that substituted carbons are reached first in the lettering system. [c] δ, in ppm; J, in Hz. [d] Abbreviations used: s = singlet, nc = not comparable, (d)dd = (doublet of) doublet of doublets, 0 = not measured due to overlap. [e] The resonances of the protons of rings 3 and 7 overlap and are very pH dependent; assignments are ambiguous.

Table III. Chemical Shifts and Temperature Dependences of Amide Protons of T-A3-2 with Analogous Data on Temperature Dependences for Ristocetin A[26] and Ristocetin ψ-Aglycon[27]

	T-A3-2		ristocetin A	ristocetin ψ-aglycon
proton	δ[a]	$\Delta\delta/\Delta T$[b]	$\Delta\delta/\Delta T$	$\Delta\delta/\Delta T$
w_2	7.41	−3	−1.5	0.7
w_3	7.57	−2.5[c]	−0.5	0
w_4	7.46	−4.5[c]	−3	−2.5
w_5	8.30	−6	−6	−7
w_6	5.99	−0.85	−5.5	−3
w_7	8.24	−2.4	−3	−2.5
w_8[d]	7.67	−3		

[a] Data obtained at 60 °C. [b] ppm/(K × 10^3). [c] Approximate due to overlap. [d] w_8 = sugar NH; not found in ristocetin.

(16) Barna, J. C. J.; Williams, D. H.; Strazzolini, P.; Malabarba, A.; Leung, T.-W.C., manuscript in preparation.
(17) Malabarba, A.; Strazzolini, P.; Depaoli, A.; Landi, M.; Berti, M.; Cavalleri, B. *J. Antibiot.*, submitted for publication.
(18) Coronelli, C.; Bardone, M. R.; Depaoli, A.; Ferarri, P.; Tuan, G.; Gallo, G. G., manuscript in preparation.
(19) Smith, K. A.; Williams, D. H.; Smith, G. A. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2369–2376.
(20) Harris, T. M.; Fehlner, J. R.; Raabe, A. B.; Tarbell, D. S. *Tetrahedron Lett.* **1975**, *31*, 2655–2658.
(21) Hunt, A. H.; Debono, M.; Merkel, K. E.; Barnhart, M. *J. Org. Chem.* **1984**, *49*, 635–640.
(22) Debono, M.; Malloy, R. M.; Barnhart, M.; Dorman, D. E. *J. Antibiot.* **1980**, *33*, 1407–1416.
(23) Williams, D. H.; Kalman, J. R. *J. Am. Chem. Soc.* **1977**, *99*, 2768–2774.



T-aglycone R=R_1=R_2=H

T-A3-2 R=N-acetyl-β-D-glucosamine ; R_1=R_2=H

T-A2 factors (1 to 5) R=N-acetyl-β-D-glucosamine ; R_1=N-acyl-β-D-glucosamine ; R_2=α-D-mannose

Figure 4. Structure of teicoplanin and its acid hydrolysis products, showing the proton nomenclature.

structure with key to the proton nomenclature in Figure 4. Discussion of the elucidation of the nature and position of attachment of the sugar is given separately below, for clarity, though in practice the aglycon and sugar structures were determined concurrently.

Table II shows first that there is a striking agreement between the chemical shifts of analogous carbon-bound protons in T-A3-2, ristocetin, and vancomycin. Most shifts agree to within 0.2 ppm except where there are obvious structural differences such as the absence of chlorine atoms in ristocetin. Secondly, the coupling constants $^3J_{HH}$ between the NH and α-CH protons of the peptide backbone of T-A3-2 are as similar to those of either vancomycin or ristocetin as are the values for the latter two compounds to each other.

The absolute values of the NH chemical shifts (δ) (Table III) are not compared with those for ristocetin or vancomycin as these shifts are always very pH dependent. However, the temperature dependences ($\Delta\delta/\Delta T$) are compared with those for ristocetin and its ψ-aglycon. Comparison of the figures for the latter two compounds shows that the parameter $\Delta\delta/\Delta T$ is less constant with respect to structural similarities than is δ for carbon-bound protons. It is, however, usual when considering the parameter $\Delta\delta/\Delta T$ for peptide NHs in dimethyl sulfoxide solution to classify values as "small" (0 to −3 ppm/(K × 10^3)) or "large" (−5 or more ppm/(K × 10^3)) sometimes with a "medium" range inserted for equivocal values. Thus small changes in δ indicate shielding of the proton from solvent while large changes indicate exposure to solvent. Thus proton ω_5 in T-A3-2 is at the exposed "back" of the molecule as is the analogous proton in ristocetin; while ω_7, ω_3, ω_4, and ω_2 are in the "front" cleft, shielded to varying degrees from the solvent. Proton ω_6 does not behave like its ristocetin analogue, for reasons discussed below.

Thus the values of $^3J_{HH}$ and $\Delta\delta/\Delta T$ obtained provide very good evidence that the stereochemistry of the teicoplanin molecule is as depicted in Figure 4; that is, the absolute configurations at the chiral centers are the same as those of ristocetin, vancomycin, and the recently studied A35512B.[25] Further evidence for the stereochemistry at most positions in the molecule was obtained by studies of nOes between protons, including their rate of growth, that is, time-dependent or kinetic nOes. The strategy followed

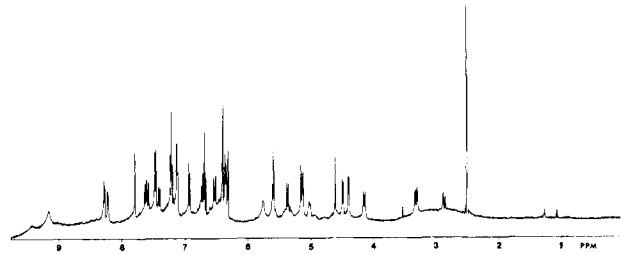


Figure 5. 400-MHz ^{1}H NMR spectrum of T-aglycon in [$^2H_6$]Me_2SO at ~60 °C.

has been described previously.[28] NOES were measured for both normal solutions of T-A3-2 in dimethyl sulfoxide and for samples in which about 90% of exchangeable protons were previously replaced by deuterium by lyophilization from D_2O. Close examination of earlier work[28] shows that, under the conditions used in our work, any $t_{1/2}$ (half-life for the exponential buildup of an nOe) of about 0.2–1 s must indicate that the protons involved are about 2.0–2.7-Å apart. Thus we have, in the present work, used the observation of any such fast growing nOe to indicate such proximity without attempting to derive exact distances. This approach has shown that patterns of observed nOes are very similar to those for vancomycin[28] and ristocetin,[24] particularly among the "nest" of protons on the left of the molecule (Figure 4, protons z_6, 6b, x_6, x_5, 5b, w_7). This evidence, added to that derived from δ, $^3J_{HH}$, and $\Delta\delta/\Delta T$ values means that we can be confident that the stereochemistry is the same for all analogous asymmetric centers. The stereochemistry of the problematic N-terminus has recently been proven chemically for ristocetin,[29] and for completeness this may profitably be done for teicoplanin. (This is the asymmetric center in these two antibiotics for which a value of $^3J_{HH}$ is not available and for which nOe evidence is not very clear). For all other asymmetric centers, we consider the evidence obtained using ^{1}H NMR to be satisfactory.

One feature of the aglycon not yet discussed is the methylene group on the top right of the molecule (Figure 4). This group, of which only one proton gave a clear resonance in the ^{1}H spectrum of T-A3-2, was at first thought to be part of the unidentified sugar, presumed to be analogous to ristosamine. In the course of decoupling and nOe experiments, it became obvious that the structure was, however, as shown. First, the two protons of the group, z_2 and z_2', are coupled to proton x_2, which thus gives rise to an octet (doublet of doublets of doublets) rather than a doublet of doublets as for ristocetin. Second, protons z_2 and z_2' gave quickly growing nOes to protons 2f and 2b, respectively, and to proton x_2. Third, proton z_2, hidden under the sugar region, was revealed both in the nOe difference spectra of T-A3-2 and when T-aglycon was eventually obtained. The spectrum of T-aglycon is given in Figure 5 where it can be seen that the signals in question are clearly revealed (2.9 and 3.3 ppm).

Lastly, the DEPT ^{13}C NMR spectrum showed a methylene group at 37.1 ppm, very similar to that of an analogous signal from a model compound.[30] An analogous methylene group has been found in actaplanin[21] but in no other member of the vancomycin group. The proof of the position of the methylene group had obvious consequences for the identity of the sugar, given that the molecular weight of T-A3-2 was known to be 1400.

Structure and Position of the Sugar in T-A3-2. Evidence for the nature of the sugar has already been alluded to: First, the molecular weight of T-A3-2 was larger than that of the postulated aglycon and ^{1}H and ^{13}C NMR spectra had shown signals ascribed to a sugar. It should be noted also that no sugar other than D-glucosamine and D-mannose had been found in a hydrolysis of T-A2.[2] Having found that the aglycon contained a methylene group, it was now clear that the sugar was not a deoxy sugar.

(24) Kalman, J. R.; Williams, D. H. *J. Am. Chem. Soc.* **1980**, *102*, 897–905.
(25) Hunt, A. H. *J. Am. Chem. Soc.* **1983**, *105*, 446–468.
(26) Kalman, J. R.; Williams, D. H. *J. Am. Chem. Soc.* **1980**, *102*, 906–912.
(27) Rajananda, V. Ph.D. Thesis, Cambridge, 1979.
(28) Williamson, M. P.; Williams, D. H. *J. Am. Chem. Soc.* **1981**, *103*, 6580–6585.
(29) Harris, C. M.; Harris, T. M. *J. Am. Chem. Soc.* **1982**, *104*, 363–365.
(30) Johnson, L. F.; Jankowski, W. C. "Carbon-13 NMR Spectra"; Wiley-Interscience: New York, 1972; spectrum 435.

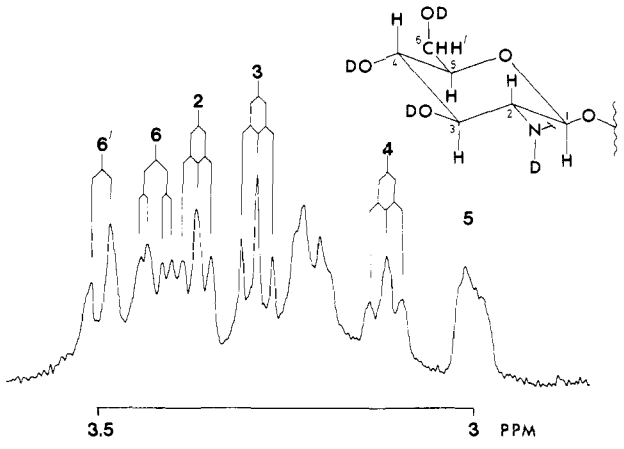


Figure 6. Assignment of the "sugar region" of the ^1H NMR spectrum of T-A3-2 (previously lyophilized from D_2O) to *N*-acetyl-β-D-glucosamine.

Second, the ^{13}C NMR spectrum of T-A3-2 showed eight carbonyl resonances rather than the expected seven and a methyl signal at 23.5 ppm. Third, the ^1H NMR spectrum contained not only a complex sugar envelope, but an anomeric proton, H_1 (δ 4.38, J = 7.8 Hz) coupled to this region. Fourth, the ^1H NMR spectrum contained an amide proton signal (w_8, Table II; J = 8 Hz) coupled to the same region and a methyl resonance at 1.87 ppm.

It seemed likely, therefore, that the sugar contained an acetylamino group. Fortuitously, when T-A3-2 was lyophilized from D_2O, the resulting spectrum rendered an analyzable sugar region (Figure 6) and the sugar was thus determined, by ^1H NMR alone, to be *N*-acetylglucosamine. This was subsequently confirmed by hydrolyses of T-A3-2, which gave at first glucosamine (Experimental Section) and eventually intact *N*-acetyl-D-glucosamine.[17] The molecular weight of T-A3-2, compared with that of the postulated aglycon (and later with that of T-aglycon itself), showed that there was indeed no difference in the molecular weights except that due to "condensation" of an *N*-acetylglucosamine with an OH group of the aglycon (Table I).

The sugar is clearly linked as the β anomer as only this configuration would give the large (trans-diaxial) coupling constant observed for the anomeric proton, H_1, and H_2 (Figure 6). This is the most conclusive evidence, but it is supported by the value of $^1J_{CH}$ = 164 Hz for the anomeric carbon, C_1, seen in a fully proton coupled ^{13}C NMR spectrum, which can be compared with values for model sugars.[31] Lastly, 1,3-diaxial nOes were seen between H_1 and H_3 and H_5 (Figure 6), which can only occur in the β anomer.

The position of the sugar was readily determined to be at the β-hydroxytyrosine oxygen, as had been tentatively expected by analogy with the ψ-aglycon of ristocetin. First, the anomeric proton gave a clear nOe to proton z_6, Figure 4 (and hence, by spin diffusion, to the "nest" of protons mentioned above). Second, it was noted above that proton w_6 does not have the large temperature dependence expected by analogy with ristocetin. It also has an unusually upfield chemical shift. These two pieces of data for w_6 were ascribed to shielding of this proton by the *N*-acetylglucosamine moiety, and this hypothesis was confirmed when T-aglycon became available. It was found that the proton analogous to w_6 now resonated at 6.7 rather than 6.0 ppm, and, more importantly, its $\Delta\delta/\Delta T$ value was now −5 ppm/(K × 10^3), showing it was now exposed to solvent. Lastly, in the ^1H NMR spectrum of T-aglycon an OH proton was found at 5.8 ppm (Figure 5) coupled to the analogue of z_6 (to which the anomeric H_1 had given an nOe in T-A3-2).

At this stage, therefore, the structures of T-aglycon and the ψ-aglycon, T-A3-2, were established. As both compounds were formed by hydrolysis of the *whole complex*, T-A2, we could be confident that we also knew the structures of the aglycon and ψ-aglycon of *each individual* T-A2 factor. Using similar arguments, it appeared that study of T-A3-1 would show the position of the D-mannose in all factors, since this sugar was known to be lost on formation of T-A3-2 from T-A3-1 (Figure 1) and the concomitant change in molecular weight exactly fitted the loss of such a sugar (Table I). In fact, T-A3-1, although appearing homogeneous by HPLC, FAB MS, or even ^{13}C NMR, appeared to be inhomogeneous by ^1H NMR.

It seemed that some mannose may have migrated during the mild hydrolysis conditions used to isolate T-A3-1, thus producing isomers. The isomerism was clearly not due to differing positions of the mannose in the individual factors for two reasons. First, the same type of mixture was seen in the ^1H NMR spectrum of T-A3-1 prepared[17] specifically from T-A2-2. Second, inhomogeneity was not found in the case of epi-T-A3-1 (the basic hydrolysis product known to have the same molecular weight as T-A3-1[16]) when it was prepared directly from teicoplanin.

Anomeric Configuration and Position of the Mannose. As a result of the inhomogeneity problem mentioned above, proof of the mannose configuration and position was made harder than anticipated. The anomeric proton gave rise to a singlet in the ^1H NMR spectra of epi-T-A3-1 and the T-A2 factors at ~5 ppm. The anomeric carbon gave a signal in the ^{13}C NMR spectrum of T-A3-1 at 97.5 ppm. At this stage, the possibility existed that the mannose was either attached to the *N*-acetylglucosamine to make a disaccharide or to one of the phenolic positions. Acetylation and methylation (Experimental Section) showed there were only five free phenolic OHs in T-A3-1, so the mannose had to be attached to a phenolic oxygen. The hydrolysis indicated that in T-A3-1 the mannose was on the biphenyl unit but suggested a minor proportion was on the diphenyl ether.

In view of this inhomogeneity, evidence for the exact position of the mannose had to be sought from the T-A2 factors and later from epi-T-A3-1. Hydrolysis of protected T-A2-2 had shown that a sugar was attached to the biphenyl, though this did not prove it was mannose rather than the additional glucosamine.

However, in the case of the factors pure enough to give clean ^1H NMR spectra (T-A2-3 is contaminated with T-A2-2, and T-A2-4 and T-A2-5 are slightly mutually contaminated) and for epi-T-A3-1, nOe evidence could be used to prove the position of the mannose.

For all these compounds, the mannose anomeric proton gave a clear mutual nOe to a proton identified as either 7d or 7f (Figure 4). It was not found possible to prove to which phenolic oxygen of ring 7 the mannose was attached by using irradiation of phenolic OHs and observations of nOes from them. (This had been achieved for ristocetin.)[24] However, proof of the position of the mannose was obtained by using ^1H NMR studies of the binding between T-A2-2 and the bacterial cell wall analogue *N*-Ac-D-Ala-D-Ala. The mannose anomeric proton and the N-terminal alanyl methyl gave clear mutual intermolecular nOes exactly analogous to those recently described for ristocetin binding to *N*,*N*′-Ac_2-L-Lys-D-Ala-D-Ala.[32] It was thus concluded that the mannose is in an exactly analogous position to that in ristocetin, namely, as depicted in Figure 4. Moreover, this nOe evidence helped prove the anomeric configuration of the sugar; models show that only the α anomer (found in ristocetin) can give such an nOe. Further evidence was obtained from the value of $^1J_{CH}$ = 173 Hz for the anomeric carbon, which can be compared with values for models[31] or ristocetin.[33] It is shown elsewhere[16] that the aglycon of epi-T-A3-1 is isomeric with that of the T-A2 factors, and hence, strictly, nOe evidence derived form epi-T-A3-1 is not applicable to the factors. However, it is certain that the isomerism does not affect that portion of the molecule containing the mannose. Hence, having seen nOes for epi-T-A3-1 analogous to those seen for the relatively pure factors, we could assume that the mannose is in

(31) Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.

(32) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332–1339.

(33) Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 1* **1981**, 1483–1491.

Table IV. Application of Empirical Chemical Shift Calculations[34] to ^{13}C NMR Signals of Postulated Fatty Acid Residues

assignment	obsd[a]	calcd
	8-Methylnonanoyl	
C_2	37.1	34 ± 5[b]
C_3	29.6	25 ± 2
C_4	36.8	30 ± 3
C_5	29.8	29.96
C_6	27.6	27.02
C_7	39.4	39.35
C_8(CH)	28.3[c]	27.62
C_9 + 8-Me	22.9[c]	23.13
	8-Methyldecanoyl	
C_8(CH)	34.7	34.59
8-Me	19.0	19.14
C_{10}(Me)	11.5	10.87
	(*Z*)-4-Decenoyl	
C_{10}(Me)	14.2	13.50

[a] In ppm relative to CD_3CN = 1.2. [b] Assuming effects of CONH are $\alpha = +20 \pm 5$, $\beta = +3 \pm 2$, $\gamma = -2 \pm 3$. [c] Shifts for C_9 and C_{10} + 9-Me in 9-methyldecanoyl (i.e., T-A2-5) are very similar, as expected.

the same place in all factors, as the epi-T-A3-1 studied was prepared from the T-A2 complex. Thus we were now confident that we knew the positions of both *N*-acetyl-β-D-glucosamine and α-D-mannose in all factors.

Structures of Additional Moieties in the T-A2 Factors. FAB MS showed that the molecular weights of the T-A2 factors were larger than the molecular weight of T-A3-1 by varying amounts, all of which were larger than could be accounted for by the "condensation" of one more molecule of glucosamine (known to be lost on formation of T-A3-1 from T-A2). When our studies were initiated there was, however, no chemical evidence at all for the structures of the fragments giving rise to such large molecular weights.

In solving this problem, T-A2-2 was considered first as it is the most abundant factor. ^{13}C NMR proved to be extremely useful in this instance. Apart from signals consistent with the additional glucosamine moiety, signals from two equivalent methyls, six methylenes, one methine,and one amide-like carbonyl carbon were seen. The ^1H NMR spectrum showed a methyl doublet at 0.85 ppm, a typical "methylene envelope", and an additional NH at similar δ to that of the *N*-acetylglucosamine, coupled to the sugar region. It was hypothesized that T-A2-2 might contain an *N*-acylglucosamine, analogous to the *N*-acetylglucosamine already found in all factors.

Application of the Lindeman–Adams Rule[34] to the observed ^{13}C shifts showed they were consistent with the presence of an *N*-(8-methylnonanoyl) residue, although at positions α, β, or γ to the amide link the empirical calculations become very crude as only approximate substituent shifts can be used. Agreement for methylenes at the alkane-like end of the moiety may be fortuitously good (Table IV) as the assignments are not rigorous. With this spectroscopic evidence in hand, chemical evidence for this fatty acid in T-A2-2, and for analogues in the other factors, was sought. Mild hydrolysis of T-A2 complex or any isolated factor was followed by derivatization and analyses by GCMS (Experimental Section). The hydrolysis of T-A2 gave nine fatty acids of which five represented over 99% of the total and corresponded closely in relative proportions to the five major factors of the T-A2 complex. Initial identification of these acids was made by comparison of fragmentation patterns of the methyl esters with those of standards in a computer library available in conjunction with the GCMS facilities. Assignments of acids to individual factors (Table V) were made using hydrolyses of T-A2-2, T-A2-1, and T-A2-5. Because of the known identity of pairs of molecular weights (Table I), these hydrolyses were sufficient to assign unambiguously also the acid moieties from T-A2-3 and T-A2-4. The assignments were confirmed by using standards (Experimental Section). The *N*-pyrrolidides of the fatty acids were examined also because these derivatives are known[9,35,36] to give valuable information about the positions of branch points and double bonds within fatty acids. The absolute position of the double bond in the acid from T-A2-1 was confirmed by ozonolysis. The stereochemistry, i.e., *Z*, was determined by co-elution of the methyl ester of the acid derived from T-A2-1, in a capillary column, with the ester of the synthetic *Z* isomer and not the synthetic *E* isomer (Experimental Section). NMR studies did not help significantly in the identification of fatty acid residues in the other factors, but it was noted that the methine and methyl ^{13}C chemical shifts were very characteristic (Table IV) of the type of branching found.

Table V. Fatty Acids Derived from T-A2 Factors

factor	acid	M_r	factor	acid	M_r
1	(*Z*)-4-decenoic	184	4	8-methyldecanoic	200
2	8-methylnonanoic	186	5	9-methyldecanoic	200
3	*n*-decanoic	186			

Since this work was completed, intact *N*-acylglucosamines having C_{10} and C_{11} acyl groups have been isolated.[17]

The fatty acid moieties found account for the measured molecular weights of the five T-A2 factors exactly. All the *N*-acylglucosamine moieties must be present as the β anomers, as in the ^1H NMR spectra of all factors it can be seen that the anomeric proton concerned resonates as a doublet as was found for the *N*-acetylglucosamine anomeric proton. Thus the structures of all parts of the antibiotic factors had been found and only the positions of the *N*-acylglucosamine moieties remained to be determined.

Positions of Attachment of the *N*-Acylglucosamines. Acetylation, methylation, and hydrolysis of T-A2-2 (Experimental Section) showed that there were only four free phenolic OH groups and that only one sugar, shown above to be mannose, was attached to any of the rings 1, 3, 5, or 7 (Figure 4). Hence, by elimination, the *N*-(8-methylnonanoyl)-β-D-glucosamine must be attached to the phenolic position of ring 4. ^1H NMR evidence for this was negative; no nOe was seen, in binding studies of T-A2-2 with *N*-Ac-D-Ala-D-Ala, between the glucosamine anomeric proton and the C-terminal alanyl methyl. An analogous nOe was observed for ristocetin but not for vancomycin,[32] and its absence can mean simply that the anomeric proton is not at the "front" of the molecule.

Positive chemical evidence for the position of the *N*-acylglucosamines could not be found for all the factors separately as a result of the small amounts available, but it was found for T-A2-2 and T-A2 complex. This was achieved by using oxidative degradation (Experimental Section) with careful alternation of the use of CD_3I and CH_3I in the protective methylation steps. This showed that a sugar, which must be the *N*-acylglucosamine, was attached to the triphenyl ether moiety in T-A2-2. Since no significant amount of the molecular ion due to triphenyl ether having no sugar attached was found for T-A2 complex, we can assume that all the factors have the *N*-acylglucosamine on ring 4. (A trace of compound of Ia, Figure 2, having incorporation of CD_3– when CD_3I is used for the first methylation is expected rom the small amount of T-A3-1 present in T-A2 complex.) An incidental finding from the oxidative degradation was additional evidence that mannose is on ring 7 and not ring 5 of the diphenyl ether (since CD_3– was incorporated in compound V, Figure 2, when CD_3I was used for the first methylation).

Conclusions

The structural elucidation of the teicoplanin antibiotic factors has only been made possible by the combination of classical chemical and modern spectroscopic techniques. The presence in each factor of a different fatty acid group is an interesting feature, which may account, through its effect on the transport of the

(34) Wehrli, F. W.; Wirthlin, T. "Interpretation of Carbon-13 NMR Spectra"; Heyden: London, 1978; pp 41–42.

(35) Andersson, B. A.; Holman, R. T. *Lipids.* **1975**, *10*, 716–717.

(36) Andersson, B. A.; Christie, W. W.; Holman, R. T. *Lipids* **1975**, *10*, 215–219.

antibiotics in the body, for the greater activity of teicoplanin as compared with vancomycin.

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Registry No. T-A2-1, 91032-34-7; T-A2-2, 91032-35-8; T-A2-3, 91032-36-9; T-A2-4, 91032-37-0; T-A2-5, 91032-38-1; T-A3-2, 91032-39-2; T-aglycone, 89139-42-4; teicoplanin, 61036-64-4.

Steric Course of Ketopantoate Hydroxymethyltransferase in *E. coli*†

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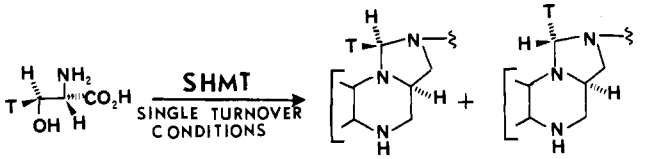
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received December 12, 1983*

Abstract: The conversion of α-ketoisovaleric acid (α-KIVA) to ketopantoate by the 5,10-methylenetetrahydrofolate-dependent enzyme ketopantoate hydroxymethyltransferase (KHMT) in *E. coli* has been shown to proceed in a retention mode at the β-position of α-KIVA. 5,10-Methylenetetrahydrofolate formed in vivo by serine hydroxymethyltransferase (SHMT) from stereospecifically deuterated (3*S*-d_1) serine was converted by KHMT into an ca. 3:1 ratio of deuterated ketopantoates with the 4*S* isomer predominating. The results indicate that KHMT and SHMT have the same overall steric course in *E. coli*.

Ketopantoate hydroxymethyltransferase (5,10-methylenetetrahydrofolate: α-ketoisovalerate hydroxymethyltransferase; KHMT) catalyzes the reversible condensation of α-ketoisovaleric acid (α-KIVA) (**1**) with the biochemical equivalent of formaldehyde, N^5, N^{10}-methylenetetrahydrofolate (5,10-CH_2-H_4folate), to produce ketopantoate (**2**).[1,2] This reaction constitutes the first *committed* step in the biosynthesis of pantothenate and coenzyme A. KHMT obtained from *E. coli* K-12 was recently purified to apparent homogeneity by Snell et al.[3] They showed that it is this enzyme, and *not* a 5,10-CH_2-H_4folate-*independent* enzyme[4] in *E. coli*, capable of catalyzing the condensation of formaldehyde with α-KIVA, which is responsible for ketopantoate formation in vivo. Unlike the related 5,10-CH_2-H_4folate-dependent enzyme, serine hydroxymethyltransferase[5] (SHMT), KHMT is a class II aldolase and does not require pyridoxal phosphate as cofactor.

1 **2**

SHMT has been the subject of extensive stereochemical studies[6-13] (Scheme I). These studies have revealed that, in the conversion of glycine to (2*S*)-serine, the 2-H_{si}[14] atom is removed and the 2-H_{re} atom retained, **3** → **4**.[6-9] Thus the reaction proceeds stereospecifically with retention at C-2 of glycine. In contrast, studies by Biellmann et al.[10,11] of the steric course of formation of the hydroxymethyl group of serine revealed a most unusual partially stereospecific process. Incubation of tritiated formate (**5**) with rat liver slices led, via tritiated 5,10-methenyltetrahydrofolate (5,10-C^3H^+-H_4folate) (**6**) and tritiated 5,10-$C^3H_1{}^1H_1$-H_4folate (**7**), to serine having 72% of the tritium in the 3-H_{si} position (**8**) and 28% in the 3-H_{re} position (**9**). More detailed studies by Benkovic and Floss et al.[12] using purified SHMT also revealed a partially stereospecific course in the reverse process leading from serine (stereospecifically tritiated at C-3) to 5,10-CH_2-H_4folate. Under single-turnover conditions, from (2*S*,3*S*)-[3-$^3H_1$]serine (**8**) a ca. 76:24 ratio of tritiated 5,10-$C^3H_1{}^1H_1$-H_4folates (**10**) and (**11**), respectively[12b] (trapped by in situ dehydrogenation by 5,10-CH_2-H_4folate dehydrogenase to 5,10-CH^+-H_4folate), was formed. However, if the reversible SHMT reaction was allowed to proceed through many turnovers, essentially complete racemization of both the resultant 5,10-$C^3H_1{}^1H_1$-H_4folate at C-11 and the resultant serine at the C-3 prochiral center was observed.



8 **10** (76%) **11** (24%)

In view of the apparent similarity, at least in terms of reaction type, of the SHMT and KHMT reactions, it was of interest to compare the steric courses of the two reactions. We therefore undertook a stereochemical investigation of the KHMT-catalyzed reaction and now report the results of that investigation.

† This paper is dedicated to Dr. Eliahu Caspi on the occasion of his 71st birthday.

(1) Brown, G. M. "Comprehensive Biochemistry"; Florkin, M., Stotz, E. H., Eds.; Elsevier: New York, 1971; Vol. 21, pp 73-80.
(2) Abiko, Y. In "Metabolic Pathways"; Greenberg, D. M., Ed.; Academic Press: New York, 1975; Vol. 7, pp 1-25.
(3) (a) Teller, J. H.; Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3780-3785. (b) Powers, S. G.; Snell, E. E. *J. Biol. Chem.* **1976**, *251*, 3786-3793. (c) Powers, S. G.; Snell, E. E. *Methods Enzymol. 62*, 204-209.
(4) McIntosh, E. N.; Purko, M.; Wood, W. A. *J. Biol. Chem.* **1957**, *228*, 499-510.
(5) Schirch, L. *Adv. Enzymol.* **1982**, *53*, 83-112.
(6) Besmer, P.; Arigoni, D. *Chimia* **1968**, *23*, 190.
(7) Besmer, P. Ph.D. Dissertation No. 4435, ETH, Zurich, 1970.
(8) Jordan, P. M.; Akhtar, M. *Biochem. J.* **1970**, *116*, 277-286.
(9) Akhtar, M.; El-Obeid, H. A.; Jordan, P. M. *Biochem. J.* **1975**, *145*, 159-168.
(10) Biellmann, J. F.; Schuber, F. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 517-522.
(11) Biellmann, J. F.; Schuber, F. J. *Bull. Soc. Chim. Biol.* **1970**, *52*, 211-227.
(12) (a) Tatum, C. M.; Benkovic, P. A.; Benkovic, S. J.; Potts, R.; Schleicher, E.; Floss, H. G. *Biochemistry* **1977**, *16*, 1093-1102. (b) Slieker, L.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 1833-1838.
(13) Reviews on SHMT stereochemistry: (a) Rētey, J.; Robinson, J. A. "Stereospecificity in Organic Chemistry and Enzymology"; Verlag Chemie: Weinheim, 1982; pp 160-167; (b) Floss, H. G.; Vederas, J. C. In "Comprehensive Biochemistry"; Tamm, C. Ed.; Elsevier: Amsterdam, 1982; Vol. 3, pp 175-177.
(14) Nomenclature: Prelog, V.; Helmchen, G. *Helv. Chim. Acta* **1972**, *55*, 2581-2598. See also ref 13a, pp 1-16.

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