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The acarbose biosynthetic enzyme AcbO from *Actinoplanes* sp. SE50/110 is a 2-*epi*-5-*epi*-valiolone-7-phosphate 2-epimerase

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Abstract The C7-cyclitol 2-*epi*-5-*epi*-valiolone is the first precursor of the cyclitol moiety of the α-glucosidase inhibitor acarbose in *Actinoplanes* sp. SE50. The 2-*epi*-5-*epi*-valiolone becomes phosphorylated at C7 by the ATP dependent kinase AcbM prior to the next modifications. Preliminary data gave evidences that the AcbO protein could catalyse the first modification step of 2-*epi*-5-*epi*-valiolone-7-phosphate. Therefore, the AcbO protein, the encoding gene of which is also part of the *acbKMLNOC* operon, was overproduced and purified. Indeed the purified protein catalysed the 2-epimerisation of 2-*epi*-5-*epi*-valiolone-7-phosphate. The chemical structure of the purified reaction product was proven by nuclear magnetic resonance spectroscopy to be 5-*epi*-valiolone-7-phosphate.

Key words: *Actinoplanes* sp.; Acarbose; Biosynthesis; C7-cyclitol

1. Introduction

The α-glucosidase inhibitor acarbose (a component of the amylostatin complex; Fig. 1) is produced by strains of the genera *Actinoplanes* and *Streptomyces* and inhibits various α-glucosidases, especially in the intestine [1,2]. Acarbose is produced industrially using developed strains of *Actinoplanes* sp. SE50/110. It is built-up from three distinct moieties, an unsaturated cyclitol (valienol), a 4-amino-4,6-dideoxyglucose and a maltose unit, linked by (pseudo-)α-1,4 linkages. The valienol and the 4-amino-4,6-dideoxyglucose are linked via 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 C-sources in the fermentation medium, *Actinoplanes* sp. SE50/110 produces also higher homologues of acarbose, which differ in the numbers of glucose residues that are linked to the reducing and/or non-reducing ends of the acarviosyl moiety. In our combined genetic and biochemical studies of the biosynthesis of the pseudotetrasaccharide acarbose we could show that 2-*epi*-5-*epi*-valiolone-7-phosphate and dTDP-4-amino-4,6-dideoxyglucose are precursors and that an activated pseudodisaccharide-phosphate, dTDP-acarviosine-7-phosphate, is a possible intermediate in the pathway (cf. Fig. 1) [5–8].

The C7-cyclitols like valienol, which can be regarded as unsaturated sugar analogues, so called carbsugars, occur both as secreted monomers (e.g. 2,3,4-trihydroxy-6-methylcyclohexanone and cyclophellitol) or as components in the natural compounds of the amylostatin-(acarbose) and validamycin complexes were considered to be derived from similar pathways; but recent evidence indicates also some differences [4,9–12]. The transition from the primary- to the secondary metabolism in the cyclitol pathway in *Actinoplanes* sp. is catalysed by the AcbC protein, a member of the dehydroquinate synthase-related enzymes, in this case cyclising *sedo*-heptulose-7-phosphate under formation of the C7-cyclitol 2-*epi*-5-*epi*-valiolone [5]. This product is further processed by a kinase, AcbM, to yield 2-*epi*-5-*epi*-valiolone-7-phosphate [6]. Here we demonstrate that the next step in the biosynthesis of the valienol (or valienamine) unit of acarbose is the epimerisation to 5-*epi*-valiolone-7-phosphate, catalysed by the epimerase AcbO.

2. Materials and methods

2.1. Bacterial strains, medium and growth conditions

Streptomyces lividans TK64 harbouring plasmids pCW4123M (expressing AcbM under control of the thiostrepton-inducible *ptipA* promoter) or pMJO7 (expressing AcbO under control of the thiostrepton-inducible *ptipA* promoter) were used in this study [6]. The strains were grown in YEME [13] at 30°C for 2 days and then 10 μg/ml thiostrepton was added in order to induce the overproduction of the AcbM or AcbO proteins for another 24 h. Culture volumes of 100 ml were used for enzyme preparations (see below) and constantly yielded 5 g of cells (wet weight).

2.2. Preparation of cell free extracts

Cells of *S. lividans* overproducing AcbM or AcbO proteins were harvested by centrifugation, washed twice and resuspended in the disruption buffer (25 mM Tris–HCl, 10 mM $MgCl_2$, 20 mM NH_4Cl, 0.5 mM DTT, pH 7.6; 7.5 ml/5 g cells, wet weight). 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 dialysed against 5 l of the disruption buffer overnight at 4°C. Protein concentrations were determined according to Bradford [14] and were constantly in the range of 5 mg/ml in the crude extract. The overproduction of Acb proteins was monitored by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously [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 2-*epi*-5-*epi*-valiolone, adjusted to pH 7.6. In each

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assay (final volume 20 μl), 16 μl of the cell free extracts as prepared above were used. The assays were incubated at 30°C for 2–6 h. The reaction was monitored by thin-layer chromatography (TLC).

2.4. TLC

Samples of the enzyme reactions were chromatographed on silica thin-layer sheets (Merck, Darmstadt, Germany) using solvent I (butanol/ethanol/water, 9:7:4). The substrates were detected as blue spots after development by use of a cerium- and molybdate-containing reagent [6].

2.5. Purification of His-tag AcbM and AcbO proteins

The purification of the His-tagged AcbM and AcbO proteins was performed by use of Ni^{2+}-HiTrap chelating columns (Amersham Biosciences, Freiburg, Germany) as described previously [6].

2.6. Preparation and purification of enzymatically formed 5-epi-*valiolone-7-phosphate*

Using the method described earlier [6], about 30 mg of enzymatically synthesised and purified 2-*epi*-5-*epi*-valiolone was obtained. 30 mg of 2-*epi*-5-*epi*-valiolone was incubated with 6 ml of His-tag AcbM protein enriched by Ni^{2+}-chelating (see above) in a buffer containing 25 mM Tris–HCl, 5 mM $MgCl_2$, 20 mM NH_4Cl, 15 mM ATP, 0.5 mM DTT, adjusted to pH 7.6, in a total volume of 10 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 concentrated to nearly 5 ml by lyophilisation. A sample of 10 ml of the Ni^{2+}-trapped His-tag AcbO protein was added and the mixture was incubated at 30°C for another 6 h. The reaction was monitored by TLC. After heating at 95°C for 5 min and centrifugation (5000 rpm, 20 min), the reaction mixture was 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 chromatography on Dowex 1×8, Cl^- form (mesh 100–200; Serva, Heidelberg, Germany) in a SR 25/50 column (Amersham-Biosciences, Freiburg, Germany). The column was washed with an excess amount of water and the product was subsequently eluted with a linear gradient of 0–600 mM of NaCl (flow rate, 2 ml/min). The elution was collected as 5 ml fractions and analysed by TLC. Fractions containing the desired product were pooled (total volume 45 ml) and concentrated to about 5 ml 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, Freiburg, Germany). The product was eluted with water (flow-rate, 1.5 ml/min). The fractions containing 5-*epi*-valiolone-7-phosphate were pooled (total volume of 50 ml). After lyophilisation a total recovery of 15 mg of 5-*epi*-valiolone-7-phosphate was obtained as a light-yellow powder.

2.7. Nuclear magnetic resonance (NMR) spectroscopic characterisation

All NMR spectra were recorded on a Bruker ARX 400 (400 MHz) spectrometer. Besides ^1H-, ^{13}C-and ^{31}P-experiments, also 2D COSY-(^1H–^1H, ^1H–^{13}C as well as ^1H–^{31}P) and DEPT spectra for the correlation of the hydrogen, carbon and phosphorus atoms were recorded. The chemical shifts are given in ppm, related to the solvents as internal standard. External standards were used for ^{31}P- (85% phosphoric acid) and ^{13}C NMR (TMS).

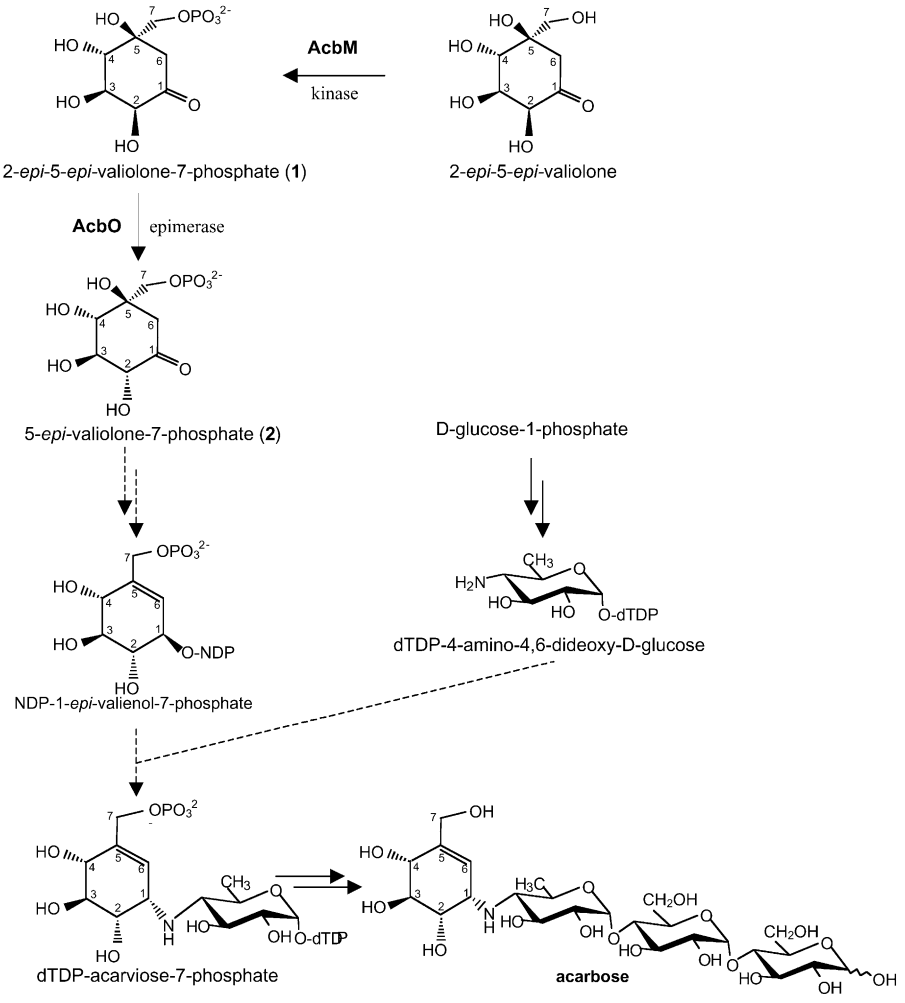

Fig. 1. Acarbose biosynthesis in *Actinoplanes* sp. SE50/110. The scheme shows the postulated biosynthetic route [6]. The still unknown postulated steps are symbolised by broken arrows.

The multiplicity is given by the following symbols: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), Ψt (pseudotriplet for unresolved dd) and br (broad). The coupling constant J is given in Hz.

NMR spectra: 5-*epi*-valiolone-7-phosphate. ^{1}H–^{31}P NMR (D_2O, pH = 6.7, 400 MHz): δ = 2.73 (s, br, 2 H, exchange in D_2O, H-6a and H-6b), 3.58 (d, 1 H, J = 10.2 Hz, H-7a), 3.88 (d, 1 H, J = 10.2 Hz, H-7b), 3.89 (d, 1 H, J = 9.7 Hz, H-4), 3.94 (Ψt, 1 H, H-3), 4.18 (d, 1 H, J = 8.7 Hz, H-2). ^{13}C NMR (D_2O, pH = 6.7, 101 MHz): δ = 49.13 (C-6), 66.87 (d, J = 5.1 Hz, C-7), 75.33 (d, J = 10.17 Hz, C-5), 75.88, 79.81 (C-3, C-4), 80.63 (C-2), 208.98 (C-1). ^{31}P–^{1}H NMR (D_2O, pH = 6.7, 162 MHz): δ = 3.02 (PC-7). ^{1}H NMR (D_2O, pH adjusted to 3.5 with d_4-AcOH, 400 MHz): δ = 2.75 (s, br, 2 H, exchange in D_2O, H-6a and H-6b), 3.60 (dd, 1 H, J = 4.3 Hz, J = 9.9 Hz, H-7a), 3.81 (Ψt, 1 H, J = 9.9 Hz, H-3), 3.92 (d, 1 H, J = 10.2 Hz, H-4), 3.96 (dd, 1 H, J = 4.1 Hz, J = 10.6 Hz, H-7b), 4.17 (d, 1 H, J = 9.2 Hz, H-2).

3. Results and discussion

3.1. Purification and testing of an N-terminal His-tag AcbO fusion protein

In a preceding publication we had noted that the product of the kinase AcbM, 2-*epi*-5-*epi*-valiolone-7-phosphate (cf. Fig. 1), was further converted by a crude extract from a strain producing recombinant AcbO protein to another C7-cyclitol-phosphate with unchanged mass [6]. The construction of a recombinant overexpressing strain of *S. lividans* TK64/pMJO7, producing an *N*-terminally His-tagged AcbO protein had also been described. We preliminarily postulated that the AcbO product was an epimer, 5-*epi*-valiolone-7-phosphate, of the substrate rather than being another epimer or a cyclitol in which the phosphate group had been shifted to another hydroxyl group. Therefore, we sat out to verify this hypothesis by using purified enzyme and preparing the product in a larger scale for further structural analysis. The His-tag AcbO protein was expressed under the control of an inducible promoter (p*tipA*) which only led to moderate but visible overproduction (Fig. 2). The protein was largely enriched via passage over Ni^{2+}-chelation and elution; minor bands were still to be seen in the SDS–PAGE analyses (cf. Fig. 2). The apparent molecular weight of the purified His-tag AcbO protein (29.5 kDa) was in good accordance with the estimation from the protein sequence of 30.616 kDa. With this enzyme preparation coupled assays were performed including chemically synthesised racemic 2-*epi*-5-*epi*-valiolone and His-tag AcbM kinase (crude extract or purified from *S. lividans* TK64/pCW4123M), in combination without or with His-tag AcbO protein. These assays clearly demonstrated that the purified His-tag AcbO protein is actively converting the product of the AcbM-catalysed reaction in absence of any other cofactor or coenzyme, as can be seen from the TLC analyses (Fig. 3).

3.2. The AcbO protein is a 2-epi-5-epi-valiolone-7-phosphate 2-epimerase

Preparative isolation of the AcbO product was achieved by scaling-up the assay for conversion of 30 mg 2-*epi*-5-*epi*-valiolone in the coupled 2-step reaction (see Section 2). The yield after the final purification was a total of 15 mg of C7-cyclitol phosphates. A ^{1}H-NMR spectrum of the isolated reaction product besides the educt showed a new product in a ratio of 2:8. Obviously, the AcbO-catalysed conversion was at equilibrium at 80% conversion of 2-*epi*-5-*epi*-valiolone-7-phosphate as further treatment with more enzyme did not change this ratio. Subsequent extensive NMR analysis by monitoring

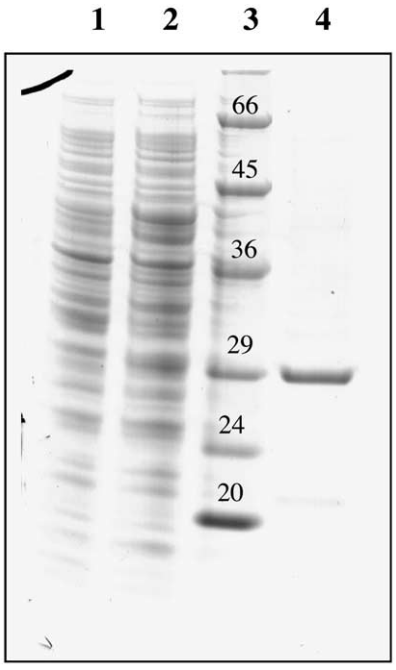

Fig. 2. Purification of the His-tagged AcbM protein. The SDS-PAGE analysis of cell free extracts and of the purified AcbO protein is shown (see Section 2). The lanes contain: 1, *S. lividans* TK64/pIJ4123 (control); 2, *S. lividans* TK64/pMJO7 (*acbO*); 3, molecular weight marker, the respective molar masses of the protein bands are shown; 4, purified His-tagged AcbO.

^{1}H-, ^{13}C-, ^{31}P-, and two-dimensional ^{1}H–^{1}H, ^{1}H–^{13}C, and ^{1}H–^{31}P COSY spectra of the sodium salt unequivocally demonstrated that the C7-phosphate group had been retained and that the coupling pattern of the new product was consistent with an epimerisation at the C-2 position by AcbO resulting in 5-*epi*-valiolone-7-phosphate as the major component in the resulting mixture. In detail the results of a comparative NMR spectroscopic analysis of the product and educt were the following: In the product the cross-peaks from the ^{1}H–^{31}P COSY experiment with the product proved that the phosphate at 3.02 ppm was correlated with H-7a and H-7b and, therefore, still was attached to the branching methylene group. Thus no shift of the phosphate ester grouping, e.g. by a mutase-type reaction, had occurred. The signal pattern for the ring protons of the product in the ^{1}H NMR, however, had changed significantly as compared to the educt (Fig. 4). Resonances for all groups, except for the methylene group had been strongly shifted upfield. All ring protons show coupling constants around ^{3}J = 9–10 Hz, which is indicative for the protons H-2, H-3, and H-4 being in axial orientation. This proved that the conformation 2b is then selected because it is clearly favoured over 2a (cf. Fig. 4). This phenomenon was already observed in previous studies [7,11]. In the ^{1}H NMR the H-2 and H-3 occurred as a doublet at 4.18 ppm (coupling constant ^{3}J = 8.7 Hz) and as a pseudotriplet at 3.94 ppm, respectively. For better resolution of the coupling pattern of all signals the pH of the NMR probe was shifted to 3.5, at which the H-3 and H-4 signals were clearer separated from the others and, therefore, could more easily be analysed. Under such conditions a NOE irradiation at H-2 ($\approx$4.2 ppm) enhanced the H-4 signal confirming the spatially close correlation between the two protons and ruling out the possibility that 2-*epi*-5-*epi*-valiolone-7-phosphate had been converted to

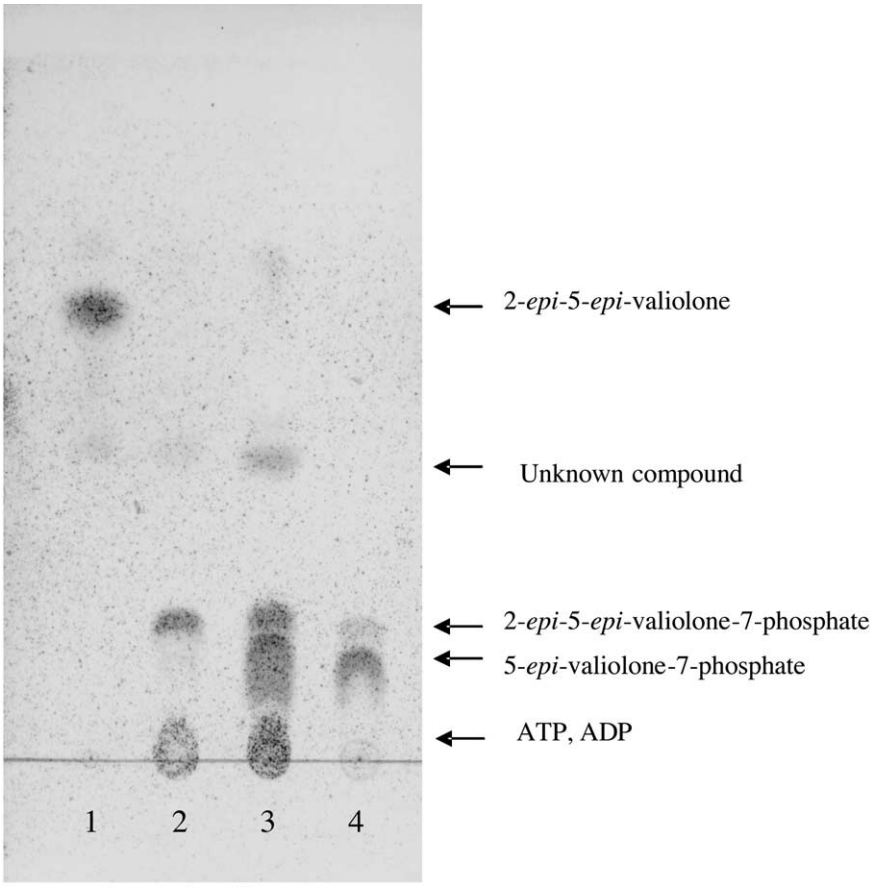

Fig. 3. TLC analysis of the AcbO-catalysed epimerisation of 2-*epi*-5-*epi*-valiolone-7-phosphate. The details are described in Section 2. The lanes contain: 1, purified 2-*epi*-5-*epi*-valiolone; 2, AcbM-catalysed phosphorylation by the purified His-tagged AcbM protein (cf. [6]); 3, reaction from lane 2 plus purified AcbO protein; 4, the purified reaction product from lane 3 after gel filtration. Arrows mark the respective compounds; a small impurity of unknown structure (degradation product?) was detected in lanes 1–3.

2-*epi*-valiolone-7-phosphate (via a retroaldol and recyclisation reaction), which could probably adopt a boat conformation, where the coupling constants of all ring protons should also be about 9–10 Hz.

3.3. Conclusions

Taken together, the *acbO* gene of *Actinoplanes* sp. SE50/110 encodes a 2-*epi*-5-*epi*-valiolone-7-phosphate 2-epimerase, which clearly seems to catalyse an essential step in the conversion of the primary metabolic precursor *sedo*-heptulose-7-phosphate to the valienol moiety of the secondary metabolite acarbose (cf. Fig. 1) [6,7]. Structurally these C7-cyclitol (phosphate) intermediates, with a keto group in position 1 of the ring, obviously have a high stability, such that no measurable keto-enol tautomerisation occurs which could spontaneously epimerise the adjacent hydroxyl in ring position 2 under physiological conditions. The primary structure of the AcbO protein (cf. accession number Y18523 in the EMBL data base) does not show any significant similarity to other proteins with known function and does not give any indication to the use of a cofactor or a coenzyme, such as, e.g. $NAD(P)^+$. Therefore, with the AcbO protein a new subclass of epimerases acting on carbohydrates and derivatives (EC 5.1.3.–) is described. The exact mechanism of this epimerisation remains unclear so far. However, the presence of the ketogroup at C1 might be necessary for this epimerisation mechanism.

For the specific design of the acarbose pathway this particular step could have several meanings: (i) the 5-*epi*-valiolone-7-phosphate could be more stable than its 2-epimer; (ii) this configuration of the cyclitol precursor is necessary to be preformed in this stage in order to create the immediate valienol precursor; (iii) the AcbO product is structurally closer related to other, more widely distributed natural ketocyclitols, such as, e.g. (2-deoxy)*scyllo*-inososе occurring in both anabolic and catabolic C6-cyclitol pathways [4]. Therefore it could be an analogous substrate of an enzyme available by chance during the individual evolution of this pathway [15]; for instance, it likely was a better substrate for the ancestral downstream enzyme, which either should have been a cyclitol 5,6-dehydratase or 2-keto-reductase according to the proposed pathway and was adopted to change its substrate profile to the particular types of C7-cyclitols we see here [6,8].

The description of the procedures for gaining preparative amounts of the products of the AcbC-, AcbM-, and AcbO-catalysed biosynthetic steps, the 'upper 2-*epi*-5-*epi*-valiolone pathway' (cf. Fig. 1) ([5,6]; this paper), will largely facilitate the further analysis of this unusual biosynthetic route yielding a carbsugar unit structurally homologous to the universal hexose β-D-glucose and replacing it in natural products which are structural analogues of either dextrins (the amylostatins; acarbose) or trehalose (validamycins).

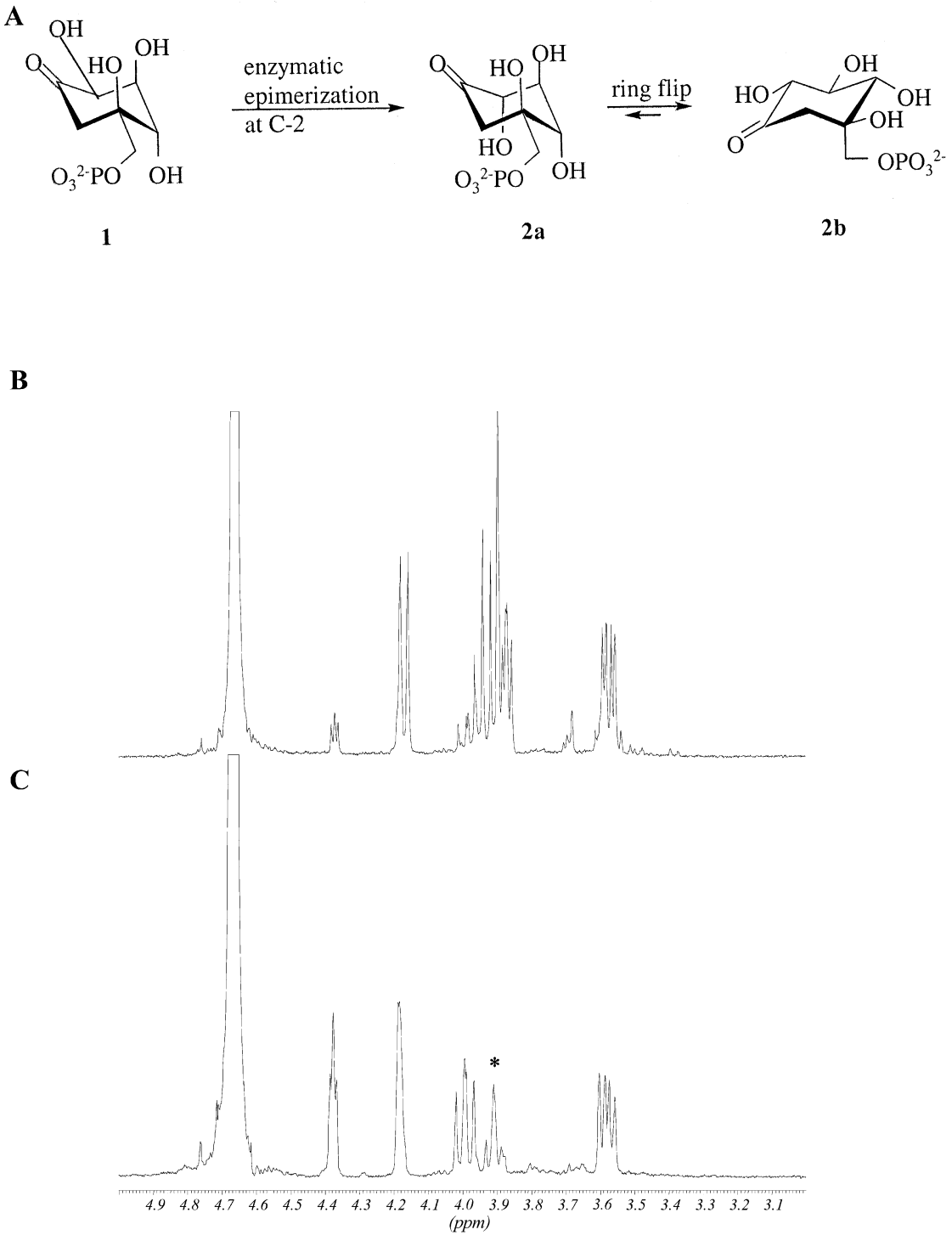

Fig. 4. Possible chair conformations as a result of ring flipping of 5-*epi*-valiolone-7-phosphate and NMR spectra. A: 2-*epi*-5-*epi*-valiolone-7-phosphate (structure 1, see Fig. 1) is epimerised by AcbO and the possible conformations of 5-*epi*-valiolone-7-phosphate (structures 2a and 2b) are shown. B,C: The ^{1}H-NMR spectra of 5-*epi*-valiolone-7-phosphate (B) and 2-*epi*-5-*epi*-valiolone-7-phosphate (C) (pH $\approx$ 7) are shown for comparison. The asterisk marks an unknown impurity (at 3.92 ppm) in the spectrum of 2-*epi*-5-*epi*-valiolone-7-phosphate (C).

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