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Biosynthetic Studies on the α-Glucosidase Inhibitor Acarbose in *Actinoplanes* sp.: 2-*epi*-5-*epi*-Valiolone Is the Direct Precursor of the Valienamine Moiety

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Abstract: The biosynthetic pathway leading to the *m*C_7N cyclitol (valienamine) moiety of acarbose (**1**) in *Actinoplanes* sp. strain SN 223/29 has been studied using ^{3}H-, ^{2}H-, and ^{13}C-labeled cyclitols. These precursors were synthesized from D-glucose or D-mannose as starting materials. The feeding experiments demonstrated that cyclitols having the same stereochemistry at C-2 as the valienamine moiety of acarbose; i.e., valienone, valienamine, valiolone, valiolamine, and 1-*epi*-valienol, were not incorporated and thus are not plausible intermediates in **1** biosynthesis. 2-*epi*-Valiolone (**10b**), which has the same stereochemistry as the presumed open-chain precursor, sedoheptulose 7-phosphate, was also not incorporated. However, its C-5 epimer (**10a**) was incorporated efficiently and specifically into the valienamine moiety of **1**. Surprisingly, the dehydrated form of 2-*epi*-5-*epi*-valiolone, 2-*epi*-valienone, was not incorporated. This suggests that 2-*epi*-5-*epi*-valiolone must be converted directly into the pseudodisaccharide moiety of acarbose without the intervention of other free cyclitol intermediates. This may occur by linkage to the amino group of TDP-4-amino-4,6-dideoxyglucose to form the imine, epimerization at C-2 to the correct stereochemistry, dehydration between C-5 and C-6 aided by enamine formation, and finally reduction to the amine. It is proposed that these reaction steps all take place on a single enzyme without free intermediates. Alternative mechanistic possibilities are also discussed.

Introduction

Acarbose (**1**), known as an α-glucosidase inhibitor and a clinically useful drug for the treatment of type II insulin-independent diabetes, is isolated from the fermentation broth of *Actinoplanes* sp.[1] Its structure (Figure 1) consists of an unsaturated amino-cyclitol, valienamine (**7**, Scheme 1), a deoxyhexose, and a maltose. The valienamine moiety, which

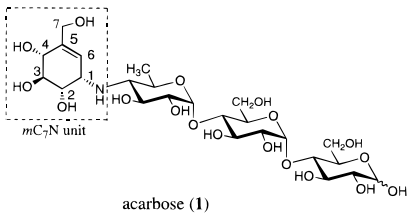

Figure 1. Structure of acarbose (**1**).

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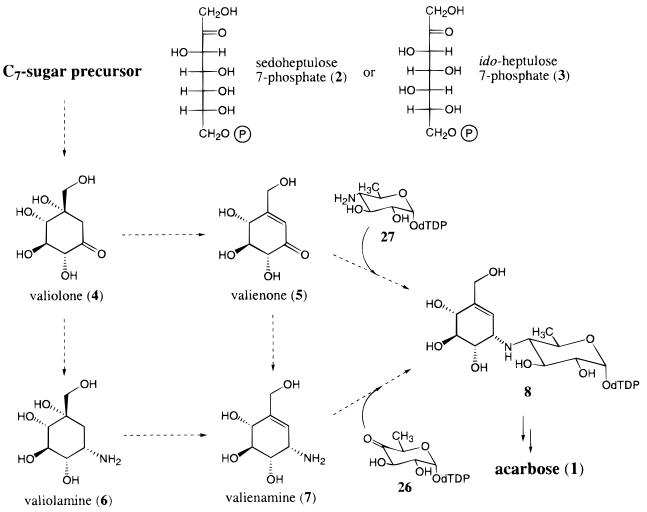
Scheme 1. Original Hypotheses for the Biosynthetic Pathway to Acarbose



is primarily responsible for the inhibition of α-glucosidase,[2] is also a structural component of other secondary metabolites, such as the adiposins,[3] amylostatins,[4] salbostatin,[5] trestatin,[6] and validamycins.[7] Valienamine and related amino-cyclitol structures can be viewed[8] as aliphatic analogues of *m*C_7N units which are found in many secondary metabolites,[9] particularly the ansamycins[10] and mitomycins.[11] The *m*C_7N unit in those metabolites is synthesized via a branch of the shikimate pathway,[12] whereas feeding experiments with stable isotope-labeled precursors have demonstrate that the *m*C_7N unit of acarbose[13] and validamycin A[14] is derived from the pentose phosphate pathway. Specifically, it was predicted that either sedoheptulose 7-phosphate (**2**), which has the same stereochemistry as **7** at all carbons except C-5, or its C-5 epimer, *ido*-heptulose 7-phosphate (**3**), is a proximate precursor of the *m*C_7N unit of acarbose (**1**) and validamycins. Genetic information[15] suggests that the cyclization of the heptulose 7-phosphate involves an enzyme resembling dehydroquinate (DHQ) synthase, which catalyzes the formation of DHQ from 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP),[16] and this notion has since been confirmed.[17]

The biosynthesis of the deoxysugar moiety of **1** conforms to that of other deoxyhexoses which are found in many secondary metabolites.[18,19] It involves the conversion of dTDP-D-glucose into dTDP-4-keto-6-deoxy-D-glucose through a stereospecific intramolecular hydride transfer[20] catalyzed by the pyridine nucleotide-containing enzyme dTDP-glucose 4,6-dehydratase. The two terminal glucose moieties of acarbose are derived not from free glucose but from maltose and maltotriose in the culture medium.[13] Using ^{3}H- and ^{2}H-labeled maltose and maltotriose, it was recently found that there are two metabolic routes from maltotriose to the maltose unit of **1**. Sixty percent of the acarbose is formed by attachment of maltose, produced by removing a glucose from the nonreducing end of maltotriose, to the pseudodisaccharide core unit. The other 40% of the acarbose is formed by direct attachment of maltotriose to the core unit, followed by loss of the terminal glucose from the reducing end.[21]

In contrast, little was known about the mode of formation of the valienamine moiety of acarbose from an initial pentose phosphate pathway-derived precursor, except that feeding experiments with ^{15}N-labeled precursors had demonstrated that glutamate is the primary source of the nitrogen.[22] Valienamine (**7**) itself was first predicted as a direct precursor of **1**, which through reductive condensation with dTDP-4-keto-6-deoxyglucose would give the pseudodisaccharide core unit **8** (Scheme 1). However, **8** could also be formed from valienone (**5**, also called gabosine[23]), the keto analogue of **7**, by connection with dTDP-4-amino-4,6-dideoxyglucose (**27**). Either **5** or **7** could presumably be derived from valiolone (**4**), which was considered a plausible first cyclization product of a linear seven-carbon sugar, sedoheptulose 7-phosphate (**2**) or *ido*-heptulose 7-phosphate (**3**). In this paper we report the synthesis of a series of isotopically labeled cyclitols as precursors and their use in feeding experiments to elucidate the biosynthetic pathway to the valienamine moiety of acarbose.

Results

Feeding Experiments with Tritiated Precursors. To probe which, if any, of the cyclitols **4**, **5**, **6**, and/or **7** shown in Scheme 1 are intermediates in the biosynthesis of acarbose, we prepared tritiated versions of these compounds and evaluated their incorporation into **1** by *Actinoplanes* sp. The tritiated precursors shown in Figure 2, [7-^{3}H]valienamine ([7-^{3}H]-**7**), [7-^{3}H]valienone ([7-^{3}H]-**5**), [7-^{3}H]valiolamine ([7-^{3}H]-**6**), and [7-^{3}H]-valiolone ([7-^{3}H]-**4**), were synthesized from valienamine as described by us previously.[24] In a first experiment, [7-^{3}H]-(**7**) (40 μCi, specific radioactivity = 100 mCi/mmol) was fed to a production culture of *Actinoplanes* sp. SN223/29, and it was found that less than 0.1% of the radioactivity was incorporated into **1**. Most of the radioactivity (75%) evaporated during the concentration of the sample solution in preparation for the CM-25 cation-exchange column chromatography, and the rest of it (25%) eluted much earlier than acarbose from the CM-25 column. This observation suggested the possibility that, at high specific radioactivity, i.e., low concentration, the labeled valienamine was metabolized extensively with formation of

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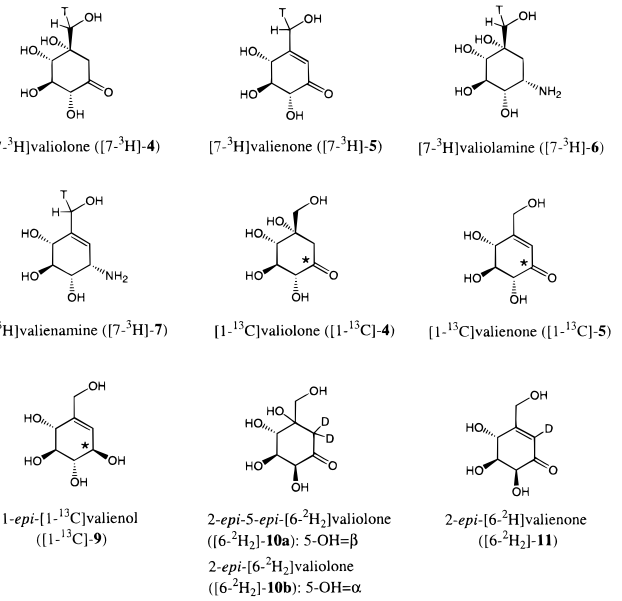


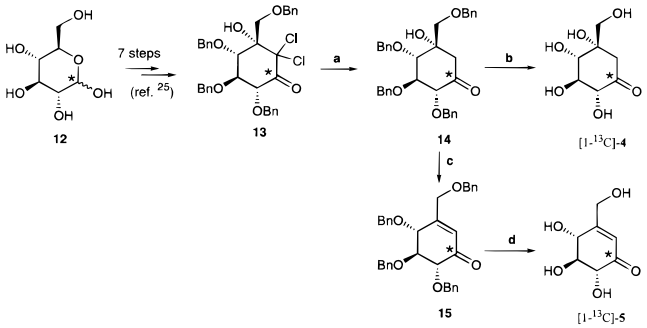
Figure 2. Structures of the ^{3}H-, ^{2}H-, and ^{13}C-labeled cyclitols synthesized and evaluated as acarbose precursors.

tritiated water and/or converted to a neutral compound. In an attempt to cut down on this presumed metabolic decomposition, the tritiated valienamine was diluted to 1 mCi/mmol and fed again (80 μCi) to a production culture. The incorporation of tritium into **1** was again below 0.1%. These results strongly suggest that valienamine is not an intermediate on the biosynthetic pathway to acarbose.

In view of the negative results with tritiated valienamine, we next considered the alternative scenario, in which valienone **5**, the keto compound corresponding to **7**, is connected to dTDP-4-amino-4,6-dideoxyglucose to give the pseudodisaccharide nucleotide **8**. [7-^{3}H]-**5** was therefore tested for incorporation into acarbose by feeding it (50 μCi, 10 mCi/mmol) to production cultures of *Actinoplanes* sp. strain SN223/29. Surprisingly, the incorporation of radioactivity into **1** was again only about 0.1%, suggesting that valienone was not a precursor in acarbose biosynthesis either. In the CM-25 cation-exchange column chromatography of the extract from this feeding experiment, some fractions preceding acarbose contained a substantial amount of radioactivity. These fractions were lyophilized and then analyzed by TLC and radioautography. The major radioactive spot had the same R_f value as valienone, and about 15 μCi of [7-^{3}H]-**5** (30%) was recovered from these fractions. This provided assurance that the tritium was not completely washed out from the precursor.

Since the feeding experiments with both tritiated valienamine and valienone had shown no incorporation of radioactivity into **1**, we next considered the possibility that the C−C double bond in the cyclitol moiety is introduced only after the linkage to the deoxyhexose moiety. In that case, valiolone **4** or valiolamine **6** would be plausible precursors of acarbose. **4** was a plausible candidate to be the first intermediate resulting from the cyclization of a heptulose 7-phosphate. **6** was a less likely choice as a precursor because, in this compound or its condensation product with dTDP-4-keto-6-deoxyglucose, there is no driving force for the elimination of the tertiary hydroxy group and the adjacent unactivated hydrogen to generate the double bond of the cyclitol moiety. Despite this reservation, feeding experiments were carried out with both [7-^{3}H]-**4** and [7-^{3}H]-**6**. Thus, three labeled cyclitols, [7-^{3}H]-**4** (22 μCi), [7-^{3}H]-**6** (22 μCi), and [7-^{3}H]-**7** (28 μCi) as a reference (all at 2.5 mCi/mmol specific radioactivity), were each fed to production cultures of *Actinoplanes* sp. SN223/29. Again, none of the compounds showed any significant incorporation into **1**. The resulting acarbose was purified to constant specific radioactivity by cocrystallization with carrier material, and the incorporations were determined as 0.016% for valiolone, 0% for valiolamine, and 0.01% for valienamine. In all cases, a large fraction (45−75%) of the radioactivity was released into the water of the medium during the fermentation and/or workup procedures. The fractions eluting from the CM-25 cation-exchange column with water or 0.5 N NH_4OH were assayed by TLC and found to contain the unchanged substrates. Totals of 14% of [7-^{3}H]-**4**, 6% of [7-^{3}H]-**6**, and 36% of [7-^{3}H]-**7**, respectively, were recovered from these fractions. This is in agreement with the recovery of 30% of unchanged tritiated valienone in the earlier experiment with [7-^{3}H]-**5**.

Scheme 2. Syntheses of [1-^{13}C]Valiolone and [1-^{13}C]Valienone[a]



[a] Conditions: (a) Bu_3SnH, AIBN, toluene, reflux, 70%. (b) 10% Pd−C, H_2, room temperature, 99%. (c) MsCl, Et_3N, TBME, room temperature, 85%. (d) BBr_3, CH_2Cl_2, −40 °C, 91%.

The negative results from the feeding experiments with the tritiated cyclitols in the acarbose producer can be interpreted in at least three different ways: (a) the investigated cyclitols are not intermediates on the biosynthetic pathway to **1**, (b) these substrates are not taken up by the cells, or (c) the tritium is completely washed out during the fermentation at the stage of the product **1** or a biosynthetic intermediate, but not the substrates. The second explanation seems unlikely given the metabolic tritium exchange, leaving mainly explanations a and c. We had considered the possibility of tritium exchange from the precursors and judged it to be unlikely; however, exchange from an intermediate or the product **1**, e.g., through a fast exchange process made possible by redox reactions at the C−N linkage, was a possibility. We therefore concluded that it was necessary to reexamine the biosynthetic pathway to **1** with stable isotope-labeled precursors and decided to synthesize ^{13}C-labeled valiolone and valienone.

Synthesis of [1-^{13}C]Valiolone ([1-^{13}C]-4) and [1-^{13}C]Valienone ([1-^{13}C]-5). The synthesis started from [1-^{13}C]glucose (**12**) and followed the procedure developed by Fukase and Horii,[25] allowing the preparation of 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valiolone (**14**), which was then converted to both [1-^{13}C]-valiolone ([1-^{13}C]-**4**) and [1-^{13}C]valienone ([1-^{13}C]-**5**) in a parallel fashion as shown in Scheme 2. The conversion of [1-^{13}C]-**4** into [1-^{13}C]-**5** was expected to occur easily under acidic conditions on the basis of our experience in the preparation of valiolone by oxidative deamination of valiolamine.[24] Only valiolone was detected in that work when the intermediate imine was hydrolyzed with oxalic acid at pH 4, whereas

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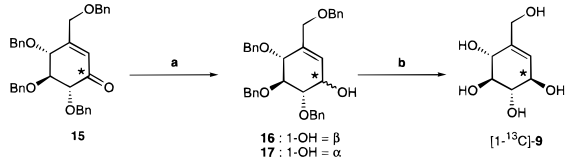
valienone and lesser amounts of valiolone were formed when the hydrolysis was conducted at a more acidic pH. However, valiolone itself was not dehydrated under strongly acidic conditions (pH 1) and even at elevated temperature (up to 80 °C). We then tried to synthesize [1-^{13}C]valienone from 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valiolone (**14**) by carrying out the dehydration first and then attempting debenzylation of the resulting 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valienone (**15**) without interfering with the α,*β*-unsaturated carbonyl system. Eventually, the conversion of **14** into **15** was carried out via a single-step dehydration using an excess of mesyl chloride and Et_3N, and the removal of the benzyl moieties from **15** was accomplished with BBr_3 in CH_2Cl_2 (Scheme 2).

Feeding Experiments with [1-^{13}C]Valiolone ([1-^{13}C]-4) and [1-^{13}C]Valienone ([1-^{13}C]-5). Both [1-^{13}C]-**4** and [1-^{13}C]-**5** (15 mg each) were fed to 100-mL production cultures of the acarbose producer. After isolation and purification by the standard procedure, the **1** samples were analyzed by ^{13}C NMR. No significant enrichment of ^{13}C was found at C-1 of acarbose in either experiment. At this point, we suspected that the labeled valiolone and valienone might be diluted with too much unlabeled material in the complex medium, and we therefore repeated the feeding experiment with [1-^{13}C]-**5** in resting cells of *Actinoplanes* sp SN223/29. Parallel experiments with a known precursor, [U-$^{13}C_3$]glycerol, and with [7-^{3}H]valienamine ([7-^{3}H]-**7**) were carried out simultaneously. [U-$^{13}C_3$]Glycerol was incorporated into **1** as expected,[13] but again [1-^{13}C]-**5** and [7-^{3}H]-**7** were not incorporated, and 65% of the [7-^{3}H]-**7** was recovered unchanged from the fermentation. These results leave little doubt that the nonincorporation into **1** of [1-^{13}C]-**5** and, by inference, of [1-^{13}C]-**4** in the previous experiments with production cultures was not an artifact of dilution of the labeled precursors with nutrients from the complex medium. The negative outcome of the experiments with the stable isotope-labeled cyclitols **4** and **5** also ruled out the possibility that the washout of tritium from a biosynthetic intermediate or from acarbose was responsible for the lack of incorporation of the tritiated cyclitols into **1**. Thus, it can be concluded that none of the four cyclitols tested, i.e., valiolone, valienone, valiolamine, and valienamine, is an intermediate on the biosynthetic pathway to acarbose. These results seemed to cast doubt on the proposed mechanism for the linkage of the cyclitol to the deoxysugar moiety by reduction of an imine formed between an amine and a ketone and caused us to consider alternatives.

Experiments with 1-*epi*-Valienol (9). In parallel to the feeding experiments described above, we also tried to obtain information on the biosynthetic pathway to **1** by analyzing the cyclitols in the fermentation broth and cell-free extracts of *Actinoplanes* sp SN223/29.[26] A compound was detected by ES-MS in both the fermentation broth and the cell-free extracts which had the same molecular weight, 176 Da, as valienol, the reduction product of **5** with the same C-1 stereochemistry as **7**. To identify the compound, the cell-free extracts were lyophilized and silylated, followed by GC−MS analysis. The GC−MS experiments revealed the presence of two compounds of molecular weight 176, showing virtually the same mass spectral fragmentation pattern. One was identical with valienol (T_{ret} = 11.12 min), while the other, having a higher retention time (T_{ret} = 11.48 min), was identified by co-injection with silylated synthetic [6-^{2}H]-**9** as 1-*epi*-valienol **9**.[27] Analyses of the time course of the concentrations of these two compounds in the cultures indicated that valienol appeared rather late in the fermentation, after substantial amounts of **1** had accumulated, suggesting that it might be a degradation product of **1**, whereas **9** appeared early in the fermentation, consistent with a possible role as a precursor of **1**.

Scheme 3. Synthesis of 1-*epi*-[1-^{13}C]Valienol[a]



[a] Conditions: (a) $NaBH_4$, $CeCl_3$, EtOH, 95% (**16**:**17** = 81:19). (b) BBr_3, CH_2Cl_2, −40 °C, 60%.

Mechanistic considerations also suggested the possibility that 1-*epi*-valienol might be a precursor of the valienamine moiety of **1**. C-1 of **9** could be linked to the C-4′ nitrogen of TDP-4-amino-4,6-dideoxyglucose by an S_N2 displacement of the C-1 oxygen, possibly after activation (e.g., as the phosphate or pyrophosphate), to give the correct stereochemistry of the pseudodisaccharide unit of **1**. Evidence for C−N bond formation by such a displacement has been obtained in studies on the biosynthesis of aristeromycin and neplanocin A in *Streptomyces citricolor*.[29] We therefore synthesized 1-*epi*-[1-^{13}C]valienol ([1-^{13}C]-**9**) by reduction of 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valienone (**15**) using $NaBH_4$ and $CeCl_3$, followed by debenzylation with BBr_3 in CH_2Cl_2 (Scheme 3). The product was fed to resting cells of *Actinoplanes* sp. SN223/29. The experiment gave no ^{13}C incorporation into **1**, indicating that 1-*epi*-valienol is not an intermediate in the formation of the cyclitol moiety of acarbose and ruling out the S_N2 displacement mechanism discussed above.

Synthesis of 2-*epi*-5-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10a), 2-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10b), and 2-*epi*-[6-^{2}H]Valienone ([6-^{2}H]-11). The original choice of cyclitols having the same C-2 stereochemistry as the valienamine moiety of **1**, such as **4** or **5**, as the precursors to be tested was predicated on the assumption that either *ido*-heptulose 7-phosphate is the open-chain substrate of the cyclase generating the cyclitol, or, if the substrate is sedoheptulose 7-phosphate, it undergoes epimerization at C-5 during cyclization. The nonincorporation of valiolone, valienone, and 1-*epi*-valienol suggested that this assumption may not be correct and that the immediate cyclization product generated by the cyclase may be 2-*epi*-valiolone (**10b**). 2-*epi*-Valiolone has the same C-2 configuration as **2**, making **2** the plausible substrate of the cyclase; the intermediacy of **10b** would require an epimerization during its further conversion into the valienamine moiety of **1**. **10b** also has the same C-5 configuration as natural valiolamine. To test whether **10b** was a precursor of **1**, the synthesis of 2-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**10b**) was pursued.

The synthesis of [6-$^2H_2$]-**10a**, its C-5 isomer [6-$^2H_2$]-**10b**, and its dehydration product, 2-*epi*-[6-$^2H_1$]valienone ([6-$^2H_2$]-**11**) (Scheme 4), proceeded from the commercially available starting material, 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (**18**), by the same route used to prepare [1-^{13}C]-**4** from **12** (cf. Scheme 2). Reduction of **20** with sodium borohydride yielded a mixture of isomers of acyclic heptitol derivative **21**. In contrast to the oxidation of the acyclic heptitol derived from glucose, which

(26) Lee, S.; Floss, H. G., unpublished results.

(27) [6-^{2}H]-**9** was synthesized[28] from 2,3,4,7-tetra-*O*-benzyl-D-glucose by adopting the procedure of Fukase and Horii.[25] The deuterium was introduced in the same way as in the preparation of [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** (Scheme 4).

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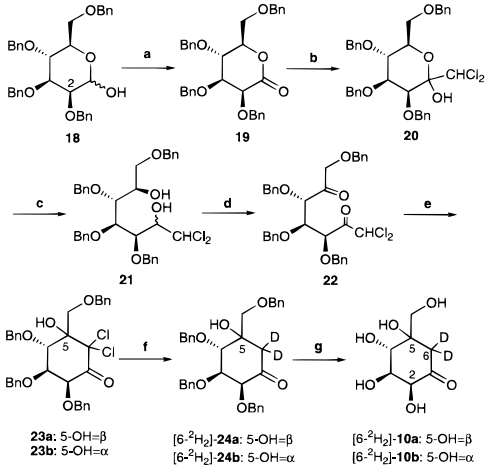
Scheme 4. Synthesis of 2-*epi*-5-*epi*-[6-$^2H_2$]Valiolone and 2-*epi*-[6-$^2H_2$]Valiolone[a]



[a] Conditions: (a) Ac_2O, DMSO, room temperature, quantitative. (b) LDA, CH_2Cl_2, −78 °C. (c) $NaBH_4$, THF/diglyme, room temperature. (d) TFAA, DMSO, Et_3N, CH_2Cl_2, −78 °C, three steps, 70%. (e) Sodium acetate, 18-crown-6, CH_2Cl_2, room temperature, 90%. (f) Bu_3SnD, AIBN, toluene, reflux, 80%. (g) 10% Pd−C, H_2, room temperature, quantitative.

was followed by spontaneous intramolecular aldol condensation to give a single cyclic dichloroinosose product, the oxidation of the diol **21** with DMSO, trifluoroacetic anhydride, and Et_3N unexpectedly gave only a stable 1,5-dioxoheptose, **22**. This result is probably due to **23a** or **23b** adopting a boat or a twist boat conformation ($J_{2,3}$ = 3.5 Hz, $J_{3,4}$ = 3.5 Hz), which is higher in energy than the chair form found in the glucose-derived dichloroinosose derivative **13**. Eventually, the intramolecular cyclization was accomplished with sodium acetate in the presence of 18-crown-6 but gave a mixture of diastereomers (**23a** and **23b**) in a 1.5:1 ratio, which could be separated by reversed-phase HPLC. As an alternative, the mixture of **23a** and **23b** was directly converted, via a free-radical reduction using Bu_3SnD/AIBN, to the deuterium-labeled 2,3,4,7-tetra-*O*-benzyl-2-*epi*-5-*epi*-valiolone ([6-$^2H_2$]-**24a**) and its 5-epimer [6-$^2H_2$]-**24b**, which showed a better separation profile on reversed-phase HPLC than the mixture of **23a** and **23b**.

Further experiments to assign the C-5 configurations in **23a** and **23b**, as well as [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b**, have been attempted, including chemical derivatizations and crystallization of **23a**, **23b**, [6-$^2H_2$]-**24a**, and [6-$^2H_2$]-**24b**, all of which were unsuccessful. In contrast to their 2-epimers derived from glucose, which are easily crystallized, these compounds were only obtained as syrups. Several trials to induce epimerization at C-2 via keto−enol tautomerization in **23a** and [6-$^2H_2$]-**24a** by treating them with LDA/THF, TEA/DMF, and *t*-BuOK/*t*-BuOH gave only the retro-aldol products. Compound **23a** was also found to be very stable under acidic conditions. We then attempted to assign the stereochemistry of [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** using NOE experiments, reasoning that the *S* configuration (**24b**) at C-5 would yield an NOE between 5-OH and 2-H and/or 3-H, whereas the corresponding *R* configuration would not show the enhancement. Thus, irradiation at 5-OH (2.61 ppm, s, disappeared in $CDCl_3$ + D_2O) of **24b** gave a strong NOE effect at 3-H and 2-H, indicating that **24b** has *S* configuration. On the other hand, irradiation at 5-OH (4.32 ppm, d, J = 1.5 Hz) of **24a** only showed an NOE at 4-H, which suggested the *R* configuration and indicated that **24a** was more likely to exist in a half-chair conformation, as also supported by the coupling constants between 2-H, 3-H, and 4-H ($J_{2,3}$= 3.5 Hz and$J_{3,4}$= 4 Hz) compared to the twist boat form of **24b** ($J_{2,3}$= 8 Hz and $J_{3,4}$= 3 Hz) (Figure 4).

Finally, both [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** were deprotected to the free cyclitols by catalytic hydrogenation using wet 10% Pd/C as the catalyst, giving [6-$^2H_2$]-**10a** and [6-$^2H_2$]-**10b**, respectively, in quantitative yields.

Next, [6-^2H]-**11**, a dehydrated form of both [6-$^2H_2$]-**10a** and [6-$^2H_2$]-**10b**, was prepared from 2,3,4,7-tetra-*O*-benzyl-2-*epi*-[6-^2H]valienone (**25**). A single-step dehydration of both [6-$^2H_2$]-**24a** and its 5-epimer [6-$^2H_2$]-**24b** using mesyl chloride and Et_3N produced **25** (Scheme 5). Monitoring of the reaction by HPLC indicated that the dehydration of [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** to **25** occurred immediately after the mesyl group attaches to the C-5 tertiary hydroxyl group in both isomers. In addition, the conversion rate of both isomers to **25** seems to be unaffected by the stereochemical and conformational differences between [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b**. To maintain the olefinic moiety in [6-$^2H_2$]-**11**, the debenzylation of **25** was carried out using 6 equiv of BBr_3 in CH_2Cl_2.

Feeding Experiments with 2-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10b), 2-*epi*-5-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10a), and 2-*epi*-[6-^2H]Valienone ([6-^2H]-11). To evaluate the role of the 2-*epi*-valiolones in acarbose biosynthesis, the synthesized [6-$^2H_2$]-**10a** and its C-5-epimer [6-$^2H_2$]-**10b** were each fed to resting cell cultures. A parallel experiment was carried out with [U-$^{13}C_3$]-glycerol as a standard. Analysis of the resulting acarbose by ES-MS, ^1H NMR, and ^2H NMR (Figure 3) showed that 2-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-**10b**) was not at all incorporated. However, its C-5 epimer, [6-$^2H_2$]-**10a**, was efficiently incorporated into acarbose (15% enrichment). Recovery of [6-$^2H_2$]-**10a** was relatively poor compared to that of other labeled cyclitols, which were usually recovered to the extent of 30−50%. These results provide strong evidence that 2-*epi*-5-*epi*-valiolone is a key intermediate in the biosynthesis of acarbose. This makes it likely that **10a** is the first cyclized compound on the pathway and that sedoheptulose 7-phosphate is the substrate of the cyclase generating it.

The next question was whether **10a** is first dehydrated to form 2-*epi*-valienone **11** and then connected to TDP-4-amino-4,6-dideoxyglucose and epimerized at C-2 to the correct stereochemistry, or whether all three steps occur on one enzyme. The nonincorporation of valiolone had already ruled out that epimerization precedes dehydration, and reductive amination of 2-*epi*-5-*epi*-valiolone as the first step would make epimerization and dehydration mechanistically implausible. We therefore fed [6-$^2H_2$]-**11** to the resting cells (10 mg to 60 mL). Analysis of the resulting **1** revealed no incorporation of deuterium, demonstrating that 2-*epi*-valienone is not an intermediate in the formation of acarbose.

Discussion

The results of the present investigation identify 2-*epi*-5-*epi*-valiolone **10a** as the only precursor of the valienamine moiety of **1** among all the cyclitols tested. This finding suggests that **10a** is the product of the cyclase, i.e., the first cyclitol generated, and it implies that the substrate of the enzyme must be sedoheptulose 7-phosphate **2**, which has the same stereochemistry as **10a**. Both of these conclusions have recently been verified by studies on the recombinant enzyme from *Actinoplanes* sp. catalyzing this cyclization.[17] This enzyme, which has pronounced sequence homology to dehydroquinate (DHQ) synthases, catalyzes the cyclization of **2**, but not that of *ido*-heptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone. In this cyclization, carbons 1−7 of **2** give rise to carbons 7, 5, 4, 3, 2, 1, and

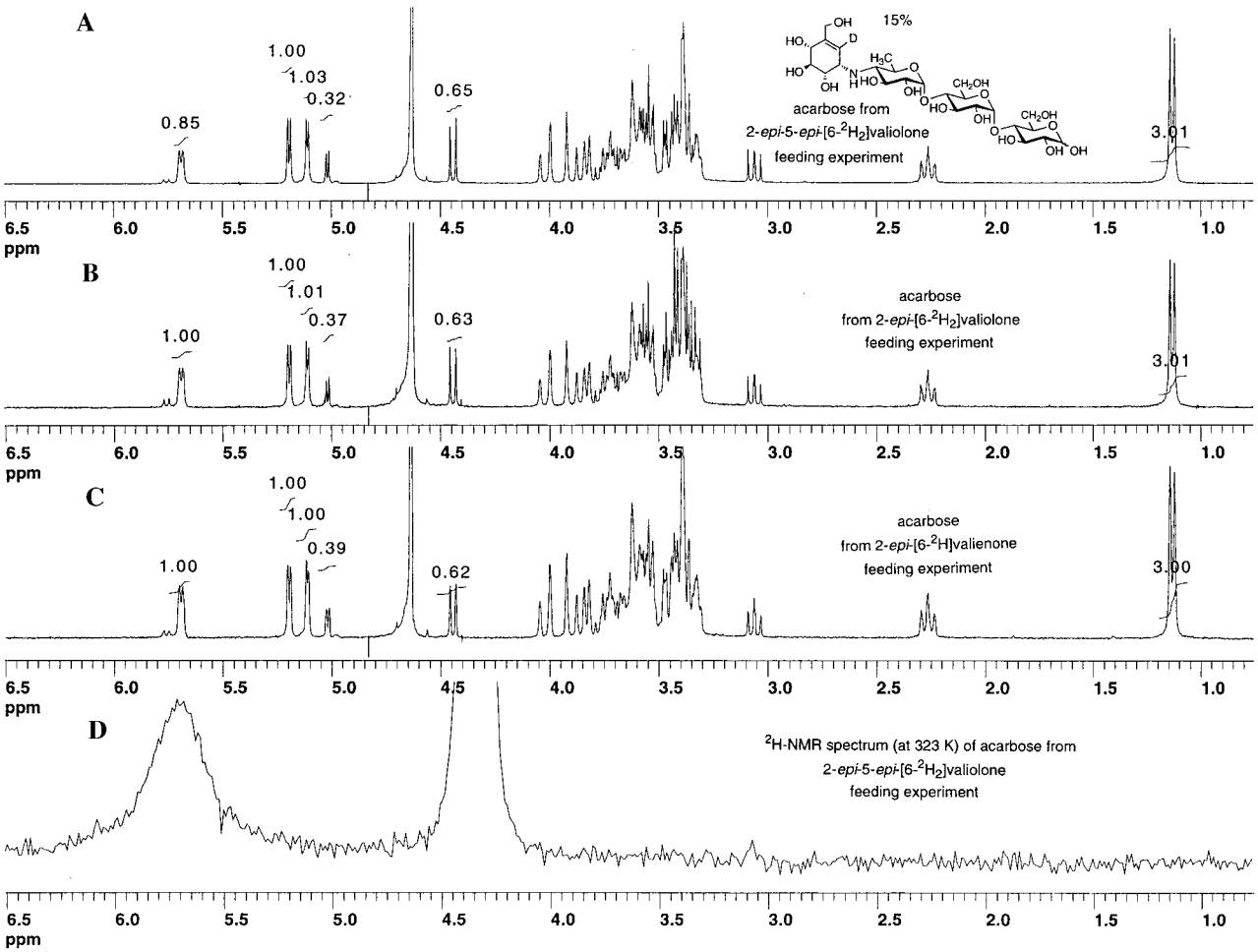


Figure 3. ^{1}H and ^{2}H NMR spectra of acarbose isolated from feeding experiments with deuterated precursors. (A) ^{1}H NMR of acarbose from 2-*epi*-5-*epi*-[6-^{2}H$_2$]valiolone ([6-^{2}H$_2$]-**10a**) feeding experiment. (B) ^{1}H NMR of acarbose from 2-*epi*-[6-^{2}H$_2$]valiolone ([6-^{2}H$_2$]-**10b**) feeding experiment. (C) ^{1}H NMR of acarbose from 2-*epi*-[6-^{2}H$_1$]valienone ([6-^{2}H$_2$]-**11**) feeding experiment. (D) ^{2}H NMR of acarbose from 2-*epi*-5-*epi*-[6-^{2}H$_2$]valiolone ([6-^{2}H$_2$]-**10a**) feeding experiment.

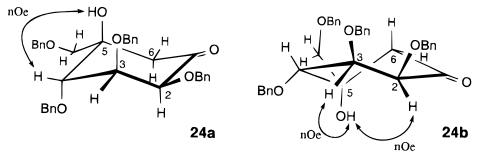
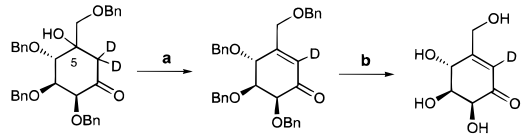


Figure 4. NOE experiments on compounds [6-^{2}H$_2$]-**24a** and [6-^{2}H$_2$]-**24b**.

Scheme 5. Synthesis of 2-*epi*-[6-^{2}H]Valienone[a]



[a] Conditions: (a) MsCl, Et_3N, TBME, room temperature, 90%. (b) BBr_3, CH_2Cl_2, −40 °C, 70%.

6, respectively, of the resulting cyclitol. The substrate stereochemistry at C-5 and C-6 conforms to that of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate, the substrate of dehydroquinate synthase, and as in the formation of DHQ, the configuration at C-5 is maintained, despite the fact that this carbon undergoes transient oxidation to the ketone.[30] The fact that, of the two C-5 epimers of **10**, it is the 5*R* isomer **10a** which is incorporated, and not the 5*S* isomer **10b**, was at first unexpected because the C-5 stereochemistry of **10b** matches that of natural valiolamine, which has been isolated from the validamycin producer, *Streptomyces hygroscopicus*.[31] However, the C-5 configuration of **10a** matches that of dehydroquinic acid at the corresponding carbon and is thus the stereochemical outcome predicted for a cyclization mediated by a DHQ synthase-like enzyme. Evidently, the naturally occurring valiolamine is not involved in the biosynthesis of validamycin; rather, it may be derived from a metabolic breakdown product of validamycin, such as valienamine.

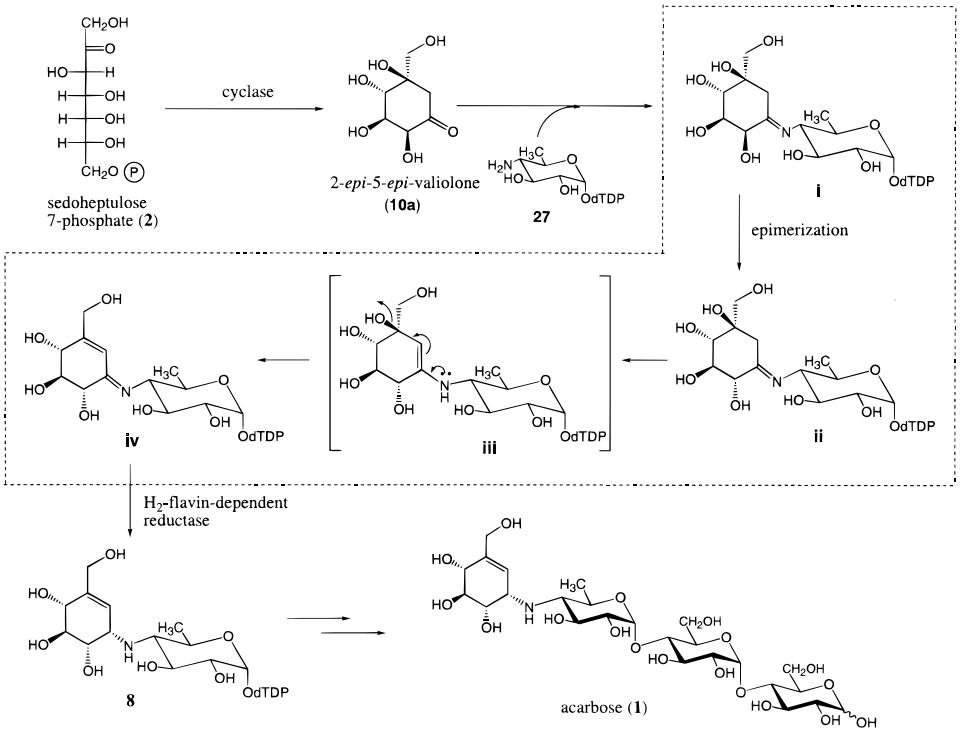
It came as a considerable surprise that none of the cyclitols having the same C-2 stereochemistry as the valienamine moiety of acarbose was incorporated into **1**. Hence, the biosynthesis of **1** does not proceed via any of the keto- or amino-cyclitols **4**, **5**, **6**, or **7** as free intermediates. This is particularly remarkable since parallel investigations on the biosynthesis of the antibiotic validamycin have revealed incorporation of not only **10a** but also valienone **5** into its valienamine moiety.[32] Evidently, there are significant differences in the pathways from **10a** to the valienamine moieties of acarbose and validamycin. The non-incorporation of 1-*epi*-valienol **9** into **1** is also noteworthy, as it rules out a biosynthetic process in which the cyclitol and the

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

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

(32) Dong, H., Ph.D. thesis, University of Washington, Seattle, 1998.

Scheme 6. Proposed Pathway for the Biosynthesis of Acarbose



deoxysugar moieties are connected by an S_N2 displacement of the 1-OH group of **9**, possibly in activated form, by the nitrogen of a 4-amino-6-deoxysugar derivative, as precedented in the biosynthesis of aristeromycin and neplanocin A.[29] This leaves as the most plausible alternative a process in which the two components are connected by formation and subsequent reduction of imine intermediates.

The structures of the two compounds undergoing reductive condensation to give the pseudodisaccharide nucleotide and the exact nature of this process cannot yet be specified, but the results do provide certain insights. The amino nitrogen of glutamate has been identified as the most proximate nitrogen source.[22] This suggests, as the simplest interpretation, that the nitrogen is first introduced into one of the keto precursors, either a keto-cyclitol or dTDP-4-keto-6-deoxyglucose (**26**, Scheme 1), followed by Schiff's base formation with the second keto component and reduction of the imine. The fact that neither valiolamine **6** nor valienamine **7** is incorporated into **1**, provided it is not due to permeability barriers, rules out transamination at the stage of the keto-cyclitols **4** or **5**. Transamination of 2-*epi*-5-*epi*-valiolone **10a** cannot be ruled out as the point of first introduction of the nitrogen, because we have not examined the incorporation of 2-*epi*-5-*epi*-valiolamine. However, the conversion of **10a** into **1** requires both epimerization at C-2 and dehydration to give the Δ-5,6 double bond. Prior transamination would leave no plausible mechanistic path for these essential reaction steps; hence, the intermediacy of 2-*epi*-5-*epi*-valiolamine seems very unlikely. Transamination of **26** to dTDP-4-amino-4,6-dideoxy-D-glucose **27**, on the other hand, is not encumbered by such mechanistic complications. The reaction is precedented in *Escherichia coli*, from which Strominger's laboratory partially purified an enzyme catalyzing the 4-transamination of dTDP-4-keto-6-deoxy-D-glucose to its 4-amino derivative.[33] An enzyme activity catalyzing the transamination of **26** to **27** has also been demonstrated in cell-free extracts of the **1**-producing *Actinoplanes* strain.[34] We therefore propose that the nitrogen is first introduced by transamination into **26** and that the resulting **27** then forms a Schiff's base with a keto-cyclitol, which is eventually reduced to the unique saturated C−N−C linkage found in **1**. However, more evidence at the enzymatic level is required in order to firmly establish this pathway and to rule out a more complicated alternative three-component process in which **26**, a keto-cyclitol, and the nitrogen are reductively condensed on a single enzyme to give the pseudodisaccharide moiety of **1**.

The conversion of **10a** into the pseudodisaccharide nucleotide **8** requires the following four reaction steps: (i) epimerization at C-2, (ii) dehydration between C-5 and C-6, (iii) condensation between the C-1 keto group and the amino group of **27**, and (iv) reduction of the resulting imine. A key question concerns the order in which these reaction steps take place. The nonincorporation into **1** of valiolone **4** rules out epimerization as the first step, and the nonincorporation of 2-*epi*-valienone rules out dehydration. Thus, Schiff's base formation between **10a** and the amine component, presumably **27**, must be the first reaction step. Mechanistic considerations dictate that epimerization and dehydration must occur prior to reduction of the imine double bond, because the amine resulting from this reduction would not provide any plausible mechanistic path for epimerization and dehydration. Thus, we propose that steps i and ii take place on the imine formed from **10a** and **27**, making use of the imine−enamine tautomerism to promote both of these reactions. The order of the epimerization and dehydration steps cannot be decided without further evidence; however, epimerization before dehydration would avoid the transient formation of a cross-conjugated enol and is thus slightly preferred. The final reduction of the imine to give **8** is likely to involve a reduced flavin as the enzymatic cofactor.

The mechanism we propose for the formation of the presumed **1** precursor, the pseudodisaccharide nucleotide **8**, based on the available information is shown in Scheme 6. Whether the

(33) Matsuhashi, M.; Strominger, J. L. *J. Biol. Chem.* **1966**, *241*, 4738.

(34) Lee, S.; Egelkrout, E.; Niggemann, J., unpublished results.

Schiff's bases **i** through **iv** are free intermediates in the process—i.e., whether the four steps i−iv listed above are each catalyzed by distinct, separate enzymes, or whether all the reactions occur on one single enzyme or enzyme complex, with **i** − **iv** only existing as enzyme-bound intermediates—remains a matter of speculation. Given that the diphosphate group provides a built-in acid catalyst for their hydrolysis and formation, compounds **i** − **iv** may be expected to be relatively unstable in the aqueous environment of the cell and to equilibrate with their components, **27** and the cyclitols **4**, **5**, and **10a**, respectively. Thus, the fact that none of the ketones **4**, **5**, and **11** showed even traces of incorporation into **1** may be taken as circumstantial evidence against the intermediacy of free **i**−**iv** in acarbose biosynthesis. Instead we favor the hypothesis that all the reactions from **10a** plus **27** to **8** take place on a single enzyme or enzyme complex, with Schiff's bases **i**−**iv** as enzyme-bound intermediates. Experiments are underway to examine this hypothesis specifically and **1** biosynthesis in general at the enzymatic level.

Experimental Section

General. The ^{1}H, ^{2}H, and ^{13}C NMR spectra were recorded on a Bruker AF-300 NMR spectrometer with *Mac*NMR 5.5 PCI as the instrument controller and data processor. HMBC and HMQC were recorded on a Bruker DRX-499 with SGI O2 computer. Infrared spectra were recorded on a Perkin-Elmer 1720 infrared Fourier transform spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Low-resolution electrospray mass spectra were recorded on a Kratos Profile double-focusing magnetic sector mass spectrometer. A Fison VG Quattro II electrospray ionization mass spectrometer was used to measure the isotope enrichment of acarbose. FAB-MS was recorded on a Micromass 70SEQ tandem hybrid mass spectrometer (EbqQ) with an RSX-11 operating system. A Hewlett-Packard 5890 GC with 5971A mass-selective detector was used for the GC−MS analysis of cyclitols. A ISF-4-V shaker, Adolf Kuhner AG, was used for the fermentations. Tritiated samples were counted in Bio-Safe II biodegradable counting cocktail (Research Products International Corp.) with a Beckman LS 1801 scintillation counter. Reactions were monitored by TLC on silica, and detection was accomplished by UV light or by alkaline permanganate spray and Ce-$(SO_4)_2/H_2SO_4$ solution.

Materials. *Actinoplanes* sp. SN223/29 was obtained from Bayer AG, Wuppertal, Germany. Tritiated valienamine, valienone, valiolamine, and valiolone were prepared in our laboratory from validamycin A as previously described.[24] All chemicals were purchased from Aldrich or Sigma and used without further purification unless otherwise noted. Ingredients for fermentations, soybean meal (fat free), maltzin powder, and yeast extract, were obtained from Bayer AG, Germany, and NZ-Amine A from ICN Biochemicals. Sephadex CM-25 was purchased from Pharmacia and Sephadex LH-20 from Sigma. Dianion WK-100 and Amberlite IR-68 were obtained from Supelco, and Amberlite CG-50, Dowex 50, and Dowex 1 were from Aldrich. Bio-Rad AG 1-8 was purchased from Bio-Rad. Surface autoradiography enhancer (EN^3-HANCE Spray) was obtained from DuPont and X-ray films from Amersham Life Science.

Culture Conditions for Feeding Experiments in Complex Medium. The inoculum suspension of *Actinoplanes* sp. SN223/29 (1 mL) was transferred into a 250-mL flask containing 50 mL of vegetative medium consisting of glycerol (2.5%), soybean meal (3%, fat free), and $CaCO_3$ (0.2%), pH 7.2, before sterilization. After incubation of the suspension for 3 days on a rotary shaker at 28 °C and 200 rpm, 5 mL of preculture was transferred to a 500-mL flask containing 45 mL of complex medium, consisting of maltzin powder (7.5%), yeast extract (0.7%), NZ-Amine A (0.3%), and K_2HPO_4 (0.3%), and incubated with shaking as above. All labeled compounds were fed as filter-sterilized solutions during the fermentation. The ^{3}H-labeled compounds were each added in two portions (half at 40 h, half at 50 h) to 50 mL of the production culture, harvesting after 90−96 h of total fermentation. [1-^{13}C]Valiolone and [1-^{13}C]valienone (15 mg) were each fed to 100-mL cultures, half at 38 h and half at 48 h, harvesting at 100 h of fermentation.

Culture Conditions for Feeding Experiments in Resting Cells. For the resting cell experiments, production cultures in complex medium were incubated for 28−30 h and centrifuged (4000*g* for 5 min) to remove the supernatant. The remaining cells were washed twice with cold 100 mM potassium phosphate buffer (pH 6.9) and then suspended in 50 mM potassium phosphate (pH 6.9) containing maltose (1.5%) and bactopeptone (0.15%) to make 75 mL of cell suspension from 50 mL of production culture. Each 60−75 mL of resting cells suspension in a 500-mL flask was incubated for 22−24 h on a rotary shaker at 220 rpm and 28 °C, with labeled substrates added at 0 h (one third) and the other two thirds at 8 h. Precursors fed were [1-^{13}C]valienone, 10 mg/75 mL; [U-$^{13}C_3$]glycerol, 100 mg/75 mL; [7-^{3}H]valienamine, 60 μCi/75 mL (specific radioactivity 2.5 mCi/mmol); 1-*epi*-[1-^{13}C]valienol, 15 mg/60 mL; 2-*epi*-5-*epi*-[6-$^{2}H_2$]valiolone, 15 mg/60 mL; 2-*epi*-[6-$^{2}H_2$]valiolone, 15 mg/60 mL; and 2-*epi*-[6-^{2}H]valienone, 10 mg/60 mL.

Isolation and Purification of Acarbose. Following incubation, the cultures from the complex medium and resting cells were harvested by centrifugation (4000*g* × 20 min). The supernatant containing acarbose was treated with an equal volume of methanol to prevent bumping and concentrated under reduced pressure to a syrup. The residue was dissolved in water (20 mL) and treated with methanol (80 mL). The mixture was stirred at room temperature for 2 h and centrifuged. The supernatant was concentrated to dryness under reduced pressure. The residue was dissolved in 5 mL of water, centrifuged to remove insoluble material, and applied to a CM-25 weak cation-exchange column (1.5 × 60 cm). The column was eluted with deionized water, and the fractions (each 7.5−8 mL) containing acarbose (usually between fractions 65 and 80), as detected by UV monitoring at 210 nm, were pooled. The acarbose-containing fractions were evaporated to dryness under reduced pressure for further analysis by ^{1}H-, ^{2}H-, and ^{13}C NMR or electrospray mass spectrometry (ES-MS). The radioactivity of acarbose was determined by liquid scintillation counting. The amounts of acarbose isolated from the production cultures were about 30−40 mg from the incubations with ^{3}H-cyclitols (50-mL cultures), 60 mg from that with [1-^{13}C]-**4**, and 70 mg from the incubation with [1-^{13}C]-**5** (100-mL cultures). In the resting cell experiments, the amounts were 9 mg from [1-^{13}C]-**5**, 8 mg from [U-$^{13}C_3$]glycerol (2−3% incorporation), 4 mg from [1-^{13}C]-**9**, 7 mg from [6-$^{2}H_2$]-**10a**, 6.5 mg from [6-$^{2}H_2$]-**10b**, and 6 mg from [6-^{2}H]-**11**, respectively.

Reisolation of Labeled Substrates. The earlier fractions (fractions 10−25) from the isolation of acarbose by CM-25 cation-exchange column chromatography contained stable isotope-labeled neutral substrates, i.e., [1-^{13}C]-**4**, [1-^{13}C]-**5**, [1-^{13}C]-**9**, [6-$^{2}H_2$]-**10a**, [6-$^{2}H_2$]-**10b**, or [6-^{2}H]-**11**. These fractions were lyophilized (100 μL each) and analyzed by both TLC and GC−MS; R_f values on TLC (*n*-butanol/ethanol/water = 9:7:4) were valiolone, 0.45; valienone, 0.64; 1-*epi*-valienol, 0.54; 2-*epi*-valiolone and its C5-epimer, 0.56; and 2-*epi*-valienone, 0.66. GC−MS (T_{ret} in min): valiolone, 11.61; valienone, 11.51; 1-*epi*-valienol, 11.48; 2-*epi*-valiolone, 11.02; 2-*epi*-5-*epi*-valiolone, 11.06; and 2-*epi*-valienone, 10.90. In the case of radiolabeled neutral substrates, [7-^{3}H]-**4** and [7-^{3}H]-**5**, the fractions showing high radioactivity were lyophilized, and then they were analyzed by thin-layer autoradiography (treated with autoradiography enhancer). The amine substrates, [1-^{3}H]-**7** and [1-^{3}H]-**6**, were recovered by elution of the CM-25 column with 0.5 N NH_4OH after **1** had been eluted with water. They were identified by radio-TLC and GC−MS: R_f values on TLC (2-propanol/AcOH/H_2O): valienamine, 0.39; valiolamine, 0.30. GC−MS (T_{ret} in min): valienamine, 11.33; valiolamine, 12.24.

Isolation and Identification of Valienol and 1-*Epi*-valienol from *Actinoplanes* sp. SN223/29. (A) From the Fermentation Broth. The culture (10 mL) from the complex medium was centrifuged (4000*g* × 20 min), and the supernatant was passed through a Dowex 50 (H^+ form, 1.5 × 5 cm) column. The column was eluted with water, and the eluate was concentrated under reduced pressure. The residue was dissolved in water (0.5 mL) and applied to a Sephadex G-15 column (1.5 × 30 cm). The column was eluted with water, and the fractions containing valienol and 1-*epi*-valienol were pooled. An aliquot (100 μL) was lyophilized and silylated with Sigma-sil A (50 μL) prior to GC−MS

analysis. GC−MS (T_{ret} in min): TMS-valienol, 11.12; TMS-1-*epi*-valienol, 11.48.

(B) From the Cells. The cells collected from the above culture were resuspended in water (10 mL) and disrupted by sonication. The cell debris was removed by centrifugation (10000*g* × 10 min), and the supernatant was subjected to filtration using a Centricon-10. The filtrate was passed through a Dowex 50 (H^+ form) column, which was eluted with water. The eluate was concentrated and subjected to Sephadex G-15 column chromatography with elution with water. The fractions containing valienol and 1-*epi*-valienol were pooled, and an aliquot (100 μL) was lyophilized and silylated with Sigma-sil A (50 μL) prior to GC−MS analysis.

Synthesis of Labeled Substrates. 2,3,4,7-Tetra-*O*-benzyl-[1-^{13}C]-valiolone (14). To a solution of **13** (3 g, 4.8 mmol) in toluene (40 mL) were added tributyltin hydride (5 mL, 18.6 mmol) and AIBN (300 mg, 1.8 mmol), and the mixture was refluxed for 2 h and cooled to room temperature. The product was extracted with EtOAc (50 mL) and washed sequentially with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic solvent was evaporated under reduced pressure and the residue purified by silica gel column chromatography (*n*-hexane/CH_2Cl_2/MeOH = 12:4:1) to give **14** (1.85 g, 70%).

14. Colorless syrup, $[\alpha]_D$ + 44.6° (*c* = 0.35, $CHCl_3$, 22 °C). FAB-MS: *m*/*z* 576 $(M + Na)^+$. HR FAB-MS: *m*/*z* calcd for $^{12}C_{34}{}^{13}C_1H_{36}O_6$-Na $(M + Na)^+$ 576.2443, found 576.2468. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.37 (d, *J* = 2 Hz, 5-OH), 2.46 (dd, *J* = 7, 15 Hz, 6-Heq), 2.85 (bdd, *J* ≈ 5, 15 Hz, 6-Hax), 3.15 (d, *J* = 9 Hz, 7-Ha), 3.53 (dd, *J* = 1, 9 Hz, 7-Hb), 4.01−4.15 (m, H-2, H-3, H-4), 4.38−5.00 (Ph-CH_2- × 4), 7.15−7.45 (m, C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 45.5 (t, J_{C-C} = 35.0 Hz, C-5), 72.7 (t, J_{C-C} = 3.6 Hz, C-7), 73.3, 74.1, 75.7, 76.0 (all t, Ph-CH_2- × 4), 80.0 (d, C-4), 85.5 (d, J_{C-C} = 35.0 Hz, C-2), 83.1 (d, J_{C-C} = 2.5 Hz, C-3), 127.6−128.4 (all d), 137.4, 137.7, 137.8, 138.4 (all s, C_6H_5- × 4), 203.9 (^{13}C-labeled C═O).

[1-^{13}C]Valiolone ([1-^{13}C]-4). To a solution of **14** (55 mg, 0.1 mmol) in 95% aqueous ethanol (5 mL) was added 10% wet Pd/C (55 mg), and the mixture was stirred at room temperature under an H_2 atmosphere overnight. The suspension was passed through a Celite column to remove the catalyst and subsequently was filtered through a 0.2-μm membrane filter. The solvent was evaporated under reduced pressure to give pure [1-^{13}C]-**4** (19.2 mg, 99%).

Colorless syrup, $[\alpha]_D$ + 0.30° (*c* = 0.54, MeOH, 22 °C). FAB-MS: *m*/*z* 216 $(M + Na)^+$. HR FAB-MS: *m*/*z* calcd for $^{12}C_6{}^{13}C_1H_{12}O_6Na$ $(M + Na)^+$ 216.0565, found 216.0557. ^{1}H NMR (CD_3OD, 500 MHz): δ 2.36 (dd, *J* = 6, 14 Hz, H-6a), 2.82 (ddd, *J* = 1, 5,5, 14 Hz, H-6b), 3.30 (d, *J* = 11 Hz, H-7a), 3.69 (t, *J* = 9.5 Hz, H-3), 3.72 (d, *J* = 11 Hz, H-7b), 3.84 (d, *J* = 9.5 Hz, H-4), 4.06 (ddd, *J* = 1, 3, 9.5 Hz, H-2). ^{13}C NMR (CD_3OD, 125 MHz): δ 46.3 (t, J_{C-C} = 41 Hz, C-6), 66.4 (t, J_{C-C} = 3.9 Hz, C-7), 73.7 (d), 70.6 (d), 77.3 (d), 79.6 (d, J_{C-C} = 37 Hz, C-2), 207.8 (s, ^{13}C-labeled C═O).

2,3,4,7-Tetra-*O*-benzyl-[1-^{13}C]valienone (15). To a solution of **14** (425 mg, 0.76 mmol) in TBME (30 mL) were added mesyl chloride (0.58 mL) and triethylamine (2.1 mL), and the mixture was stirred under argon at room temperature for 4 h. The reaction was quenched by addition of saturated aqueous Na_2CO_3 (12 mL), and stirring was continued for 30 min. The reaction mixture was partitioned between TBME and H_2O. The organic layer was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine. After the solution was dried over Na_2SO_4, the solvent was evaporated. The residue was chromatographed on a silica gel column with *n*-hexane/EtOAc (6:1) to give **15** (348 mg, 85%).

Colorless syrup, $[\alpha]_D$ −9.6° (*c* = 0.49, $CHCl_3$, 22 °C). FAB-MS: *m*/*z* 536 $(M + H)^+$. HR FAB-MS: *m*/*z* calcd for $^{12}C_{34}{}^{13}C_1H_{35}O_5Na$ $(M + H)^+$ 536.2518, found 536.2519. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.98−4.09 (m, 2-H, 3-H), 4.08 (br d, *J* ≈ 15 Hz, 7-Ha), 4.26 (br d, *J* ≈ 15 Hz, 7-Hb), 4.37 (br d, *J* ≈ 7 Hz, 4-H), 4.51 (s, Ph-CH_2-), 4.66−5.13 (Ph-CH_2- × 3), 6.22 (dd, *J* = 1.5, 2 Hz, 6-H), 7.21−7.45 (m, C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 68.9 (t, J_{C-C} = 4.9 Hz, C-7), 73.1, 74.4, 75.6, 75.7 (all t, Ph-CH_2- × 4), 79.1 (d, C-4), 83.9 (d, J_{C-C} = 40.3 Hz, C-2), 84.9 (d, J_{C-C} = 2.5 Hz, C-3), 123.9 (d, J_{C-C} = 51.3 Hz, C-6), 159.1 (s, C-5), 127.7−128.5 (all d), 137.4−138.0 (all s, C_6H_5- × 4).

[1-^{13}C]Valienone ([1-^{13}C]-5). To a solution of **15** (145 mg, 0.27 mmol) in CH_2Cl_2 (7 mL) was added of 1.0 M BBr_3 in hexane (1.6 mL) dropwise with stirring under argon at −40 °C, and stirring was continued for 30 min. The reaction mixture was allowed to warm to 0 °C, MeOH (3 mL) and H_2O (3 mL) were added, and the mixture was warmed to room temperature with stirring for 15 min. The reaction mixture was transferred to a separatory funnel and partitioned between CH_2Cl_2 and H_2O. The H_2O layer was directly subjected to anion-exchange chromatography (Bio-Rad AG1-X8, formate form, 20 mL), and the product was lyophilized to dryness. The residue was dissolved in MeOH and repurified over Sephadex LH-20 (50 mL, MeOH) to give [1-^{13}C]-**5** (43 mg, 91%).

Colorless syrup, $[\alpha]_D$ −61.7° (*c* = 0.25, MeOH, 22 °C). ESI-MS: *m*/*z* 198 $(M + Na)^+$. ^{1}H NMR (300 MHz, D_2O): δ 3.58 (ddd, *J* = 1, 9, 11 Hz, H-3), 4.05 (dd, *J* = 4, 11 Hz, H-2), 4.26, 4.33 (ABq, *J* = 19 Hz, H-7a,b), 4.36 (br d, *J* ≈ 9 Hz, H-4), 6.00 (dd, *J* = 2, 4 Hz, H-6). ^{13}C NMR (75 MHz, D_2O): δ 59.1 (t, J_{C-C} = 4.8 Hz, C-7), 70.4 (d, C-4), 74.5 (d, J_{C-C} = 40.3 Hz, C-2), 75.5 (d, J_{C-C} = 2.5 Hz, C-3), 119.0 (d, J_{C-C} = 52.5 Hz, C-6), 164.6 (s, C-5), 198.1 (s, C-1).

2,3,4,7-Tetra-*O*-benzyl-1-*epi*-[1-^{13}C]valienol (16). A suspension of $CeCl_3$ (138 mg, 0.56 mmol) in ethanol (3.3 mL) was cooled to −78 °C, and sodium borohydride (42 mg, 1.12 mmol) was added. The mixture was stirred at − 78 °C for 1 h. A solution of 2,3,4,7-tetra-*O*-benzyl-[1-^{13}C]valienone (**15**) (200 mg, 0.37 mmol) in ethanol/THF (1:1, 2 mL) was added dropwise with stirring under argon at −78 °C, and stirring was continued for 1 h. The reaction mixture was allowed to warm to 0 °C, and cold EtOAc (10 mL) and 2 N HCl (5 mL) were added. The organic layer was washed with H_2O, saturated aqueous $NaHCO_3$, and brine. The organic solvent was removed under vacuum, and the residue was subjected to silica gel column chromatography (*n*-hexane/EtOAc = 5:1−4:1) to give **16** (163 mg, 81%).

White solid, $[\alpha]_D$ −73.0° (*c* = 0.38, $CHCl_3$, 22 °C). FAB-MS: *m*/*z* 560 $(M + Na)^+$. HR FAB-MS: *m*/*z* calcd for $^{12}C_{34}{}^{13}C_1H_{36}O_5Na$ $(M + Na)^+$ 560.2494, found 560.2479. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.55 (ddd, *J* = 5, 8, 9.5 Hz, 2-H), 3.83 (ddd, *J* = 2.5, 7.0, 9.5 Hz, 3-H), 3.89 (br d, *J* ≈ 12 Hz, 7-Ha), 4.22 (br d, *J* ≈ 12 Hz, 7-Hb), 4.27−4.34 (2H, m, 1-H and 4-H), 4.42−4.97 (Ph-CH_2-), 5.71 (br s, H-6), 7.22−7.33 (m, C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 69.9 (t, J_{C-C} = 6.1 Hz, C-7), 71.2 (d, C-1), 79.6 (d, C-3), 83.5 (d, C-4), 83.8 (d, J_{C-C} = 34.2 Hz, C-2), 127.1 (d, J_{C-C} = 45 Hz, C-6), 135.9 (s, J_{C-C} = 2.44 Hz, C-5).

1-*epi*-[1-^{13}C]Valienol ([1-^{13}C]-9). To a solution of **16** (115 mg, 0.21 mmol) in CH_2Cl_2 (5.4 mL) was added 1.0 M BBr_3 in hexane (1.28 mL) dropwise with stirring under argon at −40 °C, and stirring was continued for 30 min. The reaction mixture was allowed to warm to 0 °C, MeOH (2.5 mL) and H_2O (2.5 mL) were added, and the mixture was warmed to room temperature with stirring for 15 min. The reaction mixture was transferred to a separatory funnel and partitioned between CH_2Cl_2 and H_2O. The H_2O layer was subjected to anion-exchange chromatography (Bio-Rad AG1-X8, formate form, 20 mL), and the product was lyophilized to dryness. The residue was dissolved in MeOH and repurified over Sephadex LH-20 (50 mL, MeOH) to give [1-^{13}C]-**9** (23 mg, 61%).

Colorless syrup, $[\alpha]_D$ −32.5° (*c* = 0.22, MeOH, 22 °C). ESI-MS: *m*/*z* 200 $(M + Na)^+$. ^{1}H NMR (300 MHz, CD_3OD): δ 3.36−3.47 (m, 2-H and 3-H), 3.81 (m), 4.35 (md, J_{C-H} ≈ 160 Hz, H-1), 4.05−4.20 (3H, m, 4-H and 7-H), 5.57 (dd, *J* = 1.5, 3.5 Hz, 6-H). ^{13}C NMR (75 MHz, CD_3OD): δ 69.3 (^{13}C-labeled C-1).

Lactone 19. To a solution of 2,3,4,7-tetra-*O*-benzyl-D-mannose (**18**) (5.0 g, 9.25 mmol) in DMSO (50 mL) was added acetic anhydride (35 mL), and the mixture was stirred overnight at room temperature. The reaction mixture was poured into ice water (1 L) and extracted with TBME (3 × 150 mL). The organic layer was washed with 2 N HCl, H_2O, saturated aqueous $NaHCO_3$, and brine. After the solution was dried over Na_2SO_4, the solvent was evaporated under vacuum. The residue was chromatographed on a silica gel column (*n*-hexane/EtOAc = 3:1), and the product was recrystallized from *n*-hexane/TBME to give **19** in quantitative yield.

White needles, $[\alpha]_D$ + 0.37° (*c* = 0.10, $CHCl_3$, 22 °C). FAB-MS: *m*/*z* 539 $(M + Na)^+$. HR FAB-MS: *m*/*z* calcd for $C_{34}H_{36}O_5Na$ $(M + Na)^+$ 539.2433, found 539.2408. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.63 (d, *J* = 5 Hz), 3.77 (dd, *J* = 7, 1.5 Hz), 4.05 (m), 4.24 (m), 4.23 (d, *J* = 11 Hz), 6.32 (s, *J* = 11 Hz), 4.35 (d, *J* = 2.5 Hz), 4.53 (br s), 4.58

(d, J = 12 Hz), 4.63 (d, J = 12 Hz), 4.83 (d, J = 12 Hz), 5.05 (d, J = 12 Hz), 7.06−7.10 (m), 7.27−7.37 (m).

2,3,4,6-Tetra-*O*-benzyl-1-*C*-(dichloromethyl)-D-mannopyranose (20). A 2.0 M solution of lithium diethylamide (LDA) in THF/heptane/ethylbenzene (12.5 mL) was added dropwise to a solution of **19** (4.5 g) in CH_2Cl_2 (17 mL) with stirring under argon at −78 °C, and stirring was continued for 1 h at the same temperature. The mixture was poured into ice-cooled CH_2Cl_2 and 2 N HCl (150 mL of each). The organic layer was washed with H_2O, saturated aqueous $NaHCO_3$, and brine, and then dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on a silica gel column with *n*-hexane/ethyl acetate (4:1) to give **20** (4.7 g).

Colorless syrup, $[\alpha]_D$ −14.0° (c = 0.50, $CHCl_3$, 22 °C). FAB-MS: m/z 640 $(M + NH_4)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{40}NO_6{}^{35}Cl_2$ $(M + NH_4)^+$ 640.2232, found 640.2203. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.41 (br s), 3.70 (br s), 3.97 (s), 3.98 (d, J = 7 Hz), 4.13 (m), 4.28 (d, J = 2.5 Hz), 4.49−4.61 (m, 4H), 4.76 and 4.82 (AB q, J = 12 Hz), 4.86 (d, J = 11 Hz), 5.13 (d, J = 11 Hz), 6.07 (d, J = 1 Hz), 7.18−7.37 (m, C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 60.4 (t), 69.0 (t), 72.8 (t), 73.3 (t), 74.4 (d), 75.1 (t), 75.9 (d), 81.9 (d), 98.3 (s), 127.1−129.7 (all d) and 138.2−138.3 (all s) (C_6H_5 × 4).

(1*R*)- and (1*S*)-Isomer of 2,3,4,6-Tetra-*O*-benzyl-1-*C*-(dichloromethyl)-D-mannitol (21). To a solution of **20** (4.5 g, 7.2 mmol) in diglyme/THF (1:1; 58 mL) was added $NaBH_4$ (2.25 g, 59.5 mmol), and the mixture was stirred overnight at room temperature. The mixture was concentrated and partitioned between EtOAc (200 mL) and water (100 mL). The organic layer was washed with 2 N HCl, water, saturated aqueous $NaHCO_3$, and brine and dried over Na_2SO_4. The organic solvent was evaporated under low pressure, and the residue was chromatographed on a silica gel column (*n*-hexane/CH_2Cl_2/MeOH = 10:4:1) to give **21** (4.77 g).

Yellowish syrup. FAB-MS: m/z 625 $(M + H)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{39}O_6{}^{35}Cl_2$ $(M + H)^+$ 625.2124, found 625.2100. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.52 (dd, J = 6.3, 9.7 Hz), 3.61 (dd, J = 5.5, 9.1 Hz), 3.68 (m), 3.77 (dd, J = 4.4, 7.3 Hz), 3.89 (m), 4.07−4.39 (m), 4.43−4.79 (Ph-CH_2- × 4), 5.68 (d, J = 7.3 Hz), 6.15 (d, J = 1.9 Hz), 7.17−7.30 (C_6H_5- × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 70.0, 70.7, 71.0, 73.1, 73.1, 73.2, 73.3, 74.0, 74.3, 74.7, 75.1, 75.5, 76.1, 76.5, 78.5, 79.3, 79.8, 80.1, 80.7, 126.8−128.4 (all d) and 137.2−137.9 (all s) (C_6H_5 × 4).

Dioxoheptose 22. To a solution of DMSO (1.4 mL, 26 mmol) in CH_2Cl_2 (15 mL) was added trifluoroacetic anhydride (1.8 mL, 13 mmol) in CH_2Cl_2 (7.5 mL) dropwise at −78 °C, and stirring was continued for 30 min. Then a solution of **21** (2 g, 3.2 mmol) in CH_2Cl_2 (11 mL) was added dropwise to the reaction mixture at −78 °C, and stirring was continued for 1 h at the same temperature. To the reaction mixture was added triethylamine (3.6 mL, 26 mmol) in CH_2Cl_2 (15 mL) dropwise at −78 °C, and stirring was continued for 30 min. The reaction mixture was allowed to warm to 0 °C and then poured into cold CH_2Cl_2 (40 mL) and water (20 mL), and stirring was continued for 30 min. The organic layer was washed with 2 N HCl, water, saturated aqueous $NaHCO_3$, and brine and then dried over Na_2SO_4, and the solvent was evaporated in vacuo. The extract was subjected to silica gel column chromatography (*n*-hexane/EtOAc = 4:1) to give **22**.

Yellowish syrup, $[\alpha]_D$ −19.1° (c = 0.16, $CHCl_3$, 22 °C). FAB-MS: m/z 638 $(M + NH_4)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{38}NO_6{}^{35}Cl_2$ $(M + NH_4)^+$ 638.2076 found 638.2049. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.15 (dd, J = 4, 4.5 Hz, 3-H), 4.30 (dd, J = 11, 1.5 Hz, 7-Ha), 4.47 (d, J = 11 Hz, 7-Hb), 4.36 (d, J = 4.5 Hz, 4-H), 4.58 (d, J = 4 Hz, 2-H), 6.40 (s, 6-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 68.6 (t, C-7), 74.6 (t, −$CHCl_2$), 79.5 (d, C-3), 81.8 (d, C-2 and C-4), 73.1, 73.2, 73.9, 94.6 (all t, PhCH_2 × 4), 127.5−129.0, 136.1 (s), 136.2 (s), 136.4 (s), 137.1 (s) (C_6H_5 × 4), 194.7, 206.2 (both s, C-1 and C-5).

(5*R*)- and (5*S*)-Isomer of (2*S*,3*S*,4*S*)-2,3,4-Tri-*O*-benzyl-5-*C*-[(benzyloxy)methyl]-6,6-dichloro-1-oxo-2,3,4,5-cyclohexanetetrol (23a and 23b). To a solution of **22** (1.6 g, 2.58 mmol) in toluene (50 mL) were added sodium acetate (1.48 g, 18 mmol) and 18-crown-6 (24 mg, 0.09 mmol). The mixture was stirred at room temperature for 3 days. The residue was filtered, and the filtrate was washed with 2 N HCl, water, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The extract was subjected to silica gel column chromatography (*n*-hexane/EtOAc = 6:1) to give a mixture of isomers **23a** and **23b** (1.0 g, 62%). Compounds **23a** and **23b** were separated on an analytical scale using reversed-phase HPLC (Econosil C_{18} 10U 250 × 10 mm, MeOH/H_2O 85:15).

23a: yellowish syrup. FAB-MS: m/z 621 $(M + H)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{35}O_6{}^{35}Cl_2$ $(M + H)^+$ 621.1810, found 621.1804. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.54 (dd, J = 11, 2.5 Hz, 7-Ha), 3.85 (d, J = 11 Hz, 7-Hb), 3.94 (d, J = 2.5 Hz, 5-OH), 4.00 (t, J = 4 Hz, 3-H), 4.51 (d, J = 4 Hz, 4-H), 5.19 (d, J = 4 Hz, 2-H), 4.36−4.87 (PhCH_2- × 4), 7.09−7.36 (C_6H_5 × 4).

23b: yellowish syrup. FAB-MS: m/z 638 $(M + NH_4)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{38}NO_6{}^{35}Cl_2$ $(M + NH_4)^+$ 638.2076, found 638.2057. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.71 (dd, J = 10, 2.5 Hz, 7-Ha), 4.02 (t, J = 3.5 Hz, H-3), 4.11 (d, J = 4 Hz, 4-H), 4.26 (br d, J = ca. 10 Hz, 7-Hb), 4.45 (d, J = 3.5 Hz, 2-H), 4.31−4.81 (PhCH_2- × 4), 6.99−7.28 (C_6H_5 × 4).

2,3,4,7-Tetra-*O*-benzyl-2-*epi*-5-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-24a) and 2,3,4,7-Tetra-*O*-benzyl-2-*epi*-[6-$^2H_2$]valiolone ([6-$^2H_2$]-24b). To a solution of the mixture of **23a** and **23b** (1 g, 1.6 mmol) in toluene (10 mL) were added tributyltin deuteride (1.72 mL, 6.4 mmol) and AIBN (104 mg, 0.64 mmol), and the mixture was refluxed for 2 h and then cooled to room temperature. The products were extracted with EtOAc (50 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 (*n*-hexane/EtOAc = 6:1−3:1) to give a mixture of [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** (550 mg, 62%). The products were separated by reversed-phase HPLC (Econosil C_{18} 10U 250 × 10 mm, MeOH/H_2O 80:20) to give [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** in a 1.5:1 ratio.

[6-$^2H_2$]-24a: colorless syrup, $[\alpha]_D$ −76.4° (c = 0.45, $CHCl_3$, 25 °C). IR (NaCl): ν_{max} (cm^{-1}) 3463 (OH), 1729 (C=O). FAB-MS: m/z 577 $(M + Na)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{34}O_6D_2Na$ $(M + Na)^+$ 577.2535, found 577.2510. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.22 (d, J = 10 Hz, 7-Ha), 3.60 (dd, J = 10, 2 Hz, 7-Hb), 3.96 (d, J = 4 Hz, 4-H), 4.19 (dd, J = 3.5, 4 Hz, 3-H), 4.32 (d, J = 1.5 Hz, 5-OH), 4.45 (d, J = 3.5 Hz, 2-H), 4.38−4.88 (PhCH_2- × 4), 7.05−7.37 (C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 60.3 (t, C-7), 72.4, 73.6, 73.7, 74.4 (all t, PhCH_2 × 4), 76.0 (d, C-4), 79.4 (s, C-5), 81.1 (d, C-3), 81.2 (d, C-2), 127.6−128.5 (all d) and 136.8−138.1 (all s, C_6H_5 × 4), 204.8 (s, C-1).

[6-$^2H_2$]-24b: colorless syrup, $[\alpha]_D$ −48.1° (c = 0.018, $CHCl_3$, 25 °C). IR (NaCl): ν_{max} (cm^{-1}) 3449 (OH), 1728 (C=O). FAB-MS: m/z 572 $(M + NH_4)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{38}NO_6D_2$ $(M + NH_4)^+$ 572.2981, found 572.2954. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.61 (s, 5-OH), 3.23 (d, J = 9 Hz, 7-Ha), 3.49 (d, J = 9 Hz, 7-Hb), 3.92 (dd, J = 8, 3 Hz, 3-H), 4.10 (d, J = 3 Hz, 4-H), 4.30 (d, J = 8 Hz, 2-H), 4.40−4.88 (PhCH_2- × 4), 7.15−7.40 (C_6H_5 × 4). ^{13}C NMR (75 MHz, $CDCl_3$): δ 60.4 (t, C-7), 72.1, 72.5, 73.2, 75.1 (all t, PhCH_2 × 4), 74.0 (d, C-4), 77.4 (s, C-5), 79.5 (d, C-3), 81.0 (d, C-2), 127.6−128.4 (all d) and 137.2−137.9 (all s, C_6H_5 × 4), 206.6 (s, C-1).

2-*epi*-5-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10a) and 2-*epi*-[6-$^2H_2$]Valiolone ([6-$^2H_2$]-10b). To a solution of 75 mg of either [6-$^2H_2$]-**24a** or [6-$^2H_2$]-**24b** in 95% aqueous ethanol (7.5 mL) was added wet 10% Pd/C (75 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 pure [6-$^2H_2$]-**10a** or [6-$^2H_2$]-**10b** in quantitative yield, respectively.

[6-$^2H_2$]-10a: white solid, $[\alpha]_D$ + 24.2° (c = 0.23, MeOH, 20 °C). IR (NaCl): ν_{max} (cm^{-1}) 3380 (OH), 1722 (C=O). FAB-MS: m/z 217 $(M + Na)^+$. HR FAB-MS: m/z calcd for $C_7H_{10}O_6D_2Na$ $(M + Na)^+$ 217.0657, found 217.0666. ^{1}H NMR (300 MHz, CD_3OD): δ 3.44 (d, J = 11 Hz, 7-Ha), 3.66 (d, J = 11 Hz, 7-Hb), 4.05 (d, J = 4 Hz, 4-H), 4.29 (t, J = 4 Hz, 3-H), 4.62 (d, J = 4 Hz, 2-H). ^{13}C NMR (75 MHz, CD_3OD): δ 67.5 (t, C-7), 70.7 (d, C-4), 76.0 (d, C-2), 79.7 (d, C-3), 81.5 (s, C-5), 209.9 (s, C-1).

[6-$^2H_2$]-10b: white solid, $[\alpha]_D$ + 20.4° (c = 0.23, MeOH, 20 °C). IR (NaCl): ν_{max} (cm^{-1}) 3380 (OH), 1722 (C=O). FAB-MS: m/z 217 $(M + Na)^+$. HR FAB-MS: m/z calcd for $C_7H_{10}O_6D_2Na$ $(M + Na)^+$ 217.0657, found 217.0653. ^{1}H NMR (300 MHz, CD_3OD): δ 3.41 (d,

J = 11 Hz, 7-Ha), 3.63 (d, J = 11 Hz, 7-Hb), 3.98−4.02 (m, 3-H and 4-H), 4.31 (d, J = 3.5 Hz, 2-H). ^{13}C NMR (75 MHz, CD_3OD): δ 67.0 (t, C-7), 72.4 (d, C-4), 74.8 (d, C-2), 76.2 (s, C-5), 76.6 (d, C-3), 209.7 (s, C-1).

2,3,4,7-Tetra-*O*-benzyl-2-*epi*-[6-^{2}H]valienone (25). To the solution of a mixture of [6-$^2H_2$]-**24a** and [6-$^2H_2$]-**24b** (200 mg, 0.36 mmol) in TBME (15 mL) were added mesyl chloride (0.28 mL, 3.6 mmol) and triethylamine (1 mL, 7.2 mmol), and the reaction mixture was stirred under argon at room temperature for 4 h. The reaction was quenched by addition of saturated aqueous Na_2CO_3, and stirring was continued for 30 min. The reaction mixture was partitioned between TBME and H_2O. The organic layer was washed with 2 N HCl, saturated aqueous $NaHCO_3$, and brine and dried over Na_2SO_4, and the solvent was evaporated. The residue was chromatographed on a silica gel column with *n*-hexane/EtOAc (6:1) to give **25** (90%).

Colorless syrup, $[\alpha]_D$ −79.2° (c = 0.26, $CHCl_3$, 20 °C). FAB-MS: m/z 536 $(M + H)^+$. HR FAB-MS: m/z calcd for $C_{35}H_{34}O_5D_1$ $(M + H)^+$ 536.2547, found 536.2528. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.04 (dd, J = 2, 4.5 Hz, 3-H), 4.08 (s, 7-H_2), 4.20 (d, J = 4.5 Hz, 4-H), 4.36 (d, J = 2 Hz, 2-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 69.3 (t, C-7), 74.7 (d, C-3), 77.2 (d, C-4), 78.7 (d, C-2), 72.5, 72.6, 72.8, 74.1 (all t, Ph*C*H_2 × 4), 127.7−128.5, 137.3 (s), 137.5 (s), 137.7 (s), 137.9 (s) (C_6H_5 × 4), 195.8 (s, C-1).

2-*epi*-[6-^{2}H]Valienone ([6-^{2}H]-11). To a solution of **25** (50 mg, 0.09 mmol) in CH_2Cl_2 (3 mL) was added 6 equiv of 1.0 M BBr_3 in hexane. After being stirred at −40 °C for 30 min and warming to 0 °C, the reaction mixture was quenched with MeOH/H_2O (1:2) and partitioned between CH_2Cl_2 and H_2O. The H_2O layer was subjected to anion-exchange column chromatography (Bio-Rad, AG-1 × 8, formate form) with elution with H_2O. The fractions containing [6-^{2}H]-**11** were collected, lyophilized, and subsequently purified on a Sephadex LH-20 column (MeOH) to give the product in 70% yield.

Colorless syrup, $[\alpha]_D$ −31.7° (c = 0.56, MeOH, 20 °C). IR (NaCl): ν_{max} (cm^{-1}) 3404 (OH), 1679 (enone). FAB-MS: m/z 176 $(M + H)^+$. HR FAB-MS: m/z calcd for $C_7H_{10}D_1O_5$ $(M + H)^+$ 176.0669, found 176.0665. ^{1}H NMR (300 MHz, CD_3OD): δ 4.16 (dd, J = 2.5, 3.5 Hz, 3-H), 4.19 (d, J = 3.5 Hz, 4-H), 4.21 (d, J = 17 Hz, 7-Ha), 4.40 (d, J = 17 Hz, 7-Hb), 4.54 (d, J = 2.5 Hz, 2-H). ^{13}C NMR (75 MHz, CD_3OD): δ 63.2 (t, C-7), 69.8 (d, C-3), 73.8 (d, C-4), 76.5 (d, C-2), 162.1 (s, C-5), 199.8 (s, C-1).

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