# STUDIES ON THE BIOSYNTHESIS OF THE α-GLUCOSIDASE INHIBITOR ACARBOSE: VALIENAMINE, A *m*-$C_7N$ UNIT NOT DERIVED FROM THE SHIKIMATE PATHWAY

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Feeding experiments with *Actinoplanes* sp. SN223/29 showed that 3-amino-5-hydroxy-[7-$^{13}$C]benzoic acid is not incorporated into acarbose (I). The valienamine moiety of I is thus not derived in the same way, from the shikimate pathway, as the *m*-$C_7N$ units in the ansamycin, mitomycin and ansamitocin antibiotics. Feeding experiments with [*U*-$^{13}C_3$]-glycerol followed by analysis of I by multiple quantum NMR spectroscopy support this conclusion and point to formation of the valienamine moiety by cyclization of a heptulose phosphate which arises from a triose phosphate *via* successive transfer of two 2-carbon fragments by transketolase, as proposed by PAPE and co-workers.

The α-D-glucosidase inhibitor acarbose (I)[1,2] is a pseudotetrasaccharide containing an unsaturated cyclitol moiety. This cyclitol is identical with valienamine, a component of the antibiotic validamycin A[3], and carries on the cyclohexene ring a hydroxymethyl and an amino group in a 1,3 (*meta*) arrangement. This substitution pattern is reminiscent of the "*m*-$C_7N$" unit found in various antibiotics[4], *e.g.*, the rifamycins, the mitomycins, the ansamitocins, geldanamycin, pactamycin, and actamycin[5]. Studies on several of these systems have shown that the origin of the *m*-$C_7N$ unit is related to the shikimate pathway, although shikimate itself is not incorporated[5]. RICKARDS, NÜESCH and their co-workers have established 3-amino-5-hydroxybenzoic acid as an intermediate in the formation of the *m*-$C_7N$ unit of actamycin[6], mitomycin[7], rifamycin[8] and ansamitocin[9], whereas the *m*-$C_7N$ unit of pactamycin arises differently *via* 3-aminobenzoic acid[5,10].

In this paper we report the results of some experiments on the biosynthesis of I which were designed to probe the relationship of the valienamine moiety to the other *m*-$C_7N$ units mentioned and to distinguish a biosynthetic origin *via* the shikimate pathway from other plausible alternatives, *e.g.*, cyclization of a heptulose phosphate or methylation of a six-carbon cyclitol.

## Materials and Methods

3-Amino-5-hydroxy-[7-$^{13}$C]benzoic acid was synthesized from K$^{13}$CN (99% $^{13}$C, Los Alamos Stable Isotope Resource) as described by HERLT *et al.*[11], and 3-amino-[7-$^{13}$C]benzoic acid was prepared by carboxylation of phenylmagnesium bromide with $^{13}CO_2$ (99% $^{13}$C, Mound Laboratories) followed by nitration and hydrogenation over 10% Pd-C. [*U*-$^{13}C_3$]Glycerol was synthesized from K$^{13}$CN and [1,2-$^{13}C_2$]acetic acid (99% $^{13}$C, Los Alamos Stable Isotope Resource) *via* diethyl malonate and diethyl 2-acetoxymalonate.

Fermentations were conducted with *Actinoplanes* sp. SN 223/29 using a previously described[12] three-stage protocol. The fermentation times were: preculture 72 hours, main culture 24 hours, incubation culture 22 hours. Labeled precursors were added to the incubation cultures (150 ml in a 1,000-ml Erlenmeyer flask) at time 0 (expt 1: 3-amino-[7-$^{13}$C]benzoic acid, 400 mg; expt 2: 3-amino-5-hydroxy-[7-$^{13}$C]benzoic acid, 400 mg) or in equal portions at 0, 3 and 6 hours (expt 3: [*U*-$^{13}C_3$]glycerol, 300 mg; expt 4: [*U*-$^{13}C_3$]glycerol, 200 mg plus 375 mg unlabeled glucose at time 0). Acarbose was isolated by chromatography on CM-Sephadex C-25 and quantitated both by HPLC (UV, 215 nm) and enzymatic assay (inhibition of sucrase)[12]. The yields of acarbose were (HPLC/inhibition test): expt 1, 5/6 mg; expt 2, 51/44 mg; expt 3, 32/28 mg; expt 4, 22/25 mg.

$^{13}$C NMR spectra were recorded on a Bruker WM 300 spectrometer operating at a field strength of 7.1 T; the triple quantum spectra were obtained on a Bruker AM 500 spectrometer operating at 11.75 T. Spectra were recorded in $D_2O$ using the most upfield signal in the acarbose spectrum ($\delta_c$ 18.0 ppm vs. 3-(trimethylsilyl)propane sulfonic acid (TSP) as standard). Previously determined[12,13] chemical shift assignments for acarbose were used; most of these were independently confirmed by the carbon-carbon connectivities established in this work.

## Results

Since 3-amino-5-hydroxybenzoic acid has been established as the direct precursor for the *m*-$C_7N$ units in several antibiotics[6~9], we examined the incorporation of this compound into the aminocyclitol moiety of acarbose. In view of the precursor role of 3-aminobenzoic acid in pactamycin biosynthesis[10], this compound was also tested. In the $^{13}$C NMR spectra of the samples of **I** derived from these two precursors, both labeled with $^{13}$C in the carboxyl group, no significant enhancement was seen of the signal at $\delta$ 62.3 ppm, corresponding to C-7(a), the carbon atom expected to be labeled by the carboxyl carbon of the precursors. Likewise, none of the other signals in the spectrum demonstrated significant enhancement. Under the experimental conditions, an enrichment of 1% over natural abundance would have easily been detected. Since under comparable conditions 3-amino-5-hydroxy-[7-$^{13}$C]-benzoic acid gave very high enrichments in the appropriate carbon atoms of the *m*-$C_7N$ units of naphthomycin (64%) and ansatrienine (48%)[14], we feel justified in concluding that the aminocyclitol moiety of **I** is not formed *via* either 3-amino-5-hydroxybenzoic acid or 3-aminobenzoic acid.

To obtain further insight into the biosynthetic origin of the *m*-$C_7N$ unit of **I**, we carried out two feeding experiments with [*U*-$^{13}C_3$]glycerol, one in the absence (expt 3) and one in the presence (expt 4) of non-labeled glucose. The samples of **I** from these two experiments were first analyzed by recording the inverse-gated proton decoupled $^{13}$C NMR spectra. Both samples showed virtually the same spectra, except for an overall lower $^{13}$C enrichment in the sample from expt 4. The two glucose moieties were not significantly enriched, consistent with the observation[12] that maltose is incorporated as an intact unit into this part of the acarbose molecule and the fact that the incubation culture medium contained 20 g/liter

Table 1. Relative $^{13}$C-enrichment in acarbose biosynthesized from [*U*-$^{13}C_3$]glycerol.

| Carbon No. | Chemical shift (ppm) | Relative $^{13}$C abundance[a] |
|---|---|---|
| 1(a) | 56.5 | 17 |
| 2(a) | 71.4 | 16[b] |
| 3(a) | 73.5 | 16[b] |
| 4(a) | 71.7 | 9[b] |
| 5(a) | 139.6 | 9 |
| 6(a) | 124.5 | 17 |
| 7(a) | 62.3 | 9 |
| 1(b) | 100.6 | 8 |
| 2(b) | 71.8 | 8[b] |
| 3(b) | 73.5 | 8[b] |
| 4(b) | 65.5 | 14 |
| 5(b) | 70.2 | 16 |
| 6(b) | 18.0 | 14 |

[a] Natural abundance = 1.0. Reference: Average of maltose carbon signals.
[b] Estimated value.

unlabeled maltose. The relative $^{13}$C-enrichments in I from expt 3 are shown in Table 1. Several of the values are only estimates because the complex coupling patterns combined with chemical shift overlaps made accurate quantitative determinations impossible. Although all carbons in rings a and b are labeled, clear differences in the levels of enrichment are seen. This is particularly striking in the two halves of the deoxyhexose moiety (ring b), which clearly must arise from different triose phosphate pools.

As a means of analyzing the $^{13}$C-$^{13}$C coupling patterns in order to establish the biochemical connectivities resulting from the intact incorporation of two- and three-carbon segments of the precursor, we resorted to various 2D multiple quantum experiments. The 2D-INADEQUATE spectrum[15] of I from expt 3 revealed double quantum frequencies giving the connectivities shown in Table 2. The signals for the 4(a)-5(a) pair are much weaker than the other double quantum signals; hence these signals probably reflect only statistical pairing of $^{13}$C-labels rather than intact incorporation of glycerol. The signals for carbons 3(a), 2(b), 4(a) and 3(b) are degenerate; the data therefore do not reveal whether both pairs are coupled or only one. In ring b no coupling is seen between C-3 and C-4, indicative of the formation of a deoxyhexose from glucose 6-phosphate, which in turn arises from two molecules of triose phosphate. In ring a coupling is virtually absent between C-2 and C-3 as well as C-5 and C-6 and is very weak between C-4 and C-5. A zero quantum coherence experiment[16], in

Table 2. Data from double quantum coherence experiment on acarbose sample from expt 3.

| Carbons | | $\delta_c$ in Hz from transmitter[a] | | Double quantum frequency (Hz) | |
|---|---|---|---|---|---|
| A | B | A | B | Predicted A+(B) | Observed |
| 5(a) | 7(a) | +4,528 | −1,306 | +3,222 | +3,200 |
| 5(a) | 4(a) | +4,528 | −596 | +3,932 | +3,900 |
| 3(a) | 4(a) | −460 | −596 | −1,056 | −1,100[b] |
| 1(a) | 6(a) | −1,743 | +3,389 | +1,646 | +1,600 |
| 1(a) | 2(a) | −1,743 | −619 | −2,362 | −2,400 |
| 5(b) | 6(b) | −709 | −4,649 | −5,358 | −5,400 |
| 5(b) | 4(b) | −709 | −1,064 | −1,773 | −1,800 |
| 2(b) | 1(b) | −589 | +1,585 | +996 | +1,020 |
| 2(b) | 3(b) | −589 | −460 | −1,049 | −1,100[b] |

[a] Transmitter set at 79.5892 ppm.
[b] Degenerate signal.

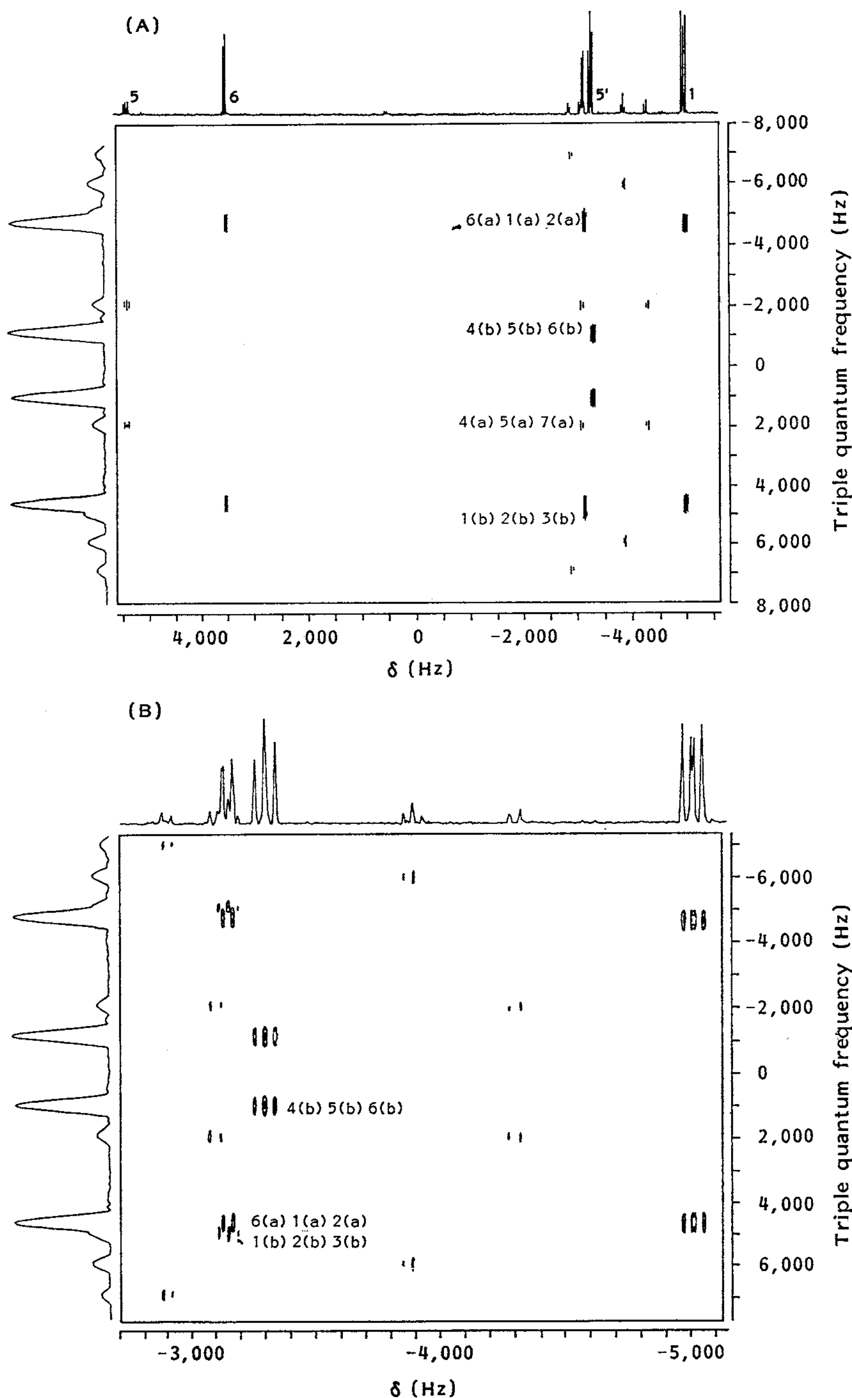
Table 3. Data from zero quantum coherence experiment on acarbose sample from expt 3.

| Carbons | | $\delta_c$ in Hz from transmitter[a] | | Zero quantum frequency (Hz) | |
|---|---|---|---|---|---|
| A | B | A | B | Predicted A−(B) | Observed |
| 5(a) | 7(a) | +4,695 | −1,139 | 5,827 | 5,800 |
| 5(a) | 4(a) | +4,695 | −430 | 5,127 | — |
| 3(a) | 4(a) | −294 | −430 | 136 | 100[b] |
| 1(a) | 6(a) | −1,577 | +3,355 | 5,131 | 5,200 |
| 1(a) | 2(a) | −1,577 | −452 | 1,125 | 1,100 |
| 5(b) | 6(b) | −543 | −4,482 | 3,939 | 3,900 |
| 5(b) | 4(b) | −543 | −898 | 355 | 300 |
| 2(b) | 1(b) | −422 | +1,751 | 2,173 | 2,100 |
| 2(b) | 3(b) | −422 | −294 | 128 | 100[b] |

[a] Transmitter set at 77.395 ppm.
[b] Degenerate signal.

Fig. 1. Triple quantum 2D-INADEQUATE spectrum of acarbose biosynthesized from [$U$-$^{13}C_3$]glycerol (expt 3).

(A) Full spectrum, (B) F2 expanded spectrum.



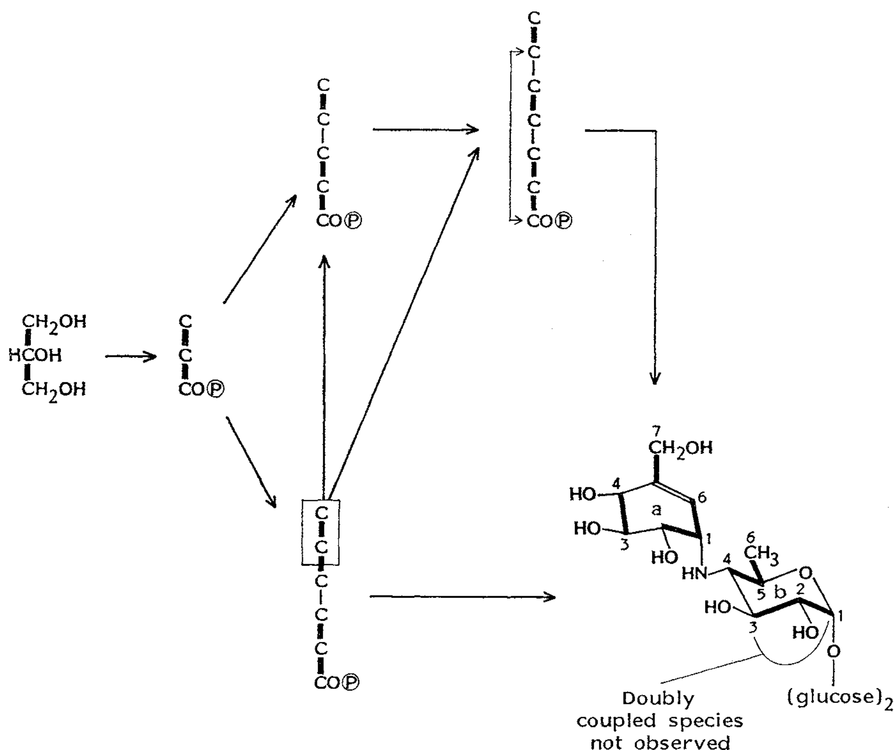

which $\alpha\beta$ to $\beta\alpha$ rather than $\alpha\alpha$ to $\beta\beta$ transitions are observed, confirmed the connectivities deduced from the double quantum experiment, except for the 4(a)-5(a) signal which was absent in the zero quantum coherence spectrum (Table 3).

Table 4. Data from triple quantum coherence experiment on acarbose sample from expt 3.

| Carbons | | | $\delta_c$ in Hz from transmitter[a] | | | Triple quantum frequency (Hz) | |
|---|---|---|---|---|---|---|---|
| A | B | C | A | B | C | Predicted (A+B+C) | Observed |
| 6(a) | 1(a) | 2(a) | +3,522 | −5,031 | −3,157 | −4,666 | 4,665 |
| 1(a) | 2(a) | 3(a) | −5,031 | −3,157 | −2,893 | −11,081 (4,912)[b] | 4,900[b] |
| 2(a) | 3(a) | 4(a) | −3,157 | −2,893 | −3,121 | −9,171 (6,829)[b] | 6,830[b] |
| 4(a) | 5(a) | 7(a) | −3,121 | +5,421 | −4,302 | −2,002 | 1,995 |
| 4(b) | 5(b) | 6(b) | −3,899 | −3,308 | −9,874 | −17,081 (1,081)[b] | 1,085[b] |
| 1(b) | 2(b) | 3(b) | +513 | −3,089 | −2,812 | −5,388 | 5,375 |

[a] Transmitter set at 96.50 ppm.
[b] These signals are folded into the spectrum since they are outside the ±8,000 Hz spectral window in F1.

Fig. 2. Predominant coupling pattern observed in acarbose biosynthesized from [$U$-$^{13}C_3$]glycerol, and its proposed origin from glycerol *via* carbohydrate metabolism.




To discern further which of the pairs of coupled carbon atoms are part of contiguously coupled 3-carbon segments resulting from intact incorporation of all three carbon atoms of glycerol, we carried out a triple quantum version of the 2D-INADEQUATE experiment[17]. This involves coherence transfer from an $\alpha\alpha\alpha$ to $\beta\beta\beta$ energy level in a three spin system; assemblies of three contiguous $^{13}C$ atoms appear as signals sharing the same triple quantum coherence frequency. Fig. 1 shows the triple quantum 2D-INADEQUATE spectrum of **I** from expt 3 and Table 4 lists the relevant data. Strong triple quantum coherence is seen at ±4,660 Hz, which is the algebraic sum of the chemical shifts (referenced to the transmitter) of C-6/C-1/C-2 in ring a, thus indicating a contiguously coupled assembly of these atoms. Another strong signal at ±1,070 Hz shows contiguous coupling between C-4, C-5

and C-6 in ring b. An expected signal for the assembly C-1(b)/C-2(b)/C-3(b) at $\pm$5,375 Hz is only observed for the center carbon of this AMX system, which appears as a triplet; the triple quantum transition is not reported at the resonance frequencies of the two "outer" carbons because transmission of triple quantum coherence from MX to A and AM to X is forbidden by the selection rules. Weak signals are seen for biochemical connectivities in ring a of C-1/C-2/C-3, C-2/C-3/C-4 and C-4/C-5/C-7. These presumably represent minor species. The predominant coupling patterns established for acarbose formed from [$U$-$^{13}C_3$]glycerol are summarized in Fig. 2.

## Discussion

The most important result of the work presented here is the conclusion that the valienamine moiety of acarbose, and therefore presumably also of validamycin, is not of the same biosynthetic origin as the $m$-$C_7N$ unit in antibiotics like the ansamycins and mitomycins, as had been proposed[4,11]. The non-incorporation of 3-aminobenzoic acid also rules out the variant of the shikimate pathway which seems to operate in the formation of pactamycin[10].

All the available data point to formation of the valienamine moiety by cyclization of a heptulose, possibly L-glucoheptulose 7-phosphate, rather than *via* the shikimate pathway. While this work was in progress, VAN HÜLST *et al.*[12] carried out feeding experiments with $^{13}$C-labeled D-glucose, L-arabinose and L-ribose. They concluded that the valienamine moiety is formed from a $C_7$ sugar which arises by transfer of 2 carbon atoms, C-1 and C-2 of a hexulose or pentulose phosphate, by transketolase to a pentose phosphate. The resulting heptulose phosphate cyclizes such that its C-1 becomes C-7 and its C-7 gives rise to C-6 of valienamine. As a result, the label from C-1 of L-arabinose, for example, appears predominantly at C-7 and C-4 of the valienamine moiety of I, and that from C-1 of glucose predominantly at C-4[12]. Despite some ambiguities in the analysis of the coupling pattern, the results of the [$U$-$^{13}C_3$]glycerol feeding experiment are consistent with the above biosynthetic pathway. As expected, ring a shows pronounced coupling between carbons 6, 1 and 2 due to intact incorporation of a triose phosphate into the "bottom" part of the heptulose phosphate (Fig. 2). The same pattern would be expected for formation *via* the shikimate route, but in this case a second intact 3-carbon fragment should be prominently incorporated into carbons 4, 5 and 7 of ring a. The triple quantum signal for this transition is very weak, on the same order as several others which result from secondary transformations and statistical coupling, and the double quantum and zero quantum spectra show strong coupling between C-7(a) and C-5(a), but little or no coupling between carbons 4 and 5 of ring a. The data are also consistent with strong coupling between C-4(a) and C-3(a), although the degeneracy of the signals for this pair and the pair C-2(b)/C-3(b) does not allow us to exclude the possibility that only one of the pairs is strongly coupled. Nevertheless, the best interpretation is that ring a arises *via* successive transfer of two coupled 2-carbon pieces onto a 3-carbon fragment which arises intact from glycerol. Mixed in with the predominant species of acarbose molecules showing the coupling pattern depicted in Fig. 2 are smaller amounts of molecules showing different coupling patterns, *e.g.*, ones showing coupling in ring a between carbons 1, 2 and 3, between 2, 3 and 4 and between 4, 5 and 7. Their presence reflects more extensive metabolism of the administered glycerol, which very likely enters the various carbohydrate pools rapidly upon addition to the cultures, rather than evenly throughout the entire fermentation period. This results in greater statistical coupling, in some instances, than would be expected from the average $^{13}$C enrichments, and probably also accounts for the differences in absolute enrichment values. This is particularly pronounced in ring b of I. Deoxyhexoses are well-known to arise intact from glucose 6-phosphate[18], and hence, the labeling of ring b should reflect the time average of the labeling of the hexose phosphate pool. The surprising observation that carbons 1, 2 and 3 of ring b are less enriched than carbons 4, 5 and 6 seems to indicate that the "top" and the "bottom" half of hexose phosphates are formed from triose phosphate pools which are not in equilibrium. Since glycerol enters metabolism by phosphorylation followed by dehydrogenation at C-2, dihydroxyacetone phosphate is formed first, followed by isomerization to phosphoglyceraldehyde. Aldolase condenses the two to produce fructose 1,6-diphosphate, with carbons 1, 2 and 3 arising direct-

ly from dihydroxyacetone phosphate. The lower enrichment of the "top" part of the hexose, despite its closer metabolic proximity to glycerol, suggests that the added glycerol pulse-labels the carbohydrate pools, and that over the entire time of the fermentation more label recycles into the phosphoglyceraldehyde than into the dihydroxyacetone phosphate pool.

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