Amino Acid Constituents of Ristocetin A

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Abstract: The glycopeptide antibiotic ristocetin A, elaborated by *Nocardia lurida*, is one of a class which interferes with biosynthesis of bacterial cell wall by complexing with peptide precursors terminating in D-Ala-D-Ala. The site of activity within the antibiotic lies in the peptide fragment. The heptapeptide from ristocetin A has been shown to contain ristomycinic acid (**12**) and actinoidinic acid (**17**), which are dimeric coupling products between hydroxylated phenylglycines, and dechlorovancomycinic acid (**23a**), which is a trimeric coupling product between *p*-hydroxyphenylglycine and two β-hydroxytyrosines. The structure of ristomycinic acid has been established from spectra of the bis(amino acid) and derivatives and by oxidation of the O-methylated derivative to give substituted oxybis(benzoates) **4** and **5** which were independently synthesized by Ullmann condensations. The structure of actinoidinic acid was established similarly using an independent synthesis of substituted bibenzoate **8**. Comparison was also made with a sample of actinoidinic acid obtained from vancomycin. Dechlorovancomycinic acid was destroyed both during acidic and basic hydrolysis of ristocetin; the structure assignment of **23a** is based upon indirect evidence. Oxidation of O-methylated aglycoristocetin yielded tribenzoate **7**; base hydrolysis in the presence of $NaBH_4$ yielded **9** and glycine. Compound **9** arises by reduction of aldehydes resulting from retro-aldol reactions of the β-hydroxytyrosine residues.

Ristocetin A is an antibiotic produced by *Nocardia lurida*[1] which interferes with formation of peptidoglycan in the cell walls of Gram-positive bacteria by a mechanism involving complexation with peptide precursors ending in D-Ala-D-Ala.[2] It belongs to a group of antibiotics which includes ristomycin,[3] actinoidin,[4] avoparcin,[5] and vancomycin,[6] all of which are characterized by containing novel and complex aromatic amino acids. Although Lomakina and her colleagues[7] had shown some time ago that some of the amino acids from actinoidin and ristomycin were hydroxylated dimeric phenylglycine derivatives, it was not until the recent elegant work on vancomycin by Williams and co-workers[8] at Cambridge that the detailed structure of any antibiotic in this group was known. In this paper we would like to report the results of our studies on the amino acid constituents of the aglycone, or peptide moiety, of ristocetin A;[9] the closely related antibiotic ristocetin B differs in its carbohydrate content.[10]

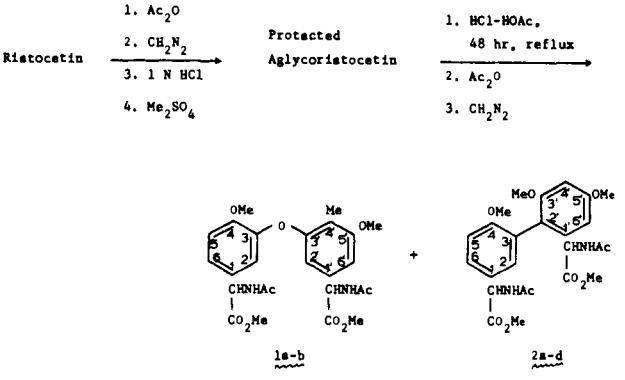
Early structural studies on ristocetin, carried out by workers at Abbott Laboratories,[10] revealed that the antibiotic is a glycopeptide of several thousand molecular weight. The active site lies in the peptide portion which constitutes about one-half of the molecule; the composition of the nonessential carbohydrate portion is known but not the structure.[7c,10] Preliminary work on the peptide revealed the presence of phenolic amino acids of uncertain structure.[11] Subsequent investigations by Tarbell and co-workers resulted in the isolation of two bis(amino acids).[12] Two stereoforms of each could be separated by ion-exchange chromatography. Although detailed studies of the NMR spectra were made, complete assignment of structures was not possible.

While the studies of ristocetin by Tarbell and earlier workers relied heavily on ion exchange chromatography and electrophoretic methods for separation of the amino acids, the approach which we have taken has depended upon derivatization of the amino acids to render them soluble in nonaqueous solvents, then making separations on silica gel with advantage being taken of the superior resolving capabilities of LC systems. Structural assignments have been made on the basis of spectra of derivatives and of degradation products of the amino acids. Independent syntheses have been used to confirm the structures of the degradation products, which, in general, are more amenable to synthesis than the amino acids. Three types of degradation have been employed on the antibiotic: acid hydrolysis, oxidation, and base hydrolysis. A brief description of these experiments is given below, followed by a more detailed discussion of the results in terms of the individual components.

Acid Hydrolysis. Ristocetin, N-acetylated with acetic anhydride and methylated with diazomethane, was treated with 1 N HCl to remove the sugars; the resulting peptide was treated with dimethyl sulfate to effect more complete protection of the phenolic groups. The N-acetylated, O-methylated peptide was hydrolyzed with 6 N HCl/acetic acid or with 4 N methanesulfonic acid. It should be noted that the conditions employed are sufficient to racemize phenylglycines. The resulting amino acids were acetylated with acetic anhydride and esterified with diazomethane prior to chromatography on an open column of silica gel followed by LC on μ-Porasil. The separation yielded two diastereoisomeric forms of diphenyl ether type bis(*N*-acetylamino acid ester) **1a** and **b** and four forms of biphenyl type bis(*N*-acetylamino acid ester) **2a–d** (Scheme I). The sequence of elution was **2a** followed by **1a, 1b, 2b,** and **2c,d.** The chromatographic system did not resolve **2c** from **2d; 2b** was only slightly more mobile than **2c,d** but could be separated from them. On ion-exchange chromatography using Aminex Q-150S resin the diastereoisomers of the diphenyl ether type bis(amino acid) (**12**) are eluted much more slowly than the biphenyl type bis(amino acid) (**17**);[12] the separation of the diastereoisomers of **12** from each other was relatively good but the separation of the forms of **17** was incomplete.

Stereoisomers **1a,b** were readily distinguished from **2a–d** by NMR but not by mass spectrometry because all forms have empirical formula $C_{25}H_{30}N_2O_9$ and their mass spectra are

Scheme I



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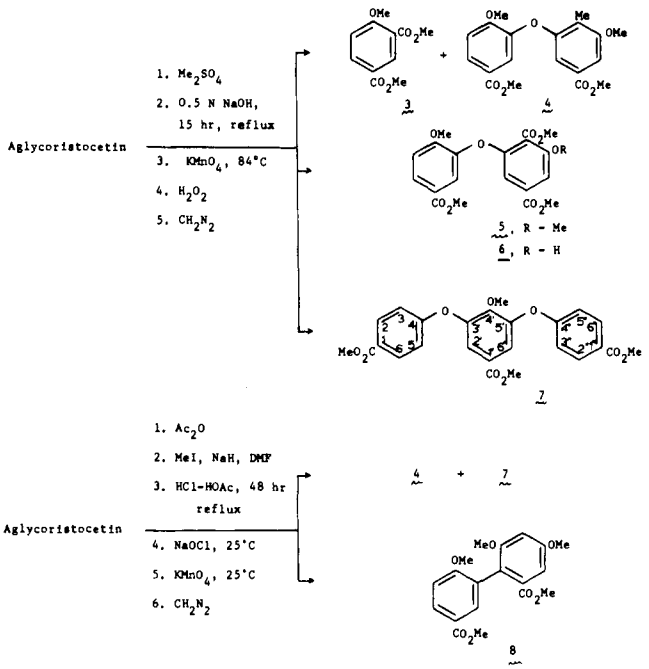
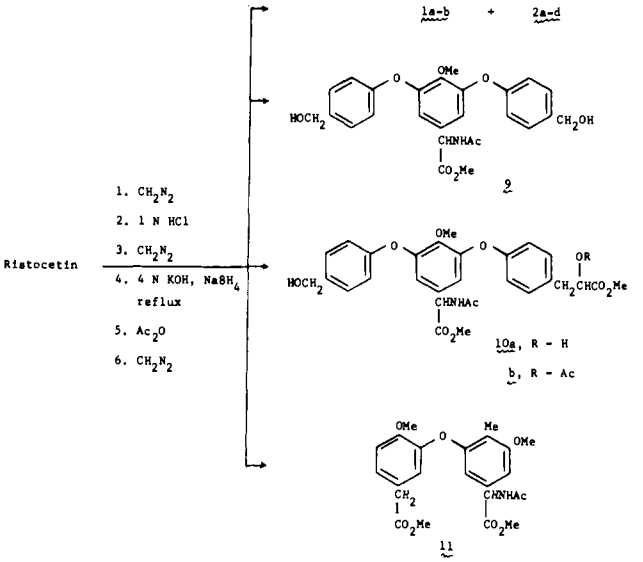
Scheme II



Scheme III



dominated by cleavages at the glycyl residues. No aliphatic or other aromatic residues could be isolated from the hydrolysis mixtures, although there was considerable insoluble material, the NMR of which (in Me_2SO-d_6) showed very broad, poorly resolved aromatic signals, suggesting that it was polymeric. Further hydrolysis of the insoluble material did not release any more amino acids.

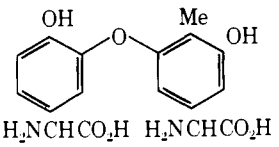
Oxidative Degradation. Oxidation studies were undertaken in an attempt to identify the arrangement of substituents on the aromatic amino acids, since NMR spectra of the amino acids themselves and of simple derivatives had been ambiguous.[9a] The phenolic hydroxyl groups were protected as methyl ethers to avoid nuclear oxidation in the course of the degradation sequence. Methylated aglycoristocetin was hydrolyzed in base and the resulting mixture of amino acids oxidized with hot (84 °C) $KMnO_4$. The acids obtained from this treatment were methylated with diazomethane and separated by LC (Scheme II). The products included dimethyl 4-methoxyisophthalate (**3**), which was identified by comparison with an authentic sample, and three novel aromatic esters: diester **4**, triester **5**, and triester **7**. In a subsequent experiment,[9b] methylated aglycoristocetin was hydrolyzed in acid and the crude hydrolysate oxidized at 25 °C with NaOCl, followed by treatment with neutral $KMnO_4$ at 25 °C (Scheme II). Under these milder conditions a second diester, **8**, was found in addition to the compounds previously isolated. Compounds **4** and **5** were also obtained from oxidation of amino acid **12** (see below) which had been isolated by ion exchange chromatography and subsequently methylated to protect the phenolic hydroxyl groups. Evidence for the structures of these compounds and for their relationship to the amino acid constituents of ristocetin is presented below.

Base Hydrolysis. Peptides are not commonly hydrolyzed with base because the resulting amino acids undergo racemization under alkaline conditions, but the procedure seemed warranted in the present case because no tris(amino acid) or other compound related to tribenzoate **7** had been found during acid hydrolysis. One possible explanation for such a failure would be that the precursor of **7** contained a carbonyl or other reactive group that underwent acid-catalyzed condensation with the activated aromatic nuclei. The base hydrolysis was carried out on O-methylated aglycoristocetin in the presence of $NaBH_4$ to reduce such carbonyl groups to alcohols and thus avoid other reactions of carbonyl groups which might occur in base such as Cannizzaro reactions. This approach had previously been used by Williams in his studies of vancomycin.[8e]

O-Methylated aglycoristocetin was treated with a mixture of KOH and $NaBH_4$; the products of the reaction were isolated by TLC and LC after acetylation and esterification (Scheme III). In addition to compounds **1a,b** and **2a–d**, three triaryl compounds were isolated: compound **9** and two others tentatively identified as **10a** and **10b**. The reaction also gave a deaminated product, **11**, related to **1a,b**. One aliphatic amino acid, glycine, was also found; it was isolated as the *N*-dinitrophenyl derivative.

Ristomycinic Acid (12). The studies by Fehlner et al.[12] of the diphenyl ether type bis(amino acid) had led them to conclude that the compound contained two phenylglycines linked via an oxygen bridge between the aromatic rings, that each ring bore one hydroxyl group, and that one ring also was methylated. They assigned the two oxy substituents in the unmethylated ring to positions 3 and 4. A meta relationship between the two protons in the other ring was evident from NMR; the placement of the remaining groups was not established, although NOE measurements led to a tentative assignment of the ring as a 3,4-dioxy-5-methylphenylglycine.

We conclude that this amino acid has structure **12** on the



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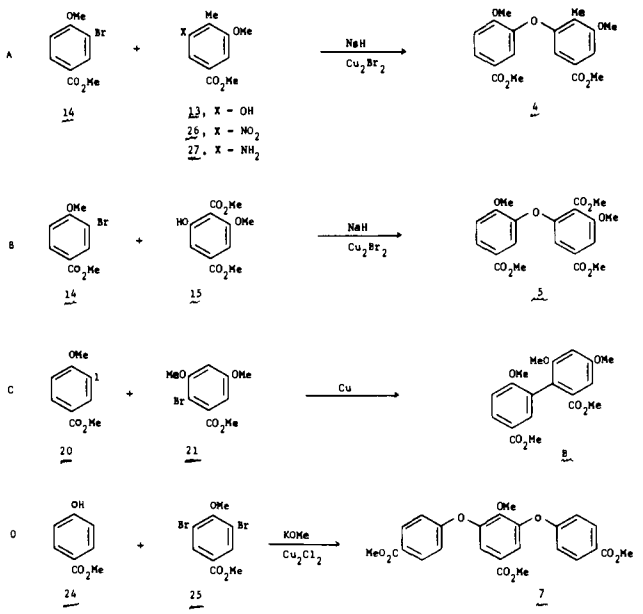
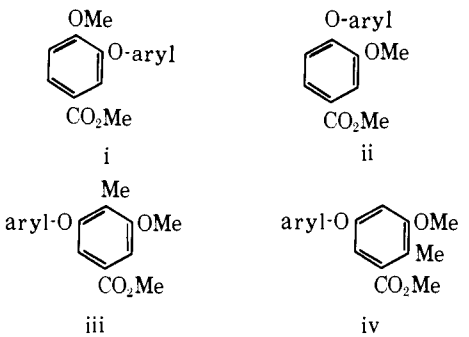
basis of the following considerations. Exact mass measurement on derivatives **1a** and **1b** established the empirical formula of **1** which, by taking into account the presence of four *O*-methyl groups and two *N*-acetyl groups indicated by the NMR spectrum, yielded the empirical formula $C_{17}H_{18}N_2O_7$ for **12**. Fehlner et al. had not been able to obtain a parent ion in the mass spectra of derivatives they had prepared but assigned the same empirical formula on the basis of exact mass measurement of a fragment ion. The NMR spectra of **1a** and **1b** established that **12** had two hydroxyl groups on the diphenyl ether system, but were no more useful than the spectra of the amino acids themselves for assignment of the locations of substituents.

The oxidation studies on **12** led to diester **4** and triester **5**, the latter arising by oxidation of the *C*-methyl group. The

Scheme IV



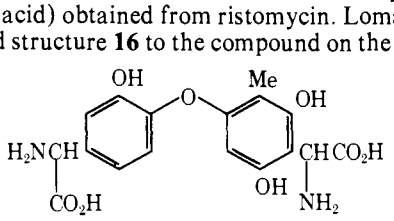
NMR spectra of **4** and **5** fully confirmed Tarbell's proposal that the trisubstituted ring had oxy substituents at positions 3 and 4 (partial structure i or ii) and that the protons in the other ring were meta to each other. The chemical shifts of the protons in the tetrasubstituted rings of **4** and **5** were hard to reconcile with ortho dioxy substitution in that ring but were consistent with meta. The possible arrangements of substituents on the tetrasubstituted ring of **4** were limited to two (partial structure iii or iv) by the finding that **4** was inert to



BCl_3 but **5** underwent demethylation to give **6** under conditions with which *o*-methoxybenzoates are selectively demethylated.

Of the four possible structures for **4** (i with iii, i with iv, ii with iii, and ii with iv), the first of these was shown to be the correct formulation by independent syntheses of **4** and **5** (Scheme IV, A and B) using Ullmann condensations of methyl 3-bromoanisate (**14**) with the sodium salts of methyl 3-hydroxy-5-methoxy-*p*-toluate (**13**) and dimethyl 2-hydroxy-6-methoxyterephthalate (**15**), respectively, thus establishing structure **12**.

Following the appearance of our preliminary communication on the use of oxidative degradation and independent synthesis to assign the structure of bis(amino acid) **12**,[9a] Katrukha et al.[13] reexamined the structure of ristomycinic acid, a bis(amino acid) obtained from ristomycin. Lomakina et al. had assigned structure **16** to the compound on the basis of elemental analysis, NMR, and certain chemical transformations. Lomakina has claimed[7a] that ristomycinic acid is also present in ristocetin. Katrukha et al. showed by mass spectrometry of the peracetyl dimethyl ester that the empirical formula of the underivatized bis(amino acid) is $C_{17}H_{18}N_2O_7$ rather than $C_{17}H_{18}N_2O_8$ which had been proposed by Lomakina. On the basis of that observation coupled with a detailed assignment of the mass spectrum, Katrukha has revised the structure in agreement with structure **12**. We will, therefore, call the bis(amino acid) from ristocetin ristomycinic acid, no other diphenyl ether type bis(amino acid) having been found in ristocetin. It should, however, be pointed out that the Russian workers have not yet *proven* the orientation of all substituents in the compound from ristomycin nor that the compound obtained from ristocetin is identical with it.[36]



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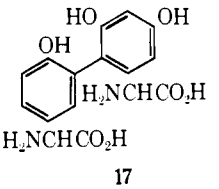
The deaminated product arising from base hydrolysis in the presence of $NaBH_4$ was assigned structure **11** on the basis of mass spectrum, NMR, and the presumed mechanism of formation. The NMR spectrum showed the presence of one *N*-acetyl, one *C*-methyl, four OMe's, a methylene group at 3.45 ppm, one α hydrogen, and five aromatic protons, which were almost identical with those of **1a,b**. The compound is believed to arise from incompletely protected aglycone. Although the peptide had been treated with diazomethane prior to hydrolysis, methylation of the phenols must not have been complete. Methylation of aglycoristocetin under more vigorous conditions (methyl iodide and sodium hydride in DMF) gave material which was resistant to hydrolysis, possibly because of N-methylation of amide linkages. Compound **11** is probably formed by deamination of the *p*-hydroxyphenylglycine moiety in the presence of base to form a quinone methide which is reduced by $NaBH_4$ (Scheme V).[14] When the hydrolysis was carried out in the presence of $NaBD_4$, compound **11** did not show incorporation of deuterium because the methylene position of the phenylacetic acid residue underwent base-catalyzed exchange with solvent.

Actinoidinic Acid (17). The structure of the second dinuclear amino acid is assigned as **17**.[9b] The empirical formula of **17**



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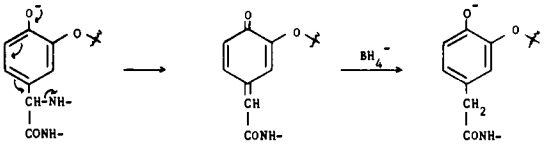
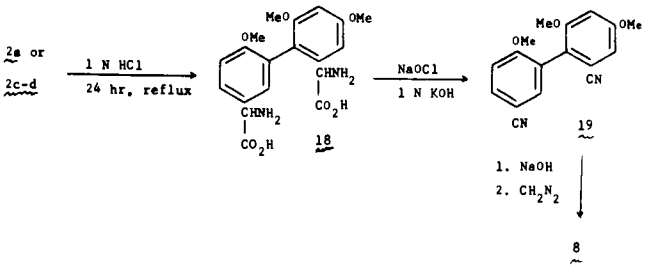
($C_{16}H_{16}N_2O_7$) was obtained by exact mass measurement on **2a** and on the mixture of **2c** and **2d**. The NMR spectra of the unprotected amino acid and simple derivatives did not permit unambiguous assignment of structure. The NMR spectrum of **2a** did, however, show that the compound was not a diphenyl

Scheme V



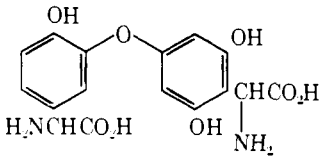
Scheme VI



ether (see ref 12); the presence of five methoxyl groups, including the esters, indicated a biphenyl nucleus. The occurrence of four stereoisomers, two obtained in pure form (**2a** and **2b**) and the other two as an unresolved mixture (**2c** and **2d**), supported this assignment. In addition to the asymmetric centers in the two amino acid groups of **2**, slow rotation about the biphenyl linkage provided a third site of asymmetry, thus leading to four racemic mixtures in epimerized material.

The structure of **2** was established by oxidative degradation of **2a** and of a mixture of **2c** and **2d**. Hydrolysis of both samples of free O-methylated amino acids **18**, followed by oxidation with NaOCl, yielded dinitrile **19** (Scheme VI). The dinitrile was then hydrolyzed and esterified to give diester **8**. The NMR spectrum of **8** indicated that one ring was substituted 1,3,4 and the other 1,2,3,5. The formation of dimethyl 4-methoxyisophthalate (**3**), as well as **8**, during oxidative degradations of O-methylated aglycoristocetin suggested that **3** might be a further degradation product of **8**. Thus one ring of **8** must be a 4-methoxybenzoate coupled at position 3 or a 2-methoxybenzoate coupled at position 5. Chemical shifts dictated a meta relationship between the two hydroxy substituents on the other ring. Structure **8** was established for the diester and thereby **17** for the bis(amino acid) by an independent synthesis of **8** involving cross-coupling of methyl 3-iodoanisate (**20**) with methyl 2-bromo-3,5-dimethoxybenzoate (**21**) (Scheme IV, C).

Actinoidinic acid is a name given by Lomakina et al.[7b] to a bis(amino acid) isolated from actinoidin hydrolysates; they claimed that the compound was also present in vancomycin, ristomycin, and ristocetin. Their studies of the structure of actinoidinic acid became complicated by an error in assignment of the empirical formula which led them to the conclusion that the aromatic rings were joined via a diphenyl ether linkage, structure **22**, similar to ristomycinic acid. Williams and co-



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workers subsequently assigned the compound from vancomycin as biphenyl-type bis(amino acid) **17**.[8c] On the basis of different evidence we reached the same conclusion concerning the compound from ristocetin. Confirmation that the amino acids in vancomycin and ristocetin are identical was obtained by converting **2a** and **2c,d** derived from vancomycin to a dinitrile by hydrolysis followed by oxidative degradation with NaOCl. The dinitrile was identical with **19** from ristocetin. Recently, Katrukha et al.[13,15] reexamined the compound from ristomycin obtaining mass spectral evidence for the empirical formula $C_{16}H_{16}N_2O_7$; this finding led them to conclude that Williams' structure is correct for that material as well. Although proof is still lacking that the compound present in ristomycin and actinoidin is identical with the compound present in vancomycin and ristocetin, we tentatively conclude that all four antibiotics contain the same biphenyl compound. Thus, we will call **17** actinoidinic acid.

Dechlorovancomycinic Acid (23a). The remaining constituent of the peptide portion of ristocetin was deduced to be tris(amino acid) **23a**, although direct isolation of the compound was not possible. The first evidence for the presence of such a compound came from the oxidation studies which gave tribenzoate ester **7** along with esters derived from ristomycinic acid and actinoidinic acid. The structure of **7** was indicated by the NMR spectrum and was established by an independent synthesis involving Ullmann condensation of the potassium salt of methyl 4-hydroxybenzoate (**24**) with methyl 3,5-dibromo-4-methoxybenzoate (**25**) (Scheme IV, D). No tris(amino acids) have been found among the products of acid hydrolysis of the peptide, which argues against **7** being the oxidation product of a simple trimeric coupling product of *p*-hydroxyphenylglycines and/or tyrosines.

23a, X = H
b, X = Cl

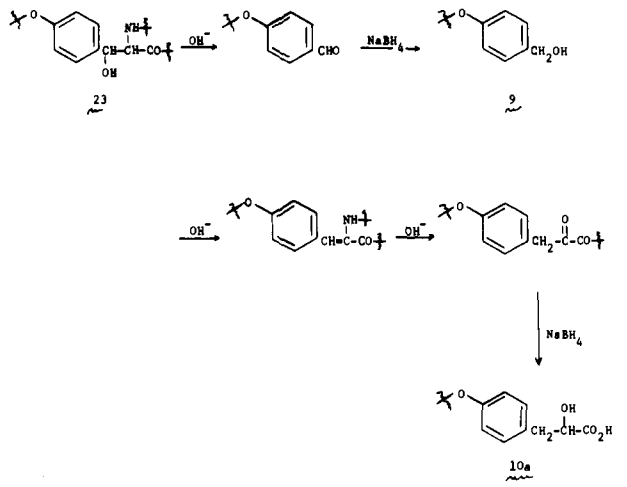
Evidence for structure **23a** was obtained from base hydrolysis of protected peptide carried out in the presence of $NaBH_4$ (Scheme III). Triaryl compound **9** containing two benzyl alcohol residues was one of the products. The empirical formula of **9** ($C_{26}H_{27}NO_8$) was assigned mass spectroscopically; the symmetrical structure was indicated by the NMR spectrum in which protons 3, 5, 3″, and 5″ appeared as a doublet at 6.97 ppm and protons 2, 6, 2″, and 6″ as a doublet at 7.27 ppm with J = 8 Hz in both cases. The 2′ and 6′ protons on the pyrogallol ring appeared as a singlet at 6.81 ppm, and the benzyl alcohol methylene groups as a broadened singlet at 4.65 ppm. Base hydrolysis was also carried out in the presence of $NaBD_4$ leading to **9** containing two deuterons. The signal for the benzylic methylene group was reduced to half its previous relative intensity and simultaneously broadened by geminal deuterium coupling indicating that one deuteron had been introduced into each methylene group.

Glycine is a product of peptide hydrolysis in base but could not be detected after acid hydrolysis.[16] This finding coupled with isolation of **9** leads to the proposal that the tris(amino acid) is **23a** containing two β-hydroxytyrosine moieties which undergo retro-aldol cleavage during treatment with base to give glycine plus benzaldehyde residues, the latter undergoing reduction by $NaBH_4$ to benzyl alcohols. The retro-aldol process can be detected in the intact antibiotic by UV. Brief heating of a solution of ristocetin in 1 M KOH at 60–70 °C led to a rapid increase in absorption at 280 nm which returned to its original value upon addition of $NaBH_4$. Extensive heating or higher temperatures led to irreversible changes in the UV spectrum. The retro-aldol reaction may be the major cause of rapid loss of biological activity which is observed when ristocetin is heated with base.[1]

Further substantiation for structure **23a** was obtained from two additional products which arose during base hydrolysis carried out in the presence of $NaBH_4$. Insufficient material was available for complete characterization but the structures of the two compounds are tentatively assigned as **10a** and **10b**. Compound **10a** (mol wt 553) exhibited in its NMR spectrum an *N*-acetyl methyl signal at 2.0 ppm, a multiplet at 3.0–3.1 ppm assigned to the phenyllactate methylene group, three methoxyls, a benzylic methylene group at 4.66 ppm, a phenylglycyl α hydrogen at 5.40 ppm, and aromatic protons similar to **9** but more complex. Compound **10b** (mol wt 595) had an NMR spectrum similar to that of **10a** but showed a second acetyl methyl signal at 2.10 ppm and thus appeared to be an *O*-acetyl derivative of **10a**; the benzylic methylene group was still at 4.6 ppm, suggesting that the other hydroxyl group had been acetylated. These compounds may arise from **23a** by dehydration and subsequent hydrolytic deamination followed by reduction of the resulting phenylpyruvate (Scheme VII).

In Williams' study of vancomycin,[8c] a triaryl product was also found after base hydrolysis which had not been detected in acid hydrolysates; compound **26** was isolated when the base hydrolysis was carried out in the presence of $NaBH_4$. Following the same line of reasoning used above he concluded that

Scheme VII



26 arose from tris(amino acid) **23b**. His structural proposal

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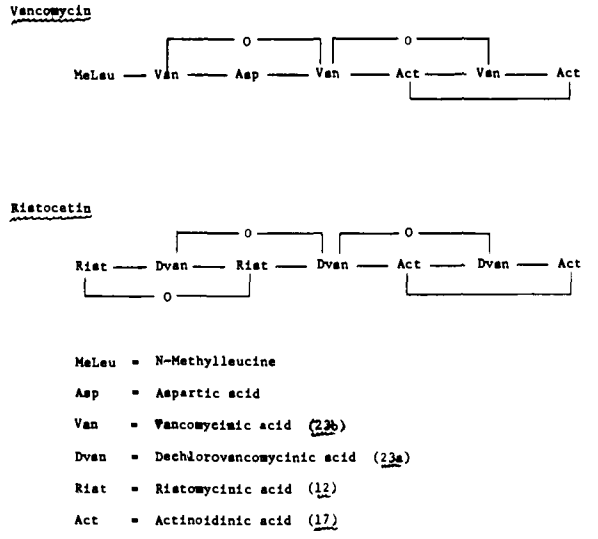
was subsequently confirmed by the X-ray crystallographic structure determination on the peptide.[8f] It is worth noting that the X-ray structure showed that the two β-hydroxytyrosyl residues in **23b** are epimeric; in addition the amino group of one is involved in an unusual cis amide linkage.[8f] We have chosen to call **23b** vancomycinic acid; accordingly **23a** is dechlorovancomycinic acid.

At present we believe that the peptide portion of ristocetin A is made up of ristomycinic acid (**12**), actinoidinic acid (**17**), and dechlorovancomycinic acid (**23a**), although the presence of a low molecular weight amino acid, particularly an aromatic one, could possibly have escaped detection. It should be pointed out that only single stereoisomers of the amino acids are thought to occur in the native antibiotic; epimerization of phenylglycines occurs during both basic and acidic hydrolyses.

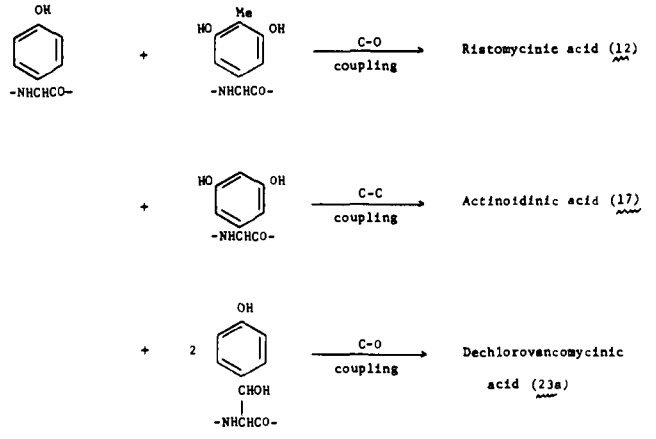
The estimates of molecular weight of the ristocetins have ranged from 4000 to 1800;[1,17] Lomakina et al.[18] reported an equivalent weight of 1163 based on NH_2 titration and DNP derivatization, giving a mol wt of 2326 on the basis of two free amino groups. They further showed that the aglycone had one titratable amino group; if one subtracts the known carbohydrates in ristocetin a mol wt of 1453 is obtained for the aglycone. By the same procedure they calculated a mol wt for vancomycin of 1600–1700, which is approximately 14% higher than that obtained from X-ray analysis. The difference may be caused by the difficulty in obtaining an unhydrated sample of antibiotic. The constituents reported herein for ristocetin A aglycone give a mol wt of 1159; with the methyl ester, reported to occur at the C terminus,[19] a mol wt of 1173 is obtained for the aglycone, or 2066 for the intact antibiotic.

Unfortunately it has not yet been possible to obtain definitive NMR spectra for ristocetin, aglycoristocetin, or derivatives. In the 100-MHz proton spectrum the peaks are broad even at elevated temperatures and the aromatic region is poorly resolved. In the 15-MHz ^{13}C NMR spectrum of the intact antibiotic seven carbonyls can be discerned but overlap of two or more carbonyl resonances cannot be excluded. Many of the aromatic carbons can be distinguished but some of them clearly overlap and detailed analysis of the spectrum has not yet been achieved. The absence of aliphatic residues is apparent in both

Scheme VIII



Scheme IX



^1H and ^{13}C spectra but better resolution will be required before the aromatic signals can be assigned. The spectra, while in agreement with the constituents proposed, do not provide unequivocal confirmation.

With the X-ray structure of a derivative of vancomycin now available[8f] and given the similarity in peptide-combining specificity between vancomycin and ristocetin,[2d] it is quite possible that both possess the same arrangement of the actinoidinic- and vancomycinic-type residues (Scheme VIII). Indeed, this may be the common feature of all members of this class of antibiotics.

Even if the sequence in Scheme VIII is not the correct structure for ristocetin, many other sequences can be eliminated on the basis of steric constraints on the coupled amino acids; for example, a peptide bond cannot occur between the two amino acid moieties of ristomycinic acid. These constraints are unusually severe in ristocetin because four cross-links are present in a peptide containing only seven amino acids.

In conclusion it can be pointed out that, with regard to biosynthesis, ristocetin can be considered to be the product of intramolecular oxidative coupling of a heptapeptide containing hydroxylated aromatic amino acids with C–O coupling occurring in three cases and C–C coupling occurring in one (Scheme IX).[20]

Experimental Section

Melting points are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 727 spectrophotometer. NMR spectra were obtained with a JEOL MH-100 100-MHz spectrometer operating in the external lock mode. With organic solutions Me_4Si was used as the in-

ternal solvent, with aqueous solutions CH_3CN (δ 2.00). The signals for amide protons are not reported because they were often difficult to observe on account of high noise level or partial overlap with the aromatic multiplet. Low-resolution mass spectra were obtained on a LKB-9000A mass spectrometer (70 eV) using the direct inlet. High-pressure liquid chromatography was carried out on a Waters Associates liquid chromatograph with UV and differential RI detectors. TLC was performed with Merck precoated silica gel 60 F-254 plates with detection by UV, exposure to I_2, or spraying with diazotized benzidine. Open column chromatography was carried out using MCB silica gel, grade 62, or, in cases where noted, Merck TLC silica gel (60) HF-254 without binder. Organic solutions were routinely dried with $MgSO_4$ before evaporation. Elemental analyses were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn. The ristocetin used in these experiments was the sulfate salt; it was essentially pure ristocetin A containing less than 5% of the B form.

Acid Hydrolysis Using HCl. Acetic anhydride (10 mL) was added dropwise over 15 min to a refluxing solution of ristocetin (1.0 g), NH_4OAc (5.0 g), and NaOAc (2.0 g) in H_2O (20 mL) under N_2. After heating for additional 10 min, the reaction mixture was cooled and H_2O, HOAc, and most of the NH_4OAc were removed in vacuo. The residue was dissolved in 50 mL of MeOH–H_2O and treated with 150 mL (35 mmol) of ethereal CH_2N_2 for 16 h. The solvents were removed in vacuo. The residue was treated for 45 min with refluxing 1 N HCl; after cooling, partially protected aglycone was isolated by filtration. This material was suspended in H_2O (30 mL) under N_2; aqueous KOH was added until the aglycone dissolved. The solution was treated with Me_2SO_4 (1.4 g, 10 mmol) and KOH (0.62 g in 1 mL of H_2O) which were added simultaneously through two syringes at 0 °C. After 30 min, the addition was repeated at 25 °C. After an additional 1 h, the mixture was acidified and the methylated aglycone isolated by filtration. Hydrolysis was carried out in a refluxing mixture of concentrated HCl (15 mL), H_2O (15 mL), and HOAc (15 mL) for 48 h under N_2. The solvents were removed in vacuo; the residue was dissolved in aqueous NaOH at 0 °C and treated with Ac_2O (5 mL) added in 0.5-mL portions over 1 h. After an additional 1 h, concentrated HCl (20 mL) mixed with ice was added and solvents were removed in vacuo. The brown residue was dissolved in MeOH and treated for 16 h with excess CH_2N_2 (15 mmol). After removal of solvents, the residue was suspended in MeOH and filtered to give 0.432 g of tan powder. The material (0.338 g) which was soluble in 4 mL of CH_2Cl_2–MeOH (4:1) was chromatographed on silica gel. The fraction which eluted with 5% MeOH in CH_2Cl_2 was further purified by LC (2-ft μ-Porasil, CH_2Cl_2–MeOH, 97:3). The protected amino acids eluted in the following order: **2a**, **1a**, **1b**, **2b**, **2c**, and **2d**. All of the compounds were well separated from one another with the exception of **2c** and **2d**, which were insufficiently resolved to be collected individually.

Compound **1a** (11.1 mg): calcd for $C_{25}H_{30}N_2O_9$ 502.1950, found (HRMS)[21a] 502.1979; IR ($CHCl_3$) 3425, 3000, 2950, 1740 (s), 1670 (s), 1580, 1505, 1140 cm^{-1}; NMR ($CDCl_3$) δ 2.00 (s, 6 H, $COCH_3$), 2.12 (s, 3 H, CMe), 3.65 (s, 3 H, OMe), 3.68 (s, 3 H, OMe), 3.86 (s, 6 H, OMe), 5.52 (d, 2 H, α-CH), 6.47–6.98 (m, 5 H, aryl); MS *m/e* 502 (parent, 23%), 470 (38), 459 (46), 443 (23), 401 (100), 368 (23), 357 (23).

Compound **1b** (16.3 mg): calcd for $C_{25}H_{30}N_2O_9$ 502.1950, found (HRMS)[21a] 502.1973; IR ($CHCl_3$) 3430, 3000, 2950, 1740 (s), 1680 (s), 1510, 1140 cm^{-1}; NMR ($CDCl_3$) δ 1.98 (s, 6 H, $COCH_3$), 2.12 (s, 3 H, CMe), 3.70 (s, 6 H, OMe), 3.85 (s, 6 H, OMe), 5.44 (d, 2 H, α-CH), 6.39–7.0 (m, 5 H, aryl); MS *m/e* 502 (parent, 36%), 470 (46), 459 (86), 443 (38), 401 (100), 368 (38), 357 (19).

Compound **2a** (13.7 mg): calcd for $C_{25}H_{30}N_2O_9$ 502.1950, found (HRMS)[21a] 502.1944; IR ($CHCl_3$) 3450, 3350, 3000, 2950, 1740 (s), 1660 (s), 1505, 1414 cm^{-1}; NMR ($CDCl_3$) δ 2.04 (s, 6 H, $COCH_3$), 3.62 (s, 3 H, OMe), 3.65 (s, 3 H, OMe), 3.68 (s, 3 H, OMe), 3.76 (s, 3 H, OMe), 3.82 (s, 3 H, OMe), 5.40 (d, 2 H, α-CH), 6.45–7.35 (m, 5 H, aryl); MS *m/e* 502 (parent, 37%), 470 (84), 459 (87), 443 (73), 401 (100), 351 (94).

Compound **2b** (7.6 mg): NMR ($CDCl_3$) δ 1.96 (s, 3 H, $COCH_3$), 2.04 (s, 3 H, $COCH_3$), 3.55 (s, 3H, OMe), 3.72 (s, 3 H, OMe), 3.76 (s, 3 H, OMe), 3.84 (s, 3 H, OMe), 5.40 (m, 2 H, α-CH), 6.55–7.3 (m, 5 H, aryl); MS *m/e* 502 (parent, 39%), 470 (61), 459 (65), 443 (51), 401 (100), 351 (77).

Mixture of compounds **2c** and **2d** (10.5 mg): calcd for $C_{25}H_{30}N_2O_9$ 502.1950, found (HRMS)[21a] 502.1958; IR ($CHCl_3$) 3450, 3000, 2950, 1740 (s), 1670 (s), 1600, 1500–1490 cm^{-1}; NMR ($CDCl_3$) δ 1.90, 1.95, 2.00, and 2.05 (4 s, total 6 H, $COCH_3$), 3.58, 3.68, 3.72, 3.73, 3.75, 3.77, and 3.85 (7 s, total 15 H, OMe), 5.40 (m, 2 H, α-CH), 6.58–7.3 (m, 5 H, aryl); MS *m/e* 502 (parent, 36%), 470 (73), 459 (68), 443 (36), 401 (100), 351 (64).

Hydrolysis with Methanesulfonic Acid. Acetylated, methylated aglycoristocetin (0.422 g) was treated for 66 h with refluxing 4 N methanesulfonic acid (30 mL) under N_2. Considerable insoluble material remained so 5 mL of HOAc was added and refluxing continued for 24 h. The reaction mixture was cooled and washed with Et_2O. The aqueous layer was adjusted to pH 9.5 by addition of solid K_2CO_3; Ac_2O (3.0 g) was added dropwise to the cooled (ice bath) solution with the pH maintained at 9.5 by addition of 4 N KOH. After stirring for 1 h, the solution was acidified to pH 2 with 6 N HCl and extracted with EtOAc–MeOH (9:1). The organic extract was dried and evaporated. The residue was dissolved in MeOH and treated with excess CH_2N_2 (14 mmol). After evaporation of the solvents, the portion of the residue which was soluble in CH_2Cl_2 was chromatographed on 5 g of TLC silica gel with elution by $CHCl_3$–MeOH (98:2). A total of 115 mg of protected amino acids **1a,b** and **2a–d** were recovered.

Isolation of 2a and 2b–d from Aglycovancomycin. Acetylated, methylated aglycovancomycin (0.406 g, prepared as described for ristocetin) was treated with refluxing 4 N methanesulfonic acid (30 mL) for 66 h under N_2. Protected **2a–d** were prepared and isolated as described for ristocetin. The diastereoisomeric **2a** (13.3 mg) and **2b–d** (27.6 mg) were separated by chromatography on 5 g of TLC silica gel with CH_2Cl_2–MeOH (98:2) elution.

Degradation of Biphenyl Derivative 2c,d to Dinitrile 19. Compound **2c,d** (22 mg, 0.044 mmol) which had been obtained from ristocetin was hydrolyzed to the free amino acid by treatment with refluxing 1 N HCl (5 mL) for 18 h to give, after removal of the solvent, bis(amino acid) **18**: NMR (D_2O–DCl) δ 3.72, 3.74, 3.80, 3.88 (4 s, total 9 H, OMe), 5.1 (broad s, 2 H, α-CH), 6.75–7.6 (m, 5 H, aryl). Compound **18**, dissolved in 1 mL of 1 N KOH, was added dropwise to an ice-cold 5% solution of NaOCl (Clorox, 0.6 mL)[22] and 1 N KOH (0.5 mL). The reaction mixture was stirred for 1 h as it warmed to room temperature. Excess NaOCl was destroyed with Na_2SO_3. The reaction mixture was heated at 55 °C for 5 min to ensure decomposition of the chloro imine intermediate, cooled, and extracted twice with EtOAc. The extracts were combined, washed with H_2O, dried, and evaporated to yield 11.5 mg (89%) of dinitrile **19** which was further purified by LC (μ-Porasil, pentane–EtOAc, 3:1): calcd for $C_{17}H_{14}N_2O_3$ 294.1003, found (HRMS)[21a] 294.0999; IR ($CHCl_3$) 2240 (s), 1601 (s), 1460, 1340, 1160 cm^{-1}; NMR (CD_3COCD_3) δ 3.81 (s, 3 H, OMe), 3.88 (s, 3 H, OMe), 3.93 (s, 3 H, OMe), 6.96 (s, 2 H, 4′- and 6′-H's), 7.30 (d, J = 8 Hz, 1 H, 5-H), 7.59 (d, J = 2 Hz, 1 H, 2-H), 7.82 (dd, J = 8 + 2 Hz, 1 H, 6-H); in $CDCl_3$ the 4′ and 6′ protons were no longer equivalent (6.77 and 6.85 ppm, J = 2 Hz); MS *m/e* 294 (parent, 100%).

Similar degradations were carried out on **2a** from ristocetin and on **2a** and **2c,d** obtained from vancomycin; in each case the oxidation yielded dinitrile **19**.

Hydrolysis of Dinitrile 19 to Diester 7. Dinitrile **19** (8.3 mg, 0.028 mmol), obtained from ristocetin, was treated for 6 h with refluxing 30% NaOH (2 mL). The mixture was diluted with 2 mL of H_2O, cooled, neutralized with 50% H_2SO_4, and extracted with EtOAc–MeOH (9:1). After evaporation of the organic phase, the residue was treated with excess CH_2N_2 and purified by TLC on silica gel (EtOAc–pentane, 1:1) to give 8.7 mg (85%) of diester **7**, identical by mass spectrum, TLC, and NMR with material isolated by permanganate oxidation of ristocetin (see below).

Oxidation of the Products Obtained from Base Hydrolysis of Ristocetin. Aglycoristocetin (prepared from 1.0 g of ristocetin by 2-h reflux in 20 mL of 1 N H_2SO_4) was dissolved in 25 mL of H_2O under N_2. To this solution was added 10 N NaOH (3 mL) and Me_2SO_4 (1.8 mL) and after 6 h additional 10 N NaOH (1.5 mL) and Me_2SO_4 (0.6 mL) were added. After an additional 12 h, the solution was treated with NH_4OH to destroy excess Me_2SO_4 and acidified and the methylated aglycone which precipitated (0.54 g) was isolated by filtration. One-half of this material was refluxed with 0.5 N NaOH (25 mL) for 15 h. The crude hydrolysate was treated with $KMnO_4$ (3 g) for 1 h at 84 °C. Excess $KMnO_4$ was destroyed with 2-propanol and the reaction mixture then refluxed for an additional 12 h. The mixture was filtered and the filtrate treated with 30% H_2O_2 (3 mL) for 20 h, acidified, and extracted with Et_2O and EtOAc. Both organic fractions were treated with excess CH_2N_2.

Dimethyl 4-methoxyisophthalate (**3**) was isolated from the Et_2O

fraction and identified by comparison with authentic material: mp 94–94.2 °C (lit.[23] mp 94 °C); NMR (CCl_4) δ 3.79 (s, 3 H, OMe), 3.89 (s, 6 H, 2 OMe), 6.84 (d, J = 9 Hz, 1 H, 5-H), 7.97 (dd, J = 9 + 2 Hz, 1 H, 6-H), 8.26 (d, J = 2 Hz, 1 H, 2-H).

The constituents of the EtOAc fraction (38 mg) were separated by LC (16-ft Corasil II, Et_2O–pentane, 1:1) to give three fractions containing **4**, **5**, and **7**. Diester **4**: mp 132–135 °C; calcd for $C_{19}H_{20}O_7$ 360.1209, found (HRMS)[21c] 360.1208; NMR ($CDCl_3$) δ 2.22 (s, 3 H, CMe), 3.87 (s, 6 H, 2 OMe), 3.95 (s, 6 H, 2 OMe), 7.02 (d, J = 8 Hz, 1 H, 5-H), 7.08 (br s, 1 H, 2′- or 6′-H), 7.32 (br s, 1 H, 6′- or 2′-H), 7.44 (d, J = 2 Hz, 1 H, 2-H), 7.85 (dd, J = 8 + 2 Hz, 1 H, 6-H); MS *m/e* 360 (parent, 100%), 329 (42), 193 (35). Triester **5**: mp 129 °C; calcd for $C_{20}H_{20}O_9$ 404.1107, found (HRMS)[21c] 404.1101; NMR ($CDCl_3$) δ 3.81 (s, 3 H, OMe), 3.82 (s, 3 H, OMe), 3.84 (s, 3 H, OMe), 3.88 (s, 3 H, OMe), 3.89 (s, 3 H, OMe), 6.90 (d, J = 2 Hz, 1 H, 2′- or 6′-H), 6.95 (d, J = 9 Hz, 1 H, 5-H), 7.27 (d, J = 2 Hz, 1 H, 6′- or 2′-H), 7.66 (d, J = 2 Hz, 1 H, 2-H), 7.86 (dd, J = 9 + 2 Hz, 1 H, 6-H); MS *m/e* 404 (parent, 100%), 373 (52), 335 (52), 231 (100). Demethylation of **5** by BCl_3 (11 h at 0 °C and 3 h at 20 °C in CH_2Cl_2) gave phenolic ester **6**: NMR ($CDCl_3$) δ 3.83 (s, 3 H, OMe), 3.85 (s, 3 H, OMe), 3.88 (s, 3 H, OMe), 3.89 (s, 3 H, OMe), 6.82 (d, J = 2 Hz, 1 H, 2′- or 6′-H), 7.00 (d, J = 8 Hz, 1 H, 5-H), 7.34 (d, J = 2 Hz, 1 H, 6′- or 2′-H), 7.51 (d, J = 2 Hz, 1 H, 2-H), 7.85 (dd, J = 8 + 2 Hz, 1 H, 6-H), 11.29 (br s, 1 H, OH); MS *m/e* 390 (parent, 80%), 359 (80), 326 (90), 299 (100). Triester **7**: mp 67 °C; NMR (CCl_4) δ 3.71 (s, 3 H, OMe), 3.83 (s, 3 H, OMe), 3.85 (s, 6 H, 2 OMe), 6.89 (d, J = 8 Hz, 4 H, 3-, 5-, 3″-, and 5″-H's), 7.55 (s, 2 H, 2′- and 6′-H's), 7.94 (d, J = 8 Hz, 4 H, 2-, 6-, 2″-, and 6″-H's); MS *m/e* 466 (parent, 100%), 435 (29), 202 (20).

The oxidation products **3**, **4**, and **7** were also isolated from $KMnO_4$ oxidation of unhydrolyzed, methylated aglycone but in lower yields.

Isolation of Diester 8 from Ristocetin. Aglycoristocetin (0.5 g, prepared by hydrolysis of ristocetin for 45 min in refluxing 0.5 M H_2SO_4) was acetylated (Ac_2O/H_2O, 100 °C), methylated (MeI, NaH, DMF), and hydrolyzed for 48 h under N_2 in a refluxing mixture of HOAc and 6 N HCl (1:1). The solvents were removed in vacuo; the residue was dissolved in aqueous $KHCO_3$, and the pH was adjusted to 8. A slight excess of NaOCl was added.[22] After standing for 24 h at 25 °C, the solution was treated with Na_2SO_3 to destroy excess NaOCl. The pH was adjusted to 14 with 50% NaOH and the solution refluxed for 4 h to hydrolyze any nitriles that might be present. The solution was cooled, the pH was adjusted to 7.5, and excess dilute $KMnO_4$ was added at 25 °C until consumption ceased. Excess $KMnO_4$ was destroyed with Na_2SO_3 and the mixture was acidified and extracted with EtOAc–MeOH (9:1). The extract was dried and evaporated; the residue was methylated with excess CH_2N_2. Preparative TLC (silica gel, EtOAc–pentane, 1:1) followed by LC (2-ft μ-Porasil, EtOAc–pentane, 1:9) gave **4** (15 mg), **7** (2 mg), and **8** (4 mg). Compound **8**: calcd for $C_{19}H_{20}O_7$ 360.1209, found (HRMS)[21d] 360.1211; NMR ($CDCl_3$) δ 3.56 (s, 3 H, OMe), 3.70 (s, 3 H, OMe), 3.76 (s, 3 H, OMe), 3.85 (s, 3 H, OMe), 3.86 (s, 3 H, OMe), 6.70 (d, J = 2 Hz, 1 H, 4′-H), 6.96 (d, J = 8 Hz, 1 H, 5-H), 7.06 (d, J = 2 Hz, 1 H, 6′-H), 7.82 (d, J = 2 Hz, 1 H, 2-H), 8.04 (dd, J = 8 + 2 Hz, 1 H, 6-H).

Isolation of Diester 8 from Vancomycin. Aglycovancomycin (1.5 g, dried in vacuo over KOH pellets) was dissolved in 40 mL of DMF under N_2, MeI (6 g) was added, and the solution was cooled to −10 °C. NaH (1 g) was added in portions over 20 min and stirring continued for 3 h. The mixture was poured cautiously into ice water and extracted with $CHCl_3$. Evaporation of the organic phase and removal of residual DMF under vacuum (30 °C, 2 mm) gave methylated aglycone (1.2 g) as a pale yellow powder which was then treated with a refluxing mixture of dioxane (40 mL) and 6 N HCl (60 mL) for 17 h under N_2. Workup as above gave diester **8** (3 mg) as white crystals having mass spectrum and NMR identical with those of material isolated from ristocetin.

Oxidation of Bis(amino acid) 12 to Esters 4 and 5. A desalted mixture of the stereoisomeric amino acids **12**[24] (ca. 40 mg) dissolved in H_2O (2.5 mL) was treated at 95 °C with Ac_2O (2.0 mL) added in several aliquots. Heating was continued for 1 h. The reaction mixture was evaporated in vacuo and the residue was taken up in H_2O (5 mL) and neutralized with K_2CO_3. KOH (0.56 g, 1 mmol) and Me_2SO_4 (0.63 g, 0.5 mmol) were added and the mixture was stirred at room temperature for 1 h. Concentrated NH_4OH (1 mL) was added, followed by brief warming of the mixture and by evaporation of solvents. The residue was diluted to 5 mL, additional KOH (0.56 g) added, and the reaction mixture heated for 60 h at 95 °C. After cooling, 5% NaOCl (2 mL) was added in aliquots over 24 h until a permanent starch–iodide test was obtained. The reaction mixture was flushed briefly with N_2 to remove amines, and then treated for 90 min at room temperature with $KMnO_4$ (0.4 g) added in two portions. The reaction mixture was quenched with MeOH, filtered through Celite, acidified with HCl, and extracted with Et_2O. The Et_2O solution was dried and evaporated. The residue was treated with excess CH_2N_2 and the resulting ester mixture separated by LC (16-ft Corsil II, Et_2O–pentane, 1:1). The major fraction was identified as diester **4** by mass spectrum and NMR; triester **5** was also isolated.

Synthesis of Diester 4. Ethyl 3-amino-5-nitro-*p*-toluate,[25] mp 147–148.5 °C, was converted to 3-hydroxy-5-nitro-*p*-toluic acid via the diazonium tetrafluoroborate salt.[26] The acid was treated with excess CH_2N_2 in MeOH to give methyl 3-methoxy-5-nitro-*p*-toluate (**26**): mp 75–76.5 °C; NMR ($CDCl_3$) δ 2.40 (s, 3 H, CMe), 3.95 (s, 6 H, 2 OMe), 7.64 (d, J = 2 Hz, 1 H, aryl), 8.00 (d, J = 2 Hz, 1 H, aryl). Anal. Calcd for $C_{10}H_{11}NO_5$: C, 53.33; H, 4.76. Found: C, 53.26; H, 4.92.

Ester **26** (1.5 g, 6.67 mmol) was reduced with H_2 (1 atm) and PtO_2 (0.4 g) in 95% EtOH (50 mL). The reduction was stopped when the calculated amount of H_2 had been taken up. Filtration, evaporation, and chromatography on silica gel (EtOAc–pentane mixtures) gave methyl 3-amino-5-methoxytoluate (**27**) in essentially quantitative yield. Recrystallization from EtOAc gave needles: mp 85–86.5 °C; IR (neat) 3320, 3250, 1708 cm^{-1}; NMR ($CDCl_3$) δ 2.08 (s, 3 H, CMe), 3.16 (br s, 2 H, NH_2), 3.87 (s, 3 H, OMe), 3.92 (s, 3 H, OMe), 7.10 (d, J = 1.5 Hz, 1 H, aryl), 7.14 (d, J = 1.5 Hz, 1 H, aryl). Anal. Calcd for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71. Found: C, 61.52; H, 6.66.

Ester **27** (1.3 g, 6.7 mmol) was diazotized by treatment of an aqueous (12 mL) solution containing HBF_4 (1.75 g) with $NaNO_2$ (0.6 g, 8.6 mmol). The diazonium salt was decomposed in situ by addition to a solution of $Cu(NO_3)_2$ (1.25 g) and Cu_2O (1.8 g) in 100 mL of H_2O. After N_2 evolution had ceased, the aqueous suspension was extracted with Et_2O; the extract was washed with dilute HCl and with H_2O and evaporated to give methyl 3-hydroxy-5-methoxy-*p*-toluate (**13**) in 60% yield. Chromatography on silica gel (EtOAc–pentane mixtures) followed by recrystallization from EtOAc–pentane gave needles: mp 132–133.5 °C; IR (Nujol) 3340, 1705 cm^{-1}; NMR ($CDCl_3$) δ 2.18 (s, 3 H, CMe), 3.90 (s, 3 H, OMe), 3.94 (s, 3 H, OMe), 5.92 (s, 1 H, OH), 7.21 (d, J = 2 Hz, 1 H, aryl), 7.32 (d, J = 2 Hz, 1 H, aryl). Anal. Calcd for $C_{10}H_{12}O_4$: C, 61.20; H, 6.15; Found: C, 61.40; H, 6.26.

A procedure modeled upon a condensation reported by Bacon[27] was employed for the preparation of **4**. Ester **13** (1.96 g, 10 mmol) was treated with NaH (0.218 g, 9.5 mmol) in Et_2O. After H_2 evolution had ceased, the precipitated phenoxide salt was isolated by filtration and dried under vacuum. A mixture of the phenoxide salt (0.436 g, 2 mmol), methyl 3-bromoanisate (**14**, 0.5 g, 2 mmol), and Cu_2Br_2 (0.8 g) in dimethylacetamide (8 mL) was refluxed under N_2 with stirring for 48 h. The mixture was poured into dilute HCl and extracted with EtOAc. The organic solution was washed with dilute HCl and with saturated NaCl solution. After evaporation and removal of dimethylacetamide in vacuo, the residue was dissolved in MeOH and treated with CH_2N_2. Evaporation followed by chromatography on silica gel (EtOAc–pentane mixtures) gave diester **4** in 10% yield. Further purification by LC (2-ft, μ-Porasil, EtOAc–pentane, 1:9) and recrystallization from EtOAc–pentane gave needles, mp 145–146 °C, having NMR and mass spectrum identical with those of material derived from ristocetin. Anal. Calcd for $C_{19}H_{20}O_7$: C, 63.32; H, 5.59. Found: C, 63.18; H, 5.69.

Synthesis of Triester 5. 3,5-Dihydroxybenzoic acid (4.0 g, 25 mmol), K_2CO_3 (8.0 g, 57 mmol), glycerol (25 g), and dry ice (ca. 5 g) were sealed in a bomb equipped with a magnetic stirrer and heated at 150 °C for 16 h (autogenous CO_2 pressure: 80 atm). After cooling, the solid residue was taken up in water, which was acidified and extracted with Et_2O–EtOAc. The extract was washed with saturated NaCl solution, dried, and evaporated to give 2.0 g (40%) of 2,6-dihydroxyterephthalic acid, mp 273–277 °C (lit.[28] mp 277 °C). Treatment of 1.15 g of the acid with CH_2N_2 in MeOH gave 1.54 g (100%) of dimethyl 2,6-dimethoxyterephthalate, mp 119–121 °C (lit.[28] mp 122 °C). This material was treated with excess BCl_3 in CH_2Cl_2 at 0 °C for 2 h followed by 20 °C for 2 h. After evaporation of solvent the yellow, crystalline residue was shaken with Et_2O and H_2O. The Et_2O layer yielded 1.17 g (84%) of diester **15**, mp 92–94 °C. Recrystallization from EtOH–H_2O followed by Et_2O–hexane

and then by sublimation (90 °C, 0.1 mm) gave white microcrystals: mp 96–96.5 °C; NMR ($CDCl_3$) δ 3.97 (s, 3 H, OMe), 3.99 (s, 6 H, 2 OMe), 7.10 (d, J = 2.3 Hz, 1 H, aryl), 7.27 (d, J = 2.3 Hz, 1 H, aryl), 11.36 (s, 1 H, OH). Anal. Calcd for $C_{11}H_{12}O_6$: C, 55.00; H, 5.04. Found: C, 55.32; H, 5.12.

Phenolic ester **15** (240 mg, 1 mmol) and NaH (50 mg) were warmed together in a tube until H_2 evolution ceased. Methyl 3-bromoanisate (**14**, 245 mg, 1.0 mmol) was added and heating continued at 150 °C for 10 min; Cu_2Br_2 (20 mg) was added and the tube was sealed. After heating at 190 °C for 12 h, the tube was opened and the contents partitioned between dilute HCl and Et_2O–CH_2Cl_2. The organic phase was evaporated and large quantities of starting materials were removed by sublimation (100–120 °C, 0.1 mm). The residue was treated with CH_2N_2 and additional material removed by sublimation. The remainder was passed through a short column of silica gel (CH_2Cl_2–Et_2O mixtures) and further purified by LC (16-ft Corasil II, Et_2O–pentane, 1:1) to yield as the last major peak a small quantity (1%) of **5**, identical by mass spectrum and NMR with **5** obtained by oxidation of ristocetin.

Synthesis of Diester 8. Methyl 3-iodoanisate[29] (**20**, 292 mg, 1 mmol), methyl 2-bromo-3,5-dimethoxybenzoate[30] (**21**, 92 mg, 0.33 mmol), and Cu powder (pretreated with 0.02 M Na_2EDTA solution)[31] were intimately combined in a 10 × 77 mm fushion tube and heated in a sand bath for 30 min at 220 °C. On cooling, the resulting cake was extracted with CH_2Cl_2; the extract was washed with 0.02 M Na_2EDTA, dried, and evaporated. TLC analysis (silica gel, EtOAc–pentane, 1:9) indicated three major products together with unreacted ester **20**. Integration of the NMR spectrum ($CDCl_3$) of the crude mixture suggested ca. 70% yield of coupling product **8**. Separation by LC (2-ft μ-Porasil, EtOAc–pentane, 1:5) gave compound **8** along with symmetrical coupling products, which were identified as dimethyl 4,4′-dimethoxy-3,3′-bibenzoate[32] and dimethyl 3,3′,5,5′-tetramethoxy-2,2′-bibenzoate[33] by NMR and mass spectrum. Compound **8**, prepared in this way, was spectroscopically identical with **8** derived from ristocetin and from vancomycin.

Synthesis of Triester 7. Methyl 4-hydroxybenzoate (**24**, 1.5 g, 10 mmol), methyl 3,5-dibromo-4-methoxybenzoate[34] (**25**, 1.5 g, 5 mmol), KOMe (10 mmol, prepared from 0.39 g of K metal), and Cu_2Cl_2 (0.3 g) were heated to 100 °C while permitting MeOH to distill out and then heated at 180 °C for 8 h under N_2. After cooling, the reaction mass was dissolved in CH_2Cl_2, decolorized with charcoal, and washed with dilute NaOH and with H_2O. After drying, the solvent was evaporated and the residue was chromatographed on silica gel (CH_2Cl_2). The fractions containing **7** were chosen by mass spectral analysis and further purified by LC (16-ft Corasil II, Et_2O–pentane, 15:85) to give 30 mg of triester **7**, mp 67 °C, which was identical with material prepared from ristocetin. Anal. Calcd for $C_{25}H_{22}O_9$: C, 64.38; H, 4.75. Found: C, 64.20; H, 4.71.

Base Hydrolysis of Ristocetin. Partially methylated aglycoristocetin (0.327 g, prepared by treatment of ristocetin with CH_2N_2, followed by removal of sugars by acid treatment and retreatment with CH_2N_2) was dissolved in 4 N KOH (15 mL) containing $NaBH_4$ (1.0 g) and refluxed under N_2 for 23 h. The colorless solution was cooled, adjusted to pH 9 with 6 N HCl, and treated with Ac_2O (3.0 mL) added dropwise with alternate addition of base to maintain alkaline pH. After 2 h, the reaction mixture was acidified to pH 1 and extracted three times with EtOAc–MeOH (9:1). The combined extracts were dried and evaporated; the residue was dissolved in MeOH and treated with CH_2N_2 (20 mmol). After 30 min, the solvents were evaporated and the residue (196 mg) chromatographed on a column containing 5 g of TLC silica gel. Elution with $CHCl_3$–MeOH (98:2) yielded compounds **1a,b** and **2a–d** plus compound **11** (25 mg) [calcd for $C_{23}H_{27}NO_8$ 445.1737, found (HRMS)[21b] 445.1744; NMR ($CDCl_3$) δ 1.96 (s, 3 H, CMe), 2.09 (s, 3 H, $COCH_3$), 3.45 (s, 2 H, CH_2), 3.62 (s, 3 H, OMe), 3.64 (s, 3 H, OMe), 3.82 (s, 3 H, OMe), 3.84 (s, 3 H, OMe), 5.45 (s, 1 H, α-CH), 6.44–6.96 (m, 5 H, aryl); MS m/e 445 (parent, 34%), 413 (100), 402 (86), 386 (25)] and another compound tentatively identified as **10b** (4.8 mg, repurified by LC on μ-Porasil, CH_2Cl_2–MeOH, 97:3) [NMR ($CDCl_3$) δ 2.00 (s, 3 H, $NHCOCH_3$), 2.10 (s, 3 H, $OCOCH_3$), 3.73 (s, 3 H, OMe), 3.77 (s, 3 H, OMe), 4.63 (s, 2 H, CH_2OH), 5.40 (d, J = 8 Hz, $CHNHCOCH_3$), 6.76–7.33 (m, 10 H, aryl) (the resonance for the $-CH_2CH-$ protons could not be identified with certainty owing to the high noise level of the spectrum); MS m/e 595 (parent)]. Further elution of the column with CH_2Cl_2–MeOH (95:5) yielded a mixture (35 mg) of compounds which was separated by preparative TLC (silica gel, $CHCl_3$–MeOH, 95:5). Compound **9**, the slowest moving component, was repurified by LC (2-ft μ-Porasil, CH_2Cl_2–MeOH, 96:4): calcd for $C_{26}H_{27}NO_8$ 481.1737, found (HRMS)[21b] 481.1741; NMR ($CDCl_3$) δ 2.05 (s, 3 H, $COCH_3$), 3.70 (s, 3 H, OMe), 3.78 (s, 3 H, OMe), 4.65 (br s, 4 H, 2 CH_2), 5.44 (d, 1 H, α-CH), 6.81 (s, 2 H, 2′- and 6′-H's), 6.97 (d, J = 8 Hz, 4 H, 3-, 5-, 3″-, and 5″-H's), 7.27 (d, J = 8 Hz, 4 H, 2-, 6-, 2″, and 6″-H's) (in CD_3COCD_3 the peaks at 6.81 and 6.97 ppm coalesce at 7.05 ppm); MS m/e 481 (parent, 35%), 463 (60), 438 (50), 421 (100). When the hydrolysis reaction was carried out in the presence of $NaBD_4$ the parent ion of compound **9** appeared at m/e 483 indicating the introduction of two deuterium atoms; the NMR spectrum of deuterated **9** showed the benzylic methylene signal to be further broadened and of diminished intensity. Another compound, which was the fastest moving band on the preparative TLC plate, was tentatively identified as **10a** after further purification by LC (2-ft μ-Porasil, CH_2Cl_2–MeOH, 97:3): NMR ($CDCl_3$) δ 2.0 (s, 3 H, $COCH_3$), 3.07–3.19 (m, 2 H, CH_2CH), 3.74 (s, 3 H, OMe), 3.78 (s, 3 H, OMe), 3.80 (s, 3 H, OMe), 4.66 (s, 2 H, CH_2), 5.40 (d, J = 8 Hz, 1 H, $CHNHCOCH_3$), 6.78–7.12 (m, 10 H, aryl) (the resonance for the $-CH_2CH-$ proton could not be identified with certainty owing to the high noise level of the spectrum); MS m/e 553 (parent).

Isolation of Glycine from Base Hydrolysis of Ristocetin. Ristocetin (216 mg) was hydrolyzed in refluxing 4 N KOH (15 mL) for 24 h under N_2. After cooling, the solution was adjusted to pH 10, 2,4-dinitrofluorobenzene (150 mg in 3 mL of EtOH) was added, and the mixture was stirred for 2 h at room temperature. The mixture was extracted with Et_2O, acidified, and reextracted with Et_2O. The second extract was treated with CH_2N_2 and evaporated. The residue was chromatographed on 5 g of silica gel (EtOAc–pentane mixtures); with a 3:7 solvent mixture 23.2 mg (1.06 mol/mol ristocetin)[35] of the methyl ester of N-(2,4-dinitrophenyl)glycine was eluted. The identity of this derivative was established by NMR, mass spectrum, and TLC comparison with synthetic material.

When ristocetin was hydrolyzed with 4 N methanesulfonic acid for 48 h and the hydrolysate treated with 2,4-dinitrofluorobenzene as above, no methyl dinitrophenylglycinate was detected by TLC or NMR, although a small parent ion (m/e 255) was seen in the mass spectrum of the appropriate chromatographic fraction.

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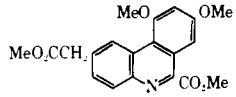

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Crystal and Molecular Structure of the Cyclic Hexapeptide *cyclo*-(Gly-Pro-*d*-Phe)$_2$

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Abstract: The crystal structure of *cyclo*-(Gly-Pro-*d*-Phe)$_2$ (GPF) has been determined by single-crystal X-ray diffraction and refined by block-diagonal least squares to an R value of 0.099. The crystals are monoclinic, $P2_1$, with cell constants of a = 19.694 (1) Å, b = 9.005 (1) Å, c = 10.357 (1) Å, and β = 104.05 (1)°. Although the crystal structure contains dimethyl sulfoxide, the conformation of GPF is similar to that of the hexapeptide *cyclo*-(Ala-Pro-*d*-Phe)$_2$, which has a crystal structure containing water. The structure of GPF consists of two type II β turns without strong 4 → 1 hydrogen bonds.

Introduction

In recent years there has been a steady increase in the frequency of reports in the literature of conformational studies of oligopeptides by either NMR experiments or energy calculations. Although several classes of compounds have been considered, the cyclic hexapeptides have been found amenable to both approaches since they have fewer degrees of freedom than do the analogous acyclic peptides yet still retain sufficient flexibility so that their conformation is not strictly dominated by nearest-neighbor interactions.

Since cyclic hexapeptides without other constraints still possess too many degrees of freedom, we have been interested in the restricted conformations of compounds with the sequence *cyclo*-(1-X-1-Pro-*d*-Phe)$_2$ in which the existence of two Pro residues restricts the available conformational space the peptide may occupy. These compounds have been postulated[1,2] to possess C_2 symmetry with the 1-Pro in the 2 position of a type II β turn which is stabilized by a 4 → 1 hydrogen bond between the N-H of X in position 1 and the C=O of the symmetry-related X in position 4. We have previously determined the crystal structure of *cyclo*-(1-Ala-1-Pro-*d*-Phe)$_2$,[3] hereafter referred to as APF, which has a conformation qualitatively similar to that postulated by NMR[1] but does not possess the anticipated strong 4 → 1 hydrogen bonds, although it does adapt a conformation characteristic of double type II β turns.

However, the more interesting result from the crystal structure of APF was not the agreement of the X-ray and NMR experiments but rather the hydration that accompanied the crystal structure. Since the peptide literally lies in a sea of solvent such that there are no peptide–peptide intermolecular hydrogen bonds, it is a prime example for studying the influence of water on its conformation. In order to more fully understand the influence of hydration (which must be the dominating intermolecular force in solution) on peptide conformation, we have continued to examine crystals of the sequence *cyclo*-(1-X-1-Pro-*d*-Phe)$_2$ and report in this paper the crystal

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