Biosynthetic Studies on the α-Glucosidase Inhibitor Acarbose in *Actinoplanes* sp.: Glutamate is the Primary Source of the Nitrogen in Acarbose

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Acarbose[1] (Figure 1) was isolated from the fermentation broth of *Actinoplanes* as a pseudooligosaccharide α-glucosidase inhibitor and serves as a clinically useful drug for the treatment of type II insulin-independent diabetes. The core unit of this compound responsible for the pharmacological function of α-glucosidase inhibition consists of an unsaturated cyclitol connected to a hexose *via* a nitrogen. The structure of the unsaturated cyclitol is identical with valienamine which is also found in other secondary metabolites, such as the amylostatins[2], adiposins[3], trestatins[4], validamycins[5] and salbostatin[6] isolated from *Streptomyces* species. Biosynthetic studies have demonstrated that the cyclitol moieties of acarbose[7] and validamycin[8] are derived from the pentose phosphate pathway, presumably *via* sedoheptulose 7-phosphate or *ido*-heptulose 7-phosphate as intermediate.

Many studies have been reported on the effects of nitrogen, carbon, phosphate, trace elements, or temperature on antibiotic production and cell growth in Actinomycetes[9]. In the case of acarbose, production occurs only in the presence of certain carbon sources such as maltose or higher oligosaccharides[10]. Furthermore, it was found recently that there are two metabolic routes from maltotriose to the maltose unit of acarbose[11]. However, the source of the nitrogen of acarbose and the mechanisms of its introduction into a biosynthetic precursor of acarbose and of the linkage between the cyclitol and the deoxyhexose moieties have not yet been characterized. In this paper, therefore, we report the results of feeding experiments using various ^{15}N-labeled compounds to determine the source of the nitrogen of acarbose, predicting the mechanism of the nitrogen introduction into a biosynthetic intermediate of acarbose.

Materials and Methods

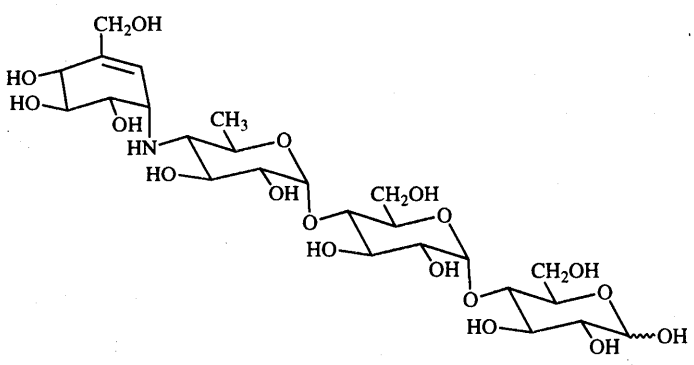
Materials

$[^{15}N_2]$Urea, [α-^{15}N]glutamine, [amide-^{15}N]glutamine and [α-^{15}N]aspartate were purchased from Cambridge Isotope Laboratory at ^{15}N-enrichments of 99.0%. [α-^{15}N]Glutamate (99.9%), [α-^{15}N]asparagine (99.0%), [amide-^{15}N]asparagine (99.0%) and $^{15}NH_4Cl$ (99.5%) were obtained from Isotec. Soybean meal (fat free), maltzin powder and yeast extract were obtained from Bayer AG, Germany. NZ-Amine A was purchased from ICN Biochemicals. Maltose was obtained from Calbiochem and glucose from Aldrich.

Organism and Culture Conditions for Feeding Experiments in Complex Medium

Actinoplanes sp. SN223/29 obtained from Bayer AG, Wuppertal, Germany, was used for all the experiments. Precultures were prepared in 250 ml flasks containing 50 ml of a medium consisting of glycerol (2.5%), soybean

Fig. 1. Structure of acarbose.



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meal (3%, fat free) and $CaCO_3$ (0.2%), pH 7.2 before sterilization. After incubation on a rotary shaker at 200 rpm (ISF-4-V shaker, Adolf Kühner AG) and 28°C for 3 days, 5 ml of preculture was transferred to a 500 ml flask containing 45 ml of complex medium consisting of maltzin powder (7.5%), yeast extract (0.7%), NZ-amine A (0.3%) and K_2HPO_4 (0.3%). The ^{15}N-labeled precursors were added in equal molar amounts (0.5 mmol) either all at 0 hour, or 1/3 at 30 hours and 2/3 at 50 hours, with harvesting at 96 hours.

Culture Conditions for Feeding Experiments in Resting Cells

For the resting cell experiments, 1 ml of preculture was transferred into each 250 ml flask containing 50 ml of medium consisting of glucose (0.5%), maltose (1%), sodium pyruvate (0.2%), NZ-Amine A (0.3%), yeast extract (0.6%), $CaCO_3$ (0.1%), and KH_2PO_4 (0.1%). The cultures were incubated on a rotary shaker at 200 rpm and 28°C for 40 hours. The contents of 3 flasks were combined and the cells were collected by centrifugation (4000 $g \times 20$ minutes). The cells were washed twice with 100 mM cold potassium phosphate (pH 6.9) and then suspended in 50 mM potassium phosphate, pH 6.9, containing maltose (1%), glucose (0.1%) and bactopeptone (0.06%), to make 150 ml of cell suspension. Each 75 ml of resting cell suspension in a 500 ml flask was incubated for 22~24 hours on a rotary shaker at 220 rpm and 28°C with an ^{15}N-labeled substrate added at an equal molar amount (0.75 mmol), 1/3 at 0 hour and 2/3 at 8 hours.

Isolation and Purification of Acarbose

The harvested cultures from the complex medium and the resting cells were centrifuged (4000 $g \times 20$ minutes) and the supernatant containing ^{15}N-acarbose was removed. The supernatant was treated with 50 ml of MeOH to prevent bumping and concentrated *in vacuo* to a syrup. The residue was treated with 100 ml of 80% MeOH, stirred for 2~3 hours at RT and centrifuged. The supernatant was evaporated to dryness *in vacuo*. The residue was dissolved in 5 ml H_2O, centrifuged to remove insoluble material and applied to a CM-25 cation-exchange column (1.5 × 60 cm) preequilibrated with 0.2 M NaOAc (pH 5). The column was eluted with deionized water with monitoring at 210 nm, and the fractions containing acarbose were pooled and evaporated to dryness for further analysis by electrospray mass spectrometry (ES-MS).

Enrichment Determination of Acarbose by ES-MS

The purified acarbose was analyzed in an electrospray ionization mass spectrometer (Fisons VG Quattro II). Five to 10 μl of sample (5~10 nmol) in water were injected at a flow rate of 10~20 μl/minute using 50% CH_3CN. The enrichment of each sample was determined from the data obtained by selective ion monitoring (SIM) at M + 1 (647.25) and M (646.25). The relative enrichment (Er) of each sample was calculated as {[M + 1/M of purified ^{15}N acarbose] − [M + 1/M of standard acarbose]} × 100 [%]. The value of M + 1/M for standard acarbose was determined as 28.9 ± 0.6% (cal. 29.2). The absolute enrichment (Ea) of each sample was calculated from [Er / (100 + Er)] × 100 (%).

Results and Discussion

To investigate the nitrogen source and to establish the mode of nitrogen introduction into a biosynthetic precursor of acarbose, we first tested ^{15}N-labeled precursors, [α-^{15}N]glutamate, [amide-^{15}N]glutamine, $^{15}NH_4Cl$ and $^{15}N_2$-urea using the complex medium which is employed for the fermentation to produce acarbose. As shown in Table 1, expt. C1, the enrichment from glutamate was higher than that from other precursors, suggesting that the nitrogen may be introduced by a PLP-dependent transamination. This result caused us to test other amino acids, [α-^{15}N]glutamine, [α-^{15}N]asparagine and [α-^{15}N]aspartate which are also potential substrates for transamination, along with [α-^{15}N]glutamate, [amide-^{15}N]glutamine, [amide-^{15}N]asparagine, $^{15}NH_4Cl$ and $^{15}N_2$-urea for comparison (Table 1, expt. C2). [α-^{15}N]Aspartate, [α-^{15}N]glutamine and [α-^{15}N]asparagine were found to be comparably good nitrogen sources as [α-^{15}N]glutamate, even though the enrichment from [α-^{15}N]asparagine was lower than from [α-^{15}N]aspartate. The enrichment levels from the amide nitrogens of asparagine and glutamine were only about half of those from their α-nitrogens, suggesting that the amide nitrogens of these compounds are incorporated only indirectly into acarbose. This could involve hydrolysis of asparagine and glutamine to NH_4^+ and then transfer of the NH_4^+ to α-ketoglutarate to form glutamate. Ammonium chloride was also found to be a nitrogen source for acarbose, but the enrichment is lower than that from [α-^{15}N]amino acids, indicating that NH_4^+ is incorporated indirectly *via* conversion to glutamate by reductive amination of α-ketoglutarate.

Since the above experiments were carried out using a complex medium containing most of the amino acids,

Table 1. Absolute ^{15}N-enrichments of acarbose from different nitrogen sources (%).

Source of nitrogen[a]	Complex medium		Resting cells[d]
	C1[b]	C2[c]	
[α-^{15}N]Asp		20.9	32.1
[α-^{15}N]Asn		11.4	23.1
[amide-^{15}N]Asn		7.2	19.9
[α-^{15}N]Glu	17.0	14.7	40.2
[α-^{15}N]Gln		14.4	31.1
[amide-^{15}N]Gln	8.9	7.8	22.1
$^{15}NH_4Cl$	6.4	10.0	38.9
$^{15}N_2$Urea	3.4	2.6	2.8

[a] Asp, aspartate; Asn, asparagine; Glu, glutamate; Gln, glutamine. Each ^{15}N precursor (enrichment: 99%) was fed at 10 mM.
[b] The ^{15}N precursors were fed all at 0 hour, harvesting at 96 hours.
[c] All ^{15}N precursors were fed 1/3 at 30 hours and 2/3 at 50 hours, harvesting at 96 hours.
[d] All ^{15}N precursors were fed 1/3 at 0 hour and 2/3 at 8 hours, harvesting at 22 hours.

the tested ^{15}N-substrates might have been diluted with unknown amounts of unlabeled material. Thus, the results obtained in the complex medium may underestimate the actual values of ^{15}N incorporation. Therefore, the ^{15}N-substrates were also tested in resting cells under conditions of negligible dilution. As shown in Table 1, the enrichments from all tested substrates except urea were higher than those obtained in the complex medium, and glutamate was again found to be the preferred nitrogen source. It was also observed that aspartate and the α-nitrogens of asparagine and glutamine are good nitrogen sources, comparable to glutamate, as in the experiments in the complex medium, although the enrichments from the α-nitrogens of asparagine and glutamine were about 25% lower than those from aspartate and glutamate, respectively. Unexpectedly, ammonium chloride was found to be a nitrogen source as efficient as glutamate in the resting cell system. The resting cell experiments demonstrate more clearly even than the ones in complex medium that glutamate is the most efficient nitrogen source among the substrates tested, and that the mode of nitrogen introduction into a biosynthetic precursor of acarbose must be a PLP-dependent transamination. In addition, ammonium chloride is a good alternative primary nitrogen source under conditions of glutamate shortage, either by direct reductive amination of a biosynthetic intermediate or, more likely, by transfer of the NH_4^+ to α-ketoglutarate by glutamate dehydrogenase/NADPH to form glutamate.

Further experiments have to be done to elucidate whether the nitrogen is introduced first into the unsaturated cyclitol or the deoxyhexose moiety, and how the two units are connected to each other to form the pseudodisaccharide core unit responsible for the α-glycosidase inhibition exhibited by acarbose.

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