Purification and Identification of dTDP-oleandrose, the Precursor of the Oleandrose Units of the Avermectins*

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The nucleotide sugar precursor of the oleandrose units of the avermectins has been purified from a mutant of Streptomyces avermitilis, which does not synthesize any avermectins but which converts avermectin aglycones to their respective disaccharides. This precursor has been identified as dTDP-oleandrose. The purification was achieved by anion exchange and reverse phase high performance liquid chromatography. The purified nucleotide sugar had an absorption spectrum characteristic of thymidine, released dTMP when treated with phosphodiesterase, and possessed an NMR spectrum in which three resonances characteristic of oleandrose were seen in addition to the thymidine signals.

The enzyme, avermectin aglycone dTDP-oleandrose glycosyltransferase, which catalyzes the stepwise addition of oleandrose to the avermectin aglycones, has been demonstrated in cell-free extracts and (NH₄)_2SO₄ fractions of cell-free extracts of S. avermitilis. The enzyme is specific for dTDP-oleandrose as the glycosyl donor but utilizes all avermectin aglycones as glycosyl acceptors. The stoichiometry between dTDP-oleandrose and the avermectin aglycones was found to be 1:1.

The avermectins are a group of derivatives of 16-membered pentacyclic lactones with potent anthelmintic and insecticidal activity produced by Streptomyces avermitilis (1–4). Fig. 1 presents a generalized structure of the avermectins. All of the avermectins produced by wild type cultures possess a disaccharide composed of a(1-4)-linked L-oleandrose (2,6-dideoxy-L-arabinohexose) units attached to the macrolide ring at C13. It is believed that the oleandrose units are derived directly from glucose. Studies in which glucose labeled with 1H/14C at C6 (5), with 13C at C1, or with 13C uniformly (6) demonstrated that the oleandrose units are derived directly from glucose.

The formation of 6-deoxy sugars from their nucleotide sugar precursors has been extensively studied (7–12), and the dTDP-glucose-4,6-dehydratase from Escherichia coli has been purified and characterized (13). The enzymes involved in the formation of 3,6-dideoxy sugars (14–18) and 4-acetamido-4,6-dideoxyhexoses (19–21) have also been investigated by a variety of workers. In contrast, very little work has been done on the formation of 2,6-dideoxy sugars which are commonly found in macrolide antibiotics. Pape and Brillinger (22) found dTDP-glucose-4,6-dehydratase in Streptomyces rimosus, which produces the antibiotic tylosin, and described the in vitro formation of a nucleotide sugar which on hydrolysis yielded a sugar with an R₈ similar to that of mycarose. Recently, Vara and Hutchinson (23) reported the presence of dTDP-glucose-4,6-dehydratase in an erythromycin-producing strain of Saccharopolyspora erythraea. Differences in the biosynthesis of 2,6-dideoxy sugars among the antibiotic-producing bacteria have, however, been observed. In avermectin biosynthesis, methylation of the sugars occurs after their attachment to the macrolide ring (24), while in tylosin and erythromycin biosynthesis, methylation of the sugars occurs after their attachment to the ring and is a terminal step (25–27).

In the course of our studies on avermectin biosynthesis, we discovered that a mutant of S. avermitilis (MA90/8) which does not produce any avermectins, was capable of converting avermectin aglycones to their respective disaccharides. Cell-free extracts of this organism also catalyzed these reactions indicating that both the glycosyltransferase(s) and their substrate, presumably a nucleotide sugar, were present in these extracts.

In this paper, we report the isolation and identification of dTDP-oleandrose as the direct precursor of the oleandrose units of the avermectins and demonstrate the stepwise glycosylation of avermectin aglycone to the mono- and disaccharide by the enzyme avermectin aglycone glycosyltransferase in cell-free extracts of S. avermitilis.

EXPERIMENTAL PROCEDURES

Materials

Purified unlabeled avermectins and [1,2,5,6,9,10,15,16,17,18,19,20,21,22,23]C-avermectins (prepared by feeding uniformly labeled acetate to S. avermitilis), free oleandrose, and oleandrose methyl glycoside, each greater than 95%, were from Merck & Co. ADP-glucose, ADP-ribose, ADP-mannose, CDP-glucose, GDP-fucose, GDP-glucose, GDP-mannose, dTDP-glucose, UDP-glucose, UDP-galactose, UDP-mannose, UDP-xylene, UDP-glucuronic acid, UDP-galacturonic acid, and UDP-N-acetylglucosamine were from Sigma; Dowex 50W-X4 (200–400 mesh) and Chelex 100 (100–200 mesh) were from Bio-Rad Laboratories. Preparative (50 × 0.8 cm) and analytical (30 × 0.4 cm) AX-10 HPLC1 anion exchange columns were from Varian

1 The abbreviations used are: HPLC, high performance liquid chromatography; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid.
Oleandrose Disaccharide
Avermectin Aglycone

![Diagram of avermectins](https://example.com/avermectins.png)

**Fig. 1. General structure of the avermectins.** Avermectin terminology is as follows: $R_1$ = H cell component; $R_2$ = CH$_3$ in A component; $x$ = CH in 1 component; $y$ = CH CHO in 2 component; $R_3$ = CH$_2$CH$_3$ in a component; $R_4$ = CH$_3$ in b component.

**Methods**

**Avermectin Aglycone**

dTDP-oleandrose Glycosyltransferase Preparation—All operations were carried out at 5°C unless otherwise indicated. Cells of S. avermitilis (MA6078 or strain D) at 168 h were harvested by centrifugation at 1,000 x g for 10 min, washed twice in cold distilled water, and once in 50 mM Tris hydrochloride (pH 7.3) containing 1 mM diethytoletol, 1 mM ortho-phenantraline, and 0.1 mM phenylmethyl sulfonyl fluoride. The mycelial pellet was weighed, suspended in the above buffer (1 g of cells/5 ml), and ruptured by two passes through a French pressure cell at 12,000 p.s.i. The homogenate from the French pressure cell was centrifuged at 102,000 x g for 20 min at 0°C and the supernatant was adjusted to 60% saturation with (NH$_4$)$_2$SO$_4$ at 0°C, stirred for 30 min, and centrifuged at 27,000 x g. The pellet was weighed, suspended in the above buffer (1 g of cells/5 ml), and centrifuged at 27,000 x g for 1 h in a Beckman L8-80 centrifuge. The supernatant was dialyzed (M, cutoff = 3,500) against 15 volumes of cold distilled water and once in 50 mM Tris hydrochloride (pH 7.3) at 5°C.

**Avermectin Disaccharides**

The resulting homogenate was centrifuged at 102,000 x g for 1 h, and the supernatant was dialyzed (M, cutoff = 3,500) against 15 volumes of cold distilled water to separate the nucleosides from the cellular proteins and nucleic acids. The dialysate containing the small molecules was lyophilized and the dry powder, designated the small molecule fraction, was stored under vacuum in a desiccator over anhydrous CaCl$_2$ at 5°C.

**Preparation of Crude dTDP-oleandrose**—Cells of MA6078 were grown and harvested at 168 h as described above. The cells were washed twice in distilled water and once in 30 mM Tris hydrochloride (pH 7.3) containing 1 mM cytoxine and 1 mM EDTA. The mycelial pellet was weighed, suspended in the above buffer (1 g of cells/5 ml), and centrifuged at 27,000 x g for 1 h, and the supernatant was dialyzed (M, cutoff = 3,500) against 15 volumes of cold distilled water to separate the nucleosides from the cellular proteins and nucleic acids. The dialysate containing the small molecules was lyophilized and the dry powder, designated the small molecule fraction, was stored under vacuum in a desiccator over anhydrous CaCl$_2$ at 5°C.

**dTDP-oleandrose Assay**—The reaction mixture (0.5 ml) contained the following: 50 mM Tris hydrochloride (pH 7.0); 3.4 mM [¹⁴C] avermectin A₄ aglycone (specific activity = 6.7 x 10⁶ dpm/mmol); 0.2-1.0 µM dTDP-oleandrose, either as the small molecule preparation (20-40 A at 265 nm) or the purified compound (0.002-0.005 A at 265 nm); and the (NH$_4$)$_2$SO$_4$ fraction (2-5 mg of protein) from MA6076 or strain D as enzyme.

The [¹⁴C] avermectin A₄ aglycone solution was prepared by suspending 1 mg of [¹⁴C] avermectin A₄ aglycone in 50 mM Tris hydrochloride (pH 7.0) containing 0.1% CHAPS and stirring the suspension vigorously at room temperature for 4 to 8 h. The solids were removed by centrifugation at 192,000 x g for 1 h at 5°C in a Beckman L8-80 centrifuge. The concentration of [¹⁴C]A₄ aglycone in the clarified supernatant was determined as described below.

**RESULTS**

**dTDP-oleandrose Assay**—This assay was developed to allow detection and quantification of the substrate for avermectin glycosylation during its isolation. Fig. 2 presents a biosynthetic trace of a typical assay for dTDP-oleandrose. At zero time (Fig. 2A) and in control extracts which did not contain either the small molecule preparation or purified dTDP-oleandrose, A₄ aglycone (peak 1) is the only radioactive component present; at 10 min (Fig. 2B), small amounts of A₄ mono- (peak 2) and disaccharide (peak 3) are formed; at 20 min (Fig. 2C), in-
dTDP-oleandrose, Precursor of Avermectin Disaccharides

Increased amounts of disaccharide are formed and there is a concomitant decrease in the amount of aglycone; at 30 min (Fig. 2D), the disaccharide is the major component and only small amounts of the aglycone and monosaccharide remain. At longer incubation times (not shown), conversion to the disaccharide is complete. Under the conditions used, the substrate rather than the enzyme is rate-limiting. Virtually identical time courses for the reaction were obtained with purified dTDP-oleandrose (data not shown). These results demonstrate that the precursor of the oleandrose units of avermectin is present in the small molecule preparation, that the 30-60% (NH₄)₂SO₄ fraction contains the enzyme(s) which glycosylate the avermectin aglycone, and that the oleandrose units are added stepwise to form a monosaccharide and then a disaccharide.

The specificity of the glycosylation was investigated using other avermectin aglycones as glycosyl acceptors and all commercially available nucleotide sugars as glycosyl donors. All of the avermectin aglycones tested, B,a, B,a, A,a, A,a, and 22, 23-dihydro B,a (at 3.4 to 5 nM) were active substrates. There was no activity however with any of the nucleotide sugars (at 0.1 to 5.0 mM in 1-h incubations) which included: ADP-glucose, ADP-ribose, ADP-mannose, CDP-glucose, GDP-fucose, GDP-glucose, GDP-mannose, dTDP-glucose, UDP-glucose, UDP-galactose, UDP-mannose, UDP-xylene, UDP-glucuronic acid, UDP-galacturonic acid, and UDP-N-acetylgalcosamine.

Purification of the substrate for avermectin glycosylation was undertaken using this assay to locate and quantify the nucleotide sugar. The sensitivity of the assay is dependent upon the specific activity of the radiolabeled avermectin aglycone. Using the [³⁵S]A,a aglycone described above, 0.25 pmol of precursor could be detected.

Isolation of dTDP-oleandrose—dTDP-oleandrose was isolated from S. avermitilis MA6078, a mutant which does not produce any avermectins. Fig. 3 outlines the isolation procedure. Portions of the small molecule preparation were dissolved in cold water to yield a solution with an absorbance at 260 nm of approximately 300 A/mg (1 cm light path). One-ml aliquots of this solution were chromatographed via HPLC on a preparative AX-10 ion exchange column using a gradient of KH₂PO₄ (pH 4.5) from 50 to 400 mM at 4 ml/min for 40 min. The column was held at 400 mM KH₂PO₄ for 15 min prior to re-equilibration with 50 mM KH₂PO₄. The absorbance of the eluant was monitored at 260 nm. Fig. 4 presents a typical elution profile of this column. All of the fractions were assayed for dTDP-oleandrose. Only one active peak with a retention time of 21.15 min was found. The inset in Fig. 4 is an enlargement of this region of the chromatograph. This peak represented approximately 0.2% of the absorbance applied to the column.

The active fractions from 30 successive AX-10 columns were combined, lyophilized, dissolved in 1 ml of cold water, and applied to a preparative PRP-1 reverse phase column using tetrabutylammonium phosphate as an ion pair (29). The column was equilibrated with solution A (30 mM KH₂PO₄, 5 mM tetrabutylammonium phosphate, 4% aceto-
Fig. 4. Preparative HPLC of dTDP-oleandrose on AX-10 anion exchange column. One ml containing 300 A units at 260 nm was chromatographed as described under "Isolation of dTDP-oleandrose." The left abscissa is the percent 0.4 M KH$_2$PO$_4$ in the eluant; the starting buffer is 0.05 M KH$_2$PO$_4$. A linear gradient from 0-100% 0.4 M KH$_2$PO$_4$ was used for 40 min, and the eluant was then held at 100% for 15 min. The right-hand abscissa is a trace of the absorbance in the eluant at 260 nm. The inset is an enlargement of the region of interest. The arrow indicates the peak containing dTDP-oleandrose.

Fig. 5. Preparative HPLC of dTDP-oleandrose on PRP-1 reverse phase column. Pooled fractions from the AX-10 column were concentrated and chromatographed on a PRP-1 column as described under "Isolation of dTDP-oleandrose." dTDP-oleandrose was the major peak which eluted at 38.4 min.

The isolation of dTDP-oleandrose is very laborious and the yield is low. Approximately 140 µg (0.25 nmol) were obtained from 500 g of cells. dTDP-oleandrose is very labile in both acid and alkalai. Below pH 4, all activity was lost in 10 min while above pH 9, activity was lost in 30 min. This instability prevented the use of acid precipitation of proteins or adsorption of the nucleotide sugar on charcoal during the purification. In the purification procedure described, the active fractions from the AX-10 column did not need to be desalted before chromatography on PRP-1. The dTDP-oleandrose eluted from the reverse phase column is essentially pure, i.e. it is the only nucleotide present. It was however necessary to remove the tetrabutylammonium ions and paramagnetic ions to allow analysis of the sample by NMR spectroscopy. The remaining steps were carried out to remove these ions. Dowex-50 NH$_4^+$ was used to exchange NH$_3^+$ for the tetrabutylammonium ion, and Chelex 100 was used to remove metals. Dowex 50 NH$_4^+$ could not be used because of the instability of dTDP-oleandrose to acid. The failure of Dowex 50 NH$_4^+$ to remove the metals suggests they were tightly bound to the dTDP-oleandrose. The purified dTDP-oleandrose from the Chelex column still contained high levels of (NH$_4$)$_2$PO$_4$ and was not stable. It decomposed at a rate of approximately 20%/week when stored at -20 °C either as a lyophilized powder or in solution at pH 7.0.

Characteristics of dTDP-oleandrose—The uv absorption spectrum of the purified dTDP-oleandrose at pH 7.0 had an $E_{265}$ at 265 nm which is characteristic of thymidine. A sample of the purified compound was treated with snake venom phosphodiesterase, and the products were identified by HPLC chromatography (Fig. 6). Exposure to phosphodiesterase yielded a new peak (Fig. 6B) with a retention time (9.7 min) identical with that of dTMP (Fig. 6C) which was not present in the untreated sample (Fig. 6A). This peak was resolved.
from that of the AMP, UMP, GMP, and CMP standards.

NMR Analysis of dTDP-oleandrose—The NMR spectrum of the purified nucleotide sugar (Fig. 7) supports its identification as dTDP-oleandrose (Fig. 8). This is based on the similarities of the spectrum with those of dTDP-glucose, oleandrose methyl glycoside, and free oleandrose. In addition to the recognizable thymidine signals, the spectrum shows three resonances that are characteristic of oleandrose. The methyl doublet at 1.32 ppm (5'-CH3) and the methoxyl peak at 3.43 ppm (3'-OCH3) are crucial. The multiplet at 2.22 ppm corresponds to one of the 2-deoxymethylene protons.

Stoichiometry of dTDP-oleandrose and Avermectin Glycosylation—Table I presents the stoichiometry between purified dTDP-oleandrose and the formation of avermectin monosaccharide catalyzed by the (NH4)2SO4 fraction containing dTDP-oleandrose avermectin aglycone glycosyltransferase. In these reactions, the concentration of [14C]avermectin Aα aglycone was 20 μM. All of the added dTDP-oleandrose was consumed in both reactions. The relative amounts of mono- and disaccharide formed varied with the amount of dTDP-oleandrose added. At the lower level, the ratio of monosaccharide to disaccharide was 2:1, while at the higher level, the ratio was reduced to 1.3:1. Since there are 2 oleandrose units in the disaccharide and 1 in the monosaccharide, this represents an equal distribution of oleandrose between the mono- and disaccharide at the lower level and a 67–33% distribution in favor of the disaccharide at the higher level.

DISCUSSION

It was previously shown that attachment of the oleandrose units to the aglycone are the terminal steps in avermectin biosynthesis (24). Avermectin biosynthesis thus differs from that of tylosin and erythromycin. With these macrolides, demethyl sugars are attached to the macrolide ring and methylation of these sugars are the terminal steps (25–27). During our continuing investigation of the biosynthesis of the avermectins, we discovered that a nonproducing mutant of S. avermitilis (MA6078) was capable of converting avermectin aglycones in vivo to their respective disaccharides. Further investigation revealed that these glycosylations were catalyzed by cell-free extracts of this organism. This finding indicated that both the glycosyltransferase and its substrate (dTDP-oleandrose), the direct precursor of the oleandrose units in the product, were present. Efforts to purify and identify the substrate and to isolate the glycosyltransferase were consequently undertaken.

An assay to detect the precursor of the oleandrose units of the avermectins was developed using a 30–60% (NH4)2SO4 fraction as a source of glycosyltransferase. Enzyme from S. avermitilis mutant MA6078 was used initially but it had a significant background in the absence of added substrate due to the presence of endogenous dTDP-oleandrose in the preparation. To circumvent this problem, enzyme from high producing strain D (28), which does not accumulate precursor, was subsequently used. The reaction products are not only dependent on time as shown in Fig. 2 but also on the substrate concentration. At very low levels of dTDP-oleandrose (<0.1 nmol), the monosaccharide is the only product formed (data not shown). This is presumed to be due to the fact that both the monosaccharide formed and the dTDP-oleandrose remaining are far below their respective Kₘ values. Addition of more dTDP-oleandrose allows conversion of the monoglycosyl avermectin to the disaccharide. It was not possible to do any kinetic studies or characterization of the glycosyltransferase(s) because of the limited quantity of dTDP-oleandrose available. At 10 μM dTDP-oleandrose, the highest concentration tested (Aα aglycone was at 50 μM), the reaction was still not linear with either time or enzyme concentration. These studies clearly require much larger quantities of dTDP-oleandrose. Since the isolation procedure is so laborious, we have undertaken a chemical synthesis of dTDP-oleandrose. Purification and characterization of the enzyme will proceed as

![FIG. 7. NMR spectrum of dTDP-oleandrose. A purified sample of dTDP-oleandrose (∼0.5 μmol) was dissolved in D2O or CDC13 and analyzed on Varian XL-400 spectrometer.](http://www.jbc.org/figs/fig7.jpg)

![FIG. 8. Structure of dTDP-oleandrose.](http://www.jbc.org/figs/fig8.jpg)

<table>
<thead>
<tr>
<th>TDP-oleandrose added*</th>
<th>Aα</th>
<th>Aα MS</th>
<th>Total Oleandrose</th>
<th>Transferred %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles</td>
<td>nmoles</td>
<td>Formed</td>
<td>Oleandrose</td>
</tr>
<tr>
<td>1.11</td>
<td>0.31</td>
<td>0.62</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>2.22</td>
<td>0.71</td>
<td>1.42</td>
<td>0.91</td>
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</table>

* Calculated using the E₅₅₀ of dTMP of 9.6 × 10⁹.
* Calculated on the basis of ¹⁴C present and the specific radioactivity of the [¹⁴C]Aα aglycone.
reduced avermectin aglycones are readily converted to the version studies have shown that desfurano and chemically avermectin aglycones tested. This was expected since biocon- soon as sufficient dTDP-oleandrose is available.

terase for the nucleotide sugar donor appears to be more disaccharide in vivo (30). The specificity of the glycosyltrans- rigorous; activity has not been observed with any of the readily

termination as dTDP-oleandrose. Its uv absorption spec-

trum had an $E_{max}$ characteristic of thymidine and treatment

was present in the molecule. The NMR studies conducted in

high salt showed three signals critical to establishing the

relationship. As discussed above, at low concentrations of

the presence of oleandrose: the C5" methyl, the C3" methoxyl,

however, not yet been tested.

The properties of the purified nucleotide sugar support its

determination as dTDP-oleandrose. Transfer of the oleandrose is stepwise

to be dTDP-oleandrose. Transfer of the oleandrose is stepwise

been overlaid by the solvent HDO and by the glycerol contam-

and the C2" methylene. (The inability to see the oleandrose

signal would have been expected since these signals would have

the solvent HDO and by the glycerol contaminant.) The determined stoichiometry between added dTDP-

more monosaccharide than disaccharide was formed while at higher concentrations more disaccharide

glycosyltransferase (Fig. 9). It is not yet known whether

one or two enzymes catalyze the addition of the oleandrose units.

REFERENCES


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