flushed with nitrogen, 10 g (250 mmol) of potassium was added, and the mixture was heated under reflux until all of the potassium had reacted (10 h). The reflux condenser was exchanged for a 30-cm Vigreux column, and *tert*-butyl alcohol was distilled from the reaction mixture until crystals appeared in the bottom of the reaction flask. One liter of heptane was added and the distillation was continued until the temperature at the still head had increased to 98 °C and the reaction mixture volume had decreased to 750 mL. The reaction mixture was cooled to 0–5 °C and 65.5 g (250 mmol) of triphenylphosphine was added in a single portion, followed by a solution of 29.8 g (250 mmol) of dry, alcohol-free chloroform over a period of 1 h. The reaction mixture was warmed to 15–20 °C, and the *tert*-butyl alcohol which had been produced during the reaction was distilled from the reaction mixture under reduced pressure until the reaction mixture volume had been reduced to 400 mL. The reaction mixture was cooled to a temperature below 10 °C and 49.1 g (500 mmol) of dry cyclohexanone was added in six portions over a period of 30 min. The reaction mixture was stirred for an additional 2 h at 10 °C, then for 5 h at 25 °C, and allowed to stand overnight. The reaction mixture was filtered, and the filtrate was reduced in volume on a rotary evaporator. The residue was distilled through a 25-cm Vigreux column at a gradually diminishing pressure. Gas-chromatographic analysis revealed the presence of considerable cyclohexanone; the distillate was carefully redistilled to afford 18.5 g (45%) of dichloride **64** as a colorless liquid: bp 77–79 °C (12 mm); ν_{max} (CCl_4) 2923, 2847, 1616, 1447, 1228, 986, 925, 917, 858 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.33 (m, 4, $(CH_2)_2C{=}C$), 1.51 (m, 6, 3 × CH_2); λ_{max} (pentane) 200 (shoulder, ϵ 11 000), 204 (ϵ 12 000), 208 nm (shoulder, ϵ 11 000); λ_{max} (methanol) 207 nm (ϵ 8400). The IR and ^1H NMR spectra were in agreement with the reported values.[30,31]

B. Irradiation. From the irradiation described in Table IX, the following products were isolated as colorless liquids by preparative gas chromatography. Methylenecyclohexane (**52**) was identified by direct comparison with a commercial specimen. (Chloromethylene)cyclohexane (**69**) was identified by comparison of its spectral properties with those previously reported.[32,33]

Acknowledgment. Generous financial assistance by the National Science Foundation and the donors of the Petroleum Research Fund, administered by the American Chemical Society, is gratefully acknowledged. We thank Robert I. Davidson for the preparation and irradiation of iodide **39**.

Registry No. 2, 35895-37-5; **3**, 21981-08-8; **6**, 1000-86-8; **11**, 17497-54-0; **12**, 49565-03-9; **13**, 17497-53-9; **14**, 17497-52-8; **17**, 26819-54-5; **23**, 50438-51-2; **26**, 31059-39-9; **29**, 13294-30-9; **30**, 4927-03-1; **32**, 50438-50-1; **34a**, 177-10-6; **34b**, 933-40-4; **37a**, 931-57-7; **37b**, 930-66-5; **39**, 40648-08-6; **40**, 16642-49-2; **43**, 931-94-2; 44, 930-29-0; **45**, 23904-33-8; **46**, 23904-34-9; **47**, 27784-30-1; **48**, 1121-49-9; **49**, 184-26-9; **51**, 27784-29-8; **61**, 871-28-3; **6i**, 67091-34-3; **63**, 60014-85-9; **64**, 1122-55-0; **67**, 87372-64-3; **68**, 87372-65-4.

Vancomycin: Structure and Transformation to CDP-I

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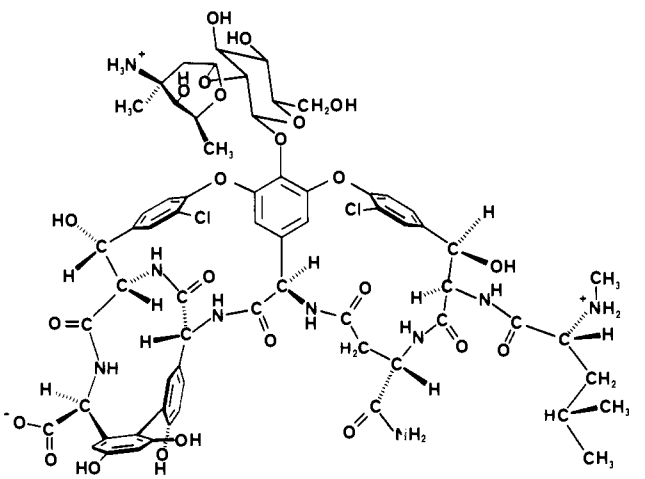
Abstract: The structure of vancomycin is assigned as **7** in which aspartate is present as asparagine, rather than isoasparagine (i.e., **4**) as had been proposed previously (Williamson, M. P.; Williams, D. H. *J. Am. Chem. Soc.* **1981**, *103*, 6580). The mechanism of rearrangement of vancomycin to CDP-I has been investigated. The pathway is deduced: vancomycin → succinimide **11** → CDP-I-m (**3**) ⇄ CDP-I-M (**2**). Succinimide **11** has been isolated and characterized by FT-IR and FAB-MS; these data are in agreement with an in-chain succinimide but not a seco structure. Treatment of **11** at pH 8 leads to immediate appearance of the minor form of CDP-I, which slowly yields an equilibrium mixture with the major form (~64:36 of 2:3). No major change in the equilibrium ratio or rate of equilibration was found by changing solvent (H_2O, Me_2SO, or 0.1% TFA/20% 2-propanol) or pH (in the range 2.0–8.0). High-field ^1H NMR studies of **2** and **3** indicate that they are atropisomers involving different orientations of the Cl-substituted aromatic ring of residue 2, in agreement with earlier assignments by Williamson and Williams. It is concluded that **2** and **3** can interconvert by simple reorientation of the aromatic ring rather than via a transient cleavage product. The cyclophane ring in which the Cl-substituted aromatic residue 2 is embedded is enlarged by one methylene unit in the conversion of vancomycin to CDP-I; rotation of ring 2 can occur in CDP-I but not in vancomycin. The additional methylene unit in the N-terminal cyclophane ring of CDP-I causes the ring to lie in a conformation that is significantly different from that of vancomycin.

The glycopeptide antibiotic, vancomycin, first reported in 1956,[1] has received renewed attention as a therapeutic agent in recent years, both for intravenous use in the treatment of methicillin-resistant staphylococcal infections and for oral use in the treatment of *Clostridium difficile* associated colitis.[2] The structure and mode of action of vancomycin have also been the subjects of numerous investigations.[3]

The first complete structure of vancomycin (**1**) was proposed by Sheldrick et al.[4] in 1978 on the basis of three types of evidence:



1

(1) chemical degradation studies that revealed the amino acids and the unusual amino sugar, vancosamine;[5] (2) high-field NMR

(1) McCormick, M. H.; Stark, W. M.; Pittenger, G. F.; Pittenger, R. C.; McGuire, G. M. *Antibiot. Annu.* **1955–1956**, 606.

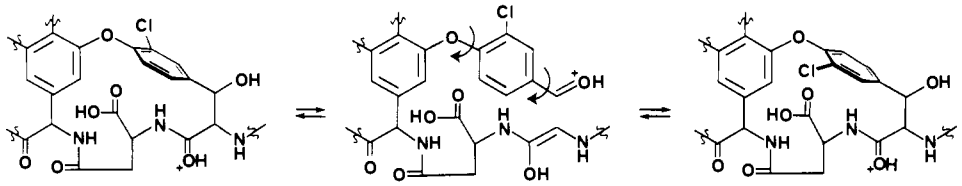
(2) (a) Cafferkey, M. T.; Hone, R.; Keane, C. T. *J. Antimicrob. Chemother.* **1982**, *9*, 69. (b) Fekety, R. *Med. Clin. North Am.* **1982**, *66*, 175. (c) Meyers, J. P.; Linnemann, C. C., Jr. *J. Infect. Dis.* **1982**, *145*, 532. (d) Craven, D. E.; Reed, C.; Kollisch, N.; DeMaria, A.; Lichtenberg, D.; Shen, K.; McCabe, W. R. *Am. J. Med.* **1981**, *71*, 53. (e) Wise, R. I.; Kory, M. *Rev. Infect. Dis.* **1981**, *3*, S199. (f) Cook, F. V.; Farrar, W. E. *Ann. Intern. Med.* **1978**, *88*, 813.

(3) For recent reviews see: (a) Williams, D. H.; Rajananda, V.; Williamson, M. P.; Bojesen, G. *Top. Antibiot. Chem.* **1980**, *5*, 119. (b) Perkins, H. R. *Pharmacol. Ther.* **1982**, *16*, 181.

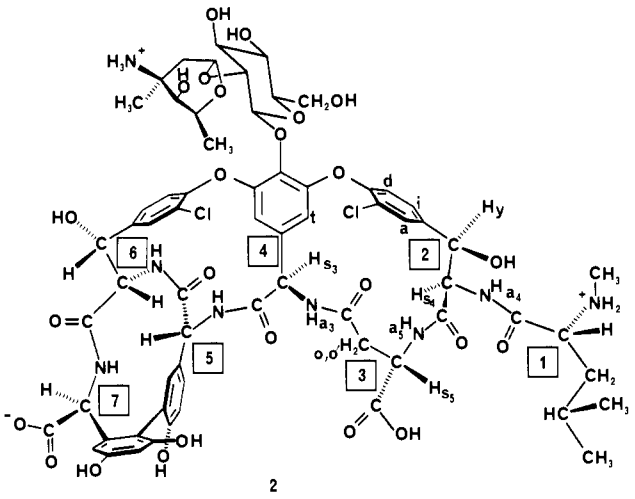
(4) Sheldrick, G. M.; Jones, P. G.; Kennard, O.; Williams, D. H.; Smith, G. A. *Nature (London)* **1978**, *271*, 223.

0002-7863/83/1505-6915$01.50/0

Scheme I

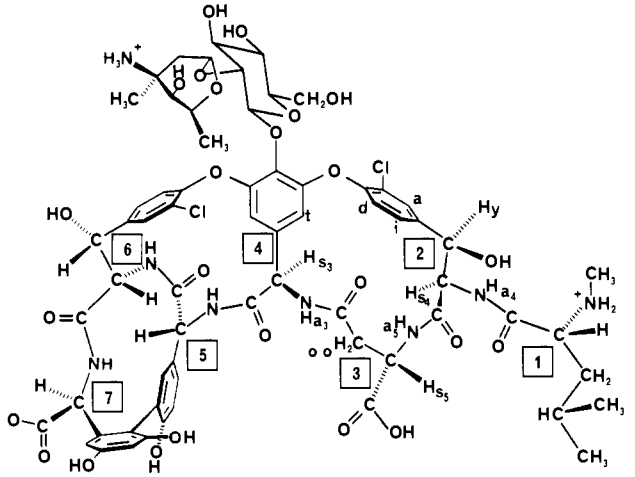


studies that confirmed the results of the chemical studies and yielded information on linkages of the individual constituents to one another and on stereochemical relationships;[6] and (3) the structure, established by X-ray diffraction,[4] of CDP-I (**2**), which



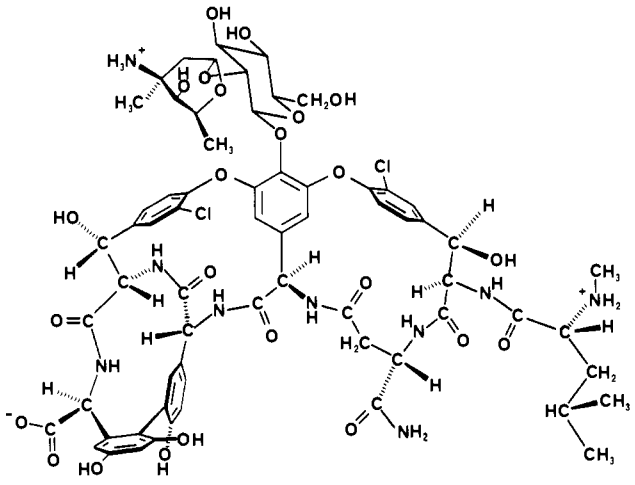
2

is a degradation product formed from vancomycin by hydrolytic loss of ammonia.[7] The structure of vancomycin was revised by Williamson and Williams in 1981,[8] on the basis of further NMR studies of vancomycin and CDP-I in which two forms of CDP-I were found. NOE and chemical shift measurements suggested that the difference between the major form (CDP-I-M, **2**) and minor form (CDP-I-m) lay primarily near residue 2. The X-ray structure determination had apparently been carried out on isomer **2**. Structure **3**, in which the orientation of the chlorine-containing



3

ring in residue 2 differs from that in **2** by 180°, was proposed for CDP-I-m. The spectrum of vancomycin itself showed NOEs similar to those in CDP-I-m for protons, a, i, s_4, and y, and thus, revised structure **4** for vancomycin was proposed with the chlorine on residue 2 on the back face.



4

From study of CPK molecular models Williamson and Williams concluded that the aromatic ring of residue 2 cannot rotate through the cyclophane ring in which it is embedded, and hence a bond-breaking reaction was proposed to explain the formation of both **2** and **3** from vancomycin. They postulated that a retro-aldol reaction occurs at residue 2 (Scheme I) accompanied by reorientation of the aromatic ring and reclosure. Weaknesses of this proposal include the following: (1) only one of the two β-hydroxytyrosines in vancomycin would be undergoing cleavage and reclosure and (2) reclosure would occur with retention of stereochemistry at both the α and β positions of the β-hydroxytyrosine residues.[8]

We regarded the aspartic acid residue at position 3 in the peptide to be a more attractive site for a cleavage–reclosure process to occur. It seemed possible that vancomycin might contain an asparagine rather than an isoasparagine, with rearrangement to isoaspartate occurring during CDP-I formation. The presence of a rearranged peptide backbone in CDP-I could also explain the observation first made by Marshall[7a] and repeated by us that CDP-I lacks antibiotic activity. Rearrangements of aspartyl residues (with either a β-ester or β-amide) to isoaspartic acid groups are well documented in the literature (Scheme II).[9] In

(5) (a) Smith, K. A.; Williams, D. H.; Smith, G. A. *J. Chem. Soc., Perkin Trans. 1* **1974**, 2369. (b) Smith, G. A.; Smith, K. A.; Williams, D. H. *Ibid.* **1975**, 2108. (c) Weringa, W. D.; Williams, D. H.; Feeney, J.; Brown, J. P.; King, D. W. *Ibid.* **1972**, 443. (d) Johnson, A. W.; Smith, R. M.; Guthrie, R. D. *J. Chem. Soc., Chem. Commun.* **1972**, 361. (e) Johnson, A. W.; Smith, R. M.; Guthrie, R. D. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2153.

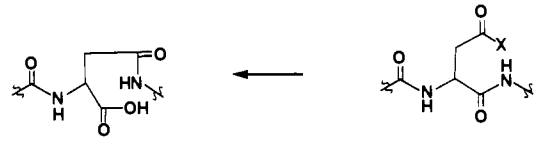
(6) Williams, D. H.; Kalman, J. R. *J. Am. Chem. Soc.* **1977**, *99*, 2768.

(7) (a) Marshall, F. J. *J. Med. Chem.* **1965**, *8*, 18. (b) Johnson, C. R. Thesis, University of Illinois, Urbana, 1962.

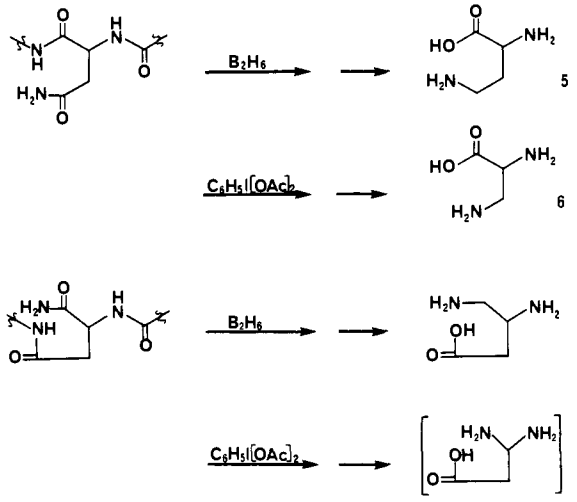
(8) Williamson, M. P.; Williams, D. H. *J. Am. Chem. Soc.* **1981**, *103*, 6580.

(9) (a) Battersby, A. R.; Robinson, J. C. *J. Chem. Soc.* **1955**, 259 (b) John, W. D.; Young, G. T. *Ibid.* **1954**, 2870. (c) Swallow, D. L.; Abraham, E. P. *Biochem. J.* **1958**, *70*, 364. (d) Sondheimer, E.; Holley, R. W. *J. Am. Chem. Soc.* **1954**, *76*, 2467. (e) Riniker, B.; Schwyzer, R. *Helv. Chim. Acta* **1964**, *47*, 2357 (f) Bernhard, S. A.; Berger, A.; Carter, J. H.; Katchalski, E.; Sela, M.; Shalitin, Y. *J. Am. Chem. Soc.* **1962**, *84*, 2421. (g) Roeske, R. *J. Org. Chem.* **1963**, *28*, 1251. (h) Marshall, G. R.; Merrifield, R. B. *Biochemistry* **1965**, *4*, 2394. (i) Baba, T.; Sugiyama, H.; Seto, S. *Chem. Pharm. Bull.* **1972**, *21*, 207. (j) Haley, E. E.; Corcoran, B. J.; Dorer, F. E.; Buchanan, D. L. *Biochemistry* **1966**, *5*, 3229 (k) Mojsov, S.; Mitchell, A. R.; Merrifield, R. B. *J. Org. Chem.* **1980**, *45*, 555. (l) Bodanszky, M.; Martinez, J. *J. Org. Chem.* **1978**, *43*, 3071. (m) Bornstein, P.; Balian, G. In "Methods in Enzymology"; Hirs, C. H. W., Timasheff, S. N., Eds.; Academic Press: New York, 1977; pp 132–145. (n) For a general discussion and additional references see: Barany, G.; Merrifield, R. B. In "The Peptides"; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1979; Vol. 2, pp 192–208.

Scheme II



Scheme III



this paper we report the results of our studies of the structure of vancomycin and of the mechanism of transformation of vancomycin to degradation product CDP-I.[10]

Results and Discussion

Two experiments were undertaken to show that vancomycin contains asparagine. In the first (Scheme III) vancomycin aglycone, treated with diazomethane to protect the phenolic hydroxyl groups (agVOMe), was reduced with diborane in THF[11] and hydrolyzed. Ion-exchange chromatography of the hydrolysate gave in low yield a component having the same retention time, ninhydrin color, and TLC behavior as 2,4-diaminobutyric acid (**5**), which is the product that would result from the reduction of the β-carboxamide group of asparagine. Preparation of the *N*,*N*-dibenzoyl methyl ester of **5** gave material identical by TLC, mass spectrum, and ^{1}H NMR with authentic material. No 3,4-diaminobutyric acid (the expected product if isoasparagine were present in the antibiotic) was detected.

In the second approach (Scheme III) a Hofmann-type oxidative degradation of the primary carboxamide in vancomycin was carried out. Treatment of agVOMe with 5 equiv of (diacetoxyiodo)benzene[12a] followed by peptide hydrolysis and ion-exchange chromatography gave the asparagine degradation product 2,3-diaminopropionic acid (**6**) identical by TLC, ninhydrin color, and ^{1}H NMR with authentic material. Quantitative amino acid analysis indicated an 18% yield of **6** and a 13% yield of unaltered aspartic acid. Hydrolysis of unoxidized agVOMe gave only a 51% yield of aspartic acid, in accordance with previous observations that a significant portion of agVOMe is resistant to hydrolysis. It was not feasible to force the oxidative degradation to completion by use of a larger excess or more vigorous conditions because the free amino group of the diaminopropionyl residue is also susceptible to oxidation. The oxidation was repeated with vancomycin itself; **6** was again obtained. Isoasparagine, had it been present in the peptide, would have undergone oxidative degradation to give the unstable 3,3-diaminopropionic acid, which would not have been detected.[13] On the basis of these results we propose structure **7** for vancomycin.

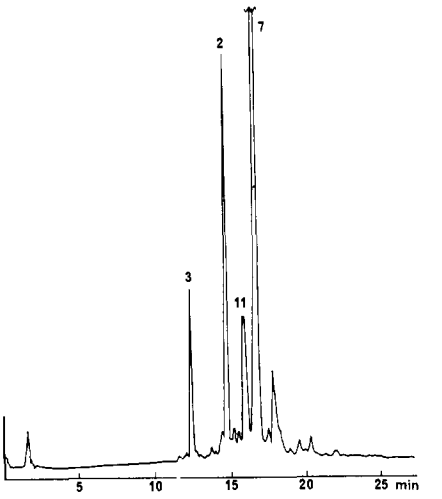
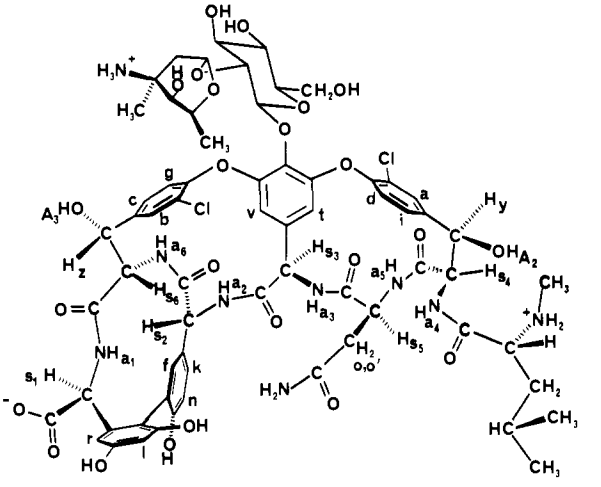


Figure 1. Elution profile of vancomycin (2 mg/mL) heated at pH 3.14, 76 °C, 18 h. Column: IBM C-18. Solvent: (A) 0.1 M ammonium formate; (B) 0.1 M ammonium formate/20% CH_3CN; gradient from 100% A to 100% B over 20 min, 2 mL/min.



7

Attention was next directed toward elucidation of the pathway for the rearrangement process. Two general pathways (Scheme IV) involving seco intermediates seemed to be consistent with a rearrangement process that would simultaneously permit CDP-I to be formed with both orientations of the Cl-substituted ring of residue 2. Path A involves attack of the side chain amide on the peptide carbonyl to form succinimide **8** with concomitant release of the amine group of residue 4; reattack of this amino group at the other carbonyl followed by hydrolysis of the resulting amide would lead to CDP-I. Path B involves attack of the side chain carbonyl on the peptide carbonyl, yielding isoimide **9** with release of the amino group of residue 4; hydrolysis of the isoimide to anhydride **10** followed by readdition of residue 4 would give CDP-I. The key feature of both path A and path B is that the peptide backbone is broken, thus allowing rotation of ring 2. It should be noted that both pathways differ from that established for aspartyl rearrangements in more conventional peptides in that in those cases rearrangement occurs by attack of the *peptide amide*

(10) A preliminary account of some of this work has appeared: Harris, C. M.; Harris, T. M. *J. Am. Chem. Soc.* **1982**, *104*, 4293.
(11) Brown, H. C.; Heim, P. *J. Org. Chem.* **1973**, *38*, 912.
(12) (a) Holt, L. A.; Milligan, B. *Aust. J. Biol. Sci.* **1981**, *34*, 395. For similar reactions employing bis(trifluoroacetoxy)iodobenzene see: (b) Radhakrishna, A. S.; Parham, M. E.; Riggs, R. M.; Loudon, G. M. *J. Org. Chem.* **1979**, *44*, 1746. (c) Soby, L. M.; Johnson, P. *Anal. Biochem.* **1981**, *113*, 149.
(13) Kovacs, J.; Kovacs, H. N.; Konyves, I.; Csaszar, J.; Vajda, T.; Mix, H. *J. Org. Chem.* **1961**, *26*, 1084. These authors used a Hofmann degradation procedure to quantitate the proportion of α and β-aspartyl residues present in thermally polymerized (anhydro)aspartic acid which had been treated with liquid ammonia.

Scheme IV

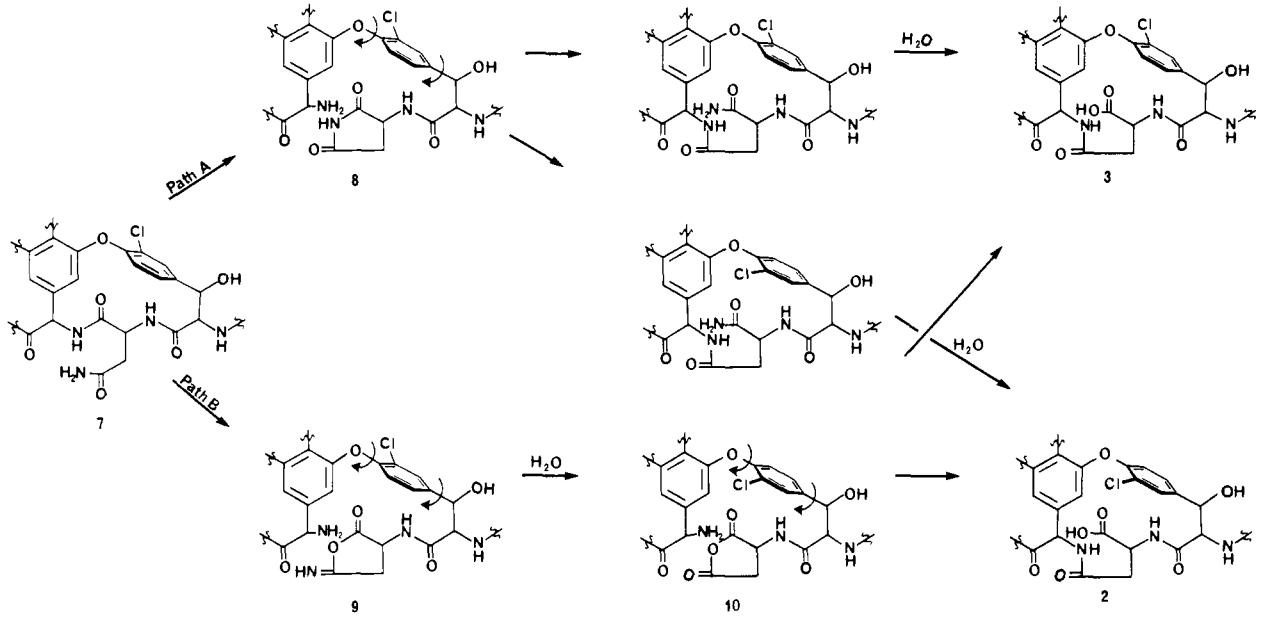


Table I. Effect of pH on Conversion of Vancomycin to CDP-I

pH	vancomycin, %[a]	CDP-I-M, %	CDP-I-m, %	11, %
2.50	26.0	17.6	7.2	12.9
3.14	47.0	15.3	6.3	10.7
4.20	55.2	16.4	7.2	6.0
5.00	57.2	16.2	6.7	5.5
6.20	59.2	16.0	6.9	<1
8.01	43.2	13.9	7.2	<1

[a] % of total area integrated; vancomycin (2 mg/mL) was adjusted to the desired pH and heated at 76 °C for 18 h. Analysis was by HPLC: Conditions as in Figure 1.

on the side chain carbonyl group to give a peptide-bound succinimide, rather than a seco-peptide having a free succinimide or isosuccinimide.

To search for intermediates in the rearrangement process a study of the conversion of vancomycin to CDP-I as a function of pH was undertaken. Reported conditions for the conversion vary as to time and temperature,[4,7] but the pH has been in the range 4.1–4.2. In the present investigation solutions of vancomycin (2 mg/mL) were adjusted to pH values ranging from 2.5 to 8.1, held at 76 °C for 18 h, and then analyzed by HPLC. The most noteworthy feature of the study is the presence of constituents other than vancomycin and CDP-I in the reactions carried out under acidic conditions. The HPLC profile of the pH 3.14 reaction is shown in Figure 1. To ascertain if any of the new peaks was an intermediate in the rearrangement process, a pH 2.43 reaction mixture was separated by HPLC and aliquots of the unknown constituents were reheated at 76 °C for 1 h. It was found that the component labeled **11** was converted to **2** and **3**; none of the other unknown components gave rise to the CDP-I isomers. Table I is a summary of the results obtained by varying the pH. It should be noted that commercial preparations of vancomycin are not totally homogeneous; some of the unidentified peaks are the other components or CDP-type reaction products derived from them.

An infrared spectrum (Figure 2) of the intermediate was obtained by FT-IR using a Fluorolube mull; a well-defined band at 1717 cm^{-1} and a broad one around 1785 cm^{-1} were observed. The polysuccinimide arising by base treatment of poly(β-benzylaspartate) has bands at 1715 and 1775 cm^{-1}.[14] Isoimides

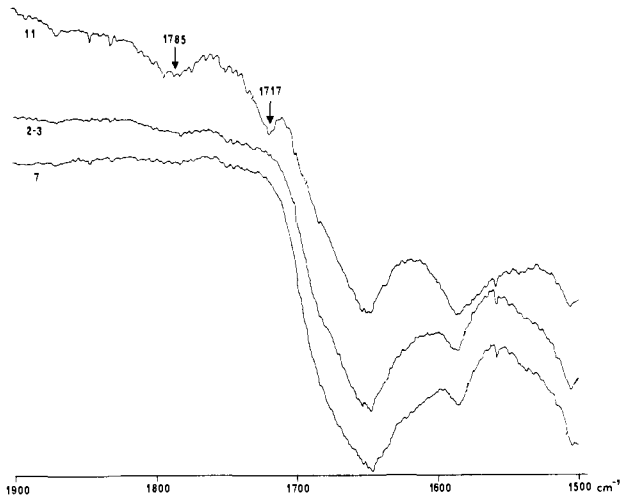


Figure 2. FT-IR spectra (Fluorolube mull) of vancomycin, CDP-I, and intermediate **11**.

are reported to have an IR band around 1785–1800 cm^{-1}, similar to a 5-member ring lactone;[15] the other intermediate in path B, succinic anhydride **10**, should have bands around 1782 and 1865 cm^{-1}.[16] Hence it appears that the intermediate is a succinimide, and path B can be eliminated.

Parent ions can be observed in the mass spectrum of glycopeptide antibiotics by using fast atom bombardment.[17] The succinimide shown in path A has a nominal molecular weight of 1447 with a cluster of other isotopic species (derived from ^{37}Cl, ^{13}C, etc.) spread over an additional 5–6 mass units. The FAB spectrum of intermediate **11** indicated not 1447 but a nominal mass of 1430, reflecting a loss of NH_3 from vancomycin. It is therefore concluded that the intermediate is structure **11** (M_r 1430) having the succinimide in-chain rather than seco compound **8**. Hence path A is also eliminated, and we are now forced to propose path C in which the peptide nitrogen attacks the side-chain carbonyl group to give **11** as the intermediate (Scheme V). This

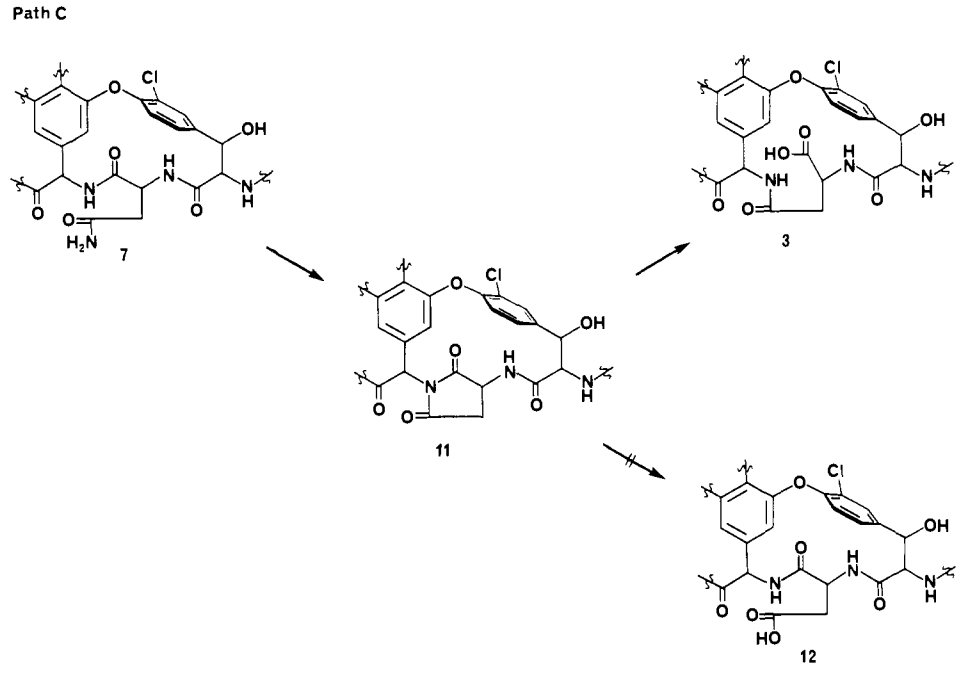
(14) Adler, A.; Fasman, G. D.; Blout, E. R. *J. Am. Chem. Soc.* **1963**, *85*, 90.

(15) Cotter, R. J.; Sauers, C. K.; Whelan, J. M. *J. Org. Chem.* **1961**, *26*, 10.

(16) "The Infrared Spectra of Complex Molecules"; Bellamy, L. J., Ed.; Methuen and Co.: London; Wiley: New York, 1958; p 128.

(17) Williams, D. H.; Bradley, C.; Bojesen, G.; Santikarn, S.; Taylor, L. C. E. *J. Am. Chem. Soc.* **1981**, *103*, 5700.

Scheme V



result, however, fails to account for the formation of both the major and minor forms of CDP-I; i.e., in path C there is no bond-breaking step to allow ring 2 to rotate.

Several possible explanations for the origin of **2** and **3** can be considered. First Williams might have erred in his assignment of the structure of **3** as an atropisomer of **2** involving reorientation of the aromatic ring of residue 2; in particular a conformational isomer of the peptide backbone might be a viable alternative. A second possible explanation is that CDP-I-m represents an isomer of **2** arising by hydrolytic opening of succinimide **11** at the β carbonyl to give unrearranged structure **12**. In rearrangements of simple aspartyl peptides the succinimide intermediates undergo ring opening to give predominantly but not exclusively the rearranged, i.e., isoaspartyl peptide. A third possibility is that the conclusion, drawn from molecular model studies, that steric constraints preclude reorientation of the aromatic ring is in error; i.e., a seco intermediate may not be required to explain the formation of **2** and **3**.

Williamson and Williams' assignment of **3** was based on NMR studies which were complicated by the fact that **3** was never isolated, but only observed as a minor component in mixtures with **2**. They had obtained **2** in relatively pure form by careful crystallization. We have developed procedures for separating the two isomers. Analytical and small-scale preparative separations can be carried out by HPLC on C-18 reverse phase columns and on a large scale by reverse phase flash chromatography on C-18 modified silica gel (LiChroprep RP-18). Thus the two species can now be studied individually.

Isomers **2** and **3** were found to be in equilibrium with each other, the equilibration being relatively slow at room temperature as demonstrated by the fact that the isomers can be separated chromatographically. Structure **12** for the minor isomer would appear to be precluded by this facile interconversion (as well as by ^{1}H NMR studies discussed below). The interconversion can be followed by optical rotation (Table II), by ^{1}H NMR, or by HPLC (Figure 3). An HPLC study showed that the constitution of the equilibrium mixture in aqueous solutions (~64:36 of **2**:**3**) is essentially independent of concentration over a range from 0.1 to 10 mg/mL (ruling out a monomer–dimer type equilibrium which had been regarded as a remote possibility).[18] Moreover

Table II. Equilibration of **2** and **3** Followed by Optical Rotation[a]

CDP-I-M		CDP-I-m	
time, h	$[\alpha]_D^{22}$, deg	time, h	$[\alpha]_D^{22}$, deg
0	−28.0	0	−15.3
2	−27.8	2	−16.5
4	−25.6	3	−19.2
5	−25.2	4	−19.5
24	−24.2	24	−22.1
70	−23.9	48	−22.5

[a] T = 22 °C; solvent: 0.1% TFA/20%/2-propanol; **2**, c, 0.802; **3**, c, 0.805.

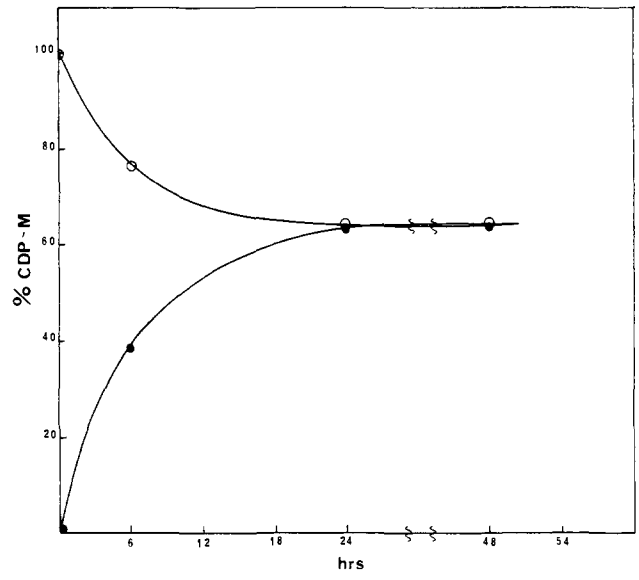


Figure 3. Equilibration of CDP-I-M and CDP-I-m; 25 °C; solvent: 0.1 M ammonium formate/8% CH_3CN, pH 6.5.

pH had little effect on the equilibrium point. The effect of pH and solvent on the rate of equilibration was investigated. At neutrality (25 °C) the $t_{1/2}$ for equilibration is approximately 3 h, increasing to 6 h at pH 4 and to about 8 h at pH 2. The properties of **2** and **3** were also investigated in Me_2SO because this is the solvent of choice for NMR investigations. Interconversion occurs more slowly than in H_2O, requiring about 3 times

(18) (a) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 773. (b) Brown, J. P.; Feeney, J.; Burgen, A. S. V. *Mol. Pharmacol.* **1975**, *11*, 119. These authors present evidence (spectrophotometric and NMR) that vancomycin aggregates at concentrations above ~1 mg/mL.

Table III. Comparison of Selected ^{1}H NMR Chemical Shifts for CDP-I Isomers and Vancomycin

proton	CDP-I-M[b]	CDP-I-m[b]	vancomycin[a]
o	1.98 (2.92)[d]	2.07 (2.40)	2.20[c]
o′	2.90 (2.92)	2.83 (2.50)	2.45[c]
y	4.98 (4.64)	4.98 (5.16)	5.15
a	7.57 (7.60)	7.58 (7.64)	7.42
i	7.41 (7.43)	7.47 (7.47)	7.57
d	7.41 (7.34)	7.15 (7.12)	7.20
t	5.68 (5.79)	5.93 (6.00)	5.63
v	5.13	5.13	5.21
b	7.87	7.89	7.87
c	7.46	7.45	7.48
g	7.36	7.30	7.28
A_2	5.80	5.76	5.85
A_3	5.97	5.96	5.85
k	6.79	6.79	6.78
n	6.69	6.70	6.73
f	7.23	7.23	7.19
l	6.40	6.43	6.44
r	6.25	6.26	6.30
s_1	4.43	4.43	4.50
s_2	4.57	4.58	4.50
s_3	5.73 (5.69)	5.76 (5.72)	5.71
s_4	4.86	4.90	4.86
s_5	4.47	4.43	4.38
s_6	4.24	4.24	4.22
a_1	8.50	8.54	8.39
a_2	8.86	8.88	8.14
a_3	8.42 (8.18)	8.20 (7.90)	8.43
a_4	7.70 br	7.6 br	8.00
a_5	6.90 (6.71)	7.20 (6.87)	6.59
a_6	6.69	6.64	6.50

[a] Taken from ref 6; spectra run in Me_2SO-d_6, 70 °C, 0.1 M. [b] Spectra run in Me_2SO-d_6, 25 °C, 7 mM. [c] Taken from ref 20a; spectrum run in Me_2SO-d_6, 60 °C, 10 mM. [d] Values in parentheses were taken from ref 8; spectra run in Me_2SO-d_6 at 70 °C.

as long to reach equilibrium ($t_{1/2}$ = 10 h). The equilibrium point is slightly more in the direction of **2** (72:28).

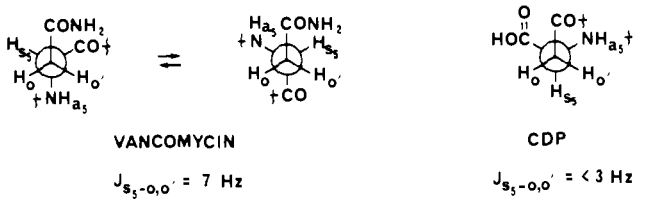
^{1}H NMR studies of the two CDP-I isomers were carried out at 300 and 360 MHz with Me_2SO solutions at room temperature. Under these conditions equilibration is slow enough that solutions of the minor isomer can be studied for ~2 h before contamination by the major isomer becomes a serious problem. The ^{1}H chemical shifts of selected protons in the two isomers are shown in Table III, along with chemical shifts reported by Williams and Kalman[6] for vancomycin in Me_2SO at 70 °C. The assignments for the CDP-I spectra are based upon decoupling and NOE studies and draw also upon analogy with the reported spectra of vancomycin and related antibiotics. The major differences between the CDP-I's themselves and between them and vancomycin lie in the region around residues 2, 3, and 4. Williamson and Williams[8] have made NMR assignments for a few of the protons in **2** and **3** by using spectra of Me_2SO solutions at 70 °C, conditions under which the isomers soon equilibrated. Their assignments are shown in parentheses in Table III. Our assignments for the CDP-I compounds differ significantly from theirs in some cases; proton y in **2** to which we assign δ 4.98 rather than 4.64 and, most significantly, protons o and o′ of the aspartyl methylene group, which they assigned as being isochronous at δ 2.92 in **2** and δ 2.40 in **3**. We find a large difference in the chemical shifts of the geminal protons ($\Delta\delta$ 0.76) in both CDP-I isomers which might be expected when the methylene group is a part of a fairly rigid ring. In vancomycin the methylene group lies in the side chain rather than within a ring structure; consequently it has considerable freedom of rotation so that differences in shielding effects on o and o′ are largely averaged out. The fact that the difference in chemical shifts of o and o′ in **3** is similar to that in **2** is further evidence against structure **12** for CDP-I-m, which would be expected to resemble vancomycin in this regard. The chemical shifts of the C-terminal end of the peptides remain remarkably constant among the three compounds. Differences in chemical shifts of amide protons between the CDP-I's and vancomycin may reflect

Table IV. Selected Vicinal Coupling Constants in Vancomycin and CDP-I Isomers

protons	CDP-I-M, Hz	CDP-I-m, Hz	vancomycin,[a] Hz
s_1–a_1	5	6	7
s_2–a_2	5	4	6
s_3–a_3	9	8	8
s_4–a_4	7	7	2
s_5–a_5	7	*b*	7
s_6–a_6	12	11	12
s_4–y	3	3	4
s_5–o,o′	3	3	7, 7[c]

[a] Taken from ref 8. [b] Obscured by overlapping peaks. [c] Taken from ref 6.

Scheme VI



in part the difference in temperatures at which the spectra were acquired. The chemical shifts and vicinal coupling constants of the NH–CH pairs along the backbone of the two isomers of CDP-I were examined (Tables III and IV) in an attempt to find support for the hypothesis that the compounds differ in peptide conformation rather than orientation of the aromatic ring. In the case of **3** it was not possible to measure the coupling constant between a_5 and s_5, but the close correspondence of the remaining vicinal coupling constants and the chemical shifts for the α protons argues against any significant differences in conformation between **2** and **3**. The major differences between **2** and **3** are at those positions most likely to be affected by the orientation of ring 2, i.e., d, t, a_3, and a_5. On the basis of these data plus the NOEs observed by Williamson and Williams for protons i, y, a, and s_4 (and repeated by us on the separated isomers), we concur with their assignment of **3** as the structure of the minor isomer of CDP-I.

Some further conclusions about the structure of vancomycin in relation to those of CDP-I-M and -m can be drawn from the ^{1}H NMR study. It can be seen from the data in Table IV that vancomycin differs from the CDP-I isomers in the geometry around several of the bonds at the N-terminal end of the molecule. For example, the coupling constant of 7 Hz for a_4–s_4 indicates a dihedral angle of ~140° in **2** and **3**,[19] much larger than in vancomycin for which Williamson and Williams estimated 40–110°. The very small coupling constant for s_4–y in **2** and **3** as compared to that in vancomycin indicates a difference in conformation around this bond also. Most significant, however, is the observation that the coupling constants from s_5 to o and o′, are too small to observe in **2** and **3** but are both ~7 Hz in vancomycin. These values are consistent with vancomycin having free rotation around the bond joining C-2 and C-3 of the asparagine, probably with preferred conformers in which the $CONH_2$ group is anti to NH_{a_5} and to the α-CO group. In CDP, however, the ring structure constrains the isoaspartyl residue in a conformation in which the β-CO group is gauche to NH_{a_5} and the α-COOH (Scheme VI). Dihedral angles estimated from the data in Table IV appear to be in reasonable agreement with the X-ray structure. The differences in geometry of the N-terminal cyclophane between vancomycin and its degradation products may, in large part, explain the lack of antibiotic activity shown by CDP-I. ^{1}H NMR studies of the binding of small aliphatic peptides, such as Ac-D-Ala-D-Ala, to vancomycin have implicated amides a_3, a_4, and a_5 in binding to the ionized carboxyl group of the C-terminal D-Ala.[20] The additional methylene group in the

(19) Dihedral angles were estimated from data in: Cung, M. T.; Marraud, M.; Neel, J. *Macromolecules* **1974**, *7*, 606.

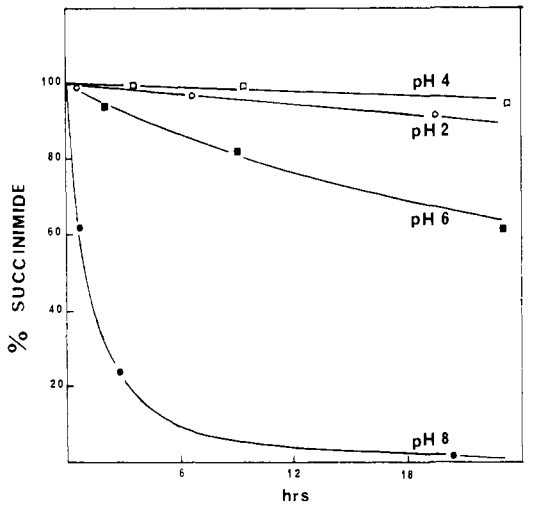


Figure 4. Conversion of intermediate **11** to CDP-I (M + m) as a function of pH; T = 25 °C.

peptide chain in CDP-I-M and -m may prevent these compounds from assuming the proper conformation for efficient peptide binding.[21]

Additional evidence for the structural relationship of vancomycin to its degradation products arose from a study of the hydrolysis of succinimide **11** as a function of pH. As might be expected, the hydrolysis process is base catalyzed (Figure 4). Under mildly acidic conditions opening of the succinimide ring is slow, accounting for the observed accumulation of **11** during the CDP-I-forming reaction. Moreover, formation of the CDP-I's is slow relative to their interconversion at neutral and acidic pH. At pH 8 hydrolysis of **11** is rapid, and exclusive formation of CDP-I-m (**3**) is observed, after which **3** equilibrates with **2**. This sequence of events, vancomycin → **11** → CDP-I-m ⇄ CDP-I-M, provides additional support for Williamson and Williams' hypothesis that the chlorine substituent on ring 2 lies on one face in vancomycin and **3** and on the other face in **2**. The observed sequence also provides further evidence against a seco intermediate being involved in the rearrangement process. If **2** and **3** were formed from a seco intermediate, they would be expected to arise simultaneously.[22,23]

We are forced to conclude that the CPK models overstate the steric barrier to rotation of the aromatic ring of residue 2 in **2** and **3**.[24] In vancomycin itself the barrier is too great for the aromatic rings of either residue 2 or 6 to undergo 180° rotation. We propose that the enlarged cyclophane ring in CDP-I permits the unsubstituted edge of aromatic ring 2 to pass by the peptide chain to effect interconversion of CDP-I-M and CDP-I-m.

Support for this hypothesis is provided by an experiment in which crude CDP-I was dehalogenated with Pd–C to give a single product that had the same HPLC retention time as the rearrangement product formed from didechlorovancomycin.[25] No evidence was found for a second stereochemical form of the dechlorinated product.

Conclusion

In summary, the above studies provide compelling evidence confirming Williams' structure assignments for CDP-I-M and m but require the revision of the structure of vancomycin to one in which a normal asparagine is present in the peptide chain. Formation of CDP-I involves a rearrangement process. Vancomycin undergoes degradation by attack of the peptide amide nitrogen of asparagine on the β-carboxamide to give a succinimide (**11**) lying in the cyclophane ring. Hydrolytic cleavage of **11** occurs exclusively by attack at the α-carbonyl group to give the rearranged isoaspartyl peptide CDP-I-m. Whereas the cyclophane ring embodying residue 2 in vancomycin (and also in **11**) is too small and rigid to permit reorientation of the chlorine-substituted ring, in CDP-I rotation becomes possible so that the chlorine which is on the back (convex) face of CDP-I-m (and the antibiotic) can reorient itself on the front face to give CDP-I-M.

At the present time vancomycin is unique among antibiotics of this group, which include ristocetin, avoparcin, A35512B, actinoidin, and others, in containing an asparagine residue. It would be of considerable interest to find another antibiotic of this class which contained asparagine (or glutamine) and to determine if it would also undergo the facile rearrangement described in this paper.

Experimental Section

General. Vancomycin was obtained as a gift from Eli Lilly Co. 2,4-Diaminobutyric acid and 2,3-diaminopropionic acid were obtained from Aldrich Chemical Co.

HPLC was carried out on an IBM Model 9533 chromatograph or a Waters Model 6000 chromatograph. Detection was by UV absorption at 254 nm. Organic solvents were HPLC grade and used without further purification. Water was purified by deionization followed by distillation in an all-glass apparatus. All solvents were filtered through a 0.4-μm filter before use. For analytical work an IBM C-18 column (4.5 × 250 mm) was used; for preparative work, an Altex Ultrasphere ODS column (10 × 250 mm). HPLC buffers were removed by lyophilization. TLC was carried out on Merck silica gel (60F-254) plates. For amino acids the following solvents were used: (A) 1-butanol–HOAc–H_2O (3:1:1) (B) pyridine–butanol–HOAc–H_2O (40:68:14:25).

Routine ^1H and ^{13}C NMR spectra were obtained on a JEOL FX-90Q spectrometer. Mass spectra were obtained on a LKB-9000 mass spectrometer. Optical rotations were measured with a Rudolph Autopol III automatic spectropolarimeter in 1-dm cells.

Peptide hydrolyses were carried out in sealed tubes for 22 h by using 1 mL of constant-boiling HCl/10 mg of peptide. Ion-exchange chromatography was performed on a 0.9 × 50 cm column of Aminex AG-50W-X2 at 35 °C with 0.1 M pyridine–acetate buffer, pH 4.50.

Reduction of Methylated Aglycovancomycin (agVOMe). Aglycovancomycin[7] (prepared from 1.0 g vancomycin hydrochloride) was dissolved in MeOH–H_2O and treated with CH_2N_2 (from 21.5 g Diazald). The product was lyophilized and vacuum-dried (56 °C) to give 0.525 g agVOMe. Dry agVOMe (0.122 g, 0.10 mmol) was placed in a 50-mL two-necked flask flushed with N_2. THF (25 mL) was added, followed by B_2H_6[11] (1.0 mL, 1.0 mmol), which was added dropwise. The reaction mixture was heated under reflux for 4 h. Hydrochloric acid (5.7 N, 5 mL) was added, and the THF was removed by distillation. Additional HCl (5.7 N, 5 mL) was added, and the mixture was heated for 23 h at 104 °C. After removal of the solvent in vacuo the resulting amino acids were separated by ion-exchange chromatography. A fraction was obtained with the same elution time, TLC behavior, and ninhydrin color as authentic 2,4-diaminobutyric acid. In a second experiment the crude hydrolysate was treated with benzoyl chloride and CH_2N_2 as described below for synthetic amino acid **5**. The derivatized amino acids were separated by TLC ($CHCl_3$–MeOH, 97:3). A fraction was obtained in very low yield that had the same TLC, mass spectrum, and ^1H NMR as

(20) (a) Convert, O.; Bongini, A.; Feeney, J. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1262. (b) Williams, D. H.; Butcher, D. W. *J. Am. Chem. Soc.* **1981**, *103*, 5697. (c) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *Ibid.* **1983**, *105*, 1332.

(21) Williamson and Williams[8] found that when Ac-D-Ala-D-Ala was added stepwise to a solution containing both **2** and **3**, isomer **3** showed greater chemical shift changes than **2**, implying that some binding does occur. The chlorine on the front of **2** might well cause **2** to bind more weakly than **3**.

(22) Still another process for conversion of the peptide backbone of vancomycin to that of CDP-I is elimination of the α-amido nitrogen from asparagine to give a maleamide followed by readdition at the β position. This seco intermediate, as with those in paths A and B would lead to simultaneous rather than consecutive formation of **2** and **3**. A referee has pointed out that all pathways involving seco intermediates are entropically unfavorable and would not be expected to go in high yield.

(23) Compounds **2** and **3** were the only products observed in hydrolyses carried out between pH 2 and 8. Apparently, succinimide **11** is cleaved *exclusively* by attack at the α-carbonyl group to give the ring-expanded cyclophane of **3**, rather than at the β-carbonyl group to give the unrearranged aspartate analogue of vancomycin. The increased selectivity in the present case may be due to relief of strain associated with the ring-expansion process.

(24) The deficiency of CPK models can be seen, for example, with 2-methoxy-2′-carboxy-2′-nitrobiphenyl which racemizes with $t_{1/2}$ = 9.4 min at 25 °C (Shriner, R. L.; Adams, R.; Marvel, C. S. In "Organic Chemistry"; Gilman, H., Ed.; Wiley: New York, 1938; Vol. I, p 281). In the CPK model of this compound the ortho substituents cannot be made to pass each other without bond rupture.

(25) The preparation and properties of mono- and didechlorovancomycin will be discussed in a later publication.

the *N,N'*-dibenzoyl methyl ester of 2,4-diaminobutyric acid.

Preparation of Synthetic Methyl Ester of 2,4-Bis(benzoylamino)butyric Acid. 2,4-Diaminobutyric acid (22.0 mg, 0.1 mmol) was dissolved in 2N NaOH (0.5 mL), and benzoyl chloride (50 mg, 0.36 mmol) was added dropwise with alternate addition of base. The mixture was stirred 1 h, acidified, and extracted 3 times with EtOAc–MeOH (9:1). The combined extracts were washed with brine, dried, and evaporated. Following removal of benzoic acid by sublimation (80 °C, 0.025 mm), the residue was dissolved in MeOH and treated with excess CH_2N_2. The crude product was purified by TLC ($CHCl_3$–MeOH, 97:3): ^1H NMR ($CDCl_3$) δ 1.87 (m, 1 H, 3-H), 2.34 (m, 1 H, 3'-H), 3.10 (m, 1 H, 4-H), 3.71 (s, 3 H, OCH_3), 4.07 (m, 1 H, 4'-H), 4.93 (m, 1 H, 2-H), 7.18 (s, 1 H, 2-NH), 7.5 (m, 7 H, aromatic, 4-NH), 7.87 (m, 4 H, aromatic); mass spectrum; *m/e* 340 (M+), 309, 308, 281, 229, 193, 160, 148, 105 (base peak), 77.

Degradation with (Diacetoxyiodo)benzene. The degradation was carried out essentially as described by Holt and Milligan.[12a] agVOMe (0.128 g, 0.104 mmol) was dissolved in CH_3CN–H_2O (1:1, 20 mL) and (diacetoxyiodo)benzene (0.151 g, 0.47 mmol) was added. The reaction mixture was stirred at room temperature for 18 h and extracted with EtOAc and Et_2O, and the aqueous layer was lyophilized. An aliquot of the lyophilized solid was hydrolyzed and subjected to quantitative amino acid analysis, which gave an 18% yield of 2,3-diaminopropionic acid (**6**) and 12.6% of aspartic acid. Analysis of the hydrolysate of an untreated sample of agVOMe gave a 51% yield of aspartic acid.

In a similar experiment the amino acids from the hydrolysate of treated agVOMe were separated by ion-exchange chromatography and the identity of the 2,3-diaminopropionic acid confirmed by ^1H NMR and TLC comparison with authentic material.

Unprotected vancomycin (0.113 g, 0.08 mmol) was treated under similar conditions with (diacetoxyiodo)benzene (0.222 g, 0.69 mmol) for 6 h. Hydrolysis followed by ion-exchange chromatography gave a 16% yield of **6**.

Effect of pH on the Formation of CDP-I. A stock solution of vancomycin (2.3 mg/mL) was prepared. Aliquots (2.0 mL) were adjusted to the desired pH by the addition of dilute HCl or NaOH. The samples were incubated in screw-capped test tubes for 18 h at 76 °C. After cooling, aliquots from each tube were analyzed by HPLC [column: IBM C-18; solvents: (A) 0.1 M ammonium formate, (B) 0.1 M ammonium formate/20% CH_3CN; a gradient from 100% A to 100% B over 20 min was used; flow rate 2 mL/min]. In cases where precipitate was present a drop of 0.1% TFA was added before the HPLC analysis to effect solution.

Isolation of Succinimide 11. Vancomycin (41.1 mg) was dissolved in H_2O (2 mL), the pH was adjusted to 2.43, and the reaction was heated at 76 °C for 18 h. The mixture was separated by HPLC [column: Altex Ultrasphere-ODS (10 × 250 mm); solvent: 0.1 M ammonium formate/9% CH_3CN; flow rate 2 mL/min]. Aliquots of the separated peaks were heated at 76 °C for 1 h and analyzed by HPLC to determine if CDP-I had been formed. The fraction eluting at 24–26 mL (2.2 mg) was the only one showing conversion to CDP-I.

Conversion of Succinimide 11 to CDP-I. Succinimide **11** (~0.4 mg) was dissolved in 0.125 mL of 0.1 M buffer at the desired pH and the mixture was allowed to stand at ambient temperature. Aliquots were analyzed by HPLC [column: IBM C-18; solvent: 0.1 M ammonium formate/8% CH_3CN; flow rate 2 mL/min].

Separation of 2 and 3. Crystalline CDP-I was prepared as described by Marshall.[7a] The initial separation was carried out by HPLC [column: IBM C-18; solvent: 0.1 M ammonium formate/8% CH_3CN; flow rate 2 mL/min]. The fractions containing isolated **2** and **3** were allowed to stand at room temperature, and aliquots from them were reanalyzed at intervals. The equilibration was also followed in Me_2SO and at various pHs by using **2** and **3** prepared as below.

Preparative separation of **2** and **3** was carried out by flash chromatography on LiChroprep RP-18 (25–40 μm) (E. Merck). Crystalline CDP-I (100 mg) was dissolved in 0.1% TFA/20% 2-propanol and stirred at room temperature for several hours to equilibrate **2** and **3**. The solution was then passed rapidly through a 2 × 12 cm column of resin. Elution was carried out with 0.1% TFA/20% 2-propanol. Fractions were pooled on the basis of UV absorption at 280 nm and HPLC analysis. CDP-I-m was eluted first. The pooled fractions were cooled and lyophilized as quickly as possible. Compounds **2** (40 mg) and **3** (20 mg) were obtained as fluffy colorless solids. Each contained less than 10% of the other isomer.

Dehalogenation of CDP-I. CDP-I (crude crystals, 10 mg) was suspended in H_2O, and the pH was adjusted to 8.3. Pd/C (40 mg, 10%, MCB) was added, and the reaction mixture was treated with H_2 (50 psi) for 20 h in a Parr shaker. The product was analyzed by HPLC in two solvent systems: (1) 0.1 M ammonium formate/6% CH_3CN and (2) gradient from 100% A (2% Et_3N–phosphate, pH 3/5% CH_3CN) to 13% B (2% Et_3N–phosphate, pH 3/40% CH_3CN) over 20 min; flow rate 2 mL/min.

Acknowledgment. We gratefully acknowledge the help of Dr. P. Polavarapu and Darlene Back in obtaining the FT-IR spectra, Dr. P. Fletcher (East Carolina State University) for the quantitative amino acid analyses, Dr. M. Nuss (Abbott Laboratories) and Dr. C. Watkins (University of Alabama—Birmingham) for the high-field NMR spectra, Dr. K. Tomer (Midwest Center for Mass Spectrometry, Lincoln, NE) for the FAB mass spectra, and the IBM Corp. for the gift of a Model 9533 chromatograph. Funds for support of this work were provided by the U.S. Public Health Service (GM-25793).

Registry No. 2, 79517-31-0; **3**, 55598-85-1; **5**, 305-62-4; **5** *N,N'*-dibenzoyl methyl ester, 87394-05-6; **6**, 515-94-6; **7**, 1404-90-6; **11**, 87394-06-7; agVOMe, 87411-95-8; aglycovancomycin, 82198-76-3.