MINI-REVIEW

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# Biotechnology and molecular biology of the α-glucosidase inhibitor acarbose

**Abstract** The α-glucosidase inhibitor acarbose, $O$-{4,6-dideoxy-4[1s-(1,4,6/5)-4,5,6-trihydroxy-3-hydroxymethyl-2-cyclohexen-1-yl]-amino-$\alpha$-D-glucopyranosyl}-(1→4)-$O$-$\alpha$-D-glucopyranosyl-(1→4)-D-glucopyranose, is produced in large-scale fermentation by the use of strains derived from *Actinoplanes* sp. SE50. It has been used since 1990 in many countries in the therapy of diabetes type II, in order to enable patients to better control blood sugar contents while living with starch-containing diets. Thus, it is one of the latest successful products of bacterial secondary metabolism to be introduced into the pharmaceutical world market. Cultures of *Actinoplanes* sp. also produce various other acarbose-like components, of which component C is hard to separate during downstream processing, which is one of the most modern work-up processes developed to date. The physiology, genetics and enzymology of acarbose biosynthesis and metabolism in the producer have been studied to some extent, leading to the proposal of a new pathway and metabolic cycle, the "carbophore". These data could give clues for further biotechnological developments, such as the suppression of side-products, enzymological or biocombinatorial production of new metabolites and the engineering of production rates via genetic regulation in future.

## Introduction

Historically, the first wave of designed screening of bacterial and other microbial secondary metabolites was aimed at the detection of anti-infective substances (antibiotics), such as antimicrobials and antivirals, and was started during world war two. Later, starting in the 1960s, other activities of potential use in many applica-

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tions were also searched for in similar screening protocols. These included in the first instance microbial inhibitors of hydrolases, such as proteases and glycosidases, but also comprised antitumor agents, immunomodulators, cardiovascular agents, inhibitors or modulators of human intracellular enzymes, signal transduction components and agents involved in the cell cycle and functions of the cell skeleton (see e.g. Demain et al. 1989; Rehm et al. 1997). Glycosidase inhibitors were suggested for pharmaceutical or agricultural applications, e.g. obesity, adipose, hyperlipidemia (arteriosclerosis), diabetes, pre-diabetes, gastritis, gastric ulcer, duodenal ulcer and caries in man, or as food additive for various purposes in farm animals (Frommer et al. 1975, 1977a, 1977b, 1979). First, α-glucosidase inhibitors from plants were tested in animal trials for use in humans, but soon turned out not to be efficient (Puls and Keup 1973, Puls et al. 1977; Bischoff et al. 1994). In screenings among actinomycetes, besides the pseudooligosaccharides described herein, polypeptide-based amylase inhibitors were also detected and developed, e.g. tendamistat (Taylor and Barker 1983; Wiegand et al. 1995). From the Bacillaceae, another type of glycosidase inhibitor, 1-deoxynojirimycin (a monosaccharide), was developed and, in form of a synthetic derivative, 3,4,5-piperidinetriol,1-(2-hydroxyethyl)-2-(hydroxymethyl)-[2R-(2a,3b,4a,5b)]-deoxynojirimycin, marketed under the trade name Miglitol (Mueller 1989).

## Detection, use and structural features of C7-aminocyclitol-containing glycosidase inhibitors

The first published group of C7-cyclitol-containing glycosidase inhibitors were the validamycins/validoxylamines obtained from *Streptomyces hygroscopicus* ssp. *limoneus*, having antibiotic activity against fungi and acting as trehalase inhibitors (Iwasa et al. 1970; Asano et al. 1990; Fig. 1B). The acarbose-related metabolites were first found at about the same time (priority date of the first patent on these inhibitors is 28 December 1970; see Frommer et al. 1975), in a screening of many strains of

various actinomycete genera for their activity as inhibitors of three mammalian intestinal glycosidases: α-amylase, saccharase, and maltase (Frommer et al. 1975, 1977a, 1977b; Truscheit et al. 1981; Mueller 1989). The same and other metabolites of the same group and further activities, e.g. against trehalases, were detected by various groups, mainly in streptomycete producers: amylostatins (Murao 1975; Murao and Ohyama 1975), oligostatins (Itoh et al. 1981), adiposins (Namiki et al. 1982), trestatins (Yokose et al. 1983) and 5,6-epoxy-C7-cyclitol-containing derivatives of amylostatins (NS complex; Takeda et al. 1983). Acarbose, $O${4,6-dideoxy-4[1s-(1,4,6/5)-4,5,6-trihydroxy-3-hydroxymethyl-2-cyclohexen-1-yl]-amino-α-D-gluco-pyranosyl}-(1→4)-$O$-α-D-glucopyranosyl-(1→4)-D-gluco-pyranose, is structurally an unusual pseudotetrasaccharide, analogous to maltotetraose, and is a component of a complex mixture of shorter or longer components, also called amylostatins (Fig. 1A). Its core structure is the pseudodisaccharide acarviosine (valienaminyl-4-amino-4,6-dideoxyglucose), which is α-1,4-bound to a maltose residue (Fig. 1A). In general, these compounds can be regarded as members of the class of aminocyclitol-containing aminoglycosides which, with few exceptions, are all produced by actinomycetes (Piepersberg 1997; Piepersberg et al. 2002).

The mode of action of the inhibitory activity of acarbose on several α-glucosidases and cyclodextrin glucanotrans-ferases has been studied, e.g. in crystals of enzyme-inhibitor complexes (e.g. Qian et al. 1994; Strokopytov et al. 1995; Gilles et al. 1996; Machius et al. 1996). The acarviosine moiety binds with high affinity to the active centers of the proteins in this enzyme family (Brayer et al. 2000). Obviously, various modes of binding and cleavage and subsequent transglycosylation with two or more acarbose molecules can also occur on α-amylases and related members of the COG family of enzymes (Tatusov et al. 2001). Transglycosylation creates elongated deriva-tives with two or more acarviosyl residues, both in the presence and absence of additional maltooligosaccharides, and forms inhibitors with much stronger affinity to the enzyme molecule. In α-amylases, these efficient inhibitor molecules bind mostly with one of their aminoglycosidic bonds, which cannot be hydrolytically split, in the hydrolase center and thereby block the enzyme almost irreversibly. Thus, acarbose acts at least in some of the blocked hydrolases as a prodrug.

The α-glucosidase inhibitor acarbose (Glucobay) is now marketed worldwide (launched in 1990 in Germany, launched in the USA in 1995) and is used in the therapy of diabetes type II (non-insulin-dependent), in order to enable patients to better control blood sugar contents while living with starch-containing diets (Ahr et al. 1989; Bischoff et al. 1994). It was selected from the amylostatin complex because of its higher inhibitory activity towards intestinal sucrase, maltase, dextrinase and glucoamylase, relative to its weaker inhibition of α-amylase. These properties, together with the specific pharmacokinetics and toxicology of the substance, make it an ideal therapeutic.
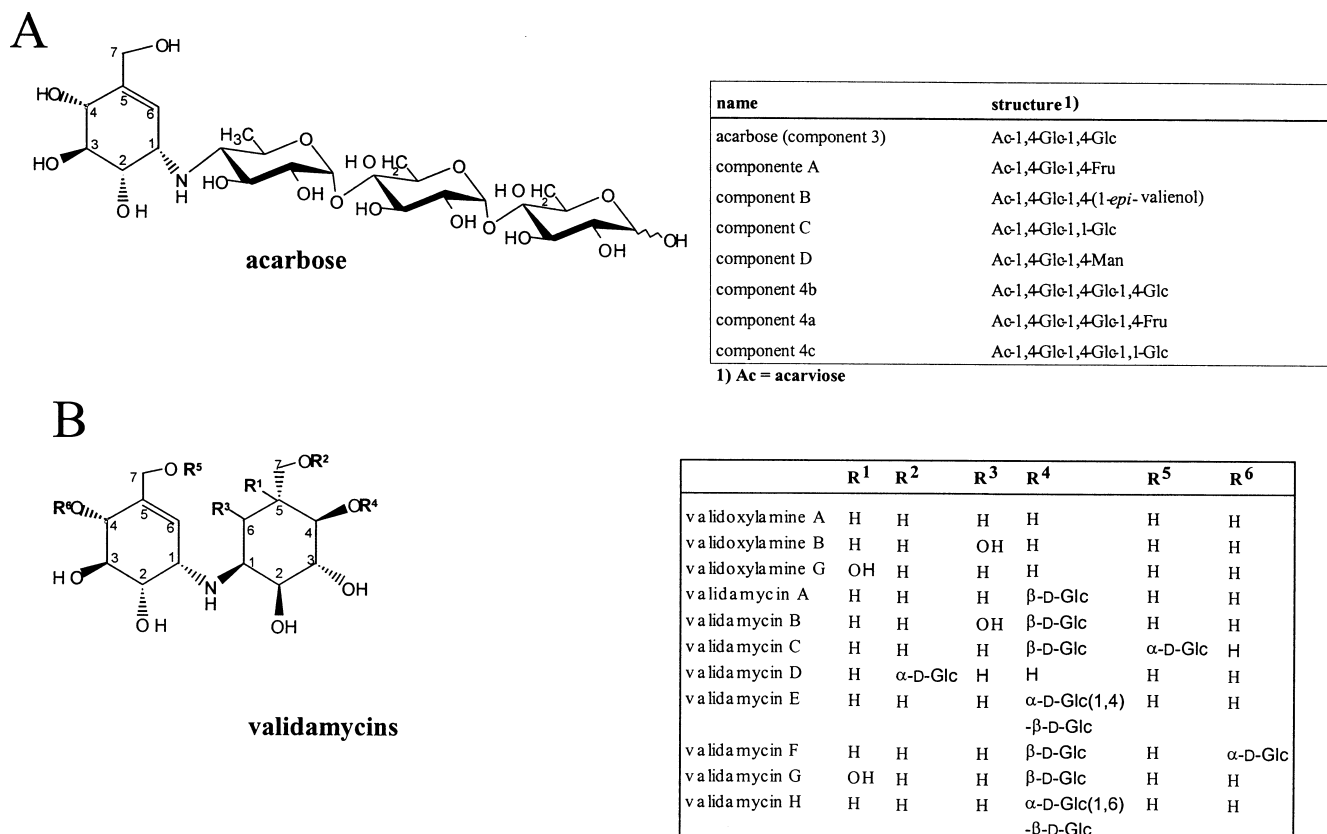


A

acarbose

| name | structure[1] |
|---|---|
| acarbose (component 3) | Ac-1,4-Glc-1,4-Glc |
| componente A | Ac-1,4-Glc-1,4-Fru |
| component B | Ac-1,4-Glc-1,4-(1-*epi*-valienol) |
| component C | Ac-1,4-Glc-1,1-Glc |
| component D | Ac-1,4-Glc-1,4-Man |
| component 4b | Ac-1,4-Glc-1,4-Glc-1,4-Glc |
| component 4a | Ac-1,4-Glc-1,4-Glc-1,4-Fru |
| component 4c | Ac-1,4-Glc-1,4-Glc-1,1-Glc |

[1] Ac = acarviose

B

validamycins

| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ |
|---|---|---|---|---|---|---|
| validoxylamine A | H | H | H | H | H | H |
| validoxylamine B | H | H | OH | H | H | H |
| validoxylamine G | OH | H | H | H | H | H |
| validamycin A | H | H | H | β-D-Glc | H | H |
| validamycin B | H | H | OH | β-D-Glc | H | H |
| validamycin C | H | H | H | β-D-Glc | α-D-Glc | H |
| validamycin D | H | α-D-Glc | H | H | H | H |
| validamycin E | H | H | H | α-D-Glc(1,4)-β-D-Glc | H | H |
| validamycin F | H | H | H | β-D-Glc | H | α-D-Glc |
| validamycin G | OH | H | H | β-D-Glc | H | H |
| validamycin H | H | H | H | α-D-Glc(1,6)-β-D-Glc | H | H |

**Fig. 1** Structure of acarbose (**A**), validamycins (**B**) and related natural products

In short, since the membrane-bound oligo- and disaccharidases at the surface of the intestinal microvilli are mainly responsible for the absorption of glucose into the blood, acarbose is preferred relative to the higher order amylostatins. Acarbose acts as a competitive inhibitor, without interfering with the glucose uptake directly. Only very little acarbose (1–4%) is absorbed itself and it practically does not penetrate into tissue. Instead, this amount is secreted rapidly via the kidneys. Also, the main fraction remaining in the intestinal tract stays mostly unaltered, with the occurrence of some microbial metabolism and conversion of intestinal glucosidases. This latter effect is in part due to the fact that e.g. pancreatic α-amylase converts acarbose to the longer products mentioned above. In general, acarbose behaves like other hardly digestible polysaccharides and has no detectable toxicity. The overall effect of this on the diabetic patient is that the starch-containing diet is degraded more slowly and along the full length of the small intestine. Thus, in type II diabetics the post-prandial increase of blood glucose levels is significantly reduced. In man, a dose of 1–1.5 mg acarbose/kg body weight reduces the post-prandial hyperglycemia after ingestion of a carbohydrate-containing diet by 50% and can help to avoid glucosuria and other deleterious effects (Truscheit et al. 1988).

## Technology of acarbose production

Acarbose-type compounds were found in a screening at Bayer AG in a program designed for the detection of inhibitors for mammalian intestinal α-amylase, sucrase and maltase activities among the products of various actinomycetes (first claimed in a patent in 1970; Frommer et al. 1975). The active ingredient of Glucobay, a weakly basic acarbose, was produced in a multi-step batch fermentation with developed strains derived from *Actinoplanes* sp. SE50 (CBS 961.70; ATCC 31042) and by use of feeding protocols. A multi-stage purification process was carried out at the Bayer plant at Wuppertal–Elberfeld (Fig. 2). The sterile fermentation took place in 30–100 m$^3$ fermenters in a high-concentration medium, mainly based on starch hydrolysates (10% or higher) with or without added maltose (low glucose levels) under continuous control and applying a feed of nutrients to keep the main C-source (poly-, oligomaltodextrins) at a high concentration throughout (Frommer et al. 1977a; Rauenbusch and Schmidt 1978; Beunink et al. 1997). The basic downstream processing of the industrial production process involved seven steps:

1. The ion content of the culture broth was lowered by mixing it with both a highly acidic cation exchanger and an anion exchanger. Under this condition, most (more than 80%) of the acarbose bound to the cation exchanger.
2. The resins were separated from the broth and mycelia by passing the mixture through a sieve screw centri-
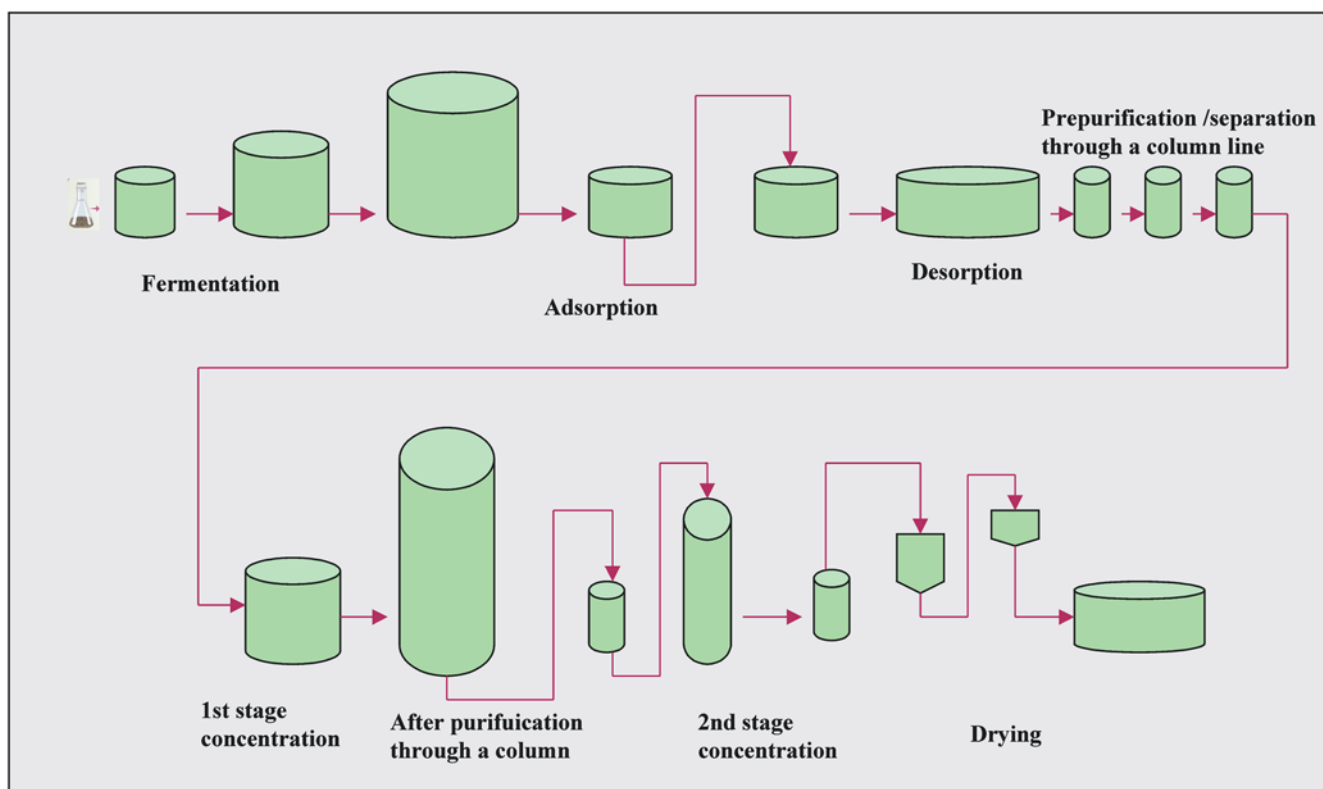


**Fig. 2** Schematic flow-sheet for the large-scale production of acarbose (according to Bayer AG, brochure PH-TO/WP, PH 5)

fuge, in which a washing step (deionized water) also took place.

3. Elution by 0.1 M sodium acetate occurred in kettles containing a suction head with nozzle sieves in the bottom, connected via a pump and rotameter to a column line.

4. Both inhibitor and contaminants were adsorbed and thereby separated via a column line of three ion-exchange columns (cation/anion/cation exchangers in acidic/basic/acidic forms, respectively) of which the first was used for desalination, the second for elevating the pH to higher than 3.0 and the third for binding the acarbose and its related components.

5. The last column was eluted by a gradient of 0.01–0.05 M mineral acids and a fractionated collection was made of the different components, the acarbose fraction of which was cut off.

6. The final creation of a slightly colored powder was achieved by volume reduction in a vacuum and the pH was brought to a value of about pH 5–7 by a mixture of cation and anion exchange resins, filtration through a sterile filter and freeze- or spray-drying (Rauenbusch and Schmidt 1978).

7. Later, several improvements of the fermentation process and downstream processing were published, e.g. replacement of step 6 of the above procedure by an upscale of a quasi-HPLC procedure (e.g. columns of 11 $m^3$ bed volume, 2.5 m diameter, 3 m height) via the development of a sulfonated bead cation exchanger using specially developed macroporous, hard and resistant polymers based on aromatic compounds with at least one vinyl group and one hydrophilic monomer (e.g. 6–20% divinylbenzene, 5-25% methoxymethacrylamide, among others; Lange and Rauenbusch 1986). This allowed a more rapid flow and smaller elution volumes, combined with a stronger separation.

Other improvements included: (a) the refined purification and separation of the acarbose fraction from the related components and other impurities after step 6 of the



Fig. 3 Chemical structures of C7-cyclitols used in studies of the biosynthesis of acarbose and validamycin
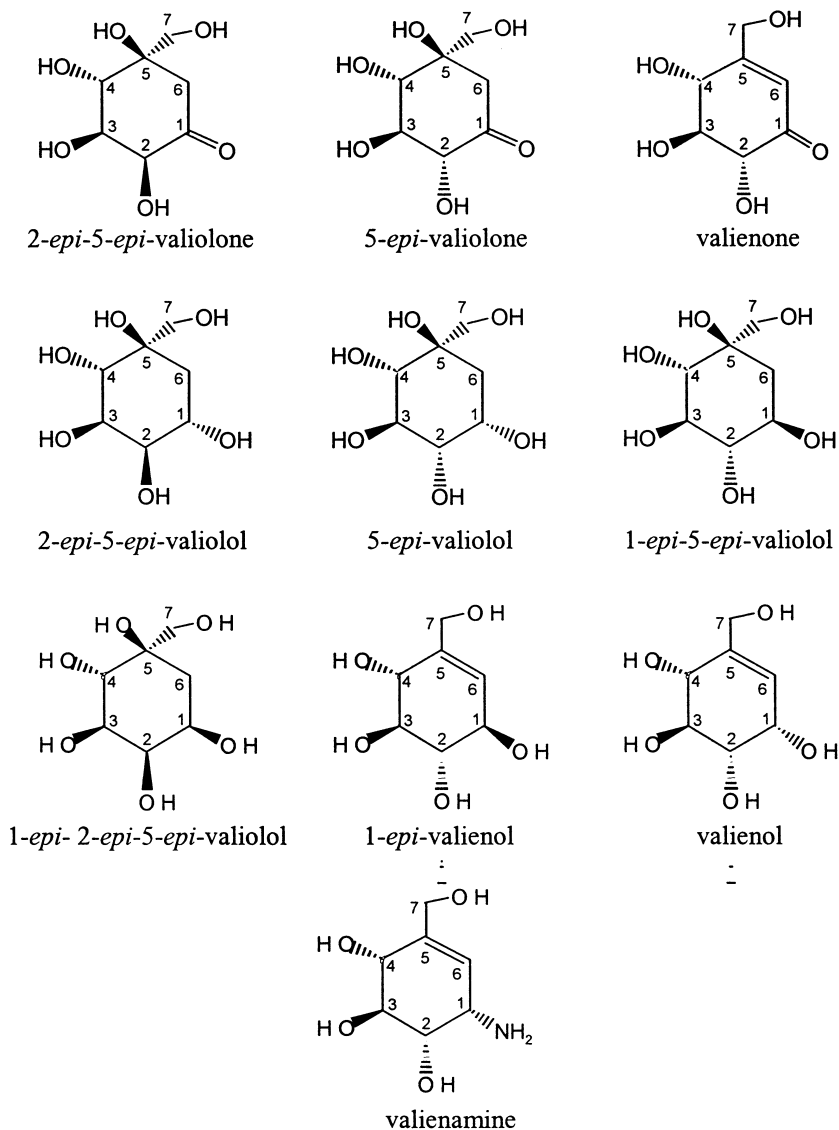
above procedure, using a special weakly acidic cation exchanger based on carboxymethyl and yielding about 98% pure acarbose (Rauenbusch 1987) and (b) controlling the osmolality of the fermentation broth at an optimal value around a relatively sharp optimum of 400 mosmol/kg. This was achieved by feeding protocols or controlling the nutrient and salt supply in continuous culture (Beunink et al. 1997), both lower and higher osmolalities having severely negative effects. No exact values for the final acarbose concentrations in the harvested broth of the fermentation have been reported for the actual production strains. The deposited derivative *Actinoplanes* sp. SE50/110 produces up to 1 g acarbose/l under optimal culture conditions (Rauenbusch and Schmidt 1978). As a side-product, with increasing relative concentration during the elongated fermentation, component C (cf. Fig. 1) seems to be unavoidable. Due to its high structural similarity to acarbose, component C is hard to separate. The biotechnological production process requires 2–3 weeks; and the purified product still contains some contamination by component C (below 2%).

## Physiology and biogenetics of acarbose formation in the producer, *Actinoplanes* sp. SE50

During the development of derivatives of *Actinoplanes* sp. SE50 for production, it soon became apparent that glucose concentration (negative control) and, relative to that, maltose and maltooligodextrin contents up to higher order hydrolysates of starch (positive control) play a key role in determining the productivity (Frommer et al. 1975; Frommer et al. 1977a, 1977b; Rauenbusch and Schmidt 1978). Further evidence for direct positive regulation (induction) by maltooligosaccharides, especially by maltotriose, came from the finding of carbohydrate-regulated operator sites, the so-called dyadic symmetry element (DSE) boxes, upstream of some of the key acarbose production genes, namely *acbA*, *acbB*, *acbD* and *acbE*, which are pairwise transcribed (*acbA* with *acbB*, *acbD* with *acbE*) from two common, divergently oriented promoter regions (see below; Stratmann 1997). The *acbD* and *acbE* genes, when heterologously expressed in strains of *S. lividans* 66, were largely inducible by maltotriose. Similar putative DSE-type operator sites mediating induction and glucose repression were reported for α-amylase genes in different *Streptomycetes* strains; and a pleiotropic regulator gene, *reg1*, exists in *S. lividans* 66, where it potentially could act heterologously on *acb* genes (Virolle and Gagnat 1994; Nguyen et al. 1997).

The biogenesis of the valienamine moiety and the acarbose molecule was investigated during the 1980s by feeding studies with various labeled precursors (Fig. 3) in whole-cell experiments with strains derived from *Actinoplanes* sp. SE50 (Degwert et al. 1987; Rinehart et al. 1992; Lee et al. 1997; Lee and Egelkrout 1998; Mahmud et al. 1999; Mahmud 2003). Biogenetic studies were conducted from the starting hypothesis that the C7N moiety of acarbose could be introduced via valienamine (cf. Fig. 3) as an intermediate and use a shikimate pathway for its biogenesis. This was tested first, but found to be not the case. Instead, a cyclization of a heptulosephosphate derived from transketolase reactions was proposed. The amino-*N* was shown to be most likely introduced from L-glutamate. Surprisingly, the labeled C7-cyclitol precursors which could be introduced into the valienamine moiety of validamycin (Dong et al. 2001; Mahmud et al. 2001) could not be incorporated into acarbose, except for one compound, 2-*epi*-5-*epi*-valiolone, which is the starting precursor of the biosynthetic pathway and is derived from *sedo*-heptulose-7-phosphate (see below). In contrast, the presumed C7-cyclitol intermediates 5-*epi*-valiolone, valienone and validone are efficiently incorporated into validamycin in its producer, *S. hygroscopicus* ssp. *limoneus*. The maltose unit in acarbose was shown to be directly incorporated from maltose or maltotriose, rather than via successive addition of glucose residues.

The enzymology of acarbose metabolism in the producer *Actinoplanes* sp. SE50 was extensively investigated by Pape and coworkers (Drepper and Pape 1996; Drepper et al. 1996; Goeke et al. 1996; Hemker 1997; Hemker et al. 2001; Stolpe 2001), who showed that an intracellular phosphotransferase phosphorylated the C7-hydroxyl of the cyclitol moiety (acarbose-7-kinase), and an unusual intracellular maltose-1-kinase and an acarviosyltransferase which (α-1,4)-transglycosylated the acarviosyl residue to several sugars and oligosaccharides with a glucose-like configuration around the receptor 4-OH group. The acarviosyltransferase first was found cell-attached, but was later also found in the fermentation supernatant. Some of this enzymology could also be attributed to encoding genes in the *acb* cluster (see below).

## Genetics of acarbose metabolism in the producer *Actinoplanes* sp. SE50

For the isolation of gene clusters of polyketides in actinomycetes, well established and highly conserved tag sequences which have existed since the mid-1980s can now efficiently be used in heterologous PCR protocols. The findings that many antibiotics and other secondary metabolites contain 6-deoxyhexoses (6DOHs; which are made via the dTDP-glucose pathway) and that the key enzyme, dTDP-D-glucose 4,6-dehydratase, is also highly conserved allowed the screening of DNA libraries with the respective probes (Stockmann and Piepersberg 1992; Piepersberg 1994). Studies on the genetics of amylostatin (acarbose) production were the first to be started using heterologous PCR in screening and analyzing DNA-fragments around the dTDP-D-glucose 4,6-dehydratase-encoding genes in the two organisms *Actinoplanes* sp. SE50 [mainly in strain SE50/110 (CBS 674.73, ATCC 31044); Crueger et al. 1996, 1997; Stratmann 1997; Stratmann et al. 1999] and *S. glaucescens* GLA.0 (Decker 1997; Fig. 4; Table 1). This method also proved to be useful in many other cases (e.g. Decker et al. 1996).

Testing other gene sequences commonly observed in secondary metabolic gene clusters, such as the secondary metabolic aminotransferase (SMAT) members which occur as aminocyclitol aminotransferases (Piepersberg 1994; Ahlert et al. 1997; Ota et al. 2000; Tamegai et al. 2002), failed to identify the *acb* cluster in *Actinoplanes* sp. SE50/110. This was later explained by the fact that the biosynthetic aminotransferase of the acarbose pathway, AcbV, is a member of another aminotransferase family (see below; Table 1).

Of the proteins encoded by the six genes analyzed in the partial *acb* gene cluster from *S. glaucescens* GLA.0, only the AcbA(*Sgl*) and AcbB(*Sgl*) proteins showed a strong conservation relative to the homologous proteins AcbW (*Asp*) and AcbV(*Asp*), respectively, from *Actinoplanes* sp. SE50. This could mean that gene clusters and production abilities for amylostatin-like compounds are both old and widespread features in the Actinomycetales. However, related gene sets do not occur in all strains; e.g. they are absent from the full-length genomes of *S. coelicolor* A3(2) and *S. avermitilis* (Piepersberg 2001; Bentley et al. 2002; Ikeda et al. 2003). Evidence for the functionality of these *acb* clusters to represent the real production genes for the compounds of the amylostatin complex stems from: (1) the enzymology of individual Acb proteins (see below), (2) mutant studies (Decker 1997; W. Wohlleben, personal communication) and (3) the expression of a cosmid containing the full-length *acb* cluster of *Actinoplanes* sp. SE50 in *S. lividans* 66 strain TK23 resulting in acarbose production in this heterologous organism (Wehmeier et al., unpublished data).

The *acb* gene cluster from the producer strain *Actinoplanes* sp. SE50 has been fully analyzed (Fig. 4; Table 1). The 25 *acb* genes are organized in several transcription units (at least eight units, three of which are operons). They encode a unique mixture of functions of which, besides biosynthetic enzymes, more than one-third should be involved in α-glucoside metabolism and transport, according to their similarity to known proteins. Other known or postulated functions of Acb proteins prove the existence of a characteristic biosynthetic pathway for the

C7-cyclitol and 6DOH moieties and their condensation in the formation of the acarviosyl residue of acarbose (cf. Fig. 1A). Especially surprising is the fact that, besides cytoplasmically localized anabolic enzymes and export systems, as usually combined in the biosynthetic gene clusters for other secondary metabolites, catabolic (both cytoplasmic and extracellular) and import systems also seem to participate in this metabolic compartment. These puzzling observations can be interpreted in a hypothetical metabolic cycle for the acarbose-like compounds and its competitive use for the removal of the C-source (α-glucosides) from the surrounding environment of the producing cells. The known or hypothetical functions of the individual gene sets are described in more detail in the following section.

## The putative biosynthetic genes *acbAB* and *acbVUSRPIJQKMLNOC*

The two genes *acbA*(*Act*) and *acbB*(*Act*) for the two first steps of 6-deoxyhexose formation (see above; Liu and Thorson 1994; Piepersberg 1994) are not located in tandem in an operon as usual, but in two adjacent and divergently oriented transcription units (cf. Fig. 4). They might be transcriptionally controlled (via so-called DSE sequences as operators) by a Reg1-like regulatory protein (Virolle and Gagnat 1994; Stratmann 1997). Their organization in the cluster and sequence similarities deviate remarkably from those observed for the equivalent genes from *S. glaucescens* GLA.0: *acbC*(*Sgl*) and *acbD*(*Sgl*). The genes *acbVUSRPIJQKMLNOC*, all oriented in the same direction, could form one or several operons. Here, for practical reasons, we treat them as two groups, *acbQKMLNOC* and *acbVUSRPIJ*, which are presumed to be mainly involved in cyclitol biosynthesis (besides intracellular metabolism, the "upper pathway") and deoxysugar and condensation pathway (the "lower pathway"), respectively. The gene *acbQ* encodes a protein which has significant similarity throughout the full-length polypeptide chain with that of the amylomaltase MalQ of



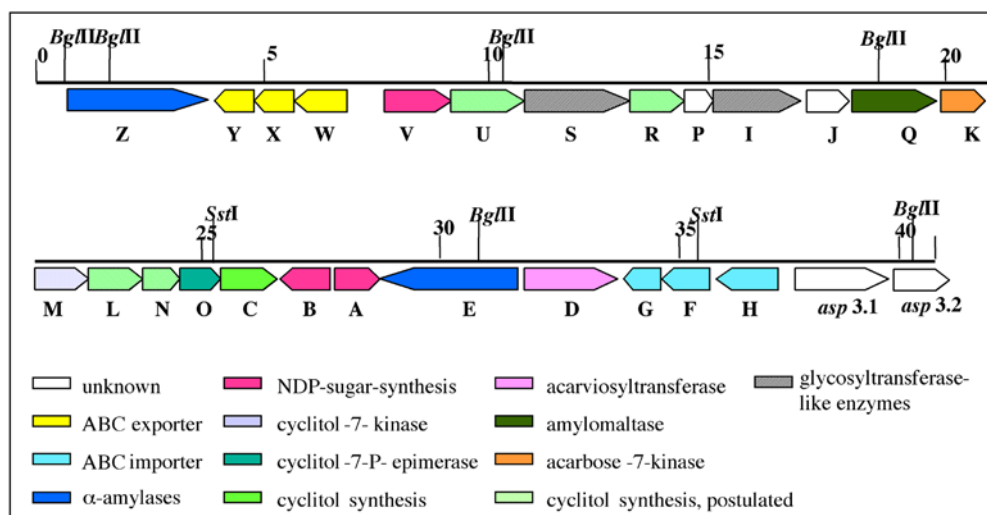**Fig. 4** The *acb* cluster of *Actinoplanes* sp. SE50 (Apeler et al. 2001)

**Table 1** Proteins encoded in the *acb* cluster of *Actinoplanes* sp. SE 50/110 and their putative functions. *AA* Coding capacity (number of amino acid residues), *COG* cluster of orthologous groups of proteins most closely related to the *acb* gene products. The putative function/cellular compartmentation of the encoded proteins is indicated as: *e* extracellular, *i* intracellular (cytoplasmic), *e-l* extracytoplasmic lipoprotein, *m* membrane-integrated

| Protein | AA | Closest similarity to protein (accession number in database) | COG | Postulated function, remarksc |
|---|---|---|---|---|
| Gene products from *Actinoplanes* sp. SE50 | | | | |
| AcbA | 307 | RfbA protein of *Shigella flexneri* (L14842) | 1,209 | dTDP-glucose synthase (i) |
| AcbB | 341 | MtmE protein of *Streptomyces argillaceus* (Y10907) | 1,088 | dTDP-glucose 4,6-dehydratase (i) |
| AcbC | 381 | AroB protein of *Mycobacterium tuberculosis* (X59509) | 337 | C7-cyclitol cyclase (i) |
| AcbD | 724 | CdgT protein of *Bacillus circulans* (X68326) | 366 | Acarviosyltransferase (e) |
| AcbE | 1,021 | Alpha-amylase of *S. lividans* (X70255) | 366 | Alpha-amylase (e) |
| AcbF | 359 | LacF protein of *Synechocystis* sp. (D90905) | 1,175 | Membrane component of carbohydrate ABC transporter (m) |
| AcbG | 239 | Orf2 protein of thermophilic bacterium RT8.B4 (L18965) | 395 | Membrane component of carbohydrate ABC transporter (m) |
| AcbH | 416 | MsmE protein of *Streptococcus mutans* (M77351) | 2,182 | Extracellular binding protein of carbohydrate ABC transporter (e-l) |
| AcbI | 696 | No significant similarity | | Unknown (i) |
| AcbJ | 283 | Conserved hypothetical protein | 561 | Unknown (i) |
| AcbK | 300 | Ribokinase of *Methanobacterium thermoautotrophicum* (AE000666) | 524 | Acarbose-7-kinase (i) |
| AcbL | 403 | HI0053 protein of *Haemophilus influenzae* (L42023) | | (Polyol) dehydrogenase (i) |
| AcbM | 359 | Conserved hypothetical protein | 1,940 | Cyclitol 7-kinase (i) |
| AcbN | 257 | Possible oxidoreductase from *Staphylococcus aureus* (SA2266) | 1,028 | Oxidoreductase (i) |
| AcbO | 270 | No significant similarity | – | Cyclitol-7-phosphate 2-epimerase (i) |
| AcbP | 195 | *Thermotoga maritima* (AE001774) | – | Unknown (i) |
| AcbQ | 698 | MalQ of *H. influenzae* (L45989) | 1,640 | Amylomaltase (i) |
| AcbR | 363 | GlgC of *B. stearothermophilus* (D87026) | 448 | GlgC-related NDP-polyol synthase |
| AcbS | 708 | *Pyrococcus abyssis* PAB2292 (AJ248283) | 297 | glycosyltransferase (?) |
| AcbU | 491 | Pep2 *S. coelicolor* (AJ001205) | – | Unknown (cyclitol kinase?) (i) |
| AcbV | 449 | SC8B7.02 of *S. coelicolor* (AL031225) | 160 | GabT-like aminotransferase (i) |
| AcbW | 357 | DR0205 of *Deinococcus radiodurans* (AE001882) | 1,131 | ATP-binding component of ABC-exporter (i, m, ?) |
| AcbX | 267 | DR0203 of *D. radiodurans* (AE001882) | 842 | Transmembrane-protein of ABC-exporter (m, ?) |
| AcbY | 267 | DR0204 of *D. radiodurans* (AE001882) | – | Unknown component of ABC-exporter (?) |
| AcbZ | 1,072 | Alpha-amylase of *S. lividans* (X70255) | 366 | Alpha-amylase (e) |
| Gene products from *Streptomyces glaucescens* GLA.0 | | | | |
| AcbA | 1,072 | DR0205 of *D. radiodurans* (AE001882); highly similar to AcbWAsp | 1,131 | ATP-binding component of ABC-exporter (i) |
| AcbB | 429 | SC8B7.02 of *Staphylococcus coelicolor* (AL031225); highly similar to AcbVAsp | 160 | GabT-like aminotransferase (i) |
| AcbC | 355 | StrD of *Streptomyces griseus* (P08075); equivalent to AcbAAsp | 1,209 | dTDP-glucose synthase (i) |
| AcbD | 325 | StrE of *S. griseus* (P29782); equivalent to AcbBAsp | 1,088 | dTDP-glucose 4,6-dehydratase (i) |
| AcbE | 345 | DegA of *B. subtilis* (P37947); no counterpart in *acb* cluster of *Actinoplanes* sp. SE50 | 1,609 | DNA-binding activator |
| AcbF | 396 | MalE of *Escherichia coli* (P02928); distantly similar to AcbHAsp | 2,182 | Extracellular binding protein of carbohydrate ABC transporter (e-l) |

*H. influenzae* (L45989). This enzyme is a typical constituent of the intracellular metabolism of maltose and other maltooligodextrins. The genes *acbK*, *acbC*, *acbM*, *acbO* and *acbL* encode the enzymes acarbose-7-kinase, 2-*epi*-5-*epi*-valiolone synthase (C7-cyclitol cyclase), 2-*epi*-5-*epi*-valiolone 7-kinase, 2-*epi*-5-*epi*-valiolone-7-phosphate 2-epimerase and putatively 2-*epi*-valiolone-7-phosphate 1-reductase, respectively, as was shown by in vitro enzyme assays with the individual purified enzymes, heterologously expressed in strains of *S. lividans* 66 (Stratmann et al. 1999; Zhang et al. 2002, 2003a, 2003b). The protein product of the *acbK* gene has acarbose 7-phosphotransferase activity. In primary structure, the acarbose-7-kinase protein resembles other members of a family of sugar kinases, such as fructo- and ribokinases and, interestingly, it shows the highest degree of similarity to an unknown protein (57.9% identity in a 297-amino acid overlap) encoded by a gene which lies adjacent to an α-amylase gene in a *Streptomyces* sp. (accession number U08602). The AcbM polypeptide chain shows distant similarity to some members of the hexokinase family of sugar phosphotransferases, which form a cluster of orthologues in complete genome sequences: COG1940 (Tatusov et al. 2001). This similarity is intriguing, since both these 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. The AcbL and AcbN proteins belong to two different families of the dinucleotide-binding oxidoreductases. The AcbL protein shows high similarities to zinc-dependent alcohol dehydrogenases and AcbN is a member of the family of short-chain dehydrogenases (cf. Table 1). AcbO shows no significant similarity to any known enzyme family. The protein product of the *acbC* gene is distantly similar to the AroB proteins encoded by the respective dehydroquinate synthase genes in various bacterial organisms (Stratmann et al. 1999).

## The genes for extracellular α-glucoside and acarbose metabolism, *acbD*, *acbE*, *acbZ*

The two genes *acbD* and *acbE* form another set of divergent mono-cistronic transcription units sharing a common promoter region (cf. Fig. 2). The protein products of the *acbD*, *acbE* and *acbZ* genes exhibit typical signal peptide motifs at their N-termini and show strong similarity to members of the α-amylase family of proteins, which are mostly extracellular α-glucosidases or α-glucoside transglycosylases (e.g. cyclodextrin synthases). Especially, the catalytic triad of acidic residues (D–E–D) is also conserved in the equivalent positions of the AcbD, AcbE and AcbZ polypeptide chains.

## The putative export and import genes, *acbWXY* and *acbFGH*

These two operons, each consisting of three genes, obviously form common transcription units. Both encode ABC-type transporter systems, however of completely different structure and functionality (see below). The proteins encoded by the *acbFGH* genes represent the trans-membrane and extra-cytoplasmic components of a typical binding protein-dependent ABC transporter, with the highest degree of similarity to the MsmEFG importer of *S. mutans* (Russell et al. 1992) and the MalEFG maltodextrin importers of enterobacteria (Boos and Lucht 1996). However, a gene for a cytoplasmic ATP-binding component, similar to MalK or MsmK and typical for this type of ABC-sugar importers (Boos and Lucht 1996), is lacking in the *acb* gene cluster, since unrelated genes are located upstream of the *acbHFG* operon of *Actinoplanes* sp. (our unpublished observations). Thus, one can speculate that a protein homologous to the general ATP-binding protein MsiK (Hurtubise et al. 1995), which was found to be involved in a number of ATP-dependent transport systems in streptomycetes, is the ATP-binding subunit of the AcbFGH transport system. The presence of genes for a putative sugar transporter in the vicinity of those for the metabolism of acarbose-like products in the genome of *Actinoplanes* sp. suggests that there is a strong metabolic link between this secondary metabolite and the import of extracellular carbohydrates (see below).

## Proposal of a biosynthetic pathway for acarbose

Our proposal for the biosynthetic pathway for acarbose is shown in Fig. 5 and is described here only briefly. We have already mentioned that 2-*epi*-5-*epi*-valiolone is the first precursor for the cyclitol moiety of acarbose. Further conversion of the cyclitol precursor, 2-*epi*-5-*epi*-valiolone-7-phosphate, is catalyzed by the epimerase AcbO. The 2-*epi*-5-*epi*-valiolone-7-phosphate-epimerase AcbO catalyzes the formation of 5-*epi*-valiolone-7-phosphate (Zhang et al. 2003a). The AcbO reaction product, 5-epi-valiolone, has been characterized by mass-spectroscopic and NMR-spectroscopic methods. The enzyme AcbO is active without any cofactor and the deduced protein exhibits no similarities with any known epimerases so far. Therefore, AcbO is obviously the first representative of a new class of epimerases (Zhang et al. 2003a). Recent experiments gave first hints that 5-*epi*-valiolol-7-phosphate is the next intermediate obtained after reduction at the C-1 keto-group, catalyzed by a NADH-dependent dehydrogenase, AcbL (Wehmeier and Zhang, unpublished data). The next steps in the biosynthesis are speculative at the moment. The AcbN protein could catalyze the dehydratase reaction necessary for the synthesis of 1-*epi*-valienol-7-phosphate. A phosphorylation at C-1 (the respective enzyme has not been identified) would be a prerequisite for a subsequent nucleotidylation step. In the *acb* cluster, we identified the gene *acbR*, which encodes GlgC (COG0448; Tatusov et al. 2001), an ADP–glucose

synthase-like protein, which could catalyze the nucleotidylation of the likely precursor, 1-*epi*-valienol-1,7-diphosphate.

The 7-phosphorylation of the C7-cyclitol moiety during the *de novo* synthesis of acarbose might be necessary to prevent an inhibitory effect of the C7-cyclitol- and acarviosyl-containing intracellular metabolites on cytoplasmic enzymes of the producer sensitive to those inhibitors, e. g. α-glucosidases or glucomaltases. For instance, after 7-phosporylation of acarbose by AcbK, the modified inhibitor does not inhibit the cytoplasmic and acarbose-sensitive amylomaltase activity of *Actinoplanes* sp. SE50/110 (Drepper and Pape 1996). Therefore, this phosphorylation can be regarded as a resistance-like self-protection mechanism similar to those in other aminogly-

coside producers (Piepersberg and Distler 1997; He and Liu 1997; Piepersberg et al. 2002).

Recently, a new kinase activity in *Actinoplanes* was characterized which phosphorylates 1-*epi*-valienol to 1-*epi*-valienol-7-phosphate (and also displays weak activity with valienol; Zhang et al. 2003b). In early fermentation phases with derivatives of *Actinoplanes* sp. SE50 (up to 30 h growth), 1-*epi*-valienol and valienol were found in the supernatant; but thereafter the concentration of these intermediates in the supernatant decreased again (Mahmud et al. 1999). Therefore, the 1-*epi*-valienol-7-kinase activity might play a role in a salvage pathway which could reintroduce these cyclitols to the biosynthetic route (not shown in Fig. 5). This kinase activity could not be attributed to any gene of the *acb* gene cluster, since *S.*



**Fig. 5** Postulated pathway of acarbose biosynthesis in *Actinoplanes* sp. SE50 (Zhang et al. 2002)
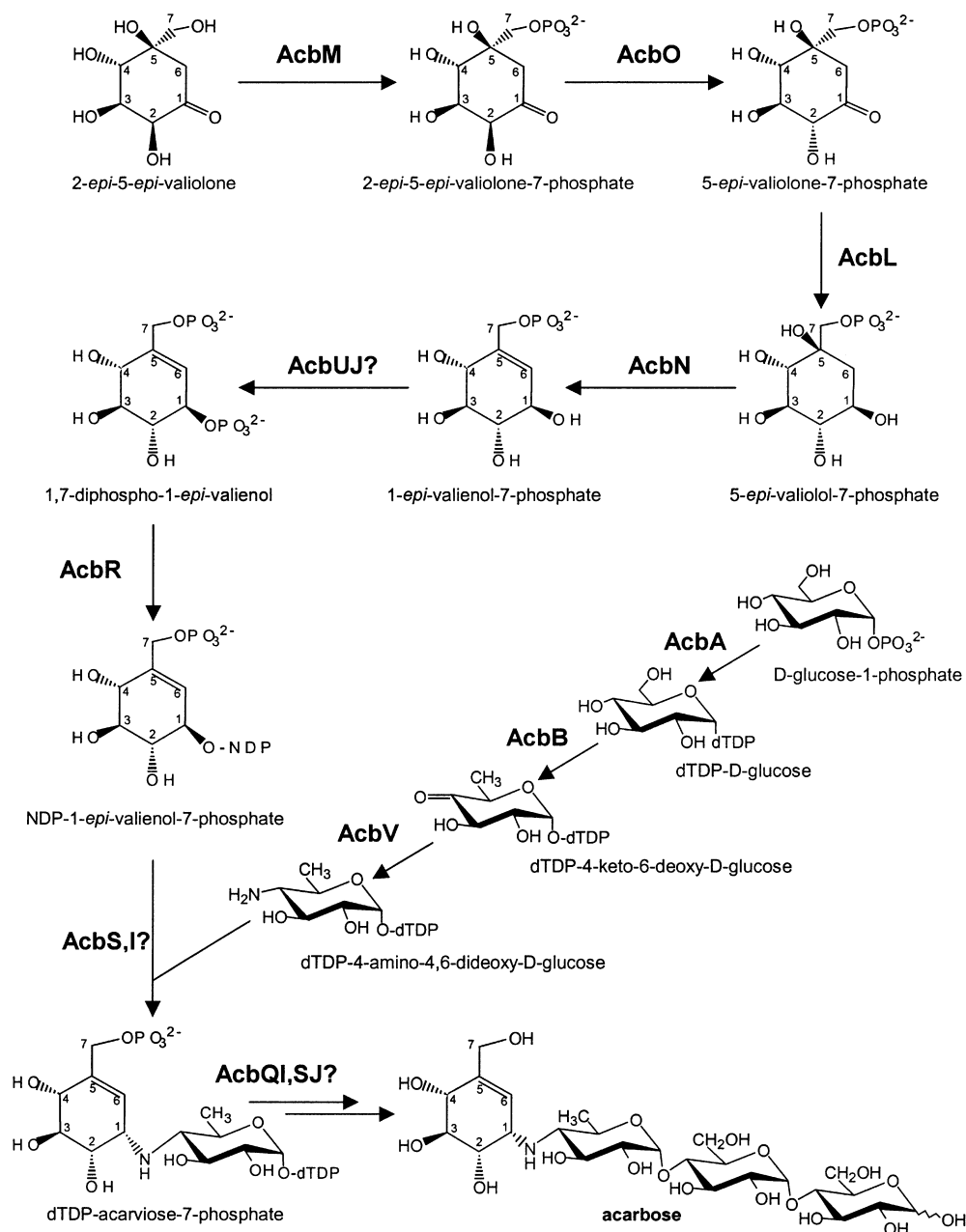
*lividans* strains harboring the cosmid pHTWCos6 (including all *acb* genes, as shown in Fig. 4; Thomas 2001) did not exhibit this 1-*epi*-valienol-7-kinase activity. Therefore, this enzyme is probably not encoded by the *acb* gene cluster (Zhang et al. 2003b).

The synthesis of the deoxysugar moiety of acarbose follows the 6DOH route mentioned above (Liu and Thorson 1994; Piepersberg and Distler 1997). It starts with glucose-1-phosphate, which is first activated by a nucleotidylation step catalyzed by a dTDP-glucose synthase (AcbA) and then further modified by a dTDP-glucose-4,6-dehydratase (AcbB) which leads to dTDP-4-keto-6-deoxy-glucose. dTDP-4-keto-6-deoxy-glucose is the substrate for the subsequent aminotransferase reaction. The aminotransferase AcbV is a member of the class III aminotransferases. When the protein was heterologously expressed in *S. lividans* 66, it catalyzed an L-glutamic acid-dependent amination of dTDP-4-keto-6-deoxy-D-glucose, probably resulting in dTDP-4-amino-4,6-dideoxy-D-glucose (Diaz-Guardamino Uribe 2000; Piepersberg et al. 2002). Interestingly, the protein AcbV is related to aminotransferases of the primary metabolism (being a member of the GabT-like aminotransferases) and it does not belong to the SMATs (Piepersberg 1994, 1997), which are normally found in the biosynthetic pathways of

antibiotics and other secondary metabolites (Piepersberg 1997; Piepersberg and Distler 1997; Piepersberg et al. 2002).

The deduced proteins AcbI and AcbS are related to glycogen and sucrose synthases. The two glycosyltransferase-like reactions postulated in the pathway (formation of the dTDP-acarviose-7-phosphate, synthesis of acarbose-7-phosphate; cf. Figs. 5, 6) thus may be catalyzed by AcbI and AcbS. However, it is not clear whether acarbose-7-phosphate or some other compound instead of acarbose, e.g. the mono- or diglucosylated acarviosine-7-phosphate, is the end-product of the cytoplasmatic pathway.

## Intra- and extracellular metabolism of amylostatins and maltooligosaccharides

As described above, the *acb* cluster encodes proteins for the biosynthetic pathway of acarbose, extracellular starch-degrading enzymes, intracellular maltose-degrading enzymes, transport systems (import/export) and a acarbose-7-kinase. All these activities are needed for a cyclic acarbose/amylostatin metabolism (Fig. 6). In this cyclic metabolism, the three extracellular proteins encoded by the *acb* cluster of *Actinoplanes* sp. SE 50/110 (AcbE, AcbZ,
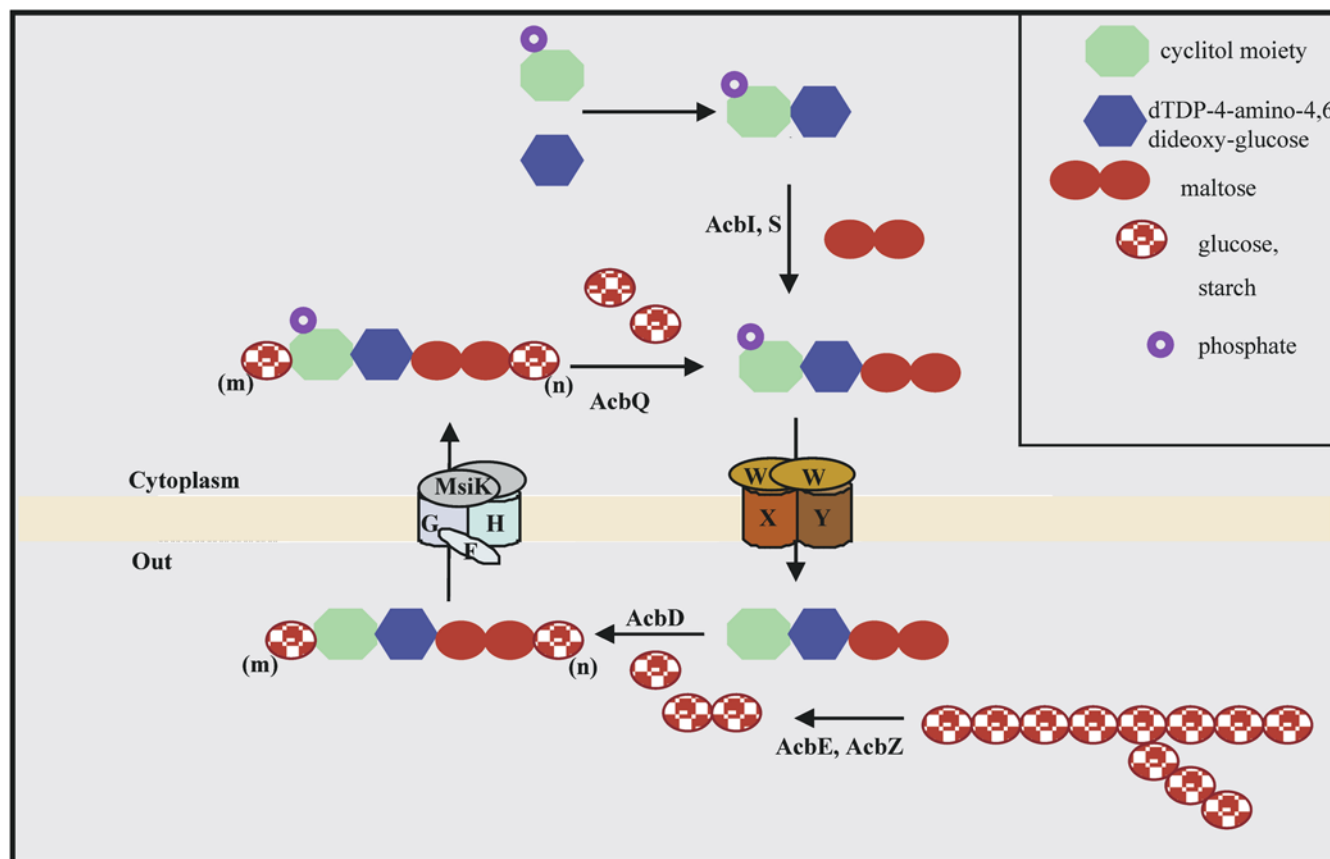


**Fig. 6** Acarbose as a possible carbophore. The possible cycling of acarbose-derived metabolites between intra- and extracellular pools is shown. Re-uptake and intracellular phosphorylation and unloading of acarbose derivatives via a specific glucomaltase activity (which have been elongated extracellularly via transglycosylation by additional glucose residues) could lead to a cyclic pool separation and net glucose uptake

AcbD) allow the degradation of starch-containing substrates and the subsequent transglucosylation of acarbose to form higher-order products (AcbE, AcbZ); and the transfer of acarviosine residues to maltose or higher maltooligosaccharides catalyzed by AcbD forms elongated species relative to acarbose. The formation of the higher-glucosylated components of the amylostatin complex yields the mixture of products observed in culture supernatants. This mixture of products includes some acarbose-related compounds, especially component C, which is not synthesized by AcbD and does not serve as a substrate for the enzyme (Hemker 1997, Hemker et al. 2001). It was also shown that the formation of component C is not catalyzed by an extracellular enzyme. Therefore, it is most likely a product of an enzyme which is released from lysed cells during the late fermentation phases. The enzymes of trehalose metabolism, which were identified in *Actinoplanes* sp., are probably involved in the formation of component C, which is obviously formed from acarbose (Stolpe 2001). The combination of an active export (promoted by AcbXYZ), with dephosphorylation and conversion by α-1,4-glucosyltransferases, such as α-amylases and related proteins (AcbE, AcbZ, AcbD), re-uptake by a specific importer (AcbFGH with MsiK), rephosphorylation by AcbK and deglucosylation by an amylomaltase-equivalent enzyme (AcbQ), could form a cycle. In *Actinoplanes* sp., a maltase activity can be detected in the cell-free extract (Pape and collaborators, unpublished data). The identity of the measured enzyme(s) and its correlation with the AcbQ gene product has not yet been demonstrated. The enzyme was sensitive to acarbose but not to acarbose-7-phosphate, an observation that fits exactly to our model.

## Hypotheses on the functions of acarbose-like alpha-glycosidase inhibitors in nature: are they "carbophores"?

It is hard to imagine how the production of an extracellular inhibitor of α-glucosidases, such as extracellular α-amylases, could facilitate the life of a bacterium in an ecological niche rich in C-sources such as starch and other α-1,4-glucans, if an inhibitory action on hydrolases is the only function provided. Therefore, additional functions have to be envisaged, e.g. selective inhibition of maltose- or maltodextrin-uptake systems in competitors. First experiments have shown that the α-amylase activity of *Bacillus subtilis* strains on starch plates is strongly inhibited when the strains are grown together with *Actinoplanes* on the same plate (Wehmeier and Rockser, unpublished data). This result is supported by the finding that the *Escherichia coli* maltose importer (MalEFGK-system) is specifically blocked by acarbose, which strongly binds to the periplasmic binding protein, MalE (Brunkhorst et al. 1999). Also, the fact that the α-amylase AcbE is not inhibited by acarbose supports this hypothesis, since an extracellular degradation of starch-like nutrients in the environment of the producer *Actinoplanes*

sp. SE50 would also feed other cells, particularly in the soil habitat where it was isolated.

From all these observations, one could suggest further ecological functions for this remarkable group of secondary metabolites: (1) a trap for extracellular glucose and (2) tagging of extracellular glucose pools for import.

Thus, in the cycle described above, acarbose or its core subunit plays a multifunctional role as both a sink for glucose-containing C-sources and a transport vehicle (Fig. 6). This could elegantly explain how acarbose-like compounds facilitate the life of microorganisms in a community competing for the same C-sources as major nutrients; and the scheme could be extended to similar structures, such as trestatins or validamycins. An intracellular maltooligosyltrehalose synthase activity was found in several actinomycetes and in other bacterial genera; and a gene was also detected in *Actinoplanes* sp. SE50 which could be responsible for its production (Kim et al. 2000; Stolpe 2001).

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