Biosynthetic Studies on the α-Glucosidase Inhibitor Acarbose in *Actinoplanes* sp.: Source of the Maltose Unit

Sungsook Lee*, Bernd Sauerbrei[a], Jutta Niggemann[b]
and Erin Egelkrout[c]

Department of Chemistry Box 351700, University of Washington,
Seattle, WA 98195-1700

To investigate the source of the maltose unit in acarbose, feeding experiments using [3]H- or [2]H-labeled maltose or maltotriose were carried out with resting cells of *Actinoplanes* sp. SN223/29. It was found by experiments with [6″-^{3}H]- and [1-^{3}H]maltotriose that a maltose unit from the nonreducing end of maltotriose is incorporated into acarbose more efficiently than from the reducing end. However, experiments with [6″-^{2}H]- and [2-^{2}H]maltotriose showed that maltose from either the reducing end or from the nonreducing end of maltotriose was incorporated into acarbose. The results established that acarbose is formed from maltotriose by two routes; (1) Sixty percent of the acarbose are formed by attachment of maltose, produced by removing a glucose exclusively from the nonreducing end of maltotriose, to the pseudodisaccharide core unit. (2) The other 40% of the acarbose are formed by direct attachment of maltotriose to the core unit followed by loss of the terminal glucose from the reducing end. Furthermore, it was observed that there is no scrambling of label between the two glucose moieties of acarbose, that maltotriose is a comparably efficient precursor of acarbose as is maltose, and that the core unit is enriched up to 50% from the [2]H-glucose liberated from the deuterated maltotrioses.

Acarbose (Figure 1), a pseudooligosaccharide known as an α-glucosidase inhibitor and clinically useful drug for the treatment of type II insulin-independent diabetes, was found in *Actinoplanes* during screening for metabolites useful in metabolic disorder like diabetes, hyperglycemia or obesity[1]. Acarbose is the major and most active component among its analogs isolated from the fermentation broth of *Actinoplanes*, and the compound consists of an unsaturated aminocyclitol, a deoxyhexose and a maltose. The unsaturated aminocyclitol moiety is primarily responsible for the inhibition of α-glucosidase[2], and its structure is identical with that of valienamine which is also found in other secondary metabolites such as the validamycins[3], salbostatin[4], adiposin[5], amylostatin[6] and trestatin[7]. These cyclitol moieties are aliphatic analogs of *m*-C7N units which are found in many antibiotics, *e.g.*, the ansamycins[8] and mitomycins[9]. The *m*-C7N unit in those antibiotics is synthesized *via* a branch of the shikimate pathway[10]. However, experiments using stable isotope-labeled precursors have established that the *m*-C7N units of acarbose[11] and validamycin[12] are derived from the

pentose phosphate pathway. The biosynthesis of the deoxysugar moiety is very likely similar to that of other deoxyhexoses which are found in many secondary metabolites[13]. The conversion of hexoses to the corresponding 4-keto-6-deoxyhexoses, as their nucleotide diphosphates, by a stereospecific intramolecular oxidoreductase reaction[14] which is catalyzed by a NAD^+-dependent 4,6-dehydratase, plays an important role in the biosynthesis of various D- and L-deoxysugars[13].

The production of acarbose is strongly dependent on fermentation conditions, especially on carbon source. Acarbose is produced at higher levels in media containing glucose plus maltose rather than only glycerol or monosaccharides[15]. In addition, it was reported that ^{14}C-glucose was incorporated only into the pseudodisaccharide core unit but not into the maltose unit[16]. Those experiments demonstrated that the maltose unit in acarbose is not derived from glucose but from maltose or higher oligomers. However, it was not known yet how higher oligomers of maltose are attached to the core unit to produce acarbose. Thus, we report in this paper the results of feeding experiments with [3]H- and [2]H-labeled

[a]　Present address: Institute for Organische Chemie, Universität Karlsruhe, D-76049 Karlsruhe, Germany.
[b]　Present address: Bijvoet Center, Department of Bioorganic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands.
[c]　Present address: Department of Biochemistry Box 7622, North Carolina State University, Raleigh, NC 27695-7622.

Fig. 1. Structure and degradation of acarbose.
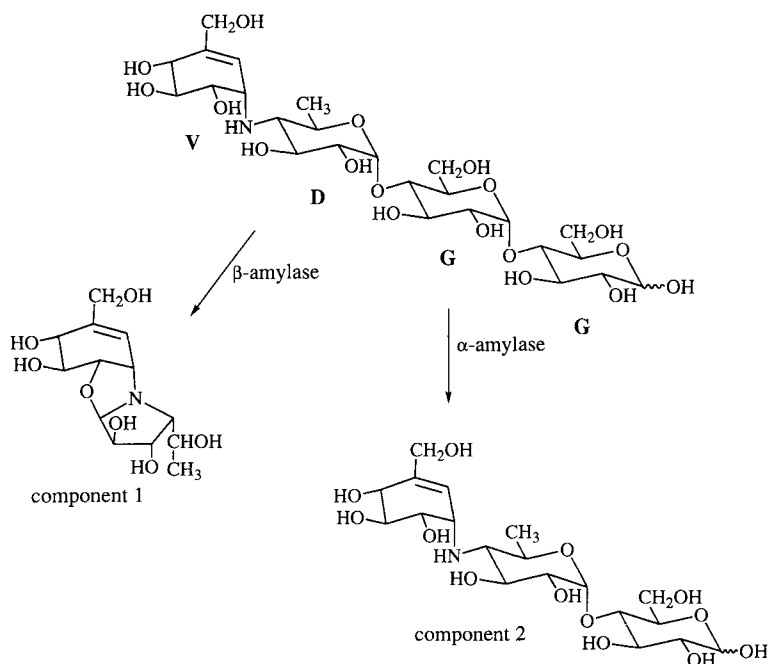
maltose/maltotriose using resting cells of *Actinoplanes* sp. SN223/29 designed to elucidate the biosynthetic route(s) from maltotriose, a model oligosaccharide, to acarbose.

Materials and Methods

Materials

[1-^{3}H]Maltotriose was obtained from the National Tritium Labelling Facility, Berkeley, CA[17]. [6''-^{3}H]-, [6''-^{2}H]-, and [2-^{2}H]maltotriose and [2-^{2}H]maltose were synthesized in our laboratory[18]. Soybean meal (fat free) and yeast extract were obtained from Bayer AG, Germany. NZ-Amine A was purchased from ICN Biochemicals, and sodium pyruvate, α- and β-amylase from Sigma. CM-Sephadex C-25 was obtained from Pharmacia and the HPLC column packed with Microsorb-NH$_2$ from Rainin.

Organisms and Culture Conditions for Feeding Experiments

Actinoplanes sp. SN223/29 provided by Bayer AG, Wuppertal, Germany, was used for all the experiments. The inoculum suspension of this microorganism (1 ml) was transferred into a 250 ml flask containing 50 ml of vegetative medium consisting of glycerol 2.5%, soybean meal (fat free) 3.0% and CaCO$_3$ 0.2%, pH 7.2 before sterilization. After incubation on a rotary shaker at

200 rpm (ISF-4-V shaker, Adolf Kuhner AG) and 28°C for 3 days, 1 ml of vegetative culture was inoculated 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$_2$PO$_4$ 0.1%. The cultures were incubated on a rotary shaker at 200 rpm and 28°C. After 40 hours incubation, the cultures from 3 flasks were combined and the cells were collected by centrifugation (4000 g × 20 minutes). The cells were washed twice with 100 mM cold potassium phosphate (pH 6.9) and were suspended in a medium consisting of 50 mM potassium phosphate (pH 6.9), glucose 0.1% and bactopeptone 0.06% to make 150 ml cell suspension. Each 50 ml of resting cells suspension in a 500 ml flask was incubated for 22 hours on a rotary shaker at 220 rpm and 28°C in the presence of the labeled compounds.

Isolation and Purification of Acarbose

After the 22 hours incubation the cultures were harvested and centrifuged (4000 g × 20 minutes). The supernatant was removed to isolate acarbose, treated with 50 ml of MeOH and concentrated *in vacuo*. To residues from experiments with ^{3}H-labeled compounds 10 mg of acarbose was added as carrier material. The residue was treated with 100 ml of 80% MeOH, stirred for 2 hours and centrifuged. The supernatant was concentrated to dryness under reduced pressure. The

residue was dissolved in 3 ml of H_2O and centrifuged to remove insoluble materials. The supernatant was applied to a CM-25 cation-exchange column (1×60 cm). The column was eluted with deionized water and the fractions containing acarbose were identified by monitoring the effluent at 210 nm. The acarbose-containing fractions were combined and concentrated to dryness under reduced pressure for further analysis. The radiolabeled acarbose obtained from the CM-25 column was purified further by HPLC on a Rainin Microsorb-NH_2 column (4.6×250 mm) using 75% CH_3CN at 1 ml/minute flow rate and monitoring at 210 nm. The radioactivity of acarbose was determined by scintillation counting (Beckman LS 1801) using Bio-Safe II biodegradable counting cocktail (Research Products International Corp). In the case of ^2H-labeled acarbose, the product was purified further on a CM-25 column instead of HPLC to separate it from acarbosylglucose (acarbose + glucose).

α- and β-Amylase Digestions of ^2H-Labeled Acarbose

The purified acarbose (~ 2 mg each from the ^2H-labeled precursors) was dissolved in 200 μl of water and 10 μl each of the solution (0.15 μmole) was digested with α- or β-amylase. The solution (10 μl) was diluted with 90 μl of 20 mM potassium phosphate buffer (pH 7.0) containing 6 mM NaCl, and incubated with α-amylase (1 U) at RT for 2 hours. For the incubation with β-amylase (0.1 U), the acarbose solution (10 μl) was mixed with 90 μl of 10 mM acetate buffer (pH 4.8) containing 3 mM NaCl and incubated at RT for 2 hours. Ten microliter of each sample was subjected to electrospray ionization mass spectrometry to determine the deuterium enrichment of the products.

Analysis of ^2H-Labeled Compounds by ES-MS

The isolated compounds, acarbose, acarbosylglucose, maltose, maltotriose and acarbose digested by α- or β-amylase were analyzed using a Fisons VG Quattro II

electrospray ionization mass spectrometer. For routine analysis, each sample was diluted with water to contain $5 \sim 10$ nmole/10 μl for an injection at a flow rate of 20 μl/minute using 50% CH_3CN. The SIM (selective ion monitoring) method for the molecular ions and MRM (molecular extract monitoring) method for the daughter ions were used to determine the absolute enrichments of target compounds. The daughter ions of acarbose and acarbosylglucose were observed as m/z 304 (V + D); the collision energy of 20 eV produced an intensity ratio, molecular ion ($M + H^+$; 646 for acarbose and 808 for acarbosylglucose) : daughter ion $(304) = 1:9$. The absolute enrichment of each sample was calculated by the following equation:

$$\frac{[(M + 1/M)\text{enr} - (M + 1/M)\text{st}]}{1 + [(M + 1/M)\text{enr} - (M + 1/M)\text{st}]} \times 100 \ (\%)$$

Results and Discussion

Generally oligo- or polysaccharides are hydrolyzed by glucosidases (amylases, sucrase, glucoamylase, amyloglucosidase, etc.) to lower saccharides, e.g., glucose, fructose or maltose, for their utilization in the cells. Therefore, we selected maltotriose, the simplest saccharide to produce maltose, as an example of oligosaccharides to search for the source of the maltose unit of acarbose. We first tested radiolabeled [1-^3H]- and [6″-^3H]maltotriose with resting cells of Actinoplanes sp. SN223/29 with and without 10 μmole of acarbose as carrier in the absence of unlabeled maltose. As shown in Table 1, incubations of ^3H-maltotriose with acarbose showed higher incorporation than those without acarbose, suggesting that acarbose itself is a very efficient substrate for a glycosyltransferase, possibly the same enzyme connecting the core unit (V + D in Figure 1) and maltose to produce acarbose. It was found that both labeled maltotrioses were incorporated into the acarbose fraction at similar levels when measured after the purification by

Table 1.   Incorporation of [1-^3H]- and [6″-^3H]maltotriose into acarbose.

Substrates [1-^3H]-, [6″-^3H] maltotriose (μCi)[a]	Unlabeled acarbose (μmole)	Specific activity of acarbose plus acarbosylglucose purified by CM-25 (nCi/mmole)	Specific activity of acarbose purified by HPLC (nCi/mmole)[b]
10, 0		1.63	0.38
10, 0	10	7.85	0.95
0, 10		1.55	1.40
0, 10	10	5.27	4.05

[a] Specific activity of [1-^3H]- and [6″-^3H]maltotriose: 40 μCi/μmole.
[b] HPLC with a microsorb-NH_2 column was used after the CM-25 cation exchange column to purify acarbose.

the CM-25 cation exchange column. However, in the subsequent purification by HPLC the specific radioactivity of acarbose isolated from the incubation with [1-^{3}H]maltotriose decreased dramatically, while the specific radioactivity of the acarbose from [6"-^{3}H]maltotriose decreased only slightly. These results demonstrate that the purification by CM-25 cation-exchange column does not effectively separate ^{3}H-acarbose from its homolog, ^{3}H-acarbosylglucose, but the HPLC method is sufficient to obtain pure acarbose. In addition, the results led to the postulate that maltose from the nonreducing end of maltotriose could be the source of the maltose unit in acarbose *via* two different pathways, either cleavage of maltose from the nonreducing end of maltotriose followed by attachment to the core unit, or attachment of maltotriose directly to the core unit followed by loss of the terminal glucose at the reducing end.

Based on the above results with radiolabeled maltotriose, experiments using stable isotope-labeled maltotriose were designed to elucidate the route(s) by which maltotriose provides the maltose unit. Therefore, [2-^{2}H]maltose (to use as reference), and [6"-^{2}H]- and [2-^{2}H]maltotriose were synthesized in our laboratory[18], and tested with the resting cell system in the absence of unlabeled maltose. Compounds isolated from the CM-25 cation exchange column were analyzed by electrospray ionization mass spectrometry, a powerful tool to analyze polar compounds such as carbohydrates or proteins[19]. We used the SIM and MRM methods to determine the percentages of deuterium enrichment of the components of interest. As shown in Table 2, both [6"-^{2}H]- and [2-^{2}H]maltotriose were incorporated into acarbose at

similar levels but lower than [2-^{2}H]maltose. Maltose was isolated from all incubations but maltotriose only from the one with [6"-^{2}H]maltotriose. [2-^{2}H]Maltotriose was degraded completely to maltose and glucose during the 22 hours incubation period (all compounds were added at 0 hour) and the enrichment of the isolated maltose was almost the same as that of the substrate, [2-^{2}H]-maltotriose. In the case of the incubation with [2-^{2}H]-maltose, the substrate was not degraded completely to glucose during the same length of time, despite the fact that it was used at the same molar concentration and enrichment as the [2-^{2}H]maltotriose, and the enrichment of the reisolated maltose was also similar to that of the substrate [2-^{2}H]maltose. However, the enrichment of the isolated maltose from the incubation with [6"-^{2}H]maltotriose (14%) was much lower than those from the [2-^{2}H]maltotriose and the [2-^{2}H]maltose (over 90%), while the enrichment of the reisolated maltotriose was found to be the same as that of the substrate [6"-^{2}H]maltotriose. The maltotriose was probably not degraded completely to maltose and glucose in this incubation due to a different feeding schedule (addition in two portions, half at 0 hour and half at 9 hours). The results also indicate that maltotriose was not significantly reformed from maltose + glucose produced by the maltotriose degradation. Since [6-^{2}H]glucose generated by hydrolysis from the [6"-^{2}H]maltotriose is diluted with unlabeled glucose present as an ingredient of the resting cell medium, the enrichment of the reisolated maltotriose should have been lower than that of the substrate [6"-^{2}H]maltotriose if maltotriose had been reformed. Furthermore, no maltotriose was detected at the end in the experiment with [2-^{2}H]maltotriose.

Table 2. Absolute deuterium enrichments (%)[a].

| Precursor[e] | Isolated compounds after incubation with labeled precursors[b] | | | |
	Acarbose	Acarbosylglucose[c]	Maltose	Maltotriose
[2-^{2}H]Maltose[f]	67		90	
[2-^{2}H]Maltotriose[g]	46	84	92	ND[d]
[6"-^{2}H]Maltotriose[h]	41	97	14	94

[a] The absolute D-enrichments were calculated by the equation given in the Materials and Methods.

[b] M + 1(647)/M(646) in standard acarbose (H$^+$) = 29.8%.
   M + 1(381)/M(382) in standard maltose (K$^+$) = 15.1%.
   M + 1(528)/M(527) in standard maltotriose (Na$^+$) = 21.7%.

[c] No standard M + 1/M is available; calculated value 37.4%.

[d] No maltotriose was detected.

[e] 0.6 mmole of each was fed to 50 ml of resting cell culture : [2-^{2}H]Maltose and [2-^{2}H]maltotriose were fed all at 0 hour, but [6"-^{2}H]maltotriose was fed 1/2 at 0 hour and 1/2 at 9 hours. Total incubation time for each substrate was 22 hours.

[f] Absolute enrichment: 90%.

[g] Absolute enrichment: 92%.

[h] Absolute enrichment: 94%.

The results demonstrate that maltotriose is degraded to maltose, exclusively by removal of a glucose from the nonreducing end, predicting that the "right" two glucose units are the source of the maltose unit of acarbose. However, it was found that acarbose isolated from the incubation with [6''-²H]maltotriose was enriched at almost the same level as that from [2-²H]maltotriose. Therefore, two possible routes to produce acarbose from maltotriose are suggested (Figure 2). One route is that maltotriose is degraded to maltose retaining the "right" two glucose units and then the maltose is attached to the core unit. The other route is that maltotriose is attached directly to the core unit followed by removal of the terminal glucose from the reducing end to form acarbose. The glucoside cleavage pattern indicated by these experiments is different from that known for β-amylase[1] which cleaves off maltose units from the nonreducing

end of polysaccharides.

The two possible routes described above at first glance appear inconsistent with the results of the radiolabeling experiments. In those experiments, however, the ³H-maltotrioses were incubated in the presence of 10 μmole of acarbose. Thus, the ³H-maltotriose could exchange, faster than being degraded to maltose, with a maltose unit of the external acarbose (in the absence of unlabeled maltose) followed by removal of the terminal glucose to produce acarbose. Therefore, the specific radioactivity of the acarbose, purified by HPLC, from the incubation with [6''-³H]maltotriose was much higher than that from [1-³H]maltotriose although the specific radioactivities of the acarbose fractions after purification by CM-25 were similar due to the presence of acarbosylglucose (Table 1).

The deuterated acarbose and acarbosylglucose samples were further analyzed to determine the percentages of enrichment of the core unit, because it was known that glucose, which was produced by the degradation of the labeled substrates, is a precursor of the core unit in acarbose[11]. Fortunately, the core unit was detected as the only daughter ion in electrospray MS-MS. It was found, as shown in Table 3, that the core units were enriched about 20～28%. This enrichment was confirmed by β-amylase degradation of the isolated acarbose. Acarbose is cleaved by β-amylase to maltose and a tricyclic compound (modified core unit, component 1, Figure 1) and the enzymatic reaction is not reversible[20]. Acarbose was also analyzed by treatment with α-amylase to check whether the two glucose units of acarbose have undergone isotope scrambling. Acarbose is an efficient α-amylase inhibitor but is itself degraded to component 2 (VDG, Figure 1) by α-amylase at low levels[20]. However, component 2 is not degraded by either α- or β-amylase. As shown in Table 3, the enrichments of

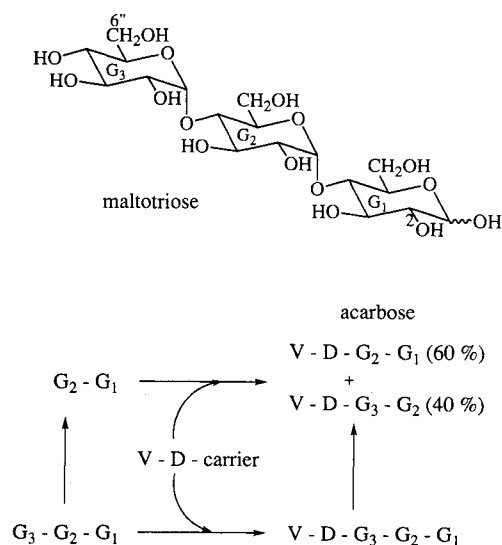Fig. 2.   Biosynthetic routes from maltotriose to acarbose.

Table 3.   Absolute enrichments in monodeuterated species of acarbose and acarbosylglucose[a].

	Total molecule	Core unit (VD)		VDG by α-amylase
		by MRM	by β-amylase	
Acarbose from:	(VDGG)			
2D-Maltose	67 [100%]	24 [36%]av[b]	24	25 [38%]
2D-Maltotriose	46 [100%]	20 [39%]av	20	20 [42%]
6''D-Maltotriose	41 [100%]	28 [54%]av	24	60 [100%][c]
[Acarbose + Glucose] from:	(VDGGG)			
2D-Maltotriose	84 [100%]	7 [8%]	—	—
6D''-Maltotriose	97	—	—	—

ᵃ   Figures without brackets are absolute enrichments in monodeuterated species (abs.% D₁); figures in square brackets are percentage deuterium distribution within a molecule.

ᵇ   Average between MRM and β-amylase.

ᶜ   The absolute enrichment of VDG, 60, is calculated to be larger than the enrichment of acarbose, 41.

VDG from the incubations with [2-^{2}H]maltotriose and [2-^{2}H]maltose were close to those of the core units (VD), whereas the enrichment of VDG from [6″-^{2}H]maltotriose was much higher than that of VD. The enrichment of VDG (60%) from [6″-^{2}H]maltotriose was calculated to be higher than that of the acarbose (41%) from which it was derived. This result, which is obviously physically impossible, is an artefact due to the low levels of acarbose degradation by α-amylase. The α- and β-amylase experiments demonstrate that there is no isotope scrambling between the two glucose units in acarbose.

It was found that the acarbosylglucose samples generated from the labeled maltotrioses were almost as highly enriched (84% from [2-^{2}H]maltotriose and 97% from [6″-^{2}H]maltotriose, respectively) as their substrates. However, the enrichment of the acarbose (67%) from [2-^{2}H]maltose was not so high compared to those of acarbosylglucose from [2-^{2}H]maltotriose and [6″-^{2}H]maltotriose (see above). The lower enrichment is probably due to dilution of the labeled maltose with unlabeled maltose remaining inside the cells from their growth in the medium containing maltose (see Materials and Methods), even though the cells were washed twice with 100 mM of potassium phosphate buffer prior to the incubation with labeled maltose.

Based on all the analyses discussed above, the distributions of deuterium in acarbose are summarized in Figure 3. The ratio of acarbose production (Figure 2) by the two pathways (40:60) was calculated from the deuterium percentage in the GG portion of acarbose derived from [6″-^{2}H]maltotriose, 41 × (1−0.54) = 19 and from [2-^{2}H]maltotriose, 46 × (1−0.39) = 28. Comparative rates of acarbose production from maltose and from maltotriose were calculated from how much of the GG unit of acarbose was synthesized from each precursor. For [2-^{2}H]maltose this is 67 − 24 = 43, for [2-^{2}H]maltotriose it is (46 − 20) ÷ 0.6 = 43 and for [6″-^{2}H]maltotriose it is (41 − 26) ÷ 0.4 = 38, respectively, indicating that maltotriose is a comparably good precursor of the maltose unit of acarbose as maltose.

The conclusions can summarize as follows: (1) Maltotriose can be degraded to maltose; this occurs almost exclusively (>90%) by removal of a glucose (G$_3$) from the non-reducing end. (2) The maltose unit of acarbose is formed from maltotriose by two routes. On one route, which accounts for 60% of the acarbose formed, maltotriose is degraded to maltose retaining the "right hand" two glucose units (G$_2$-G$_1$); this maltose is then attached to the core unit. The other 40% of the acarbose are formed by direct attachment of maltotriose to the

Fig. 3. Deuterium distribution within acarbose.
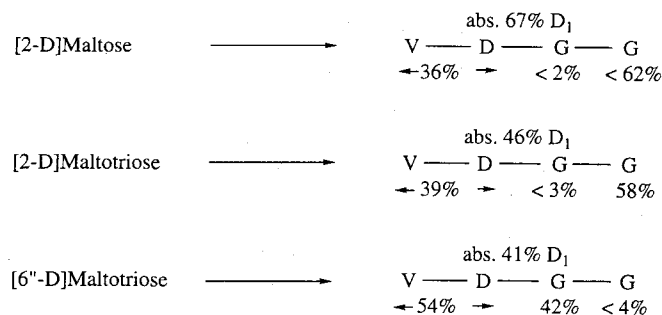
core unit, followed by loss of the terminal glucose unit from the "right hand" end (G$_1$). (3) Maltotriose is a comparably good precursor of the maltose unit of acarbose as maltose. (4) The core unit is enriched appreciably, up to about 50%, from the glucose liberated either from [6″-^{2}H]maltotriose (G$_3$) or from acarbosyl-[2-^{2}H]glucose (G$_1$). Maltose is also cleaved to glucose which labels the core unit. (5) No scrambling of isotope between the two glucose moieties of acarbose is observed.

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