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Biosynthesis of the Cyclitol Moiety of Pyralomicin 1a in *Nonomuraea spiralis* MI178-34F18

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The biosynthetic pathway leading to the cyclitol moiety of pyralomicin 1a (**1**) in *Nonomuraea spiralis* MI178-34F18 has been studied using a series of ^{2}H-labeled potential precursors. The results demonstrate that 2-*epi*-5-*epi*-valiolone (**7**), a common precursor for acarbose (**4**) and validamycin A (**5**) biosynthesis, is an immediate precursor of pyralomicin 1a. 5-*epi*-Valiolone (**8**) was also incorporated into **1**, albeit less efficiently than **7**. Other potential intermediates, such as valiolone (**9**), valienone (**10**), valienol (**11**), 1-*epi*-valienol (**12**), 5-*epi*-valiolol (**13**), and 1-*epi*-5-*epi*-valiolol (**14**) were not incorporated into pyralomicin 1a. To explain this surprising observation, it is proposed that either 2-*epi*-5-*epi*-valiolone (**7**) is specifically activated (*e.g.*, to its phosphate) and that the further transformations take place on activated intermediates (which can not be generated directly from their unactivated counterparts), or that the transformation of **7** into **1** involves a substrate-channeling mechanism in which enzyme-bound intermediates are directly transferred from one enzyme active site to the next in a multi-enzyme complex.

The antibiotic pyralomicin 1a (**1**) is the major component of the pyralomicin complex isolated from the cultured broth of *Nonomuraea spiralis* MI178-34F18[1]. Former *Microtetraspora spiralis* MI178-34F18 was renamed as *Nonomuraea* sp. according to a new classification[2]. This compound has a unique structure, a benzopyranopyrrole that is *N*-glycosylated by a carbasugar (cyclitol) (Figure 1)[3]. Biosynthetically, the benzopyranopyrrole core unit in **1** is derived from two units of acetate, one unit of propionate and one unit of proline arranged as shown in Scheme 1[4]. It was proposed that the formation of the antibiotic proceeds *via* the cyclization of a simple tetraketide intermediate which is generated from the coenzyme A thioester of pyrrole-2-carboxylate as primer[5]. The cyclitol structure in **1** is similar to the valienamine moieties of the anti-diabetic drug, acarbose (**4**)[6], and the anti-fungal antibiotic, validamycin A (**5**)[7], the only difference being the opposite stereochemistry at the C-1′ position. Valienamine (**3**) and related aminocyclitol structures can be viewed as aliphatic analogues of mC_7N units which are found in many secondary metabolites, such as the ansamycins and mitomycins[8] (as a six membered carbocyclic ring bearing a carbon and a nitrogen substituents in 1,3(*meta*) arrangement). The biosynthetic pathways to the aminocyclitol moieties in acarbose (**4**) and validamycin A (**5**) have been partially deduced based on the results of incorporation experiments with labeled precursors and of biochemical approches[9~13]. Although the valienamine moiety in both **4** and **5** originates from the same precursor, D-sedoheptulose 7-phosphate (**6**), which is cyclized *via* dehydroquinate (DHQ) synthase-like mechanism[13] to yield 2-*epi*-5-*epi*-valiolone (**7**), further downstream the pathways of their formation seem to be

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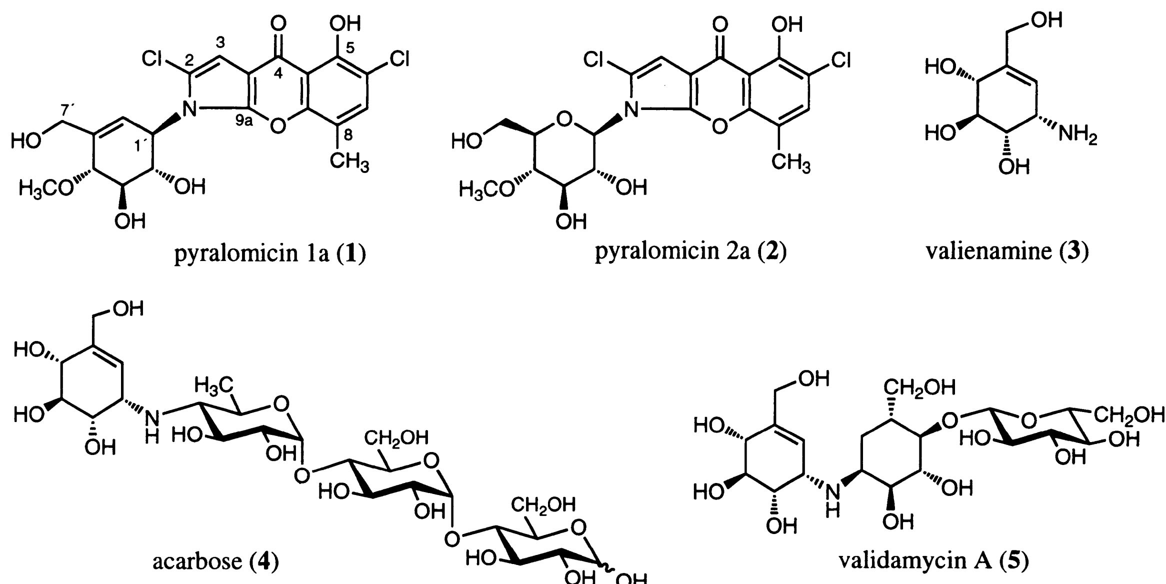
Fig. 1. Structures of pyralomicins 1a and 2a, valienamine, acarbose and validamycin A.



substantially different[14].

Initial incorporation experiments with D-[U-$^{13}C_6$]glucose on pyralomicin 1a (**1**) suggested that the cyclitol in **1** is derived from the pentose phosphate pathway[4], the same pathway utilized in the biosynthesis of the cyclitol moieties of **4** and **5**. However, the opposite stereochemistry at C-1′ of **1** suggests an essential biosynthetic divergence between the cyclitol in **1** and that in **4** or **5**. This could take place either during the formation of the cyclitol or during the condensation of the cyclitol and the core benzopyranopyrrole unit. In the present paper we describe further studies on the biosynthesis of the cyclitol moiety of **1**, in which a series of potential precursors, in deuterium-labeled form, were fed to *Nonomuraea spiralis* MI178-34F18 in order to evaluate their roles in the biosynthetic pathway.

Materials and Methods

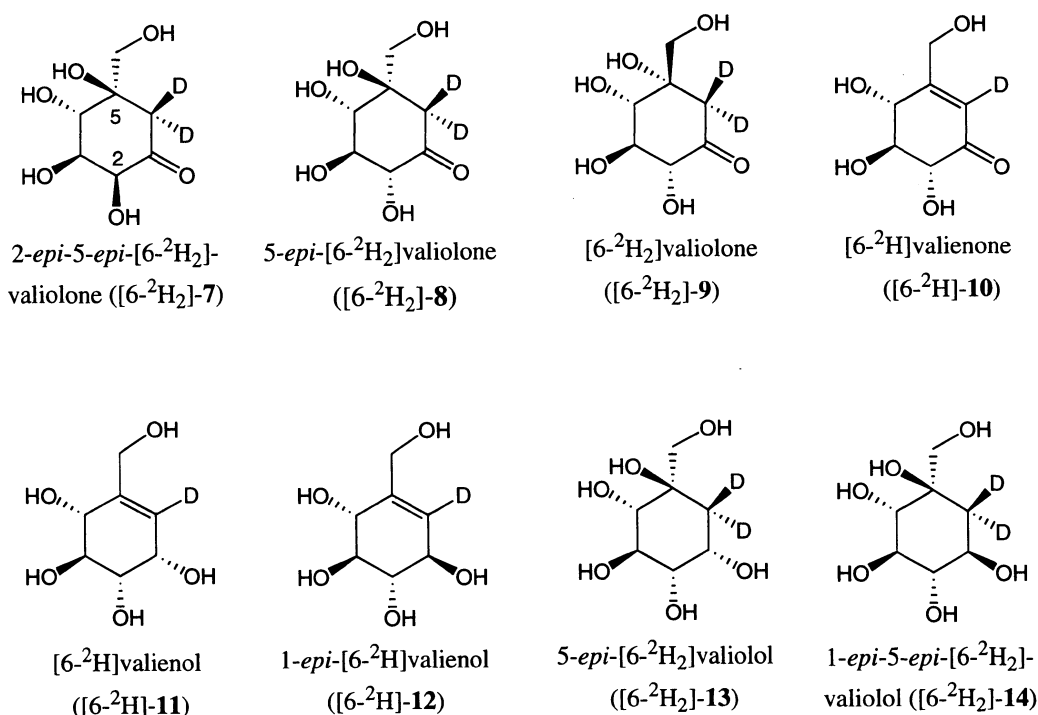
Synthesis of Labeled Precursors

The labeled compounds used in this study are shown in Figure 2. The preparation of 2-*epi*-5-*epi*-[6-$^2H_2$]valiolone from 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose and 5-*epi*-[6-$^2H_2$]valiolone from 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose has been reported earlier[11,15]. [6-$^2H_2$]Valiolone, [6-^{2}H]valienone, [6-^{2}H]valienol and its epimer, 1-*epi*-[6-^{2}H]valienol, were prepared using the same method as previously reported for their ^{13}C-labeled analogs[11], except that deuterium was introduced by carrying out the reductive dehalogenation with tributyltin deuteride (Scheme 2). The preparation of 5-*epi*-[6-$^2H_2$]valiolol and its isomer, 1-*epi*-5-*epi*-[6-$^2H_2$]valiolol will be reported in an upcoming publication (manuscript in preparation).

Fermentation and Incorporation Experiments with Labeled Precursors

An agar slant culture of *Nonomuraea spiralis* MI178-34F18 was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of seed medium [galactose 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki) 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, and a drop of silicone oil (adjusted to pH 7.4 before sterilization)]. The culture was incubated at 27°C on a rotary shaker (180 rpm) for 72 hours. Two ml of the seed culture was transferred into a 500 ml Erlenmeyer flask containing 110 ml of production medium [starch 1.5%, toast soya (Nisshin) 0.75%, corn steep liquor (Iwaki) 0.25%, yeast extract 0.1%, $MgSO_4 \cdot 7H_2O$ 0.025%, NaCl 0.15%, $CoCl_2 \cdot H_2O$ 0.0005%, $CaCO_3$ 0.15% and a drop of silicone oil (adjusted to pH 7.4 before sterilization)]. This production culture was incubated at 27°C on a rotary shaker (180 rpm). Each labeled precursor was dissolved in water (2 ml) and sterilized using a Millipore filter. Each solution was added to a production culture in two equal portions at the 48th and 52nd hours of

Fig. 2. Structures of the ^{2}H-labeled compounds used in this study.



cultivation. The culture was harvested at the 72nd hour of cultivation.

Isolation and Analysis of Pyralomicin 1a

Each harvested fermentation broth (98 ml) from the feeding experiments with ^{2}H-labeled precursors was centrifuged and the supernatant was extracted with butyl acetate (80 ml and 40 ml). The mycelial cake was extracted further with methanol (5×30 ml). The methanol extract was concentrated under reduced pressure, and the resulting material was suspended in water (30 ml). This suspension was subsequently extracted with butyl acetate (5×30 ml). Both butyl acetate extracts were combined and evaporated to dryness. This crude material containing pyralomicin 1a was chromatographed on a silica gel column (Wakogel C-300, 3 g) using *n*-hexane - ethyl acetate (from 1 : 1 to 1 : 4, v/v) as the eluant. Pyralomicin 1a was eluted with a solvent ratio of 1 : 2 (*n*-hexane/ethyl acetate, v/v).

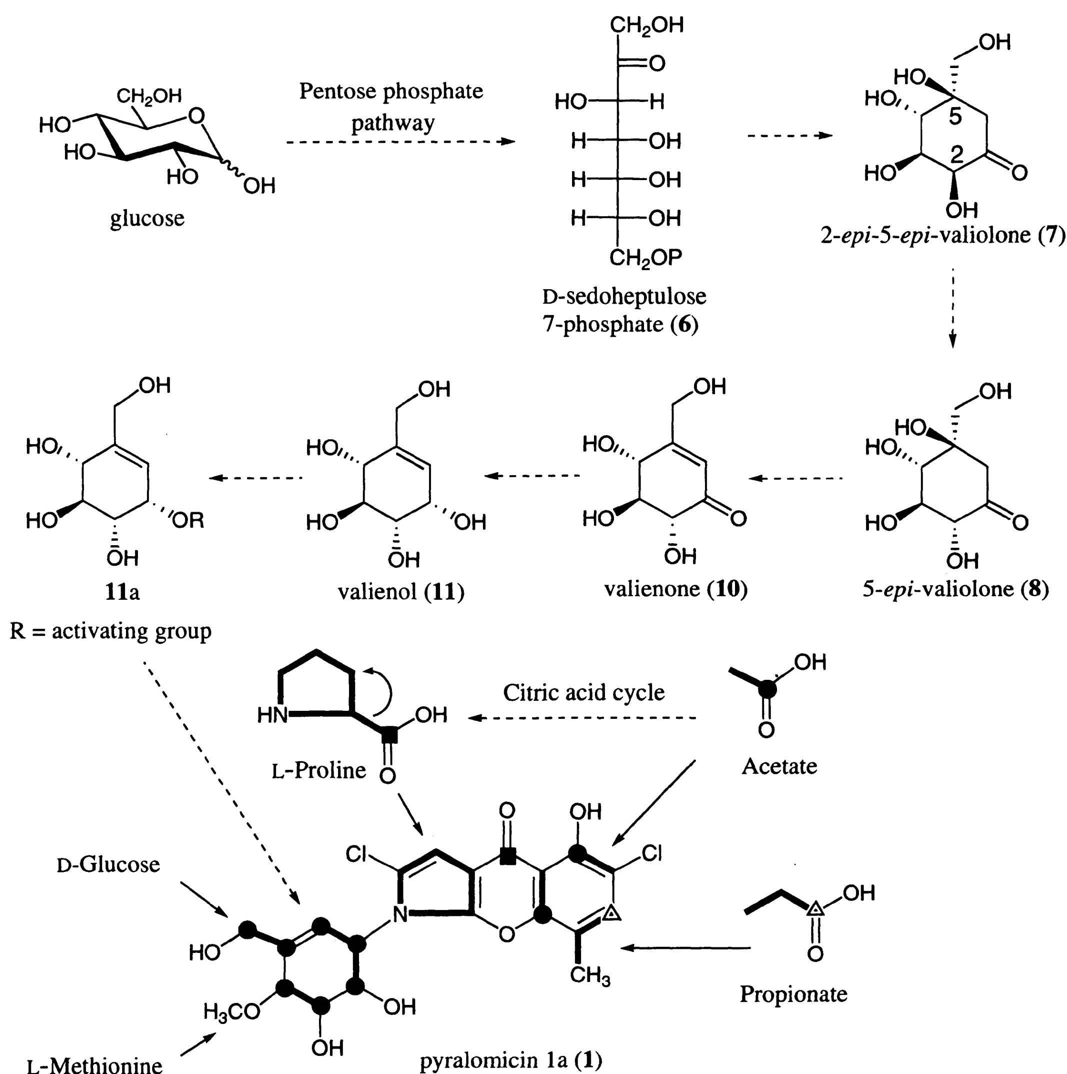
^{1}H and ^{2}H NMR spectra were recorded on a JEOL JNM-A500 spectrometer, FAB-MS on a JEOL JMS-SX102 spectrometer and EI-MS on a Hitachi M-80H spectrometer.

Results and Discussion

Eight ^{2}H-labeled cyclitols (Figure 2) were fed to cultures of the pyralomicin 1a producer, *Nonomuraea spiralis* MI178-34F18, as potential precursors of the cyclitol moiety of **1**. It was found that only 2-*epi*-5-*epi*-valiolone (**7**) and 5-*epi*-valiolone (**8**) were incorporated into pyralomicin 1a and other compounds (**9**~**14**) were not incorporated into **1** (Table 1). 2-*epi*-5-*epi*-Valiolone (**7**) has been recognized as the immediate cyclization product of D-sedoheptulose 7-phosphate (**6**)[13] and is the only free cyclitol intermediate identified in the biosynthesis of the valienamine moiety of acarbose[11]. The incorporation of **7**, but not of **9**, suggests that the immediate precursor of the cyclitol in pyralomicin 1a biosynthesis is D-sedoheptulose 7-phosphate. In validamycin A (**5**) biosynthesis, epimerization at C-2 of 2-*epi*-5-*epi*-valiolone (**7**) gives 5-*epi*-valiolone (**8**), which was also efficiently incorporated into **5**. In the case of validamycin A biosynthesis, **8** is then dehydrated *via* a *syn*-elimination of water[15] to give valienone (**10**), another efficient precursor of **5**[12]. However, feeding of [6-$^2H_2$]valienone to the pyralomicin 1a producer, surprisingly, showed no incorporation of this compound into **1**.

Although the mechanism of the coupling reaction

Scheme 1. Biosynthesis of pyralomicin 1a.



between the components of the pseudodisaccharide moieties of acarbose and validamycin A is not clearly understood, the condensation between the core benzopyranopyrrole unit and the cyclitol in **1** can be predicted with some confidence to proceed *via* a nucleophilic displacement. This prediction is based on the fact that the nitrogen atom in **1** originates from the pyrrole nitrogen of L-proline, and on the plausible suggestion that formation of the core benzopyranopyrrole unit proceeds *via* a pyrrolomycin derivative[4,5], leaving the possibility for glycosylation processes to take place at a late stage in the biosynthesis, that is, after benzopyranopyrrole ring formation. The isolation of the *N*-glucoside analogs, pyralomicins 2a~2c, from the same organism[1,2], and the production of unglycosylated benzopyranopyrrole antibiotics, TAN-876A and TAN-876B[16], by *Streptomyces* sp. C-70899 also suggests that glycosylation of the core unit of pyralomicin 1a must occur at a late stage in the biosynthesis, and therefore presumably *via* a nucleophilic displacement reaction. The evidence cited above argues against an alternative mechanism involving transamination of the cyclitol to its amino derivative and incorporation of this nitrogen into the pyrrole ring *via* Schiff's base intermediates.

Glycosylation of natural products *via* nucleophilic replacement reactions normally requires activation of the C-1 position of the sugar. The most common glycosyl donors in nature are nucleoside diphosphosugars, which are derived from sugar 1-phosphates. For such a process to be involved in pyralomicin 1a biosynthesis, reduction of the

Scheme 2. Synthesis of [6-$^{2}H_{2}$]valiolone([6-$^{2}H_{2}$]-**9**), [6-^{2}H]valienone([6-^{2}H]-**10**), [6-^{2}H]valienol([6-^{2}H]-**11**) and 1-*epi*-[6-^{2}H]valienol([6-^{2}H]-**12**).

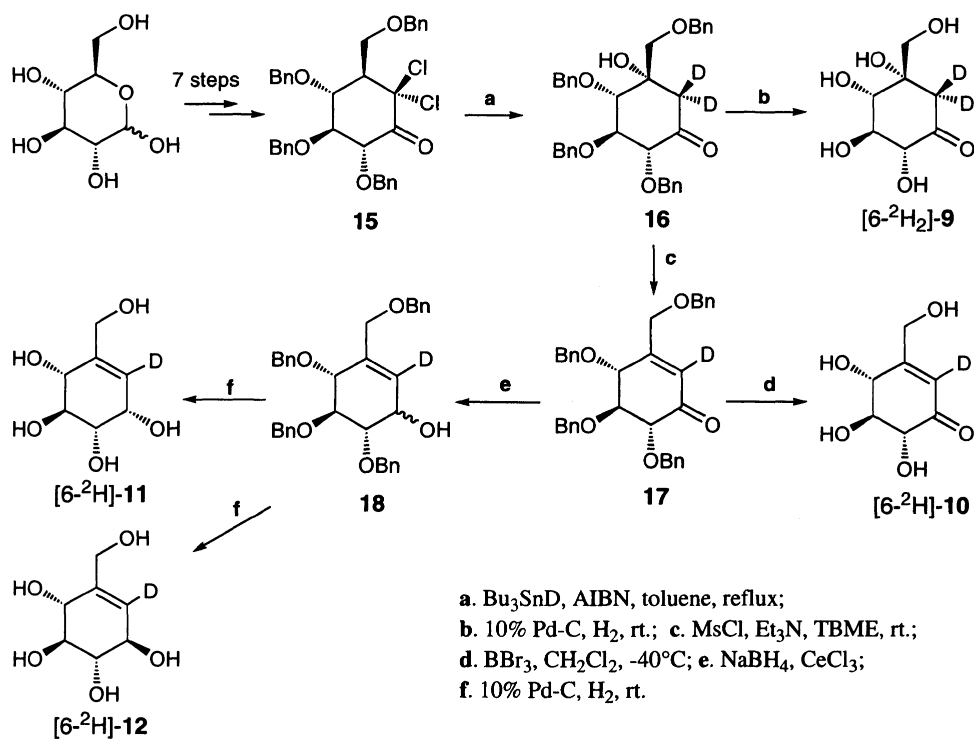


Table 1. Results of the incorporation experiments with the cyclitols to **1** producer.

Precursor	Amount Fed	Production Yield of **1**	Specific Incorporation
2-*epi*-5-*epi*-[6-$^{2}H_{2}$]valiolone (**7**)†	16.8 mg	0.2 mg	23%
5-*epi*-[6-$^{2}H_{2}$]valiolone (**8**)†	16.7 mg	0.2 mg	10%
[6-$^{2}H_{2}$]valiolone (**9**)	15.2 mg	1.1 mg	-
[6-$^{2}H_{1}$]valienone (**10**)	18.0 mg	2.4 mg	-
[6-$^{2}H_{1}$]valienol (**11**)	18.0 mg	1.5 mg	-
1-*epi*-[6-$^{2}H_{1}$]valienol (**12**)	19.0 mg	2.0 mg	-
5-*epi*-[6-$^{2}H_{2}$]valiolol (**13**)	17.0 mg	1.0 mg	-
1-*epi*-5-*epi*-[6-$^{2}H_{2}$]valiolol (**14**)	16.0 mg	1.0 mg	-

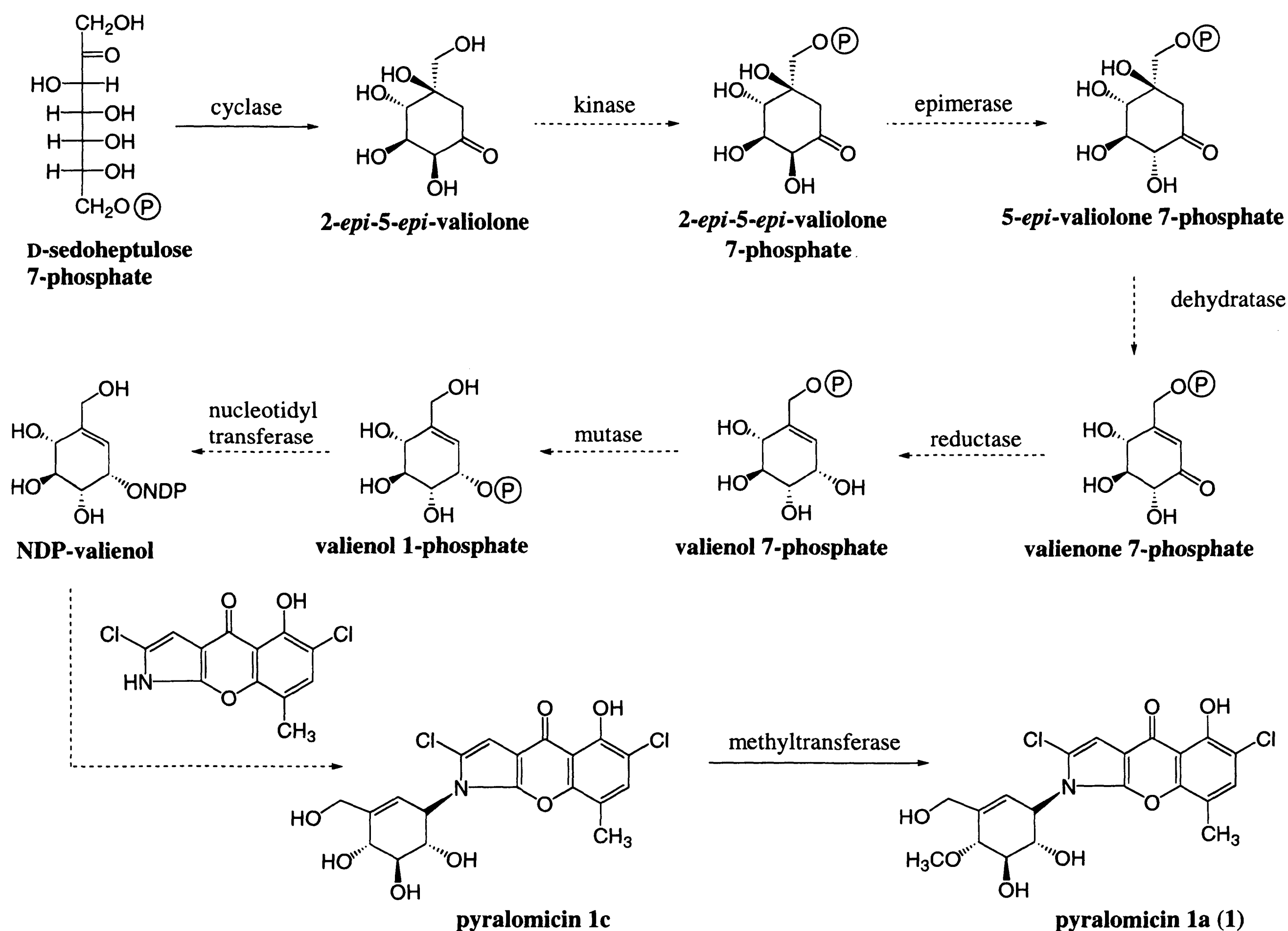
$$\text{Spec. Incorp.} = \frac{\text{Atom \% excess D in product*}}{\text{Atom \% excess D in precursor}} \times 100\ [\%]$$

* corrected for mandatory loss of one D from [6-$^{2}H_{2}$] precursors
† 98+ atom % D

keto function of the cyclitol and activation of the resulting C-1 alcohol (*e.g.*, as phosphate or nucleosidyl diphosphate) must occur. If reduction of the ketone takes place at the stage of 5-*epi*-valiolone (**8**), the immediate product would be 5-*epi*-valiolol (**13**) or 1-*epi*-5-*epi*-valiolol (**14**). This early reduction of the keto group, however, would remove the most obvious mechanistic driving force for the dehydration between C-5 and C-6. On the other hand, if dehydration of 5-*epi*-valiolone (**8**) takes place first, prior to the reduction, then the product after reduction would either be valienol (**11**) or 1-*epi*-valienol (**12**). Surprisingly, our feeding experiments revealed that neither 5-*epi*-valiolol (**13**), 1-*epi*-5-*epi*-valiolol (**14**), valienol (**11**), or 1-*epi*-valienol (**12**) were incorporated into pyralomicin 1a at a detectable level. This result contradicts the above hypothesis with respect to the involvement of the alcohol derivatives in the biosynthesis of **1**, and leaves the path from 5-*epi*-valiolone (**8**) to the cyclitol moiety of **1** unexplained.

One possible explanation for these surprising results would be that the cyclitol intermediates prior to the condensation with the aglycone are tightly enzyme-bound and are involved in a substrate-channeling process by which intermediates are directly transferred from one enzyme active site to the next in a multi-enzyme complex. In other words, reaction steps such as dehydration between C-5 and C-6, reduction of the C-1 ketone, and activation of the C-1 alcohol, would all take place on a complex of enzymes without free intermediates. As an alternative possibility, the reaction steps from 2-*epi*-5-*epi*-valiolone (**7**) or 5-*epi*-valiolone (**8**) to **1** might take place on activated intermediates, *e.g.*, phosphate derivatives, which cannot be generated directly from their unactivated counterparts. Scheme 3 shows an attractive version of such a pathway. In analogy to the metabolism of glucose and mannose, isosteres of **8** and **7**, it is proposed that **7** is phosphorylated at C-7, followed by epimerization, dehydration and ketoreduction. Migration of the phosphate from C-7 to C-1, catalyzed by an enzyme similar to phosphoglucomutase, would then set the stage for the activation of the cyclitol to

Scheme 3. Proposed biosynthetic pathway to pyralomicin 1a.



a nucleotidyl diphospho-valienol (NDP-valienol) and transfer of the pseudosugar moiety to the aglycone.

Although there is no hard evidence to validate either of these hypotheses, the lower incorporation of **8** (10%) into **1** compared to that of **7** (23%) can be taken as an indication for the involvement of either activated or enzyme-bound intermediates which are not in equilibrium with their free non-activated counterparts. If **8** were a free intermediate on the biosynthetic pathway (Scheme 1), it would have to be closer to the final product, **1**, in the sequence than **7**, and **8** should therefore be incorporated into **1** more efficiently than **7**[12]. Hence, the lower incorporation of **8** compared to **7** is more likely due to inefficient equilibration of external **8** with either the enzyme-bound **8** or an activated form of **8** within the cell. If the biosynthesis involves channeling of tightly enzyme-bound intermediates, the system may still allow some binding of external **8** to the cognate active site in competition with internally generated **8**, whereas intermediates further downstream are excluded completely from binding. In the biosynthetic pathway proposed in Scheme 3, 2-*epi*-5-*epi*-valiolone (**7**), but not 5-*epi*-valiolone (**8**), would presumably be the natural substrate for the phosphorylation to give 2-*epi*-5-*epi*-valiolone 7-phosphate, which then undergoes epimerization, dehydration, and reduction. The low incorporation of **8** could then be due to unspecific utilization of 5-*epi*-valiolone by the kinase, albeit less efficiently than **7**, to generate the next intermediate, 5-*epi*-valiolone 7-phosphate. Further experimentation will be required to unravel the pathway of pyralomicin 1a biosynthesis completely and to determine which of the scenarios discussed above applies in this process.

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