The effect of tsushimycin on the synthesis of lipid-linked saccharides in aorta

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The antibiotic, tsushimycin, inhibits the formation of dolichyl phosphate mannose, dolichyl phosphate glucose and dolichyl pyrophosphate *N*-acetylglucosamine in the particulate enzyme preparation from pig aorta. Although this antibiotic also inhibits the incorporation of mannose and glucose into lipid-linked oligosaccharides, these reactions are less sensitive to antibiotic than those involved in the synthesis of lipid-linked monosaccharides. In the presence of tsushimycin, most of the mannose incorporated into lipid-linked oligosaccharides is into one oligosaccharide that has the properties of the heptasaccharide $\text{Man}_3\text{GlcNAc}_2$, whereas in the absence of antibiotic most of the mannose is in larger-sized oligosaccharides. On the other hand, the glucose-labelled lipid-linked oligosaccharides appear to be similar in size in the presence or absence of antibiotic. Tsushimycin also inhibits the formation of lipid-linked monosaccharides by the solubilized enzyme preparation of aorta. Various concentrations of dolichyl phosphate or the detergent, Nonidet P40, had no effect on antibiotic inhibition. Some evidence indicates that tsushimycin binds to the particulate enzyme.

The biosynthesis of asparagine-linked oligosaccharides involves a series of membrane-bound glycosyltransferases that utilize dolichol-linked saccharide intermediates (Hemming, 1974; Waechter & Lennarz, 1976; Elbein, 1979). These enzymes catalyse the transfer of *N*-acetylglucosamine, mannose and glucose to dolichyl phosphate to form a dolichyl pyrophosphate oligosaccharide having the composition, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Spiro *et al.*, 1976; Li *et al.*, 1978; Liu *et al.*, 1979). This oligosaccharide is then transferred to protein (Pless & Lennarz, 1977; Turco *et al.*, 1978; Spiro *et al.*, 1979; Das & Heath, 1980).

Although the general pathway of assembly of the lipid-linked saccharides is known, the details of formation of the various lipid intermediates remains to be established. For example, some of the questions to be answered are how many glycosyltransferases are involved, what are the glycosyl donors and the structures and properties of the lipid acceptors and what are the sequences of reactions? But many of these questions are difficult to answer in particulate enzyme systems. One approach to such questions is the use of inhibitors to block specific steps in the pathway, which could lead to the accumulation of various lipid intermediates.

In the present paper, we show that the antibiotic tsushimycin blocks the transfer of sugars from sugar

nucleotides to dolichyl phosphate, preventing the formation of dolichol-linked monosaccharides. Tsushimycin is a peptide antibiotic that was isolated from the culture filtrate of a *Streptomyces* strain Z-237 (Shoji *et al.*, 1968; Shoji, 1978). Although the complete structure of this antibiotic has not been established, it is an acidic acylpeptide related to the amphotycin-glutamycin group but differs in the amino-acid and fatty-acid moieties. High concentrations of tsushimycin block the formation of Dol-*P*-Man and Dol-*P*-Glc, but still allow the incorporation of sugars into the lipid-linked oligosaccharides. The major oligosaccharide formed from GDP-[^{14}C]mannose in the presence of tsushimycin is $\text{Man}_3\text{GlcNAc}_2$.

Materials and methods

Materials

GDP-[^{14}C]mannose (sp.radioactivity 278 Ci/mol), UDP-[^3H]GlcNAc (sp.radioactivity 6.6 Ci/mmol) and UDP-[^3H]glucose (sp.radioactivity 1–5 Ci/mmol) were purchased from New England Nuclear. Dolichyl phosphate and unlabelled sugar nucleotides were from Sigma Chemical Co. and silica-gel F254 thin-layer plates were from Brinkmann Instruments. Bio-Gel P-4 and P-6 were purchased from Bio-Rad Laboratories. Tsushimycin was a generous gift from Dr. J. Shoji, Shionogi Chemical Co., Tokyo, Japan.

Preparation and assay of aorta enzyme

The intimal layer of pig aorta was removed and homogenized in 50 mM-Tris buffer, pH 7.5, as described in previous publications (Chambers & Elbein, 1975; Chambers *et al.*, 1977). The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 5000g for 10 min to remove large particles. The supernatant liquid from this centrifugation was centrifuged at 100000g for 90 min to obtain a 'high-speed pellet'. This pellet was suspended by gentle homogenization in 50 mM-Tris buffer at about one-tenth of the original volume and used as the particulate enzyme fraction.

The particulate enzyme fraction was solubilized by vigorous mixing in 0.5% Nonidet P40 for 2–3 min followed by centrifugation at 100000g for 45 min. The supernatant was removed and used as the 'solubilized' enzyme (Heifetz & Elbein, 1977).

Incubation mixtures for the assay of the particulate enzyme contained the following components in a final volume of 0.4 ml: 10 μ mol of Tris/HCl buffer, pH 7.5; 2.5 μ mol of MnCl₂; 50000 c.p.m. of either GDP-[¹⁴C]mannose, UDP-[³H]GlcNAc or UDP-[³H]glucose; various concentrations of tsushimycin as indicated; 100 μ l of particulate enzyme (usually 1–2 mg of protein). After incubation at 37°C for the indicated times, reactions were stopped by the addition of 2 ml of chloroform/methanol (1:1, v/v) and 0.6 ml of water. The reactions were mixed vigorously and centrifuged to separate the layers. Lipid-linked monosaccharides and lipid-linked oligosaccharides were isolated by the multiple extraction method described previously (Chambers & Elbein, 1975; Chambers *et al.*, 1977).

When the solubilized enzyme was used, incubation mixtures contained the same components as above except that dolichyl phosphate had to be added as an acceptor. Thus in these experiments, 5 μ g of dolichyl phosphate (usually in 25 μ l of chloroform) was added to the incubation tube and the solvent was removed under a stream of air. Nonidet P40 (1%; 100 μ l) was added to suspend the lipid, and after vigorous mixing, 100 μ l of 50 mM-Tris buffer, 150 μ l of water and 20 μ l of 50 mM-MnCl₂ were added in that order with vigorous mixing after each addition. Tsushimycin, sugar nucleotide and soluble enzyme (100 μ l; 0.5–1 mg of protein) were then added and tubes were incubated for the indicated times. After incubations, reactions were stopped with chloroform/methanol and the lipids were isolated as described above.

Analytical, chemical and chromatographic methods

Radioactivity was measured in a Beckman liquid-scintillation spectrometer using either a Scintilene or a Scintiverse 'cocktail' (Fisher Scientific Co.). Paper chromatograms were scanned with a Packard radiochromatogram scanner. Mild acid hydrolysis of

lipid-linked saccharides was done in 50% propanol in 0.02 M-HCl at 95°C for 15 min. After this hydrolysis, the reaction mixtures were partitioned by the addition of chloroform to give chloroform/propanol/water mixture of 1:1:1 (by vol.). The aqueous phase, containing the oligosaccharides, was removed, taken to dryness and chromatographed.

Descending paper chromatography of radioactive oligosaccharides was done on Whatman 3MM paper in solvent A [n-butanol/pyridine/water (4:3:4, by vol.)]. Standard sugars were detected with the silver nitrate dip (Trevelyan *et al.*, 1950). T.l.c. of the lipid products was done on silica-gel plates (2 mm thickness) in solvent B chloroform/methanol/water (65:25:4, by vol.) or solvent C [chloroform/methanol/NH₃ (75:25:4, by vol.)]. Samples were evaporated with a stream of air, resuspended in 100 μ l of chloroform/methanol (1:1, v/v) and applied to the plate. Lipids were detected with I₂ vapour and radioactive areas were identified by cutting the plates into 1 cm sections and placing in scintillation vials for counting the radioactivity.

Polyprenyl-linked saccharides were purified on DEAE-cellulose as previously reported (Chambers & Elbein, 1975). The lipid-linked oligosaccharides were chromatographed on DEAE-cellulose, packed and eluted with chloroform/methanol/water (10:10:3, by vol.). The lipid-linked saccharides were eluted with a gradient of 0–250 mM-ammonium formate. The peak of radioactivity was pooled, saponified, extracted into chloroform and re-chromatographed on DEAE-cellulose. This purified material was used for further studies.

Results

The effect of tsushimycin on mannose incorporation by the particulate enzyme

The aorta particulate enzyme catalyses the transfer of mannose from GDP-[¹⁴C]mannose into Dol-*P*-Man and into lipid-linked oligosaccharides. Most of the radioactivity in the lipid-linked oligosaccharides is into oligosaccharides having nine to twelve or more sugar residues (Chambers *et al.*, 1977).

The antibiotic, tsushimycin, inhibits the transfer of mannose from GDP-[¹⁴C]mannose to Dol-*P*-Man in the particulate enzyme (Fig. 1), with 50% inhibition requiring about 100 μ g of antibiotic. The incorporation of glucose and *N*-acetylglucosamine into the lipid-linked monosaccharides was also inhibited by tsushimycin (Fig. 1; see below for discussion). In control experiments, tsushimycin was added at various concentrations, at the end of the incubation, to be certain that it had no effect on the extraction of the lipid intermediates. The incorporation in these experiments was the same as in incubations without antibiotic. In Fig. 1, the incubations were done for

Fig. 1. Effect of tsushimycin concentration on the formation of lipid-linked monosaccharides by the aorta particulate enzyme

Incubations were as described in the text and contained either GDP-[14 C]mannose, UDP-[3 H]-glucose or UDP-[3 H]GlcNAc. After incubation for 20 min, lipid-linked monosaccharides were extracted with chloroform/methanol/water (1:1:1, by vol.) and the chloroform layer was measured to determine its radioactive content. Antibiotic concentrations are given in μ g/incubation mixture (0.4 ml).

20 min, but in other experiments various times of incubation were used. Thus a time course of mannose incorporation is shown in Figs. 2(a) and 2(b). Mannose incorporation into both Dol-P-Man (Fig. 2a) and into lipid-linked oligosaccharides (Fig. 2b) was followed at several concentrations of antibiotic. In the control (without tsushimycin) the formation of Dol-P-Man reached a maximum in the first 5 min and then declined over the next 15 min. In the presence of 50 or 200 μ g of antibiotic, there was a slow increase in the radioactivity in Dol-P-Man, but at early times the incubation was inhibited by 75% or greater. On the other hand, incorporation of mannose into lipid-linked oligosaccharides was much less effected by tsushimycin (Fig. 2b). Thus 50 μ g of antibiotic only inhibited lipid-linked oligosaccharides formation about 10 or 15% and even at 200 μ g there was only a 50% inhibition of incorporation.

The [14 C]mannose-labelled lipids extracted with chloroform/methanol/water (1:1:1, by vol.) were examined by t.l.c. to determine if tsushimycin affected the nature of the products. Only one radioactive band was observed in any of these incubations and this band migrated in solvents B and C with authentic Dol-P-Man. Thus tsushimycin greatly decreased the radioactivity in this band, but it did not give rise to any other detectable products.

To determine the lipid-linked oligosaccharide products formed in the presence of tsushimycin, large-scale incubations were prepared containing GDP-[14 C]mannose, Mn^{2+} , particulate enzyme and various concentrations of tsushimycin. After an

Fig. 2. Time course of incorporation of mannose into Dol-P-Man and lipid-linked oligosaccharides by the particulate enzyme

(a) Shows the formation of Dol-P-Man; (b) shows the formation of lipid-linked oligosaccharides. Incubation mixtures were as described in the text with GDP-[14 C]mannose and the indicated amounts of tsushimycin. At the times indicated, Dol-P-Man and lipid-linked oligosaccharides were isolated and their radioactive content was determined.

incubation of 10 min, Dol-P-Man and lipid-linked oligosaccharides were isolated and a portion of each was taken for the determination of radioactivity. Fig. 3 shows that at high concentrations of antibiotic, Dol-P-Man formation was inhibited up to 90%, but incorporation into lipid-linked oligosaccharides was only inhibited about 50%. The lipid-linked oligosaccharides formed at various tsushimycin concentrations were concentrated to dryness and hydrolysed in 0.02 M-HCl in 50% propanol at 95°C for 15 min. The samples were adjusted to a 1:1:1 mixture of chloroform/propanol/water and, after thorough mixing, the aqueous phase was removed, concentrated to a small volume and chromatographed on Whatman 3 mm paper in solvent A. Fig. 4 shows the scans of radioactivity of these paper chromatograms. In the control incubation (Fig. 4a),

most of the radioactivity in the lipid-linked oligosaccharides was in large oligosaccharides that moved more slowly than the stachyose standard.

Fig. 3. Large-scale incubations for the formation of [^{14}C]mannose-labelled products

Large-scale incubations were prepared (10-fold over normal incubations) containing the amounts of antibiotic shown. After incubation for 20 min, lipid-linked monosaccharide and lipid-linked oligosaccharide were isolated and one-twentieth of the solution (by vol.) was taken for the determination of radioactivity.

Fig. 4. Paper chromatography of the oligosaccharides formed from $\text{GDP-}[^{14}\text{C}]\text{mannose}$ by the particulate enzyme in incubations described in Fig. 3

Papers were run in solvent A and scanned for radioactivity. (a), Control without antibiotic; (b), 250 μg of tsushimycin; (c), 1 mg of tsushimycin; (d), 500 μg of amphomycin; (e), 0.06 M-EDTA. S indicates the migration of a stachyose standard and M indicates the migration of a mannose standard.

However, with increasing amounts of tsushimycin (Figs. 4b and 4c) more and more of the mannose incorporated was into a single oligosaccharide that had an $R_{\text{stachyose}}$ of 0.7. Based on this migration rate as well as the elution from Bio-Gel P-4, this oligosaccharide is apparently a heptasaccharide of composition, $\text{Man}_7\text{GlcNAc}_2$. Thus this oligosaccharide co-migrated on paper in several solvents with a $\text{Man}_7\text{GlcNAc}_2$ oligosaccharide kindly supplied by Dr. S. Kornfeld (Chapman *et al.*, 1980). These oligosaccharides were also eluted from a Bio-Gel P-4 column in the same position. Almost all of the radioactivity was released from this oligosaccharide as free mannose on digestion with α -mannosidase. As shown in scans (d) and (e) of Fig. 4, an oligosaccharide with $R_{\text{stachyose}} = 0.7$ also accumulates when incubation mixtures are inhibited with EDTA (Chambers *et al.*, 1977) or with the antibiotic amphomycin (Kang *et al.*, 1978).

The effect of tsushimycin on glucose incorporation by the particulate enzyme

Fig. 1 showed that tsushimycin inhibits the transfer of glucose from $\text{UDP-}[^3\text{H}]\text{glucose}$ into Dol-P-Glc as well as the transfer of N -acetylglucosamine from $\text{UDP-}[^3\text{H}]\text{GlcNAc}$ into dolichyl pyrophosphate N -acetylglucosamine. Glucose incorporation into lipid was apparently more susceptible to antibiotic than was mannose or N -acetylglucosamine incorporation. However, even high concentrations of antibiotic could not completely inhibit glucose or N -acetylglucosamine incorporation, probably because more than one product is formed from these sugar nucleotides (see below).

A time course of glucose incorporation into the lipid-linked saccharides is shown in Figs. 5(a) and 5(b). Fig. 5(a) demonstrates the incorporation of glucose into Dol-P-Glc , and Fig. 5(b) shows the formation of glucose-labelled lipid-linked oligosaccharides. Both of these reactions are inhibited by tsushimycin, but the formation of Dol-P-Glc is somewhat more sensitive. Thus at 50 μg of antibiotic, Dol-P-Glc formation is inhibited more than 80%, whereas lipid-linked oligosaccharides is inhibited about 50%. The lipid-linked monosaccharides formed from $\text{UDP-}[^3\text{H}]\text{glucose}$ in the presence and absence of antibiotic were extracted with chloroform/methanol/water (1:1:1, by vol.) and were examined by t.l.c. in solvents B and C. Two radioactive bands were observed in control incubations; the major one representing 75–80% of the radioactivity migrated with Dol-P-Man in both solvents, whereas the other migrated more rapidly. Based on other studies (Kang *et al.*, 1978), the faster component is probably a glucosylceramide. In the presence of tsushimycin, the radioactivity in Dol-P-Glc decreased, whereas the other radioactive band remained fairly constant.

Fig. 5. Time course of incorporation of glucose from UDP-[^3H]glucose into Dol-P-Glc and lipid-linked oligosaccharides

(a) Shows the formation of Dol-P-Glc; (b) shows the formation of lipid-linked oligosaccharides. Incubation mixtures were as described in the text and contained the indicated amounts of antibiotic. At the times shown, Dol-P-Glc and lipid-linked oligosaccharides were isolated.

The glucose-labelled lipid-linked oligosaccharides were examined to see whether tsushimycin had affected the nature of the oligosaccharide products.

Fig. 6. Paper chromatography of the oligosaccharides formed from UDP-[^{14}C]glucose in the presence (b) or absence (a) of tsushimycin

Incubation mixtures were scaled up 10-fold and contained 500 μg of antibiotic. Oligosaccharides were run in solvent A and scanned for radioactivity. O, origin; S, stachyose; M, mannose.

Thus the oligosaccharides were released by mild acid hydrolysis and separated by paper chromatography as shown in Fig. 6. In the control, the radioactivity was found in several large oligosaccharides, one of which remained at the origin and another of which migrated slower than stachyose. In the incubation containing tsushimycin, there was much less radioactivity in the lipid-linked oligosaccharides but this radioactivity appeared to be in the same products as in control incubations.

The effect of tsushimycin on the incorporation of N-acetylglucosamine by the particulate enzyme

A time course of N-acetylglucosamine incorporation into lipid is shown in Fig. 7. As seen in other cases, tsushimycin strongly inhibited the incorporation of N-acetylglucosamine by more than 60% at 50 μg /incubation. However, in this case, as with glucose, complete inhibition could not be observed even at high antibiotic concentrations. When the lipids were examined by t.l.c. two bands were observed. The major band migrated with dolichyl pyrophosphate N-acetylglucosamine and accounted for 75% of the activity, whereas the second band migrated more slowly and is probably dolichyl pyrophosphate NN'-diacetylchitobiose. In the presence of increasing amounts of antibiotic, the radioactivity in the fastest moving band decreased most rapidly, whereas that in the slower band also declined but not as markedly.

Binding of tsushimycin to the particulate enzyme

To determine whether the antibiotic bound to the particulate enzyme, particles were mixed with several concentrations of antibiotic and allowed to

stand in ice for 10 min. At the end of this time, 10 vol. of Tris buffer were added to dilute the antibiotic and the particles were re-isolated by centrifugation. The particles were then resuspended and assayed for activity with either UDP-[3 H]-GlcNAc or UDP-[3 H]glucose. Table 1 shows the

Fig. 7. Effect of tsushimycin on the incorporation of *N*-acetylglucosamine into lipid by particulate enzyme. Incubation mixtures containing UDP-[3 H]GlcNAc were as described in the text. At the times indicated, the lipids were extracted by the addition of chloroform/methanol (1:1, v/v) to give a chloroform/methanol/water mixture of 1:1:1 (by vol.). The chloroform-soluble lipids were removed and their radioactive content was determined.

Table 1. Binding of tsushimycin to the particulate enzyme

Particulate enzyme (400 μ l) was incubated for 10 min in ice with 250 μ g or 1 mg of tsushimycin. Tris buffer (5 ml) was added and, after thorough mixing, the incubations were centrifuged at 10 000 *g* for 45 min. The pellets were resuspended in 0.5 ml of buffer and 100 μ l portions were taken for assay. Controls were incubated and centrifuged in the absence of antibiotic.

Amount of tsushimycin added	Radioactivity incorporated (c.p.m.) into:	
	Dolichyl pyrophosphate <i>N</i> -acetylglucosamine	Dol- <i>P</i> -Glc
0	2311	1433
250 μ g	1706	961
1 mg	806	613

results of this experiment. The particles incubated with various concentrations of antibiotic, were considerably less active after centrifugation than control particles incubated and centrifuged in the absence of antibiotic. Thus, the tsushimycin apparently binds to the transferases and inactivates them.

The effect of tsushimycin on the soluble enzyme

Effect on mannose, glucose and *N*-acetylglucosamine incorporation. The incorporation of sugars from their sugar nucleotides into the lipid-linked monosaccharides was studied with the soluble aorta enzyme. Fig. 8 shows the incorporation of mannose into Dol-*P*-Man as a function of time in the presence of several concentrations of antibiotic. Somewhat lower concentrations of tsushimycin were required with the soluble enzyme so that greater than 60% inhibition was observed at 10 μ g of antibiotic. Similar types of curves were seen with respect to glucose and *N*-acetylglucosamine incorporation (results not shown). Thus the control incubations showed a linear increase with time for about 5 or 10 min before levelling off, but there was considerable inhibition with 10–50 μ g of antibiotic.

Concentration of dolichyl phosphate and Nonidet P40 on mannose incorporation. The effect of increasing concentrations of dolichyl phosphate on

Fig. 8. Time course of mannose incorporation into Dol-*P*-Man by the solubilized enzyme fraction. Incubations and assays were as described in the text and in other Figures. The amount of antibiotic added is indicated in the Figure.

the incorporation of mannose into Dol-*P*-Man by the soluble enzyme is shown in Fig. 9. The optimum concentration of dolichyl phosphate was about 5–6 μg /incubation (i.e. 10 μg /ml). In the presence of tsushimycin, there was inhibition observed in the presence and absence of dolichyl phosphate and this inhibition was not overcome even at 25 μg /incubation.

Fig. 9. Effect of dolichyl phosphate concentration on mannose incorporation in the presence and absence of tsushimycin

Incubations were as described, except that the concentration of dolichyl phosphate was varied as indicated (10 μl of dolichyl phosphate) contains 2.5 μg of phosphate and solubilized enzyme was used. The amount of tsushimycin in each incubation is shown. After incubation for 10 min, Dol-*P*-Man was isolated.

Fig. 10. Effect of Nonidet P-40 concentration on the tsushimycin inhibition of Dol-*P*-Man

Incubation mixtures with the soluble enzyme were as described, except that the amount of Nonidet P40 (NP-40) was varied. Incubations contained the indicated amounts of antibiotic. After an incubation of 10 min, Dol-*P*-Man was isolated and its radioactivity was measured.

Since these reactions with soluble enzyme require detergent to suspend the dolichyl phosphate, the effect of Nonidet P40 concentration on mannose activity was examined as shown in Fig. 10. In the control incubation (0.1% Nonidet P40) incorporation of mannose decreased with increasing amounts of tsushimycin, being decreased by 50% at about 25 μg of antibiotic/incubation mixture. As the amount of Nonidet P40 in the incubation was increased, incorporation of mannose into Dol-*P*-Man decreased even in the absence of tsushimycin. But as the antibiotic concentration increased there was a progressive loss of activity and in each case (i.e. various Nonidet P40 concentrations) the curves had the same shape as the control. Thus detergent does not appear to affect the tsushimycin inhibition.

Discussion

The results show that tsushimycin inhibits the glycosyltransferases that are involved in the formation of Dol-*P*-Man, Dol-*P*-Glc and dolichyl pyrophosphate *N*-acetylglucosamine. Although the formation of lipid-linked oligosaccharides labelled with either glucose or mannose is also inhibited by tsushimycin, the inhibition in these cases is much less than that of monosaccharide-lipids. Whether the inhibition of lipid-linked oligosaccharides is due to a direct effect of tsushimycin on the enzymes of lipid-linked oligosaccharide synthesis, or is due to a lack of the substrates, Dol-*P*-Man and Dol-*P*-Glc, is not clear at present. However, in the presence of tsushimycin concentrations that block Dol-*P*-Man formation by greater than 90%, the lipid-linked oligosaccharides are composed of a single radioactive oligosaccharide that appears to be the heptasaccharide, $\text{Man}_7\text{GlcNAc}_2$. Thus these studies indicate that the first five mannose residues in this heptasaccharide come directly from GDP-mannose, whereas the next four mannoses may come from Dol-*P*-Man.

The initial evidence to suggest that some of the mannose residues in the lipid-linked oligosaccharides came directly from GDP-mannose was the observation that in the presence of EDTA the formation of Dol-*P*-Man was almost completely inhibited, but mannose was still incorporated into lipid-linked oligosaccharides (Chambers *et al.*, 1977). Later studies showed that in the presence of amphotycin, little Dol-*P*-Man is formed but lipid-linked oligosaccharides still become labelled with mannose. Whereas normal incubations form large oligosaccharides of nine to twelve sugars, the oligosaccharide produced in the presence of amphotycin appears to be $\text{Man}_5\text{GlcNAc}_2$ (Kang *et al.*, 1978). The hypothesis that the first five mannoses come directly from GDP-mannose is supported by studies of Schutzbach *et al.* (1980) with a

solubilized enzyme that adds the two terminal ($\alpha 1 \rightarrow 2$)-mannose residues and by studies by Chapman *et al.* (1980), who found a mutant mouse lymphoma (class E thy-1 negative) cell line that cannot synthesize Dol-*P*-Man. This mutant accumulated the Man₅GlcNAc₂-lipid intermediate, but is not able to synthesize the Glc₃Man₅GlcNAc₂-lipid unless supplied with exogenous Dol-*P*-Man.

In terms of glucose incorporation into the lipid-linked oligosaccharides, recent studies have indicated that Dol-*P*-Glc is the glucosyl donor (Murphy & Spiro, 1980; Staneloni *et al.*, 1980). Tsushimycin did decrease the radioactivity into both Dol-*P*-Glc and lipid-linked oligosaccharides, but it did not appear to affect the nature of the radioactive products in either case. The problem *in vitro* is that glucose incorporation into lipid-linked monosaccharides cannot be completely inhibited by tsushimycin so that it is still possible that enough Dol-*P*-Glc is formed to give rise to normal lipid-linked oligosaccharide. These results may also imply that (a) there is more normal oligosaccharide acceptor than Man₅GlcNAc₂ acceptor for glycosylation, or (b) that the glucosyltransferase is specific for the longer normal acceptor or (c) that the shorter oligosaccharide acceptor is glycosylated to a greater extent than the longer acceptor.

Tsushimycin also inhibited the formation of lipid-linked monosaccharides with the solubilized enzyme preparation. In this case less antibiotic was required for the same degree of inhibition, probably because the particulate enzyme contains more protein and possibly other components that may bind antibiotic. That tsushimycin does bind to protein, or at least to the glucosyltransferases, was shown by incubating particulate enzyme and antibiotic and then re-isolating particles by centrifugation. The re-isolated particles still showed inhibition of lipid-linked saccharide formation, suggesting that tsushimycin was still bound to these particles. With the solubilized mannosyltransferase, increasing the concentration of dolichyl phosphate had no effect on the tsushimycin inhibition, nor did varying the concentration of detergent. The mechanism of action of this antibiotic is not known, but apparently polypeptide antibiotics, such as

tsushimycin and amphomycin, bind to the transferases and inhibit them.

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