A35512, A COMPLEX OF NEW ANTIBACTERIAL ANTIBIOTICS PRODUCED BY *STREPTOMYCES CANDIDUS*

II. CHEMICAL STUDIES ON A35512B

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(Received for publication September 9, 1980)

The glycopeptide antibiotic A35512B was isolated from *Streptomyces candidus* NRRL 8156 as the major active factor. Chemical degradation studies showed that mild acid hydrolysis resulted in the release, one molecule each, of four neutral sugars: rhamnose, fucose, glucose and mannose, as well as the liberation of a complex peptide core which retained all the amino acids and from which 3-amino-2,3,6-trideoxy-3-C-methyl-L-*xylo*-hexopyranose, a new amino sugar, was isolated (**2**). Oxidative degradation of A35512B resulted in the isolation of a chlorodiphenylether (**5**), dimethyl 4-methoxyisophthalate (**7**) and methyl 3,5-bis-(4-methoxycarbonylphenoxy)-4-methoxybenzoate (**6**). The structure of **5** could not be conclusively elucidated but was shown to be either 5-chloro-2′,3-dimethoxy-2,5′-dicarbomethoxy diphenylether (**5a**) or 2-chloro-2′,3-dimethoxy-5,5′-dicarbomethoxy diphenylether (**5b**) by physical methods. This halogenated fragment was shown to arise from oxidation of constituent amino acid (**10**) which has the aromatic substitution pattern of fragment (**5a** or **5b**). Base hydrolysis resulted in the isolation of a phenanthridine (**9**) which arose from 2′,4,6-trihydroxybiphenyl-2,5′-diyldiglycine. These chemical degradation studies on A35512B showed that this antibiotic is closely related to the ristocetin class of antibiotics.

The antibacterial antibiotic complex A35512 is produced by *Streptomyces candidus* NRRL 8156 from which several closely related factors have been isolated and characterized by MICHEL.[1a] These antibiotics possess high antimicrobial activity against Gram-positive bacteria and are ineffective against Gram-negative organisms[2].

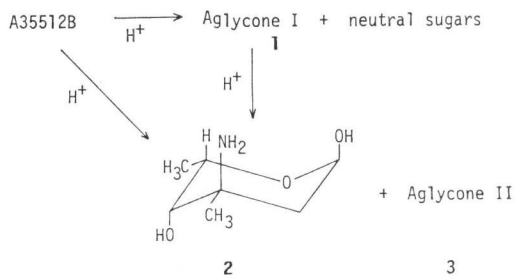
The A35512 complex has been shown to be a member of the glycopeptide family of antibiotics of which vancomycin, ristocetin and actinoidin are prominent members[3]. Characterization work by MICHEL showed that this complex was composed of six closely related factors.[1a] Each of these factors contained the identical complex amino acids (amino acid analysis of 6 N HCl total hydrolyzate). The complex released neutral sugars and a complex peptide upon mild acid hydrolysis. The antibiotic complex had several phenolic groups (UV spectrum) and a complex titration curve. These data were consistent with the conclusion that A35512 represented a complex of new glycopeptide antibiotics.

The major factor, A35512B, was obtained in crystalline form. The present paper will summarize the chemical studies on this antibiotic and relate it to other members of the glycopeptide class of antimicrobial agents.

Chemical Properties of A35512B

When A35512B was hydrolyzed under mild conditions (0.5 N HCl, 4 hours, reflux) an antimicrobially active aglycone I (**1**) was formed, and a number of neutral sugars were released. These sugars were identified by paper and thin-layer chromatography as rhamnose, glucose, fucose and mannose. This

identification was verified by comparison of the gas chromatography retention times of the trimethylsilyl (TMS) derivatives of the sugars from A35512B with those of the derivatives of authentic monosaccharides. This analysis also showed each sugar to be present in an equimolar quantity.



The aglycone was isolated by pH adjustment of the reaction mixture to 3.2 with a basic resin followed by neutralization with an inorganic base. The precipitated aglycone I was purified and characterized as its hydrochloride. Analytical data indicated an empirical formula of $C_{71}H_{66}N_8O_{25}Cl$ for the aglycone. Titration data showed at least six ionizable groups between pKa 7.4 and 12.4 for this aglycone.

The presence of two alkyl methyl groups in **1** was indicated by its ^1H NMR spectrum (δ 1.40 d, J=6 Hz, δ 1.58 [s] each 3H) and two methyl signals in the ^{13}C NMR spectrum (17.1 and 23.7 Hz). The chemical shifts of these methyl groups were identical with those observed in the spectrum of the constituent amino sugar **2**. Previous structural studies showed **2** to be 3-amino-2,3,6-trideoxy-3-C-methyl-L-*xylo*-hexopyranose[1b] thus confirming that A35512B contained an amino sugar constituent as do most of the known glycopeptide antibiotics.[7~11] Extended mild acid hydrolysis of **1** gave a new peptide **3** (aglycone II) whose NMR spectrum lacked these methyl signals. Aglycones I and II each gave the same amino acid analysis as parent A35512B. Aglycone I appeared to be formed from A35512B by loss of the neutral sugars while longer hydrolysis times resulted in the formation of **3** by loss of amino sugar **2**.[21]

The *in vitro* antimicrobial activity of **1** was essentially the same as parent A35512B and is shown in Table 1. However, compound **1** appeared to be somewhat less active *in vivo*. Aglycone II retains approximately one-third of the *in vitro* antimicrobial activity of A35512B (data not shown).

Table 1. Antimicrobial activity—A35512B aglycone I.

1. *In vitro*

	No. of strains examined	MIC (μg/ml)		
		A35512B	A35512B aglycone I	Vancomycin
S. aureus	11	1.0~4.0	0.25~1.0	0.5~1.0
Group D *Strep.*	7	1.0~2.0	1.0	1.0~2.0
S. pyogenes C-203	1	1.0	2.0	1.0
S. pneumoniae Park I	1	2.0	2.0	1.0

2. *In vivo*

	ED_{50}—s.c. (mg/kg)×2		
	A35512B	A35512B aglycone I	Vancomycin
S. aureus 3055	2.98	1.04	0.52
S. pyogenes C 203	0.71	5.8	0.38
S. pneumoniae Park I	3.72	7.0	1.1

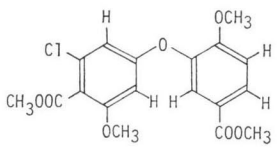
Structural Features of the Constituent Amino Acids of A35512B

The nature of the aromatic rings in A35512B was revealed by oxidative degradation of the methylated aglycone I with basic permanganate.[12,13] This oxidation gave a mixture of extractable acids which after esterification were examined by field desorption mass spectrometry (FDMS) and which showed three major products to be present: **5**, *m/z* 380; **6**, *m/z* 466, and **7**, *m/z* 224. This mixture of esters was purified by chromatography to give three pure compounds whose structures were determined.

Structure of 5

The mass spectrum of **5** showed molecular ions at *m/z* 380 and 382 indicating the presence of a single chlorine atom. Peak matching experiments established that **5** had an empirical formula of $C_{18}H_{17}O_7Cl$ which was verified by microanalysis. The NMR spectrum of **5** shows four equivalent methoxyl groups (δ 3.88 [12 H]). Addition of europium shift reagent (Eu[fod]$_3$), shifted these signals to δ 3.90, 3.93, 4.02 and 4.95 (s, 3H). The latter two resonances are assigned to the carbomethoxyl group and the magnitudes of the shifts indicate that one ester group (δ 4.95) is complexed substantially more strongly than the other (δ 4.02) by the shift reagent. The aromatic protons appeared as two groups of signals. The first group was an ABX system with signals at δ 7.03 (d, J=10 Hz), 7.91 (q, J=10, 2 Hz) and 7.69 (d, J=2 Hz). The signal at δ 7.03 shifted 0.29 ppm with Eu(fod)$_3$ and showed a 14% NOE when the methoxyl region (δ 3.90, 3.93) was irradiated, while the other two signals shifted 1.1 and 0.8 ppm to δ 9.01 and δ 8.57, respectively, and neither showed an NOE upon methoxyl irradiation. These data indicate that the signals at δ 7.91 and 7.69 ppm arise from aromatic protons adjacent to the strongly complexed (δ 4.95, shifted spectra) carbomethoxy group. A second group of aromatic protons at δ 6.73 ppm (s, 2H) became non-equivalent in the presence of shift reagent to give two separate signals at δ 6.88 (d, 1 H, J=2 Hz) and δ 7.03 (d, 1 H, J=2 Hz) with only the former signal showing an NOE (32%) upon methoxyl irradiation (δ 3.90~3.93). The splitting pattern observed for these signals is consistent with the assignment of these protons to positions *meta* to each other. The small effect of Eu(fod)$_3$ on the position of these signals indicated that either these signals were not adjacent to a Eu(fod)$_3$-COOMe binding site or that they are adjacent to a carbomethoxy group which is not extensively bound by shift reagent.[12] The latter possibility is strongly suggested by the observation that one of the ester groups in **5** experienced only a 0.16 ppm shift in the methoxyl resonance with this reagent.

The spin decoupling experiments with and without shift reagent indicated that **5** was composed of two distinct aromatic nuclei: a 2-methoxy-5-carbomethoxy benzene ring and a tetra-substituted aromatic ring bearing a pair of *meta* protons, a methoxyl, a carbomethoxyl, and a chloro group leaving an oxygen atom to be accounted for. The lack of fragmentation typical of ketones in the mass spectrum of **5** permitted the assignment of an ether bridge between these aromatic rings.[14] The following structures were consistent with the NMR and mass spectral data discussed above.



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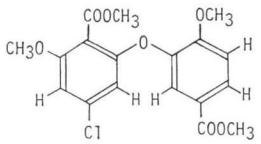
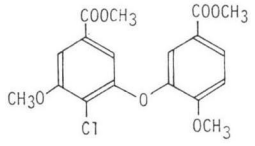


5a



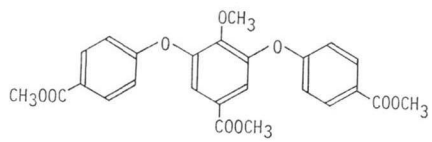
5b

Structure **4** was differentiated from **5a** and **5b** by a single NOE experiment in presence of $Eu(fod)_3$. Irradiation of the δ 8.57 signal caused only one of the two protons in the tetra-substituted ring to show an NOE. Structure **4** would be expected to show an NOE at both protons flanking the ether bridge on the chlorinated ring. Structure **5** (**a** or **b**) satisfied all the available data for this degradation product; however, a choice between these structures could not be made on spectral grounds.

Of the two structures **5b** is favored on biogenetic arguments. The aromatic halogenation patterns normally favored in microbial products are usually *ortho* to an oxygenated position.* Structure **5a**, on the other hand, is halogenated at a position *meta* to the nearest oxygen function. Also, **5b** is an analog of ristomycinic acid, the corresponding diphenylether amino acid found and recently described by HARRIS[20]. Therefore, from model building experiments **5b** is also favored over **5a**. However, clear differentiation between these structures must await structural studies, in progress on the intact antibiotic.

Structure of 6

The high resolution mass spectrum of degradation product **6** gave a molecular ion *m/z* 466 ($C_{25}H_{22}O_9$). The NMR spectrum of this substance showed 4 methoxyl signals, two chemically equivalent at δ 3.90 and two at δ 3.77 and 3.85. Likewise, the aromatic region of the spectrum showed 10 protons: two different AB types of 4 protons each, δ 6.96 and δ 8.05 (d, J=9 Hz) and a 2 proton singlet at δ 7.58. This spectrum corresponded to the presence of two equivalent 1,4-disubstituted aromatic rings and a third aromatic ring with two chemically equivalent aromatic protons (δ 7.58). These spectral data are consistent with structure **6** which was also isolated by TARBELL from degradation of ristocetin.[13]



6

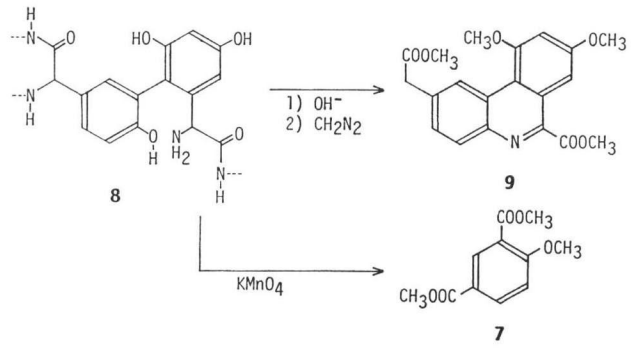
The isolation of this type of bis-diphenyl ether derivative from five members of the glycopeptide class of antibiotics A35512B, vancomycin, ristocetin, avoparcin and actinoidin[12,13,18,19] suggests that this fragment constitutes a characteristic structural unit in this family. Hence the isolation and characterization of this structural unit is of diagnostic value for the preliminary chemical characterization of new members of this class as they are discovered.

Product 7

The third oxidation product isolated, product **7**, had the identical NMR and physical characteristics as methyl 4-methoxy-isophthalate **7**[12,15].

This degradation product had previously been isolated by other workers from the oxidative degradation of vancomycin and ristocetin and shown to arise from the oxidation of the complex biphenylamino acid **8** which WILLIAMS showed also to be present in vancomycin[15,16]. That **8** was also present in A35512B was confirmed by the isolation of the base degradation product **9** whose properties were identical with those reported by WILLIAMS[12]. Further confirmation of the presence of **8** was obtained by isolation from acetylation-methylation of A35512B hydrolyzates of a compound ($C_{25}H_{30}N_2O_9$) which corresponded to the N-acetyl-O-methylated derivative of **8**[15].

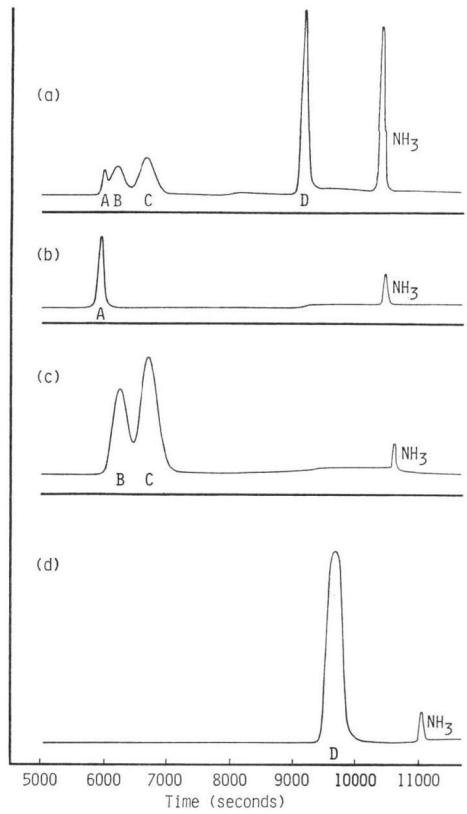
* This biogenetic argument against the assignment of structure **5a** was initially raised in a helpful discussion with Dr. T. M. HARRIS, Vanderbilt University.



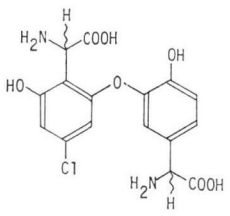
Structure of Amino Acid D

Total hydrolysis of A35512B was conveniently achieved by refluxing the antibiotic in 0.05 N HCl containing a strongly acidic ion exchange resin. Elution of the absorbed amino acids from the resin with ammonium hydroxide gave a mixture of the constituent amino acids. The NMR spectrum of this mixture showed only aromatic protons and amino acid side chains of the glycine type. Fractionation of these amino acids on AG 50×12 was attempted using increasing concentrations of hydrochloric acid. The results of this separation are shown in Fig. 1. Only glycine and amino acid D could be obtained in sufficient purity for characterization. The structure of amino acid D is discussed below.

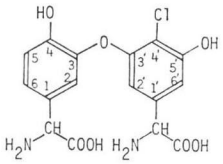
Fig. 1. Amino acid analysis of A35512B hydrolyzates: (a) amino acid analysis before AG 50×12 chromatography showing amino acid A, B, C and D. AG 50×12 ion exchange chromatography gave: (b) elution with 0.36 N HCl; (c) elution with 2 N HCl; (d) amino acid D eluted with 6 N HCl.



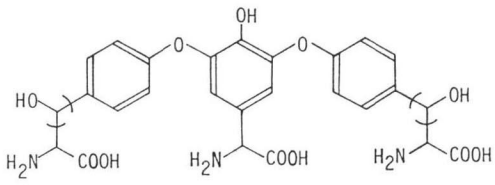
The NMR spectrum of amino acid D (D_2O + DCl) showed two sets of signals in the aromatic region: δ 7.18 ppm (dd, 2H, J=2.5 Hz) assigned to a pair of *meta* protons and a 3 proton ABC multiplet at δ 7.70 ppm. These data indicate the presence of two aromatic rings; one tetra-, the other trisubstituted. Amino acid D was derivatized (N-acetylation, methylation) resulting in the isolation of a compound whose composition was $C_{24}H_{27}N_2O_9Cl$. The NMR spectrum of this derivative showed signals for two acetyl groups and four methoxyl groups. Oxidation of this derivative with permanganate under the conditions used for degradation of the parent antibiotic gave rise to **5**, previously isolated from similar treatment of A35512B. These data taken together demonstrated that amino acid D was the source of degradation product **5** and that the substitution pattern in **5** was the same as in the



10a



10b



11

amino acid D. Furthermore, the carboxyl groups in **5** are the remnants of glycine side chains. On the basis of the discussion of the structure of degradation product **5** the structure of the amino acid D will be assigned **10a** or **10b**. Note that **10b** corresponds to 4′-chloro-4′-desmethyl-ristomycinic acid, an analog of ristomycinic acid, found in ristocetin[20].

The isolation of glycine from the acid hydrolysis of A35512B in appreciable quantities was in agreement with the observation made by WILLIAMS that vancomycin yielded two moles of glycine upon acid hydrolysis by reverse aldol condensation from hydroxylated tyrosine side chains[15]. A reaction scheme designed to prove that amino acid **11** also had the hydroxy alanine side chains failed in our hands and its presence could not be proved conclusively in this manner.

From our studies we conclude that A35512B is a complex glycopeptide antibiotic made up of three complex amino acids **8**, **10** (**a** or **b**) and **11**, amino sugar **2** and neutral sugars rhamnose, fucose, mannose and glucose.

The sequence in which these amino acids are arranged is the subject of on-going investigations.

Experimental Section

The following instruments were used in this study: NMR spectrometer: Varian HA 100; UV spectrophotometer: Cary 15; ORD and CD spectropolarimeter: Perkin Elmer 241 and Jasco J40AS; IR spectrophotometer: Beckman IR426; high resolution mass spectrometer: Varian MAT731; amino acid analyses: Beckman 120C amino acid analyzer equipped with a Bio-Cal autosample applicator and programmer and single Durrum BC-1A resin; gas chromatograph: F and M 402 equipped with a 10.5 ft column of 3% OV-101 column on Chromosorb W operated at 200°C.

Preparation of A35512B Aglycone I (1)

A solution of A35512B (10 g) in 100 ml of 0.5 N hydrochloric acid was refluxed for 4 hours. Upon cooling to room temperature the pH was adjusted to 3.2 by the addition of Bio-Rad 1×4 (OH^-) and the mixture was filtered. The filtrate was neutralized by the addition of 5 N NaOH and the precipitate formed was collected (4.25 g) while the aqueous filtrate was set aside. This solid [Rf 0.2: cellulose, butanol - pyridine - HOAc - H_2O, 15 : 10 : 3 : 12 (BPAW)] had the following properties:

UV: λ_{max}^{EtOH} 282 nm (ε 7,184) neutral; 303 nm (ε 1,300) base.

Titration: pKa (66% DMF) 5.0, 7.58, 9.88, 11.90.

Anal. Calcd. for: $C_{71}H_{66}O_{25}N_8Cl \cdot \frac{1}{2}HCl$: C, 57.41; H, 4.52; N, 7.54; Cl, 3.58; Cl^-, 1.12.

Found: C, 57.52; H, 4.55; N, 7.80; Cl, 3.89; Cl^-, 0.88.

NMR: ^1H NMR: (CD_3OD) 1.4 (d, 3H, J=6 Hz), 1.6 (s, 3H). ^{13}C NMR (DMSO) showed two methyl resonances at 17.15 and 23.85 Hz. Amino acid analysis showed the identical composition as parent A35512B.

The aqueous filtrate was passed over an Amberlite 120 (NH_4^+) column. The initial fractions (500 ml) containing the neutral sugars were concentrated and the residue was examined by paper chromatography (Whatman 1: EtOAc - HOAc - H_2O, 60 : 26 : 14) and thin-layer chromatography (silica gel plates buffered with NaOAc; 3% aqueous acetone). Both methods employed a *p*-anisidine oxalate

visualization spray. Both methods showed the presence of rhamnose, fucose, mannose and glucose.

The solution of neutral sugars was concentrated under reduced pressure and the resulting syrup silylated with trimethylsilyl imidazole (0.5 ml). This solution was analyzed by gas liquid chromatography (10.5 ft-3% OV-101 on Chromosorb W column at 200°C). The sugars were detected as a mixture of their anomers. The same sugars detected by paper and TLC were found by this method. Integration of the areas beneath the curves showed each sugar to be present in equimolar quantities.

Preparation of Aglycone II (3)

A35512B (1.0 g) was dissolved in 40 ml of 0.15 N HCl and refluxed for 24 hours under an atmosphere of nitrogen. The reaction mixture was cooled and concentrated to half of its original volume (temperature maintained below 50°C). The pH was adjusted to 6.5 with 2 N NaOH to give 620 mg of a white precipitate. This solid was dissolved in 2 : 1 H_2O - MeOH and passed over Duolite S-30 (NH_4^+). Elution with water resulted in partial desalting. Further elution with 0.5 N NH_4OH gave 340 mg of a solid which was rechromatographed on Duolite S-30 (H^+ form). Elution with H_2O gave 140 mg of a solid with an additional 43 mg eluting with 0.1 N HCl. This material had the following properties: λ_{max}^{EtOH}, 225 nm (ε 26,077), 280 nm (ε 4,000). NMR: alkyl methyl groups present in A35512B have been lost in **3**. Amino acid analysis showed that **3** retained the same amino acids found in A35512B.

Titration data showed the following pKa (80% DMF): 6.58, 7.9, 11.08, (AMW: 1206).

The same substance could be produced by hydrolysis of aglycone I under the same conditions.

Permanganate Oxidation of Methylated A35512B Aglycone I

A35512B aglycone I (2 g) was dissolved in 360 ml of MeOH and 7.2 g of K_2CO_3 was added. This mixture was treated with 90 g of methyl iodide and refluxed for 3 hours. Upon cooling, the solvents were removed under reduced pressure, the residue suspended in H_2O, the methylated aglycone collected by filtration and dried in a desiccator (1.92 g). Addition of base showed no wavelength shifts in the UV spectrum.

The methylated aglycone (1.66 g) was suspended in 100 ml of H_2O, 35 ml of 2 N NaOH was added and the temperature was raised to 70°C. A solution of $KMnO_4$ (10 g in 230 ml of H_2O) was added dropwise over a 4-hour period and stirring continued for an additional 1.5 hours. The solution was filtered through Hiflo Supercel and excess $KMnO_4$ destroyed with a minimal amount of $NaHSO_3$. After acidification with hydrochloric acid the reaction mixture was extracted with EtOAc and the organic layer dried ($MgSO_4$) and evaporated to an oil. The extracted acids were esterified with excess diazomethane by the usual procedure. TLC showed a mixture of five spots (petroleum ether - acetone, 5 : 1). The mixture of esters were applied to a preparative TLC plate and all materials except those remaining at origin were scraped from the plate and eluted from the silica gel with methanol. This mixture (290 mg) was rechromatographed on a silica gel column (20 g) and eluted with benzene-ethyl acetate mixtures. The column was eluted stepwise from 100% benzene to 5% EtOAc - benzene. Two closely moving bands were collected. The first band A (80 mg) crystallized on standing. The product **5** was recrystallized from petroleum ether: m.p. 136~137°C.

Anal. Calcd. for $C_{18}H_{17}O_7Cl$: C, 56.78; H, 4.50; Cl, 9.31.
Found: C, 56.55; H, 4.51; Cl, 9.57.

UV: λ_{max}^{EtOH} 214 nm (ε 43,911), 287 nm (ε 6,000)

IR: ($CHCl_3$) 1710 cm^{-1}

NMR: ($CDCl_3$), 100 MHz, δ 3.86 (s, 12H), 6.73 (s, 2H), 7.03 (d, 1H, J=2.5 Hz), 7.91 (dd, 1H, J=9.25 Hz); $CDCl_3$ +0.5 mg Eu(fod)$_3$; δ 3.90 (s, 3H), 3.93 (s, 3H), 4.02 (s, 3H), 4.95 (s, 3H), 6.88 (q, 1H, J=8.0, 2.0 Hz), 7.15 (d, 1H, J=9 Hz), 8.57 (d, 1H, J=2 Hz), 9.01 (q, 1H, J=8, 2 Hz).

Mass spectrum: m/z 380, 382, 349, 351—isotope peaks confirm monochlorination and peak matching confirm above empirical formula.

A second band, **6**, was eluted (80 mg) and also crystallized on standing, m.p. 93~95°C (MeOH).

Anal. Calcd. for: $C_{25}H_{22}O_9$: C, 64.38; H, 4.75.
Found: C, 64.64; H, 4.98.

Mass spectral data: M^+: m/z 466, 435 (M^+-31)

IR: 1700 cm^{-1}

UV: λ_{max}^{EtOH} 254 nm (ε 50,944), 296 (sh)

NMR: ($CDCl_3$) δ 3.77 (s, 3H), 3.90 (s, 6H), 6.97 (d, 4H, J=9 Hz), 7.61 (s, 2H), 8.02 (d, 4H, J=9 Hz).

Isolation of Product **7** (Dimethyl 4-Methoxyisophthalate)

A35512B aglycone (1 g) was permethylated and oxidized by the procedure outlined above. The extractable acids were esterified with excess diazomethane, dissolved in toluene and chromatographed on a column of silica gel (20 g). The column was eluted with 1%, 2%, 5%, 10%, 20% and 30% EtOAc in toluene mixtures. The individual fractions were combined on the basis of their TLC (silica gel, 30% EtOAc - toluene). The fractions eluted between 2% and 10% EtOAc - toluene were composed primarily of two components (Rf 0.61 and 0.74). These fractions were combined and rechromatographed on silica gel (20 g) and eluted with 5% EtOAc - toluene. The component eluting first (Rf 0.71) had a mass spectrum and NMR spectrum identical with **6**. The slower component (Rf 0.57) gave a mass spectrum (M^+ m/z 224) and an NMR: δ 3.85 (s, 6H), 3.96 (s, 3H), 7.01 (d, 1H, J=9 Hz), 8.15 (dd, 1H, J=2.0, 9.0 Hz), 8.48 (d, 1H, J=2 Hz). These data are identical with those reported in the literature for dimethyl 4-methoxyisophthalate, **7**[2].

Total Hydrolysis of A35512B

A solution of A35512B (2.98 g) in 150 ml of 0.05 N HCl was treated with 29 g of IR-120 (H^+) ion exchange resin and refluxed 24 hours. The resin was collected on a glass filter and eluted with 160 ml of 10 N NH_4OH. The eluate was freeze-dried and 170 mg of a mixture on ninhydrin-positive material was obtained. TLC showed three prominent spots (cellulose, BPAW). Amino acid analysis confirmed that these represented all of the amino acids present in the parent antibiotic.

An aqueous solution of the A35512B hydrolyzate (918 mg) was applied to an AG 50W×8 (50~100 mesh) cation exchange column (2.5 cm×30 cm). Stepwise elution was carried out from 0.12 N HCl to 6 N HCl. No appreciable elution with 500 ml each 0.12 N and 0.24 N HCl occurred. 0.36 N HCl (700 ml) eluted a single amino acid identified as glycine (65 mg). No appreciable elution occurred with 0.48 N HCl (500 ml) while 1 N HCl (500 ml) and 2 N HCl (40 ml) eluted mixtures of amino acids Rf 0.16 and 0.17 (cellulose, BPAW). Elution with 6 N HCl gave a single amino acid Rf 0.30 (cellulose, BPAW). This amino acid could not be crystallized but was purified on a column of cellulose eluting with BPAW. TLC and amino acid analysis showed a single amino acid corresponding to amino acid D (**10**).

Anal. Calcd. for $C_{16}H_{15}O_7N_2Cl\cdot\frac{1}{2}H_2O$: C, 49.09; H, 4.12; N, 7.15.
Found: C, 49.46; H, 3.86; N, 7.16.

UV: λ_{max}^{EtOH} 285 nm (ε 4,828), neutral; 304 nm (ε 8,000) base

IR: (KBr) 1710 cm^{-1} (broad).

Titration: pKa (66% DMF): 4.12, 9.0, 10.2, 12.6.

NMR: (D_2O+DCl) 7.18 (dd, 2H, J=2.5 Hz), 7.70 ppm (m, 3H: ABX).

Amino acid **10** was converted to its N-acetyl-methyl ester derivative (Ac_2O - MeOH, followed by CH_2N_2). The resulting non-crystalline derivative showed 6 CH_3-C=O protons (δ 2.0 ppm) 12 CH_3O protons δ 3.70 (6H), 3.82 (6H) ppm:

Mass spec.: m/z 522, composition: $C_{24}H_{27}N_2O_9Cl$. Calcd: 522.1045. Found: 522.1404.

Data are consistent with N-acetyl-dimethoxy-dimethyl ester of amino acid D (**10**).

Permanganate Oxidation of Amino Acid D (**10**)

Amino acid **10** (16 mg) was dissolved in a few ml of MeOH and treated with an excess of CH_2N_2 for two days. After destruction of reagent with a few drops of HOAc, solvents were removed under reduced pressure. The residue was suspended in 2 ml H_2O and heated to 70°C; 2 N NaOH (0.4 ml) was added followed by portionwise addition of 120 mg of $KMnO_4$. The reaction mixture was heated for 3 hours, then cooled and excess $KMnO_4$ was destroyed ($NaHSO_3$). After acidification to pH 1.2 the reaction mixture was filtered through Hiflo Supercel and then extracted with ethyl acetate (3×). The residue was esterified with CH_2N_2. The product had the identical Rf (silica gel TLC, 30% EtOAc - benzene) as 5-chloro-2,3'-dicarbomethoxy-3,6-dimethoxy diphenyl ether (obtained from the oxidation of intact antibiotic) **5**. The mass spectrum (m/z 380, 217) was also identical with that of latter degrada-

tion product.

N-Acetyl-methyl Ester of Amino Acid (**8**)

The crude mixture of amino acids derived from total hydrolysis of A35512B (46 mg) were converted to the corresponding N-acetyl-methyl esters (CH_3OH - Ac_2O followed by CH_2N_2). After lyophilization of the reaction product, the residue was-chromatographed on silica gel (methanol - ethyl acetate mixtures). Fractions eluting with 15~20% MeOH - EtOAc gave a single spot whose UV spectrum showed free phenolic groups. These fractions were remethylated with CH_2N_2 to give a product whose UV spectrum was no longer shifted by base addition (λ_{max}^{EtOH} 282, 312 (sh) nm).

The high resolution mass spectrum of this derivative gave the following empirical formulas:

M^+: *m/z* 502. : $C_{25}H_{30}N_2O_9$ Calcd: 502.1951. Found: 502.1961

M−32: *m/z* 470. : $C_{24}H_{26}N_2O_8$ Calcd: 470.1689. Found: 470.1684

M−43: *m/z* 459. : $C_{23}H_{27}N_2O_8$ Calcd: 459.1767. Found: 459.1767

These results correspond exactly with the mass spectral fragmentation of dimethyl N,N′-diacetyl-2′,4,6-trimethoxy-biphenyl-2,3′-diyldiglycinate (N-acetyl-methyl ester of **8**) reported by SMITH *et al.*[15].

Base Degradation of A35512B

Using the procedure employed by WILLIAMS for base degradation of vancomycin, A35512B (500 mg) was dissolved in 100 ml of 4 N NaOH and refluxed for 24 hours[12]. The reaction mixture was cooled and pH adjusted to 9 (12 N HCl). An inorganic precipitate formed and was discarded. The filtrate was acidified (pH 2) and extracted 3× with 200 ml of EtOAc. The extract was evaporated to dryness and gave 68 mg of an oil. The aqueous filtrate showed presence of glycine and A35512B constituent amino acids in the amino acid analyses. The organic extract was treated with excess ethereal CH_2N_2 by the usual procedure (18 hours). The reaction mixture was diluted with water and reextracted into 100 ml of EtOAc, washed with H_2O, 10% $NaHCO_3$, H_2O and dried over $MgSO_4$. The organic solvent was evaporated to give 18 mg of a crude product whose mass spectrum showed a series of peaks at *m/z* 369 (M^+), 311. This sample was purified by preparative TLC (silica gel, 4 : 1, petroleum ether B - acetone). The band moving at Rf 0.25 was extracted from the plate and repurified by double elution on a preparative silica gel plate ($CHCl_3$) which removed some polar components (Rf 0) and material at Rf 0.1 was extracted from the plate (acetone) to give 2.5 mg of an oil. The composition of this material was obtained by high resolution mass spectroscopy. M^+ *m/z* 369, calcd. for $C_{20}H_{19}NO_6$: 369.12123, found 369.12103; $C_{18}H_{17}NO_4$: 311.11575, found 311.11556.

UV: λ_{max}^{EtOH} 253, 275, 315, 370 (sh) nm.

NMR data matched reported data for phenanthridine **9** isolated by WILLIAMS from alkaline degradation of vancomycin.[12]

Isolation of Amino Sugar Derivatives from A35512B Aglycone

A solution of A35512B aglycone (1 g) in 5 ml of pyridine was treated with 2 ml of benzoyl chloride for 22 hours at room temperature. Excess methanol was added and the solvents removed under reduced pressure. Methyl benzoate was removed by trituration 3× with ether and decantation of the ether solution. The ether-insoluble mass was dissolved in 250 ml of 1.5 N methanolic HCl and refluxed for 7 hours. This reaction mixture was taken to dryness under reduced pressure, water added and extracted twice with EtOAc. The solvent was again removed *in vacuo* and the residue was extracted with 3~100 ml portions of ether by decantation. The combined ether extract gave 378 mg of an oil which was chromatographed on a column of Grade I neutral aluminum (Woelhm) (40 g) starting with petroleum ether B. One hundred percent benzene eluted 58 mg of an impurity, 10% EtOAc - benzene eluted 82 mg of a solid, m.p. 158~169°C (Et_2O - petroleum ether). The NMR and TLC properties of this material corresponded to a mixture of α (72%) and β (28%) anomers of the methyl glycoside of N,O-dibenzoyl adduct of amino sugar **2**. Further elution with 100% EtOAc gave 109 mg of a solid which crystallized (EtOAc - petroleum ether, 1: 2), m.p. 173~178°C, whose m.p., NMR, TLC and mass spectral properties corresponded (α or β) to the N,O-dibenzoyl derivative of amino sugar **2**.[11b,21]

Acknowledgments

We wish to thank Mr. JOHN OCCOLOWITZ for valuable assistance in obtaining and interpretation of the mass spectral data cited here and Mr. THOMAS ELZEY for obtaining key ^{1}H NMR spectra in the course of these studies.

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