Biosynthesis of the C7-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 C7-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., Mahmoud, 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, acbKLNO, 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 C7-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 C7-amino cyclitol 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 (dehydroquinase synthase, AroB) proteins. Its product was shown to be a C7-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. 1B) is an antifungal antibiotic used as a crop protectant. Validamycins are produced by Streptomyces hygroscopicus spp. limoneus and consist of two similar C7-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, valiennone, 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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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-

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**FIG. 1.** Chemical structures of two α-glucosidase inhibitors containing C₇-cyclitols. A, acarbose; B, validamycin A; C, C₇-cyclitol. Valienamine is not shown as an extra structure because it is represented by the cyclitol moiety of acarbose including the NH₂ group at C-1.
Biosynthesis of the C₇-cyclitol Moiety

TABLE I

| 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 *sp-*                                                   | 11          |
| *S. lividans* TK64                           | Actinorhodin, prodigiosin *pa* - *str*                                            | 11          |
| E. coli BI2.1(DE3)pLysS                      | TT RNA polymerase, cat                                                             | 21          |
| E. coli DH5α                                  | F′ ϕ80d, lacZΔ15, endA1, recA1, hsdR17 (rK, m−), supE44, thi-1, ·, gyrA96, relA1, DlacZα-argF/U169 | 22          |
| E. coli JM109                                  | F′ troD36, lacZΔ15, proA2 B′ lacZ Δ lacI Δ recA1, hsdR17 (rK, m−), supE44, thi-1, gyrA96, relA1, ΔlacZα-argF/U169 | 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          |
| pJO2702                                     | blacrZB, rha-p                                                                   | 26          |
| pJL4123                                     | kan, tsr, tipA                                                                    | 13          |
| pJL6021                                     | kan, tsr, tipA                                                                    | 13          |
| pUC18                                       | bla, lacZ-α                                                                       | 23          |
| pUWL201RBSA                                  | bla, lacZ-α, tsr, ermEp                                                            | 27          |
| pUCW49                                      | blacZ-α, tsr, ermEp, eryBIV                                                        | 27          |
| pUCW50                                      | blacZ-α, tsr, ermEp, eryBIV                                                        | 27          |
| pAS8/7                                      | acbC in pJL6021                                                                   | 5           |
| pCW1L1                                      | 1.42-kb NdelI/SstI fragment from pMJL1 in pET16bP (NdelI/SstI)                     | This work   |
| pCWN16                                      | 0.86-kb NdelI/BamHI fragment from pMJN1 in pET16bP (NdelIBamHI)                    | This work   |
| pCW1O1                                      | 0.82-kb NdelI/HindIII acbO fragment in pUWL201RBSA (NdelI/HindIII)                 | This work   |
| pCW201L                                     | 1.1-kb NdelI/HindIII acbO fragment in pUWL201RBSA (NdelI/HindIII)                  | This work   |
| pCW201K6                                    | 1.98-kb NdelI/HindIII acbKM fragment in pUWL201RBSA (NdelI/HindIII)                | This work   |
| pCW4123L                                    | 1.42-kb NdelI/EcoRI acbL fragment in pJL4123 (NdelI/EcoRI)                         | This work   |
| pCW4123M                                    | 1.07-kb NdelI/EcoRI acbM fragment in pJL4123 (NdelI/EcoRI)                         | This work   |
| pMJJ1                                       | 1.42-kb acbL PCR fragment in pUC18 (SmaI)                                         | This work   |
| pMJM1                                       | 0.86-kb acbN PCR fragment in pBluescript SK (EcoRV)                                | This work   |
| pMJM2                                       | 0.86-kb NdelI/BamHI acbN fragment in pET11aP (NdelI/HindIII)                       | This work   |
| pMJN1                                       | 0.86-kb NdelI/BamHI acbN fragment in pUWL201RBSA (NdelI/HindIII)                   | This work   |
| pMJO1                                       | 0.87-kb acbO PCR fragment in pBluescript SK (EcoRV)                               | This work   |
| pMJO7                                       | 0.87-kb NdelI/BglIII acbO fragment in pJL4123 (NdelI/HindIII)                     | 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 flour-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 pJL4123 and pJL6021 and their recombinant derivatives, media were supplemented with kanamycin (50 μg/ml). 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 μg/ml).

**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 acbKLMO 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 MgCl₂, 20 mM NH₄Cl, 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).
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 MgCl₂, 20 mM NH₄Cl, 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, NAPH, FAD, or FADH₂) were used in final concentrations of 10 mM in the test volumes.

Method a—The acbK gene was amplified by Drebber and Pape (18), but without NH₄Cl in the buffer.

Method b—The acbM gene was amplified by Drebber and Pape (18), but without NH₄Cl in the buffer.

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 flow-through was collected, concentrated 10-fold by freeze-drying, and then applied to an 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

30 ml of the AcbC reaction solution were heated at 90 °C for 5 min, centrifuged (5000 rpm, 20 min), and then applied to a ultratitration 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 obtained. 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.

NMR

All NMR spectra were recorded on a Bruker ARX 400 spectrometer (400 MHz). In addition to ¹H, ¹³C, and ³¹P experiments, also COSY (¹H-¹H, ¹H-¹³C, and ¹H-³¹P) and distortionless enhancement of polarization transfer (DEPT) spectra for the unequivocal correlation of the hydroxyl, 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), ²J (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: ¹H NMR (d₆-MeOH, 400 MHz): 8 = 2.33 (dd, 1H, J = 13.7, 1.7 Hz, H-6₅), 2.84 (d,
RESULTS

Identification of AcbK as the Acarbose 7-Kinase—The gene cluster for the synthesis and metabolism of acarbose and related components of the amylosstatin complex of the producer Actinoplanes sp. SE50/110 has been cloned on a single cosmid and fully sequenced recently. 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 acarosyltransferase 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 (GenBankTM/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. 3A). 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/pCW2101KMe6) (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. 4A). 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. 1C).

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 dinucleotided-binding fold, the zinc-dependent and short-chain alcohol dehydrogenases, respectively (data not shown). AcbM exhibited a distant similarity to GlcK from Bacillus subtilis (GenBankTM/EBI accession number P54495) and other glucokinases, being a member of the hexose 6-kinase family of phosphotransferases (Fig. 3B). 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, and AcbO 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 dinucleotided-binding fold, the zinc-dependent and short-chain alcohol dehydrogenases, respectively (data not shown). AcbM exhibited a distant similarity to GlcK from Bacillus subtilis (GenBankTM/EBI accession number P54495) and other glucokinases, being a member of the hexose 6-kinase family of phosphotransferases (Fig. 3B). No data base entries significantly similar to AcbO were found with all methods of similarity search used (BLAST and fasta 3).

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In subsequent assays using [$\gamma^32$P]ATP, we determined that the new product was indeed radioactively labeled. This clearly showed that 2-epi-5-epi-valiolone phosphate was phosphorylated. We also tested the phosphorylation of other cyclitols (2-epi-valiolol, 5-epi-valiolol, 1-epi-2-epi-5-epi-valiolol, 1-epi-5-epi-valiolol, 1-epi-valienol, and valienol) (Fig. 1B). To overcome this problem, we synthesized enantiomerically pure 2-epi-5-epi-valiolone phosphate. Then 1H, 13C, and 31P NMR data for chemically synthesized and purified 2-epi-5-epi-valiolone were used in scaled-up phosphorylation assays. From these assays, the phosphorylated product was isolated and purified 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 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 phosphorylation, the substrate 2-epi-5-epi-valiolone was enzymatically phosphorylated. In a coupled assay (see “Materials and Methods”), 2-epi-5-epi-valiolone was synthesized, and the purified product was incubated with AchM-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.

Fig. 3. Alignment of the AchK and AchB protein sequences with those of other kinases. A, the AchK 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 AchB sequence is distantly related to members of the glucokinase family (COG1940). A consensus sequence is given for residues identical in all three aligned sequences. The sequences aligned are from the following sources: AchK sp. SE50/110 (GenBank TM/Glka4; accession code AAK25695); GlkASL, putative glucokinase from S. lividans TK24 (accession code AAF42869); GlkCBSU, putative glucokinase from B. subtilis 168 (accession code F54465); AchMsp, AchM protein from Actinoplanes sp. SE50/110 (accession code Y18035).
Acarbose

**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 pTLW201 (control; lane 1) or pCW201KM6 (AcbK; lane 2). B, production of AcbM and AcbL in S. lividans TK64. Soluble proteins from cells harboring plasmid pJL4123 (control; lane 1), pCW122M (N-terminal His-tagged AcbL; lane 2), or pCW122M (N-terminal His-tagged AcbM; lane 3) were subjected to PAGE. C, production of AcbL in S. lividans TK23 harboring pMNN5 (N-terminal His-tagged AcbN; lane 1). D, production of AcbM in S. lividans TK64 harboring plasmid pMJ07 (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**

| 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                   | +                 | –                 |

Phosphorylation assays with AcbK and AcbM

All substrates were used in phosphorylation assays with unlabeled ATP and [γ-32P]ATP as described under “Materials and Methods.” +, phosphorylation; ±, weak phosphorylation, detectable only with [γ-32P]ATP, –, no phosphorylation.

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 13C spectrum, C-7 appears as a doublet at 70.2 ppm with coupling constant J_C7,C7_P = 5.1 Hz. Instead of the expected carbonyl resonance, C-1 shows a resonance for a quaternary carbon at 101.23 ppm, indicating a hydration 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.

**FIG. 5. TLC analyses of AcbM and AcbO assays.** Extracts from S. lividans TK64 with pIJ4123 (control), pCW122M (AcbM), or pMJ07 (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 cesium/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 ATP (10 mM); lanes 2-11, 4 μl of the reaction mixtures of complete assays with cell extracts from strains containing plasmids pJL4123, pMJ07, pCW122M, pCW122M (without ATP), pCW122M/pMJ07, pCW122M/pMJ07, pCW122M (without ATP), pMJ07, 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 C7-cyclitol unit of the acarvisoyl 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 acarvisone. 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
Biosynthesis of the C_7-cyclitol Moiety

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. 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 sp. 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 a-glucosidases and glucomaltases of the producer. For instance, after 7-phosphorylation 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...
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 Strep-
tomyces griseus strains, which also are catalyzed by a biosynthetic 6-kinase (probably StrN) acting on a monomeric cyclitol inter-
mediate (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 produc-
ers 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 me-
tabolites (the intracellular end product has not yet been identi-
fied) has, in addition, a role in their export to the environment.

Acarbose can be regarded both physiologically and structur-
ally as a member of the aminglucoside group of bacterial
products (3–5). For instance, the three subunits of streptomy-
cin, before condensation, are either phosphorylated (strepti-
dine 6-phosphate) or nucleotidylated (dTDP-1-dihydrostreptose
or dTDP-1-rhamnose and nucleoside diphosphate-N-methyl-
-glucosamine). Condensation occurs via typical glycosyltrans-
ferase-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 trans-
fer, 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 biosyn-
thesis of the acarboxyl unit of acarbose (Fig. 8). The main
characteristics of our suggestion for this biosynthesis are (i)
the phosphorylation of 2-epi-5-epi-valiollone (5) prior to its.mod-
fication; (ii) the further modification of the cyclitol 7-phosphate
intermediate by 2-epimerization, 5,6-dehydration, and 1-reduc-
tion, resulting in 1-epi-valienol 7-phosphate; (iii) a second phos-
phorylation 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-valiollone
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 Acti-
noplanes sp. SE50/110 (data not shown); experiments with
1-epi-valienol 7-phosphate as the likely substrate (be-
cause 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-

FIG. 8. Proposed pathway for the biosynthesis of the pseudodisaccha-
ride acarviosyl moiety of acarbose. The scheme shows the postulated biosyn-
thesis for the acarviol moiety starting from the first known intermediate, 2-epi-
5-epi-valiollone (5). Postulated steps are symbolized by enzyme names with
question marks; for unknown enzymatic steps, all putative enzymes are given. If one
gene product with a question mark is given for one step (AcbR and AcbS), then
this enzyme exhibits similarities to en-
zymes with similar functions and there-
fore 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
dTTP-4-amino-4,6-dideoxy-4-amino-b-
hexose by catalysis of amino transfer from
L-glutamic acid to dTTP-4-keto-6-deoxy-
N-glucosamine by AcbV was shown to occur by
Diaz-Guardamino and Piepersberg (see
Footnote 3).
sor 1-epi-valienol 1,7-diphosphate. We also have experimental evidence that the gene acbV encodes a GBt-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. 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 acarviose 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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