The Structures of Oligosaccharides Excreted by Sheep with Swainsonine Toxicosis*

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Christopher D. Warren‡, Peter F. Daniel§, Birgitte Bugge, James E. Evans§, Lynn F. James§, and Roger W. Jeanloz

From the Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114; ‡The Eunice Kennedy Shriver Center for Mental Retardation, Inc., Waltham, Massachusetts 02254, and §The Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, Utah 84321

Eleven oligosaccharides were purified from the urine of sheep with swainsonine toxicosis induced by the feeding of Astragalus lentiginosus. Oligosaccharides were extracted by charcoal adsorption, chromatographed on Bio-Gel P-2, and partially fractionated by preparative-layer chromatography. Separation into individual compounds was completed by semi-preparative high pressure liquid chromatography.

Structures were determined by a combination of high pressure liquid chromatography and exo- and endo-glycosidase action, methanalysis followed by gas-liquid chromatography, methylation analysis, and high resolution nuclear magnetic resonance spectroscopy. Two homologous series of oligosaccharides were identified: (a) α-D-Manp-(1→6)-β-D-Manp-(1→4)-D-GlcNAc, α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-β-D-Manp-(1→4)-D-GlcNAc, α-D-Manp-(1→2)-α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-β-D-Manp-(1→4)-D-GlcNAc, and α-D-Manp-(1→2)-α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-β-D-Manp-(1→4)-D-GlcNAc (minor series); (b) α-D-Manp-(1→6)-β-D-Manp-(1→4)-[α-D-Manp-(1→3)]-β-D-GlcNAc-(1→4)-D-GlcNAc, α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-β-D-Manp-(1→4)-D-GlcNAc, α-D-Manp-(1→6)-β-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc, α-D-Manp-(1→6)-α-D-Manp-(1→4)-β-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc, α-D-Manp-(1→3)-α-D-Manp-(1→6)-[α-D-Manp-(1→3)]-β-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc, α-D-Manp-(1→2)-α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-α-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc, α-D-Manp-(1→2)-α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-α-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc, and α-D-Manp-(1→3)-[α-D-Manp-(1→6)]-α-D-Manp-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc (major series).

The poisoning of livestock that ingest leguminous plants of the genera Astragalus or Oxytropis (locoweeds) in the United States, or Swainsona (Australia), is a major agricultural problem (1, 2). The principal symptoms of intoxication are neurological and resemble those of bovine α-mannosidosis (2, 3), which results from a genetic deficiency of lysosomal α-mannosidase activity. The toxic principle of locoweeds is swainsonine (together with the N-oxide) (4), a potent reversible inhibitor of certain α-mannosidase activities including the lysosomal enzyme. Swainsonine toxicosis can therefore be considered an induced α-mannosidosis, and poisoned animals would be expected to accumulate glycoprotein-derived "high mannose" oligosaccharides in tissues (5) and body fluids, and to excrete them in urine (6). Oligosaccharides of this type were required in our laboratory for the synthesis of dolichol intermediates (7, 8). For this reason, oligosaccharides were isolated from the urine of locoweed-poisoned sheep and the structures determined as reported in this paper and in a preliminary report (9). During the course of the study, the characterization of the excreted oligosaccharides was shown to be important for the early diagnosis of locoism and for the development of swainsonine toxicity as a reversible animal model for α-mannosidosis (10, 11).

EXPERIMENTAL PROCEDURES AND RESULTS*

The determination of the structures of 11 oligosaccharides from the urine of sheep with swainsonine toxicosis (see Table IV) allows a detailed comparison with the oligosaccharides of human (24, 25) and bovine (13)α-mannosidosis. It also provides important information regarding the pathogenicity of swainsonine, and on the pathways of degradation of mannosyl oligosaccharides by lysosomal α-mannosidase.

The initial step in the structure determination of the purified oligosaccharides was high resolution HPLC, using synthetic compounds (14–17) or oligosaccharides isolated from human (12) or bovine (13) α-mannosidosis urine as the reference materials. This HPLC analysis was performed on a mixture of oligosaccharides with or without appropriate de-
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TABLE IV
Structures of oligosaccharides from loco-sheep urine, susceptibility to the action of Endo D and Endo H, and potential ability to bind to concanavalin A-Sepharose

| HPLC peak | Structure | Abbreviation | Endo D | Endo H | Concanavalin A |
|-----------|-----------|--------------|--------|--------|---------------|
| 1         | Manα1→6Manβ1→4GlcNAc | ManαGlcNAc | NA     | NA     | ND            |
| 2         | Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2 | –      | –      | ND            |
| 3         | Manα1→6Manβ1→4GlcNAc | ManαGlcNAc | NA     | NA     | +             |
| 4         | Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2·Ia | +      | –      | +             |
| 5         | Manα1→3Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2·Ib | –      | +      | –             |
| 6         | Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2·II | –      | –      | +             |
| 7         | Manα1→6Manβ1→4GlcNAc | ManαGlcNAc | NA     | NA     | ND            |
| 8         | Manα1→3Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2·I | +      | +      | –             |
| 9         | Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2·II | –      | +      | +             |
| 10        | Manα1→6Manβ1→4GlcNAc | ManαGlcNAc | NA     | NA     | ND            |
| 11        | Manα1→6Manβ1→4GlcNAcβ1→4GlcNAc | ManαGlcNAc2 | +      | +      | +             |

*Compounds are listed in HPLC elution order (reversed phase HPLC after perbenzoylation; for details of chromatography see “Experimental Procedures,” Miniprint Section).

Sugar residues are in pyranose form: Man = D-mannose; GlcNAc = 2-acetamido-2-deoxy-D-glucose.

Digestions with Endo D and Endo H were performed as described in Ref. 9.

For definition of binding and conditions of chromatography on concanavalin A-Sepharose, see Table VI (Miniprint Section) and Ref. 32. NA, not applicable; ND, not determined.

rification (18), and the next step was digestion of the mixture with Endo H or Endo D. Initially, reduction to the aldolts was routinely performed prior to the digestions. Later it was discovered that at least one of the oligosaccharides, ManαGlcNAc2·Ib (for structures see Table IV) is unexpectedly resistant to the action of Endo H when it has been reduced to the aldol (32). Therefore, the reduction was performed after the incubation and prior to HPLC. By HPLC analysis of the digest, including the identification, where possible, of new peaks, preliminary structural data could be obtained for some of the compounds. As a result of these experiments, it became apparent that the urine oligosaccharides of swainsonine-intoxicated sheep, like those of bovine mannosidosis, consist of two homologous series, (a) a major series, with an intact di-N-acetylchitobiose residue (G2 series), and (b) a minor series, with a single “reducing” terminal 2-acetamido-2-deoxy-D-glucose residue (G1 series). When individual oligosaccharides had been purified, compositional analyses of per(trimethyl)silylated methyl glycosides, following methanalysis, confirmed these findings and provided a molecular formula for each compound. In some cases, this was also confirmed by chemical-ionization mass spectrometry (9). Further analysis of the purified oligosaccharides again involved high resolution HPLC, combined, in the case of the G2 series, with Endo D and Endo H digestions. It is noteworthy that, knowing the substrate requirements of these glycosidases (26, 27), this combination of techniques was a convenient and rapid way of obtaining a provisional structure for the compounds, because the products of the digestions could often be identified by HPLC comparison with appropriate "standards." The potential pitfall of this approach is the reliability of HPLC separations (see comments below), but this problem has been largely resolved by modern column-packing technology, resulting in superior performance, and by performing the HPLC after perbenzoylation (18). The other analytical techniques employed in this study were chemical ionization mass spectrometry of permethylated alditol acetates (22) (methylation analysis) and high resolution (500 MHz) 1H NMR spectroscopy. In the following discussion, individual oligosaccharides will be referred to by their abbreviated formula (see Table IV, which shows the structure determined for each compound).

The presence of a "nonreducing" terminal α(1→6)-linked D-mannose residue in triasaccharide ManαGlcNAc, tetrascarharide ManαGlcNAc2, and in the corresponding compounds from bovine mannosidosis urine (13), instead of a (1→3) linkage as found in the smallest oligosaccharide of human mannosidosis urine (α-D-Manp→(1→3)-β-D-Manp→(1→4)-D-GlcNAc) (24, 25), suggested a different substrate specificity for the residual α-mannosidase activity in humans and ru-
minants. By studying swainsonine-induced mannosidosis in human fibroblasts, Cenci Di Bello et al. (33) concluded that a residual α-mannosidase activity, specific for α(1→6) linkages, is inhibited by swainsonine in human cells, because the swainsonine-treated normal and mannosidosis cells both accumulate Man₅GlcNAc₂ instead of the usual trisaccharide. The lack of removal of the last α-linked mannose residue in most types of genetic and induced α-mannosidosis suggests that compounds containing this single α-linked residue are very poor substrates for lysosomal α-mannosidases.

The presence of an intact di-N-acetylchitobiose moiety in Man₉GlcNAc₂, Man₇GlcNAc₂, Man₅GlcNAc₂, and Man₃GlcNAc₂, and the corresponding oligosaccharides of bovine mannosidosis (13), suggests a fundamental difference in the catabolic pathway of oligosaccharide catabolism in humans and other mammals. This is supported by studies on α-mannosidosis in cats (31, 34), β-mannosidosis in goats (35), swainsonine toxicity in pigs (36), and GM₁ gangliosidosis in dogs (37) and cats (38). The difference could be due to a deficiency or inhibition of an endo-β-N-acetylglucosaminidase activity (39) in non-human species, or an enhanced activity in these species of a peptide N-glycanase (39). The recently reported absence of an endo-β-N-acetylglucosaminidase activity in kidney of sheep, pigs, and cattle supports the former conclusion (40). Furthermore, ovine and bovine tissues apparently contain an endo-β-N-acetylglucosaminidase activity with a different substrate specificity (see comments below). Such an activity could be responsible for the production of tetrasaccharide Man₃GlcNAc by the formation of hybrid chains in cultured fibroblasts (43) or from the catabolism of oligosaccharides into species with 2-5 mannose residues (33, 43). However, it should be emphasized that the distribution of oligosaccharides into species with 2-5 mannose residues is completely different for the swainsonine-induced and genetic conditions. This is not a species difference, but reflects the dual role of swainsonine as an inhibitor of both lysosomal α-mannosidase and Golgi mannidosidase II (42). Thus, swainsonine inhibits the processing of newly formed N-glycoprotein saccharide chains at the Man₉GlcNAc₂ stage (44) and causes the formation of hybrid chains in cultured fibroblasts (43) or hepatocytes (45). If similar hybrid chains are assumed to be formed in swainsonine-fed animals, they would undergo normal catabolism in the presence of lysosomal fucosidase, N-acetylenuraminidase, β-galactosidase, and N-acetylglucosaminidase until Man₃GlcNAc₂ is formed. The conversion of Man₃GlcNAc₂ to Man₅GlcNAc₂-II, which becomes the major excreted compound in fully established toxicosis (11), is presumed to be an inhibitor of both lysosomal α-mannosidase and Golgi mannidosidase II (42). Two other heptasaccharides were identified, both having an intact chitobiosyl residue. In earlier work (9), a compound, Man₅GlcNAc₂-I, was isolated and characterized by digestion with Endo D and Endo H, resistance to β-N-acetylglucosaminidase, and digestion by α-mannosidase. Its structure was determined to be that shown in Table IV. This is now known to be a minor isomer, its isolation being due to the limited resolving capabilities of the analytical and preparative HPLC columns employed in the initial phase of this work. The major isomer, Man₅GlcNAc₂-II has the structure shown in Table IV. This structure was confirmed by 600-MHz two-dimensional J-correlated ¹H NMR spectroscopy (collaboration with A. A. Bothner-By and R. L. Stephens, Carnegie-Mellon University, Pittsburgh, PA). The heptasaccharides Man₅GlcNAc₂-I and Man₅GlcNAc₂-II could be separated by chromatography on concanavalin A-Sepharose (32), and the structures were consistent with their assigned structures. It also allowed a calculation of the relative proportions as approximately 1:9, Man₅GlcNAc₂-I to Man₅GlcNAc₂-II. This was in reasonably good agreement with the ratio obtained by perbenzoylation and reversed phase HPLC. These isomers presumably represent minor and major pathways of degradation of Man₅GlcNAc₂ by residual lysosomal α-mannosidase activity (33), the major pathway involving initial cleavage of the α(1→3)-linked D-mannose residue of the trimannosyl core.

The heptasaccharide Man₅GlcNAc₂ was examined by the same procedures as those employed for Man₅GlcNAc₂-I, and shown to have the double-branched structure shown in Table IV. Therefore, it can be concluded that all the major oligosaccharides from locoweeds-intoxicated sheep urine have the same structures as those found in bovine α-mannosidosis (13). However, it should be emphasized that the distribution of oligosaccharides into species with 2-5 mannose residues is completely different for the swainsonine-induced and genetic conditions. This is not a species difference, but reflects the dual role of swainsonine as an inhibitor of both lysosomal α-mannosidase and Golgi mannidosidase II (42). Thus, swainsonine inhibits the processing of newly formed N-glycoprotein saccharide chains at the Man₅GlcNAc₂ stage (44) and causes the formation of hybrid chains in cultured fibroblasts (43) or hepatocytes (45). If similar hybrid chains are assumed to be formed in swainsonine-fed animals, they would undergo normal catabolism in the presence of lysosomal fucosidase, N-acetylenuraminidase, β-galactosidase, and N-acetylglucosaminidase until Man₅GlcNAc₂ is formed. The conversion of Man₅GlcNAc₂ to Man₅GlcNAc₂-II, which becomes the major excreted compound in fully established toxicosis (11), is presumably the result of either residual lysosomal α-mannosidase activity or of the activity of another mannidosidase not totally inhibited by swainsonine. The accumulation and excretion of high levels of Man₅GlcNAc₂ and Man₅GlcNAc₂ contrasts to the situation in bovine mannosidosis, where Man₅GlcNAc₂ and Man₅GlcNAc₂ are relatively minor components, being derived from "high mannose" chains. On the other hand, the identical nature of the oligosaccharide structures in the induced ovine and genetic bovine mannosidosis conditions, and the similarity of clinical and pathological changes that occur in affected animals, suggests that deficiency of the lysosomal
degradation of glycoprotein oligosaccharides is the primary cause of swainsonine toxicosis (6). It is interesting to note that gyro-asparagines, having the same structures as ManGlcNAc, ManGlcNAc2, and ManGlcNAc3, were isolated from the urine of patients with Gaucher’s disease (46). Their accumulation was said to arise from an obstruction of lysosomal function by gross storage of glucocerebroside.

The comparison of the relative abundance of individual oligosaccharides in urine pooled from sheep that had ingested locoweed over a 7-week period with that in urine collected when swainsonine toxicosis was fully established (a single urine sample collected 6 weeks after the start of locoweed feeding) allowed some conclusions to be drawn concerning how the animals adapt to chronic intoxication. Thus, after long-term treatment it appears that sheep attempt to compensate for loss of normal lysosomal α-mannosidase activity by synthesis of an α-mannosidase that is only partially inhibited by swainsonine. Hence the relative abundance of ManGlcNAc is greatly decreased in the long-term treatment, and that of ManGlcNAc2 and ManGlcNAc3 is increased.

The proportion of “G series” oligosaccharides (e.g. ManGlcNAc) is also significantly increased from 5 to 15%, indicating increased endo-α-N-acetylglucosaminidase activity or increased flux through the alternate glycosylation pathway.

A preliminary investigation of the oligosaccharides accumulated in the tissues of rams and pregnant ewes showed that each tissue is characterized by a unique pattern of oligosaccharides, and that fetal and adult tissues differ with regard to the pattern and level of stored oligosaccharides.6 The continuation of this study, and correlation of the results with tissue α-mannosidase levels (Ref. 36, collaboration with O. Touster and D. R. P. Tulsiani, Vanderbilt University, Nashville, TN) is in progress. Finally, it is important to note that the high mannose oligosaccharides described here are excellent reference compounds for studies of N-glycoprotein saccharide chain structure and biosynthesis (42, 43).

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When loco-sheep oligosaccharides (Fig. 1A) were compared on TLC with those from human α-mannosidosis (Fig. 1C) it was apparent that most of the major spots for the ovine and human oligosaccharides did not correspond. In contrast, the spots for bovine (Fig. 1B) and ovine (Fig. 1A) compounds had similar migrations, but were of markedly different intensity distribution.

RESULTS

When loco-sheep oligosaccharides (Fig. 1A) were compared on TLC with those from human α-mannosidosis (Fig. 1C) it was apparent that most of the major spots for the ovine and human oligosaccharides did not correspond. In contrast, the spots for bovine (Fig. 1B) and ovine (Fig. 1A) compounds had similar migrations, but were of markedly different intensity distribution.

These similarities and differences could be seen much more clearly by LC. For the sheep oligosaccharides, this was performed either on a normal-phase “Hain” column (Fig. 2) or on a C-4 reversed phase column after perbenzoylation (18) (Fig. 3). The comparison of two species (e.g., ovine and bovine) was best performed on the C-4 column (Fig. 3), and the same reversed phase LC could also be employed to quantify the individual oligosaccharides in the sheep urine (Table 2).
Oligosaccharides from Swainsonine-intoxicated Sheep

Comparison of Oligosaccharide Abundance in Pooled Sheep Liver Urine and Urine from a Sheep After Long-term Treatment

| Oligosaccharide | Pooled urine | Long-term treatment |
|-----------------|--------------|---------------------|
| Man2GlcNAc     | 1.4          | 2.3                 |
| Man3GlcNAc     | 14.5         | 21.4                |
| Man3GlcNAc     | 0.4          | 3.2                 |
| Man4GlcNAc     | 2.7          | 1.2                 |
| Man6GlcNAc     | 13.6         | 16.8                |
| Man6GlcNAc2-1  | 0.2          | 2.1                 |
| Man8GlcNAc     | 0.4          | 1.9                 |
| Man8GlcNAc2-1  | 21.5         | 20.9                |
| Man8GlcNAc     | 1.3          | 1.3                 |
| Man8GlcNAc     | 17.8         | 10.8                |
| Man8GlcNAc     | 0.7          | 0.7                 |
| Man8GlcNAc     | 0.8          | 0.7                 |
| Man8GlcNAc     | 3.3          | 1.7                 |
| Man8GlcNAc     | 0.9          | 0.7                 |
| Man8GlcNAc     | 12.7         | 12.2                |

* From concentrations (nanomoles) determined by incorporation of oligosaccharides isolated from urine collected from two sheep over a 7-week period (Pooled urine) or from a single sheep urine collected after 9 weeks of treatment (Long-term treatment), following the procedures outlined in Experimental Procedures. Integrated peak areas were compared with the areas of the peaks for standards, purchased and characterized under identical conditions, on the basis that abundance at 230 nm is proportional to the number of hexose groups (13). For explanation of abreviated formulae, see Table IV. 10 mg/ml urine.

Analysis of oligosaccharide by electrophoresis followed by gas-liquid chromatography of permethylated (silanized) methyl ester hydrolysates

| Component | Expected | Found |
|-----------|----------|-------|
| 1 Man5GlcNAc | 1.00     | 1.06  |
| 2 Man5GlcNAc | 3.00     | 3.05  |
| 3 Man6GlcNAc | 1.50     | 1.55  |
| 4 Man6GlcNAc | 1.50     | 1.26  |
| 5 Man6GlcNAc | 1.00     | 1.05  |
| 6 Man6GlcNAc | 1.00     | 1.05  |
| 7 Man6GlcNAc | 1.00     | 0.95  |
| 8 Man6GlcNAc | 1.00     | 1.05  |
| 9 Man6GlcNAc | 1.00     | 1.05  |
| 10 Man6GlcNAc | 1.00    | 1.05  |
| 11 Man6GlcNAc | 1.00    | 1.05  |

These results are shown for sheep (10 mg/ml urine).

In agreement with the TLC results, the major series of oligosaccharides (LC peaks 1-11) did not correspond to the major oligosaccharides of human monosaccharide urine, but four of the minor peaks (1, 3, 7, 8) did migrate similarly to the human series, suggesting some structural similarity (data not shown). A higher chromatochromatographic resolution of the sheep urine oligosaccharides and detailed comparison with estruria-5 monosaccharides, 5 was performed on a reversed phase column, after permethylation of the oligosaccharides mixture. This LC also afforded a quantification of the different oligosaccharides, both in pooled urine, and in a sample of urine collected from a sheep that had been inoculated for six months, and two new oligosaccharides (Fig. 3b), which were not observed in the urine collected (Fig. 3a). Examination of the reversed phase LC (Fig. 3b) showed that all the major peaks in the urine and estruria-5 series had the same retention times, suggesting that they had the same structure. However, the peak areas were quite different, the most intense peaks in the sheep urine (1-11) corresponding to minor peaks in the sheep urine. Based on comparison with the structures of oligosaccharides present in human monosaccharide urine (24,25), and in sheep monosaccharide urine (13), it was preliminarily concluded that the major series (LC peaks 1-11) were series of oligosaccharides in sheep monosaccharide urine with two residues of N-acetylglucosamine in the reducing end and a 6-6 (asparaginyl) residue as the non-reducing end (Fig. 3b). The major series contained a single residue (12) series. On the same basis, both major and minor series would contain the same two and three monosaccharide residues. To confirm these conclusions, the mixture of oligosaccharides from sheep urine was digested with endo B or endo R, and then re-chromatographed by LC. Peak 1 was digested by endo B to yield a product showing a time-shift of one residue of N-acetylglucosamine in LC (data not shown). Similarly peaks 2, 3, and 5 were digested by endo R, and peaks 1 and 12 by endo B and endo R. Peaks 2 and 12 of the O series were not affected by either enzyme, and no expected peak of the 6 series was affected. Based on the known carbohydrate composition of endo B and endo R (24,25), the possible structures for the G series of oligosaccharides were restricted by this experiment. Thus when composition had been determined (see below), some of the structures could be assigned with a good deal of certainty, even without further work.

For quantification of individual components, a large sample isolation of oligosaccharides from sheep liver urine, consisting of 5 ml of concentrated specimen (long-term treatment) followed by reversed phase chromatography and propane-gas chromatography, provided pure samples of the oligosaccharides. The structures of these have been determined, where possible, by comprehensive analysis by gas-liquid chromatography of trimethylsilyl methyl glucoside, mass-spectrometry of methoxylated alditol acetates (23), and 1H-NMR spectroscopy. In addition, the structures of two minor components have been determined from the results of LC and NMR responses. The results for individual components (snamed according to their electrophoretic LC) are as follows:

Table 1: Oligosaccharide Names

4 Fig. 2. Relationship between retention times and number of hexose residues in LC of sheep monosaccharides. A = oligosaccharides, 10 mg/ml urine. C = urine. G = urine. 8 = urine. 12 = urine. The retention times were derived from an internal standard of a mixture of oligosaccharides similar to that shown in Fig. 1, but with a larger injection of oligosaccharide mixture.
| Chemical shifts and coupling constants for 1H and 13C resonances of low-molecular-weight oligosaccharides |  |
|---|---|---|---|
| Compound | Proton | Residue | Signal \& J value (Hz) |
| NAc-glucosamine | H-4 | d | 5.190 2.9 |
| H-3 | d | 4.915 1.6 |
| H-2 | d | 4.773 2.8 |
| H-1 | d | 5.15 8.6 |
| NAc-Sialyl | H-2 | d | 6.02 2.0 |
| H-1 | d | 5.97 3.5 |
| H-4 | d | 6.915 3.5 |
| NAc-Sialyl | H-1 | 3 | d | 6.770 1.5 |
| H-2 | 3 | d | 6.611 8.0 |
| H-3 | 3 | d | 4.616 4.4 (0.2)7 |
| NAc-Sialyl | H-2 | 4 | d | 6.25 2.9 |
| H-1 | 4 | d | 5.94 3.4 |
| NAc-Sialyl | H-1 | 4 | d | 5.95 1.0 |
| H-4 | 4 | d | 5.99 1.6 |
| H-3 | 4 | d | 5.79 3.3 |
| NAc-Sialyl | H-2 | 3 | d | 5.96 2.9 |
| H-1 | 3 | d | 4.90 1.6 |
| H-4 | 3 | d | 4.79 1.5 |
| NAc-Sialyl | H-1 | 3 | d | 6.24 3.5 |
| H-2 | 3 | d | 6.24 3.5 |
| H-4 | 3 | d | 6.09 3.5 |
| NAc-Sialyl | H-2 | 2 | d | 4.91 1.5 |
| H-1 | 2 | d | 4.83 1.4 |
| NAc-Sialyl | H-1 | 2 | d | 4.79 1.7 |
| H-2 | 2 | d | 4.54 7.8 |
| H-1 | 2 | d | 4.54 7.8 |
| NAc-Sialyl | H-2 | 1 | d | 4.26 5.0 (11.2)5 |
| H-1 | 1 | d | 4.13 2.2 |
| NAc-Sialyl | H-1 | 1 | d | 4.06 3.3 |
| H-2 | 1 | d | 4.06 3.3 |

For N-2 resonances, only J2,3 is quoted (except as noted in cl).
1 Sample analyzed as the tetrasaccharide compound.
2 Value in parentheses is J2,3 (Hz).
3 Spectrum shows low-intensity signals from NAc-glucosamine (see Table II).
4 Two experimentally determined samples were isolated and prepared for spectroscopy, and spectra recorded as described in Experimental Procedures. For measurement of the 1H/13C resonances, the following formulae were used: N = monosaccharide, and C = L-arabinose. For the experimental procedures, see Table III. The 1H/13C resonances are numbered according to the following formulae, where M = monosaccharide, and G = L-arabinose-1-deoxy-
3.1. \( H-1 \) 4.06 3.3
2.1. \( H-2 \) 4.06 3.3
1.1. \( H-3 \) 4.06 3.3

The presence of other poorly-defined resonances in the 9.5-7.3 and 3.3-2.1 region showed that this sample was less pure than the standard. However, a summary of the compositional, chromatographic, and 1H/13C data was assigned to the structure shown in Table IV.

**Table II:** Oligosaccharides from Swainsonine-intoxicated Sheep

### Table II Cont.

| Component | Proton | Residue | Signal \& J value (Hz) |
|---|---|---|---|
| NAc-glucosamine | H-4 | d | 5.100 1.5 |
| H-3 | d | 5.097 1.4 |
| H-2 | 3 | d | 4.806 1.5 |
| H-1 | 3 | d | 4.777 1.5 |
| NAc-Sialyl | H-2 | 2 | d | 4.36 2.8 |
| H-1 | 2 | d | 4.24 2.8 |
| H-4 | 2 | d | 4.24 2.8 |
| NAc-Sialyl | H-1 | 2 | d | 4.06 3.7 |
| H-2 | 2 | d | 4.06 3.7 |
| H-4 | 2 | d | 3.93 1.3 |

The experimental procedures for sample preparation, isolation, and 1H/13C analysis were described in Experimental Procedures. The 1H/13C spectra were recorded using a Varian VXR-500 spectrometer. The 1H resonances were assigned in the order shown in Table II. The spectra were recorded using a Varian VXR-500 spectrometer. The 1H resonances were assigned in the order shown in Table II.
Table V

| Ratio | δ-Methyl aldito acid | δ-Methyl aldito acid | δ-Methyl aldito acid | δ-Methyl aldito acid | δ-Methyl aldito acid | δ-Methyl aldito acid |
|-------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|       | a                  | b                   | c                   | d                   | e                   | f                   |
| 1:3:4:6 | 0.13 (1)            | 0.10 (1)             | 0.07 (1)             | 0.32 (1)             | 0.32 (1)             | 0.32 (1)             |
| 1:3:6   | 0.10 (1)            | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             |
| 1:4:6   | 0.10 (1)            | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             |
| 1:4:6   | 0.10 (1)            | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             | 0.10 (1)             |

*HPLC ratio was calculated relative to the amount of 2,4:6-oligogluc.*

*HPLC ratio was calculated relative to the amount of 1,3,6-tri-oligogluc.*

*HPLC ratio was calculated relative to the amount of 3,6-di-oligogluc.*

*Shades (-→-) signify that the isolated derivative was not detected. Expected ratios are given in parentheses.*

*The response factor for this compound was not known, therefore the presence of this derivative is shown as a problem; countable the quenchability recovery of methylated derivatives of 1,3,6-tri-2-deoxymannose were discussed in a recent review (79).*

*In the abovementioned formula, M = d-mannose, and k = 2,4:6-di-2-deoxymannose.*

There was also evidence for the presence of a contaminating linear tetra-oligosaccharide containing a 3-linked α-d-galactose residue, whose structure is probably analogous to ManGal(1→4)GalMove(1→).

This compound co-chromatographed (LC) with chemically synthesized Nα-ManGal(1→4)GalMove(1→3)Gal(1→3)Gal(1→4)GlcNAc(1→6)GlcNAc(1→6), and with ManGal(1→4)GalMove(1→3)Gal(1→3)Gal(1→4)GlcNAc(1→6)GlcNAc(1→6), from the same source. It was digested by Endo H and Endo E, and the product from Endo E digestion did not co-chromatograph with the product from the digestion of synthetic Nα-ManGal(1→4)GalMove(1→3)Gal(1→3)Gal(1→4)GlcNAc(1→6)GlcNAc(1→6) with Endo 9L.

The 1H NMR spectrum (Table III) showed the expected doublets for H-2 of the β-galactosylacetamidomethyl residue, and H-1 (10) signal for the β-GlcNAc(1→6) residue. The spectrum also showed H-2 and H-2' signals for a 3-linked mannose residue substituted only at O-4, and an α-1,6-linked mannose residue unsubstituted at O-3, and an "external" α-1,3-linked mannose residue (83).

Fractionation analysis (see Table VI) indicated that the most abundant mannose derivatives were trans-α-Me and 3,4,6-tri-α-Me and 3,4,6,tri-α-Me and 3,4,6-tri-α-Me, present in approximately equal ratio. In conjunction with the recoverability to Endo B, this confirmed the linear structure with a childlike core as shown in Table VI.

This compound co-chromatographed (LC) with ManGal(1→4)GalMove(1→3)Gal(1→3)Gal(1→4)GlcNAc(1→6)GlcNAc(1→6), from the same source (reversed phase LC). It was not digested by Endo B or Endo D. The 1H NMR spectrum (Table III) showed the expected signals for H-2 of the β-galactosylacetamidomethyl residue, and H-1 (10) signal for a 2-linked GlcNAc residue. The spectrum also showed H-2 and H-2' signals for a 3-linked mannose residue substituted only at O-4, and one "internal" α-1,3-linked mannose residue, the H-2 resonance appearing as a very similar signal value, and the H-2' signal being actually superimposed. In the 8-2 region, a low intensity signal at δ 5.46 showed that this compound was slightly contaminated with ManGal(1→6). Fractionation analysis (see Table VI) indicated that 2,4,6-tri-α-Me and 3,4,6-tri-α-Me were the most abundant derivatives and confirmed that this compound had a linear structure with two 8-linked mannose residues and a chitobiose core, as shown in Table IV. The presence of small amounts of 2,4,6-tri-α-Me and 3,4,6-tri-α-Me was consistent with a single amount of contamination from ManGal(1→4)GalMove(1→3)Gal(1→3)Gal(1→4)GlcNAc(1→6)GlcNAc(1→6), respectively.
Oligosaccharides from Swainsonine-intoxicated Sheep

The oligosaccharide fraction containing Man$_{2}$GlcNAc$_{2}$-olomers was labeled by reaction with 3-[14C]-labeled sodium periodate (36). A column (1 x 2.5 cm) of concanavalin A-Sepharose was filled with a buffer containing 1 M NaCl, 1 M Na$_{2}$CO$_3$, and 0.01 M Tris (37). The sample, 170,000 cpm in 455 ml buffer, was applied to the column, rinsed with 10 ml buffer, and the eluent counted ("cope not retained"). The column was then rinsed with 10 ml buffer containing 0.1 M methyl a-Man-N-glycososamine ("cope retained and eluted..."). Each fraction was applied to a column (2 x 4.5 cm) of Bio-Gel P-2, and the Man$_{2}$GlcNAc$_{2}$ fraction collected (for structure see Table V). Finally, each fraction from the P-2 column was purified by LC on an acetonitrile column as described in Chromatographic Methods, with an internal standard of low-reducing uronyl oligosaccharides. The fraction corresponding to the Man$_{2}$GlcNAc$_{2}$ peak was collected and counted (GC percolation). The radioactive peak corresponding to the Man$_{2}$GlcNAc$_{2}$ fraction eluted approximately 29 seconds earlier than the peak corresponding to Man$_{3}$GlcNAc$_{3}$-II (acetamidomethyl water fraction). Flow rate 2.26 ml/min.

**Table V.**

| Oligosaccharide | Cope not retained | Cope retained and eluted | Not recorded |
|-----------------|------------------|--------------------------|-------------|
| Man$_{2}$GlcNAc$_{2}$-I | 41,086 | 47,400 |
| Man$_{2}$GlcNAc$_{2}$-I1 | 10,700 | 40,000 |

Note: a-Man-N-glycososamine

6 Consists of Man$_{2}$GlcNAc$_{2}$-I eluted from a non-specific "coping".

7 Mean of the determinations, calculated from chromatography of an elixer (22, 2700).