poured into saturated NaCl solution, and isolated with ether. Crystallization from ether gave 2.41 g (79.5%) as a first crop and 470 mg (14.7%) as a second crop: mp 109–110 °C; $[\alpha]^{25}_D$ +3.80° (c 1.06, $CHCl_3$); IR (film) 3500, 2990, 1805, 1250, 1190, 1080, 980, 780 cm^{-1}; NMR ($CDCl_3$) δ 4.24 (AB_q, $\Delta\nu$ = 140.0 Hz, J_{AB} = 8.47, 2 H), 4.12 (d of d, J = 6.81 Hz, J = 8.20 Hz, 1 H), 3.73 (m, 1 H), 3.50 (AB_q split into doublet, $\Delta\nu$ 129.35 Hz, J_{AB} = 10.38, J = 2.00 Hz, 2 H), 2.19 (m, 1 H), 2.07 (m, 1 H), 1.72 (m, 2 H), 1.47 (s, 3 H), 1.21 (s, 3 H). Anal. Calcd for $C_{11}H_{17}O_4I$: C, 37.10; H, 4.81; I, 35.63. Found: C, 37.16; H, 4.87; I, 35.57.

Preparation of Alcohol 30. A solution of 2.41 g (7.07 mmol) of iodide **29** in 20.0 mL of 95% EtOH at room temperature was treated with 1.87 mL (7.09 mmol) of tri-*n*-butyltin hydride. The mixture was stirred at room temperature for 2 h, and the EtOH was removed under reduced pressure and purified by flash chromatography with 50% EtOAc/hexane to afford 1.60 g (98.4%) of the alcohol **30** after Kugelrohr distillation (130 °C (0.1 torr)): IR (film) 3350, 2990, 1800, 1305, 1300, 1115, 1100, 1075, 980, 880 cm^{-1}; NMR ($CDCl_3$) δ 4.25 (AB_q, $\Delta\nu$ = 133.8 Hz, J_{AB} = 8.45 Hz, 2 H), 4.13 (t, 7.08 Hz, 1 H), 3.78 (q, J = 6.59 Hz, 1 H), 1.48 (s, 3 H), 1.15 (s, 3 H), 1.14 (d, J = 6.59 Hz, 3 H). anal. Calcd for $C_{11}H_{18}O_5$: C, 57.38; H, 7.88. Found: C, 57.46; H, 7.70.

Preparation of Silyl Ether 31. A solution of 1.60 g (6.95 mmol) of alcohol **30**, 2.08 mL (8.00 mmol) of *tert*-butyldiphenylchlorosilane, 1.09 g (16.00 mmol) of imidazole, and 6.0 mL of DMF was heated to 60 °C for 3 h. The mixture was poured into saturated NaCl and extracted with ether (3 times). The combined ether layers were dried over $MgSO_4$ and the solvent was removed under reduced pressure. Flash chromatography with 25% EtOAc/hexane gave the product silyl ether **31** which was crystallized from EtOAc/hexane to give 1.97 g (60.6%) as a first crop and 880 mg (27.1%) as a second crop: mp 154–155 °C; $[\alpha]^{25}_D$ +5.2° (c 2.8, $CHCl_3$); IR (film) 3007, 1810, 1120, 1100, 1075, 710 cm^{-1}; NMR ($CDCl_3$) δ 7.63 (m, 4 H), 7.42 (m, 6 H), 4.00 (AB_q, $\Delta\nu$ = 94.90, J_{AB} =v 8.33 Hz, 2 H), 4.03 (d of d, J = 6.70 Hz, J = 8.20 Hz, 1 H), 3.55 (q, J = 6.20 Hz, 1 H), 1.26 (s, 3 H), 1.25 (s, 3 H), 1.05 (s, 9 H), 1.04 (d, J = 6.20 Hz).

Preparation of Epoxide 34. A solution of 1.80 g (3.84 mmol) of carbonate **31** in 50 mL of ether at 0 °C was treated with 146 mg (3.84 mmol) of $LiAlH_4$. The mixture was stirred at 0 °C for 3 h and then treated successively with 0.19 mL of H_2O, 0.38 mL of 10% NaOH, and 0.38 mL of H_2O. The resulting white suspension was filtered and concentrated to afford 1.79 g of crude diol which was flash chromatographed with 100% EtOAc to give 1.61 g (95.3%) of pure diol. An analytical sample was secured by Kugelrohr distillation (150 °C (0.1 torr): IR (film) 3400, 3008, 2990, 2930, 1595, 1435, 1380, 1100, 1050, 1025, 830, 785, 745, 710 cm^{-1}; NMR ($CDCl_3$) δ 7.70 (m, Ar), 7.40 (m, Ar), 3.76 (d of d, J = 6.48 Hz, J = 8.35 Hz, 1 H), 3.60 (q, J = 6.2 Hz, 1 H), 2.54 (AB_q, $\Delta\nu$ = 37.5 Hz, J_{AB} = 4.90 Hz, 2 H), 2.00 (m, 1 H), 1.79 1.25 (s, 3 H), 1.12 (s, 3 H), 1.06 (d, J = 6.28 Hz, 3 H), 1.01 (s, 9 H). Anal. Calcd for $C_{24}H_{38}O_4Si$: C, 70.55; H, 8.65. Found: C, 70.50; H, 8.65.

A solution of 1.59 g (3.61 mmol) of the above diol in 5.0 mL of anhydrous pyridine at 0 °C was treated with 705 mg (3.70 mmol) of *p*-toluenesulfonyl chloride. The mixture was stirred at 0 °C overnight, poured into saturated $CuSO_4$ solution, extracted with ether, and concentrated under reduced pressure to afford the crude tosylate **33** which was dissolved in 100 mL of anhydrous MeOH and treated with 600 mg (4.35 mmol) of K_2CO_3. After the mixture was stirred for 1 h at room temperature, the solution was poured into saturated NaCl solution, and extracted with ether (3 times). The combined ether layers were concentrated under reduced pressure and purified by flash chromatography with 25% EtOAc/hexane to afford 1.51 g (99%) of pure epoxide **34**: IR (film) 3045, 2990, 1585, 1435, 1380, 1120, 830, 790, 745, 710 cm^{-1}; NMR ($CHCl_3$) δ 7.69 (m, 4 H), 7.38 (m, 6 H), 3.76 (d of d, J 6.48 Hz, J = 8.35 Hz, 1 H), 3.62 (q, J = 6.27 Hz, 1 H), 2.532 (AB_q, $\Delta\nu$ = 37.47 Hz, J = 4.9 Hz, 2 H), 1.25 (s, 3 H), 1.12 (s, 3 H), 1.05 (d, J = 6.27, 3 H), 1.05 (s, 9 H). Anal. Calcd for $C_{26}H_{36}O_3Si$: C, 73.54; H, 8.54. Found: C, 73.62; H, 8.52.

Preparation of Alkene 35. A solution of 1.50 g (3.58 mmol) of epoxide **34**, 15.0 mL of THF, and 3.0 mL of a 2.0 M solution of allylmagnesium chloride was stirred at room temperature of 36 h. The mixture was poured into saturated NH_4Cl solution and extracted with ether (3 times). The combined ether layers were dried over $MgSO_4$ and filtered, and the solvent was removed under reduced pressure to afford the crude alkene. Chromatography on silica gel with 15% EtOAc/hexane gave 1.49 g (91%) of the pure alkene **35** as a viscous oil, after Kugelrohr distillation (bp 150 °C (0.1 torr)): IR (film) 3045, 3008, 2990, 1650, 1600, 1380, 1120, 940, 830, 750, 710 cm^{-1}; NMR ($CDCl_3$) δ 7.68 (m, 4 H), 7.40 (m, 6 H), 5.78 (m, 1 H), 4.94 (m, 2 H), 3.77 (t, J = 7.20 Hz, 1 H), 3.65 (q, J = 6.20 Hz, 1 H), 1.23 (s, 3 H), 1.10 (s, 3 H), 1.04 (s, 9 H), 1.00 (d, J = 6.20 Hz, 3 H). Anal. Calcd for $C_{29}H_{42}O_3Si$: C, 73.25; H, 9.56; Si, 6.34. Found: C, 73.89; H, 8.97; Si, 6.41.

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Supplementary Material Available: Tables of bond distances, bond angles, atomic coordinates, and thermal parameters, (4 pages). Ordering information is given on any current masthead page.

Biosynthesis of Antibiotics of the Virginiamycin Family. 4.[1] Biosynthesis of A2315A

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The biosynthesis of A2315A, **1**, has been studied by using stable isotope techniques. The basic skeleton of the antibiotic is constructed from seven acetate units together with the amino acids valine, glycine, alanine, and serine and a methyl group from methionine. The uncommon D-alanine unit is shown to arise from both D- and L-alanine with equal facility.

The antibiotic A2315A,[2] identical with madumycin II,[3] A15104V,[4] A17002F,[4] and CP-35,763,[5] is a complex macrocyclic compound isolated from *Actinomadura flava*[3] and various *Actinoplanes* species.[2,4-5] It is a member of the

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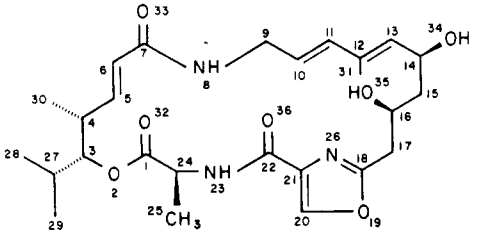
virginiamycin or streptogramin family of antibiotics, being closely related to virginiamycin M_1 (ostreogrycin A) (2).[6,7]



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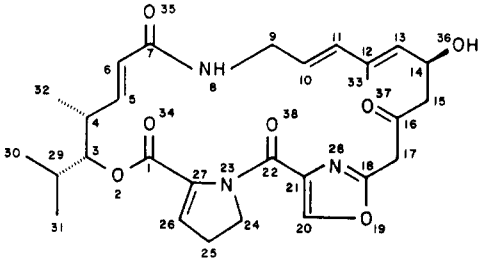

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Members of this family normally occur as synergistic mixtures of two distinct types of antibiotic. The group A antibiotics, of which A2315A is a member, are polyunsaturated cyclic peptides with molecular weights around 500, while the cooccurring group B antibiotics are cyclic peptidolactones of about 800 molecular weight.[8] A2315A is unusual in that it occurs alone, without any synergistic group B component; it is active mainly against Gram-positive organisms.[3]

The isolation and structure elucidation of A2315A has been reported by several groups,[2-5] its solution conformation has been studied,[9] and its ^{13}C NMR spectrum has been assigned.[10] Structurally, A2315A differs from virginiamycin M_1 in only two respects: A2315A bears a hydroxyl group at C-16 instead of a carbonyl oxygen and a D-alanine unit replaces the dehydroproline unit of virginiamycin M_1. Both molecules contain the unusual oxazole ring system, found elsewhere in only a few naturally occurring compounds such as the antibiotics berninamycin[11] and nocobactin NA[12] and in the alkaloid annuloline.[13]

In this paper we report results that reveal the biosynthetic origin of the D-alanine unit of A2315A and confirm the anticipated biosynthetic pathways to the remainder of the molecule.

Results and Discussion

The antibiotic used in our studies was produced by *Actinoplanes philippinensis* grown in baffled shake flasks containing a complex medium and incubated at 30 °C. Three days after inoculation the appropriate ^{14}C-, ^{3}H-, or ^{13}C-labeled precursors were added. The cultures were worked up 36 h later by filtration to remove the mycelium and extraction of the filtrate with hexane followed by ethyl acetate. Pure A2315A could be isolated from the crude ethyl acetate extract by preparative reversed-phase high-performance liquid chromatography. For incorporation of radioactive precursors (^{14}C and ^{3}H) the extent of incorporation and the $^{3}H/^{14}C$ ratios for doubly labeled samples were determined by counting the chromatographically homogeneous product. For ^{13}C precursors the incorporations were observed directly by ^{13}C NMR spectroscopy.

Incorporation of [1,2-$^{13}C_2$]Acetate. Sodium [1,2-$^{13}C_2$]acetate was incorporated in order to establish the biosynthesis of the major portion of the carbon skeleton of A2315A and to verify the anticipated parallel with the labeling pattern of the closely related antibiotic virginiamycin M_1. ^{13}C NMR spectroscopy of the isolated antibiotic showed couplings between the pairs of carbons 4 and 5, 6 and 7, 15 and 16, and 17 and 18 (Table I), indicating that these pairs of carbons arise from an intact acetate unit. These pairings agree with those found in virginiamycin M_1. Presumably the two pairs of carbons 11 and 12 and 13 and 14 also derive from two intact acetate units as in virginiamycin M_1, but overlapping signals prevented the determination of the coupling constants for three of the four resonances due to these carbons. In virginiamycin M_1 the C-33 methyl group was found to arise from the methyl group of an acetate molecule by a novel pathway involving addition of acetate to a pre-formed polyketide chain and subsequent decarboxylation.[1] A similar pathway is presumably operating in the case of A2315A, since carbon-31 showed no coupling to carbon-12, and a slight enrichment of this carbon is observed in antibiotic biosynthesized from [2-^{13}C]acetate derived from L-[3-^{13}C,3,3,3-$^{2}H_3$]alanine (see below). The lack of enrichment of carbon-31 in A2315A biosynthesized from [1,2-$^{13}C_2$]acetate is probably due to the fact that this carbon is added at a later stage of the biosynthesis, after the pool of labeled acetate has been depleted.

Incorporation of [2-^{13}C]Valine. Since virginiamycin M_1 efficiently incorporated DL-[2-^{13}C]valine (synthesized from [2-^{13}C]glycine),[1] it was probable that carbons 3, 27, 28, and 29 of A2315A also arose from this unit. Feeding of DL-[2-^{13}C]valine under the standard conditions resulted in a high enrichment of only carbon 3 by 22.2%, indicating that, as in virginiamycin M_1, valine serves as the chain starter unit for the biosynthesis of the western portion of A2315A, presumably after conversion to isobutyryl coenzyme A.

Origin of the D-Alanine Residue. Although D-amino acids are rare in nature, they occur relatively frequently in antibiotics, and some 29 D-amino acids have been identified as components of antibiotics and other peptides derived from microorganisms.[14] The biosynthesis of D-

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Table I. ^{13}C NMR Spectral Data for A2315A, Including Enrichments from Labeled Precursors

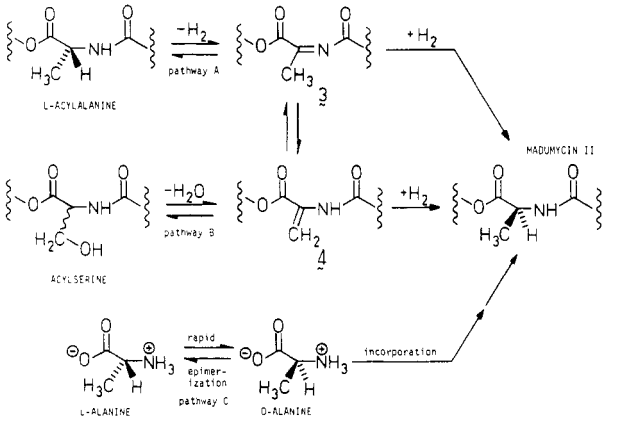
carbon no.	δ_c[a]	I unlabeled/I unlabeled[b]				J_{C-C}, Hz	
		[1,2-$^{13}C_2$]Ac	[2-^{13}C]Val	[U-$^{13}C_3$]Ser	[3-^{13}C,3,3,3-$^2H_3$]Ala	c	d
1	172.6	1.0	0.4	1.3	0.5		
27	29.6	1.0[e]	0.8	1.0[e]	1.2		
28	18.6[f]	1.0	0.9	1.0	*h*		
29	19.7[f]	1.0	0.7	1.0	1.1		
3	83.2	0.9	22.2	1.0	1.2		
4	36.8	1.8	0.7	1.0	2.0	35	
30	10.5	0.8	0.6	3.7	0.7		
5	145.1	1.1	0.7	0.8	0.7	40	
6	124.4	1.4	0.8	1.0	1.7	65	
7	166.3	1.2	0.4	0.8	0.4	63	
9	40.7	1.0	1.0[e]	4.5	1.0[e]		40
10	124.7	1.1	1.0	3.3	1.0		40
11	134.7[g]	0.8	1.0	1.5	*i*	*j*	
12	134.5	1.0	1.1	1.2	*i*	*j*	
31	13.1	1.0	0.7	0.7	1.2		
13	134.0[g]	1.4	1.1	1.1	1.9	*j*	
14	67.4	1.7	1.3	1.0	1.0	40	
15	43.2	1.8	1.2	1.1	2.2	35	
16	67.7	1.1	1.1	1.3	1.1	40	
17	36.1	1.4	1.0	0.9	1.9	56	
18	152.2	1.2	0.6	0.8	0.5	58	
20	140.7	0.8	0.9	5.2	0.8		71
21	135.6	0.9	0.5	5.7	0.7		70, 80
22	160.1	0.7	0.5	3.9	0.4		80
24	47.3	0.8	0.9	1.0	0.6		
25	19.1	1.0	0.9	1.1	2.9[k]		

[a] Chemical shifts in ppm are downfield from internal Me_4Si in $CDCl_3$. [b] Intensity of each peak in the labeled antibiotic divided by that of the corresponding peak in the unlabeled antibiotic, normalized to give a ratio of 1.0 for an unenriched peak, either C-9 or C-27. [c] Carbon–carbon coupling observed when [1,2-$^{13}C_2$]acetate was incorporated into A2315A. [d] Carbon–carbon coupling observed when [U-$^{13}C_3$]serine was incorporated into A2315A. [e] Peak selected as the unenriched standard for this spectrum. [f,g] These signals may be interchanged. [h] Overlapping signals due to ^2H–^{13}C coupling prevented accurate determination of this signal. [i] Overlapping signals prevented determination of intensities of these signals. [j] Overlapping signals prevented detection of coupling for these resonances. [k] Determined from the ^1H,^2H, decoupled ^{13}C NMR spectrum.

amino acids has been the subject of much study, and various schemes have been proposed: these have been reviewed.[15] In general, it is found that the L-amino acid serves as the precursor of the D acid and that the D-amino acid itself is not incorporated into the microbial peptide.[16] It has also been noted that D-amino acids with a second chiral center are almost always diastereomers rather than enantiomers of the natural form, indicating that the D acid is formed by α-epimerization rather than by some more complex process.[16]

In considering ways in which the D-alanine residue of A2315A could be formed, we were attracted initially to the suggestion made by Bycroft that D-amino acids could be formed by dehydrogenation of an L-amino acid already incorporated into a peptide chain to an acylimine **3** followed by stereospecific rehydrogenation to the α-enantiomer (Scheme I, pathway A).[17] This idea is supported by two lines of evidence. First, as already noted, in microbial peptides in general the D-amino acid residues arise from the corresponding L isomers, implying that α-epimerization occurs during or after incorporation of the amino acid into the metabolite. Secondly, tautomerization of the imine **3** to the enamine **4** (Scheme I) would explain the rather common occurrence of dehydroamino acids in both microbial and plant peptides.[18,19] In the particular case

Scheme I. Possible Pathways for the Formation of the D-Alanine Residue in A2315A



of A2315A we were also intrigued by the observation that the closely related antibiotic virginiamycin M_1 (formerly ostreogrycin A) contains a dehydroproline residue,[6] while virginiamycin M_2 (formerly ostreogrycin G) contains a D-proline unit at the same position.[20] It thus appeared reasonable to suppose that the D-proline unit could have been biosynthesized by hydrogenation of an intermediate α,β-dehydroproline unit, and extension of this reasoning to A2315A suggested that the enamine **4** could possibly serve as a precursor of the D-alanine unit. Although some recent studies have cast doubt on the involvement of α,β-dehydro intermediates,[14] we decided to carry out a

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Table II. Incorporations of Labeled Alanines into A2315A

precursor	$^3H/^{14}C$ ratio of precursor[a]	$^3H/^{14}C$ ratio of A2315A	$^3H/^{14}C$ ratio of isolated *N*-benzoylalanine
DL-[3-^{3}H]alanine and L-[1-^{14}C]alanine	5.5	ND[b]	5.5
DL-[3-^{3}H]alanine and D-[1-^{14}C]alanine	5.9	5.5	6.0
DL-[3-^{3}H]alanine and DL-[1-^{14}C]alanine	6.3	6.4	6.2

[a] Measured as a mixture of *N*-benzoyl derivatives. [b] Not determined.

definitive study to determine whether α,β-dehydroalanine was involved in the formation of the D-alanine unit of A2315A.

If α,β-dehydroalanine (presumably as its acyl derivative **4**) is involved in the biosynthesis of D-alanine, then D-alanine could in principle arise either from an acylserine intermediate via dehydration to **4** and hydrogenation (Scheme I, pathway B) or from an acylalanine intermediate via dehydrogenation to the imine **3**, tautomerization to the enamine **4**, and finally hydrogenation to an acylated D-alanine derivative. The dehydroalanine units of berninamycin have been shown to arise from L-serine,[11] and hence we first investigated the possibility that serine could be the precursor of the D-alanine unit of A2315A.

Growth of A2315A in a medium enriched in DL-[U-$^{13}C_3$]serine yielded an antibiotic in which carbons 9, 10, 20, 21, 22, and 30 contained ^{13}C label derived from the serine (Table I). The labeling of carbons 20, 21, and 22 is consistent with formation of the oxazole ring from serine,[1] while the labeling of carbons 9 and 10 is explained by the conversion of L-serine to glycine by the action of N^5,N^{10}-methylenetetrahydrofolate and serine hydroxymethyltransferase, followed by incorporation of glycine into the N-8, C-9, and C-10 unit.[1] The labeling of C-30 is consistent with the formation of the C-30 methyl group from methionine, derived from L-serine via N^5-methyltetrahydrofolate.[21]

In spite of this extensive labeling of A2315A by serine, there was *no detectable label* in the D-alanine portion of the molecule represented by carbons 1, 24, and 25, even when incorporation was analyzed for by the sensitive method of analysis of satellite peaks due to ^{13}C–^{13}C coupling of resonances derived from carbons incorporated from an intact serine unit. This clear absence of label in the D-alanine unit conclusively indicates that D-alanine does not arise from an α,β-dehydroalanine unit derived by dehydration of serine.

Although the preceding experiment indicated that the D-alanine residue in A2315A does not derive from DL-serine, it was still possible to consider a pathway involving an acyldehydroamino acid intermediate such as **4** (Scheme I) derived from L-alanine. A preliminary experiment with DL-[1-^{14}C]alanine indicated that this amino acid was efficiently incorporated into the antibiotic, and hence a double labeling experiment was carried out with L-[3-^{13}C,3,3,3-$^2H_3$]alanine. If an intermediate such as **4** were involved in the formation of D-alanine, then the resulting D-alanine unit formed from the labeled precursor would have $^{13}C^2H_2{}^1H$ as the composition of its methyl group, while D-alanine formed by simple loss of an α-hydrogen without concomitant loss of a β-hydrogen would have an intact $^{13}C^2H_3$ methyl group. The isolated antibiotic gave a ^{13}C NMR spectrum (obtained with simultaneous ^{1}H and ^{2}H decoupling) that showed two resonances for the C-25 carbon (Figure 1). The first resonance, at 19.04 ppm, was due to natural abundance ^{13}C. The second signal, at 18.25 ppm, was approximately twice as intense as that at 19.04 ppm, and was assigned to the methyl carbon of the D-alanine unit with three deuterium atoms directly bonded, since each deuterium atom is known to cause an upfield shift of 0.25–0.30 ppm in the ^{13}C NMR spectrum of its attached carbon.[22-27] There were no detectable signals for a C-25 carbon bearing one or two deuterium atoms, although the spectral region of interest is complicated by an overlapping signal due to C-28 and small peaks corresponding to mono- or dideuterated species would have been undetected. When the ^{13}C NMR spectrum was run with only ^{1}H decoupling, the signal at 18.25 ppm broadened into a complex multiplet due to ^{2}H coupling and the signal due to C28(29) remained. This result clearly eliminates any consideration of α,β-dehydroalanine intermediates of type **4** as the major precursors of D-alanine in A2315A.

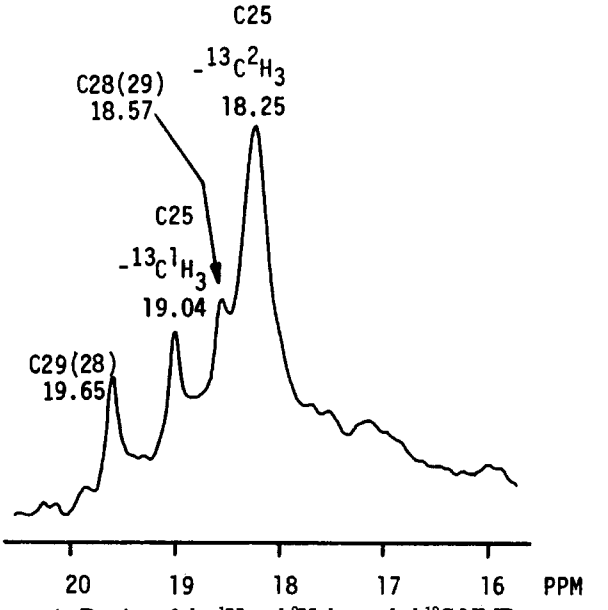

Figure 1. Portion of the ^{1}H and ^{2}H decoupled ^{13}C NMR spectrum of A2315A biosynthesized from L-[3-^{13}C,3,3,3-$^2H_3$]alanine.

In addition to the enrichment of C-25 described above, carbons 4, 6, 13, 15, and 17 were enriched in ^{13}C by approximately 1% above natural abundance, and carbon 31 was enriched by approximately 0.2%. This is consistent with their formation from the methyl carbon of acetyl coenzyme A derived from the known conversion of alanine to acetyl coenzyme A.[21]

Having shown that DL-[1-^{14}C]alanine and L-[3-^{13}C,3,3,3-$^2H_3$]alanine are both incorporated into A2315A,

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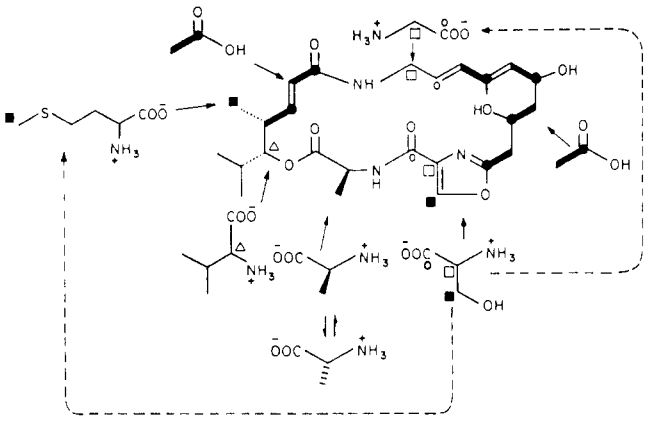
Scheme II. Major Units Involved in the Biosynthesis of A2315A



it became of interest to determine whether L-alanine alone could serve as a precursor of the D-alanine unit of the antibiotic or whether both L- and D-alanines could serve this function. To this end we carried out a rigorous analysis of precursor–product relationships by the method of Spenser and his co-workers.[28] DL-[3-^{3}H]Alanine was prepared from L-[2,3-$^3H_2$]alanine by a modification of a literature method,[30] and three separate mixtures were prepared of it with L-[1-^{14}C]alanine, D-[1-^{14}C]alanine, and DL-[1-^{14}C]alanine. The $^3H/^{14}C$ ratio for each precursor mixture was determined, and each mixture was then separately incorporated into A2315A. The resulting labeled antibiotics were isolated and subjected to hydrolysis, and the hydrolysates were derivatized with benzoyl chloride to yield *N*-benzoylalanines which were purified by HPLC. The three purified *N*-benzoylalanines were then analyzed for their radioactivity, with results which are indicated in Table II.

These results show clearly that there is *no preference for incorporation of* L-*alanine as opposed to* D-*alanine*. This result is unexpected in the light of the evidence already cited that D-amino acids are not normally incorporated into microbial peptides,[14,16] and it can be rationalized in one of two different ways. The first possibility is that the biosynthetic enzyme system accepts both L- and D-alanine and that the L-amino acid is inverted after incorporation into a suitable precursor. The second possibility is that the biosynthetic enzymes accept only D-alanine and that a separate alanine racemase is responsible for converting L-alanine to D-alanine prior to incorporation (Scheme I, pathway C). Although we have no evidence to distinguish between the possibilities, we incline toward the second one since it requires the fewer ad hoc hypotheses.

Conclusions

The results presented above establish the major biosynthetic precursors to A2315A, and these are summarized in Scheme II. As with the related antibiotic virginiamycin M_1,[1] the carbon skeleton arises from valine, seven acetate units, glycine, and serine, with serine being the precursor of the oxazole ring. The D-alanine residue is shown to arise both from L-alanine and from D-alanine with equal facility, and thus suggests the occurrence of an efficient alanine racemase system in the biosynthetic enzyme system.

Experimental Section

General Methods. The melting point of DL-*N*-benzoylalanine was determined on a Kofler block and was uncorrected. The ^{13}C NMR spectra were taken on a JEOL FX-200 spectrometer at 46 kG. All spectra were proton noise decoupled (pnd) and were obtained at ambient temperature in 10-mm tubes. The typical pulse width was 7.5 μs. The racemization of alanine was followed by ^{1}H NMR on an EM 390 90-Mz spectrometer. HPLC was performed on equipment consisting of a Waters Associates M6000A pump, a Valco six-port injection valve, and a Waters Associates Model 441 absorbance detector operating at 254 nm. The columns used were EM Hibar packed with LiChrosorb RP-8, 250 × 4.6 mm (analytical) and 250 × 10 mm (preparative). Evaporation in vacuo refers to solvent removal on a rotary evaporator at aspirator pressure and 25–45 °C.

L-[1-^{14}C]Alanine (specific activity 56.8 mCi/mmol, lot number 1377105), D-[1-^{14}C]alanine (specific activity 56.8 mCi/mmol, lot number 1377105), and DL-[1-^{14}C]alanine (specific activity 56.2 mCi/mmol, lot number 945785) were purchased from ICN Chemical and Radioisotope Division. L-[2,3-$^3H_2$]Alanine (specific activity 36 Ci/mmol, batch number 16) was purchased from Amersham Corp. L-[U-$^{13}C_3$]Serine was provided by the Los Alamos Stable Isotope Resource, sodium [1,2-$^{13}C_2$]acetate and L-[3-^{13}C,3,3,3-$^2H_3$]alanine were purchased from MSD Isotopes, and DL-[2-^{13}C]valine was prepared as described previously.[1] A strain of *A. philippinensis* and a sample of antibiotic A2315A were gifts from Dr. Robert L. Hamill of Lilly Research Laboratories.

Samples for liquid scintillation counting were evaporated in vacuo and dried in a vacuum desiccator for several hours to remove traces of any solvent which might give rise to quenching. Counting was carried out on a Beckman LS-100 liquid scintillation counter, using a xylene emulsifier cocktail.[29]

Culture Conditions. *Actinoplanes philippinensis* was first grown from a pellet on agar slants composed of the following ingredients: distilled water (1000 mL), dry precooked oatmeal (60 g), autolyzed yeast (2.5 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), $FeSO_4$ (0.01 g), and agar (25.0 g). The pH was adjusted to 7.3 with 6 N NaOH before sterilization. The slants were incubated 7 days at 30 °C following inoculation. Spores from an agar slant were then used to inoculate 35 mL of seed culture prepared in the following manner. To 1000 mL of distilled H_2O were added soybean meal (20 g) and cottonseed meal (20 g). At this point the suspension was boiled for 2 min, cooled, and filtered through Hyflo Super-Cel. To the filtrate were then added dextrose (10 g), dextrin (30 g), and $CaCO_3$ (2 g). The seed culture was grown in a baffled 250-mL Erlenmeyer flask for 4 days at 30 °C on a Lab-Line Orbit Environ shaker at 325 rpm. The production medium contained distilled water (1000 mL), dextrose (40 g), black strap molasses (5 g), Amber EHC (10 g), Amber OM Peptone (10 g), and $CaCO_3$ (4 g); 35 mL of this medium in a baffled 250-mL Erlenmeyer flask was inoculated with 2 mL of the seed culture and incubated for 5–6 days at 30 °C and 325 rpm. For ^{14}C and ^{3}H feedings either 280 mL of production medium distributed over 8 flasks or 175 mL distributed over 5 flasks was used, while for ^{13}C feedings 875 mL distributed over 25 flasks was utilized. The precursors were first filtered through a disposable Millipore filter before addition to the sterile cultures at approximately 3 days after inoculation with seed culture. For ^{13}C incorporation experiments 250 mg of ^{13}C-labeled precursor was used.

Isolation of A2315A. The cultures were filtered through Hyflo Super Cel and the filtrate was extracted one time with one-fourth volume of hexane to remove oils. The aqueous layer was then extracted 3 times with one-fourth volume ethyl acetate. The ethyl acetate layers were combined and washed 1 time with an equal volume of distilled H_2O. The ethyl acetate layer was then dried with anhydrous Na_2SO_4 and evaporated in vacuo to yield a yellow residue. This residue was dissolved in acetonitrile for purification by preparative HPLC using the preparative column previously described, a solvent system of CH_3CN:H_2O, 32:68, and a flow rate of 6.5 mL/min. The A2315A peak had a k' value of 5.5 and an elution volume (peak maximum) of 54.6 mL. The isolated antibiotics were checked by analytical HPLC using the analytical column previously described, the same solvent system, and a flow

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rate of 2.0 mL/min. The purity was routinely >99% based on HPLC. The A2315A had a k' of 5.8 and an elution volume (peak maximum) of 11.9 mL. The yields of A2315A obtained in ^{13}C runs by using 875 mL of medium were in the range 10–20 mg.

Incorporation of DL-[1-^{14}C]Alanine. A culture of *Actinoplanes philippinensis* was used to inoculate a seed culture as described earlier. Following incubation for four days at 30 °C this inoculum was used to inoculate 280 mL of fermentation medium distributed over eight 250-mL baffled flasks. These flasks were incubated at 30 °C for 87 h and each flask was then given 1 μCi of DL-[1-^{14}C]alanine. The cultures were harvested after another 27–48 h of incubation after which the antibiotic was isolated and purified as described above. The A2315A had a specific activity of 2.51×10^3 μCi/mol. A portion of the antibiotic was subjected to hydrolysis in 6 N HCl (1.5 mL) for 17 h at 100 °C. The hydrolysate was made 1 N in NaOH by addition of solid NaOH (0.42 g). To the basic solution were added 1.5 mL of diethyl ether and 1.2 equiv of benzoyl chloride (~6 μL). The heterogeneous solution was stirred at room temperature for 24 h in a capped vial. It was later found that less basic conditions (~pH 10) and shorter reaction time (2 h) increased significantly the yield of *N*-benzoylalanine. The aqueous layer was separated and made acidic to pH paper with a few drops of 6 N HCl. The acidic solution was then extracted 2 times with 1.5 mL of ethyl acetate. The ethyl acetate layers were combined, dried with anhydrous $MgSO_4$, and evaporated to dryness.

The resulting pale yellow solid was dissolved in 0.5 mL of CH_3CN:H_2O:formic acid, 20:79:1, for HPLC purification using a LiChrosorb RP-8 analytical column and the same solvent system as above. The peak corresponding to *N*-benzoylalanine was collected and quantified by comparison with a standard solution of *N*-benzoylalanine. The specific activity of the *N*-benzoylalanine was 2.03×10^3 μCi/mol. The peak corresponding to *N*-benzoylalanine had a K' value of 2.2 and an elution volume (peak maximum) of 9.0 mL.

DL-[3-^{3}H]Alanine. A sample of L-alanine (60 mg) in [*hydroxy*]-^{2}H]acetic acid (3.0 mL) was heated at 122 °C and the progress of racemization was followed by the disappearance of the ^{1}H NMR signal at 4.1 ppm due to the α-proton;[30] racemization under these conditions was complete after 24 h. When the reaction was monitored in acetic acid, however, a new signal was observed at 4.5 ppm, and after 24 h it was the only one observed in this region of the spectrum. This new signal is assigned to the α-protons of the diketopiperazine formed by dimerization of two alanine molecules. The racemized diketopiperazine was hydrolyzed by treatment with acetic acid:6 N HCl, 2:1, for 1 h under reflux; the ^{1}H NMR spectrum of the resulting DL-alanine was identical with that of a standard sample measured under the same conditions.

These racemization conditions were then applied to L-[2,3-$^{3}H_2$]alanine. An aqueous solution of the radioactive amino acid (1 mCi) was evaporated in vacuo and the residual solid dissolved in acetic acid (1 mL). The resulting solution was heated at 122 °C for 24 h, treated with 0.5 mL acetic acid:6 N HCl, 2:1, heated under reflux for 75 min, and evaporated in vacuo.

Incorporation of [^{14}C,^{3}H]Alanine Mixtures. DL-[3-^{3}H]-Alanine (ca. 300 μCi) was mixed with either L-, D-, or DL-[1-^{14}C]alanine (ca. 25 μCi), and the three resulting mixtures were treated in two separate ways. A portion of each mixture (10%) was converted to its *N*-benzoyl derivative as described earlier, and *N*-benzoyl-DL-alanine (1.2 g) was added. The resulting mixture of *N*-benzoylalanines was then recrystallized to constant specific activity (Table II). The remaining portion of each mixture (90%) was added to growing cultures of *A. philippinensis* as described earlier, and the resulting antibiotic was isolated and hydrolyzed. The hydrolysate was treated with benzoyl chloride to form *N*-benzoylalanine, which was isolated by HPLC using an analytical column[31] as previously described. The radioactivities of the *N*-benzoylalanines from each experiment, together with those of the isolated A2315A in two cases, are given in Table II.

***N*-Benzoyl-DL-alanine.** DL-Alanine (5 g) in 0.1 N NaOH (100 mL) was treated with benzoyl chloride (7.8 mL, 1.2 equiv) in diethyl ether (100 mL). The heterogeneous mixture was stirred at 25 °C for 21 h. The aqueous layer was then separated and acidified with 6 N HCl to yield a white precipitate. The acidic suspension was extracted thrice with ethyl acetate, and the organic layers were combined, washed, dried ($MgSO_4$), and evaporated in vacuo to yield a crude product which was recrystallized from ethyl acetate to yield 5.3 g (49%) *N*-benzoyl-DL-alanine, mp 162–164 °C.

Acknowledgment. We thank Dr. R. L. Hamill for gifts of a producing strain of *A. philippinensis* and of a sample of antibiotic A2315A and Professor H. C. Dorn and T. Glass for assistance with ^{13}C NMR spectra. The L-[U-$^{13}C_3$]serine was provided by the Los Alamos Stable Isotopes Resource under grant P41RR00962. Financial support from Lilly Research Laboratories and the National Science Foundation (PCM8111508) is gratefully acknowledged.

Registry No. 1, 58717-24-1; acetate, 64-19-7; valine, 72-18-4; D-alanine, 338-69-2.

(31) The *N*-benzoylalanine collected in each case was rigorously purified (2 times) by analytical HPLC to ensure purity, and it was thus not necessary to recrystallize to constant specific activity. The A2315A used for the determination of $^3H/^{14}C$ ratios was also chromatographically homogeneous and there was not sufficient pure antibiotic on hand to attempt recrystallization. All earlier attempts in our laboratory to crystallize the closely related antibiotic virginiamycin M_1 had failed.