Synthesis of 5-*epi*-[6-$^2H_2$]Valiolone and Stereospecifically Monodeuterated 5-*epi*-Valiolones: Exploring the Steric Course of 5-*epi*-Valiolone Dehydratase in Validamycin A Biosynthesis

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In validamycin A biosynthesis, as well as that of acarbose, the valienamine and validamine moieties are ultimately derived from a C_7 sugar, sedoheptulose 7-phosphate, which is cyclized to 2-*epi*-5-*epi*-valiolone by a cyclase that operates via a dehydroquinate (DHQ) synthase-like mechanism. 2-*epi*-5-*epi*-Valiolone is first epimerized at C-2 to give 5-*epi*-valiolone and then dehydrated between C-5 and C-6 to yield valienone. To probe the dehydration mechanism of 5-*epi*-valiolone to valienone, stereospecifically 6α- and 6*β*-monodeuterated 5-*epi*-valiolones were synthesized. The key step in the synthesis was desulfurization of the tetrabenzyl-6,6-bis(methylthio)-5-*epi*-valiolone and introduction of the deuterium utilizing Zn, $NiCl_2$, ND_4Cl/D_2O, and THF. Extensive studies using various combinations of protio- and deuteroreagents and solvents probed the mechanism of the reductive desulfurization, which is crucial for the preparation of stereospecifically monodeuterated 5-*epi*-valiolones. Incorporation experiments with the labeled precursors in the validamycin A producer strain, *Streptomyces hygroscopicus* var. *limoneus*, revealed that the dehydration of 5-*epi*-valiolone to valienone occurs by a *syn* elimination of water.

Introduction

Validamycin A, a major component of the validamycin complex produced by *Streptomyces hygroscopicus* var. *limoneus*,[1] is an antifungal antibiotic widely used as a crop protectant, especially to control rice sheath blight disease caused by the phytopathogenic fungus, *Rhizoctonia solani*.[2] Validamycin A is composed of a pseudodisaccharide moiety, validoxylamine A,[3] connected to glucose. The pseudodisaccharide moiety consists of two aliphatic C_7 units, valienamine and validamine, which are connected via a single nitrogen atom. The valienamine unit is also found in acarbose, an α-glucosidase inhibitor and clinically useful antidiabetic drug.[4]

In our earlier work on the biosynthesis of validamycin A in *Streptomyces hygroscopicus* var *limoneus*[5] and acarbose in *Actinoplanes* sp.,[6] we had demonstrated that the core units, valienamine and validamine, originally derived from 2-*epi*-5-*epi*-valiolone, an intramolecular aldol cyclization product of sedoheptulose 7-phosphate (Scheme 1). This cyclization process was proposed to occur via a dehydroquinate (DHQ) synthase-like cyclization mechanism.[7] In further steps, in *S. hygroscopicus* var *limoneus* 2-*epi*-5-*epi*-valiolone is epimerized at the C-2 position to give 5-*epi*-valiolone and dehydrated between C-5 and C-6 to yield valienone. The latter is a proximate precursor of the cyclitol moieties in validamycin A,[5] giving rise to the unsaturated moiety directly and to the saturated cyclitol unit indirectly via validone. The conversion of 5-*epi*-valiolone to valienone resembles the well-known dehydration of DHQ to dehydroshikimate, which is catalyzed by two evolutionarily and mechanistically unrelated types of DHQ dehydratases. The type I enzymes catalyze a *syn* elimination of the elements of water, whereas the type II enzymes catalyze an *anti* elimination. To determine what type of enzyme might be catalyzing the dehydration of 5-*epi*-valiolone, it was desirable to elucidate the steric course of this reaction. To this end we developed a synthesis of stereospecifically 6α and 6*β* monodeuterated 5-*epi*-valiolones and explored the fate of the deuterium in validamycin A biosynthesis.

Syntheses of various related cyclitol derivatives have been reported by our group[5−6,9] and other laboratories.[10−13]

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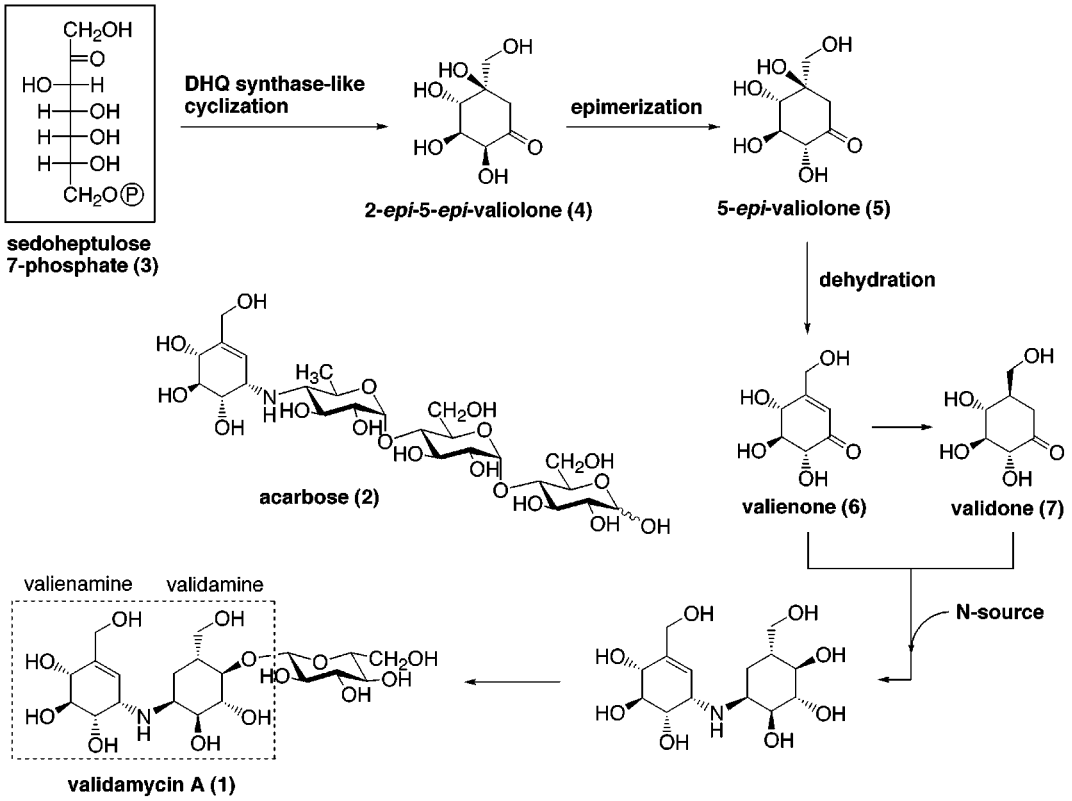
Scheme 1. Structures of Validamycin A and Its Biosynthetic Intermediates and of Acarbose



It is known that an intramolecular aldol cyclization of 2,3,4,6-tetra-*O*-benzyl-1-*C*-[bis(methylthio)methyl]-D-*xylo*-5-hexosulose (**12**) gives both C-5 stereoisomers **13a** and **13b**,[13] while that of its dichloromethyl derivative, which has been used in the preparation of valiolone,[6] gives only the 5*S* isomer. However, unlike the dichloromethyl derivative which could be easily reduced to give the dechlorinated analogue, desulfurization of **13a** seemed to be problematic. Reductive desulfurization of **13a** using Raney nickel was accompanied by elimination of the C-3 benzyloxyl group to give the 2-cyclohexenone derivative **14**.[13]

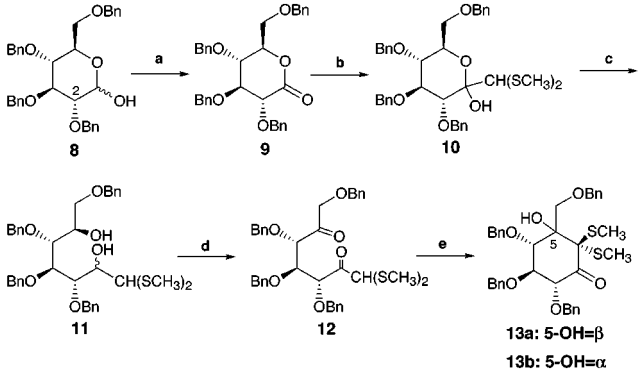
In the present paper, we describe successful attempts to selectively reduce the bis(methylthio) moiety of **13a** and introduce deuterium into the C-6 methylene group of 5-*epi*-valiolone. Using combinations of protio- and deuteroreagents and solvents, we probed the mechanism and sequencial steps of the reductive desulfurization process, which then allowed the preparation of stereospecifically 6α and 6*β* deuterated 5-*epi*-valiolones. Results on the utilization of the samples in validamycin A biosynthesis are also presented.

Results

Synthesis of 5-*epi*-[6-$^2H_2$]Valiolone. 5-*epi*-[6-$^2H_2$]-Valiolone was synthesized from 2,3,4,6-tetra-*O*-benzyl-D-glucose by a route partly adopted from methods reported by Fukase and Horii.[13] Instead of using the dichloroinososе derivative,[6,13] bis(methylthio) derivatives were employed to control the stereoselectivity of the intramolecular aldol cyclization toward the desired 5*R* stereochemistry of the final product (Scheme 2). Aldol condensation of **12** with potassium carbonate in the presence of 18-crown-6 gave an approximately 4:1 mixture of **13a** and **13b**. The former was applied for the synthesis of 5-*epi*-[6-$^2H_2$]valiolone reported here, whereas the latter had originally been used in the synthesis of *N*-substituted valiolamine analogues.[13] In contrast to the desulfurization of **13b** with Raney nickel which gives tetrabenzyl valiolone, reduction of **13a** with Raney nickel was accompanied by elimination of the 3-benzyloxy group to give the 2-cyclohexenone derivative **14** (Scheme 3).

The second method of choice to reduce the bis(methylthio) moiety in **13a** was using tri-*n*-butyltin hydride (Bu_3-

Scheme 2. Synthesis of Compounds 13a and 13b



Reagents: **a**. Ac_2O, DMSO, rt., quant.; **b**. ^{t}BuLi, $CH_2(SCH_3)_2$, −78 °C; **c**. $LiAlH_4$, THF/Diglyme, rt.; **d**. TFAA, DMSO, Et_3N, CH_2Cl_2, −78 °C, 3 steps 70%; **e**. K_2CO_3, 18-crown-6, CH_2Cl_2, rt., 90% (**13a**:**13b** = 4:1).

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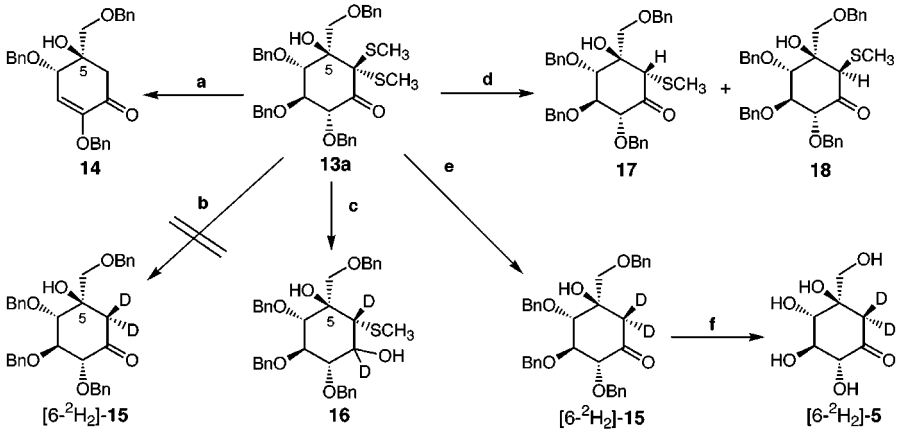
Scheme 3. Synthesis of 5-*epi*-[6-$^2H_2$]Valiolone



Reagents: **a**. Raney Ni, dioxane, reflux.; **b**. Bu_3SnD, AIBN, toluene, reflux; **c**. Ni_2BD_2, EtOD; **d**. Zn, NH_4Cl/H_2O, THF; **e**. Zn, $NiCl_2$, ND_4Cl/D_2O, THF; **f**. wet 10% Pd/C, H_2, 95% EtOH.

SnH) in the presence of AIBN.[14] With this method, we successfully converted **13a** to tetrabenzyl-5-*epi*-valiolone (**15**). However, replacement of Bu_3SnH with Bu_3SnD, to provide the deuterium-labeled derivative, surprisingly, failed. Attemps to reduce the bis(methylthio) moiety in **13a** with Ni_2BD_2[15] or with $LiAlD_4/TiCl_4$[16] were also unsuccessful, wherein Ni_2BD_2 mostly removed only one of the methylthio moieties in **13a**.

Partial desulfurization was also achieved by vigorous stirring of **13a** with activated zinc, an aqueous solution of saturated ammonium chloride, and THF at room temperature.[17] This reaction gave after 48 h of stirring compounds **17** and **18** in roughly a 2:1 ratio which can be separated by means of normal- or reversed-phase silica gel column chromatography. The stereochemistry at C-6 of **17** and **18** was determined on the basis of NOE experiments. Strong NOE correlations were observed between 6β-H (3.45 ppm, d, J = 1.3 Hz) and 2-H (4.15 ppm, dd, J = 9.2, 1.3 Hz), 4-H (3.80 ppm, d, J = 8.6 Hz), 6-SCH_3 (2.20 ppm, s) in **17**, whereas NOE correlation between 6α-H (3.31 ppm, s) and 7-H_2 (3.55 and 3.62 ppm, ABq, J = 9.0 Hz), 6-SCH_3 (2.25 ppm, s) were observed in **18**.

The complete reduction was eventually achieved by utilizing a mixture of activated zinc and nickel chloride in an aqueous (D_2O) solution of saturated ammonium-d_4 chloride and THF. After vigorous stirring overnight, about 80% of the reactant was converted to [6-$^2H_2$]-**15**. The synthesis was completed by catalytic hydrogenation of [6-$^2H_2$]-**15** with wet 10% Pd/C in a hydrogen atmosphere for 16 h to give 5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**5**) in quantitative yield.

Mechanistic Study on the Reductive Desulfurization of the Bis(methylthio) Moieties by Zinc and Nickel Chloride. Treatment of **13a** with activated zinc in saturated aqueous NH_4Cl and THF evidently only gave **17**. However, under the same reaction conditions **17** is slowly epimerized to **18**. Vigorous stirring of **17** in Zn/NH_4Cl/H_2O/THF at room temperature for 5 days yielded almost 90% of **18**, unlike treatment of **18** under the same reaction conditions, which gave only a trace amount of **17**. When a saturated solution of ND_4Cl in D_2O was used in place of NH_4Cl/H_2O in the reaction of **17**, about 50% of [6-$^2H_1$]-**18** was recovered together with 50% of [6-$^2H_1$]-**17** (Scheme 4). Interestingly, when purification of the products was conducted using silica gel column chromatography with toluene and ethyl acetate (20:1), almost all the deuterium in [6-$^2H_1$]-**17** was replaced by hydrogen. To some degree, this replacement was also observed in [6-$^2H_1$]-**18**, ranging from 0 atom % H in the earlier column fractions to about 50 atom % H in the later ones. However, this protonated species was probably not generated from the existing [6-$^2H_1$]-**18** but rather by the in situ conversion of [6-$^2H_1$]-**17** to **18** catalyzed by SiO_2. Stirring of compound **17** in a mixture of silica gel and toluene:ethyl acetate (10:1) gave **18**. Concurrent experiments were carried out by stirring each of [6-$^2H_1$]-**17** and [6-$^2H_1$]-**18** with a slurry of SiO_2 in toluene/EtOAc (10:1) at room temperature for 5 h, and the products were analyzed by ESI-MS. The results indicated that most of the deuterium in [6-$^2H_1$]-**17** was replaced by hydrogen, whereas that of [6-$^2H_1$]-**18** was retained. Moreover, exposure of **18** to Zn/ND_4Cl/D_2O/THF for 5 h did not yield any deuterium incorporation into **18**. This evidence suggests that the 6-Hβ in **17** is much more acidic and accessible to nucleophilic attack compared to the 6-Hα in **18**. The complete replacement of the deuterium by hydrogen in **17**, but not in **18**, during purifications also suggests that **17** exists in a rapid equilibrium with its enolate, some of which is converted more slowly to **18**.

The second methylthio group can be reduced using a mixture of activated zinc, $NiCl_2$, saturated aqueous ammonium chloride, and THF. Thin-layer chromatography monitoring of simultaneous reactions of **17** and **18** with Zn, $NiCl_2$, NH_4Cl/H_2O, and THF showed that **18** was directly reduced to the final product **15**, whereas **17** is first epimerized to **18** prior to reduction to yield **15**.

Synthesis of 5-*epi*-[6α-$^2H_1$]Valiolone and 5-*epi*-[6β-$^2H_1$]Valiolone. The synthesis of 5-*epi*-[6β-$^2H_1$]valiolone ([6β-$^2H_1$]-**5**) began with the reduction of the first methylthio group of **13a** with Zn/NH_4Cl/H_2O/THF at room temperature for 72 h to give **17** and its epimer **18** (4:5 ratio; total yield 83%). To provide additional product **18**, **17** was isolated and re-treated with a fresh mixture of

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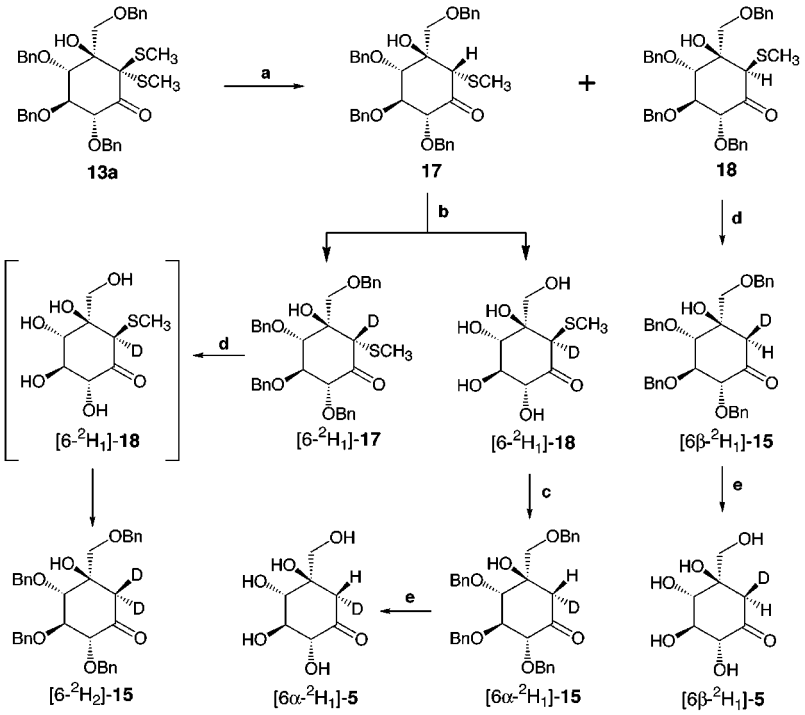
Scheme 4. Sequencial Reductions of Methylthio Moieties in 13a and Synthesis of Stereospecifically Labeled 5-*epi*-Valiolone



Reagents: **a**. Zn, NH_4Cl/H_2O, THF; **b**. Zn, ND_4Cl/D_2O, THF; **c**. Zn, $NiCl_2$, NH_4Cl/H_2O, THF; **d**. Zn, $NiCl_2$, ND_4Cl/D_2O, THF; **e**. wet 10% Pd/C, H_2, 95% EtOH.

$Zn/NH_4Cl/H_2O$/THF, and the resulting **18** was pooled. Treatment of **18** with $Zn/ND_4Cl/D_2O$/THF and $NiCl_2$ at room temperature for 16 h gave tetrabenzyl-5-*epi*-[6*β*-$^2H_1$]valiolone ([6*β*-$^2H_1$]-**15**) with 93 atom % D and 81% de, based on the integration of its ^1H NMR signals (Figure 1). The 6-Heq in [6*β*-$^2H_1$]-**15** appeared as a singlet (2.64 ppm), instead of a doublet (2.66 ppm) in **15**, shifted upfield around 0.02 ppm due to the deuterium isotope effect.[18] Purification of the synthesis product was carried out using ODS C_{18} reversed-phase column chromatography or HPLC to avoid epimerization and wash out of the isotope. Deprotection of the benzyl moieties using wet 10% Pd−C in a hydrogen atmosphere gave 5-*epi*-[6*β*-$^2H_1$]-valiolone ([6*β*-$^2H_1$]-**5**) in 66.4% yield from **18**.

Conversely, preparation of 5-*epi*-[6α-$^2H_1$]valiolone was carried out by treatment of **13a** with $Zn/ND_4Cl/D_2O$/THF to give [6-$^2H_1$]-**17** and [6-$^2H_1$]-**18**. Typically, reactions using $Zn/ND_4Cl/D_2O$/THF (deuterated reagents) gave slightly lower yields (78%) with a 2:1 ratio of [6-$^2H_1$]-**17** over [6-$^2H_1$]-**18**. [6-$^2H_1$]-**17** was isolated and re-treated with a fresh mixture of $Zn/ND_4Cl/D_2O$/THF to provide additional [6-$^2H_1$]-**18**. Treatment of [6-$^2H_1$]-**18** with $Zn/NH_4Cl/H_2O$/THF and $NiCl_2$ yielded tetrabenzyl-5-*epi*-[6α-$^2H_1$]valiolone ([6α-$^2H_1$]-**15**) with 93 atom % D and 78% de. The synthesis was completed by catalytic hydrogenolysis of the benzyl moieties of [6α-$^2H_1$]-**15** to give 5-*epi*-[6α-$^2H_1$]valiolone ([6α-$^2H_1$]-**5**) in 80% yield from [6-$^2H_1$]-**18**.

Incorporation Experiments with 5-*epi*-[6α-$^2H_1$]-Valiolone and 5-*epi*-[6*β*-$^2H_1$]Valiolone to the Validamycin A Producer. Fifteen milligrams each of 5-*epi*-[6α-$^2H_1$]valiolone ([6α-$^2H_1$]-**5**) and 5-*epi*-[6*β*-$^2H_1$]valiolone ([6*β*-$^2H_1$]-**5**) and, for comparison, 5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**5**) were fed to cultures (100 mL) of the validamycin A producer, with $^1/_3$ added at 24 h, $^1/_3$ at 48 h, and the rest at 55 h after inoculation. The cultures were harvested on the seventh day of fermentation. The fermentation broths were acidified to pH 3.0 with oxalic acid, and the cells and other solids were removed by centrifugation. The supernatants were directly subjected to cation exchange column chromatography on Dowex 50W followed by rechromatography of the validamycin complex on an anion exchange column (Dowex 1) to give, typically, about 45 mg of validamycin A from a 100 mL culture.

Selected ion monitoring (SIM) mass spectra ([M + Na]$^+$, [M + 1 + Na]$^+$, [M + 2 + Na]$^+$) of all samples together with an authentic unlabeled sample of validamycin A were recorded. Comparison of the intensities of the isotope peak [M + 1 + Na]$^+$ against the parent peak [M + Na]$^+$ of the samples and the reference sample indicated the incorporation of about 19.2% of 5-*epi*-[6-$^2H_2$]valiolone, 24.4% of 5-*epi*-[6α-$^2H_1$]valiolone, and 3.9% of 5-*epi*-[6*β*-$^2H_1$]valiolone.

The ^2H NMR spectrum of the validamycin A sample isolated from the feeding experiment with 5-*epi*-[6-$^2H_2$]-valiolone (Figure 2B) had clearly indicated the incorporation of 5-*epi*-valiolone into validamycin A.[5] The deuterium is distributed in a 3:2 ratio between the valienamine and the validamine moiety, because both derived from valienone. In the saturated cyclitol moiety the majority of the deuterium occupies the pro-6*S* position, reflecting the *anti* stereochemistry of the reduction of valienone to validone, with some scrambling to the pro-6*R* position due to reversible enolization at the validone stage.[5] The identical signal was observed in the ^2H NMR spectra of validamycin A isolated from the feeding experiment with 5-*epi*-[6α-$^2H_1$]valiolone (Figure 2C) and at much lower

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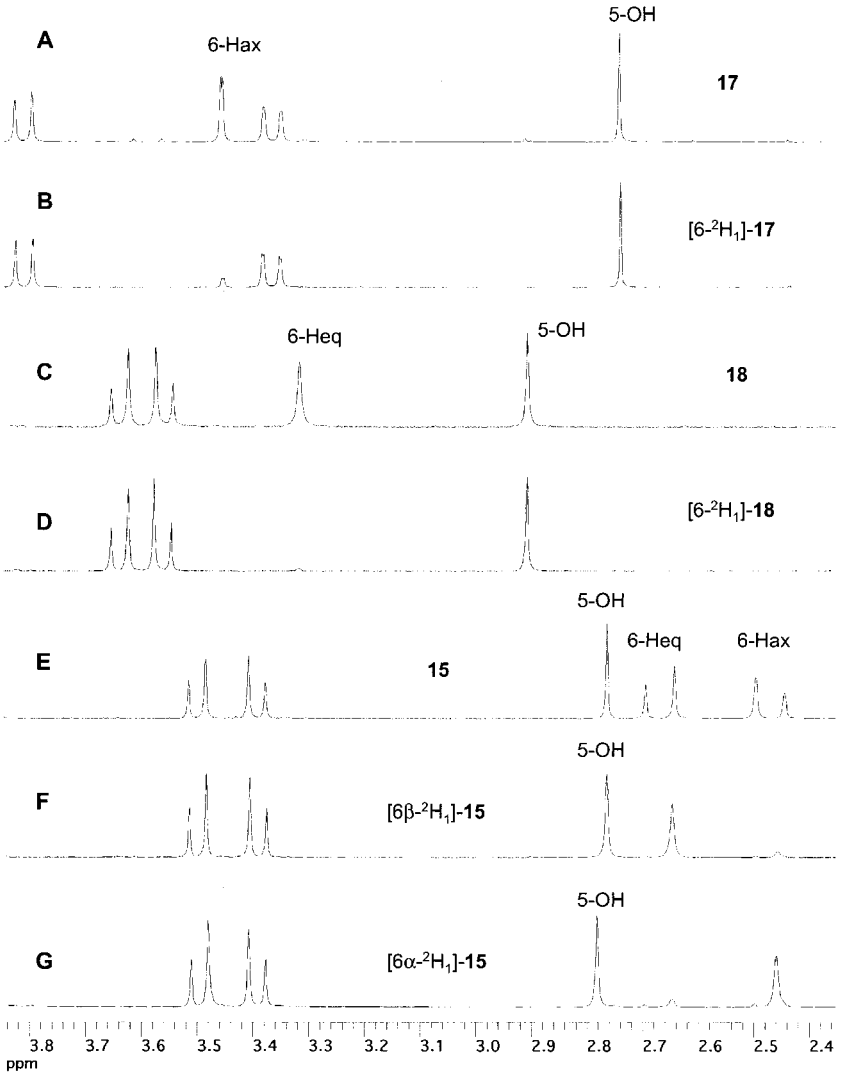

Figure 1. Partial ^{1}H NMR spectra (300 MHz, $CDCl_3$) of the compounds synthesized: (A) compound **17**, (B) [6-$^2H_1$]-**17**, (C) compound **18**, (D) [6-$^2H_1$]-**18**, (E) compound **15**, (F) [6b-$^2H_1$]-**15**, (G) [6a-$^2H_1$]-**15**.

intensity from that with 5-*epi*-[6β-$^2H_1$]valiolone (Figure 2D). These results show that the dehydration of 5-*epi*-valiolone to valienone occurs via a *syn* elimination of the elements of water.

Discussion

In the adaptation of the original Fukase and Horii chemistry to the synthesis of **5**, the reductive removal of the methylthio moieties of **13a** was surprisingly difficult. Desulfurization using a common reducing agent, Raney nickel, only gave an elimination product, **14**, as reported by Fukase and Horii.[13] The success of the approach using tri-*n*-butyltin hydride in the presence of AIBN, but its failure with Bu_3SnD, is also surprising, given that both Bu_3SnH and Bu_3SnD have been effectively used to reduce the dichloro analogues.[5,6] Although this failure is not clearly understood, kinetic isotope effects[19] could be a possible reason. The incomplete reduction of the methylthio moieties in **13a** by Ni_2BD_2, on the other hand, is easily rationalized by the concomitant reduction of the ketone function resulting in deactivation of the C-6 position and preventing epimerization of the monomethylthio product to a more reactive configuration. Moreover, it was also reported that desulfurization of the aliphatic derivative using Ni_2BH_2 is consistently more difficult than the analogous procedure with aryl ring containing substrates.[15a]

A similar phenomenon has been observed with activated zinc/NH_4Cl/H_2O/THF which was reported effective for the reductive desulfurization of α-phenylthio and α-phenylsulfinyl carbonyl compounds.[17] In the case of **13a**, this reagent reductively eliminated only one of the methylthio groups. This reaction proceeds by replacement of the methylthio moiety with a hydrogen radical, which evidently occurs solely toward the axial methylthio moiety to give **17**. The hydrogen radicals can be formed at the Zn surface by the reaction of protons with electrons released by the metal.[20] Interestingly, under the same reaction conditions **17** is slowly epimerized to **18**, presumably catalyzed by a Lewis acid, $[Zn(NH_3)_n]^{2+}$, via enol formation. Treatment of **17** with mineral acid, HCl, or saturated aqueous NH_4Cl did not give **18**. Since practically no epimerization has been observed from **18** to **17** in Zn/NH_4Cl/H_2O/THF or SiO_2/toluene/EtOAc, the steri-

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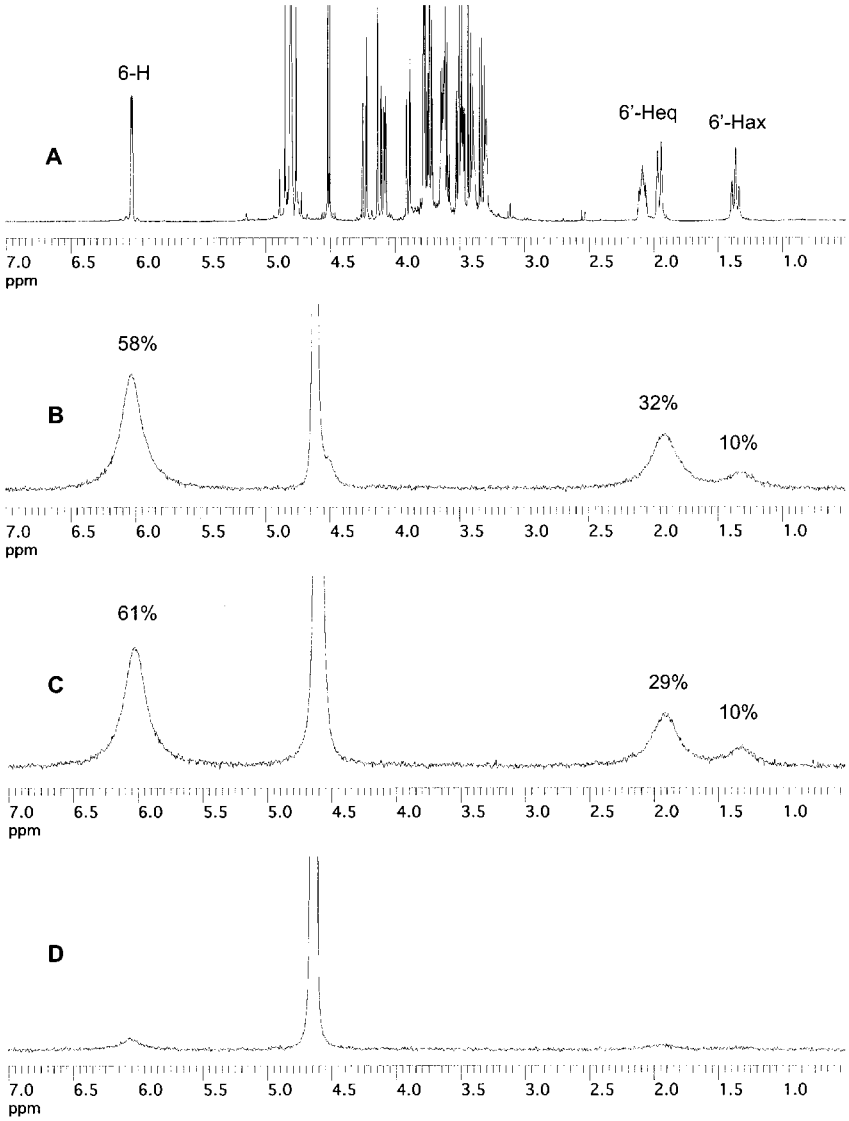

Figure 2. NMR spectra of validamycin A: (A) 500 MHz ^{1}H NMR spectrum of unlabeled **1** in D_2O, (B) 76.78 MHz ^{2}H NMR spectrum of **1** isolated from the feeding experiment with 5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**5**) in H_2O at 313 K,[5] (C) 76.78 MHz ^{2}H NMR spectrum of **1** isolated from the feeding experiment with 5-*epi*-[6α-$^2H_1$]valiolone ([6α-$^2H_1$]-**5**) in H_2O at 313 K, (D) 76.78 MHz ^{2}H NMR spectrum of **1** isolated from the feeding experiment with 5-*epi*-[6β-$^2H_1$]valiolone ([6β-$^2H_1$]-**5**) in H_2O at 313 K.

cally less accessible 6-Hα (equatorial proton) must be resistant to nucleophilic deprotonation. Therefore, once the enolate intermediate has been converted to **18**, the difficult deprotonation of 6-Hα traps this compound by preventing its conversion back to the thermodynamically more stable epimer **17**.

Epimerization of the methylthio group from an equatorial position in **17** to an axial position in **18** makes this group accessible to radical attack. The Zn/NH_4Cl/H_2O/THF reagent, however, is lacking sufficient reducing power to execute the second reduction. However, addition of nickel chloride to the reaction mixture evidently increases the reduction potential of the reagents. The first step of the reaction probably involves the reduction of the divalent nickel to a low-valent form adsorbed on the zinc surface[21] or to a free activated nickel precipitate. Isolation of **14** as a side product when the reaction was carried out at higher concentration, which resemble the reaction of **13a** with Raney nickel,[13] supports the hypothesis that nickel (Ni^0) is directly involved in the reduction reaction.

The success of the stereospecific synthesis of both 5-*epi*-[6α-$^2H_1$]valiolone and 5-*epi*-[6β-$^2H_1$]valiolone has made it possible to probe the steric course of the dehydration of 5-*epi*-valiolone to valienone in validamycin biosynthesis. The 24.4% incorporation rate of deuterium in validamycin A isolated from a 5-*epi*-[6α-$^2H_1$]valiolone feeding experiment compared to 3.9% of that from 5-*epi*-[6β-$^2H_1$]-valiolone unambiguously revealed that the dehydration reaction of 5-*epi*-valiolone to valienone occurs via a *syn* elimination. The 3.9% incorporation of that from 5-*epi*-[6β-$^2H_1$]valiolone could be rationalized by the fact that an 80% de of 5-*epi*-[6β-$^2H_1$]valiolone was used in the experiment.

The dehydration reaction of 5-*epi*-valiolone to valienone is very similar to the interconversion of dehydroquinic acid (DHQ) to dehydroshikimic acid (DHS), catalyzed by 3-dehydroquinate dehydratase (DHQase), in the shikimate pathway.[22] There are two distinct classes of DHQase enzymes known, designated as type I and type II, whose

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biophysical properties and reaction mechanisms are very different.[23−25] Type I DHQases catalyze a *syn* elimination of a molecule of water from dehydroquinate (DHQ) using an active-site lysine to form a covalent imine intermediate (Schiff's base) as part of the reaction mechanism.[26] Type II DHQases were originally identified as part of a catabolic pathway for utilization of quinic acid but in some cases are also involved in the shikimate pathway, and they catalyze an *anti* elimination of water by an unknown mechanism.[27]

In validamycin biosynthesis in *Streptomyces hygroscopicus* var. *limoneus*, the first reaction, cyclization of sedoheptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone, is catalyzed by a DHQ synthase-like enzyme.[7] 2-*epi*-5-*epi*-Valiolone is epimerized at the C-2 position to give 5-*epi*-valiolone. Dehydration of 5-*epi*-valiolone to valienone takes place via a *syn* elimination, possibly involving a type I DHQase-like mechanism. To shed more light on the mechanism and origin of the enzymes involved in the biosynthesis of validamycins, further studies at the biochemical and genetic levels will be pursued.

Experimental Section

General. ^{1}H, ^{2}H, and ^{13}C NMR spectra were recorded on Bruker AF-300 or AM500 NMR spectrometers with *Mac*NMR 5.5 PCI as the instrument controller and data processor. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Low-resolution electrospray mass spectra were recorded on a Bruker-Esquire liquid chromatography-ion trap mass spectrometer with electrospray, APCI, and nanospray ionization sources. A Fison VG Quattro II electrospray ionization mass spectrometer was used to measure the high-resolution mass spectroscopy. A ISF-4-V shaker, Adolf Kuhner AG, was used for the fermentations. All synthetic reactions were carried out under an atmosphere of dry argon at room temperature in oven-dried glasswares unless otherwise noted. Reactions were monitored by TLC (silica gel 60 F_{254}, Merck) with detection by UV light or by alkaline permanganate spray or $Ce(SO_4)_2/H_2SO_4$ solution. Column chromatography was performed on 230−400 mesh silica gel (Aldrich) or YMC*Gel 120 Å ODS 63−210 μm. Ion exchange chromatography was carried out on 100 mesh Dowex-50W and 100 mesh Dowex 1 (Sigma).

Materials. All chemicals and solvents were of reagent or HPLC grade and were used without further purification unless otherwise noted. Activated zinc was prepared by washing a 20 g portion of well-ground zinc dust in a fritted glass funnel with three 50 mL portions each of 4% aqueous HCl, water, methanol, and ether.[17] The active zinc was ground again to remove lumps and then dried first at 110 °C for 15 min and then at room temperature under reduced pressure for several hours. 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 were provided by Takeda Chemical Co. and Professor Kenneth L. Rinehart Jr., University of Illinois at Urbana.

Fermentation and Incorporation Experiments. Fermentation and isolation of validamycin A, as well as the incorporation experiments with labeled precursors, were carried out as described previously.[5]

Synthesis of Labeled Precursors. The synthesis of compounds **13a** and **13b** from tetra-*O*-benzylglucose was carried out following a method developed by Fukase and Horii[13] with some minor modifications.[28]

Tetrabenzyl-5-*epi*-valiolone (15). (a) Using Tri-*n*-butyltin Hydride/AIBN. To a solution of **13a** (100 mg, 0.155 mmol) in toluene (1 mL) were added tributyltin hydride (1.66 μL, 0.62 mmol) and AIBN (10 mg, 0.062 mmol), and the mixture was refluxed for 2 h and then cooled to room temperature. The products were extracted with EtOAc (5 mL), and the organic solution was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic solvent was evaporated under reduced pressure, and the extract was purified over a silica gel column (toluene/EtOAc = 10:1) to give **15** (60 mg, 70%).

(b) Using Zn/$NiCl_2$/NH_4Cl/H_2O/THF. To a solution of **13a** (480 mg, 0.745 mmol) in THF (50 mL) were added activated zinc (5 g), a saturated aqueous solution of NH_4Cl (10 mL), and anhydrous $NiCl_2$ (1 g), and the mixture was stirred vigorously at room temperature for 20 h. Twenty milliliters of EtOAc was added, and the mixture was filtered through a filter paper to remove the solid materials. The filtrate was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was purified over a silica gel column (toluene/EtOAc = 20:1−10:1) to give **15** (340 mg, 85%) and **17** (34 mg, 7.6%).

15: yellowish syrup, $[\alpha]_D$ +25.5° (c = 0.96, $CHCl_3$, 25 °C). ESI-MS: m/z 575 $(M + Na)^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.46 (1H, d, J = 16.0 Hz), 2.66 (1H, d, J = 16.0 Hz), 2.77 (1H, s), 3.38 (1H, d, J = 9.3 Hz), 3.49 (1H, d, J = 9.3 Hz), 3.86 (1H, d, J = 6.2 Hz), 3.91 (1H, dd, J = 6.2, 8.0 Hz), 4.41 (1H, d, J = 8.0 Hz), 4.47−4.95 (8H), 7.16−7.42 (10H).

Tetrabenzyl-5-*epi*-[6-^{2}H$_2$]valiolone ([6-^{2}H$_2$]-15). The procedure was identical with that in **b** for **15**, except ND_4Cl and D_2O were used in place of NH_4Cl/H_2O.

[6-^{2}H$_2$]-15: yellowish syrup. ESI-MS: m/z 577 $(M + Na)^+$. HR ESI-MS calcd for $C_{35}H_{34}D_2O_6Na$ $[M + Na]^+$: 577.2533, found 577.2529. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.77 (1H, s), 3.38 (1H, d, J = 9.3 Hz), 3.49 (1H, d, J = 9.3 Hz), 3.86 (1H, d, J = 6.2 Hz), 3.91 (1H, dd, J = 6.2, 8.0 Hz), 4.41 (1H, d, J = 8.0 Hz), 4.47−4.95 (8H), 7.16−7.42 (10H).

5-*epi*-[6-^{2}H$_2$]Valiolone ([6-^{2}H$_2$]-5). To a solution of 110 mg of [6-^{2}H$_2$]-**15** in 95% aqueous ethanol (8 mL) was added wet 10% Pd/C (200 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 [6-^{2}H$_2$]-**5** in quantitative yield.

[6-^{2}H$_2$]-5: yellowish syrup, $[\alpha]_D$ +6.5° (c = 0.86, MeOH, 25 °C). ESI-MS: m/z 217 $(M + Na)^+$. HR ESI-MS calcd for $C_7H_{10}D_2O_6Na$ $[M + Na]^+$: 217.0655, found 217.0655. ^{1}H NMR (500 MHz, CD_3OD) δ: 3.42 (1H, d, J = 11 Hz), 3.63 (1H, d, J = 11 Hz), 3.71 (1H, t, J = 9.3 Hz), 3.85 (1H, d, J = 9.3 Hz), 4.06 (1H, d, J = 9.3 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 64.7 (t), 74.7 (s), 75.9 (d), 79.1 (d), 79.9 (d), 206.3 (s).

Tetrabenzyl-6(*S*)-methylthio-5-*epi*-valiolone (17) and Tetrabenzyl-6(*R*)-methylthio-5-*epi*-valiolone (18). To a solution of **13a** (500 mg, 0.776 mmol) in THF (12.5 mL) were added activated zinc (2.75 g) and a saturated aqueous solution of NH_4Cl (12.5 mL), and the mixture was stirred vigorously at room temperature for 72 h. Twenty milliliters of EtOAc was

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(28) An application of ^{t}BuLi in place of *n*-BuLi in the reaction of **9** to **10** and using a silica gel column with *n*-hexane/EtOAc as solvent system to purify compound **12**, to overcome the difficulty encountered by Fukase and Horii in isolating compound **12**, which was reportedly too unstable to be isolated.

added, and the mixture was filtered through a filter paper to remove the solid materials. The filtrate was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was purified over a silica gel column (toluene/EtOAc = 20:1−10:1) to give **17** (214 mg, 46%) and **18** (172 mg, 37%).

17: yellowish syrup, $[\alpha]_D$ +1.85° (c = 0.76, $CHCl_3$, 25 °C). ESI-MS: m/z 621 $(M + Na)^+$. HR ESI-MS calcd for $C_{36}H_{38}O_6NaS$ $[M + Na]^+$: 621.2287, found 621.2257. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.20 (3H, s), 2.74 (1H, s), 3.36 (1H, d, J = 9 Hz), 3.45 (1H, d, J = 1.3 Hz), 3.80 (1H, d, J = 9.0 Hz), 3.90 (1H, d, J = 9.3 Hz), 4.15 (1H, dd, J = 1.3, 9.3 Hz), 4.25 (1H, t, J = 9.3 Hz), 4.37−5.00 (8H), 7.20−7.44 (10H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.5 (q), 60.2 (t), 62.4 (d), 69.1, 73.2, 73.3, 75.3 (all t), 75.5 (s), 81.1 (d), 85.1 (d), 85.5 (d), 127.3−128.2 (all d), 137.0, 137.8 (s), 138.2 (s), 138.3 (s), 197.4 (s).

18: yellowish syrup, $[\alpha]_D$ −44.1° (c = 0.83, MeOH, 25 °C). ESI-MS: m/z 621 $(M + Na)^+$. HR ESI-MS calcd for $C_{36}H_{38}O_6NaS$ $[M + Na]^+$: 621.2287, found 621.2298. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.09 (3H, s), 2.90 (1H, s), 3.31 (1H, brs), 3.55 (1H, d, J = 9.0 Hz), 3.62 (1H, d, J = 9.0 Hz), 3.88 (1H, dd, J = 6.2, 8.0 Hz), 3.98 (1H, d, J = 6.2 Hz), 5.04 (1H, d, J = 8.0 Hz), 4.44−4.91 (8H), 7.09−7.44 (10H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.3 (q), 57.7 (t), 71.2 (t), 73.5 (2C), 74.4, 74.5 (all t), 75.0 (s), 81.2 (d), 81.8 (d), 127.7−128.3 (all d), 137.2 (s), 137.6 (s), 137.8 (s), 138.0 (s, C_6H_5- × 4), 201.3 (s).

Tetrabenzyl-6(*S*)-methylthio-5-*epi*-[6-^{2}H$_1$]valiolone ([6-^{2}H$_1$]-17) and Tetrabenzyl-6(*R*)-methylthio-5-*epi*-[6-^{2}H$_1$]-valiolone ([6-^{2}H$_1$]-18). The procedure was identical with that for **17** and **18**, except ND_4Cl and D_2O were used in place of NH_4Cl/H_2O and the purification of the products was conducted using an ODS column (MeOH−H_2O = 80:20).

[6-^{2}H$_1$]-17: yellowish syrup. ESI-MS: m/z 622 $(M + Na)^+$. HR ESI-MS calcd for $C_{36}H_{37}DO_6NaS$ $[M + Na]^+$: 622.2348, found 622.2364. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.20 (3H, s), 2.74 (1H, s), 3.36 (1H, d, J = 9 Hz), 3.80 (1H, d, J = 9.0 Hz), 3.90 (1H, d, J = 9.3 Hz), 4.15 (1H, d, J = 9.3 Hz), 4.25 (1H, t, J = 9.3 Hz), 4.37−5.00 (8H), 7.20−7.44 (10H).

[6-^{2}H$_1$]-18: yellowish syrup. ESI-MS: m/z 622 $(M + Na)^+$. HR ESI-MS calcd for $C_{36}H_{37}DO_6NaS$ $[M + Na]^+$: 622.2348, found 622.2347. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 2.10 (3H, s), 2.90 (1H, s), 3.55 (1H, d, J = 9.0 Hz), 3.62 (1H, d, J = 9.0 Hz), 3.88 (1H, dd, J = 6.2, 8.0 Hz), 3.98 (1H, d, J = 6.2 Hz), 5.04 (1H, d, J = 8.0 Hz), 4.44−4.91 (8H), 7.09−7.44 (10H).

Tetrabenzyl-5-*epi*-[6α-^{2}H$_1$]valiolone ([6α-^{2}H$_1$]-15). To a solution of [6-^{2}H$_1$]-**18** (240 mg, 0.40 mmol) in THF (30 mL) were added activated zinc (2 g), a saturated aqueous solution of NH_4Cl (10 mL), and anhydrous $NiCl_2$ (400 mg), and the mixture was stirred vigorously at room temperature for 16 h. Twenty milliliters of EtOAc was added, and the mixture was filtered through a filter paper to remove the solid materials. The filtrate was washed with brine. The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was purified by ODS column chromatography (MeOH−H_2O = 80:20) to give ([6α-^{2}H$_1$]-**15** (143 mg, 65%) with a recovery of [6-^{2}H$_1$]-**18** (83.2 mg, 35%).

[6α-^{2}H$_1$]-15: yellowish syrup. ESI-MS: m/z 576 $(M + Na)^+$. HR ESI-MS calcd for $C_{35}H_{35}DO_6Na$ $[M + Na]^+$: 576.2471, found 576.2453. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.46 (1H, s), 2.76 (1H, s), 3.38 (1H, d, J = 9.3 Hz), 3.49 (1H, d, J = 9.3 Hz), 3.86 (1H, d, J = 6.2 Hz), 3.91 (1H, dd, J = 6.2, 8.0 Hz), 4.41 (1H, d, J = 8.0 Hz), 4.48−4.95 (8H), 7.18−7.42 (10H).

Tetrabenzyl-5-*epi*-[6β-^{2}H$_1$]valiolone ([6β-^{2}H$_1$]-15). To a solution of **18** (275 mg, 0.46 mmol) in THF (50 mL) was added activated zinc (2.1 g), a saturated D_2O solution of ND_4Cl (10 mL), and anhydrous $NiCl_2$ (415 mg), and the mixture was stirred vigorously at room temperature for 15 h. Twenty milliliters of EtOAc was added, and the mixture was filtered through a filter paper to remove the solid materials. The filtrate was washed with brine. The organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was purified over an ODS column (MeOH−H_2O = 80:20) to give ([6β-^{2}H]-**15** (110 mg, 43%) with recovery of **18** (130 mg, 47%).

[6β-^{2}H$_1$]-15: yellowish syrup. ESI-MS: m/z 576 $(M + Na)^+$. HR ESI-MS calcd for $C_{35}H_{35}DO_6Na$ $[M + Na]^+$: 576.2471, found 576.2462. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.64 (1H, s), 2.76 (1H, s), 3.39 (1H, d, J = 9.3 Hz), 3.50 (1H, d, J = 9.3 Hz), 3.86 (1H, d, J = 6.2 Hz), 3.91 (1H, dd, J = 6.2, 7.8 Hz), 4.40 (1H, d, J = 7.8 Hz), 4.48−4.95 (8H), 7.18−7.42 (10H).

5-*epi*-[6α-^{2}H$_1$]Valiolone ([6α-^{2}H$_1$]-5). To a solution of 128 mg (0.23 mmol) of [6α-^{2}H$_1$]-**15** in 95% aqueous ethanol (12.8 mL) was added wet 10% Pd/C (128 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, and the sample was passed through a Sephadex LH-20 (MeOH) to give [6α-^{2}H$_1$]-**5** (35.6 mg, 80%).

[6α-^{2}H$_1$]-5: colorless syrup. ESI-MS: m/z 216 $(M + Na)^+$. HR ESI-MS calcd for $C_7H_{11}D_1O_6Na$ $[M + Na]^+$: 216.0593, found 216.0585. ^{1}H NMR (300 MHz, CD_3OD) δ: 2.58 (1H, brs), 3.42 (1H, d, J = 11 Hz), 3.64 (1H, d, J = 11 Hz), 3.72 (1H, t, J = 9 Hz), 3.85 (1H, d, J = 9 Hz), 4.07 (1H, d, J = 9 Hz).

5-*epi*-[6β-^{2}H$_1$]Valiolone ([6β-^{2}H$_1$]-5). To a solution of 100 mg (0.18 mmol) of [6β-^{2}H$_1$]-**15** in 95% aqueous ethanol (10 mL) was added wet 10% Pd/C (100 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, and the sample was passed through a Sephadex LH-20 column (MeOH) to give [6β-^{2}H$_1$]-**5** (28.7 mg, 82%).

[6β-^{2}H$_1$]-5: colorless syrup. ESI-MS: m/z 216 $(M + Na)^+$. HR ESI-MS calcd for $C_7H_{11}D_1O_6Na$ $[M + Na]^+$: 216.0593, found 216.0584. ^{1}H NMR (300 MHz, CD_3OD) δ: 2.65 (1H, brs), 3.41 (1H, d, J = 11 Hz), 3.64 (1H, d, J = 11 Hz), 3.72 (1H, t, J = 9 Hz), 3.85 (1H, d, J = 9 Hz), 4.07 (1H, d, J = 9 Hz).

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Supporting Information Available: ^{1}H NMR spectra of compounds **15**, [6-^{2}H$_2$]-**15**, [6α-^{2}H$_1$]-**15**, [6β-^{2}H$_1$]-**15**, **17**, [6-^{2}H$_1$]-**17**, **18**, [6-^{2}H$_1$]-**18**, [6α-^{2}H$_1$]-**5**, and [6β-^{2}H$_1$]-**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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