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

V. AROMATIC CONSTITUENTS

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Oxidative and hydrolytic degradation reactions were carried out on teicoplanin in order to characterize the aromatic portion of the molecule and relate it to the other members of the class of glycopeptide antibiotics. Seven aromatic rings, obtained as triphenyl ether, diphenyl ether, and diphenyl moieties after oxidation and hydrolysis of teicoplanin, were identified. They are present in teicoplanin as aromatic amino acids and constitute the peptidic part of the molecule. The diphenyl ether and diphenyl moieties, which were isolated both as esters after oxidation and as α-amino acids after acid hydrolysis clearly indicate the nature of the corresponding amino acids in teicoplanin. The triphenyl ether moiety, which was isolated only as ester, allows the hypothesis that the corresponding amino acids are the same as those of the other glycopeptide antibiotics.

Teicoplanin, a member of the class of glycopeptide antibiotics, is produced by *Actinoplanes teichomyceticus* as a group of very similar compounds which have the same aglycone containing aromatic aminoacidic moieties as well as D-mannose and D-glucosamine[1~5]. This paper describes the completion of the oxidative and hydrolytic degradation reactions carried out in order to characterize the aromatic portion of the molecule and relate it to the other members of this class of antibiotics.

Oxidative Degradation

Teicoplanin was hydrolyzed with 2 N H_2SO_4 and the reaction mixture, which accounts for the peptidic fragments of the molecule, was adsorbed on an anionic resin column (IRA-904 free base). After elution, the mixture was methylated with CH_3I/K_2CO_3 to protect the free functions and treated with $KMnO_4/MgSO_4$ to obtain a mixture of oxidation products.

The crude mixture was methylated with diazomethane and chromatographed on a Silica gel column eluting with $CHCl_3$ - MeOH, 98: 2. Two esters were obtained in significant amount: **1**, M^+ *m/z* 534 and **2**, M^+ *m/z* 346; a third one was isolated in trace amount: **3**, M^+ *m/z* 519. When teicoplanin as such was methylated and oxidized under the same conditions two main products were obtained: **4**, M^+ *m/z* 224 and **2**. The structures of products **1** through **4** are assigned on the basis of spectral data in comparison with those of similar fragments isolated from other glycopeptide antibiotics.

Structures of Compounds **1** and **3**

The MS spectrum of **1** shows molecular ions at *m/z* 534, 536, 538, whose ratio 9: 6: 1 indicate the presence of two chlorine atoms. The exact mass measurement (534.0484) indicates the elemental composition $C_{25}H_{20}Cl_2O_9$.

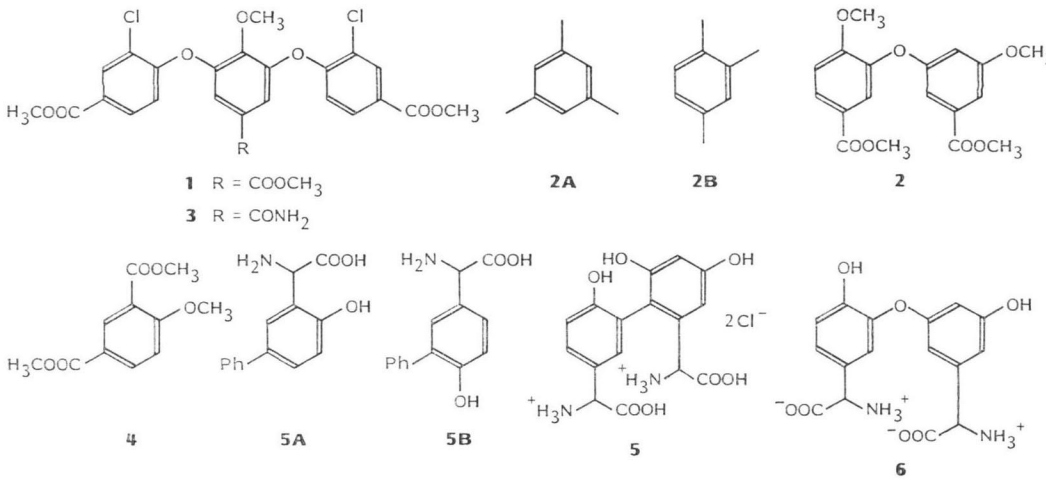
The IR spectrum of **1** shows the presence of ester functions (1730 cm^{-1}).

The ^1H NMR spectrum of **1** ($CDCl_3$) (Table 1) shows four methoxy signals, two of them equivalent,

Table 1. ^{1}H NMR data for compounds **1**, **2**, **4**, **5** and **6** at 270 MHz in $CDCl_3$.

Compound	δ (ppm)	Area H atom	Multiplicity	J(Hz)	Compound	δ (ppm)	Area H atom	Multiplicity	J(Hz)
1	3.75	3	s	—		8.56	1	d	2
	3.80	3	s	—	**5**	4.10	0.3	s	—
	3.85	6	s	—		4.24	0.7	s	—
	6.73	2	d	9		4.83	1	s	—
	7.52	2	s	—		6.34	0.3	d	2
	7.83	2	dd	9 and 3		6.37	0.7	d	2
	8.13	2	d	3		6.46	0.3	d	2
2	3.85	3	s	—		6.52	0.7	d	2
	3.90	9	s	—		6.90	0.3	d	8
	6.72	1	d	2		7.01	0.7	d	8
	7.13	1	d	2		7.2~7.4	2	m	—
	7.27	1	d	2		8.0~9.9	11	br	—
	7.13	2	d	9	**6**	4.22	1	s	—
	7.71	2	d	2		4.35	1	s	—
	7.94	2	dd	9 and 2		6.31	1	dd	2 and 2
4	3.91	6	s	—		6.53	1	dd	2 and 1.5
	4.00	3	s	—		6.60	1	dd	2 and 1.5
	7.04	1	d	9		6.9~7.1	3	m	—
	8.24	1	dd	9 and 2		6.5~10.0	8	br	—

Fig. 1. Structures of compounds **1**~**6**.



and four signals in the aromatic region, each accounting for two protons. Three of these are interrelated and correspond to two equivalent 1,2,4-trisubstituted aromatic rings. The remaining two protons are symmetrically located on a third aromatic ring. These spectral data indicate structure **1** (Fig. 1), already described for vancomycin[6].

The MS spectrum of **3** shows molecular ions at m/z 519, 521, 523 which correspond to the elemental composition $C_{24}H_{19}Cl_2NO_8$. The key fragment at m/z 256 (100) is attributable to $C_{15}H_{12}O_4^{+}$. The fragmentation patterns of compounds **1** and **3** are very similar and the a.m.u. values indicate that **3** contains an amide group instead of an ester group, while the stable fragment at m/z 256 suggests that the

amide is in position *para* to the methoxy group. Structure **3** (Fig. 1) is then deduced for compound **3** in analogy to the fragment isolated from vancomycin[8].

Structure of Compound **2**

The MS spectrum gives the molecular ion at *m/z* 346 and exact mass measurements (346.1089) indicate the elemental composition $C_{18}H_{18}O_7$. The most relevant peak is at *m/z* 315 (44%, $M-OCH_3$) and another relevant peak is at *m/z* 287 (12%, $M-COOCH_3$).

The IR spectrum shows the presence of ester functions (1730 cm^{-1}).

The ^1H NMR spectrum ($CDCl_3$) (Table 1) shows four methoxy signals and two groups of signals in the aromatic region accounting for six protons. Three signals appear as doublets with *meta* coupling constants; the other three signals show *ortho* and *meta* coupling constants. These signals are consistent with the presence of the two tri-substituted aromatic rings **2A** and **2B** (Fig. 1).

The molecular formula and the IR and ^1H NMR data indicate that two methoxy and two carbomethoxy groups are present. The functions found leave one oxygen to be accounted for which has to be the ether linkage between rings **2A** and **2B**. The relative positions of substituents OAr, OCH_3 and $COOCH_3$ in ring **2B** have been attributed on the basis of the comparison of the chemical shift values found with the calculated ones[7] and structure **2** (Fig. 1) is attributed to the compound.

The pattern of the oxygen functions of **2** is identical to that of the diphenyl ether moiety found in ristocetin[5], actaplanin[9] and A35512B[10].

Structure of Compound **4**

The MS spectrum shows the molecular ion at *m/z* 224, while the base peak at *m/z* 193 corresponds to $C_{10}H_9O_4^+$, *i.e.*, $M-OCH_3$.

The IR spectrum indicates an ester group (1730 cm^{-1}).

The ^1H NMR spectrum ($CDCl_3$) (Table 1) shows three aromatic protons and three methoxy signals. These spectral data are consistent with structure **4** (Fig. 1), already described among the degradation products of vancomycin[8], ristocetin A[5] and A35512B[10].

Hydrolytic Degradation

Teicoplanin, hydrolyzed in 6 N HCl, gave a mixture of ninhydrin positive and UV absorbing products which were separated on a Dowex-50 resin under acidic and basic conditions. Elution with acidic solutions allowed the isolation of glucosamine and one aromatic amino acid designated **5**. Elution with 1 N NaOH gave a mixture of products, which after separation on Silica gel column, yielded only one other aromatic amino acid, designated compound **6**.

Structure of Compound **5**

The MS spectrum of the molecule as such could not be obtained, while after methylation and acetylation a molecular ion at *m/z* 503, $(M+H)^+$ in positive CI and LC-MS, and at *m/z* 502 in EI was obtained. EI mass spectrum of the derivative indicates the presence of two acetylated amino groups [*m/z* 459 (M^+-COCH_3) and *m/z* 401 ($459-NHCOCH_3$)] and of two methyl ester functions [*m/z* 369 ($401-CH_3OH$), *m/z* 310 ($369-COOCH_3$)].

The IR (1735 cm^{-1}, $\nu_{C=O}$; 1615, 1595 and 1495 cm^{-1}, $\delta_{NH_3^+}$) and UV [λ_{max} nm ($E^{1\%}_{1cm}$) 0.1 N HCl: 284 (152); buffer pH 7.4: 287 (140); 0.1 N NaOH: 309 (264)] spectra of compound **5** indicate that it is the hydrochloride of an aromatic amino acid with free phenolic functions.

The ^1H NMR spectrum (DMSO-d_6) (Table 1) shows that the product is a mixture of two diastereoisomeric compounds. Two protons (δ 4.10/4.24 and 4.83) are attributable to two methine groups

of the α-amino acid functions. Two groups of signals (δ 6.3~7.4) accounting for five protons are present in the aromatic region. Two protons (δ 6.34/6.37 and 6.46/6.52) correspond to a 1,2,3,5 tetra-substituted aromatic ring, and three protons (δ 6.90/7.01 and 7.2/7.4) correspond to a tri-substituted aromatic ring. A broad band corresponding to 11 mobile H atoms is also present.

The isolation of 4-methoxy isophthalate **4** from oxidation of vancomycin and of other antibiotics of the class is reported to derive from a diphenyl moiety[11]. If compound **5** is the parent diphenyl moiety of compound **4** two possible structures can be considered for the tri-substituted ring of **5**, *i.e.*, **5A** and **5B** (Fig. 1). The comparison of the chemical shift values found with the calculated ones[7] is in favor of **5B**. The same comparison made for the chemical shift values of the tetra-substituted ring allows to propose structure **5** for the compound. This assignment is confirmed by the comparison of our data with those reported for the other antibiotics of the class[11,12].

Structure of Compound **6**

The IR spectrum (1625 cm^{-1}, ν_{COO^-}; 1600 and 1515, $\delta_{NH_3^+}$; 1170, ν_{C-O}) indicates an aromatic amino acid containing hydroxylic functions and present as an internal salt.

The ^1H NMR spectrum (Table 1) shows two protons attributable to two methine groups of the α-aminoacidic function. Two groups of aromatic protons are present: three protons (δ 6.31, 6.53 and 6.60) correspond to a 1,3,5 tri-substituted aromatic ring and three protons (δ 6.9~7.1) correspond to a second tri-substituted aromatic ring. A broad band corresponds to 8 mobile H atoms. The substitution pattern of the two aromatic rings of compound **6** corresponds to the diphenyl ether moiety of compound **2** isolated from oxidation of teicoplanin. Then, structure **6** (Fig. 1) is assigned to this amino acid.

Conclusions

Seven aromatic rings were obtained as dichlorotriphenyl ether, diphenyl ether, and diphenyl moieties after oxidation and hydrolysis of teicoplanin. They are present in teicoplanin as aromatic amino acids and constitute the peptidic part of the molecule. The diphenyl ether and diphenyl moieties, which were isolated both as esters after oxidation and as α-amino acids after acid hydrolysis indicate the presence of *p*-hydroxy- and *m*-dihydroxyphenylglycines. The dichlorotriphenyl ether moiety was isolated only as an ester and the presence of *p*-hydroxyphenylglycine and tyrosine can only be hypothesized by analogy to the structures reported for the other glycopeptide antibiotics[13].

To date the class of glycopeptide antibiotics include six representative members: vancomycin, ristocetin, actinoidin, avoparcin, A35512B and actaplanin[9,13]. All contain the basic heptapeptide backbone which derives from aromatic and aliphatic amino acids and two or more different sugars. A comparison of the results obtained from degradation studies of teicoplanin with those of the other antibiotics shows that:

1) the diphenyl moiety is common to all the antibiotics.
2) the triphenyl ether is also a common moiety but differs in the chlorine content. Teicoplanin, like vancomycin, contains two chlorine atoms.
3) the diphenyl ether moiety is present in teicoplanin, as in ristocetin, actaplanin and A35512B, with different substituents.
4) the pattern of the oxygen functions at the common aromatic portions are the same for all the antibiotics of the class, suggesting a common biosynthetic route[14].

Experimental

Instrumentation

Mass spectra were obtained on a Hitachi RMU-6L instrument at 70 eV by DIS, ion source temperature 200°C. Mass spectra in CI mode were obtained on a HP 5985 instrument.

IR spectra were run as Nujol mull with a PE 580 spectrophotometer.

UV spectra were run with a PE 320 spectrophotometer.

^{1}H NMR spectra were obtained at 270 MHz with a Bruker WH-270 cryospectrometer.

Oxidative Degradation of Teicoplanin after Acid Hydrolysis

Hydrolysis and Methylation: Teicoplanin (5 g) was treated with 230 ml of 2 N H_2SO_4 in a sealed tube at 100°C for 2 hours. The cooled mixture, filtered from an insoluble residue (350 mg), was brought to pH 5 with $Ba(OH)_2$ and the $BaSO_4$ was separated. The solution was chromatographed on a column of IRA-904 (OH^-) resin (750 ml) on which the neutral and basic sugars are not absorbed. The acidic peptidic portion absorbed on the column was eluted with 0.1 N HCl. The fractions with ninhydrin positive reaction (about 2 liters) were pooled together and concentrated to dryness giving a mixture of products (TLC: BuOH - CH_3COOH - H_2O, 8: 2: 2, Rf 0.10, 0.32, 0.50). The mixture was dissolved in 150 ml of anhydrous CH_3OH with the addition of 3 g K_2CO_3 and 30 ml CH_3I. The suspension was refluxed for 6 hours and left at room temperature overnight. The insoluble portion was filtered off and the solvent removed under reduced pressure. The residue, washed with water and dried, gave 1.1 g of ninhydrin positive products.

Oxidation and Methylation: The methylated mixture (1 g) was suspended in water containing 400 mg $MgSO_4$, the suspension was warmed at 75°C and a solution of 1 g $KMnO_4$ in 75 ml H_2O was added dropwise. The mixture was left at 75°C for 5 hours and then filtered. The excess of $KMnO_4$ was reduced by treatment with sodium metabisulfite and the solution was extracted three times with ethyl acetate at pH 2.0. The combined extracts were concentrated and precipitated by addition of excess of petroleum ether giving 180 mg. The product was dissolved in anhydrous CH_3OH and treated at 0°C with ethereal solution of CH_2N_2, the solution, left at room temperature under stirring for about 4 hours was then concentrated to dryness giving 160 mg (TLC: benzene - petroleum ether, 2: 1, mixture of several UV absorbing products). The mixture was dissolved in $CHCl_3$ and chromatographed on a Silica - Celite, 1: 1 (v/v) column eluted with $CHCl_3$ - $(C_2H_5)_2O$, 3: 1 mixture. Ten fractions were collected and separately dried under vacuum. Product could be recovered from fractions 1 and 7, the other fractions contained mixture of UV absorbing products which could not be recovered. Fraction 1 (44 mg) contained compound **1** (MS: *m/z* 534, 536, 538. ^{1}H NMR: see Table 1. *Anal* Calcd for $C_{25}H_{20}O_9Cl_2$: C 56.09, H 3.76, Cl 13.24. Found: C 56.45, H 3.72, Cl 13.34). Fraction 7 (9 mg) contained compound **3** (MS: *m/z* 519). The column was then eluted with $CHCl_3$ - CH_3OH, 98: 2 mixture, the mixture of products obtained were applied to a preparative TLC plate and eluted with benzene - petroleum ether, 2: 1 mixture. One band was collected (15 mg) which contained compound **2** (MS: *m/z* 346. ^{1}H NMR: see Table 1).

Oxidative Degradation of Intact Teicoplanin

Teicoplanin (1.5 g) was suspended in 150 ml anhydrous CH_3OH with the addition of 3 g K_2CO_3 and 30 ml CH_3I. The mixture was refluxed for 3 hours, 15 ml CH_3I were added and the mixture was refluxed for additional 4 hours and left at room temperature overnight. The suspension was concentrated to dryness, the residue was suspended in 40 ml of water, 4.8 g $KMnO_4$ in 90 ml of water containing 14 ml 2 N NH_4OH were added and the mixture was heated at 75°C for 3 hours. Additional $KMnO_4$ was added leaving the mixture for an additional hour at 75°C and overnight at room temperature. The filtered solution was extracted several times with ethyl acetate at pH 2.0, the combined extracts dried ($MgSO_4$) were concentrated to dryness (50 mg). The oxidized mixture was dissolved in CH_3OH, treated with ethereal CH_2N_2 solution at 0°C, left overnight at room temperature and concentrated to an oily residue. Preparative TLC plates eluted with $CHCl_3$ - MeOH, 95: 5 mixture gave two main products (TLC, same solvent: Rf 0.7 and 0.8). Fraction at Rf 0.7 contained compound **4** (MS: *m/z* 224. ^{1}H NMR: see Table 1. *Anal* Calcd for $C_{11}H_{12}O_5$: C 58.92, H 5.40; Found: C 58.60, H 5.35). Fraction at Rf 0.8 contained product **2** (MS: *m/z* 346).

Hydrolytic Degradation of Teicoplanin

Teicoplanin (10 g) was dissolved in 500 ml of 6 N HCl and refluxed 8 hours. The cooled solution was filtered and the clear filtrate was dried under reduced pressure. The solid residue was redissolved in 300 ml water and the pH was adjusted to 7.0 with 1 N NaOH. The precipitate formed was removed by

centrifugation and the supernatant, containing a mixture of ninhydrin-positive and UV absorbing products (TLC: CH_3OH - $CHCl_3$ - NH_4OH, 4: 4: 2) was applied to a Dowex 50X8 (H^+) cation exchange column (2×30 cm). Elution carried out with HCl (linear gradient 0~5 N HCl, 1 liter total volume) allowed the isolation of glucosamine (400 mg) and of compound **5** (100 mg, Rf 0.1, ^1H NMR: see Table 1). A mixture of two other products, obtained by eluting the column with 1 N NaOH, was further purified on silica gel column eluted stepwise with CH_3OH - $CHCl_3$ - NH_4OH from 30: 60: 10 to 50: 25: 25. Only compound **6** was collected in an amount (44 mg, Rf 0.45, ^1H NMR: see Table 1) sufficient for analysis.

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