recrystallizations, lit.[25] mp 163-165 °C, undepressed on admixture with an authentic sample: UV (pH 7) max 260 nm (ε 13100), 206 (ε 16400).

Deamination of 3a and 6a with ADA. Substrate **3a** or **6a** (2.2-2.4 μmol) was incubated with ADA (0.4 unit, 0.2 unit/mol) in 0.05 M Na_2HPO_4 (pH 7.5, 0.4 mL) at room temperature. Aliquots were periodically withdrawn, and they were examined by TLC (S_1) and UV spectroscopy. The deamination was quantitative in both cases after 6 h.

Deamination of L-Adenosine with ADA. L-Adenosine (2 μmol) was incubated with ADA as described above in 0.05 M K_2HPO_4. The withdrawn aliquots were examined by paper chromatography in 2-propanol-NH_4OH-water (7:1:2) on Whatman 3MM paper and by UV spectrophotometry of the excised spots of L-adenosine and L-inosine. The deamination was 50% complete after 6 days and quantitative after 34 days. Control experiments without the enzyme (L-adenosine) and with ADA (tubercidin) showed that both compounds were stable after 8 days of incubation. D-Adenosine was quantitatively deaminated at 43 μM concentration in 4 min.

Stability of Cytallene (3c) toward CDA. Compound **3c** (2.2 μmol) was incubated with CDA (2×10^{-3} unit) in 0.05 M Na_2HPO_4 (pH 7, 0.4 mL) for 40 h at room temperature. Aliquots which were periodically withdrawn and examined by TLC (S_1) showed only the presence of unchanged **3c**. In a control experiment (pH 8, 8×10^{-4} unit of CDA/μmol of substrate) cytidine (0.2 mM) was deaminated at a rate of 7×10^{-3} OD_{280} unit/min as estimated spectrophotometrically.

Stability of Hypoxallene (3h) toward PNP. Compound **3h** (2.4 μmol) was incubated with PNP (0.4 unit) for 24 h in 0.05 M Na_2HPO_4 (pH 7.5, 0.4 mL). TLC (S_1) and UV indicated no reaction after 24 h and then with 4.4 units of enzyme after an additional 24 h. By contrast, guanosine (0.19 mM) was converted quantitatively to guanine within 20 min (1.6 units of PNP/μmol of substrate) as shown by UV spectrophotometry at 253 nm.[60]

Acknowledgment. Thanks are due to the Central Instrumentation Facility (Department of Chemistry, Wayne State University, Dr. Robin Hood, Director) for NMR and mass spectral data. We are grateful to Dr. W. B. Butler (MCF) for computer assistance, to Dr. Barry Schoenfelner (CIF) for his help in obtaining the CD spectra, and to Dr. J. Pitha, National Institute of Aging, NIH, Baltimore, MD, for a sample of N^9-vinyladenine. L-Adenosine was obtained through courtesy of Dr. H. B. Wood, National Cancer Institute, NIH, Bethesda, MD. The work described herein was supported in part by U.S. Public Health Service Research Grant CA 32779 from the National Cancer Institute and in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit. The CD spectrometer was obtained with the aid of BRS Shared Instrumentation Grant S10 03366 from the Division of Research Resources, NIH, Bethesda, MD.

(60) **Note Added in Proof**: After submission of this paper, syntheses of compounds **6a** and **6b** by different methods were published: (a) Borcherding, D. R.; Narayanan, S.; Hasobe, M.; McKee, J. G.; Keller, B. T.; Borchardt, R. T. *J. Med. Chem.* **1988**, *31*, 1729. (b) Ashton, W.; Canning Meurer, L.; Cantone, C. L.; Field, A. K.; Hannah, J.; Karkas, J. D.; Liou, R.; Patel, G. F.; Perry, H. C.; Wagner, A. F.; Walton, E.; Tolman, R. L. *J. Med. Chem.* **1988**, *31*, 2304. Also, another inhibitor of HIV lacking the oxacyclopentane moiety (1'a-carba-2',3'-didehydro-2',3'-dideoxyguanosine, carbovir) was reported: Vince, R.; Hua, M.; Brownell, J.; Daluge, S.; Lee, F.; Shannon, W. M.; Lavelle, G. C.; Qualls, J.; Weislow, O. S.; Kiser, R.; Canonico, P. G.; Schultz, R. H.; Narayanan, V. L.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 1046.

Biosynthesis of Antibiotics of the Virginiamycin Family. 8.[1] Formation of the Dehydroproline Residue

Michael B. Purvis,[2] Joseph W. LeFevre,[2] Vickie L. Jones,[2] David G. I. Kingston,*[,2] Andrē M. Biot,[3] and Francis Gosselē[3]

Contribution from the Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0212, and Smith Kline—RIT, B-1330 Rixensart, Belgium. *Received July 20, 1988*

Abstract: The formation of the dehydroproline residue of the antibiotic virginiamycin M_1 occurs with equal facility from both (*R*)- and (*S*)-proline; the 3-*pro-R* proton of proline is lost stereospecifically in this process. *cis*-3-Hydroxyproline but not *trans*-3-hydroxyproline is incorporated into the antibiotic, although less efficiently than proline, and virginiamycin M_2 is converted into virginiamycin M_1. These results suggest that virginiamycin M_1 is most probably formed by incorporation of (*S*)-proline into virginiamycin M_2, which then undergoes hydroxylation with retention of configuration and elimination of water to yield virginiamycin M_1.

The antibiotics of the virginiamycin family contain a rich diversity of unusual and interesting amino acids. Antibiotics of group B, such as virginiamycin S_1, contain 3-hydroxypicolinic acid, which may be viewed as a dehydrolysine.[1] Antibiotics of group A, such as virginiamycin M_1 (VM_1, **1**), contain both a dehydroproline unit and an oxazole ring;[4] the latter may be viewed as a dehydroserine moiety.

The origin of α,β-dehyro amino acids in natural products has been the subject of considerable speculation, and it has variously been suggested that they arise by tautomerization of an acyl imino intermediate[5] or by dehydration of hydroxy amino acids.[6,7] The relationship between (*R*)-amino acids and α,β-dehydro amino acids has also been discussed, and the suggestion that (*R*)-amino acids arise from dehydro amino acids[5] has been refuted.[8] In this

(1) Paper 7: Reed, J. W.; Kingston, D. G. I.; Purvis, M. B.; Biot, A.; Gosselē, F. *J. Org. Chem.* **1989**, *54*, 1161-1165.

(2) Department of Chemistry, Virginia Polytechnic Institute and State University.

(3) Smith Kline-Recherche et Industrie Thērapeutiques.

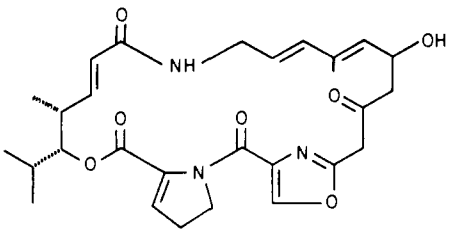
(4) Delpierre, G. R.; Eastwood, F. W.; Gream, G. E.; Kingston, D. G. I.; Todd, A. R.; Williams, D. H. *J. Chem. Soc. C* **1966**, 1653-1669. Kingston, D. G. I.; Todd, A. R.; Williams, D. H. *J. Chem Soc. C* **1966**, 1669-1676.

(5) Bycroft, B. W. *Nature (London)* **1969**, *224*, 595-597.

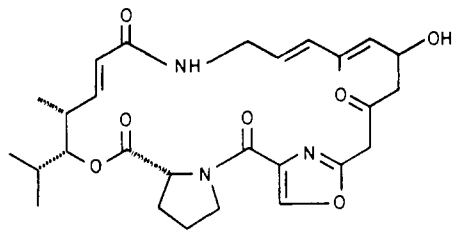
(6) Pearce, C. J.; Rinehart, K. L., Jr. *J. Am. Chem. Soc.* **1979**, *101*, 5069-5070. Rinehart, K. L., Jr.; Weller, D. D.; Pearce, C. J. *J. Nat. Prod.* **1980**, *43*, 1-20.

(7) Schmidt, U.; Häusler, J.; Ohler, E.; Poisel, H. *Fortschr. Chem. Org. Naturst.* **1979**, *37*, 251-327.

0002-7863/89/1511-5931$01.50/0 © 1989 American Chemical Society



1



2

connection it is of interest that virginiamycin M_2 (VM_2, **2**) contains an (*R*)-proline unit in place of the dehydroproline unit of VM_1.[9]

Our previous studies on the biosynthesis of VM_1 have focused on the biosynthesis of the carbon skeleton of the antibiotic[10] and on the stereochemistry of formation of the oxazole ring from serine.[11] These studies confirmed that the oxazole ring is formed from serine by an oxidative pathway of some type; the same conclusion was reached independently by Rinehart and his coworkers in studies on the biosynthesis of the antibiotic berninamycin.[6] The same workers also showed that dehydroalanine in berninamycin arises from serine,[6] and a recent study by Floss indicates that this is also true of the dehydroalanine units of nosiheptide.[12]

Although the extant studies indicate that two rather different dehydro amino acids arise from serine, there is no evidence that other dehydro amino acids also derive from hydroxy amino acids. Thus, the fact that serine is a protein amino acid may be thought to bias the pathway in favor of this precursor, since the metabolic machinery to make serine is in place; this is not necessarily true of other hydroxy amino acids. The dehydroproline residue of VM_1 provides an ideal test of the generality of the pathway from hydroxy amino acids to dehydro amino acids, since 3-hydroxyproline is not a protein amino acid. In addition, the stereochemistry associated with the formation of the double bond is more easily defined in a ring system than in an acyclic system.

For these reasons we have carried out a study of the formation of the dehydroproline residue in VM_1. Our results show that the route to VM_1 lies through VM_2, with stereospecific dehydrogenation of VM_2 to VM_1 occurring most probably via a hydroxylated intermediate.

Results and Discussion

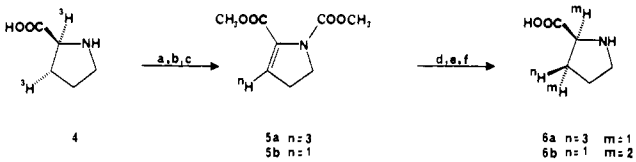
The biosynthesis of VM_1 was studied in shake cultures of *Streptomyces virginiae* strain PDT 30 or strain 5277, as previously described.[10] After addition of the appropriate labeled precursors at 24 h and harvesting 24 h later, the VM_1 was isolated by extraction and purified by high-performance liquid chromatography (HPLC). The activity of the dehydroproline portion was determined, where necessary, by hydrogenation, hydrolysis, and benzoylation of the antibiotic to yield (*R,S*)-*N*-benzoylproline, which was purified by HPLC, mixed with unlabeled material, and recrystallized to a constant specific activity ratio.

Table I. Incorporation of Labeled Prolines into Virginiamycins M_1 and M_2

			$^3H/^{14}C$ ratio			
			isolated antibiotic		*N*-Bz-Pro from	
entry	precursors added	pre-cursor	VM_1	VM_2	VM_1	VM_2
1	(2*S*,3*S*,4*R*)-[3,4-$^3H_2$]proline (**3**); (*S*)-[1-^{14}C]proline	6.8	*a*	*a*	7.5	*a*
2	(2*S*,3*S*,4*R*)-[3,4-$^3H_2$]proline (**3**); (*R,S*)-[*carboxyl*-^{14}C]-proline	15.6	32.2	*a*	17.7	*a*
3	(2*S*,3*R*)-[3-^{3}H]proline (**6a**); (*S*)-[U-^{14}C]proline	2.5	0.4	1.9	0.1	2.0

[a] Not determined.

Scheme I[a]



4 5a n=3, 5b n=1 6a n=3 m=1, 6b n=1 m=2

[a] (a) CH_3OH/HCl; (b) Bu^{t}OCl, NEt_3; (c) CH_3OCOCl, pyridine; (d) H_2/PtO_2 or D_2/PtO_2; (e) Me_3SiI, CH_3CN; (f) resolve via tartrate complex.

Incorporation of Labeled Prolines. Previous work[10] had established that both (*S*)-[U-^{14}C]proline and (2*S*)-[3,4-$^3H_2$]proline were incorporated efficiently into VM_1, although the product from the incorporation of the tritiated proline was not degraded to establish that incorporation was specific to the dehydroproline moiety. The incorporation of this tritiated proline did however enable us to determine the specificity of the biosynthetic pathway for (*S*)- versus (*R*)-proline. Following the procedure of Spenser,[13] we compared the incorporation of this tritiated proline with that of (*S*)-[1-^{14}C]proline and (*R,S*)-[*carboxyl*-^{14}C]proline (entries 1 and 2, Table I). The resulting data showed clearly that *both* (*R*)- and (*S*)-proline were incorporated with equal facility. Thus the $^3H/^{14}C$ ratio for incorporation of (*S*)-[$^3H_2$]proline versus (*S*)-[1-^{14}C]proline was essentially unchanged between the precursor mixture and the *N*-benzoylproline from VM_1 (ratios of 6.8 before and 7.5 after incorporation), and the same thing was true in the incorporation of (*S*)-[$^3H_2$]proline versus (*R,S*)-[*carboxyl*-^{14}C]proline (ratios of 15.6 before and 17.7 after incorporation).[14] This finding parallels that observed for the related antibiotic A2315A, where the (*R*)-alanine moiety, corresponding to the dehydroproline unit of VM_1, derived equally well from (*S*)-alanine and (*R*)-alanine.[15]

A second major conclusion could be drawn from this experiment. The tritiated proline was prepared by catalytic reduction of (*S*)-3,4-didehydroproline,[16] and tritiated proline prepared in this way has been shown to have predominantly the 2*S*,3*S*,4*R* stereochemistry (**3**).[17] The retention of all the tritium from this

(8) Stammer, C. H. *Chem. Biochem. Amino Acids, Pept., Proteins* **1982**, *6*, 33–74. Davies, J. S.; Foley, M. H.; Hassall, C. H.; Arroyo, V. *J. Chem. Soc., Chem. Commun.* **1973**, 782–783. Davies, J. S. *Chem. Biochem. Amino Acids, Pept., Proteins* **1977**, *4*, 1–27.

(9) Kingston, D. G. I.; Sarin, P. S.; Todd, A. R.; Williams, D. H. *J. Chem. Soc. C* **1966**, 1856–1860.

(10) Kingston, D. G. I.; Kolpak, M. X.; LeFevre, J. W.; Borup-Grochtmann, I. *J. Am. Chem. Soc.* **1983**, *105*, 5106–5110.

(11) Purvis, M. B.; Kingston, D. G. I.; Fujii, N.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1987**, 302–303.

(12) Houck, D. R.; Chen, L.-C.; Keller, P. J.; Beale, J. M.; Floss, H. G. *J. Am. Chem. Soc.* **1988**, *110*, 5800–5806.

(13) Leistner, E.; Gupta, R. N.; Spenser, I. D. *J. Am. Chem. Soc.* **1973**, *95*, 4040–4047.

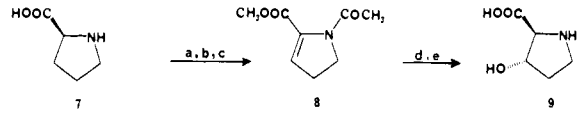
(14) The fact that the $^3H/^{14}C$ ratios both increase slightly on incorporation into virginiamycin M_1 is not readily explained. If the increase is due to a secondary isotope effect, then the effect is in the opposite direction from that expected for a reaction in which the overall change is from an sp^3 to an sp^2 hybridization (e.g., Lowry, T. H.; Richardson, K. S. *Mechanism and Theory in Organic Chemistry*, 2nd ed.; Harper & Row: New York, 1981; pp 210–212).

(15) LeFevre, J. W.; Kingston, D. G. I. *J. Org. Chem.* **1984**, *49*, 2588–2593.

(16) ICN Chemical and Radioisotope Division, Data Sheet for L-Proline, [3,4-$^3H_2$], lot no. 2006122.

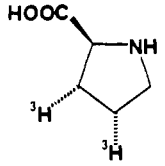
(17) Altman, L. J.; Silberman, N. *Anal. Biochem.* **1988**, *79*, 302–309.

Scheme II[a]



[a] (a) CH_3OH/HCl; (b) Bu^tOCl, NEt_3; (c) CH_3COCl, pyridine; (d) CH_3ONa, CH_3OH; (e) HBr, HOAc.

tritiated proline on incorporation into VM_1 thus indicates that the 3-*pro-S* proton is retained on the proline ring.



3

Although the preceding experiment indicates that the 3-*pro-S* proton is retained, we wished to confirm this result by proving that the 3-*pro-R* proton is lost. In order to do this, it was necessary to carry out a stereospecific synthesis of (2*S*,3*R*)-[3-^{3}H]proline (**6a**); the synthesis is shown in Scheme I.

Treatment of (2*S*,3*S*)-[2,3-$^3H_2$]proline (**4**) with methanolic hydrogen chloride followed by *tert*-butyl hypochlorite and triethylamine yielded [3-^{3}H]-1,2-didehydroproline methyl ester, which was converted to *N*-carbomethoxy[3-^{3}H]-2,3-didehydroproline methyl ester (**5a**) by treatment with methyl chloroformate.[18] The tritium at the 3-position was largely retained in this conversion because of the operation of a primary isotope effect in the isomerization of the 1,2- to the 2,3-didehydroproline.

The next step involved the catalytic hydrogenation of **5a** to generate the desired stereochemistry in the product **6a**. In order to confirm the stereospecificity of this reaction, we first carried out studies on reduction of the dehydroproline **5b** with deuterium. Reduction with deuterium over a palladium catalyst yielded a proline which was shown by ^{1}H NMR spectroscopy to have a label distribution that was extensively scrambled, presumably by a vinyl rearrangement. This result was expected, in view of the known propensity of palladium to promote vinyl rearrangement,[19] and it thus served to demonstrate that the ^{1}H NMR method could detect such scrambling in this system. Attempted reduction of **5b** with a homogeneous catalyst[20] failed to yield product, but reduction with deuterium over platinum oxide, followed by deprotection with trimethylsilyl iodide, yielded a product, **6b**, in which only 5–10% label scrambling had occurred.

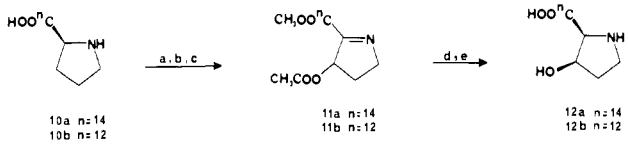
With this result in hand, we carried out catalytic hydrogenation of the tritiated dehydroproline **5a** over platinum and converted the product to racemic proline by treatment with trimethylsilyl iodide. The proline was resolved via its tartrate salt to yield (2*S*,3*R*)-[3-^{3}H]proline (**6a**).

Incorporation of the labeled proline (**6a**) into VM_1 proceeded with *almost complete loss of the tritium label* (Table I, entry 3). This finding thus confirms that the formation of the dehydroproline unit of VM_1 proceeds by a stereospecific pathway and that the 3-*pro-R* proton of proline is lost in the process.

A second important finding could be deduced from this result, since in this particular run the antibiotic VM_2 was produced in adequate amount for degradation.[21] The proline derived from VM_2 was found to have retained the greater part of the initial tritium label from the proline **6a** (Table I, entry 3). This result thus indicates conclusively that VM_2 is *not* derived by reduction of VM_1, since if it were it would have lost the tritium label

(18) Häusler, J. *Liebigs Ann. Chem.* **1981**, 1073–1088.
(19) Rylander, P. N. *Catalytic Hydrogenation over Platinum Metals*; Academic Press: New York, 1967; p 81.
(20) McQuillin, F. J. *Homogeneous Hydrogenation in Organic Chemistry*; D. Riedel: Dordrecht, The Netherlands, 1976.
(21) Normally virginiamycin M_2 is produced in only minor amounts (less than 5% of the total type A antibiotic). Recently however, for reasons that are not presently clear, the organism has been producing larger amounts of virginiamycin M_2.

Scheme III[a]



[a] (c) CH_3OH/HCl; (b) Bu^tOCl, NEt_3; (c) $Pb(OAc)_4$, CH_2Cl_2; (d) NaOH, $NaBH_4$; (e) HCl, heat.

Table II. Incorporation of Labeled Hydroxyprolines into Virginiamycin M_1

		$^3H/^{14}C$ ratio		
entry	precursors added	pre-cursor	VM_1	*N*-Bz-Pro
1	(2*S*,3*S*,4*R*)-[3,4-$^3H_2$]proline (**3**); (2*S*,3*S*) + (2*R*,3*R*)-[U-^{14}C]-3-hydroxyproline (**9**)	5.4	402	*a*
2	(2*S*,3*S*,4*R*)-[3,4-$^3H_2$]proline (**3**); (2*S*,3*R*) + (2*R*,3*S*)-[*carboxyl*-^{14}C]-3-hydroxyproline (**12a**)	9.0	41.3	19.8

[a] Not determined.

completely. A similar result was found for the formation of the (*R*)-alanine unit of antibiotic A2315A,[15] and these results thus corroborate earlier findings[8] which indicate that dehydro amino acids are not precursors of (*R*)-amino acids.

In considering the possible origin of the dehydroproline unit, we were attracted to the hypothesis that dehydro amino acids are formed by elimination of water from β-hydroxy amino acids,[7] as is the case in the formation of the dehydroalanine units of berninamycin from serine.[6] We thus elected to investigate the possible role of *cis*- and *trans*-3-hydroxyprolines as precursors of VM_1.

(2*S*,3*S*) + (2*R*,3*R*)-[U-^{14}C]-3-Hydroxyproline (*rac-trans*-3-hydroxyproline, **9**) was prepared by the pathway of Scheme II. Conversion of (*S*)-[U-^{14}C]proline to its methyl ester followed by treatment with *tert*-butyl hypochlorite and triethylamine yielded an imine that was converted to the enamine **8** by acetylation.[18] Reaction with sodium methoxide yielded a mixture of cis and trans 3-methoxy derivatives in which the trans isomer predominated; after cleavage of the ether and ester groups with hydrobromic acid, the racemic trans isomer **9** was obtained pure by ion-exchange chromatography. The cis isomer was not obtained pure by this method.

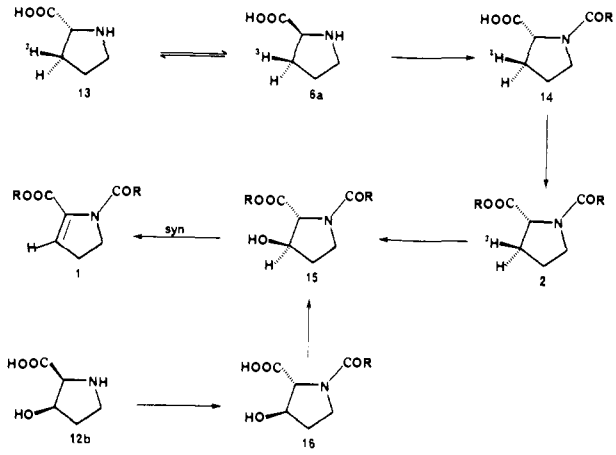
(2*S*,3*R*) + (2*R*,3*S*)-[*carboxyl*-^{14}C]-3-hydroxyproline (*rac-cis*-3-hydroxyproline, **12a**) was prepared by the pathway of Scheme III.[18,22] (2*S*)-[*carboxyl*-^{14}C]Proline (**10a**) was converted to its methyl ester and then to the imine as described previously. The imine was acetoxylated with lead tetraacetate to yield the acetoxydehydroproline **11a** which was reduced and hydrolyzed to yield **12a** as the major isomer. Although the overall yield of this sequence was low, it did yield the cis isomer as the major product: the small amount of trans isomer formed was removed by recrystallization from aqueous ethanol.

Incorporation of the *trans*- and *cis*-3-hydroxyprolines **9** and **12a** was carried out in the presence of [^{3}H]proline as an internal standard. Although for obvious reasons the starting mixture of proline and hydroxyproline could not be recrystallized to a constant specific activity ratio, the amino acids were purified or recrystallized individually to a constant specific activity and then mixed, and the specific activity ratio of the mixture was determined. With this technique, incorporation of the *trans*-3-hydroxyproline **9** did not occur to any meaningful extent (Table II, entry 1): *trans*-3-hydroxyproline was incorporated less than 2% as effectively as proline. Incorporation of *cis*-3-hydroxyproline **12a**, however (Table II, entry 2), proceeded relatively efficiently, being incorporated 20% as effectively as proline into the whole antibiotic and 45% as efficiently into the dehydroproline unit.

A final experiment was carried out to determine whether VM_2 lies on the biosynthetic pathway to VM_1. A doubly labeled sample

(22) Ewing, W. R.; Harris, B. D.; Bhat, K. L.; Joullie, M. M. *Tetrahedron* **1986**, *42*, 2421–2428.

Scheme IV



of VM_2 was prepared by biosynthesis from (*S*)-[U-^{14}C]proline and sodium [2-^{3}H]acetate; these precursors were selected to ensure labeling of both the proline unit and the main carbon skeleton of the antibiotic.[10] The VM_2 isolated had a $^3H/^{14}C$ ratio of 2.25. Feeding this material to growing cultures of *S. virginiae* yielded radioactive VM_1 with about a 2% level of incorporation. Significantly, the $^3H/^{14}C$ ratio of the isolated antibiotic was essentially unchanged at 2.42. The fact that the $^3H/^{14}C$ ratio is unchanged excludes the possibility that incorporation is by some general degradation of VM_2 to its constituent units and reincorporation of these units and *requires that VM_2 be on the biosynthetic pathway to VM_1*.

These results enable us to make a number of conclusions about the biosynthetic pathway from proline to the dehydroproline unit of virginiamycin M_1. In the first place, we note that the clear-cut difference in the incorporation of racemic *cis*- and *trans*-3-hydroxyprolines implies that there is *no epimerization* of these amino acids during the initial biosynthetic process; if epimerization occurred, these hydroxyprolines would have been incorporated equally effectively. Because there is no epimerization, it is probable that only one enantiomer of *cis*-3-hydroxyproline is incorporated, and the observed incorporation efficiency of the racemic material of 45% as compared with that of proline becomes an efficiency of 90% on the basis of the enantiomer actually incorporated. The fact that the incorporation efficiency is less than 100% suggests that free *cis*-3-hydroxyproline is not on the direct biosynthetic pathway to VM_1 but indicates that it can be incorporated by an alternate and less efficient pathway.

The fact that the 3-*pro-R* proton of proline is lost on its incorporation into VM_1 (Table I) puts additional constraints on possible biosynthetic pathways. Two basic schemes are possible, depending on whether proline undergoes hydroxylation at the 3-position with retention or with inversion of configuration. The hydroxylation of proline to 4-hydroxyproline is a well-studied reaction that is known to proceed either on a preformed peptide with retention of stereochemistry[23] or on the free imino acid,[24] and it is probable that hydroxylation to 3-hydroxyproline proceeds similarly since the enzymes involved appear to be similar.[25]

On the assumption that hydroxylation of proline does occur with retention of configuration, it is possible to propose a biosynthetic scheme for VM_1 that is consistent with all the known facts (Scheme IV). In this scheme, the labeled (*S*)-proline **6a** is incorporated as an (*R*)-prolyl unit into the previrginiamycin peptide **14**, either directly or through the (*R*)-proline **13**. If incorporation occurs directly, epimerization presumably occurs on an activated enzyme-bound intermediate, analogous to the racemization of phenylalanine during gramicidin S and tyrocidine biosynthesis.[26] Conversion of the peptide **14** to VM_2 (**2**) is followed by hydroxylation with retention of stereochemistry (and thus loss of the ^{3}H label) to give the hydroxyvirginiamycin M_2 **15**. Elimination of water from **15** occurs by a syn process, which is reasonable since syn eliminations can be facile in 5-membered rings.[27]

The incorporation of (2*S*)-*cis*-3-hydroxyproline (**12b**) can be explained by its conversion into a modified previrginiamycin peptide **16** with epimerization at C-2 and subsequent conversion of this peptide to the hydroxyvirginiamycin **15**. This process is exactly analogous to that observed by Katz for the incorporation of (2*S*,4*R*)-4-hydroxyproline (*trans*-4-hydroxy-L-proline) into the (2*R*,4*R*)-4-hydroxyproline, or *cis*-4-hydroxy-D-proline, unit of etamycin,[24] and this fact provides support for the reasonableness of the proposed pathway. The poorer incorporation of *cis*-3-hydroxyproline as compared with that of proline also has analogy in the lower incorporation of 4-hydroxyproline as compared with that of proline into the 4-hydroxyproline moiety of the actinomycins.[28]

In conclusion, this work provides unequivocal support for the stereospecific dehydrogenation of VM_2 to VM_1 in the biosynthesis of the latter antibiotic. The work also strongly suggests that the pathway from VM_2 to VM_1 involves the formation of a hydroxyvirginiamycin M_2 by a hydroxylation of the proline unit of VM_2 with retention of configuration and a subsequent syn elimination to yield VM_1. This work represents the first demonstration that dehydroproline units in nature arise from hydroxyproline intermediates, and it has important implications in current efforts to improve the microbiological production of VM_1 and minimize that of VM_2.

Experimental Section

General. General experimental procedures were as described in previous papers.[1,10] ^{1}H NMR spectra were measured in $CDCl_3$, and IR spectra were determined in $CHCl_3$ solution.

(2*S*,3*S*,4*R*)-[3,4-$^3H_2$]Proline (3). This compound was purchased from ICN Radiochemicals (lot no. 79529, 134411, and 2006122).

***N*-Carbomethoxy-2,3-didehydroproline Methyl Ester (5b).** Compound **5b** was prepared from (*S*)-proline by the method of Hausler,[18] using methyl chloroformate instead of ethyl chloroformate. The product was purified by chromatography on silica gel: yield 68%; TLC (EtOAc–hexane, 2:1) R_f 0.34; ^{1}H NMR δ 2.09 (s, 3 H), 2.70 (dt, 4 H), 3.81 (s, 3 H); IR 2990, 1760, 1740, 1720, 1458 cm^{-1}.

(2*R*,3*R*) + (2*S*,3*S*)-[2,3-$^2H_2$]Proline (6b). Compound **5b** (100 mg, 0.55 mmol) and platinum oxide (10 mg) in ethyl acetate (10 mL) were treated with deuterium for 24 h at ambient temperature and pressure. The product (obtained by filtration to remove catalyst and evaporation of solvent in vacuo) was treated with trimethylsilyl iodide (0.712 g) in dry acetonitrile (10 mL) under reflux for 20 h, followed by stirring with 1:1 ethanol–acetic acid (20 mL) at 0 °C for 20 min. The mixture was diluted with H_2O (10 mL) and extracted with dichloromethane (5 × 10 mL). The aqueous layer was purified by ion-exchange chromatography on Dowex 50 (1 × 30 cm, 10 g) in the H^+ form. Elution with deionized water until neutral and then with 4 N NH_4OH gave racemic proline **6b**, 60 mg (95%), identical (TLC, co-TLC) with authentic (*S*)-proline: ^{1}H NMR δ 1.99 (m, 2 H, 4α, 4β), 2.01 (m, 1 H, 3β), 2.32 (m, 0.1 H, 3α), 3.3 (m, 1 H, 5β), 3.40 (m, 1 H, 5α), 4.12 (t, 0.1 H, 2α).

Resolution of Proline. Racemic proline was resolved by the tartrate method[26] as follows. (*RS*)-Proline (100 mg, 0.89 mmol) and (2*S*,3*S*)-tartaric acid (68 mg, 0.45 mmol) were mixed in water (0.1 mL), and the mixture was diluted with cold absolute ethanol (1.5 mL). Addition of a seed crystal of (*S*)-proline (*S*)-tartrate and refrigeration for 24 h yielded crystals of the *S*–*S* salt, which were filtered to yield 55 mg (24%) of product: mp 154–6 °C (lit.[24] mp 154.5 °C); $[\alpha]^{25}_D$ −24.82° (*c* = 1, H_2O) [lit.[29] $[\alpha]^{25}_D$ −24.2 (*c* = 1, H_2O)].

(2*S*,3*R*)-[3-^{3}H]Proline (6a). A mixture of (*S*)-proline (303 mg, 2.29 mmol) and (2*S*)-[2,3-$^3H_2$]proline (New England Nuclear lot 2221-296,

(23) Lamport, D. T. A. *Nature (London)* **1964**, *202*, 293–294. Salzman, L. A.; Weissbach, H.; Katz, E. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *54*, 542–546.
(24) Katz, E.; Kamal, F.; Mason, K. *J. Biol. Chem.* **1979**, *254*, 6684–6690.
(25) Udenfriend, S.; Cardinale, G. In *Oxygenases and Oxygen Metabolism*; Nozaki, M.; Yamamoto, S.; Ishimura, Y.; Conn, M. J.; Ernster, L.; Estabrook, R. W., Eds.; Academic Press: New York, 1982; pp 99–107.
(26) Lipmann, F.; Gevers, W.; Kleinkauf, H.; Roskowski, R. *Adv. Emzymol. Relat. Areas Mol. Biol.* **1971**, *35*, 1–34.
(27) Reference 14, pp 550–552.
(28) Katz, E.; Prockop, D. J.; Udenfriend, S. *J. Biol. Chem.* **1962**, *237*, 1585–1588.
(29) Yamada, S.; Hongo, C.; Chibati, I. *Agric. Biol. Chem.* **1977**, *41*, 2413–2416.

3 mCi) was converted to *N*-carbomethoxy[3-^{3}H]-2,3-didehydroproline methyl ester (**5a**) as previously described in 48% yield from proline. Hydrogenation over platinum oxide, followed by deprotection and purification by ion-exchange chromatography, yielded racemic proline **6a** (100 mg, 0.87 mmol, 33%), identical (TLC, ^{1}H NMR) with authentic proline. Total activity recovered was 0.7 mCi, for a radiochemical yield of 23%.

The racemic proline **6a** was resolved as described above to give (2*S*,3*R*)-[3-^{3}H]proline **6a** as its (*S*)-tartrate salt (45 mg, 20%). The free amino acid was isolated by ion-exchange chromatography on Dowex 50; elution with 0.1 N HCl and evaporation yielded the amino acid as its hydrochloride, identical (TLC) with authentic material.

***N*-Acetyl-2,3-didehydro[U-^{14}C]proline Methyl Ester (8).** (*S*)-[U-^{14}C]Proline **7** (115 mg, 1.0 mmol, 247 μCi) was converted to the dehydroproline **8** by the method of Häusler,[18] except that final purification was by chromatography (silica gel; ethyl acetate–CH_2Cl_2, 1:9) rather than by distillation. A total of 41.5 mg (0.25 mmol, 25%) of homogeneous product was obtained, identical with an authentic unlabeled sample.

(2*S*,3*S*) + (2*R*,3*R*)-[U-^{14}C]-3-Hydroxyproline (9). Dehydroproline **8** (41.5 mg, 62 μCi), was diluted with 58.5 mg of unlabeled material. The mixture was dissolved in methanol (4 mL) containing 0.5 equiv of sodium methoxide, and the solution was refluxed for 20 min. The resulting solution was diluted with ether and filtered through a short column of silica gel, with final elution by methanol–ether, 1:1, to remove polar impurities. The resulting mixture of *cis*- and *trans*-3-methoxyproline methyl esters (88 mg, 0.44 mmol) was dissolved in 1.0 mL of 48% HBr and stirred at 100 °C in a sealed vial for 2 h. The resulting dark brown solution was evaporated to dryness at 55 °C in vacuo, dissolved in 0.6 mL of pH 3.25 citrate buffer, and added to a column (50 × 0.9 cm) of Dowex 50 resin (Na^+ form) prepared by the literature procedure.[30] Elution with citrate buffer and collection of 1-mL fractions yielded the *trans*-3-hydroxyproline **9** in fractions 62–86; the other sample collected in fractions 89–113 was shown to be a mixture of *cis*- and *trans*-3-methoxyproline and *cis*-3-hydroxyproline. The *trans*-3-hydroxyproline **9** was desalted on a 16 × 1.6 cm column of Dowex 50 resin in the H^+ form and elution with water followed by 2 N ammonium hydroxide. The product **9** (22 mg, 0.17 mmol, 14 μCi) had an ^{1}H NMR spectrum (D_2O) essentially identical with that of a standard sample of unlabeled **9**.

3-Acetoxy-1,2-didehydro[*carboxy*-^{14}C]proline Methyl Ester (11a). A mixture of (*R*,*S*)-[*carboxyl*-^{14}C]proline (Research Products Inc., lot 276-577) (**10a**, 300 μCi) and (*S*)-proline (230 mg, 2 mmol) was converted to 1,2-dehydro[*carboxyl*-^{14}C]proline methyl ester by the method of Häusler.[18] This compound (210 mg, 1.65 mmol, 83%) was dissolved in dichloromethane and treated with 814 mg (1.11 equiv) of lead tetraacetate in portions at 0 °C.[18,28] The resulting solution was stirred for 15 min at 0 °C and then heated under reflux for 5 h. Removal of the lead oxide precipitate by filtration and evaporation of solvent yielded the acetoxyproline **11a** (87%) and the methyl ester of pyrrole-2-carboxylic acid (13%).

(2*S*,3*R*) + (2*R*,3*S*)-[*carboxyl*-^{14}C]-3-Hydroxyproline (12a). The crude 1,2-didehydro-3-acetoxyproline methyl ester **11a** (167 mg) was dissolved in cold 0.502 N methanolic NaOH (1.8 mL, 1 equiv). Sodium borohydride (17.8 mg, 2.1 equiv) was added, and the solution was stirred 10 min. The pH was then adjusted to 6 with aqueous HBr; the solution was filtered, evaporated to dryness, redissolved in 2 HCl (25 mL), and refluxed for 2 h. The resulting brown solution was evaporated to 10 mL, decolorized with Norite, and evaporated to dryness. The yellow residue was dissolved in a minimum amount of water and added to a column of Dowex 50 resin (1.6 × 8 cm) in the H^+ form. After being washed with water, the column was eluted with 1 N NH_4OH. Collection and evaporation of the appropriate fraction (125 mL) with an intermediate decolorization with Norite yielded crude *cis*-3-hydroxyprolines **12a** as an off-white solid, contaminated with 15–20% of the trans isomer as determined by ^{1}H NMR spectroscopy.

The *cis*-3-hydroxyprolines **12a** were purified by recrystallization from aqueous ethanol (5 drops of water and 1.5 mL of absolute ethanol). Threefold recrystallization yielded material which was homogeneous on TLC (Whatman $MKC_{18}F$; ethanol–1% acetic acid, 7:3) and identical with an authentic sample of unlabeled material (**12b**). The *cis*-3-hydroxyprolines **12a** had an R_f of 0.73, while *trans*-3-hydroxyprolines had an R_f of 0.88 in this system. Autoradiography showed the presence of only one radioactive substance, corresponding to *cis*-3-hydroxyprolines **12a**. ^{1}H NMR (**12b**, D_2O): δ 1.99 (m, 2 H), 3.33 (m, 2 H), 3.94 (d, 1 H, J = 4), 4.51 (m, 1 H).

Culture Conditions. Incorporations of proline **3** and the hydroxyprolines **9** were carried out under culture conditions as previously described.[10] Due to problems with obtaining adequate production under these conditions in later runs, incorporations of proline **6a** and hydroxyprolines **12a** were carried out as follows. *S. virginiae* strain 5266 was grown in vegetative medium and transferred into production medium after 2 days. After 24 h the precursor solution was added, and the fermentation was ended after 3 days.

Isolation. The cultures were extracted, and the virginiamycins M_1 and M_2 were isolated as previously described,[10] except that methyl isobutyl ketone was used rather than ethyl acetate to extract the antibiotic in later runs. A Dynamax Macro C-18 column, 1 × 25 cm, was used for HPLC purification with CH_3CN–water, 40:60, and a flow rate of 6 mL/min. Virginiamycin M_2 eluted at 6.5 min and virginiamycin M_1 at 10.1 min with this system.

Preparation of Precursor Mixtures. For incorporation of the ^{3}H-labeled prolines **3** and **6a**, a mixture of ^{3}H- and ^{14}C-labeled (*S*)-prolines was prepared, and a 10% aliquot was recrystallized to a constant specific activity ratio. The remaining 90% was used for the incorporation. For incorporation of the *cis*-3-hydroxyproline **12a**, the [^{14}C]-3-hydroxyproline and [^{3}H]proline were recrystallized separately, and a mixture of the purified amino acids was then prepared and its specific activity ratio determined. In the case of *trans*-3-hydroxyproline **9**, the radiochemically homogeneous hydroxyproline **9** was mixed directly with the [^{3}H]proline and the specific activity ratio of this mixture determined.

Determination of Specific Activity Ratios of Products. Portions of the isolated antibiotics were counted on a Beckman LS 3800 liquid scintillation counter in National Diagnostics Ecoscint or Beckman Ready Solv MP cocktail. Quench corrections were carried out by the internal standard method, using [^{14}C]- and [^{3}H]hexadecane standards from Amersham Corp.

The proline unit was obtained as *N*-benzoylproline by the hydrogenation–hydrolysis–benzoylation–HPLC sequence previously described;[10] the hydrogenation step was omitted for virginiamycin M_2. The *N*-benzoylproline was then mixed with unlabeled material and recrystallized to a constant specific activity ratio for liquid scintillation counting as described above.

Preparation of [^{14}C/^{3}H]Virginiamycin M_2. A precursor solution containing (*S*)-[U-^{14}C]proline (1 mCi, sp act. 280 mCi/mmol) and sodium [2-^{3}H]acetate (25 mCi, sp act. 3.3 Ci/mmol) was added to cultures of *S. virginiae* 27 h after the start of fermentation. Crude mixed virginiamycins were isolated as previously described, and the VM_2 was purified by HPLC on Diaion HP20 (Mitsubishi) with acetone–H_2O, 40:60, as mobile phase. The fractions containing VM_2 were pooled; VM_2 was extracted with $CHCl_3$ and precipitated with hexane from the concentrated extract. The isolated antibiotic (130 mg) was chromatographically homogeneous and free of any detectable VM_1. Specific activities: ^{3}H, 0.472 mCi/g (0.247 mCi/mmol); ^{14}C, 0.209 mCi/g (0.109 mCi/mmol). ^{3}H/^{14}C ratio: 2.25.

Conversion of Virginiamycin M_2 to Virginiamycin M_1. The VM_2 prepared as described above was dissolved in DMSO and added to a growing culture of *S. virginiae* 24 h after the start of fermentation; the final concentration of VM_2 in the fermentation was 50 mg/L. The VM_1 produced was isolated and purified by preparative HPLC; the isolated antibiotic had the following specific activities: ^{3}H, 0.0085 mCi/g (0.0044 mCi/mmol); ^{14}C, 0.0035 mCi/g (0.00018 mCi/mmol). ^{3}H/^{14}C ratio: 2.42.

Acknowledgment. We thank Dr. Richard Wang for advice and encouragement and the National Science Foundation (PCM 8111508) and Smith Kline Animal Health Products for support of this work.

(30) Moore, S.; Stein, W. H. *J. Biol. Chem.* **1951**, *192*, 663–681.