Biosynthesis of the Validamycins: Identification of Intermediates in the Biosynthesis of Validamycin A by *Streptomyces hygroscopicus* var. *limoneus*

Haijun Dong,[†] Taifo Mahmud, Ingo Tornus,[‡] Sungsook Lee,[§] and Heinz G. Floss*

Contribution from the Department of Chemistry, Box 351700, University of Washington, Seattle, Washington 98195-1700

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Abstract: To study the biosynthesis of the pseudotrisaccharide antibiotic, validamycin A (**1**), a number of potential precursors of the antibiotic were synthesized in ^{2}H-, ^{3}H-, or ^{13}C-labeled form and fed to cultures of *Streptomyces hygroscopicus* var. *limoneus*. The resulting validamycin A from each of these feeding experiments was isolated, purified and analyzed by liquid scintillation counting, ^{2}H- or ^{13}C NMR or selective ion monitoring mass spectrometry (SIM-MS) techniques. The results demonstrate that 2-*epi*-5-*epi*-valiolone (**9**) is specifically incorporated into **1** and labels both cyclitol moieties. This suggests that **9** is the initial cyclization product generated from an open-chain C_7 precursor, D-sedoheptulose 7-phosphate (**5**), by a DHQ synthase-like cyclization mechanism. A more proximate precursor of **1** is valienone (**11**), which is also incorporated into both cyclitol moieties. The conversion of **9** into **11** involves first epimerization to 5-*epi*-valiolone (**10**), which is efficiently incorporated into **1**, followed by dehydration, although a low level of incorporation of 2-*epi*-valienone (**15**) is also observed. Reduction of **11** affords validone (**12**), which is also incorporated specifically into **1**, but labels only the reduced cyclitol moiety. The mode of introduction of the nitrogen atom linking the two pseudosaccharide moieties is not clear yet. 7-Tritiated valiolamine (**8**), valienamine (**2**), and validamine (**3**) were all not incorporated into **1**, although each of these amines has been isolated from the fermentation, with **3** being most prevalent. Demonstration of in vivo formation of [7-^{3}H]validamine ([7-^{3}H]-**3**) from [7-^{3}H]-**12** suggests that **3** may be a pathway intermediate and that the nonincorporation of [7-^{3}H]-**3** into **1** is due to a lack of cellular uptake. We thus propose that **3**, formed by amination of **12**, and **11** condense to form a Schiff base, which is reduced to the pseudodisaccharide unit, validoxylamine A (**13**). Transfer of a D-glucose unit to the 4′-position of **13** then completes the biosynthesis of **1**. Other possibilities for the mechanism of formation of the nitrogen bridge between the two pseudosaccharide units are also discussed.

The validamycins are a family of closely related pseudotrisaccharides isolated from *Streptomyces hygroscopicus* var. *limoneus*.[1] Validamycin A (**1**) (Figure 1), the major and most active component of the complex, is widely used in the Far East for the treatment of sheath blight disease of rice plants.[2] The structure of validamycin A contains two pseudosaccharide moieties, valienamine (**2**) and its dihydroderivative, validamine (**3**), and an additional glucose unit. The valienamine moiety is also found in acarbose (**4**), which is produced by *Actinoplanes* species[3] and exhibits potent inhibition of α-amylase; this has led to its clinical use for the treatment of Diabetes mellitus. Other secondary metabolites that contain the valienamine or validamine moiety as a structural component include the adiposins,[4] amylostatins,[5] salbostatin,[6] and trestatin.[7] In principle, valienamine and validamine could both be considered aliphatic analogues of the "*m*-C_7N" unit,[8] a biosynthetically unique structural element consisting of a six-membered carbocycle, usually aromatic or quinoid, carrying an extra carbon and a nitrogen in a meta arrangement. Such *m*-C_7N units are characteristic structural components of various antibiotics, for example, the rifamycins,[9,10] the mitomycins,[11] the ansamitocins,[12] geldanamy-

* Corresponding author: Telephone: (206)543-3791. Fax: (206)543-8318. E-mail: floss@chem.washington.edu..

[†] Present address: Drug Metabolism & Pharmacokinetics, Boehringer Ingelheim Pharmaceuticals, Inc., P.O. Box 368, Ridgefield, CT 06877-0368.

[§] Present address: Chong Kun Dang Research Institute, P.O. Box 74, Chonan, 330−600 Korea.

[‡] Present address: Schering AG, Berlin, Germany.

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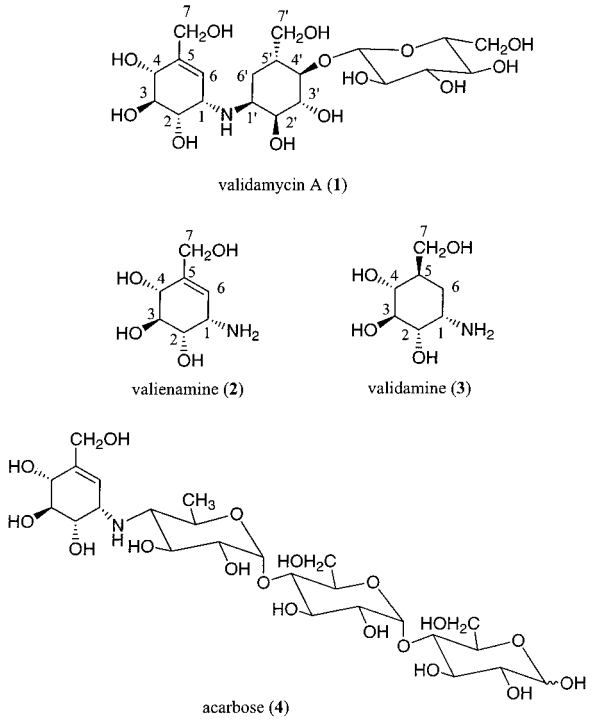
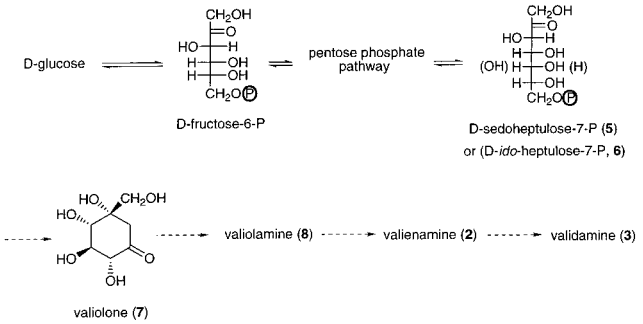


Figure 1. Structures of validamycin A, acarbose, and their component aminocyclitols.

cin,[13,14] pactamycin,[15] and actamycin.[16] Feeding and genetic experiments have indicated that the *m*-C_7N unit in most of those antibiotics originates from a branch[17] of the shikimate pathway.[18] However, previous studies by Rinehart and co-workers[19] on **1** and by our laboratory[20] on **4** using bond-labeling with [U-$^{13}C_6$]-glucose or [U-$^{13}C_3$]glycerol as well as position-labeled carbohydrates as precursors demonstrated labeling- and coupling patterns which argued for formation of both cyclitol moieties from a seven-carbon sugar which is derived by successive transfer of two glucose-derived two-carbon units onto a three-carbon unit derived intact from glucose or glycerol. Cyclization of this seven-carbon sugar between C-7 and C-2 forms the C-5/C-6 bond in the valienamine and validamine moieties. It was further proposed[19,21] that either D-sedoheptulose 7-phosphate (**5**) or D-*ido*-heptulose 7-phosphate (**6**), both derived from the pentose phosphate pathway,[22] is the seven-carbon precursor sugar (Scheme 1) and that valiolone (**7**) is probably the initial cyclization product, which then undergoes sequential amination, dehydration, and reduction reactions to give valiolamine (**8**), valienamine (**2**), and validamine (**3**).

Scheme 1. Biosynthetic Pathway to the Aliphatic *m*-C_7N Units in Validamycin A Originally Proposed by Rinehart and Coworkers[19]



In the present paper, we describe further studies on the biosynthesis of **1** in which a series of potential precursors in stable isotope (^2H and ^{13}C) or radioactively (^3H) labeled form were evaluated for their roles in the biosynthetic pathway. We have unambiguously identified four specific precursors of validamycin A, 2-*epi*-5-*epi*-valiolone (**9**), 5-*epi*-valiolone (**10**), valienone (**11**), and validone (**12**) and obtained preliminary evidence pertaining to the mechanism of formation of the pseudodisaccharide unit of **1**, validoxylamine A (**13**).

Results

Production and Isolation of Validamycin A. Two fermentation conditions for the production of validamycin A (**1**) have been reported, the original one by Iwasa and co-workers[2] and another one more recently by Rinehart and co-workers[23] (Table 1). In our hands, no **1** was produced using condition 2, whereas 18−30 mg of pure validamycin A per 100 mL of production medium were obtained from fermentations under condition 1. The highest yield, 46 mg per 100 mL of production medium, was achieved with a combination of the seed medium from condition 2 and the production medium from condition 1, and these were the conditions used for all of the subsequent feeding experiments. Although an ion-exchange procedure for the isolation of **1**, **2**, and **3** from the fermentation had been reported by Iwasa and co-workers,[1d] this used relatively expensive buffers and included some steps that seem to be unnecessary. A simpler, more economical isolation procedure was therefore developed (Scheme 2) in which H_2O and 0.5 N NH_4OH were the only solvents used. This modified procedure took advantage of the different basicities of the compounds in each of the two pairs, validamycins A and B, and valienamine and validamine, and between the two pairs, that is, the primary and secondary amines. The identity and purity of the validamycin A sample produced and isolated by the above procedures were established by ^1H- and ^{13}C NMR[23] and ES-MS. Standard **1** (40 mg) was used to test the efficiency of the isolation procedure. The standard sample was recovered essentially quantitatively.

Synthesis of Labeled Precursors. The labeled compounds used in this study are shown in Figure 2. The preparation of [7-^3H]valiolamine ([7-^3H]-**8**), [7-^3H]valiolone ([7-^3H]-**7**), [7-^3H]-valienamine ([7-^3H]-**2**), and [7-^3H]valienone ([7-^3H]-**11**) has been reported in an earlier paper.[24] [1-^{13}C]Valiolone ([1-^{13}C]-**7**) and [1-^{13}C]valienone ([1-^{13}C]-**11**) were prepared from D-[1-^{13}C]glucose by the general method of Fukase and Horii[25] as previously described.[26] Similarly, 2-*epi*-[6-$^2H_2$]valiolone ([6-

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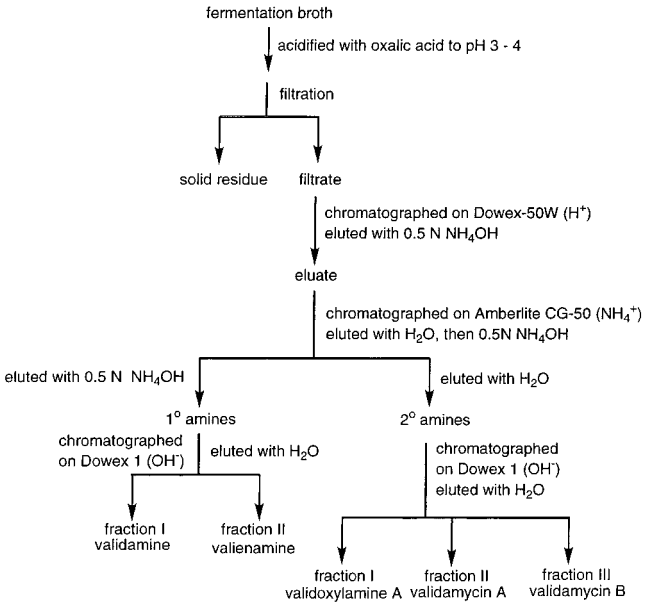
Table 1. Two Fermentation Conditions for the Production of Validamycin A

	composition		pH	
	seed medium	production medium	seed	production
condition 1, reported by Iwasa et al.[2]	3% glucose 2.2% soybean flour 0.3% peptone 0.4% $CaCO_3$	2% glucose 4% corn starch 2% corn steep liquor 4% corn gluten meal 0.5% NH_4Cl 1.5% NaCl 1.5% $CaCO_3$	7	7
condition 2, reported by Rinehart and coworkers[23]	0.5% tryptone 0.3% yeast extract 0.3% glucose	1% glucose 5% soluble starch 1.5% peptone 3% corn gluten meal 0.5% NaCl 1% $CaCO_3$	7	9.3−9.5

Scheme 2. Modified Procedure for the Isolation of Validamycins A and B, Valienamine, and Validamine from the Fermentation of *Streptomyces hygroscopicus* var. *limoneus*



$^2H_2$]-**14**), 2-*epi*-5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**9**), and 2-*epi*-[6-^{2}H]valienone ([6-^{2}H]-**15**) were synthesized as described previously[26] from commercially available 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose by an adaptation of the Fukase and Horii[25] approach. Deuterium was introduced late in the synthesis by a reductive dehalogenation with tributyltin deuteride; the configurations of **9** and **14** were deduced by nOe measurements.[26] The preparation of 5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**10**) from D-glucose involved another modification of the Fukase and Horii[25] approach and will be reported in an upcoming publication (Mahmud, T.; Xu, J., and Choi, Y. U., manuscript in preparation).

[7-^{3}H]Validamine ([7-^{3}H]-**3**) and its epimer, 5-*epi*-[7-^{3}H]-**3**, were prepared by catalytic hydrogenation of [7-^{3}H]-**2** in a ratio of 2 to 1 and an overall yield of 90% (Scheme 3). Both compounds were identified by comparison of their NMR and $[\alpha]_D$ data with those reported previously.[27] The two products were separated by preparative TLC with ^{n}PrOH/AcOH/H_2O, 4:1:1, as solvent. Oxidation of [7-^{3}H]-**3** with 3,5-di-*tert*-butyl-*O*-benzoquinone afforded [7-^{3}H]validone ([7-^{3}H]-**12**) in 61% yield.

The preparation of [1-^{13}C]validone ([1-^{13}C]-**12**) involved hydrogenation of the double bond and hydrogenolysis of the benzyl groups of 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valienone (**16**), an intermediate in the synthesis of [1-^{13}C]-**11**[26] (Scheme 4). Reaction in a single step with 10% Pd−C as catalyst gave [1-^{13}C]-**12** and 5-*epi*-[1-^{13}C]-**12** in a ratio of 4 to 1, which were separated by preparative TLC.

Feeding Experiments with Valiolone, Valienone, and Validone. The structure of validamycin A (**1**) suggests that valienone (**11**) and validone (**12**) might be precursors of the valienamine and validamine moieties of **1**, respectively. In addition, valiolone **7** seemed a plausible precursor of **11** and

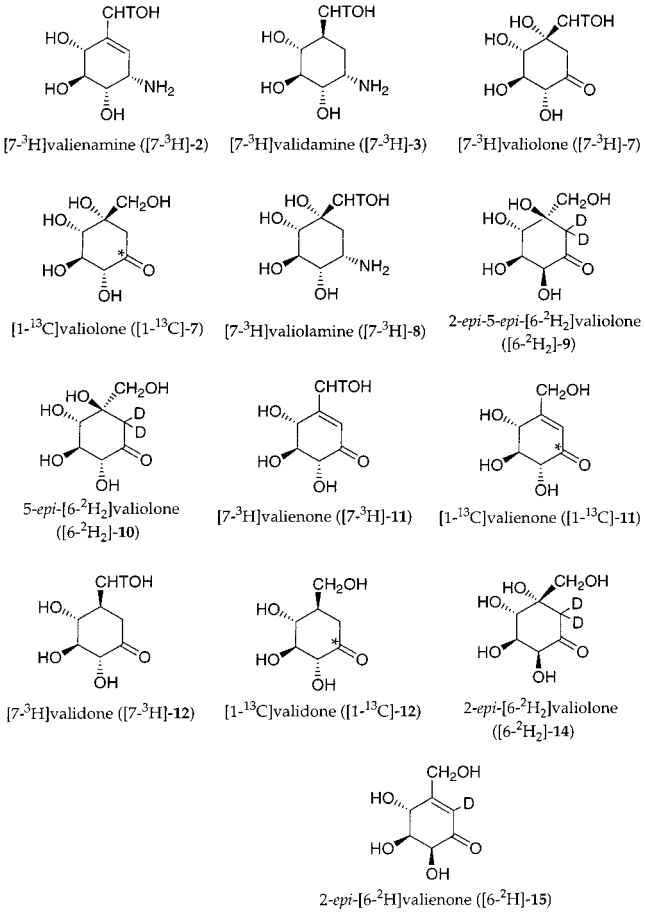


Figure 2. Structures of the ^{2}H-, ^{3}H-, and ^{13}C-labeled compounds evaluated as precursors of validamycin A.

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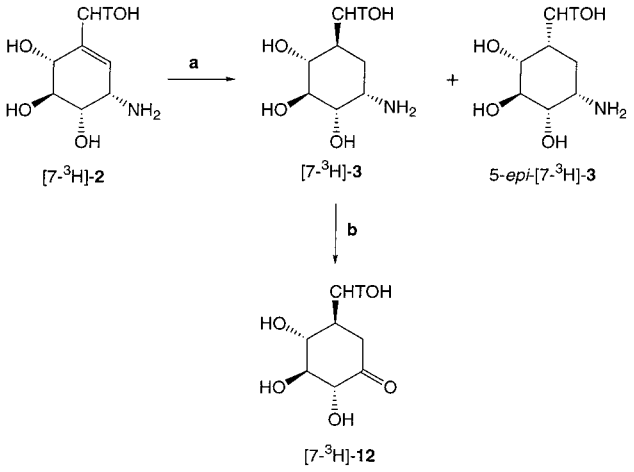
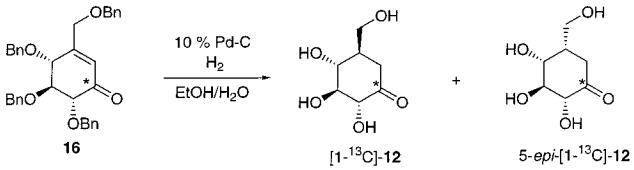
Scheme 3. Synthesis of [7-^{3}H]validamine and Its 5-epimer, and of [7-^{3}H]validone[a]



[a] **a.** H_2/Raney-Ni; **b.** 1. 3,5-di-*tert*-butyl-*O*-benzoquinone, 2. H^+

Scheme 4. Synthesis of [1-^{13}C]Validone



12 and was proposed by Rinehart and co-workers[19,21] to be the initial product of the cyclization of an open-chain precursor, a seven-carbon sugar. These notions were initially tested by feeding experiments with tritiated precursors, [7-^{3}H]-**7**, [7-^{3}H]-**11**, and [7-^{3}H]-**12** (Table 2). Radioactivity analysis of the resulting **1** indicated 30% incorporation of tritium from [7-^{3}H]-**11** and 15% from [7-^{3}H]-**12**; the radiochemical purity of the product was in each case confirmed by autoradiography. Analysis of the **1** produced in the feeding experiment with [7-^{3}H]-**7** indicated 2% incorporation of tritium. It was suspected that this low level of incorporation might reflect nonspecific labeling or might be due to the presence of traces of [7-^{3}H]-**11** in the sample of [7-^{3}H]-**7**, formed under the acidic conditions in the last step of the synthesis.[24] The feeding experiment was therefore repeated with [1-^{13}C]-**7** which had been synthesized by a different route[25,26] that did not produce the corresponding ^{13}C-labeled **11**. The ^{13}C NMR spectrum of the resulting antibiotic showed no enhanced signals, indicating that **7** was not incorporated into **1**.

The efficient incorporation of tritiated **11** and **12** strongly suggests that these two compounds are specific precursors of **1**. However, although suggestive, the data do not reveal whether these precursors label both or only one of the cyclitol moieties and if the latter, which one. To provide this information and to confirm the specificity of the incorporation, the experiments were repeated with ^{13}C-labeled samples. Thus, 15 mg of [1-^{13}C]-**11** and [1-^{13}C]-**12** were each fed to a culture of the validamycin A-producing organism. The cultures were harvested on the seventh day of the fermentation, and the antibiotic was isolated, purified, and analyzed by selective ion monitoring mass spectrometry (SIM-MS) and ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of **1** from the feeding experiment with [1-^{13}C]-**11** revealed two highly enhanced signals (54.2 ppm, C-1, and 55.8 ppm, C-1′) of approximately equal intensity and indicated 46% specific ^{13}C-incorporation into the two carbons combined (Figure 3). Thus, **11** was incorporated specifically into both cyclitol moieties and with about equal efficiency. In contrast, **1** from the experiment with [1-^{13}C]-**12** showed 20% specific ^{13}C-incorporation, and its ^{13}C NMR spectrum displayed only one enhanced signal at δ 55.8 ppm, corresponding to C-1′. Thus, **12** is incorporated exclusively into the saturated cyclitol moiety of **1**. These results establish **11** and **12** as proximate precursors of the cyclitol moieties of **1**, and they show that the formations of the saturated and the unsaturated cyclitol moieties share a common pathway at least up to the stage of valienone **11**.

Identification of 2-*epi*-5-*epi*-Valiolone as the First Precursor Cyclitol. The negative outcome of the feeding experiments with labeled valiolone (**7**) demonstrated that **7** is not the initial precursor cyclitol arising from the cyclization of an open-chain sugar. This seven-carbon precursor sugar is presumed[19,20] on biochemical and stereochemical grounds to be either sedoheptulose 7-phosphate (**5**) or idoheptulose 7-phosphate (**6**). The configuration of **6** conforms to that of valienone **11** and of the valienamine moiety of **1** at all shared stereocenters, whereas **5** is epimeric at C-5, the carbon which gives rise to C-2 of the cyclitol moiety. Since **5**, an intermediate of the pentose phosphate shunt of carbohydrate metabolism,[22] is the biochemically more plausible substrate for the initial cyclization reaction, a C-2 epimer of **7** was a reasonable alternative candidate to **7** to be the product of this reaction. The stereochemistry at C-5 of the newly formed cyclitol is established de novo in the cyclization reaction, and hence, the product could have either the same or the opposite configuration as **7** at this center. We therefore prepared both 2-*epi*-valiolone **14** and 2-*epi*-5-*epi*-valiolone **9** in deuterated form and evaluated their incorporation into **1**. By SIM-MS it was found that [6-$^2H_2$]-**9** was incorporated into **1** to the extent of 12.2%, whereas its 5-epimer, [6-$^2H_2$]-**14**, showed no incorporation. The ^{2}H NMR spectrum of **1** from the feeding experiment with [6-$^2H_2$]-**9** (Figure 4B) indicated that both cyclitol units were labeled, with the unsaturated unit (δ 6.02 ppm, H-6) enriched about 1.5 times as much as the saturated one (δ 1.95 ppm, H-6′eq and 1.36 ppm, H-6′ax). The difference in enrichment may reflect the fact that the metabolic pathway from [6-$^2H_2$]-**9** to the saturated cyclitol unit in the final product is longer than that to the unsaturated one. The sample of **1** isolated from the feeding experiment with [6-$^2H_2$]-**14** gave no signal in the ^{2}H NMR spectrum. These results establish 2-*epi*-5-*epi*-valiolone (**9**) as the initial cyclization product on the biosynthetic pathway to **1**.

Pathway from 2-*epi*-5-*epi*-Valiolone (9) to Valienone (11). The conversion of the initial cyclization product **9** into **11**, the precursor of the two cyclitol moieties of **1**, requires epimerization at C-2 and dehydration between C-5 and C-6. To try to discern the order of these two reactions we evaluated the incorporation of 2-*epi*-valienone (**15**), the compound that would result from the dehydration of **9**, and of 5-*epi*-valiolone (**10**), the intermediate that would be formed if epimerization at C-2 were the first step. The validamycin A isolated from the feeding experiment with [6-$^2H_2$]-**10** showed 19.2% deuterium enrichment by SIM-ES-MS analysis, and the ^{2}H NMR spectrum (Figure 4C) was very similar to that of **1** labeled by feeding [6-^{2}H]-**9**. Again, the unsaturated cyclitol moiety contained about 1.5 times as much deuterium as the saturated one. Careful comparison of the integrations of the mass spectral peaks for $(M + H)^+$, $(M + 1 + H)^+$, and $(M + 2 + H)^+$ of the labeled **1** from the experiment with [6-^{2}H]-**15** and an unlabeled reference sample indicated a 2.0% incorporation of [6-^{2}H]-**15** into **1**. The ^{2}H NMR spectrum (data not shown) displayed the same signal pattern as seen in **1** from the feeding experiments with [6-$^2H_2$]-**9** and [6-$^2H_2$]-**10**, albeit in much lower overall intensity. Thus, **15** clearly is incorporated specifically into **1** and labels both

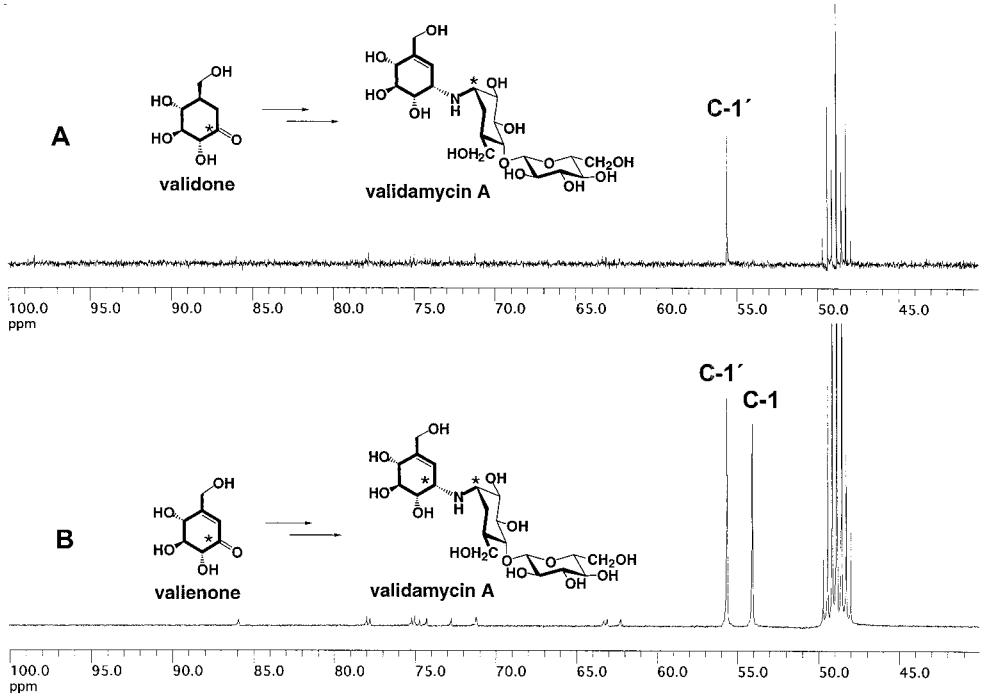


Figure 3. Partial NMR spectra of validamycin A: (A) 75 MHz ^{13}C NMR spectrum of validamycin A isolated from the feeding experiment with [1-^{13}C]validone ([1-^{13}C]-**12**) in CD_3OD, (B) 75 MHz ^{13}C NMR spectrum of validamycin A isolated from the feeding experiment with [1-^{13}C]-valienone ([1-^{13}C]-**11**) in CD_3OD.

Table 2. Results of Feeding Experiments with [7-^{3}H]Valiolone, [7-^{3}H]Valienone, [7-^{3}H]Validone, [7-^{3}H]Valienamine, [7-^{3}H]Validamine, 5-*epi*-[7-^{3}H]Validamine, and [7-^{3}H]Valiolamine

	[7-^{3}H]-**7**	[7-^{3}H]-**11**	[7-^{3}H]-**12**	[7-^{3}H]-**2**	[7-^{3}H]-**3**	5-*epi*-[7-^{3}H]-**3**	[7-^{3}H]-**8**
amount fed	23 μCi	29 μCi	30 μCi	28 μCi	30 μCi	27 μCi	20 μCi
specific radioactivity	2.0 mCi/mmol	2.0 mCi/mmol	2.0 mCi/mmol	2.5 mCi/mmol	2.0 mCi/mmol	2.0 mCi/mmol	2.5 mCi/mmol
incorporation into validamycin A	2%	30%	15%	0%	0%	0%	0%
autoradiography	+	+	+	−	−	−	−

the unsaturated and the saturated cyclitol moieties. However, the much higher incorporation of **10** compared to **15** (19.2 vs 2%) strongly suggests that 2-epimerization precedes dehydration in the conversion of **9** into **11**. Most likely **15** is not a free intermediate in the biosynthesis of **1**, rather, its low incorporation may reflect either a low level of nonenzymatic 2-epimerization of **15** under the fermentation conditions or some degree of nonspecificity of the 2-epimerase converting **9** into **10**.[28] The pathway from 2-*epi*-5-*epi*-valiolone to the valienamine and validamine moieties of **1** can thus be described as shown in Scheme 5.

Stereochemistry of Double Bond Reduction in Valienone 11. The successful incorporation experiments with [6-$^2H_2$]-**9** and [6-$^2H_2$]-**10** allow the deduction of the steric course of the enzymatic double bond reduction of **11** to give **12** during the biosynthesis of **1**. The enzymatic dehydration of [6-$^2H_2$]-**10** will produce within the cells [6-$^2H_1$]-**11**, which is then subject to the double bond reduction and further incorporation of the resulting [6-$^2H_1$]-**12** into the validamine moiety of **1**. The validamine moiety of **1** is known to have 5*R* configuration;[29] hence, reduction of [6-$^2H_1$]-**11** by *syn* addition of hydrogen will produce a validamine moiety labeled in the *pro*-6*R* position (H-6′ax deuterated), whereas *anti* addition of hydrogen will result in labeling of the *pro*-6*S* position (H-6′eq deuterated). The ^2H NMR spectra of **1** from the feeding experiments with [6-$^2H_2$]-**9** and [6-$^2H_2$]-**10** were analyzed at slightly elevated temperature to achieve baseline resolution of the signals for H-6′eq (1.95 ppm) and H-6′ax (1.36 ppm), and the assignments of these signals in the ^1H NMR spectra[23] were confirmed by nOe measurements. The analyses indicated that both samples contained the majority of the deuterium at C-6′ (63 and 77%, respectively) in the equatorial position. Thus, in the majority of molecules the newly introduced hydrogen occupies the *pro*-6′*R* position, resulting from an *anti* addition of hydrogen in the enzymatic double bond reduction step (Scheme 6). Since it is unlikely that the enzymatic reduction of the same double bond occurs with two different stereochemistries, the lesser but significant labeling of the other methylene position is probably not the result of an enzymatic *syn* addition but rather reflects nonenzymatic partial epimerization at C-6 of the intermediate **12** due to enolization of the keto group at C-1.

Introduction of the Nitrogen. While the experiments reported above clearly define the sequence of reactions by which the ketocyclitol precursors of the valienamine and validamine moieties of **1** are generated, they shed no light on the mode of introduction of the bridging nitrogen atom. A plausible scenario would involve introduction of the nitrogen into one ketocyclitol first to give the corresponding aminocyclitol, which is then reductively coupled to the second ketocyclitol to produce validoxylamine A (**13**). To try to identify the point in the

(28) An alternative explanation, that the low incorporation of [6-$^2H_2$]-**15** may be due to some contamination of the sample with [6-$^2H_2$]-**9** resulting from its preparation, is rendered unlikely by the observation that [6-$^2H_2$]-**15** prepared in the same way showed no incorporation into acarbose, which is also labeled by [6-$^2H_2$]-**9** (Mahmud, T., unpublished results).

(29) Kamiya, K.; Wada, Y.; Horii, S.; Nishikawa, M. *J. Antibiot.* **1971**, *24*, 317.

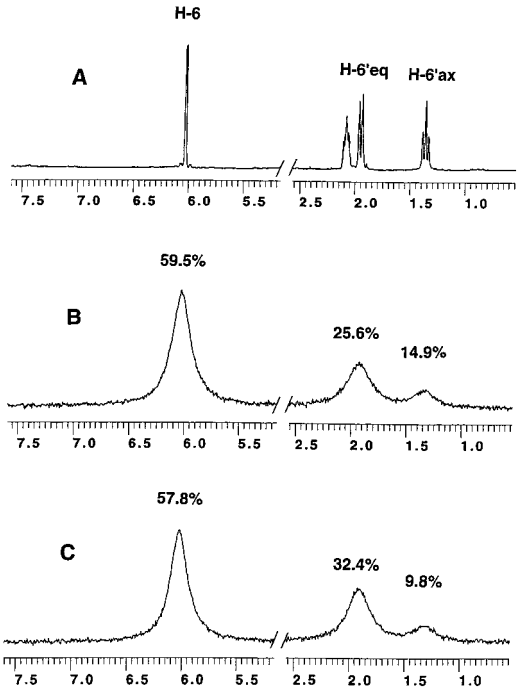
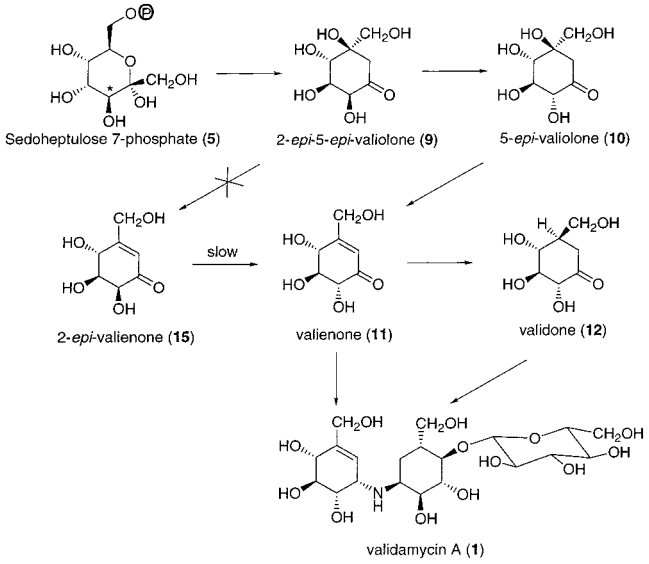


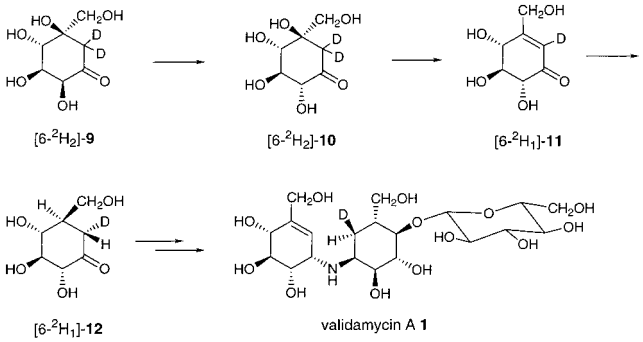
Figure 4. Partial NMR spectra of validamycin A: (A) 500 MHz ^{1}H NMR spectrum of unlabeled **1** in D_2O, (B) 76.78 MHz ^{2}H NMR spectrum of **1** isolated from the feeding experiment with 2-*epi*-5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**9**) in H_2O at 308 K, (C) 76.78 MHz ^{2}H NMR spectrum of **1** isolated from the feeding experiment with 5-*epi*-[6-$^2H_2$]-valiolone ([6-$^2H_2$]-**10**) in H_2O at 313 K.

Scheme 5. Pathway from the Acyclic Precursor Sedoheptulose 7-Phosphate to Valienone and Validone, the Ketocyclitol Precursors of Validamycin A

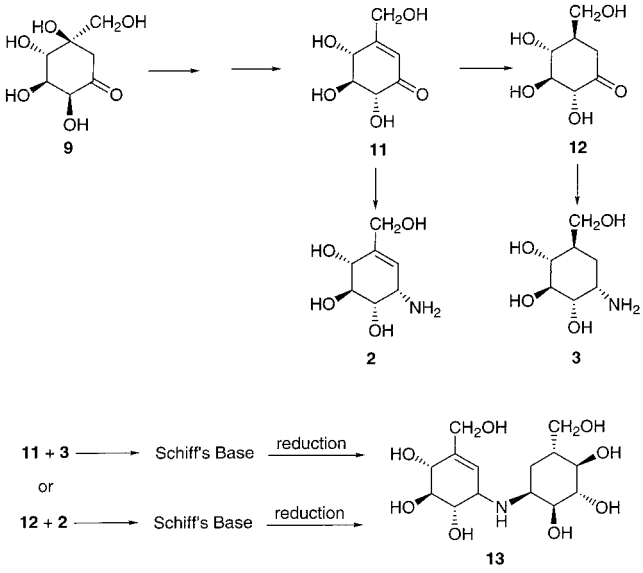


biosynthetic pathway to **1** at which a nitrogen is first introduced into a cyclitol, a series of tritiated aminocyclitols, corresponding to some of the ketocyclitols tested, were evaluated as biosynthetic precursors. The compounds fed were [7-^{3}H]valiolamine ([7-^{3}H]-**8**), [7-^{3}H]valienamine ([7-^{3}H]-**2**), and [7-^{3}H]validamine ([7-^{3}H]-**3**), which had been proposed by Rinehart and coworkers[19,21] as intermediates, and as a negative control the C-5 epimer of **3**, 5-*epi*-[7-^{3}H]-**3**. As shown in Table 2, none of these compounds was incorporated into **1** to any detectable degree. It was ascertained that no significant amount of radioactivity was released into the volatile part of the fermentation medium in any of these feeding experiments, thus eliminating the

Scheme 6. Stereochemistry of the Enzymatic Reduction of Valienone to Validone: Fate of a Deuterium Label at C-6 in the Formation of the Validamine Moiety of Validamycin A



Scheme 7. Two Plausible Scenarios for the Formation of Validoxylamine A from Valienone and Validone



possibility of metabolic washout of the tritium label during the experiment. While the nonincorporation of **8** is understandable, given that its keto analogue **7** is not a precursor of **1**, the negative results with **2** and **3** are rather surprising, particularly since all three compounds have been isolated from the validamycin fermentation.[30] Although these results could signal an entirely different mode of nitrogen introduction, more likely they may merely reflect impermeability of the cells to these ionizable molecules or the operation of an active transport system which excretes these molecules and thus prevents their uptake into the cells.

If the basic hypothesis for the mode of nitrogen introduction is still correct and the nonincorporation of **2** and **3** is due to cellular impermeability, two alternative scenarios can account for the formation of the pseudodisaccharide precursor of **1**, validoxylamine **13**, from **11** and **12** (Scheme 7). Either transamination of **11** would give **2**, which would then reductively couple with **12** to give **13**, or alternatively, **12** would be transaminated to give **3**, which would then couple with **11** to give **13**. Attempts were made to demonstrate either of these two reductive coupling reactions in a cell-free extract of *S. hygroscopicus* var. *limoneus*, but these were unsuccessful. However, circumstantial evidence in favor of the second of these two scenarios comes from observations in the feeding experi-

(30) Kameda, Y.; Asano, N.; Yoshikawa, M.; Takeuchi, M.; Yamaguchi, T.; Matsui, K.; Horii, S.; Fukase, H. *J. Antibiot.* **1984**, *37*, 1301.

ments with [7-^{3}H]-**11** and [7-^{3}H]-**12**, demonstrating their incorporation into **1**. When these experiments were conducted, particular attention was paid to the possible formation of the corresponding aminocyclitols in vivo. It was found that about 4% of the [7-^{3}H]-**12** fed to the organism was converted to [7-^{3}H]-**3**, whereas no conversion of [7-^{3}H]-**11** to [7-^{3}H]-**2** was detected. The in vivo formation of [7-^{3}H]-**3** from [7-^{3}H]-**12** was confirmed by regular autoradiography and by two-dimensional co-autoradiography of the radioactive sample isolated from the fermentation with an authentic reference sample of [7-^{3}H]-**3**, which showed a single radioactive spot. The formation within the cells specifically of **3** from **12**, but not of **2** from **11**, lends credence to the scenario of formation of validoxylamine A (**13**) by reductive coupling of **3** and **11** and to the conjecture that the negative outcome of the feeding experiments with the tritiated aminocyclitols may be due to lack of cellular uptake.

Discussion

Following the earlier work of the Rinehart group[19,21] which demonstrated the biosynthetic origin of the two cyclitol moieties of validamycin A (**1**) from a pentose phosphate pathway-derived seven-carbon sugar, proposed to be either sedoheptulose 7-phosphate (**5**) or idoheptulose 7-phosphate (**6**), the results presented here identify the first carbocyclic precursor of both cyclitol moieties of **1** as 2-*epi*-5-*epi*-valiolone (**9**). The facts that this cyclitol has the same configuration at all shared stereocenters as **5** and that valiolone (**7**), which has the same stereochemistry as **6**, is not a precursor imply that sedoheptulose 7-phosphate (**5**) must be the substrate for the cyclization reaction. These findings parallel recent results on the biosynthesis of acarbose (**4**) which have also identified **9** as the initial cyclitol precursor of the valienamine moiety of **4**.[26] Furthermore, a cyclase gene has been cloned from the acarbose biosynthetic gene cluster of the **4**-producing *Actinoplanes* species, and the recombinant protein expressed from this gene has been shown to catalyze the conversion of **5**, but not **6**, into **9**.[31] It seems very likely that a similar enzyme catalyzes the first committed step in the biosynthesis of **1**.

Two biochemical mechanisms are known for the cyclization of sugar phosphates to cyclitol derivatives. One is exemplified by the cyclization of D-glucose 6-phosphate to *myo*-inositol 1-phosphate catalyzed by *myo*-inositol 1-phosphate synthase[32,33] and the other by that of D-*arabino*-heptulosonic acid 7-phosphate to dehydroquinic acid (DHQ) catalyzed by DHQ synthase.[34] Applied to the cyclization of **5** the first mechanism would predict 2-*epi*-valienone (**15**) as the first nonphosphorylated cyclization product, whereas the DHQ synthase-like mechanism would lead to the observed precursor, 2-*epi*-5-*epi*-valiolone (**9**), as the first cyclized compound. Several other facts support the notion that the cyclization of **5** to **9** involves a DHQ synthase-like mechanism: (i) The substrate **5** has the same configuration at C-5 and C-6 as the substrate of DHQ synthase, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate, (ii) the product **9** has the same configuration at the newly generated stereocenter, C-5, as DHQ, the product of DHQ synthase, at the corresponding carbon, and (iii) the cyclase from *Actinoplanes* generating **9** for acarbose biosynthesis shows a high degree of sequence homology to DHQ synthases from different organisms.[31]

Whereas the initial cyclization step leading to the first carbocylic precursor seems to be the same or very similar in the validamycin and acarbose fermentations, there are substantial differences between the two biosynthetic pathways in the subsequent reactions. Notably, none of the cyclitols fed to the validamycin producer in the present study except **9** showed any incorporation into acarbose, leading to the proposal that all of the steps from **9** to acarviosyl-dTDP, a pseudodisaccharide nucleotide precursor of **4** containing the valienamine moiety, must occur on one enzyme or enzyme complex without any free intermediates.[26] In contrast, the results presented here demonstrate that the transformation of **9** into the two cyclitol moieties of **1** involves a series of free intermediates, and they define the sequence of reactions and identify valienone (**11**) as a proximate precursor of the two cyclitol units. The two reactions needed to transform **9** into **11**, epimerization at C-2 and dehydration to generate the $\Delta_{5,6}$ double bond, both require the carbonyl group at C-1, or an equivalent function such as an imine, for activation. The efficient incorporation of 5-*epi*-valiolone (**10**) demonstrates that C-2 epimerization apparently occurs first, followed by dehydration (Scheme 5). The low incorporation of 2-*epi*-valienone (**15**) into **1**, although significant, is best interpreted by assuming that **15** undergoes slow spontaneous epimerization under the fermentation conditions, producing small amounts of the pathway intermediate **11**. Alternatively the 2-epimerase may not be completely specific for its substrate **9** but may less efficiently also epimerize **15** when presented with this compound. However, the low overall incorporation of **15** compared to **9** and **10** argues against a second parallel pathway from **9** to **11** via **15**, suggesting that **15** may not normally be formed from **9**.

Valienone (**11**) as well as its precursors **9** and **10** are incorporated into both cyclitol moieties of **1** as demonstrated by NMR analysis of the products from feeding experiments with the ^{13}C- or deuterium-labeled compounds. The isotope distribution between the two cyclitol moieties was 1:1 in the experiment with ^{13}C-labeled **11**. A slightly higher deuterium content of the valienamine moiety than of the validamine moiety in the samples of **1** derived from [6-^{2}H$_2$]-**9** and [6-^{2}H$_2$]-**10** (60 and 58%, respectively) would be consistent with a somewhat longer pathway to the validamine unit. It may, however, merely reflect the loss of some deuterium from the latter moiety due to enolization at the validone stage, the same process thought to be responsible for the partial scrambling of deuterium between the two methylene hydrogens at C-6′ of **1**. Consistent with this explanation the slightly lower relative deuterium enrichment in the validamine moiety of **1** derived from [6-^{2}H$_2$]-**9**, compared to that from [6-^{2}H$_2$]-**10**, coincides with a somewhat higher degree of deuterium scrambling (see Figure 4).

Valienone (**11**) represents a branchpoint in the biosynthetic pathway to **1**. One molecule of **11** is incorporated into the valienamine moiety of **1**, the other is reduced to validone (**12**) which gives rise to the validamine moiety. This follows clearly from the fact that **12** is incorporated into **1** only half as efficiently as **11** and labels exclusively the validamine moiety of the antibiotic. The experiments with deuterated **9** and **10** incidentally also revealed the steric course of the reduction of **11** to **12**, because both compounds give rise to [6-^{2}H$_1$]-**11** in the cells, which is then reduced to monodeuterated **12** and incorporated into the validamine moiety of **1**. The deuterium NMR analysis of these samples of **1** revealed that the enzymatic reduction of the $\Delta_{5,6}$ double bond in **11** involves the *anti* addition of two hydrogens. This steric course is commonly observed for enzymatic reductions of C−C double bonds in α,β-un-

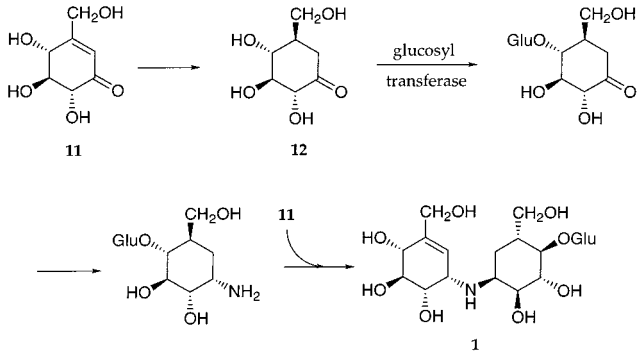
(31) Stratmann, A.; Mahmud, T.; Lee, S.; Distler, J.; Floss, H. G.; Piepersberg, W. *J. Biol. Chem.* **1999**, *274*, 10889.

(32) Loewus, M. W.; Loewus, F. A.; Brillinger, G. U.; Otsuka, H.; Floss, H. G. *J. Biol. Chem.* **1980**, *255*, 11710.

(33) Tian, F.; Migaud, M. E.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 5795.

(34) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299.

Scheme 8. Alternative Route for the Formation of Validamycin A

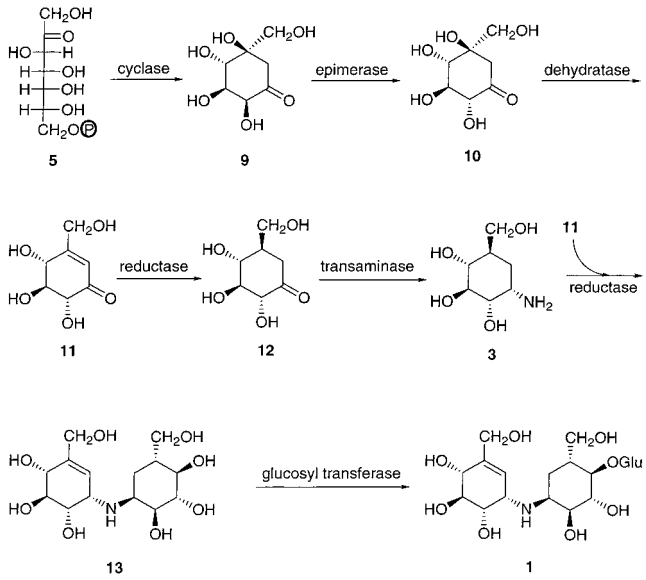


saturated carbonyl systems, such as enoates and enoyl thioesters in fatty acid biosynthesis and metabolism and in polyketide biosynthesis.[35]

A unique feature of **1** and related compounds, such as **4**, is the nitrogen bridge linking the two cyclitol moieties in **1** or the valienamine and deoxysugar moieties in **4**. Both the source of the nitrogen in **1** and the mode of formation of the nitrogen bridge remain obscure at this time. Studies in *Actinoplanes* sp. have identified glutamate, a typical substrate of transaminases, as the most efficient nitrogen donor in the biosynthesis of **4**.[36] In view of the similarity of the two systems the most plausible mechanism in the case of **1** thus is the introduction of the nitrogen into one ketocyclitol, **11** or **12**, by transamination to give either **2** or **3**, followed by imine formation with the second ketocyclitol, **12** or **11**, and reduction of the resulting Schiff's base. However, the feeding experiments with these aminocyclitols have not provided any support for such a mechanism, although this may be due to failure of the externally fed compounds to penetrate the cell membrane and reach the site of synthesis. If indeed neither **2** nor **3** are free intermediates in **1** biosynthesis, an alternative would be that glucosylation precedes the introduction of the nitrogen. In that case, as outlined in Scheme 8, validone **12** would first be glucosylated followed by transamination and reductive condensation with **11**. Since the glucosyl derivatives of **12** and **3** are not available, this alternative pathway has not yet been evaluated. As another possible alternative all of the steps of the entire process of reductively linking the nitrogen to both ketocyclitols may take place on one enzyme or enzyme complex without free intermediates. Again, no experimental evidence for or against such a process is available.

The one pertinent experimental result available, the demonstration of in vivo formation of tritiated **3** from [7-^{3}H]-**12**, is consistent with the original hypothesis for the mode of introduction of the nitrogen. We therefore consider it most likely that the negative outcome of the feeding experiment with tritiated **3** does not indicate the operation of a different mechanism but merely reflects cellular impermeability to the precursor. Since no in vivo conversion of **11** into **2** was observed, the above result favors a process involving transamination of **12** to **3** followed by reductive condensation with **11**. On the basis of these considerations and the experimental data presented we therefore propose the overall pathway shown in Scheme 9 for

Scheme 9. Proposed Biosynthetic Pathway to Validamycin A, Based on the Results of This Study



the biosynthesis of validamycin A in *Streptomyces hygroscopicus* var. *limoneus*. In addition to the major product **1** the validamycin fermentation also produces a number of minor components. Some of these, such as validamycins C, D, E, and F, are evidently derived from **1**. The formation of the validamycin congeners which differ from **1** in the structure of the second cyclitol moiety would require the transamination of other ketocyclitols, such as 6-hydroxyvalidone to **17** for validamycin B[1b] and valiolone (**7**) to (**8**) for validamycin G,[1g,i] and reductive condensation of the resulting aminocyclitols with **11** (Scheme 10).

Experimental Section

General Procedures. All synthetic reactions were carried out under an atmosphere of dry nitrogen at room temperature in oven-dried glassware unless otherwise noted. Radioactivity analyses were performed in a Beckman LS 1801 liquid scintillation counter (Beckman Instruments, Inc.) using Bio-Safe II (Research Products International Corp.) biodegradable liquid scintillation cocktail. ^{1}H-, ^{2}H-, and ^{13}C NMR spectra were recorded on a Bruker AF300 spectrometer with a *Mac*NMR 5.5 PCI instrument controller and data processor or on a Bruker WM500 spectrometer. Routine electrospray mass spectra were obtained on a Kratos Profile HV-4 double focusing magnetic sector mass spectrometer and high-resolution mass spectra on a Micromass 70SEQ tandem hybrid mass spectrometer. SIM-MS was carried out on a Micromass Quattro II tandem quadrupole mass spectrometer. Fermentations were carried out in a New Brunswick G25 controlled environment incubator/shaker. Sterilization of culture media, buffers, solutions, glassware, and pipets was done in an Amsco 2332 isothermal steam autoclave operating at 121 °C and 18 psi. All media were sterilized for 20 min.

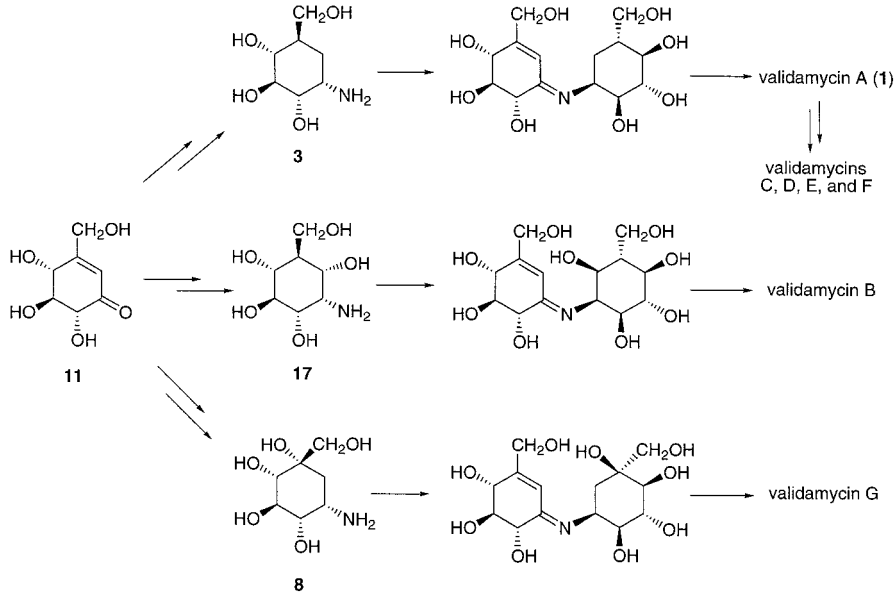
Materials. All chemicals and solvents were of reagent or HPLC grade and were used without further purification unless otherwise noted. Cultures of *Streptomyces hygroscopicus* var. *limoneus* were purchased from the American Type Culture Collection (ATCC 21431 and 21432) or obtained from Professor Eiji Higashide, Okayama University, Japan (No. T-7545). Fermentation ingredients were purchased from Difco or Sigma except corn gluten meal which was obtained from Professor Higashide and Takeda Chemical Co. Samples of validamycin A and valienamine were provided by Takeda Chemical Co.

Chromatography. Reactions were monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60F-254 aluminum sheets (0.25 mm). Compounds were visualized under UV light or by spraying with aqueous $KMnO_4$ or $Ce_2(SO_4)_3$ solution followed by heating.

(35) (a) Retey, J.; Robinson, J. *Stereospecificity in Organic Chemistry and Enzymology*; Verlag Chemie: Weinheim, 1982; pp 83−90. (b) Reynolds, K. A.; Fox, K. M.; Yuan, Z−m.; Lam, Y. *J. Am. Chem. Soc.* **1991**, *113*, 4339. (c) Hutchinson, C. R.; Shu-Wen, L.; McInnes, A. G.; Walter, J. A. *Tetrahedron* **1983**, *39*, 3507. PKS; (d) Moore, B. S.; Poralla, K.; Floss, H. G. *J. Am. Chem. Soc.* **1993**, *115*, 5267.

(36) Lee, S.; Egelkrout, E. *J. Antibiot*. **1998**, *51*, 225.

Scheme 10. Proposed Mode of Formation of Minor Components, Validamycins B through G



Preparative TLC was carried out on E. Merck silica gel 60F-254 glass plates (0.5, 1, or 2 mm). Column chromatography was performed on 230−400 mesh silica gel (Aldrich). Ion-exchange chromatography was carried out on the following resins: 100 mesh Dowex-50W and 100 mesh Dowex 1 (Sigma), 100−200 mesh Amberlite CG-50 (Aldrich), and AG 1-X8 (Bio-Rad). Autoradiography was performed on the same plates as those used for preparative TLC. The plates were developed in either ^{n}PrOH/AcOH/H_2O (4/1/1) or ^{n}BuOH/EtOH/H_2O (9/7/1), sprayed with ^{3}H enhancer (DuPont) and exposed to X-ray film (Amersham Life Sciences) for 6−7 days. In the 2D co-autoradiography analysis of the formation of [7-^{3}H]-**3** from [7-^{3}H]-**12**, the putative sample of [7-^{3}H]-**3** purified from the fermentation was co-applied on a TLC plate with an authentic reference sample of [7-^{3}H]-**3** in a radioactivity ratio of 1:1. The TLC plate was developed in ^{n}PrOH/AcOH/H_2O (4:1:1), rotated 90°, and developed again in ^{n}BuOH/EtOH/H_2O (9:7:4), followed by spraying with ^{3}H enhancer and exposure to X-ray film.

Fermentation and Isolation of Validamycin A and Related Compounds. *Streptomyces hygroscopicus* var. *limoneus* was grown in yeast malt extract agar medium on Petri plates for 7 days. A single colony selected from the plates was added to 25 mL of the following seed medium in a 125 mL Erlenmeyer flask: 0.5% tryptone, 0.3% yeast extract, 0.3% glucose, pH 7.0. The flask was shaken at 28 °C and 200 rpm for 2 days. Five milliliters of the seed culture was used to inoculate 100 mL of production medium containing 2% glucose, 4% corn starch, 2% corn steep liquor, 4% corn gluten meal, 0.5% NH_4Cl, 1.5% NaCl, 1.5% $CaCO_3$, pH adjusted to 7 with 5 N NaOH before sterilization. The production cultures were grown for 7 days at 28 °C with rotary shaking at 200 rpm.

The culture broth was acidified with oxalic acid to pH 3−4 and centrifuged. The precipitate formed, as well as the mycelium, were removed by filtration. The filtrate was applied directly to a Dowex 50W column (150 mL). The column was washed with 450 mL of H_2O. Validamycin A and other amino group-containing compounds adsorbed on the column were eluted with 0.5 N NH_4OH. The fractions that contained the desired compounds, as judged by TLC, were combined, concentrated, and chromatographed on Amberlite CG-50 (100 mL) with H_2O. Validamycin A and other secondary amino group-containing compounds that were not adsorbed on the resin were eluted with H_2O. The primary amines were then eluted with 0.5 N NH_4OH. Each of the two fractions was rechromatographed on a Dowex 1 column (100 mL) which was eluted with H_2O to give pure validoxylamine A, validamycin A, and validamycin B from the first fraction, and validamine and valienamine from the second fraction, respectively. When radioisotope-labeled precursors were fed to the fermentation, the resulting products were further purified, after addition of unlabeled material, by recrystallization from two different solvent systems, EtOH and EtOH−Et_2O, to constant specific radioactivity.

Feeding Experiments with Labeled Precursors. In general, each of the labeled precursors was dissolved in H_2O and administered through a 0.2 μm diameter pore size ultrafilter to a 500 mL Erlenmeyer flask containing 100 mL of production culture. The labeled precursors were fed in three equal portions at 24 h, 48 h, and approximately 55 h after inoculation, and the cultures were harvested on day 7 of the fermentation. The total amount fed of each of the stable isotope-labeled precursors was 15 mg. The total radioactivity and specific activity administered in each feeding experiment with the tritiated precursors are listed in Table 2. After the fermentation, approximately 2 mL of the culture broth was removed to measure the radioactivity in the volatile part. The rest of the broth was subjected to the isolation and purification procedure described above. The 2 mL of the original culture broth was centrifuged to give about 1.5 mL of supernatant. One ml of the supernatant was lyophilized, and 0.5 mL of the condensate was used to count the radioactivity in the volatile part of the fermentation broth. One milliliter of H_2O was added to the solid residue from the lyophilization, and 0.5 mL of this solution as well as 0.5 mL of the supernatant from the original broth were also subjected to scintillation counting.

In the experiments with the tritiated precursors, the purified products were analyzed by liquid scintillation counting. ^{2}H- or ^{13}C NMR and SIM-MS methods were used to analyze the products from the experiments with ^{2}H- and ^{13}C-labeled precursors.

Synthesis of Labeled Precursors. Validamine (3) and 5-*epi*-validamine (5-*epi*-3). To a solution of 253 mg (1.45 mmol) of **2** in 3 mL of H_2O were added several drops of a slurry of Raney Ni in H_2O. The mixture was placed in a Parr hydrogenator and shaken for 20 h at a H_2 pressure of 40 psi and at room temperature. The mixture was filtered through Celite and the filtrate evaporated to dryness to give a mixture of **3** and 5-*epi*-**3**. The two compounds were separated by flash chromatography (silica gel, ^{n}PrOH/AcOH/H_2O = 6/1/1) or preparative TLC (^{n}PrOH/AcOH/H_2O = 4:1:1, R_f: **3**, 0.28; 5-*epi*-**3**, 0.40). Each compound was further purified on Dowex 50W (washed with H_2O first and then eluted by 0.5 N NH_4OH) to give 75.8 mg of 5-*epi*-**3** and 152 mg of **3**. **3**: a white amorphous solid; $[\alpha]_D$ +52.8° (c = 0.17, H_2O, 26 °C); ES-MS m/z: 178.2 $(M + H)^+$; ^{1}H NMR (300 MHz, in D_2O) δ 1.36 (1H, ddd, J = 3.5, 14.5, 14.5 Hz, 6-H_{ax}), 1.57−1.69 (2H, m, 5-H and 6-H_{eq}), 3.05−3.13 (2H, m, 1-H and 4-H), 3.33 (1H, dd, J = 4.5, 10.8, 2-H), 3.36 (1H, dd, J = 9.8, 10.8 Hz, 3-H), 3.48 (1H, dd, J = 5.2, 11.4, 7-H_a), 3.56 (1H, dd, J = 3.6, 11.4 Hz, 7-H_b); ^{1}H NMR (300 MHz, in CD_3OD) δ 1.44 (1H, ddd, J = 3.5, 14.0, 14.0, 6-H_{ax}), 1.77−1.87 (2H, m, 5-H and 6-H_{eq}), 3.18 (1H, dd, J = 8.8, 12.4 Hz, 4-H), 3.19 (1H, dt, J = 3.5, 4.0 Hz, 1-H), 3.36 (1H, dd, J = 4.0, 9.8 Hz, 2-H), 3.48 (1H, dd, J = 8.8. 9.8 Hz, 3-H), 3.62 (1H, dd, J = 5.7, 10.9 Hz, 7-H_a), 3.69 (1H, dd, J = 4.2, 10.9 Hz, 7-H_b); ^{13}C NMR (300 MHz, CD_3OD) δ 30.8 (C-6), 39.7 (C-5), 51.6 (C-1), 64.4 (C-7), 75.2 (C-4),

75.4 (C-2), 75.7 (C-3). 5-*epi*-**3**: a white amorphous solid; $[\alpha]_D$ +7.6° (c = 0.75, H_2O, 26 °C); ES-MS m/z: 178.5 $(M + H)^+$; ^1H NMR (300 MHz, in D_2O) δ 1.57 (1H, ddd, J = 8.8, 10.4, 10.4, 6-H_{ax}), 1.88−1.91(2H, m, 5-H and 6-H_{eq}), 3.42 (1H, dd, J = 6.7, 10.9 Hz, 7-H_a), 3.46 (1H, m, 1-H), 3.52 (1H, dd, J = 6.7, 10.9 Hz, 7-H_b), 3.77 (1H, br t, J = .∼3 Hz, 4-H), 3.81 (1H, br t, J = .∼3 Hz, 2-H), 3.91 (1H, br t, J = .∼3 Hz, 3-H); ^1H NMR (300 MHz, in CD_3OD) δ 1.71 (1H, ddd, J = 8.8, 11.8, 11.8 Hz, 6-H_{ax}), 2.06−2.11 (2H, m, 5-H and 6-H_{eq}), 3.48 (1H, ddd, J = 2.5, 3.0, 8.8 Hz, 1-H), 3.54 (1H, dd, J = 6.8, 10.4 Hz, 7-H_a), 3.68 (1H, dd, J = 7.7, 10.4 Hz, 7-H_b), 3.88−3.91 (2H, m, 2-H and 4-H), 4.00 (1H, t, J = 3.6 Hz, 3-H); ^{13}C NMR (300 MHz, in CD_3OD) δ 23.1 (C-6), 38.6 (C-5), 50.6 (C-1), 64.2 (C-7), 70.9 (C-4), 71.0 (C-2), 71.6 (C-3).

[7-^{3}H]Validamine ([7-^{3}H]-3) and 5-*epi*-[7-^{3}H]validamine (5-*epi*-[7-^{3}H]-3). The above procedure was used to prepare 130 μCi of [7-^{3}H]-**3** and 60 μCi of [7-^{3}H]-5-*epi*-**3** from 400 μCi of [7-^{3}H]-**13** (specific radioactivity 2.0 μCi/μmol) as the starting material. The radiochemical purity of the products was confirmed by autoradiography.

Validone (12). To a solution of 30 mg (0.169 mmol) of **3** in 5 mL of CH_3OH was added 41 mg (0.186 mmol) of 3,5-di-*tert*-butyl-1,2-benzoquinone in 2 mL of CH_3OH with stirring under argon at room temperature. Stirring was continued for 1 h. About 3 mL of H_2O and 2 mL of THF were added to the mixture to make a clear solution. The pH of the solution was adjusted to about 3 by addition of crystalline oxalic acid dihydrate. The acidic solution was stirred for 2 h and neutralized by addition of saturated aqueous $NaHCO_3$. The solution was washed twice with Et_2O and evaporated to dryness. The residue was further purified over Dowex 50W and AG 1 × 8 columns (eluted in both cases with H_2O) to give a clean product (18 mg, 61%) with R_f = 0.53 (^{n}PrOH/AcOH/H_2O = 4/1/1). **11**: A white amorphous solid; $[\alpha]_D$ +17.5° (c = 0.35, H_2O, 26 °C); HR-MS m/z: calculated for $C_7H_{12}O_5Na$: 199.0582 $(M + Na)^+$, found 199.0578. ^1H NMR (300 MHz, in CD_3OD) δ 1.66 (1H, m, 5-H), 2.40 (1H, dd, J = 4.6, 14.0 Hz, 6-H_{ax}), 2.49 (1H, ddd, J = 1.5, 13.5, 14.0 Hz, 6-H_{eq}), 3.29 (1H, dd, overlapped with solvent signals, 4-H), 3.66 (1H, dd, J = 9.3, 10.5 Hz, 3-H), 3.70 (1H, dd, J = 4.1, 10.9 Hz, 7-H_a), 3.75 (1H, dd, J = 5.1, 10.9, 7-H_b); 4.06 (1H, dd, J = 1.5, 10.5 Hz, 2-H); ^{13}C NMR (CD_3OD) δ 40.0 (C-5), 42.93 (C-6), 63.0 (C-7), 73.2 (C-4), 79.4 (C-3), 80.5 (C-2), 208.8 (C-1).

[7-^{3}H]Validone ([7-^{3}H]-12). [7-^{3}H]-**12** (60 μCi, specific radioactivity 2 μCi/μmol) was prepared by the above method from 100 μCi of [7-^{3}H]-**3**. The radiochemical purity of the product was confirmed by autoradiography.

[1-^{13}C]Validone ([1-^{13}C]-12). To a solution of 120 mg of tetrabenzyl-[1-^{13}C]valienone (**16**) in 95% aqueous ethanol (10 mL) was added wet 10% Pd/C (120 mg), and the mixture was stirred at room temperature under an H_2 atmosphere for 16 h. The suspension was passed through a Celite column to remove the catalyst and then filtered through a membrane filter. The solvent was evaporated in vacuo to give a crude product which was purified further by preparative TLC to give pure [1-^{13}C]-**12** and 5-*epi*-[1-^{13}C]-**12** (4:1; total yield 90%).

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