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Biosynthesis of the C_7-cyclitol Moiety of Acarbose in *Actinoplanes* Species SE50/110

7-*O*-PHOSPHORYLATION OF THE INITIAL CYCLITOL PRECURSOR LEADS TO PROPOSAL OF A NEW BIOSYNTHETIC PATHWAY*

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We have previously demonstrated that the biosynthesis of the C_7-cyclitol, called valienol (or valienamine), of the α-glucosidase inhibitor acarbose starts from the cyclization of *sedo*-heptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone (Stratmann, A., Mahmud, T., Lee, S., Distler, J., Floss, H. G., and Piepersberg, W. (1999) *J. Biol. Chem.* 274, 10889–10896). Synthesis of the intermediate 2-*epi*-5-*epi*-valiolone is catalyzed by the cyclase AcbC encoded in the biosynthetic (*acb*) gene cluster of *Actinoplanes* sp. SE50/110. The *acbC* gene lies in a possible transcription unit, *acbKLMNOC*, cluster encompassing putative biosynthetic genes for cyclitol conversion. All genes were heterologously expressed in strains of *Streptomyces lividans* 66 strains 1326, TK23, and TK64. The AcbK protein was identified as the acarbose 7-kinase, which had been described earlier (Drepper, A., and Pape, H. (1996) *J. Antibiot.* (*Tokyo*) 49, 664–668). The multistep conversion of 2-*epi*-5-*epi*-valiolone to the final cyclitol moiety was studied by testing enzymatic mechanisms such as dehydration, reduction, epimerization, and phosphorylation. Thus, a phosphotransferase activity was identified modifying 2-*epi*-5-*epi*-valiolone by ATP-dependent phosphorylation. This activity could be attributed to the AcbM protein by verifying this activity in *S. lividans* strain TK64/pCW4123M, expressing His-tagged AcbM. The His-tagged AcbM protein was purified and subsequently characterized as a 2-*epi*-5-*epi*-valiolone 7-kinase, presumably catalyzing the first enzyme reaction in the biosynthetic route, leading to an activated form of the intermediate 1-*epi*-valienol. The AcbK protein could not catalyze the same reaction nor convert any of the other C_7-cyclitol monomers tested. The 2-*epi*-5-*epi*-valiolone 7-phosphate was further converted by the AcbO protein to another isomeric and phosphorylated intermediate, which was likely to be the 2-epimer 5-*epi*-valiolone 7-phosphate. The products of both enzyme reactions were characterized by mass spectrometric methods. The product of the AcbM-catalyzed reaction, 2-*epi*-5-*epi*-valiolone 7-phosphate, was purified on a preparative scale and identified by NMR spectroscopy. A biosynthetic pathway for the pseudodisaccharidic acarviosyl moiety of acarbose is proposed on the basis of these data.

The α-glucosidase inhibitor acarbose (part of the amylostatin complex) (Fig. 1), produced by strains of the genera *Actinoplanes* and *Streptomyces*, is a member of an unusual group of bacterial (mainly actinomycete) secondary metabolites, all of which inhibit various α-glucosidases, especially in the intestine (1, 2). Acarbose is produced industrially using developed strains of *Actinoplanes* sp. SE50/110. It is predominantly used in the treatment of diabetes patients, enabling them to better utilize starch- or sucrose-containing diets by slowing down the intestinal release of α-D-glucose. The pseudotetrasaccharide acarbose consists of an unsaturated cyclitol (valienol), a 4-amino-4,6-dideoxyglucose, and maltose. The valienol and 4-amino-4,6-dideoxyglucose are linked via an amino bridge mimicking an *N*-glycosidic bond. This acarviosyl moiety is primarily responsible for the inhibitory effect on α-glucosidases. Biosynthetically, these compounds resemble aminoglycoside antibiotics (3, 4). Dependent on the carbon sources in the fermentation medium, *Actinoplanes* sp. SE50/110 produces also higher homologs of acarbose, which differ in the numbers of glucose residues that are linked to the reducing and nonreducing end of the acarviosyl moiety (Fig. 1). The C_7-aminocyclitol units are considered to be similar to other common structural motifs observed in bacterial secondary metabolites (4). The transition from primary to secondary metabolism in the cyclitol pathway in *Actinoplanes* sp. SE50/110 is catalyzed by the AcbC protein. The *acbC* gene was expressed heterologously in *Streptomyces lividans* employing the same reaction conditions as used in *in vitro* studies on dehydroshikimate synthase (dehydroquinate synthase, AroB) proteins. Its product was shown to be a C_7-cyclitol synthase using *sedo*-heptulose 7-phosphate as substrate for the production of 2-*epi*-5-*epi*-valiolone (5). Until now, no other intermediate for the biosynthesis of acarbose has been identified.

The trehalase inhibitor validamycin A (*cf.* Fig. 1*B*) is an antifungal antibiotic used as a crop protectant. Validamycins are produced by *Streptomyces hygroscopicus* ssp. *limoneus* and consist of two similar C_7-cyclitol units, one belonging to the valienol family (valienamine) and the other to a saturated 6-hydroxy derivative thereof (called validamine). In the biosynthetic pathway for validamycin, 2-*epi*-5-*epi*-valiolone has also been identified as the first precursor for these two cyclitol units. In this pathway, the feeding of various other potential precursors had led to the identification of some intermediates, including 5-*epi*-valiolone, valienone, and valienamine (*cf.* Fig. 1) (6, 7). In contrast, similar feeding experiments revealed 2-*epi*-5-*epi*-valiolone to be the only precursor that was incorporated into acarbose (8). Therefore, fundamental differences in

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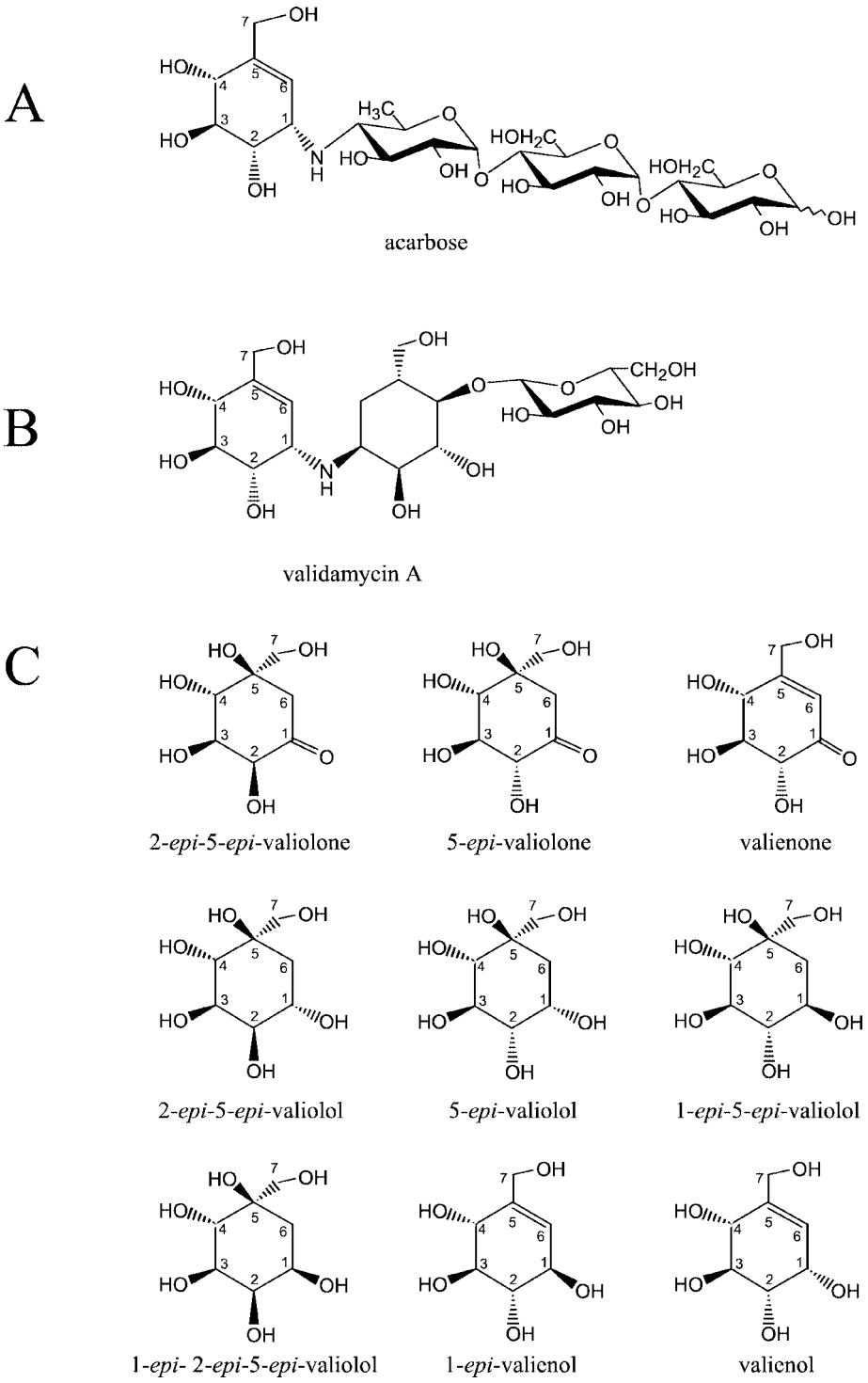

FIG. 1. **Chemical structures of two α-glucosidase inhibitors containing C_7-cyclitols.** *A*, acarbose; *B*, validamycin A; *C*, C_7-cyclitols. Valienamine is not shown as an extra structure because it is represented by the cyclitol moiety of acarbose including the NH_2 group at C-1.

the two pathways leading to the very similar end products are likely to exist.

In this study, we show that, during the biosynthesis of acarbose in *Actinoplanes* sp. SE50/110, the cyclitol precursor 2-*epi*-5-*epi*-valiolone is phosphorylated, forming the intermediate 2-*epi*-5-*epi*-valiolone 7-phosphate, by the enzyme AcbM as a first step in its conversion to the valienol moiety. Beyond this, we found that AcbO catalyzed the next conversion step, leading to an isomeric phosphorylated substance with the same molecular mass, most likely the epimerization product of 2-*epi*-5-*epi*-valiolone 7-phosphate to 5-*epi*-valiolone 7-phosphate. These findings, together with the genetic record from the *acb* gene cluster, provided evidence for the postulate of a new biosynthetic pathway for the acarviosyl moiety of acarbose, resembling those for activation (by phosphorylation and subsequent nucleotidylation) and modification of hexoses to be incorporated into oligo- or polysaccharides by glycosyl transfer (3, 4, 9). The fact of 7-*O*-phosphorylation in addition points to the need of an inactivating protection group already in the cyclitol intermediates and the oligosaccharidic end product(s) inside the producing cell. This requirement is underlined by the existence of a second 7-phosphotransferase gene, *acbK*, which is localized in the same transcription unit together with the *acbM* and other putative cyclitol biosynthetic genes and encodes a cytoplasmic acarbose 7-kinase. AcbK introduces a phosphate group into the same position of the cyclitol moiety of the oligosaccha-

TABLE I
Bacterial strains and plasmids

Strain/plasmid	Properties/product	Source/Ref.
Bacterial strains		
Actinoplanes sp. SE50/110	Acarbose	ATCC 31044
S. lividans 66 1326	Actinorhodin, prodigiosin	11
S. lividans TK23	Actinorhodin, prodigiosin *spc-1*	11
S. lividans TK64	Actinorhodin, prodigiosin *spc-2, pro-2, str-6*	11
*E. coli*BL21(DE3)/pLysS	T7 RNA polymerase, *cat*	21
E. coli DH5α	F φ80d, *lacZ*ΔM15, *endA1, recA1, hsdR17* ($r_k^- m_k^+$), *supE44, thi-1,* ·, *gyrA96, relA1*, D(*lacZYA-argF*)U169	22
E. coli JM109	*F′ traD36, lacZ*ΔM15, *proA*$^+$*B*$^+$ lac1 · λ · recA1, hsdR17 (r_k^- m_k^+), *supE44, thi-1, gyrA96, relA1,* Δ(*lac*$^-$)	22
Plasmids		
pBluescript II SK	*bla, lacZ-α, f1 ori*	24
pET11aP	*bla, lacZ-α*, T7 promoter	25
pET16bP	*bla, lacZ-α*, T7 promoter, His-tagged fusion peptide	25
pJOE2702	*bla, rrnB, rha-p*	26
pIJ4123	*kan, tsr, tipAp*	13
pIJ6021	*kan, tsr, tipAp*	13
pUC18	*bla, lacZ-α*	23
pUWL201RBSA	*bla, lacZ-α, tsr, ermEp*	27
pPWW49	*bla, lacZ-α, tsr, ermEp, eryBIV*	27
pPWW50	*bla, lacZ-α, tsr, ermEp, eryBIV*	27
pAS8/7	*acbC* in pIJ6021	5
pCWL16	1.42-kb *Nde*I/*Sst*I fragment from pMJL1 in pET16bP (*Nde*I/*Sst*I)	This work
pCWN16	0.86-kb *Nde*I/*Bam*HI fragment from pMJN1 in pET16bP (*Nde*I/*Bam*HI)	This work
pCWO16	0.87-kb *Nde*I/*Bgl*II fragment from pMJO1 in pET16bP (*Nde*I/*Bam*HI)	This work
pCWM16	1.08-kb *Nde*I/*Kpn*I fragment from pMJM1 in pET16bP (*Nde*I/*Kpn*I)	This work
pCWK11	1.0-kb *Nde*I/*Bgl*II *acbK* fragment in pET11aP (*Nde*I/*Bam*HI)	This work
pCWK16	1.0-kb *Nde*I/*Bgl*II *acbK* fragment in pET16bP (*Nde*I/*Bam*HI)	This work
pCW201L	1.1-kb *Nde*I/*Hin*dIII *acbL* fragment in pUWL201RBSA (*Nde*I/*Hin*dIII)	This work
pCW201M	1.08-kb *Nde*I/*Hin*dIII *acbM* fragment in pUWL201RBSA (*Nde*I/*Hin*dIII)	This work
pCW201O	0.82-kb *Nde*I/*Hin*dIII *acbO* fragment in pUWL201RBSA (*Nde*I/*Hin*dIII)	This work
pCW2072K	1.0-kb *Nde*I/*Bgl*II *acbK* fragment in pJOE2702 (*Nde*I/*Bam*HI)	This work
pCW201KM6	1.98-kb *Nde*I/*Hin*dIII *acbKM* fragment in pUWL201RBSA (*Nde*I/*Hin*dIII)	This work
pCW4123L	1.42-kb *Nde*I/*Eco*RI *acbL* fragment in pIJ4123 (*Nde*I/*Eco*RI)	This work
pCW4123M	1.08-kb *Nde*I/*Eco*RI *acbM* fragment in pIJ4123 (*Nde*I/*Eco*RI)	This work
pMJL1	1.42-kb *acbL* PCR fragment in pUC18 (*Sma*I)	This work
pMJM1	1.07-kb *acbM* PCR fragment in pBluescript SK$^-$ (*Eco*RV)	This work
pMJN1	0.86-kb *acbN* PCR fragment in pBluescript SK (*Eco*RV)	This work
pMJN2	0.86-kb *Nde*I/*Bam*HI *acbN* fragment in pET11aP (*Nde*I/*Hin*dIII)	This work
pMJN5	0.86-kb *Nde*I/*Bam*HI *acbN* in pPWW50 (*Nde*I/*Bam*HI)	This work
pMJO1	0.87-kb *acbO* PCR fragment in pBluescript SK (*Eco*RV)	This work
pMJO7	0.87-kb *Nde*I/*Bgl*II *acbO* fragment in pIJ4123 (*Nde*I/*Bam*HI)	This work

ridic end product, but does not use monomeric cyclitol precursors such as 2-*epi*-5-*epi*-valiolone as substrates.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table I. The following strains of *S. lividans* 66 were used as the hosts in expression experiments for the heterologous production of Acb proteins: strain TK64 for AcbL, AcbM, and AcbO; strain TK23 for AcbK and AcbN; and strain 1326 for AcbC. The recombinant strains were routinely cultured at 28 °C on soya fluor-mannitol-agar (SMA) agar plates (10), yeast extract-malt extract medium (YEME) medium with 10.3 or 34% sucrose (11), or protoplast regeneration medium (SPMR) plates (12); *Actinoplanes* sp. SE50/110 was cultured in MD50 medium (2). To maintain plasmids pIJ4123 and pIJ6021 and their recombinant derivatives, media were supplemented with kanamycin (50 mg/liter). The thiostrepton-inducible expression of the cloned *acbC*, *acbL*, *acbM*, and *acbO* genes in *S. lividans* TK64 was carried out according to Takano *et al.* (13), with the exception that thiostrepton was used at a concentration of 10 μg/ml, and the incubation time after induction was prolonged to 24 h. Recombinant *Escherichia coli* strains were grown at 37 °C in LB broth or on LB agar plates (14) supplemented with ampicillin (100 mg/liter).

Cloning, Manipulation, and Sequencing of DNA

The techniques for all manipulations and the transformation of recombinant DNA molecules and their analysis by restriction and sequencing were performed according to standard protocols or as described earlier (5, 11, 15). The general strategy for cloning the *acbKLMNO* genes into the expression vectors indicated in Table I with an N-terminal His-tagged fusion peptide was as follows. The genes were first amplified by PCR from the genomic DNA of *Actinoplanes* sp. SE50/110 using the primers listed in Table II. The PCR products were then cut by the restriction enzymes for which recognition sites were designed in the respective primer pairs (see Table II) and subsequently introduced by ligation into cut standard vectors (pUC18 or pBluescript II KS), and the inserts were inserted into pET16bP to create reading frames with N-terminal His-tagged fusions. The resulting plasmids were transformed and propagated in *E. coli* DH5α. The correctness of the nucleotide sequences of the inserts was controlled by DNA sequencing. The resulting His-tagged fusion cassettes were cut out by the enzyme pairs and further ligated to the streptomycete expression vectors given in Table I for later transformation and expression in *S. lividans* 66 strains. Automated DNA sequencing was carried out on an A.L.F.-Express machine (Amersham Biosciences, Freiburg, Germany) using the Thermosequenase DNA sequencing kit (Amersham Biosciences) and standard primers.

Preparation of Cell Extracts and Overexpression of Proteins in S. lividans

Cells were harvested by centrifugation, resuspended in 0.1 volume of disruption buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, and 1 mM β-mercaptoethanol, pH 7.6), and disrupted by sonication (2–3 min at 60 watts). Cell-free extracts were obtained after centrifugation at 13,000 × *g* for 1 h at 4 °C. The extracts were dialyzed against 5 liters of disruption buffer overnight at 4 °C. The proteins were analyzed by SDS-PAGE as described previously (5, 16). Protein concentrations were determined according to the method of Bradford (17).

TABLE II
Primers for amplification of the acb genes investigated

Primer	Nucleotide sequence[a]	Target
AcbK1	5′-CAAGGAGACATATGTCGGAGCAC-3′	*acbK* start
AcbK2	5′-GTGGTGAGATCTTCGCCCAGT-3′	*acbK* stop
2775M1	5′-GCCGGCCATATGAAGCGGC-3′	*acbM* start
pMJM1E	5′-CGGTGCCGGTACCACGATCGCGC-3′	*acbM* stop
2775L1	5′-TTGGTCGGCATATGAGCCGG-3′	*acbL* start
pMJL.1E	5′-GTACGGAATTCGTCCACCGCCAC-3′	*acbL* stop
2775N1	5′-AGAGGATCACATATGAGCGGGACTC-3′	*acbN* start
pMJN.1E	5′-GAGCTGGATCCCGTC-3′	*acbN* stop
2775O1	5′-GGTGCGCATATGACCTGCCG-3′	*acbO* start
pMJO.1E	5′-TACCGTCTCGACAGATCTCAGTCAGCTTCCT-3′	*acbO* stop

[a] Introduced recognition sites for restriction endonucleases are underlined.

Enzyme Assays

Generally, the crude extracts as prepared above were tested in assays of 20-μl final volume in a standard buffer system containing 25 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, and 10 mM 2-*epi*-5-*epi*-valiolone (or other substrates that were tested) adjusted to pH 7.6. The coenzymes and/or cosubstrates (ATP, NAD, NADH, NADP, NADPH, FAD, or $FADH_2$) were used in final concentrations of 10 mM in the test volumes.

AcbK—The acarbose 7-kinase AcbK was tested as described by Drepper and Pape (18), but without NH_4Cl in the buffer.

AcbM—AcbM tests were routinely performed in a volume of 15 μl. Each assay contained 25 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, 10 mM ATP, 10 mM 2-*epi*-5-*epi*-valiolone (or other substrates that were tested) adjusted to pH 7.6, and 12 μl of cell-free extracts. The assays were incubated at 30 °C for 2–12 h. The reaction was monitored by TLC. For radioactive assays, 1 μl of [γ-^{32}P]ATP (2.0 μCi; Amersham Biosciences) was added, and only 11 μl of cell-free extracts were used. Radioactively labeled spots were visualized after TLC by autoradiography with x-ray films (Hyperfilm, Amersham Biosciences).

AcbO—For assaying the activity of AcbO, a mixture of cells from *S. lividans*/pCW4123M and *S. lividans*/pMJO7 (1:1) was used. Cell extracts were prepared as described for the AcbM tests. The test conditions and the detection of the reaction product were also identical as for the analysis of the AcbM-catalyzed reaction.

Thin-layer Chromatography (TLC)

Samples of the enzyme reactions were chromatographed on silica thin-layer sheets (Merck, Darmstadt, Germany) using solvent I (isobutyric acid and 1 N NH_3 in water, 5:3) or solvent II (butanol/ethanol/water, 9:7:4). The substrates were detected as brown spots after heating or as blue spots after development using a cerium- and molybdate-containing reagent (19).

Purification of the His-tagged AcbM Protein

10-ml cell-free extracts from *S. lividans* TK64/pCW4123M were applied to an Ni^{2+}-HiTrap chelating column (Amersham Biosciences). The column was first washed with 10–20 ml of starting buffer (20 mM Na_3PO_4, 500 mM NaCl, 20 mM NH_4Cl, 10 mM imidazole, and 1 mM β-mercaptoethanol adjusted to pH 7.5) and then washed with a linear gradient of 10–500 mM imidazole in 10 ml of starting buffer and 10 ml of elution buffer (20 mM Na_3PO_4, 500 mM NaCl, 20 mM NH_4Cl, 500 mM imidazole, and 1 mM β-mercaptoethanol adjusted to pH 7.5). The fractions were analyzed by SDS-PAGE. The His-tagged AcbM protein was eluted at ~200–300 mM imidazole from the column. The partially purified protein was dialyzed for 24 h against 5 liters of dialysis buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, and 1 mM β-mercaptoethanol adjusted to pH 7.6).

Synthesis of 2-epi-5-epi-Valiolone

Method a—The enzyme-catalyzed synthesis of 2-*epi*-5-*epi*-valiolone was performed in a coupled assay using transketolase (EC 2.2.1.1), ribose 5-phosphate, and hydroxypyruvate to synthesize the substrate *sedo*-heptulose 7-phosphate *in situ* according to the protocol described previously (20). The cyclization of *sedo*-heptulose 7-phosphate was performed with cell-free extracts from *S. lividans* containing the AcbC protein according to the protocol of Stratmann *et al.* (5) under the following specific conditions, The preparative enzyme reaction was performed overnight at 30 °C in a total volume of 30 ml. The assay contained 2 units of transketolase (Sigma, Munich, Germany), 10 mM hydroxypyruvate (Sigma), 10 mM ribose 5-phosphate (Sigma), 0.5 mM thiamin pyrophosphate (Sigma), 1 mM $MgCl_2$, 0.025 mM $CoCl_2$, 2 mM NaF, pH 7.6, and variable amounts of cell-free extracts from strain *S. lividans* 1326/pAS8/7 containing overproduced AcbC protein (1). The reaction was monitored by TLC. The product of the AcbC reaction had the same R_F value (R_F = 0.53, solvent II) as the chemically synthesized 2-*epi*-5-*epi*-valiolone ((5*R*,2*S*,3*S*,4*S*)-5-(hydroxymethyl)cyclohexanone-2,3,4,5-tetrol).

Method b—The chemical synthesis of racemic 2-*epi*-5-*epi*-valiolone was performed according to a new protocol, the details of which will be published elsewhere.[1]

Purification of 2-epi-5-epi-Valiolone

30 ml of the AcbC reaction solution were heated at 90 °C for 5 min, centrifuged (5000 rpm, 20 min), and then applied to an ultrafiltration cell with a YM-10 ultrafiltration membrane (10,000-Da cutoff; Amicon, Witten, Germany). The flow-through was collected. After freeze-drying, ~250 mg of yellow powder were acquired. The product was dissolved in 3 ml of Milli-Q water and then subjected to anion-exchange chromatography with Dowex 1-X8 (Cl^- form, mesh 100–200; Serva, Heidelberg, Germany) on an SR25/50 column (Amersham Biosciences). The column was washed with water, and the fractions containing 2-*epi*-5-*epi*-valiolone were pooled. After lyophilization, 20 mg of 2-*epi*-5-*epi*-valiolone were obtained as a light-yellow powder.

Purification of 2-epi-5-epi-Valiolone 7-Phosphate

The partially purified AcbM protein was used in phosphorylation assays with 20 mg of purified 2-*epi*-5-*epi*-valiolone. The reaction mixture was applied to an Amicon ultrafiltration cell with a YM-10 ultrafiltration membrane (10,000-Da cutoff). The 10-ml flow-through was collected, concentrated to 3 ml by freeze-drying, and then subjected to anion-exchange chromatography with Dowex 1-X8 (Cl^- form, mesh 100–200) on a SR25/50 column. The column was washed with plenty of water, and the 2-*epi*-5-*epi*-valiolone phosphate was eluted with a linear gradient of 0–600 mM NaCl at a flow rate of 2 ml/min. The elutions were collected as 2-ml fractions and analyzed by TLC. Fractions containing the desired product (total volume of 48 ml) were pooled and concentrated 10-fold by freeze-drying. Desalting the product was carried out at 4 °C on Sephadex G-10 (5.0 × 81-cm SR25/100 column, Amersham Biosciences). The product was eluted with water at a flow rate of 1.5 ml/min. The fractions containing 2-*epi*-5-*epi*-valiolone phosphate were pooled. After lyophilization, 12 mg of 2-*epi*-5-*epi*-valiolone phosphate were obtained as a white powder.

NMR

All NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz). In addition to ^{1}H, ^{13}C, and ^{31}P experiments, also COSY (^{1}H-^{1}H, ^{1}H-^{13}C, and ^{1}H-^{31}P) and distortionless enhancement of polarization transfer (DEPT) spectra for the unequivocal correlation of the hydrogen, carbon, and phosphor atoms were recorded.

The chemical shifts are given in ppm, related to the solvents as internal standard. The multiplicity is given by the following symbols: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), Ψt (pseudotriplet for unresolved dd), and br (broad). The coupling constant *J* is given in Hz.

The NMR spectra for 2-epi-5-epi-valiolone are as follows: ^{1}H NMR (d_4-MeOH, 400 MHz): δ = 2.33 (dd, 1H, *J* = 13.7, 1.7 Hz, H-6_{ax}), 2.84 (d,

[1] O. Block and H.-J. Altenbach, unpublished data.

Gene	aa[a]	Closest similarity to protein (accession no.)	COG[b]	Postulated function, (reference)
acbK	300	Urf2 *Streptomyces* sp. (U08602)	0524	acarbose-7-kinase (18)
acbM	359	GlcK *Bacillus subtilis* (P54495)	1940	polyol kinase
acbL	403	Rv1895 *Mycobacterium tuberculosis* (O07737)	1063	dehydrogenase
acbN	257	MLR9033 *Mesorhizobium loti* (AP003015)	1028	short-chain dehydrogenase
acbO	270	No significant similarity		2-epimerase
acbC	381	AroB (Rv2538c) *Mycobacterium tuberculosis* (X59509)	0337	C7-cyclitol synthase (5)

[a] aa = coding capacity in number of amino acid residues;
[b] COG = cluster of orthologous proteins in genomes (www.ncbi.nlm.nih.gov/COG/) (30)

FIG. 2. **Genetic organization of genes *acbKLMNOC*.** The genes are symbolized by *arrows*. The putative proteins encoded by the genes and their postulated functions are listed below. The genes are part of the putative transcription unit *acbKLMNOC* in the *acb* gene cluster (GenBank™/EBI accession number Y18523). The recognition sites for the restriction endonucleases *Bgl*II and *Sst*I in this DNA segment are shown.

1H, J = 13.7 Hz, H-6_{eq}), 3.43 (d, 1H, J = 11.3 Hz, H-7a), 3.64 (d, 1H, J = 11.3 Hz, H-7b), 4.03 (m, 1H, H-4), 4.27 (Ψt, 1H, J = 4 Hz, H-3), and 4.59 (d, J = 4.0 Hz, H-2); ^{13}C NMR (d_4-MeOH, 101 MHz): δ = 46.0 (C-6), 67.7 (C-7), 70.9, 76.1, 79.7 (C-2, C-3, C-4), 81.5 (C-5), and 209.8 (C-1).

The NMR spectra for 2-epi-5-epi-valiolone 7-phosphate are as follows: ^{1}H NMR (D_2O, 400 MHz): δ = 2.38 (d, 1H, J = 13.8 Hz, H-6_{ax}), 2.89 (d, 1H, J = 14.2 Hz, H-6_{eq}), 3.58 (dd, 1H, J = 6.6, 11.7 Hz, H-7a), 3.99 (dd, 1H, J = 9.4, 11.5 Hz, H-7b), 4.18 (m, 1H, H-4), 4.38 (Ψt, 1H, J = 3.8 Hz, H-3), and 4.71 (d, under HDO, J = 4.0 Hz, H-2); ^{13}C NMR (D_2O, 101 MHz): δ = 46.38 (C-6), 70.17 (d, J = 5.1 Hz, C-7), 71.19 (C-4), 76.74 (C-2), 79.62 (C-3), 82.86 (C-5), and 101.23 (C-1); ^{31}P{^{1}H} NMR (D_2O, 162 MHz): δ = 5.55 (PC-7). The optical rotation of 2-*epi*-5-*epi*-valiolone 7-phosphate was $[\alpha]_D^{20}$ + 4.9° (*C*, 0.35, IN H_2O).

Ion Chromatography

The chromatographic part consisted of a Dionex DX-500 ion chromatography system equipped with a gradient pump (GP40), an eluent generator (EG40) with an EGC-KOH cartridge, a 25-μl injection loop, and an electrochemical conductivity detector (ED40). Chromatographic separation was performed on a Dionex Ion Pac AS11 column (250 × 2 mm) provided with a guard column (AG11). An anion trap column (ATC-1) was placed in-line with the GP40 pump and the injection valve to remove anionic contaminants from the eluent. Microfiltered (0.2 μm) water with a specific resistance of at least 18 milliohms was used. The gradient was run as shown in Table II. The Dionex ASRS-II self-regenerating chemical suppressor was housed in an LC30 chromatography oven at 35 °C and operated in the external water mode at 100 mA. The samples were analyzed in triplicate at a flow rate of 0.25 ml/min. The following KOH gradient profile was used: 0–2 min at 100 mM, 2–9 min at 1 mM (point of injection), 9–11 min at 1 mM, 11–15 min increasing from 1 to 19 mM, 15–18 min increasing from 19 to 20 mM, 18–25 min increasing from 20 to 100 mM, and 25–26 min at 100 mM.

Mass Spectrometry

The experiments were performed on a PE-Sciex API 2000 triple-quad instrument (Applied Biosystems, Langen, Germany) equipped with a Turbolon ion spray and heated nebulizer. To avoid current leakage, the connection tube between the ion chromatograph and the mass spectrometer had to be grounded directly behind the ED40 detector. All measurements were made in the negative scan mode. Hyphenation was realized with the original flow rate of 0.25 ml/min at a heater temperature of 380 °C and a Turbolon ion spray voltage of −4000 V. Optimal gas (N_2) pressures were found as follows: nebulizer gas at 30 p.s.i., heater gas at 90 p.s.i., and curtain gas at 45 p.s.i. Especially for mass-reaction-mass (MRM) measurements, the collision-added dissociation gas rate was set at 3 p.s.i.

RESULTS

Identification of AcbK as the Acarbose 7-Kinase—The gene cluster for the synthesis and metabolism of acarbose and related components of the amylostatin complex of the producer *Actinoplanes* sp. SE50/110 has been cloned on a single cosmid and fully sequenced recently.[2] Its function was proven by heterologous expression from the cosmid in *S. lividans* TK23, which formed acarbose-related substances and characteristic enzyme activities involved in acarbose metabolism such as the extracellular acarviosyltransferase AcbD (28), and the cytoplasmic acarbose 7-kinase (18, 19). The gene for the acarbose 7-kinase, *acbK*, was identified in a putative operon, *acbKLMNOC* (GenBank™/EBI accession number Y18523) (Fig. 2), by the identity of the first 20 amino acids of the N-terminal peptide sequence obtained from the purified enzyme by others (18) to that of the deduced AcbK protein sequence. Also, AcbK exhibited significant similarity to members of the ribokinase family of phosphotransferases (Fig. 3*A*). Therefore, we constructed various plasmids for the expression of AcbK in recombinant derivatives of both *E. coli* (BL21(DE3)/pLysS/pCW11K, BL21(DE3)/pLysS/pCW16K, and JM109/pCW2072K) and *S. lividans* (TK23/pCW201KM6) (*cf.* Table I). In all host strains, heterologous (over)expression of soluble AcbK protein, which was active with or without an N-terminal His tag extension, could be achieved (Fig. 4*A*). The host strains lacked any background activity for phosphorylation of acarbose. The acarbose 7-kinase AcbK did not phosphorylate 2-*epi*-5-*epi*-valiolone or any of the other possible cyclitol precursors tested in this study in both assay systems used (Table III; *cf.* Fig. 1*C*).

Identification of AcbM as a 2-epi-5-epi-Valiolone Kinase—The genes *acbLMNO*, bracketed by the functionally characterized *acbK* and *acbC* genes, were among those putative acarbose biosynthetic genes with some likelihood of being involved in the cyclitol pathway because of their location and the similarities of the encoded AcbL, AcbM, and AcbN proteins to known enzymes (*cf.* Figs. 2 and 3). The proteins AcbN and AcbL exhibited significant similarities to known oxidoreductases, belonging to two different subfamilies of the dehydrogenases with an N-terminal dinucleotide-binding fold, the zinc-dependent and short-chain alcohol dehydrogenases, respectively (data not shown). AcbM exhibited a distant similarity to GlcK from *Bacillus subtilis* (GenBank™/EBI accession number P54495) and other glucokinases, being a member of the hexose 6-kinase family of phosphotransferases (Fig. 3*B*). No data base entries significantly similar to AcbO were found with all methods of similarity search used (BLAST and fasta 3).

It is known that 2-*epi*-5-*epi*-valiolone is the first intermediate in the cyclitol pathway of acarbose, leading to valienol or valienamine (5). It was speculated that the first reaction after the formation of 2-*epi*-5-*epi*-valiolone might be catalyzed by either a dehydratase or a dehydrogenase, followed by a step catalyzed by an epimerase (5, 9). To determine whether the next conversion step was catalyzed by AcbL, AcbM, AcbN, or AcbO, the corresponding genes (*acbLMNO*) were cloned separately in various *E. coli* expression vectors (pET11a, pET11aP, or pET16bP) (Table I). However, when using these plasmids, none of the proteins could be produced as soluble proteins, although a number of variations in the culture conditions had been tested. Therefore, the genes were cloned in suitable streptomycete expression vectors (pUWL201, pPWW50, and pIJ4123) (*cf.* Table I) and overexpressed in *S. lividans* strain TK23, TK64, or 1326. All proteins were well expressed especially when the recombinant pIJ4123 derivatives for the production of N-ter-

[2] H. Thomas, U. F. Wehmeier, A. Stratmann, M. Jarling, and W. Piepersberg, unpublished data.

A

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AcbKAsp MSEHTDVLVLGGAGVDTIAYVPELPLPFQDSYVVAAIEPRAGQTGDNVALGLHTLGLRTM
MTH404    MRFDAVGLGALNMDQLHMVERIAGPDEETFVRGLVE-SCGGSAANTMIGLSRLGLRTA
PAB0280   MRFDVICMGNLNYDIVFLMEKFPG-IHEKVNAKGVFTGLGGSAGNTATWLAKLGLKVG
MTH1544 MSEDRDLLAVGHTAFDYIIHLDEFPEP-NTSTAIKRMRNLHGGAAANVALVGSRLGLRTS
CC3733  IAQCDDAFLEREGLVKGSMALIDPARAASLYDVMSAAIEASGGSAANTVAGVASFGGKAA
consens      D    G    D                          GG A N       LGL

AcbKAsp HVDVLGDDPEGDLVRAFHTRHGL-..PFAALPTAAGTKRAVNLVGPDGRRLSLWDG-SRE
MTH404  HIGKVADDREGGLLRSNLSSEGVTDFTVVA-DTGRSGRVMGFVDPDGNRALYVDPG-VND
PAB0280 FIGAVGNDDFGRLHLEFFREIGVDTSGIKVVND-ATGIAVTMVKGEDKRIVKYP-GA---
MTH1544 LVSAVGGDFEGSEYRELLESSGIDIESMILVADESTPTAFVMTDSDHNQISYFYWGAARY
CC3733  FLGKVADDQLGRVFRHDMNAIGCVFTTPPLAEGPATAQSLINVTPDAQRTMSTYLGACVE
consens     V  D  G   R      G                    V  D         G

AcbKAsp AEE-DRYPA-ALIAAHTAHAR----HVHVCITPPGQHVFGQLND-LPVTVS-TDLHNWDG
MTH404  TLRVDEVADEALNTEL-LHLTSF----AGDGINVQVEVIEALDESVTVSLDPGHIYASRG
PAB0280 NRW-KEVNTEYLKRARHLHLSSN--PIELIREAVEKA-KE-L--GLTVSFDPGEMEVPR-
MTH1544 FKDA-ETPADAIKSARAVHLA-TGDPSFNCR-CGEYEGFEVYAFARSLGKIISFDPGQDL
CC3733  LNPAD-VDPDIIEAAQISYLEGYLFDPPEARRAFAKAAALAHGSDRKIALTLSDSFVVDR
consens                  H

AcbKAsp A-YEGF--EVYAFNADLVFLSATALT------DVAATMRRVIDRG---RARLVVATGRAH
MTH404  V-SE---LSDILERTDILLTNQRELE-----LMTGSADPEEAAS--LLGIGVVVVKMGAR
PAB0280 ---------DVEKELDILMMNEDEPKAKYGSLDRIKDVKARIAIATLNGGGALVRDEKGE
MTH1544 HMYSRSQLERAVGVCDILFGNHHEIDRICSKLSVDIHGLRE------MGPGVVVKTYGKE
CC3733  H--RGALLGFVETQCDIVFANAAEVCALFETDDFDAAVKALAERC-----EIAAVTRSEK
consens               DI   N                          V

AcbKAsp GGSVLVRRRKAEVRRYAAVAPEAPVVDSNGAGDAFVSGFLFGHLAGEPLETCPRYGAIAG
MTH404  GVRAWDGESVMVDALST----E--CVDTTGAGDAFNAGFIYAWLEGFGLEVSCRPGNYIA
PAB0280 -----------VHEVRGLS-EAAKAIDTTGGGDAFNAGFLYGFLSGWDVVNSAKLGMLLA
MTH1544 GSIIYSDDVIKIDAIPR--------VDPTGAGDSYRAGFMRAYLRGADLKTCGRFASAVA
CC3733  GSVV-A-ANGQLHEISAYPV-E-KVVDTTGAGDQYAAGFLFGLSQGRPLPICGQLGSLAA
consens G                        VD TGAGD   AGF    L G  L      G   A

AcbKAsp AYACTIPATRAGAIDRAALL-RPAA
MTH404  SRCIGGYGATESLPGTGPGCPQEVR
PAB0280 YLTVQEVGARSAVRPLDEIK-KIAQ-ELKLNLPI
MTH1544 SFIVEDEGTQTNIPDTGEAV-KRFTAQWGYEPPI
CC3733  AEVIDHYGPRPQV-SLRELAEKNGL
consens       G
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B

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GlkASli         MGLTIGVDIGGTKIAAGVVDEEGNILSTHKVPTPTTPEAIVDAIASAVEGA-
GlcKBsu       MDEIWFAGIDLGGTTIKLAFINQYGEIQHKWEVPTDKTGDTITVTIAKTIDSKL
AcbMAsp    MKRPPHHPVTVADVGGTHLRWARWSPDGRTGRSAHHASPGHARRPGAGAADLQAELI
consens               D GGT          G                    A

GlkASli -----RVGHEIVA-VGIGAAGYVNRQRSTVYF-A--------------------PNIDWR
GlcKBsu DEL-QKPKHIIK-YIGMGAPGPVDMAAGVVYETV--------------------NLGWK
AcbMAsp RELASRVEPGARAGVSLGAAMDHH--SGHAYASAPLWGPQVSPFDVPAALRAARPDVHWT
consens                  GA           Y                        W

GlkASli --QEP-LKE-KV-EARV-------------------------GLPVVVENDANAAAWGE
GlcKBsu --NYA-LKN-HL-ETET-------------------------GIPAVIENDANIAALGE
AcbMAsp VVNDVTAGLLHLAEMVRDAGVRKACLVTISTGIACRTMDLRTGGIP-VDDITAGLLHLAE
consens                                        G P V    A      E

GlkASli YKFGGGKGHRNVICITLGTGLGGGIII--GNKLRRGHFGVAAEFGHIRMVP--DGLL---
GlcKBsu MWKGAGDGAKDVILVTLGTGVGGGIIA--NGEILHGINGAGGEIGHICSIP--EGGAP--
AcbMAsp MVP--NDRVPKRCLVTISTGMSCRTMDLRTGGFPVEAGGLQGEKSVTCRRPCWPNGVPVV
consens               T  TG                 G           P

GlkASli --CGCGSQGCWEQYASGRALVRYAKQR-ANATPERAEVLLALGDGTPDGIEGKHISVAAR
GlcKBsu --CNCGKTGCIETIASATGIVRIAKGKIANA--KKTTRLKAT-----EQLSARDVFEAAG
AcbMAsp TRCDCGEPGHVAASSSGPGIRRVAPSWPGVT--RPPGRARAD-----HPDDGGLGLRDAF
consens     CG  G      S     R A                A                A

GlkASli QGCPVAVDSYRELARWAGAG-LADLAS---LFDP--SAFIVGGGLSDEGDLVLDPIRKS
GlcKBsu ENDEIALEVVDYVAKHLGLV-LGNLAS---SLNP--SKIVLGGGVSRAGELLRSKVEKT
AcbMAsp RAALDDGDPVAADLLTAVTAPIADLLRTALCLDPELDLIALTGGVAHGLEPHYSAAVHD
consens                          L        P        GG

GlkASli YKRWLVGGNWRPV-ADVIAAQLGNKAGLVGAADLAREPDPIM
GlcKBsu FRK-CAFPRAAQA-ADISIAALGNDAGVIGGAWIAKNEWLKHQNC
AcbMAsp HCGAGVLYLTSEREPDWLTGRIRSSRRRRPIRWSAPVWPPGGGPVPAYSGGGREALVG
consens               D                  A
```

FIG. 3. **Alignment of the AcbK and AcbM protein sequences with those of other kinases.** *A*, the AcbK sequence is significantly related to members of the ribokinase family (COG0524; www.ncbi.nlm.nih.gov/COG) (30). A consensus (*consens*) sequence is given for residues identical in at least four of the aligned five sequences. *B*, the AcbM sequence is distantly related to members of the glucokinase family (COG1940). A consensus sequence is given for residues identical in all three sequences. The sequences aligned are from the following sources: *AcbKAsp*, AcbK protein from *Actinoplanes* sp. SE50/110 (GenBank™/EBI accession code Y18523); *MTH404*, putative ribokinase from *Methanobacterium thermoautotrophicum* (accession code AAB84910); *PAB0280*, putative ribokinase from *Pyrococcus abyssi* (accession code E75157); *MTH1544*, putative ribokinase from *M. thermoautotrophicum* (accession code AAB86018); *CC3733*, putative ribokinase from *Caulobacter crescentus* (accession code AAK25695); *GlkASli*, glucokinase from *S. lividans* TK24 (accession code AAF42869); *GlcKBsu*, putative glucokinase from *B. subtilis* 168 (accession code P54495); *AcbMAsp*, AcbM protein from *Actinoplanes* sp. SE50/110 (accession code Y18523).

minal His-tagged fusion proteins were used (Fig. 4, *B–D*).

Extracts from *S. lividans* TK23 with pMJN5 (*acbN*) and from *S. lividans* TK64 harboring pCW4123M (*acbM*), pCW4123L (*acbL*), or pMJO7 (*acbO*) were prepared and used in a number of various enzyme assays (dehydration, reduction, epimerization, or phosphorylation assays), including chemically synthesized cyclitols (*cf.* Fig. 1*C*) and combinations of the coenzymes ATP, $NAD(P)^+$, NAD(P)H, FAD^+, and $FADH_2$. In these tests, extracts containing a single overproduced Acb protein and also combinations of the various extracts were analyzed. The enzyme tests were monitored by TLC and also photometrically. In none of the assays was a dehydrogenase-, an epimerase-, or a dehydratase-like activity detected (data not shown). However, when chemically synthesized 2-*epi*-5-*epi*-valiolone was incubated with cell extracts including AcbM and ATP as a cosubstrate, a new product was observed upon TLC (Fig. 5). This product was missing in all assays lacking ATP. Therefore, we assumed that the new product was 2-*epi*-5-*epi*-valiolone phosphate. In subsequent assays using [γ-^{32}P]ATP, we determined that the new product was indeed radioactively labeled. This clearly showed that 2-*epi*-5-*epi*-valiolone was phosphorylated. We also tested the phosphorylation of other cyclitols (2-*epi*-5-*epi*-valiolol, 5-*epi*-valiolol, 1-*epi*-2-*epi*-5-*epi*-valiolol, 1-*epi*-5-*epi*-valiolol, 1-*epi*-valienol, and valienol) (Fig. 1*C* and Table III) in assays with AcbM extracts. In the presence of [γ-^{32}P]ATP, only in assays with 2-*epi*-5-*epi*-valiolol and 1-*epi*-2-*epi*-5-*epi*-valiolol were weak radioactively labeled spots detected on the autoradiogram of the TLC separation. In nonradioactive assays, these spots were not visible, indicating very low and less specific phosphorylation of these compounds (Table III). As the chemically synthesized 2-*epi*-5-*epi*-valiolone consisted of a racemic mixture, only ~50% of the substrate used in the assays was converted (*cf.* Fig. 5). To overcome this problem, we synthesized enantiomerically pure 2-*epi*-5-*epi*-valiolone enzymatically. In a coupled assay (see "Materials and Methods"), 2-*epi*-5-*epi*-valiolone was synthesized, and the purified product was incubated with AcbM-containing extracts. In these assays, the conversion of 2-*epi*-5-*epi*-valiolone to the new phosphorylated spot upon TLC was nearly 100% (*cf.* Fig. 5). This result clearly demonstrated that the enzymatically produced 2-*epi*-5-*epi*-valiolone was the substrate for this phosphorylation step.

Purification and Structural Characterization of Phosphorylated 2-epi-5-epi-Valiolone—To prepare 2-*epi*-5-*epi*-valiolone phosphate from a purified system and to identify the position of the phosphate, the substrate 2-*epi*-5-*epi*-valiolone was enzymatically synthesized and purified on a preparative scale, and the His-tagged AcbM enzyme was purified via its affinity for Ni^{2+}-agarose (see "Materials and Methods") (Fig. 6). These were used in scaled-up phosphorylation assays. From these assays, the phosphorylated product was isolated and purified as described under "Materials and Methods." The product was first characterized by ion chromatography-mass spectrometry. The ion chromatography-mass spectrometry data showed that a substance with a molecular mass of 272 Da was generated. This mass corresponds exactly to the calculated mass for 2-*epi*-5-*epi*-valiolone phosphate. Then ^{1}H, ^{13}C, and ^{31}P NMR data for the purified substance were determined (see "Materials and Methods") (Fig. 7). In the ^{1}H NMR spectrum, the coupling pattern of the former hydroxymethyl group changed significantly, indicating that it had been modified. A signal in the ^{31}P NMR at 5.55 ppm is indicative of a phosphate group, and a ^{1}H-^{31}P COSY experiment proved that the phosphate group sits at the primary position. The methylene group occurred in the ^{1}H NMR as an ABX spin system at 3.58 and 3.99 ppm with coupling constants $^3J_{\text{H-7a,P}}$ = 6.6 Hz and $^3J_{\text{H-7b,P}}$ = 9.4 Hz, respectively. Interestingly, while dissolving in D_2O, the AB

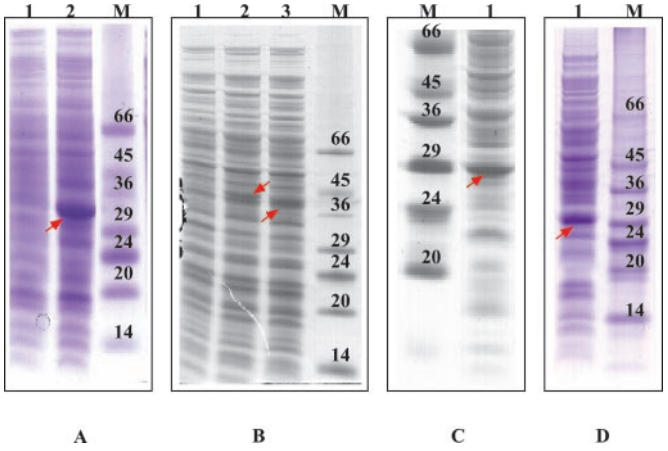

FIG. 4. **SDS-PAGE analysis of the production of proteins AcbK, AcbM, AcbL, AcbN, and AcbO in *S. lividans*.** Electrophoresis was run on a 10% polyacrylamide gel. *A*, production of AcbK in *S. lividans* TK23. Soluble proteins were separated from *S. lividans* TK23 harboring pUWL201 (control; *lane 1*) or pCW201KM6 (AcbK; *lane 2*). *B*, production of AcbM and AcbL in *S. lividans* TK64. Soluble proteins from cells harboring plasmid pIJ4123 (control; *lane 1*), pCW4123L (N-terminal His-tagged AcbL; *lane 2*), or pCW4123M (N-terminal His-tagged AcbM; *lane 3*) were subjected to PAGE. *C*, production of AcbN in *S. lividans* TK23 harboring pMJN5 (N-terminal His-tagged AcbN; *lane 1*). *D*, production of AcbO in *S. lividans* TK64 harboring plasmid pMJO7 (N-terminal His-tagged AcbO; *lane 1*). The molecular masses of the marker proteins (*lanes M*) are indicated in kDa. The respective overexpressed Acb proteins are depicted by *arrows*.

TABLE III
Phosphorylation assays with AcbK and AcbM

All substrates were used in phosphorylation assays with unlabeled ATP and [γ-^{32}P]ATP as described under "Materials and Methods" +, phosphorylation; ±, weak phosphorylation, detectable only with [γ-^{32}P]ATP; −, no phosphorylation.

Substrate	Phosphorylation by	
	AcbK	AcbM
2-*epi*-5-*epi*-Valiolone	−	+
2-*epi*-5-*epi*-Valiolol	−	±
1-*epi*-2-*epi*-5-*epi*-Valiolol	−	±
1-*epi*-5-*epi*-Valiolol	−	−
5-*epi*-Valiolol	−	−
1-*epi*-Valienol	−	−
Valienol	−	−
Acarbose	+	−

pattern of the ring methylene group was replaced by two pseudosinglets at 2.40 and 2.78 ppm within 30 min. This phenomenon can be explained by a fast exchange of the methylene protons by deuterium from the solvent and has also been observed for the 2-*epi*-5-*epi*-valiolone system itself (6). In the ^{13}C spectrum, C-7 appears as a doublet at 70.2 ppm with coupling constant $^{2}J_{C\text{-}7,P}$ = 5.1 Hz. Instead of the expected carbonyl group, C-1 shows a resonance for a quaternaric carbon at 101.23 ppm, indicating a hydratization of the ketone function. From the described results, the purified product of the AcbM reaction can be unequivocally identified as 2-*epi*-5-*epi*-valiolone 7-phosphate, the second intermediate in the cyclitol branch of the acarbose pathway.

2-epi-5-epi-Valiolone 7-Phosphate Is the Substrate of AcbO—To obtain further insight into the biosynthetic pathway for valienol, cell extracts containing AcbM were combined with extracts containing AcbO, AcbL, or AcbN. In all tests, 2-*epi*-5-*epi*-valiolone and ATP were used as substrates without or in combination with various dinucleotides as redox or epimerase coenzymes (NAD, NADH, NADP, and NADPH). Only when extracts with AcbM/ATP in combination with AcbO were used was a new spot observed on the TLC plates (Fig. 5). Without

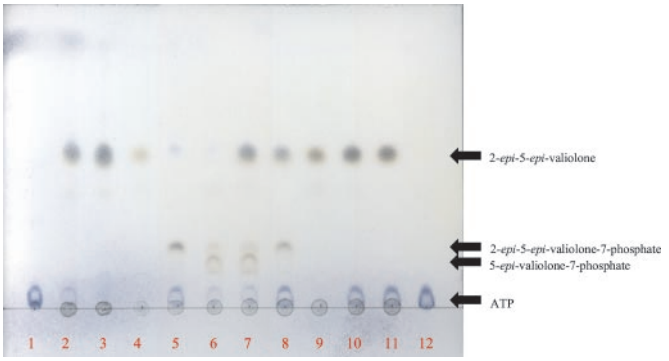

FIG. 5. **TLC analyses of AcbM and AcbO assays.** Extracts from *S. lividans* TK64 with pIJ4123 (control), pCW4123M (AcbM), or pMJO7 (AcbO) were used for these assays. Samples were prepared as described under "Materials and Methods." Samples were applied to TLC sheets and separated using solvent II (see "Materials and Methods"); spots of cyclitol components were visualized using a cerium/molybdate-containing reagent (19). The respective spots are marked by *arrows*. For assays shown in *lanes 2-6*, enzymatically synthesized 2-*epi*-5-*epi*-valiolone was used; for assays shown in *lanes 7-11*, chemically synthesized 2-*epi*-5-*epi*-valiolone was used. *Lane 1*, 4 μl of ADP (10 mM); *lanes 2–11*, 4 μl of the reaction mixtures of complete assays with cell extracts from strains containing plasmids pIJ4123, pMJO7, pCW4123M, pCW4123M (without ATP), pCW4123M/pMJO7, pCW4123M/pMJO7, pCW4123M, pCW4123M (without ATP), pMJO7, and pIJ4123, respectively; *lane 12*, 4 μl of ATP (10 mM).

AcbM and in the presence of ATP alone, no conversion of 2-*epi*-5-*epi*-valiolone by AcbO was monitored. These data give an important hint for AcbO being the next enzyme in cyclitol conversion. In these assays, no dinucleotide coenzyme was required, although extensively dialyzed cell extracts were used. This finding indicated that the reaction catalyzed by AcbO was coenzyme-independent or that if a cofactor was involved, it must have been tightly bound to the enzyme. Therefore, it was further investigated whether AcbO could be either (i) the epimerase that catalyzes the epimerization at C-2 to give 5-*epi*-valiolone phosphate or (ii) a dehydratase that catalyzes the formation of 2-*epi*-valienone 7-phosphate.

Characterization of the AcbO Product as a Probable Epimer of 2-epi-5-epi-Valiolone 7-Phosphate—To identify the conversion product from 2-*epi*-5-*epi*-valiolone 7-phosphate that was obtained in the reaction catalyzed by AcbO, the ion chromatography-mass spectrometry data of the reaction product were analyzed. In assays containing 2-*epi*-5-*epi*-valiolone/ATP and the overproduced enzyme AcbM alone or AcbM and AcbO together, three new mass peaks of phosphorylated compounds were detected: *m/z* 253 (13.27 min), 271 (12.77 min; 2:1 ratio), and 287 (12.97 min). These peaks were missing in assays without AcbM. The dominant mass peak (*m/z* 271) corresponded exactly to the expected mass for 2-*epi*-5-*epi*-valiolone 7-phosphate. As the same mass peak (*m/z* 271) was detected in assays with AcbO, we concluded that AcbO most likely catalyzed the epimerization at C-2, yielding 5-*epi*-valiolone 7-phosphate.

DISCUSSION

Earlier work has demonstrated that 2-*epi*-5-*epi*-valiolone is the precursor of the C_7-cyclitol unit of the acarviosyl moiety of acarbose (5, 8). The expectation was that a series of enzyme-catalyzed steps involving dehydration, reduction, and epimerization converted this precursor to valienol (or valienamine) before its incorporation into the pseudodisaccharide acarviosine. Although a number of feeding experiments with possible intermediates were described, all other steps and intermediates of this biosynthetic route remained unclear so far (8).

In this study, we have shown that, unexpectedly, the second step after initial formation of the cyclitol precursor by the

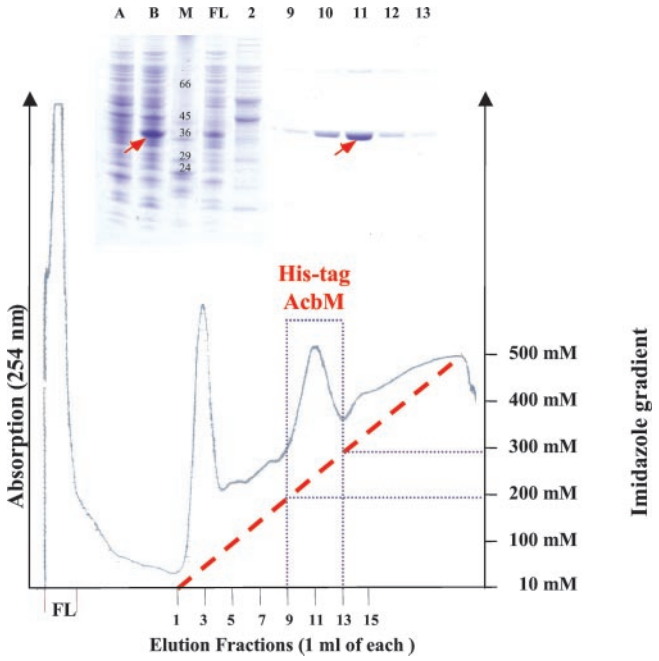

FIG. 6. **Schematic diagram of affinity chromatography and SDS-PAGE analysis of the purification of His-tagged AcbM.** Purification was performed as described under "Materials and Methods." An elution chromatogram of His-tagged AcbM from an Ni^{2+}-HiTrap chelating column is shown. The linear gradient of 10–500 mM imidazole is indicated by a *broken line*. The fractions containing His-tagged AcbM are indicated by *dotted lines*. 1-ml fractions were collected as indicated. The *inset* shows the results from SDS-PAGE analysis of the elution fractions. Electrophoresis was run on 10% protein gel. *Lane A*, cell-free extracts from *S. lividans* TK64/pIJ4123; *lane B*, cell-free extracts from *S. lividans* TK64/pCW4123M; *lane M*, protein molecular mass marker (sizes indicated in kDa); *lane FL*, flow-through; *lanes 2*, *9*, and *10–13*, 14 μl of the elution fractions corresponding to those shown in the chromatogram. The His-tagged AcbM protein is depicted by *arrows*.

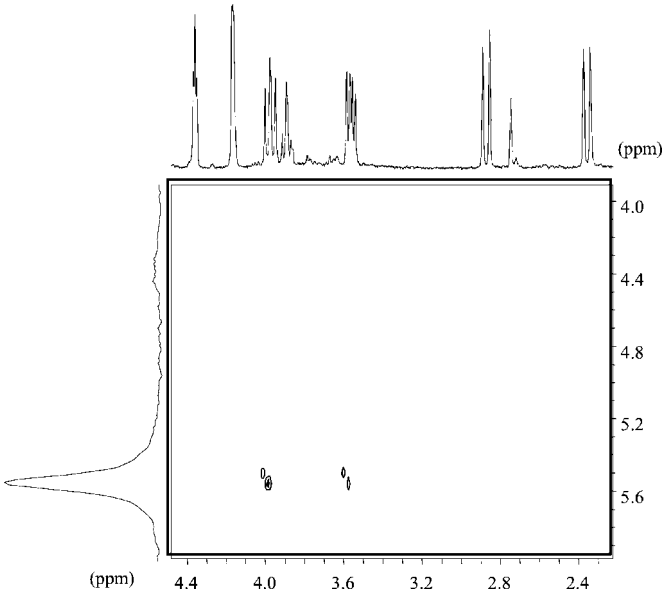

FIG. 7. **^{1}H-^{31}P COSY NMR spectrum of 2-*epi*-5-*epi*-valiolone 7-phosphate.**

cyclase AcbC represents a modification by phosphorylation. The reaction involves the conversion of 2-*epi*-5-*epi*-valiolone to 2-*epi*-5-*epi*-valiolone 7-phosphate, which is catalyzed by a novel kinase, AcbM. The AcbM polypeptide chain shows distant similarity to some members of the hexokinase family of sugar phosphotransferases, which form a cluster of orthologs in complete genome sequences (COG1940; www.ncbi.nlm.nih.gov/COG) (30). This similarity is intriguing because both enzymes D-glucose 6-kinase (GlcK) and 2-*epi*-5-*epi*-valiolone 7-kinase (AcbM) phosphorylate the primary hydroxyl group on a cyclic polyol with a six-member ring system. This might indicate that not only valienol, but also its precursor 2-*epi*-5-*epi*-valiolone, has a distinct structural resemblance to hexoses. The C_7-cyclitol unit of acarbose and its precursors behave functionally as sugar-related metabolites, which are metabolized inside cells mainly as their phosphorylated or nuleotidylated forms. The same position of the cyclitol moiety of free acarbose molecules is also phosphorylated inside cells by the acarbose 7-kinase, encoded by the *acbK* gene in the same putative transcription unit together with *acbM* and some other genes (*cf.* Fig. 2) (Refs. 19 and 29 and this work). During tests for the substrate specificity of AcbK, others had measured a weak activity also with two C_7-cyclitols not directly related to valienol metabolism and with a derivative of acarbose lacking the valienol moiety (19). These assays had been carried out with an indirect measurement of ATP consumption, and no direct determination of the phosphorylated products was achieved. However, in our hands, AcbK acted only on the oligomeric end product acarbose and not on any of the monomeric C_7-cyclitols that are likely precursors of valienol or on valienol itself. In contrast, AcbM seems to phosphorylate a monomeric cyclitol and to be specific only for the first cyclic intermediate of valienol biosynthesis, 2-*epi*-5-*epi*-valiolone, but not for any other monomeric C_7-cyclitols. Therefore, we conclude that AcbM is a biosynthetic enzyme, whereas AcbK has another, possibly protective and/or transport-prone function(s) (see below).

Further conversion of the cyclitol precursor 2-*epi*-5-*epi*-valiolone 7-phosphate is catalyzed by the isomerase AcbO, which is possibly a 2-epimerase. This conversion step was unexpected because it seemed more likely that either dehydration at positions 5 and 6 or reduction of the keto group at position 1 of the cyclitol came first. The fact that 2-*epi*-5-*epi*-valiolone is first phosphorylated and only thereafter further modified by other biosynthetic enzymes such as AcbO is in good accordance with all feeding experiments with whole cells that have been performed so far (8). In these studies, 2-*epi*-5-*epi*-valiolone was the only extracellularly provided C_7-cyclitol that was incorporated into acarbose. In contrast to these results, in addition to the identical starter metabolite 2-*epi*-5-*epi*-valiolone, some additional intermediates (5-*epi*-valiolone and valienone) were incorporated into the trehalase inhibitor validamycin A, although it shares the same cyclitol moiety, valienol (or valienamine) (*cf.* Fig. 1) (6). Therefore, the biosynthesis of the two C_7-cyclitol units (valienamine and validone) seemed to occur without initial phosphorylation by direct epimerization and dehydration or reduction (7). Also, the incorporation of the nitrogen into validamycin must occur on another route because, in acarbose, it is introduced via formation of a dideoxyaminohexose (see below). So, if phosphorylated cyclitol intermediates are used, *e.g.* before condensation, they occur in a later phase; and as compared with acarbose synthesis in *Actinoplanes* sp. SE50/110, a completely different pathway seems to be used in the validamycin producer *S. hygroscopicus* ssp. *limoneus*.

The 7-phosphorylation of the C_7-cyclitol moiety during the *de novo* synthesis of acarbose could be necessary to prevent an inhibitory effect of the C_7-cyclitol- and acarviosyl-containing intracellular metabolites on cytoplasmic enzymes sensitive to those inhibitors, such as α-glucosidases and glucomaltases of the producer. For instance, after 7-phosporylation of acarbose by AcbK, the cytoplasmic and acarbose-sensitive maltase activity of *Actinoplanes* sp. SE50/110 is no longer strongly inhibited by the modified inhibitor (19). Therefore, this phosphorylation can be regarded as a resistance-like self-protection

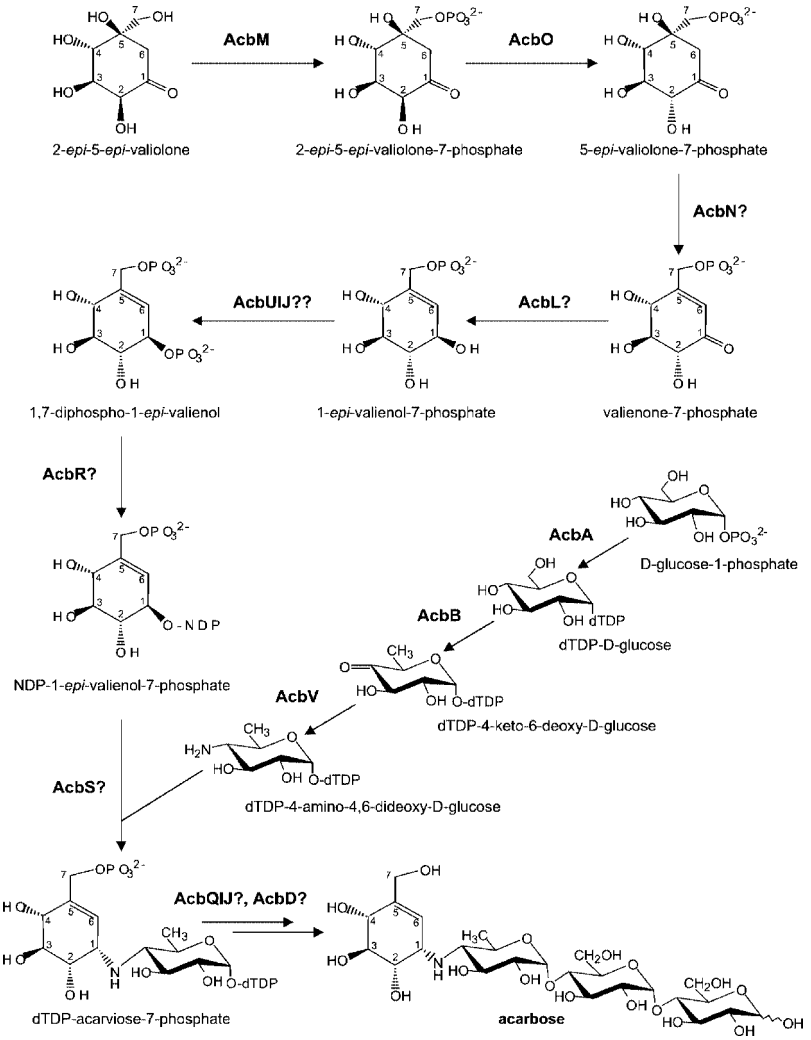

FIG. 8. **Proposed pathway for the biosynthesis of the pseudodisaccharidic acarviosyl moiety of acarbose.** The scheme shows the postulated biosynthesis for the acarviose moiety starting from the first known intermediate, 2-*epi*-5-*epi*-valiolone (5). Postulated steps are symbolized by enzyme names with *question marks*; for unknown enzymatic steps, all putative enzymes are given. If only one gene product with a *question mark* is given for one step (AcbR and AcbS), then this enzyme exhibits similarities to enzymes with similar functions and therefore is the most likely candidate for this reaction (see "Discussion"). The secondary amino-*N* is likely to be introduced via the 6-deoxyhexose unit because synthesis of dTDP-4-amino-4,6-dideoxy-4-amino-D-hexose by catalysis of amino transfer from L-glutamic acid to dTDP-4-keto-6-deoxy-D-glucose by AcbV was shown to occur by Diaz-Guardamino and Piepersberg (see Footnote 3).

mechanism similar to those in other aminoglycoside producers (3, 31). It was shown earlier that the 6-phosphorylations of both streptomycin precursors and streptomycin itself in *Streptomyces griseus* strains, which also are catalyzed by a biosynthetic 6-kinase (probably StrN) acting on a monomeric cyclitol intermediate (*N*-amidinostreptamine) and a resistance-conferring 6-kinase (AphD), always keep the bioactive metabolites in an inactivated state intracellularly (reviewed in Refs. 3 and 32). In addition, (dihydro)streptomycin 6-phosphate seems to be the export form of this antibiotic. It becomes dephosphorylated to the bioactive form only by a specific phosphatase (StrK) outside the cell (33). This coupling of resistance and active export mechanisms seems to be a widespread strategy used in producers of self-toxic metabolites. Similar modes are used also in the producers of macrolides (by glucosylation) (34) and puromycin and phosphinothricin (by acetylation) (35, 36). Therefore, we speculate that the 7-phosphorylation of acarbose-related metabolites (the intracellular end product of the underlying pathway has not yet been identified) has, in addition, a role in their export to the environment.

Acarbose can be regarded both physiologically and structurally as a member of the aminoglycoside group of bacterial products (3–5). For instance, the three subunits of streptomycin, before condensation, are either phosphorylated (streptidine 6-phosphate) or nucleotidylated (dTDP-L-dihydrostreptose or dTDP-L-rhamnose and nucleoside diphosphate-*N*-methyl-L-glucosamine). Condensation occurs via typical glycosyltransferase-catalyzed reactions. In the new pathway for acarbose, which we present here, the subunits are phosphorylated and/or activated by nucleotidylation and condensed by glycosyl transfer, too. The postulated cyclitol transferase AcbS (related to bacterial glycosyltransferases; COG0297) would catalyze the last step.

From the data presented herein and from the analysis of the whole *acb* cluster, we propose a new pathway for the biosynthesis of the acarviosyl unit of acarbose (Fig. 8). The main characteristics of our suggestion for this biosynthesis are (i) the phosphorylation of 2-*epi*-5-*epi*-valiolone at C-7 prior to its modification; (ii) the further modification of the cyclitol 7-phosphate intermediate by 2-epimerization, 5,6-dehydration, and 1-reduction, resulting in 1-*epi*-valienol 7-phosphate; (iii) a second phosphorylation and a subsequent nucleotidylation step at C-1 in 1-*epi*-valienol 7-phosphate; and (iv) the incorporation of the amino nitrogen into the sugar moiety and not into the cyclitol unit. Evidence for the phosphorylation of 2-*epi*-5-*epi*-valiolone and its further conversion products has been presented in this work. In additional experiments, we have found evidence that 1-*epi*-valienol is phosphorylated by a crude extract from *Actinoplanes* sp. SE50/110 (data not shown); experiments with 1-*epi*-valienol 7-phosphate as the more likely substrate (because extracellularly applied 1-*epi*-valienol is not incorporated) (8) in similar assays are in progress. Phosphorylation at C-1 would be a prerequisite for a subsequent nucleotidylation step. In the *acb* cluster, we have identified the gene *acbR*, which encodes a GlgC (ADP-glucose synthase; COG0448)-like protein, which could catalyze the nucleotidylation of the likely precur-

sor 1-*epi*-valienol 1,7-diphosphate. We also have experimental evidence that the gene *acbV* encodes a GabT-like aminotransferase, which uses dTDP-4-keto-6-deoxyglucose and L-glutamate as substrates for the synthesis of dTDP-4-amino-4,6-dideoxy-D-glucose.[3] From all these data, it seems most likely that the acarviose is the condensation product of nucleoside diphosphate-1-*epi*-valienol 7-phosphate and dTDP-4-amino-4,6-dideoxy-D-glucose. The putative glycosyltransferase AcbS is postulated to fulfill this function. It is still unclear whether acarviose (or activated acarviose or acarviose 7-phosphate) or acarbose is the final product that is synthesized inside the cell. So far, we do not know if the glucose or maltose moieties of acarbose are introduced inside the cell or after the export of the activated acarviose. If acarviosylglucose 7-phosphate is the exported product, the acarviosyltransferase AcbD described by Hemker *et al.* (28) could be a candidate that adds, via transglycosylation, the maltose or maltotriose residues to the acarviosyl unit outside the cell to form acarbose and the higher order oligomeric components found in the fermentation broth of *Actinoplanes* sp. SE50/110.

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