Tetrahedron Letters No. 41, pp 3905 - 3908.

0040-4039/79/1008-3905$02.00/0

DETERMINATION OF THE N-TERMINAL RESIDUE OF THE PEPTIDE IN RISTOCETIN A AND RISTOMYCIN A BY REDUCTIVE ISOPROPYLATION.

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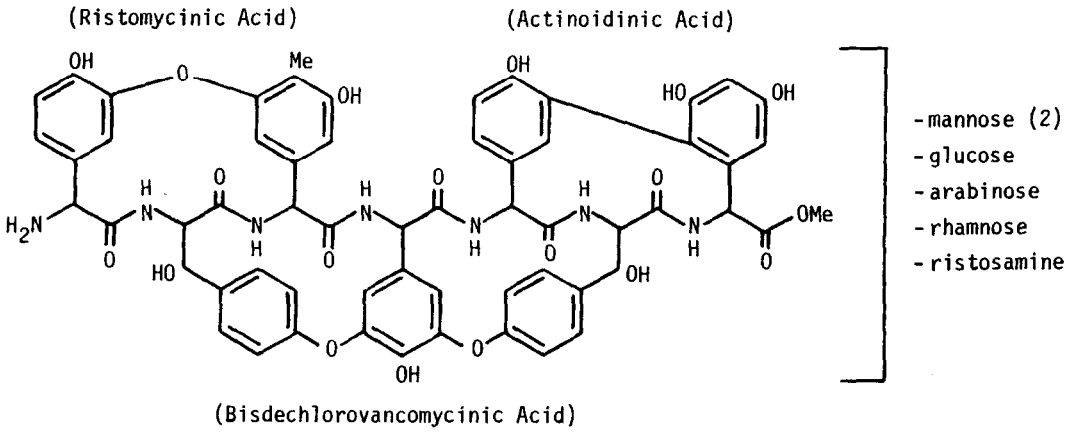
The N-terminus of ristocetin A and ristomycin A was found to be the p-hydroxyphenylglycine ring of ristomycinic acid by reductive alkylation of the peptide with acetone and $NaCNBH_3$ followed by peptide hydrolysis and separation of the two rings of the isopropyl amino acid by hydrogenolysis.

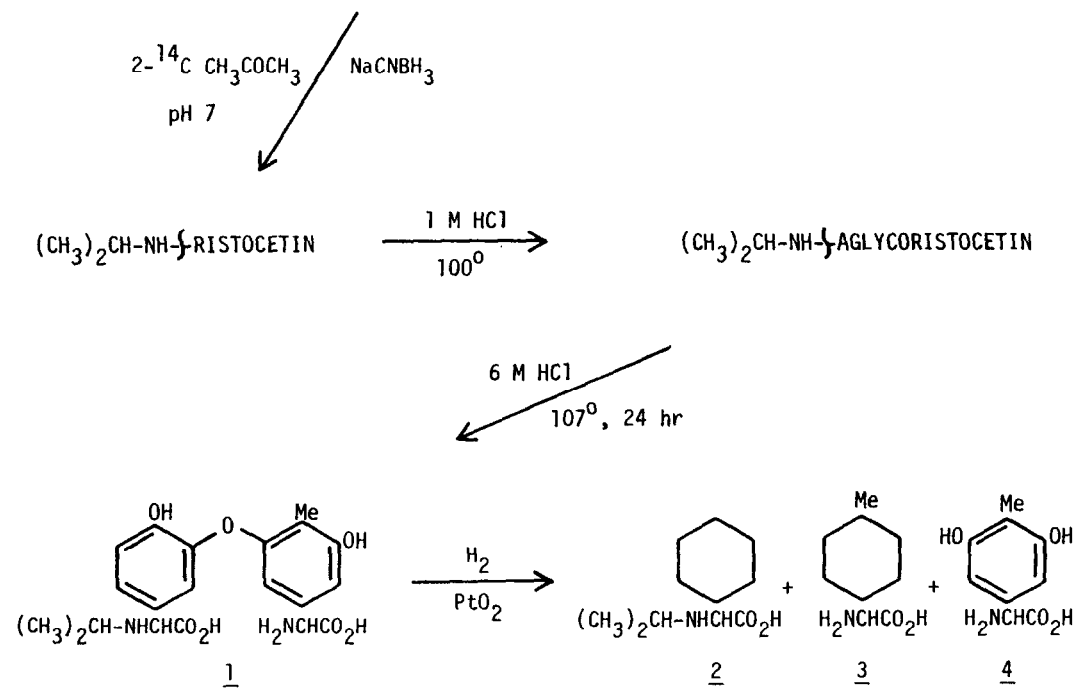
Ristocetin[1] belongs to a group of glycopeptide antibiotics which includes vancomycin,[2] ristomycin,[3] actinoidin,[4] and avoparcin.[5] Vancomycin is the only member of the group for which the full structure is known;[2c] however the presence of a biphenyl type bis(amino acid) (actinoidinic acid) and a tris(amino acid) (vancomycinic acid or nor-chloro analogs) in all members of the group has led us to propose a similar sequence for the aglycone of ristocetin A in which the diphenyl ether type bis(amino acid) (ristomycinic acid) is at the N-terminus of the peptide.[1b] Williams has independently made the same proposal.[6] As the first step to obtain supporting evidence for this structural proposal we sought to determine the N-terminus. Commonly used methods such as the Edman and Sanger procedures did not appear promising for use with peptides containing only bis and tris(amino acids). Instead a method of reductive alkylation[7,8] was chosen because an alkyl substituent on the terminal amino acid would be unreactive under hydrolytic and reductive conditions, thus providing the stability required during subsequent steps which would be needed to differentiate among the amino groups of these complex amino acids. Reductive alkylation has occasionally been used in the past as an end group method but is more widely used as a technique for modifying lysyl residues.[7] In the present study radiolabeled acetone was used as the carbonyl component to facilitate isolation and identification of the alkylated amino acid.

Ristocetin A (1.0 g) was treated with 2-^{14}C acetone (2.8 mmol, 25 μc) for 5 min at pH 7 followed by addition of $NaCNBH_3$ (0.133 g, 2.1 mmol). After 1 hr additional acetone (1.0 ml, non-radioactive) was added and the mixture allowed to stand overnight. Excess reducing agent was destroyed with HCl. Treatment of the alkylated antibiotic with 1M HCl (100°, 45 min) gave

RISTOCETIN A



(Bisdechlorovancomycinic Acid)



aglycone (0.42 g, 3.2 x 10^9 dpm/mol); an aliquot (0.11 g) was hydrolyzed in 6M HCl (107°, 24hr) Tlc of the hydrolysate (n-BuOH-HOAc-H_2O, 3:1:1) showed complete disappearance of ristomycinic acid with concomitant appearance of a new faster moving spot. The amino acids were separated by ion-exchange chromatography on Aminex 50W-X2 (0.9 x 60 cm, 0.2M NH_4OAc, pH 4.65, 8 ml fractions). The radioactivity was contained in fractions 16-18 which were combined to yield an N-isopropyl derivative (1) of ristomycinic acid: NMR (D_2O) δ 1.29 (6H, d, J = 6.6 Hz, $CH(\underline{CH_3})_2$), 2.11 (3H, s, arom CH_3), 3.28 (1H, m, J = 6.6 Hz, $\underline{CH}(CH_3)_2$), 5.09 (2H, s, HCNH), 6.61 (1H, d, J = 1.6 Hz, arom), 6.88 (1H, d, J = 1.6 Hz, arom), 6.95 (1H, arom), 7.21 (2H, arom). Esterification (MeOH/HCl) yielded the bis(methyl ester) (12.3 mg, 3.6 x 10^9 dpm/mol); NMR ($CDCl_3$) δ 1.05 (6H, d, J = 7 Hz, $CH(\underline{CH_3})_2$), 2.08 (3H, s, arom CH_3), 2.78 (1H, m, $\underline{CH}(CH_3)_2$), 3.68 (6H, s, OCH_3), 4.36 (2H, s, HCNH), 6.61 (1H, m, arom), 6.76 (2H, m, arom), 6.96 (2H, m, arom).

To ascertain which amino group of ristomycinic acid was alkylated, the rings were separated by hydrogenolysis using a procedure based on one employed by east European workers[3a,b] in the investigation of the structure of ristomycinic acid. Hydrogenation (1 atm, 25°, 18 hrs) of an aqueous solution using PtO_2 gave a mixture of amino acids. The mass spectrum after esterification with MeOH/HCl showed prominent peaks at m/e 154, 126 and 152 corresponding to P-CO_2CH_3 for the esters of amino acids 2, 3 and 4 in support of structure 1 which has the isopropyl group attached to the p-hydroxyphenylglycine fragment. No peaks corresponding to the alternative structure (i.e. m/e 194, 168 or 112) were observed. Chromatographic separation of a mixture of the reduced amino acids on Aminex 50W-X2 (0.1 M pyridine-acetate, pH 4.7) gave the individual constituents: 2: NMR (D_2O-DCl) δ 1.24 (m, cyclohexyl), 1.33 (d, J = 6.5 Hz, $CH(\underline{CH_3})_2$, 1.36 (d, J = 6.5 Hz, $CH(\underline{CH_3})_2$), 1.74 (m, cyclohexyl), 3.51 (m, J = 6.5 Hz, $\underline{CH}(CH_3)_2$), 3.97 (d, J = 4.1 Hz, HCNH), MS (methyl ester) m/e 154 (P-CO_2CH_3), 130 (P-cyclohexyl); 3: NMR (D_2O-DCl) δ 0.92 (d, J = 7 Hz, 4-CH_3), 1.50-185 (m, cyclohexyl), 4.0 (d, HCNH); MS (N-acetyl methyl ester) m/e 227 $M^{+\cdot}$); 4: NMR (D_2O-DCl) δ 2.07 (s, arom CH_3), 6.60 (s, arom, exchanged rapidly). Compound 2 was identical by TLC (3 solvents), NMR and MS (methyl ester) to an authentic sample prepared by reduction of N-isopropylphenylglycine and was the only radioactive compound detected.

It should be noted that the success of N-isopropylation as a method for determination of the N-terminal residue was not dependent upon ristomycinic acid occupying that position. If one of the amino groups of actinoidinic acid had been the terminus, the ring to which it was attached could have been identified by acetylation of the unalkylated amino group (N-isopropylphenylglycine does not acetylate under mild conditions) and oxidation of the alkylated amino acid to the aryl aldehyde with NaOCl. If the N-terminus had been one of the amino groups of the tris(amino acid), it would have been necessary to hydrolyze the isopropylated peptide under basic conditions. The amino acid decomposes during acidic hydrolysis of the aglycone to give undefined products. Decomposition also occurs in base but a major pathway is retroaldol cleavage of the phenylserine moieties to give glycine and benzaldehydes attached via ether linkages to p-hydroxyphenylglycine; the latter product can be stabilized by reduction of the aldehydes to benzyl alcohols using $NaBH_4$.[1b, 2b] Thus, if a phenylserine fragment had been

the N-terminus, N-isopropylglycine could have been isolated or, if the central amino acid had been the terminus, N-isopropyl-4-hydroxy-3,5-bis(4-hydroxymethylphenoxy)phenylglycine would have been obtained instead.

Ristomycin A contains the same amino acids and sugars as ristocetin A and substantial evidence has accumulated which indicates that the two antibiotics are identical.[3g,6] We have repeated the above procedure with ristomycin and have obtained the same result, providing further evidence that the two antibiotics are the same. The result is in agreement with the observation of the Russian workers[3d] that ristomycinic acid is not detectable in acid hydrolysates of ristomycin derivatized with 2,4-dinitrofluorobenzene.

Acknowledgement. We wish to thank Professor S. Moore for helpful advice and Professor R. Bognár for the gift of ristomycin. This study was supported by the U. S. Public Health Service (Research grant GM-23593).

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(Received in USA 4 June 1979)