Formation of Acarbose Phosphate by a Cell-free Extract from the Acarbose Producer *Actinoplanes* sp.

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The α-glucosidase inhibitor acarbose is modified during incubation with cell-free extract from the producing *Actinoplanes* strain. The formation of this product depends on the presence of ATP. Chromatographic and chemical properties of the purified transformation product indicate the presence of a phosphate ester. The structure is deduced by NMR analysis and shown to be acarbose-7-phosphate.

The α-glucosidase inhibitor acarbose[1] (Fig. 1) and structurally related adiposins[2] and trestatins[3] are secondary metabolites from Actinomycetes. Acarbose is produced by a strain of *Actinoplanes* sp., whereas adiposins and trestatins were isolated from *Streptomyces*. Acarbose is widely used as an oral antidiabetic[1]. Its pseudotetrasaccharide structure resembles maltotetraose. The presence of an aminocyclitol moiety, valienamin, relates this compound to validamycins[4] and, to a lesser extent, aminocyclitol antibiotics.

In the course of studies on the biosynthesis and metabolism of acarbose we noted the transformation of acarbose during incubation with cell-free extracts from the producing *Actinoplanes* strain. The isolation and structural characterization of this product is reported in the present paper.

Materials and Methods

Organism and Cultivation

Actinoplanes sp. SN 223/29 was used throughout this study.

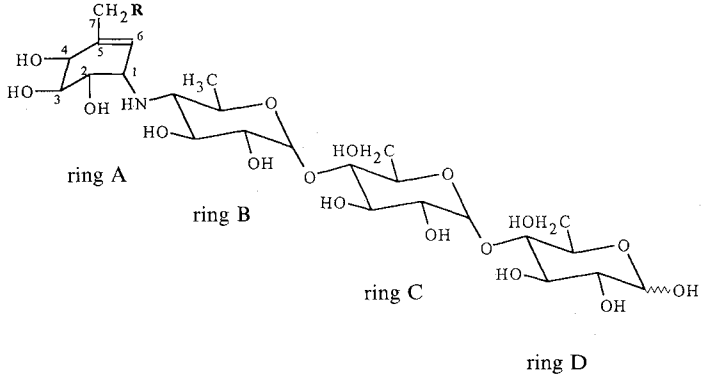
The strain was grown in a two-step fermentation procedure. Preculture: soy flour (defatted) 3%, glycerol 3%, $CaCO_3$ 0.2%, tap water, pH 7.2. Main culture: malt extract 7.5%, yeast extract 0.7%, NZ-amine 0.3%, $CaCO_3$ 0.3%, K_2HPO_4 0.3%, tap water, pH 6.9.

The preculture (120 ml in 1000 ml Erlenmeyer flasks) was grown on a rotary shaker for 72 hours at 27°C. 9 ml of this culture were used to inoculate 120 ml of main culture medium. After 120 hours at 27°C on a rotary shaker the culture was harvested by centrifugation at 3500 *g*. The sedimented mycelium was washed three times with 0.05 M potassium phosphate buffer (pH 6.8).

Preparation of Cell-free Extracts

Washed mycelium was suspended in the double volume (v/w) of Tris buffer (0.025 M Tris-HCl pH 7.8, 0.025 M $MgCl_2$, 1.5 mM 2-mercaptoethanol) and disrupted by

Fig. 1. Chemical structure of acarbose and acarbose-7-phosphate.



Acarbose: R=OH
Acarbose-7-phosphate: R=OPO_3H^-

sonication (Branson Sonfier B 12, six pulses of 30 s interrupted by 1-minute-intervals). Cell debris was sedimented by centrifugation (20000*g*, 4°C). The supernatant was used as cell-free extract.

Enzymatic Formation of Acarbose Phosphate

(a) Analytical scale: in a total volume of 14 μl, [U-^{14}C]-acarbose (0.25 mM, 24 nCi), ATP (9.1 mM), magnesium acetate (25 mM), Tris-HCl (71.4 mM, pH 7.6) and cell-free extract (10 μl) were incubated up to 2 hours at 30°C. The reaction was stopped by addition of ethanol.

(b) Preparative scale: 50 ml cell-free extract (dialyzed prior to incubation against the same Tris buffer as used for sonication), 0.38 mmol acarbose, 0.25 mmol ATP, 0.75 mmol $MgCl_2$ and 2.72 mmol Tris-HCl (pH 7.6) in a total volume of 60 ml were incubated for 90 minutes at 30°C. The reaction was stopped by the addition of 70 ml ethanol. Precipitated protein was removed by centrifugation. Ethanol was removed from the supernatant under vacuum. The remaining aqueous solution was freeze-dried.

Isolation of acarbose phosphate

Crude dried material obtained from 247 mg acarbose (see above) was dissolved in 10 ml ammonium bicarbonate (2 mM) and applied to a column of DEAE-sephadex A-25 (42 cm × 2 cm). Phosphorylated compounds were eluted by two successive linear gradients of ammonium bicarbonate (50 mM to 230 mM, 3000 ml; and 230 mM to 334 mM, 1200 ml). Fractions (15 ml) with $E_{235} > E_{260}$ containing a substance moving at $R_{acarbose} = 0.6$ (TLC) were pooled, freeze-dried and redissolved in water. This solution was applied to a column of Biogel P-2 (54 cm × 2.2 cm) and eluted with water. Fractions (8.8 ml) containing the substance were pooled, freeze-dried and the resulting material rechromatographed on Biogel P-2. To remove traces of metal ions the pooled acarbose phosphate containing fractions were passed through a small column of Dowex chelating resin A-1 (5 cm × 1.7 cm). The resulting aqueous solution was freeze-dried. From 247 mg acarbose, 48 mg acarbose phosphate were obtained by this procedure.

Analytical Methods

^{13}C NMR-spectra were recorded at 75.47 MHz (Bruker WM 300 Spectrometer). Acarbose and acarbose phosphate were dissolved in D_2O (99.95%), [d_4]-trimethylsilylpropionic acid sodium salt was used as internal standard.

Thinlayer chromatograms were run on cellulose plates using the following solvents: (I) isobutyric acid - 1 M NH_3 (5:3, v/v); (II) *n*-butanol - ethanol - water (5:3:2, v/v); (III) ethylacetate - methanol - water - 25% NH_3 (5:3:2:0.1, v/v).

Paper electrophoresis was done on Whatman 3MM paper with ammonium acetate buffer (0.1 M) pH 4.8 at 14 V/cm.

Acarbose and acarbose phosphate were located on chromatograms with alkaline silver nitrate reagent[5]. Radioactive compounds were located by autoradiography (Agfa Curix X-ray film).

For quantitation radioactive spots were scraped from the TLC plate and counted in a liquid scintillation counter.

Proof for the presence of a phosphate ester was achieved by treatment with phosphatase, phosphodiesterase or a combination of both enzymes. Radioactive modified acarbose (0.03 μmol) was incubated with 0.25 M Tris buffer (pH 8.6), 0.1 mM $MgCl_2$ and enzyme in a total volume of 4 μl. After 30 minutes at 37°C 5 μl of cold ethanol were added, protein removed by centrifugation and the supernatant applied to a TLC plate.

Results and Discussion

Formation and Isolation of Acarbose Phosphate

When cell-free extracts of *Actinoplanes* sp. were incubated with ^{14}C-labeled acarbose, a radioactive product was formed which differs from acarbose by its chromatographic and electrophoretic properties. After thinlayer chromatography a radioactive spot were evident with a significantly lower Rf-value as Acarbose ($R_{acarbose}$: 0.57 [Solvent I], 0.50 [Solvent II]). Whereas acarbose is positively charged at pH 4.8, after electrophoretic separation a negatively charged substance appeared. This product was not formed by cell-free extract after gel filtration (Sephadex G-25), indicating the participation of a low molecular cosubstrate or cofactor contained in whole cell extract. Based on the assumption of a negatively charged group in the modified acarbose, possibly a phosphate, radioactive product was treated with alkaline phosphatase. Cleavage products were separated chromatographically again (3 solvents) and only acarbose was obtained, whereas modified product disappeared. Treatment with phosphodiesterase has no effect. These data confirm that the acarbose derivative is a phosphate ester. By addition of ATP the activity of a Sephadex-treated extract was restored and the product was formed in 84% radiochemical yield, indicating a phosphorylation reaction catalyzed by a kinase.

For structural studies modification product was prepared on a larger scale and purified by combination of ion exchange and molecular sieve chromatography.

Properties of the Product and Structure Elucidation

The anionic compound shows no significant absorption at wavelengths higher than 240 nm.

Table 1. Natural abundance ^{13}C NMR signals of acarbose and acarbose-phosphate (H-decoupled).

C-atom	Chemical shift values (ppm) of acarbose (first column) and acarbose-phosphate (second column)									
	Cyclitol moiety (ring A)		$\Delta\delta$[a]	C-1′-C-6′ (ring B)		C-1″-C-6″ (ring C)		C-α-1‴-C-α-6‴ (ring D)		C-β-1‴-C-β-6‴ (ring D)	
1	58.71	58.91	+0.2	102.53	102.81	102.26	102.30	94.46	94.71	98.34	98.60
2	75.20	75.72	+0.5	73.75	73.91	74.54	75.34	74.03	74.03	76.52	76.80
3	75.20	75.56	+0.4	75.72	75.99	75.88	76.14	75.72	75.99	78.68	78.97
4	74.13	74.31 (d)[b]	+0.2	67.07	67.90	79.67	79.81	79.99	80.00	79.87	80.00
5	143.18	140.97 (d)[b]	−2.2	72.48	72.44	74.03	74.03	73.57	73.45	77.09	77.35
6	124.28	127.36	+3.1	20.00	20.14	63.10	63.29	63.25	63.48	63.10	63.29
7	64.07	67.15 (d)[b]	+3.1								

[a] $\Delta\delta$ = Change of chemical shift values; in agreement with data for α- and β-D-mannose[6] and α- and β-D-fructose[7] at C-6.
[b] (d) = Doublet: C-4 (J = 6.9 Hz); C-5 (J = 6.3 Hz); C-7 (J = 4.5 Hz).

Cleavage with alkaline phosphatase set free a cationic compound with same chromatographic and inhibitory properties as acarbose. The stoichiometric relation acarbose : phosphate = 1 : 0.95 was obtained using a saccharase inhibition assay for the quantitation of acarbose and a colorimetric assay for the determination of inorganic phosphate. This indicates that one phosphate group has been introduced into acarbose.

The ^{13}C NMR spectrum obtained from phosphorylated product is highly similar to the spectrum of acarbose (Table 1). There are only negligible differences in chemical shift values for signals representing the 18 carbon atoms of the trisaccharide portion (rings B, C and D) compared with acarbose. Ring A shows significant alterations of chemical shifts indicating an introduction of the phosphate group at the cyclitol moiety. Compared with the corresponding signals in acarbose a downfield shift of approx. 2.2 ppm at C-5 and upfield shifts of approx. 3.1 ppm at C-6 and C-7 , and rather insignificant shift differences for carbons 1 to 4 point to a positioning of the phosphate at either C-5, C-6 or C-7 (Table 1). Since the double bond between C-5 and C-6 is still present (δ = 140.97 and 127.36 ppm, respectively) phosphate ester bond must be located at the exocyclic C-7 of valienamine. Chemical shift differences observed in acarbose phosphate relative to acarbose show similar ranges and directions to hexose-6-phosphates relative to free hexoses[6,7]. Additional coupling constants of J = 2.8 to 5.0 Hz (^{13}C-O-^{31}P) and 7.0 to 8.1 Hz (^{13}C-C-O-^{31}P) were reported for hexose-6-phosphates. In acarbose phosphate significant C-P-couplings are seen at C-5 and C-7, and the coupling constants observed are J = 6.3 Hz and 4.5 Hz, respectively. These values are in good agreement with our assumption that acarbose is phosphorylated at C-7 of the cyclitol moiety (Fig. 1).

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