FEBS 27093

FEBS Letters 540 (2003) 53–57

Identification of a 1-*epi*-valienol 7-kinase activity in the producer of acarbose, *Actinoplanes* sp. SE50/110

Chang-Sheng Zhang[a], Michael Podeschwa[b], Oliver Block[b], Hans-Josef Altenbach[b], Wolfgang Piepersberg[a], Udo F. Wehmeier[a,*]

[a] *Institute of Chemical Microbiology, Bergische University, Gauss-Str. 20, D-42097 Wuppertal, Germany*
[b] *Institute of Organic Chemistry, Bergische University, Gauss-Str. 20, D-42097 Wuppertal, Germany*

Received 7 January 2003; revised 25 February 2003; accepted 27 February 2003

First published online 11 March 2003

Edited by Judit Ovádi

Abstract In the biosynthesis of the C7-cyclitol moiety, valienol, of the α-glucosidase inhibitor acarbose in *Actinoplanes* sp. SE50/110 various cyclitol phosphates, such as 1-*epi*-valienol-7-phosphate, are postulated precursors. In the cell extracts of *Actinoplanes* SE50/110 we found a new kinase activity which specifically phosphorylates 1-*epi*-valienol; other C7-cyclitol analogs were only weakly or not phosphorylated. The purified product of the kinase reaction turned out to be 1-*epi*-valienol-7-phosphate in analyses by nuclear magnetic resonance spectroscopy. The enzyme seems not to be encoded by an *acb* gene and, therefore, plays a role in a salvage pathway rather than directly in the de novo biosynthesis of acarbose.

Key words: Acarbose; Biosynthesis; 1-*epi*-Valienol; C7-cyclitol 7-kinase; *Actinoplanes* sp.

1. Introduction

The α-glucosidase inhibitor acarbose (part of the amylostatin complex, Fig. 1A) is a member of an unusual group of bacterial secondary metabolites and is produced by strains of the actinomycete genera *Actinoplanes* and *Streptomyces* [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 the 4-amino-4,6-dideoxyglucose are linked via an imino bridge mimicking an *N*-glycosidic bond. This acarviosyl moiety is primarily responsible for the inhibitory effect on α-glucosidases. The C7-aminocyclitol units are considered to be similar to other common structural motifs observed in bacterial secondary metabolites [3]. We have recently shown that 2-*epi*-5-*epi*-valiolone, the only cyclitol that was incorporated into acarbose, becomes phosphorylated prior to the next modifications [4]. This finding led to the proposal of a new pathway for the biosynthesis of acarbose. When analyzing the cell-free extracts of *Actinoplanes* sp. SE50 strains we now identified a new kinase activity, which phosphorylates the cyclitol 1-*epi*-valienol on its C7-hydroxyl group. As 1-*epi*-valienol-7-phosphate is probably one of the intermediates of the acarbose pathway, but 1-*epi*-valienol is not, we postulate that this phosphorylation step may play a role for the re-incorporation of the putative degradation or side product 1-*epi*-valienol in some kind of salvage pathway. There is evidence that 1-*epi*-valienol 7-kinase is likely not to be encoded by one of the genes in the known gene cluster (*acb*) for acarbose biosynthesis.

2. Materials and methods

2.1. Bacterial strains, medium and culture conditions

The *Actinoplanes* sp. strains SE50/110 and SN223/29 used in this study are improved acarbose producers developed from the wild-type strain SE50. The recombinant strain *Streptomyces lividans* TK23/pHTWCos6 carries a cosmid with all known *acb* genes (H. Thomas, personal communication). The strains were cultivated in acarbose production medium (MD-50 [maltodextrins], 70 g; $(NH_4)_2SO_4$, 5 g; yeast extract, 2 g; K_2HPO_4, 1 g; KH_2PO_2, 1 g; trisodium citrate, 5 g; $MgCl_2{\cdot}6H_2O$, 1 g; $FeCl_3{\cdot}6H_2O$, 0.25 g; $CaCl_2{\cdot}2H_2O$, 2 g; dissolved in 1000 ml H_2O, pH adjusted to 7.0 and sterilized by filtration [5].

2.2. Preparation of cell-free extracts

The cells of *Actinoplanes* sp. were harvested by centrifugation, washed twice and resuspended in the double volume (v/w) of disruption buffer (25 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, 0.5 mM dithiothreitol (DTT), pH 7.6; 1.5 ml/g cells). Cells were then disrupted by sonication (2–3 min at 60 W). Cell-free extracts were obtained after centrifugation at 4°C and 13 000×*g* for 1 h. The extracts were dialyzed against 5 l of the disruption buffer overnight at 4°C. Protein concentrations were determined according to Bradford [6].

2.3. Enzyme assays

Generally enzyme assays were performed in a standard buffer system containing 25 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, 10 mM ATP, 10 mM 1-*epi*-valienol (or other substrates that were tested), adjusted to pH 7.6. In each assay (final volume 20 μl), 16 μl of the cell-free extract as prepared above was used. The assays were incubated at 30°C for 2–6 h. The reaction was monitored by thin-layer chromatography (TLC). For radioactive assays, 1 μl of [γ-^{32}P]ATP (2.0 μCi, Amersham-Biosciences, Freiburg, Germany) was added and only 15 μl of the cell-free extract was used. Radioactively labeled spots were visualized after TLC by autoradiography with X-ray films (Hyperfilm; Amersham-Biosciences). The syntheses of the C7-cyclitol systems used in this study (cf. Fig. 1B) are published elsewhere [4,7].

2.4. TLC

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

*Corresponding author. Fax: (49)-202-439 2698.
E-mail address: wehmeier@uni-wuppertal.de (U.F. Wehmeier).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00222-9

2.5. Purification of enzymatically formed 1-epi-*valienolphosphate*

The reaction mixture for the enzymatic synthesis of 1-*epi*-valienolphosphate contained 20 ml of cell-free extract of *Actinoplanes* sp. SE50/110, 25 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, 10 mM ATP, 0.5 mM DTT, 10 mM racemic 1-*epi*-valienol and desalted water in a total volume of 30 ml. After incubation at 30°C for 6 h, the reaction mixture was heated at 95°C for 5 min, centrifuged (5000 rpm, 20 min) and then applied to an ultrafiltration Amicon cell with a YM-10 ultrafiltration membrane (cut-off 10 000 Da; Amicon, Witten, Germany). The flow-through was collected, concentrated to 3 ml by freeze-drying and then was applied to an anion-exchange chromatograph with Dowex 1×8, Cl^- form (mesh 100–200; Serva, Heidelberg, Germany) in a SR 25/50 column (Amersham-Biosciences). The column was washed with plenty of water and the reaction product was eluted with a linear gradient of 0–600 mM of NaCl (flow rate, 2 ml/min). The elutions were collected as 2 ml fractions and analyzed by TLC. Fractions containing the desired product were pooled (total volume 50 ml) and concentrated by freeze-drying. Desalting of the product was carried out at 4°C on a Sephadex G-10 (5.0×81 cm, SR25/100 column; Amersham-Biosciences). The product was eluted with water (flow rate, 1.5 ml/min). The fractions containing 1-*epi*-valienolphosphate were pooled. After lyophilization 38 mg of 1-*epi*-valienolphosphate was obtained as a white powder.

2.6. Nuclear magnetic resonance (NMR) spectroscopic characterization

All NMR spectra were recorded on a Bruker ARX 400 (400 MHz) spectrometer. Besides ^1H, ^{13}C and ^{31}P experiments, 2D COSY (^1H-^1H, ^1H-^{13}C as well as ^1H-^{31}P) and DEPT spectra for the unequivocal correlation of the hydrogen, carbon and phosphorus atoms were recorded. The chemical shifts are given in ppm, relative to the solvents as internal standard. External standards were used for ^{31}P- (85% phosphoric acid) and ^{13}C-NMR (TMS). 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 synthesis of racemic 1-*epi*-valienol was performed by stereocontrolled chemical transformations starting from *p*-benzoquinone [7]. Its ^1H-NMR spectrum and melting point are in accordance with data previously reported [8].

2.6.1. Analytical data of rac. 1-epi-*valienol.* m.p. 149–150°C. ^1H-NMR (D_2O, 400 MHz): δ = 3.42 (dd, 1 H, J = 7.9, J = 10.4 Hz, H-2 or H-3), 3.50 (dd, 1 H, J = 7.9, J = 10.4 Hz, H-2 or H-3), 4.06 (d, 1 H, J = 13.9 Hz, H-7a), 4.15–4.19 (m, 3 H, H-1, H-4, H-7b), 5.58 (d, 1 H, J = 1.5 Hz, H-6). ^{13}C-NMR (D_2O, 101 MHz): δ = 63.3 (C-7), 73.6, 74.2 (C-1 and C-4), 77.4, 77.8 (C-2 and C-3), 127.2 (C-6), 140.6 (C-5). MS (EI, 70 eV, *m/z* (%)): 158 (2), 116 (88), 111 (76), 98 (69), 41 (100). Anal. calcd. for $C_7H_{12}O_5$: (176.16): C 47.73, H 6.87; found C 47.75, H 6.88.

2.6.2. Analytical data of 1-epi-*valienol-7-phosphate.* ^1H-NMR (D_2O, 400 MHz, pH ≈ 7): δ = 3.44 (dd, 1 H, J = 7.6, J = 10.7 Hz, H-2 or H-3), 3.50 (dd, 1 H, J = 7.4, J = 10.4 Hz, H-2 or H-3), 4.16 (d, 1 H, J = 8.1 Hz, H-1 or H-4), 4.21 (d, 1 H, J = 9.2 Hz, H-1 or H-4), 4.30 (dd, 1 H, J = 7.1, J = 13.2 Hz, H-7a), 4.44 (dd, 1 H, J ≈ 10, J = 13.2 Hz, H-7b), 5.67 (s, br, 1 H, H-6). ^{13}C-NMR (D_2O, 101 MHz): δ = 66.81 (d, J = 5.1 Hz, C-7), 73.58, 73.82 (C-1, C-4), 77.35, 77.60 (C-2, C-3), 128.67 (C-6), 138.20 (d, J = 7.1 Hz, C-5). $^{31}P\{^1H\}$-NMR (D_2O, 162 MHz): δ = 1.93 (PC-7). HR-MS (ESI-neg., phosphoric acid 0.002%, H_2O/acetonitrile 1:1, Q-TOF): *m/z*: 255.0293 $[M-H]^-$ calcd. for $C_7H_{12}O_8P$: 255.0278.

3. Results and discussion

We have recently shown that phosphorylated cyclitol intermediates are the precursors of the valienamine moiety of the pseudooligosaccharide acarbose [4,9]. The phosphorylation of 2-*epi*-5-*epi*-valiolone to 2-*epi*-5-*epi*-valiolone-7-phosphate was catalyzed by the kinase AcbM [4]. In cell-free extracts of the acarbose-producing strains *Actinoplanes* SE50/110 and *Actinoplanes* SN223/29 and the recombinant strain *S. lividans* TK23/pHTWCos6, however, this kinase activity was not detectable, probably due to too low expression of the protein and its instability. However, the activity of the acarbose kinase AcbK, which phosphorylates acarbose to acarbose-7-phosphate, was found in cell-free extracts of the producer strains as well as in the cell-free extracts of *S. lividans* TK23/pHTWCos6 [4,10].

In contrast, among the C7-cyclitols screened (cf. Fig. 1B) racemic 1-*epi*-valienol turned out to be a good substrate for a

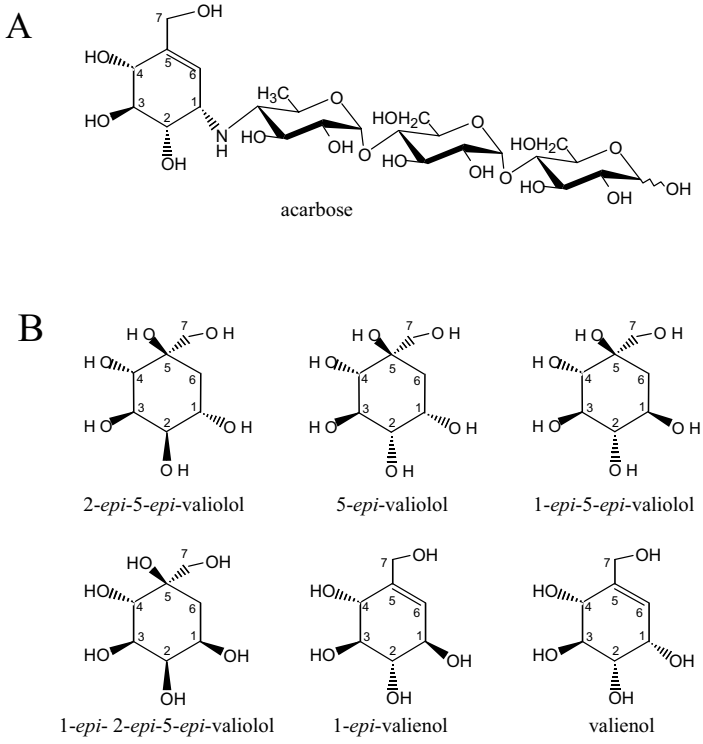

Fig. 1. Structures of acarbose (A) and of the cyclitols used in this study (B). The structure of 2-*epi*-5-*epi*-valiolone is shown in Fig. 4.

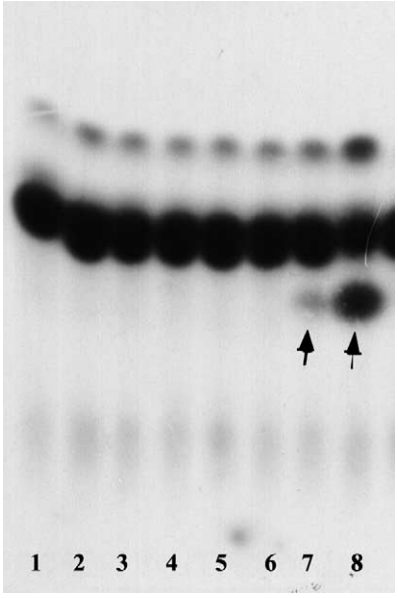

Fig. 2. Phosphorylation of racemic cyclitols by cell-free extracts of *Actinoplanes*. The figure shows an autoradiogram of a TLC. Cell-free extracts were incubated with the various cyclitols and [γ-^{32}P]ATP. The probes were applied to a TLC as described in Section 2. The arrows show the newly phosphorylated spots. Lanes: 1: no substrate; 2: 1-*epi*-2-*epi*-5-*epi*-valiolol; 3: 2-*epi*-5-*epi*-valiolol; 4: 2-*epi*-5-*epi*-valiolone; 5: 5-*epi*-valiolol; 6: 1-*epi*-5-*epi*-valiolol; 7: valienol; 8: 1-*epi*-valienol.

new kinase activity in the cell-free extracts of the *Actinoplanes* sp. strains. In these assays only about 50% of the substrate 1-*epi*-valienol was phosphorylated. This indicates that only one enantiomer of the racemic mixture was accepted by the kinase. The phosphorylation of a set of other available racemic C7-cyclitols (1-*epi*-2-*epi*-5-*epi*-valiolol, 2-*epi*-5-*epi*-valiolol, 2-*epi*-5-*epi*-valiolone, 5-*epi*-valiolol, 1-*epi*-2-*epi*-5-*epi*-valiolol, valienol, 1-*epi*-valienol; cf. Figs. 1 and 4) was further investigated in more detail using the cell-free *Actinoplanes* extracts. Besides racemic 1-*epi*-valienol, only racemic valienol was weakly phosphorylated by these cell-free extracts as detected by a sensitive radiolabel assay (Fig. 2). It remained unclear whether this weak activity indicated a conversion of the valienol epimer to valienolphosphate or whether the phosphorylated spot was caused by a contamination with 1-*epi*-valienol resulting in 1-*epi*-valienolphosphate. It might also be possible that a valienol epimerase activity existed in the cell-free extracts, such that the phosphorylated substance would result from a small amount of newly formed 1-*epi*-valienol. Surprisingly, we could not detect the 1-*epi*-valienol kinase activity in cell-free extracts of *S. lividans* TK23/pHTWCos6. From our genetic analysis we have evidence that all known *acb* genes are cloned on the cosmid pHTWCos6 ([11]; U.F. Wehmeier, H. Thomas, C.-S. Zhang and W. Piepersberg, unpublished observations). We also could show that other enzymes encoded by this cluster, e.g. the kinase AcbK, were well expressed in *S. lividans* TK23/pHTWCos6 indicating that the *acb* genes were actively expressed from the cosmid. Therefore, one can speculate that either some *acb* genes are not located in the identified gene cluster or that *Actinoplanes* sp. SE50 possesses an additional kinase, which accepts 1-*epi*-valienol as an additional substrate among others, e.g. hexoses.

In order to determine the exact structure of the 1-*epi*-valienolphosphate the product was purified (see Section 2) and ^{1}H-, ^{13}C- and ^{31}P-NMR spectra as well as two-dimensional ^{1}H-^{1}H, ^{1}H-^{13}C and ^{1}H-^{31}P COSY (Fig. 3) spectra were recorded. In the ^{1}H-NMR spectrum the coupling pattern of the former hydroxymethyl group has changed significantly. It can be noted that the signals of H-7a and H-7b are shifted downfield relative to the methylene protons of the educt which is indicative of an electron-withdrawing group. The ^{1}H-^{31}P COSY experiment proves that the phosphate group is bound at the methylene group. The methylene group occurs as an ABX spin system at 4.30 ppm and 4.44 ppm with coupling constants $^{3}J_{H-7a,P} = 7.1$ Hz and $^{3}J_{H-7b,P} \approx 10$ Hz. The coupling pattern of the ring protons did not change when compared to the educt. H-3 and H-2 occur as 'dd' with $J \approx 7.5$ and $J \approx 10.5$, respectively. This indicates that the product is still a 1-*epi*-valienol isomer. Due to these results the main product can be unequivocally identified as 1-*epi*-valienol-7-phosphate. A small signal at 5.70 ppm next to signal H-6 from 1-*epi*-

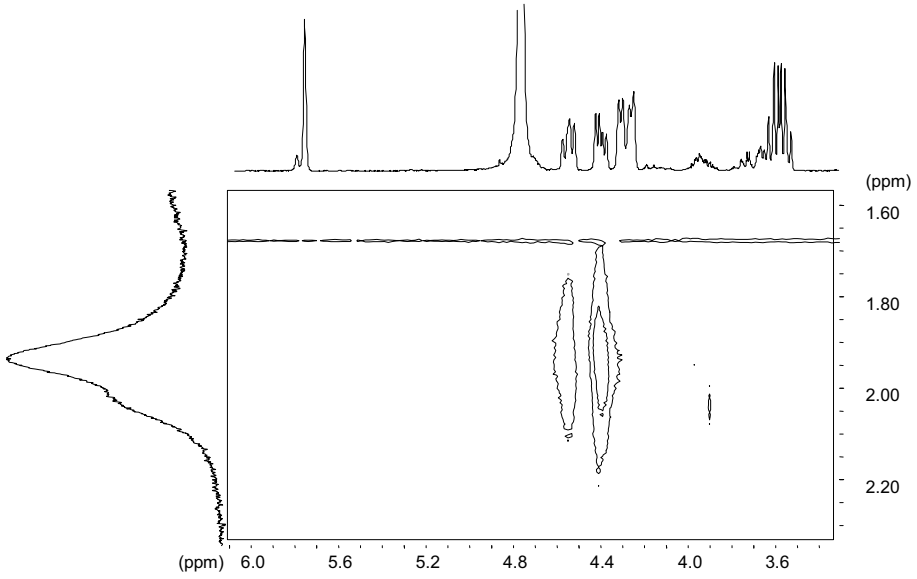

Fig. 3. ^{1}H-^{31}P COSY NMR spectrum of 1-*epi*-valienol-7-phosphate.

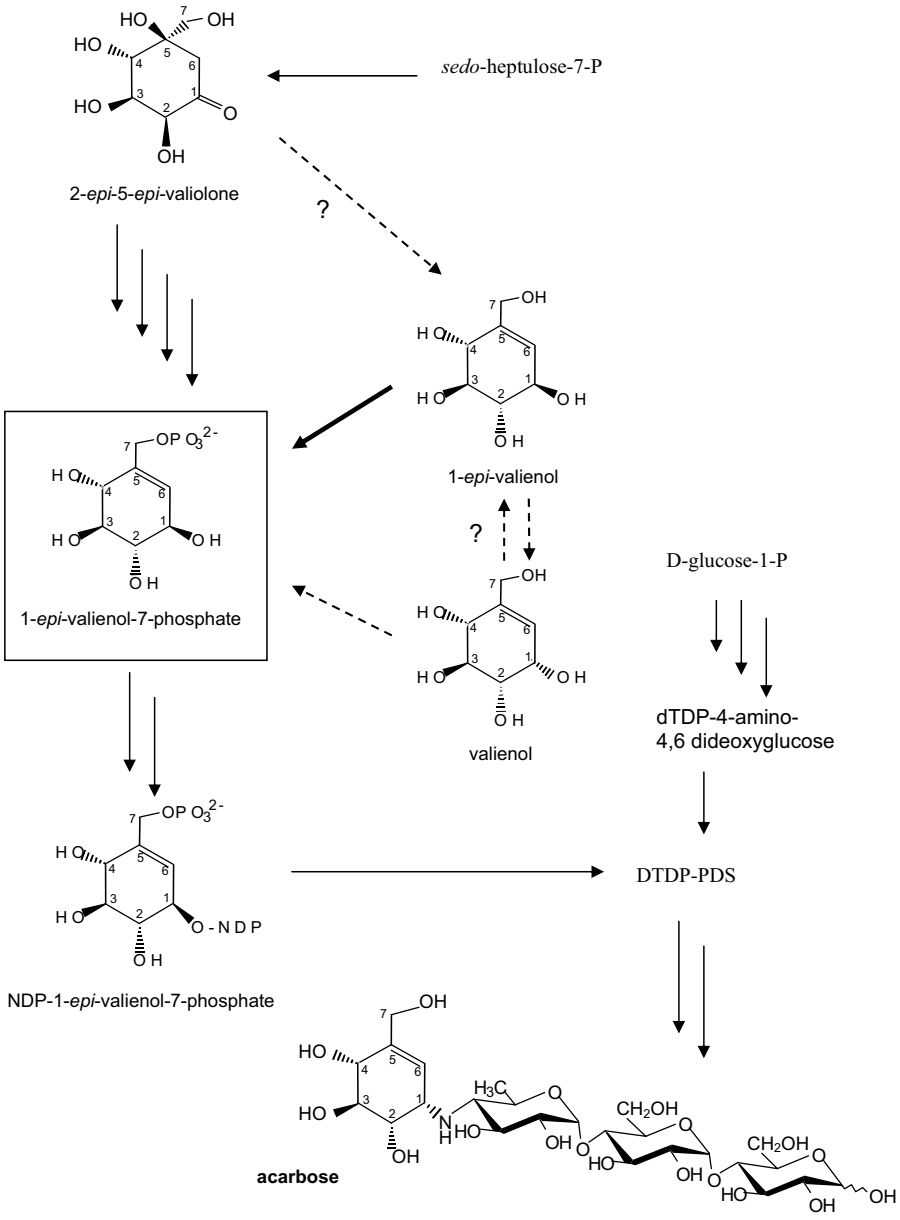

Fig. 4. Scheme of the acarbose biosynthesis. Only the structures that are relevant for this work are shown (cf. [4]). Dotted arrows symbolize hypothetical routes. dTDP-PDS = dTDP-pseudodisaccharide (dTDP-acarviose).

valienol-7-phosphate indicates the formation of a byproduct, which could not be identified, but may be a phosphorylated isomer.

The presence of a 1-*epi*-valienol 7-kinase fits well into the current picture we have from the biosynthetic pathway [4]. In this pathway 1-*epi*-valienol-7-phosphate is one intermediate within the cyclitol pathway (Fig. 4). Thus the 7-kinase we have newly identified and described in this work could be responsible for a salvage pathway for 1-*epi*-valienol, which is probably available both intra- and extracellularly as a degradation product, but not phosphorylated and which then can be (re-)directed to the acarbose biosynthesis. This would make sense, because the two intermediates 1-*epi*-valienol and valienol have been detected in successive stages during the growth of *Actinoplanes* [12]. In early fermentation phases (30 h growth) of *Actinoplanes* sp. SE50 derived strains valienol and 1-*epi*-valienol were observed in cell-free extracts, but especially in the fermentation broth: 1-*epi*-valienol appeared early, valienol at the end of the fermentation process. Both intermediates behaved as would be expected from degradation products of acarbose, one occurring directly from this metabolite or some intracellular intermediate and the other as a subsequent conversion product of the first. However, a still unanswered question remains. In feeding experiments both valienol and 1-*epi*-valienol were not introduced into acarbose, while the first cyclitol intermediate 2-*epi*-5-*epi*-valiolone was quite well incorporated [12]. If the 1-*epi*-valienol kinase is part of a salvage pathway the 1-*epi*-valienol should have been incorporated into acarbose in vivo. The failure of these feeding studies could reflect the fact that not all substrates are taken up equally well by the resting cells which were used in these experiments and generally are low in energy charge. Another possibility could be that the 1-*epi*-valienol 7-kinase was not active under the conditions used in the resting cell experi-

ments. Thus, from the presence of a highly active 1-*epi*-valienol 7-kinase in acarbose-producing cells of *Actinoplanes* sp. SE50 and its derivatives under fermentative conditions we conclude that 1-*epi*-valienol is a possible side product of the acarbose pathway or a degradation product after reuptake of the acarbose itself and that this compound (and possibly its epimer valienol via prior re-epimerization) can be reintroduced into the biosynthetic pathway via the 7-kinase (cf. Fig. 4). Since 1-*epi*-valienol is a structural analog of β-D-glucose, the 1-*epi*-valienol 7-kinase could be basically a hexokinase-like enzyme which has shifted its substrate range towards this C7-cyclitol in *Actinoplanes* sp. SE50. The preparative isolation of the 1-*epi*-valienol 7-kinase and the identification and mapping of the respective gene will be the next steps in order to answer the still unsolved questions.

Acknowledgements: We thank M. Schellenträger for HR-MS analyses.

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