The Pyrrolidine Alkaloid, 2,5-Dihydroxymethyl-3,4-dihydroxy-3-pyrrolidine, Inhibits Glycoprotein Processing*

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2,5-Dihydroxymethyl-3,4-dihydroxy-3-pyrrolidine (DMDP) is a pyrrolidine alkaloid that was isolated from the plant, Lonchocarpus sericeus. In the present study, DMDP was tested as an inhibitor of glycoprotein processing. MDCK cells were infected with influenza virus and the virus was raised in the presence of various amounts of DMDP. The glycopeptides were labeled by the addition of [2-'H]mannose or [1-'H]galactose to the medium. The virus was isolated by differential centrifugation and treated with Protase to obtain glycopeptides. These glycopeptides were isolated by chromatography on Bio-Gel P-4, then digested with endoglucoasaminidase H (Endo H) and rechromatographed on the Bio-Gel P-4 column. In the control virus, more than 70% of the glycopeptides were resistant to Endo H and were previously characterized as complex types of oligosaccharides. The remaining 20-25% are sensitive to Endo H and are high mannose type. However, in the presence of DMDP (250 μg/ml), more than 80% of the glycopeptides are susceptible to digestion by Endo H. The oligosaccharide released by this treatment sized like a hexose, 1,1,1-GlcNAc on a calibrated column of Bio-Gel P-4, and was only slightly susceptible to α-mannosidase treatment. This oligosaccharide was also labeled in the glucose moieties by growing the virus in [1-'H]galactose in the presence of DMDP. Following isolation, the oligosaccharide was subjected to complete methylation. Acid hydrolysis of the methylated oligosaccharide gave three methylated glucose derivatives, corresponding to 2,3,4,6-tetra- and 2,3,4-tri- methylglucose, and 2,4,6-trimethylglucose in almost equal amounts. These data indicate that the oligosaccharide is a GlcαMan2,GlcNAc and that DMDP inhibits glucosidase I. Similar results were obtained with the cellular glycopeptides. DMDP did not inhibit the incorporation of [3-'H]leucine into protein in MDCK cells, nor did it inhibit virus production as measured by plaque counts or hemagglutination assays. DMDP did cause some inhibition of mannose incorporation into the lipid-linked monosaccharides, but incorporation into lipid-linked oligosaccharides was not greatly affected, and incorporation into protein was stimulated. These results suggest that the pyrrolidine alkaloids are a new class of processing inhibitors.

The biosynthesis of the complex types of N-linked oligosaccharides involves a series of processing reactions whereby sugars are trimmed from the high-mannose types of structures, and other sugars are added to the oligosaccharide chains. Thus, the initial oligosaccharide that is transferred to protein is a GlcαManαGlcNAc, and this structure is rapidly trimmed by the removal of all three glucose residues by at least two membrane-bound glucosidases, called glucosidase I and glucosidase II (2-6). Following the removal of glucose, mannosidase I may remove all four of the α1,2-linked mannoses to give the Manα1,3(Manα1,5)Manα1,6(Manα1,3)-ManβGlcNAc2-protein (7-10). A GlcNAc is then added to the mannose that is linked α1,3 to the β-linked mannose (11, 12), and this GlcNAc appears to be the signal for mannosidase II to remove the α1,3- and α1,6-linked mannosides (13, 14). These reactions result in the formation of a GlcNAc-Manα1,3(Manα1,5)ManβGlcNAc2-protein, which can be further elongated to complex structures by the addition of GlcNAc, galactose, sialic acid, and fucose (15).

One mechanism for studying the biosynthesis and function of the N-linked complex chains is with the use of inhibitors that block specific steps in the processing pathway. A number of such inhibitors are now known and these have proven to be valuable tools in a variety of studies (16, 17). In this report, we describe some studies with 2,5-dihydroxymethyl-3,4-dihydroxy-3-pyrrolidine, an analog of β-D-fructofuranose, that demonstrate that this compound (DMDP) is an inhibitor of glycoprotein processing. DMDP was first isolated from the leaves of Derris elliptica (18). In the present study, DMDP was isolated from the seeds of the closely related plant, Lonchocarpus sericeus, and shown by NMR studies to be identical to the compound from Derris elliptica. In the studies described here, we tested DMDP as a processing inhibitor of the influenza virus hemagglutinin, and found that it prevented the formation of complex chains and gave rise to a new glycopeptide(s) having GlcαManαβGlcNAc structures. These results indicate that DMDP is an inhibitor of glucosidase I. Thus, DMDP represents one member of a new class of processing inhibitors, the pyrrolidine alkaloids. Such compounds may be valuable tools for functional and biosynthetic studies on N-linked glycoproteins. The structure of DMDP is shown in Structure 1.

* The abbreviations used are: DMDP, 2,5-dihydroxyethyl-3,4-dihydroxy-3-pyrrolidine; Endo H, endoglucosaminidase H.

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1 The abbreviations used are: DMDP, 2,5-dihydroxymethyl-3,4-dihydroxy-3-pyrrolidine; Endo H, endoglucosaminidase H.

2 Comparisons of the compound from the leaves of Derris elliptica (18) with that from Lonchocarpus sericeus by NMR analysis showed that they were identical and had the structure 2,5-dihydroxymethyl-3,4-dihydroxy-3-pyrrolidine. We thank Dr. G. W. Fleet, Oxford University for these analysis. This data will be published elsewhere.
MDCK cells were grown in modified Eagle's medium containing 10% fetal calf serum, and the confluent monolayers were infected with the NWS strain of influenza virus (19). After infection, the cells were placed in 2% minimal Eagle's medium, and various amounts of DMDP were added, from 1 μg/ml to 250 μg/ml. The cultures were allowed to incubate for 2 to 3 h to allow the inhibitor to take effect, and then either [2-3H]mannose or [1-3H]galactose was added to label the viral glycopeptides. In some experiments, the cultures were harvested after 3 h of incubation in the isotopes in order to determine the effect of DMDP on the formation of lipid-linked saccharide intermediates. These extractions, were digested with Pronase to obtain the cellular glycopeptides. In some experiments, the cells were harvested after 36 h and also contained 0.05 mM ZnCl₂. For the other digestions, the glycopeptide profiles from control and DMDP-treated (50 and 250 μg/ml) virus. It can be seen that there was a considerable alteration in the profiles obtained in the presence of DMDP especially at 250 μg/ml. However, these columns did not completely resolve the complex types of glycopeptides from the high-mannose (and hybrid) structures.

In order to completely separate the various types of glycopeptides, each glycopeptide peak (fractions 30–50) was pooled, concentrated to a small volume, and digested exhaustively with endoglucosaminidase H. These digests were rechromatographed on the Bio-Gel P-4 column as shown in Fig. 1. This treatment clearly resolved the control viral glycopeptide profiles from control and DMDP-treated (50 and 250 μg/ml) virus. It can be seen that there was a considerable alteration in the profiles obtained in the presence of DMDP especially at 250 μg/ml. However, these columns did not completely resolve the complex types of glycopeptides from the high-mannose (and hybrid) structures.

In order to determine the effects of DMDP as a glycoprotein processing inhibitor, MDCK cells were infected with the NWS strain of influenza virus, and various amounts of DMDP were added to the cultures. Following an incubation of 3 h to allow the inhibitor to act, [2-3H]mannose was added to label the viral glycoproteins. The cultures were allowed to incubate for about 40 h to produce mature virus, and the presence of viral particles in the medium was measured by hemagglutination assays. The virus was isolated from the medium by differential centrifugation, and the viral pellets were digested with Pronase to obtain the cellular glycoproteins.

However, in most experiments, the infected cells were allowed to incubate with the isotope and inhibitor for 48 h in order to produce mature virus. The presence of virus in the medium was determined by hemagglutination assays, and the virus was isolated by differential centrifugation. In these cases, the cell pellets were also examined for their glycoprotein content and structure.

Virus, labeled with either [2-3H]mannose or [1-3H]galactose, was digested exhaustively with Pronase to produce glycopeptides, and these glycopeptides were isolated initially on columns of Bio-Gel P-4 (1.5 x 150 cm; 200-400 mesh). Since these columns did not completely resolve the complex from the high-mannose structures, the entire glycopeptide peak from these initial runs was pooled, concentrated to a small volume, and digested with endoglucosaminidase H, as described previously (21). After digestion, the samples were rechromatographed on the Bio-Gel P-4 column. Aliquots of every other fraction were removed for the determination of radioactivity.

Susceptibility of the various oligosaccharides and glycopeptides to digestion by α-mannosidase, or to a combination of β-galactosidase and β-N-acetylgalactosaminidase, was also examined. These incubations were done in 50 mM acetate buffer, pH 5.0, under a toluene atmosphere. For α-mannosidase, 0.1 units of enzyme were added initially and again after 18 h. These incubations were done for a total of 36 h and also contained 0.05 mM ZnCl₂. For the other digestions, 50 milliunits of each enzyme were added initially and again at 18 h, for a total incubation of 36 h. Susceptibility of the various oligosaccharides to digestion was determined by chromatography of the digestion mixtures on columns of Bio-Gel P-4.

RESULTS AND DISCUSSION

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of the radioactivity was in the complex chains and about 25% in the high-mannose types. On the other hand, in the presence of DMDP at 250 µg/ml, more than 80% of the radioactive glycopeptides were susceptible to Endo H, and only a small peak of radioactivity remained in the original position (profile P). In addition, the new peak resulting from this digestion appeared to be larger in size than the high-mannose structures of the control virus, since it emerged earlier from the Bio-Gel columns. Although the resolution on this column was not sufficient for an accurate size determination, this oligosaccharide appeared to be a hexose<sub>3</sub>GlcNAc. Table I summarizes the percentage of the total radioactivity in the complex (Endo H-resistant) and high-mannose (Endo H-sensitive) structures at various concentrations of DMDP. It can be seen that the percentage of radioactivity in the complex chains continually decreased with increasing amounts of DMDP, with a corresponding increase in the percentage of [3H]mannose in the high-mannose types (Endo H-sensitive).

To determine the exact size of the DMDP-induced oligosaccharide, it was sized on a long, calibrated column of Bio-Gel P-4 as shown in Fig. 2A. On this column, the oligosaccharide migrated mostly near the hexose<sub>3</sub>GlcNAc standard, but there was some heterogeneity with larger (hexose<sub>5</sub>GlcNAc), and smaller (hexose<sub>2</sub>GlcNAc) oligosaccharides. This heterogeneity is not surprising since these studies were done in cell culture, and some processing of the oligosaccharides would be expected. The oligosaccharide peak from Fig. 2A was pooled (fractions 158-176), concentrated to a small volume, and treated exhaustively with α-mannosidase. These digestions were then chromatographed on the same Bio-Gel P-4 column as shown in Fig. 2B. It can be seen that the oligosaccharide was only partially susceptible to this enzyme and the major peak was shifted to the hexose<sub>2</sub>GlcNAc area. These results indicated removal of one or two mannose residues by the α-mannosidase treatment. On the other hand, the oligosaccharide was not susceptible to digestion by β-galactosidase or β-N-acetylhexosaminidase, either sequentially or in combination. These data suggested that the original oligosaccharide contained glucose residues that blocked or capped one or more of the mannose branches, making this structure only slightly susceptible to α-mannosidase digestion.

To demonstrate that this oligosaccharide did contain glucose and to determine the number of glucose residues present, infected MDCK cells were incubated with DMDP and labeled with [1-3H]glucose. Viral glycopeptides were isolated as described above, treated with Endo H, and rechromatographed on the Bio-Gel column. In the presence of DMDP, a radioactive oligosaccharide was obtained that migrated in the same position as the mannose-labeled oligosaccharide described above (data not shown). This oligosaccharide also migrated on the calibrated Bio-Gel column in the same position as the mannose-labeled oligosaccharide. The label was shown to be present in glucose by complete acid hydrolysis and paper chromatography. The number of glucose residues present in this oligosaccharide was determined by complete methylation and isolation of the methylated glucose derivatives by thin-layer chromatography. As shown in Fig. 3, three methylated glucose derivatives were obtained, corresponding to 2,3,4,6-tetramethylglucose, 3,4,6-trimethylglucose, and 2,4,6-trimethylglucose. These three peaks were present in the approximate ratio of 1:1:1, based on their radioactive content (actual ratio 1.00:0.75:0.86). Thus, the DMDP-induced oligosaccharide appears to contain three glucose residues and is a Glc<sub>3</sub>Man<sub>3</sub>GlcNAc. These data would be consistent with the inhibition of glucosidase I by this inhibitor. Preliminary experiments with a rat liver microsomal fraction incubated with [3H]glucose-labeled Glc<sub>3</sub>Man<sub>3</sub>GlcNAc indicated that DMDP did inhibit glucosidase I.

In order to determine whether DMDP had any effect on the formation of lipid-linked saccharide intermediates, infected MDCK cells were incubated in the presence of 10, 100, or 250 µg/ml of DMDP, and then labeled with either [2-3H]mannose or [1-3H]galactose. In one experiment, the cells were

**Table I**

| Amount of DMDP (µg/ml) | Complex chains (Endo H-resistant) | High-mannose types (Endo H-sensitive) |
|------------------------|----------------------------------|-------------------------------------|
| 0                      | 71                               | 29                                  |
| 5                      | 65                               | 35                                  |
| 50                     | 55                               | 45                                  |
| 250                    | 17                               | 83                                  |

*In control virus, 28,300 cpm were in the complex chains and 11,550 cpm in the high-mannose structures. In the presence of DMDP (250 µg/ml), 13,750 cpm were in complex chains and 66,330 in the high-mannose types.

![Fig. 2. Partial characterization of the mannose-labeled oligosaccharide formed in the presence of DMDP.](image-url)
The Endo H-released oligosaccharide from \(^{3}H\)galactose-labeled virus grown in DMDP was subjected to complete methylation (22). After hydrolysis, the methylated glucose derivatives were separated by thin-layer chromatography in benzene:acetone:water:ammonium hydroxide (50:200:3:1.5). Radioactive methylated sugars were detected by scraping the plates in 1-cm sections and counting each section in the scintillation counter. Standard methylated glucose derivatives are shown at the top and correspond to: 3,4,6, 3,4,6-trimethylglucose; 2,3,4,6, 2,3,4,6-trimethylglucose; and 2,3,4,6,2,3,4,6-tetramethylglucose.

**TABLE II**

| Isotope used | Amount of DMDP | Glyco-protein (cellular) | Glyco-protein (viral) |
|--------------|----------------|-------------------------|----------------------|
|              | \(\mu g/ml\)  | \(cpm\)                  | \(cpm\)               |
| Mannose, 3 h | 10             | 7,760                    | 60,720               |
|              | 1:1            | 7,670                    | 152,380              |
|              | 10:1:3         | 3,230                    | 155,190              |
|              | 250            | 2,615                    | 200,410              |
| Mannose, 48 h| 0              | 57,516                   | 960,013              |
|              | 10             | 24,505                   | 927,050              |
|              | 100            | 49,171                   | 1,288,590            |
|              | 250            | 38,025                   | 1,640,114            |
| Galactose, 48 h| 0     | 2,236,614                | 1,607,320            |
|              | 10             | 68,503                   | 1,599,690            |
|              | 100            | 47,705                   | 886,800              |
|              | 250            | 3,888,560                | 1,483,530            |
|              | 250            | 3,872,433                | 603,460              |

* Lipid-linked monosaccharides such as mannosyl-P-dolichol and glucosyl-P-dolichol are extracted by CHCl\(_3\)-CH\(_2\)OH-H\(_2\)O (1:1:1) which is indicated by the column 1:1. Lipid-linked oligosaccharides are extracted by CHCl\(_3\)-CH\(_2\)OH-H\(_2\)O (10:1:3).

The results reported here indicate that DMDP is an inhibitor of glycoprotein processing by virtue of the fact that it had little effect on the incorporation of mannose and galactose into the lipid-linked saccharides and glycoproteins. However, since the virus assays were only done at one time point, it is possible that this inhibitor might affect the rate of virus production.

The effect of DMDP on the incorporation of \(^{3}H\)leucine into protein in MDCK cells was examined to determine whether this compound affected protein synthesis. Confluent monolayers were preincubated with DMDP for several hours in 2% minimal Eagle's medium, and then \(^{3}H\)leucine was added to each culture. At the times shown in Fig. 4, the monolayers were washed well with saline, and the cells were removed from the dish by scraping. The cells were extracted with 5% trichloroacetic acid, and the trichloroacetic-acid-insoluble residue was suspended in Protosol and counted to determine its radioactive content. Fig. 4 shows that even at 250 \(\mu g/ml\) of DMDP, there was no change in the amount of radioactivity incorporated into protein over a 2-h incubation. In addition, we examined the yield of virus produced at various concentrations of DMDP, and the infectivity of these particles. Virus yields were determined by hemagglutination assays on the medium, and infectivity was measured by plaque counts. DMDP had no effect on either of these parameters (data not shown). Based on these studies, we assume that DMDP is not affecting protein synthesis or virus production. However, since the virus assays were only done at one time point, it is possible that this inhibitor might affect the rate of virus production.
Inhibits glucosidase I. Thus, the major oligosaccharide produced in the presence of inhibitor was susceptible to Endo H and migrated on calibrated Bio-Gel columns like a hexose$_7$GlcNAc. This oligosaccharide was only partially susceptible to α-mannosidase, but was resistant to both β-galactosidase and β-hexosaminidase, suggesting that it contained blocking glucose units. The oligosaccharide was synthesized in the presence of [H]$^1$galactose to label the glucose moieties, and this oligosaccharide was subjected to methylation analysis. Since three methylated glucose derivatives were obtained, corresponding to terminal, 3-linked, and 2-linked glucose, the oligosaccharide must contain three glucose and a Glc$_3$Man$_2$GlcNAc. Since DMDP did not affect protein synthesis and also had no effect on virus production or release, it should be a useful inhibitor for studies on glycoprotein biosynthesis and function. Perhaps the most interesting aspect of these studies is that DMDP represents a member of a new class of processing inhibitors, the pyrrolidine alkaloids. Based on studies with other processing inhibitors, it seems quite likely that other pyrrolidine alkaloids with different chirality will prove to be inhibitors of other glycosidases, and perhaps other processing enzymes.

There are a number of processing inhibitors that have now been described (16, 17). All of these compounds are glycosidase inhibitors and share several common features, i.e. 1) they all contain a nitrogen in the ring, and 2) they all contain three or more asymmetric hydroxyl groups. Thus, swainsonine (23) was the first processing inhibitor to be described (19), and this compound has shown to inhibit mannosidase II (24). In the presence of swainsonine, cells in culture produce hybrid types of oligosaccharides (25–29). Deoxynojirimycin (29–33) and castanospermine (21) inhibit glucosidase I and lead to the accumulation in cell culture of Glc$_3$Man$_7$GlcNAc$_3$ structures on the protein. Recently, the mannos analog of deoxynojirimycin (deoxymannojirimycin) was synthesized chemically (34), and shown to be an inhibitor of mannosidase I (35). This inhibitor causes the accumulation of Man$_6$GlcNAc$_2$ structures. The important point here is that all of the above inhibitors contain the nitrogen in a 6-membered ring. DMDP is the first 5-membered ring compound that has been found to be an inhibitor of glycoprotein processing. Such a structure may be of considerable importance in understanding the mechanism of glycosidase inhibition. In addition, 5-membered ring structures are likely to be easier to synthesize chemically and therefore it should be feasible to prepare various isomers as inhibitors of other glycosidases. In fact, another pyrrolidine alkaloid that inhibits α-mannosidase has been synthesized chemically.\(^3\)

**REFERENCES**

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\(^3\) G. W. Fleet, manuscript in preparation.