Biochemistry of the Sphingolipids. XIX. Studies on an Epimerization Phenomenon in the Oligosaccharide of Phytoglycolipid*

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ABSTRACT: Phytoglycolipids from various plant seeds yield on alkaline hydrolysis a mixture of oligosaccharides which on further acid hydrolysis give a common trisaccharide, glucosamidoglucuronidoinositol. In a more detailed study of the trisaccharide fraction from corn a relatively water-insoluble material was isolated amounting to 15% of the total. This material has now been identified as glucosamidoiduronidoinositol. In a study of the possible origin of the insoluble trisaccharide it was discovered that glucosamidoglucuronidoinositol and glucuronidoinositol are partially epimerized to the corresponding iduronido derivatives in yields of 15 and 27%, respectively, by treatment with hot $Ba(OH)_2$ solution. These findings are in accord with the facile 50% epimerization of unsubstituted glucuronic acid to iduronic acid. Phytoglycolipid (tri- and tetrasaccharide mixture) was esterified and reduced thus converting the hexuronic acid moiety to hexose. Hydrolysis of the reduced material yielded glucose but no idosan. Nitrous acid degradation of the tri- and tetrasaccharide mixture (obtained by alkaline hydrolysis) gave glucosylinositol but no idosylinositol was obtained. Therefore it can be concluded that iduronic acid is not present in phytoglycolipids but is an artifact produced by the alkaline treatment used in hydrolysis of the glycolipid. The facile eperimization of glucuronido derivatives under alkaline conditions is a matter of concern in dealing with various glucuronic acid containing polymers.

An earlier publication (Carter *et al.*, 1958a) described the isolation from plant seed-phosphatides of the phytosphingosine-containing glycolipids designated as phytoglycolipid. The materials contain phytosphingosine, fatty acid, phosphate, inositol, glucosamine, hexuronic acid, galactose, arabinose, and mannose.

Initial studies (Carter *et al.*, 1958b) showed that the phytosphingosine was linked through a phosphate ester to *myo*-inositol. Investigation of the carbohydrate moiety (Carter *et al.*, 1964a,b) showed that the oligosaccharide fraction obtained by alkaline hydrolysis of phytoglycolipids was a mixture of tri-, tetra-, penta-, hexa-hepta-, and octasaccharides. The crystalline trisaccharide which could be obtained readily from each of the other oligosaccharides by acid hydrolysis was shown to be glucosamidoglucuronidoinositol for which the complete structure is described in the preceding paper.

During the course of preparing a sample of trisaccharide from corn oligosaccharide a relatively water-insoluble carbohydrate material was obtained. The insoluble carbohydrate and the water-soluble trisaccharide had the same mobility on paper chromatography with Whatman No. 1 filter paper but the insoluble material had a mobility slightly greater than that of glucosamidoglucuronidoinositol on anionic exchange chromatographic paper. A Dische carbazole determination (Bitter and Ewins, 1961) showed that approximately one-third of the molecule was hexuronic acid and a hexosamine test (Elson and Morgan, 1933; Rondle and Morgan, 1955; Boas, 1953) revealed it also constituted a third of the material. The insoluble carbohydrate material was hydrolyzed with 6 N HCl; by paper chromatographic and gas–liquid partition chromatographic analysis, the presence of an equal amount of inositol and glucosamine was demonstrated. These data establish that the water-insoluble material is a trisaccharide containing inositol, hexuronic acid, and glucosamine. For convenience the water-soluble trisaccharide, glucosamidoglucuronidoinositol, is referred to as trisaccharide A, and the water-insoluble material is referred to as trisaccharide B.

Since hexuronic acids decompose during acid hydrolysis it was decided to prepare the corresponding hexosyl compound by reduction of the methyl esters. The trisaccharide B was N acetylated with acetic anhydride and aqueous sodium bicarbonate to afford a water-soluble amorphous derivative, which gave a good elemental analysis. The hexuronic acid was esterified by dissolving the *N*-acetyl derivative in dimethylformamide and adding diazomethane to the solution, allowing only a short reaction time, thus avoiding formation of methyl ethers. The methyl ester was reduced with sodium borohydride to give a semicrystalline *N*-acetylcarboxyl-reduced derivative. Deacetylation was achieved by treatment with anhydrous hydrazine (Phillips, 1963). Nitrous acid degradation of this amino trisaccharide gave as sole products chitose and a hexosylinositol. Hydroly-

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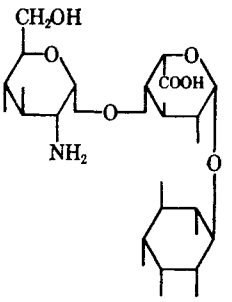
sis of the hexosylinositol with 1 N HCl for 75 min at 100° gave only inositol and idosan,[1] identified by paper chromatography and by gas–liquid partition chromatographic comparisons with authentic specimens. These data require that in the trisaccharide B inositol be linked to iduronic acid.

Since D-glucuronic acid can be epimerized in neutral solution to L-iduronic acid (Fischer and Schmidt, 1959), it seemed probable that treatment of the trisaccharide A glucosamidoglucuronidoinositol with alkali would afford some epimerization to the trisaccharide B. The trisaccharide A was refluxed for 8 hr with saturated $Ba(OH)_2$ solution (the conditions used to hydrolyze phytoglycolipid) and the trisaccharide was reisolated. Of the recovered material 15% was water insoluble. This was shown to be identical with the trisaccharide B originally isolated from the oligosaccharide as were its derivatives and its degradation products. Therefore by comparison with the trisaccharide A whose complete structure is known it can be concluded that the trisaccharide B is glucosamidoiduronidoinositol (I).



I

In order to show that the carboxyl function of the hexuronic acid is necessary for epimerization, the *N*-acetylcarboxyl-reduced trisaccharide A was treated with saturated $Ba(OH)_2$ at reflux for 8 hr. Analysis of the *N*-acetyl products showed no change had taken place.

The ready isomerization of glucuronic to iduronic acid is of interest in studies of the natural occurrence of the latter compound. In this connection it is worth noting that the equilibrium point in the alkaline epimerization is affected markedly by substituents. Thus while glucuronic acid gives a 1:1 mixture of iduronic acid and glucuronic acid, glucuronidoinositol gives only 27% and trisaccharide only 15% of the corresponding iduronido derivative.

In an attempt to determine whether iduronic acid is present in phytoglycolipid, a tri- and tetrasaccharide phytoglycolipid fraction was N acetylated and esterified with diazomethane, and the methyl ester was reduced with sodium borohydride. Any natural iduronic acid should, therefore, be present as the corresponding idose derivative. This N-acetylated, esterified, and reduced phytoglycolipid was hydrolyzed with saturated $Ba(OH)_2$ solution and the tri- and tetrasaccharides were isolated. Nitrous acid degradation of the tri- and tetrasaccharides did not reveal the presence of idosylinositol when analyzed by gas–liquid partition chromatography. Analysis of acid-hydrolyzed tri- and tetrasaccharides and acid hydrolysis of the nitrous acid degradation products showed no evidence of idosan being present. It is concluded therefore that the hexuronic acid present in the phytoglycolipid tri- and tetrasaccharide fraction is exclusively glucuronic. These results however, do not exclude the possibility that iduronic may be present in the higher phytoglycolipid oligosaccharides. It seems most likely, however, that the iduronic acid isolated from phytoglycolipid is an artifact.

An investigation of the trisaccharide fraction of soybean and flax oligosaccharides has shown the presence of the trisaccharide B to the same extent as in corn; this result is assumed to be also an artifact produced during the hydrolysis of the corresponding phytoglycolipid.

Experimental Section

Materials and Methods. The nonphosphorylated oligosaccharide was prepared from corn phytoglycolipid by the previously described method (Carter *et al.*, 1958a).

Paper chromatography was carried out at room temperature by the descending technique using Whatman No. 1 filter paper unless otherwise stated. The solvent systems employed were: (A) isopropyl alcohol–acetic acid–water (3:1:1), (B) ethyl acetate–acetic acid–water (3:1:3, upper phase), and (C) *n*-butyl alcohol–pyridine–water (6:4:5). S & S anion-exchange chromatographic paper (Carl Schleicher & Schuell Co.) containing 5% Dowex 2-X8 was used for the identification of trisaccharides and *N*-acetyl derivatives run descending in solvent 1-propanol–nitromethane–water (5:2:3) for 30 hr. Spots were located using the silver nitrate and NaOH dip method of Pizer and Ballou (1959).

Gas–liquid partition chromatography was done on an F & M Model 400 instrument with hydrogen flame ionization detector. Analyses of di- and monosaccharides were done on a 6-ft glass U-shaped column 4-mm i.d. with 3.8% S.E.-30 on Diatoport S (80–100 mesh) (F & M Corp.). The disaccharides were run at 246° and the monosaccharides at 170°.

Preparation of Trisaccharide B. Oligosaccharide (6.9 g) was refluxed with 2 N H_2SO_4 (240 ml) for 30 min. The solution was cooled to room temperature and neutralized to pH 7 with $Ba(OH)_2$ solution. The precipitate of barium sulfate was removed by centrifugation and the solution was concentrated to approximately 100 ml on a rotary evaporator. The solution was adjusted to pH 1 with 1 N H_2SO_4 and filtered through Super-Cel. The filtrate was adjusted to pH 4 with Dowex 2 (HCO_3^-) anion-exchange resin. The resin was removed and the solution was concentrated to 50 ml. Ethanol was added until a faint turbidity was produced. The solution was left for 2 hr at room temperature. The precipitate was removed and more ethanol was added to the filtrate to produce a second crop of material. The precipitated trisaccharide (2.8 g) was suspended in hot water (28 ml)

[1] The authors are grateful to Dr. J. A. Cifonelli, University of Chicago, Department of Pediatrics, for his gift of a sample of tri-*O*-acetylidosan.

and the insoluble fraction was collected by centrifugation. The precipitate was resuspended in hot water (10 ml) and the insoluble material was collected (437 mg). This material (4 mg) was hydrolyzed in a sealed tube with 6 N HCl for 6 hr at 100°. The presence of inositol and glucosamine was shown on paper chromatography in solvent system C. Gas–liquid partition chromatography of the trimethylsilyl derivatives at 160° showed approximately equal amounts of inositol and glucosamine. For further purification the trisaccharide B (the insoluble material) was dissolved in 1 equiv of 0.02 N NaOH and precipitated by neutralization with 1 equiv of 0.02 N HCl. This white solid (trisaccharide B), $[\alpha]_D^{22}$ +100° (*c* 0.35, 0.01 M NaOH), had the same mobility as glucosamidoglucuronidoinositol (trisaccharide A) on paper chromatography. On anion-exchange paper the trisaccharide A moved 16.1 cm and the trisaccharide B moved 18.9 cm. *Anal.* Calcd for $C_{18}H_{31}NO_{16}\cdot H_2O$: C, 40.37; H, 6.21; N, 2.61. Found: C, 40.58; H, 6.29; N, 2.65; glucosamine, 32:1; hexuronic acid, 29.2.

N-Acetyl Derivative of Trisaccharide B. Trisaccharide B (902 mg) was suspended in water (14 ml) containing sodium bicarbonate (2 g) cooled to 0°. To this suspension, acetic anhydride (0.9 ml) was added over 5 min with continuous stirring. The mixture was maintained at 0° for 3 hr and then allowed to stir at room temperature overnight (17 hr). The solution was passed down an IR-120 (H^+) cation-exchange resin column and the aqueous eluent was lyophilized to afford a glass (1.02 g). Paper chromatography in solvent system A revealed the presence of a small amount of nonacetylated material. The product was dissolved in water (5 ml) and passed down a column of Dowex 50 (H^+) cation-exchange resin. The eluent was lyophilized to afford a white amorphous powder, showing as a single spot on paper chromatography in solvent system A. The product (943 mg, 96%, $[\alpha]_D^{25}$ +109° (*c* 0.5 H_2O), mp 170–175°) could be distinguished from the *N*-acetyl derivative of the trisaccharide A on anion-exchange paper chromatograms (the former moves 9.4 cm and the latter 5.8 cm). *Anal.* Calcd for $C_{20}H_{33}NO_{17}$: C, 42.91; H, 5.94; N, 2.50. Found: C, 42.64; H, 5.97; N, 2.60.

Methyl Ester of N-Acetyl Trisaccharide B. *N*-Acetyl trisaccharide B (517 mg) was dissolved in dimethylformamide (20 ml). An ethereal solution of diazomethane was added dropwise until a permanent yellow coloration was obtained. The solution was allowed to remain with an excess of diazomethane for 3 min. The solution was evaporated to dryness and the product was lyophilized from water to afford a white amorphous powder. The material ($[\alpha]_D^{25}$ 101° (*c* 0.5, water) gave a single spot on paper chromatography in solvent system A. *Anal.* Calcd for $C_{21}H_{35}NO_{17}$: C, 43.96; H, 6.15; N, 2.44. Found: C, 44.33; H, 6.63; N, 2.39.

The methyl ester (100 mg) was acetylated with acetic anhydride (0.5 ml) and pyridine (0.5 ml) at 100° for 2 hr. The product was isolated by evaporation of the reactants and extraction of the material into chloroform. This chloroform extract was percolated through a short silicic acid column and the eluent was evaporated to afford an oil (156 mg) which crystallized from methanol–ether in white prisms: mp 228–229°, $[\alpha]_D^{25}$ +72° (*c* 0.5, $CHCl_3$). *Anal.* Calcd for $C_{41}H_{55}NO_{27}$: C, 49.56; H, 5.58; N, 1.41. Found: C, 49.78; H, 5.73; N, 1.39.

N-Acetylcarboxyl-Reduced Trisaccharide B. The methyl ester (320 mg) was dissolved in water (15 ml) and cooled to 0° in an ice bath. Sodium borohydride (400 mg) was added to the cooled solution. The solution was allowed to warm up to room temperature and stirred for 17 hr. The excess sodium borohydride was decomposed with glacial acetic acid and the solution was passed down a column of IR-120 (H^+) cation-exchange resin. The eluent was evaporated to dryness, methanol (10 ml) was added, and the solution was evaporated; this procedure was repeated four more times. The product was lyophilized from water and then passed down a column of Dowex 2 (HCO_3^-) anion-exchange resin. Paper chromatography showed the product to be a single spot in solvent A. This product was obtained as colorless cubes: 289 mg, 88%, $[\alpha]_D^{23}$ +96° (*c* 0.5, H_2O). *Anal.* Calcd for $C_{20}H_{35}NO_{16}$: C, 44.03; H, 6.47; N, 2.57. Found: C, 44.01; H, 6.53; N, 2.50.

The polyacetyl derivative was prepared as described above and obtained as white needles from aqueous methanol: mp 218–219°, $[\alpha]_D^{25}$ +55° (*c* 0.5, $CHCl_3$). *Anal.* Calcd for $C_{40}H_{55}NO_{26}$: C, 49.75; H, 5.74; N, 1.45. Found: C, 49.99; H, 5.69; N, 1.28.

Deacetylation of N-Acetylcarboxyl-Reduced Trisaccharide B. The *N*-acetylcarboxyl-reduced derivative (300 mg) was added to anhydrous hydrazine (10 ml) in a stoppered flask and heated at 100° for 17 hr. The solution was allowed to cool and poured into a 1:1 mixture of ether–acetone (300 ml). The oil that separated out was stirred with a glass rod and the product adhered to the sides of the vessel. This material was washed with ether (100 ml) and lyophilized from water to give a pale buff colored solid, 266 mg, 94%, $[\alpha]_D^{25}$ +79° (*c* 0.5, H_2O). *Anal.* Calcd for $C_{18}H_{33}NO_{15}\cdot 0.5H_2O$: C, 42.21; H, 6.49; N, 2.73. Found: C, 41.96; H, 6.70; N, 2.71.

Idosylinositol. Deacetylated carboxyl-reduced trisaccharide B (100 mg) was dissolved in water (10 ml) containing sodium nitrite (300 mg). The solution was adjusted to pH 3 with glacial acetic acid and stirred at room temperature for 17 hr. The solution was passed down a column of Amberlite MB-3 ion-exchange resin and the eluent was evaporated to dryness. The product was immediately chromatographed on a carbon–Celite column. Idosylinositol was obtained as a semicrystalline solid (48.1 mg, $[\alpha]_D^{25}$ +25° (*c* 0.5, H_2O)) when lyophilized from water. Paper chromatography in solvent system A showed the product to be a single spot. The chitose was also identified. *Anal.* Calcd for $C_{12}H_{22}O_{11}\cdot 0.5H_2O$: C, 41.02; H, 6.59. Found: C, 41.15; H, 6.36.

The trimethylsilyl derivative of idosylinositol has a retention time of 14.43 relative to the trimethylsilyl derivative of inositol on gas–liquid partition chromatography.

Hydrolysis of Idosylinositol. Idosylinositol (5 mg) was hydrolyzed with 1 N HCl (0.2 ml) in a sealed tube at 100° for 75 min. The products, obtained by lyophilization of the neutralized solution, were run on paper chromatography in solvent system B. The sole products were shown to be inositol and idosan by comparison with authentic samples. Gas–liquid partition chroma-

TABLE I: Comparison of Trisaccharides.

Compound	Physical Constant	Trisaccharide A from Corn Oligosaccharide	Trisaccharide B from Corn Oligosaccharide	Trisaccharide B from Epimerized Trisaccharide A
I. Trisaccharide	$[\alpha]_D^{22}$	+117[a]	+100[a]	+105[a]
II. *N*-Acetyl I	$[\alpha]_D^{25}$	+134[b]	+109[b]	+107[b]
III. Methyl ester of II	$[\alpha]_D^{25}$	+85.3[b]	+101[b]	+100[b]
IV. Carboxyl-reduced III	$[\alpha]_D^{23}$	+136[b]	+96[b]	+98[b]
V. Deacetylated IV	$[\alpha]_D^{25}$	+115[b]	+79[b]	+78[b]
VI. Nitrous acid degradation on V	$[\alpha]_D^{25}$	+83[b]	+25[b]	+27[b]
VII. Polyacetyl III	Mp (°C)	222–221	228–229	229–230
	$[\alpha]_D^{25}$	+104[c]	+72[c]	+73[c]
VIII. Polyacetyl IV	Mp (°C)	128–129	218–219	218–219
	$[\alpha]_D^{25}$	+102[c]	+55[c]	+57[c]
IX. Hydrolysis products of VI		Glucose and inositol	Idosan and inositol	Idosan and inositol

[a] $[\alpha]_D$ (*c* 0.35, 0.01 M NaOH). [b] $[\alpha]_D$ (*c* 0.5, H_2O). [c] $[\alpha]_D$ (*c* 0.5, $CHCl_3$). All $[\alpha]$ values are in degrees.

tography of the trimethylsilyl derivatives showed the products to have the same retention times as idosan and inositol trimethylsilyl derivatives. The tri-*O*-acetyl derivative of idosan had the same retention time as an authentic sample from Dr. Cifonelli. Retention time of idosan trimethylsilyl derivative relative to inositol trimethylsilyl derivative = 0.19. Retention time of tri-*O*-acetylidosan relative to α-glucose trimethylsilyl derivative = 0.32.

Epimerization of Trisaccharide A. Trisaccharide A (glucosamidoglucuronidoinositol) (5 g) was dissolved in a saturated solution of $Ba(OH)_2$ (300 ml) and refluxed for 8 hr. The solution was cooled and percolated through an IRC-50 (H^+) cation-exchange column and the eluent was concentrated to 50 ml. Ethanol was added to this solution to produce turbidity and the precipitate was collected. A second crop was obtained by adding more ethanol to the filtrate. The trisaccharide recovered (4.1 g) was a white solid. This was suspended in water (41 ml) and the insoluble material was collected by centrifugation (619 mg, 15%). This product had the same physical constants as the trisaccharide B obtained from the oligosaccharide. This trisaccharide B was N acetylated, esterified, carboxyl reduced, deacetylated, and degraded with nitrous acid. Table I shows the comparison made of the trisaccharide A (glucosamidoglucuronidoinositol), trisaccharide B from phytoglycolipid oligosaccharide, and trisaccharide B dervied from the trisaccharide A in parallel experiments.

Epimerization of Glucuronidoinositol. Glucuronidoinositol (95 mg) was dissolved in a saturated solution of barium hydroxide and refluxed for 8 hr. The cooled solution was percolated through a cation-exchange resin column of IRC-50. The eluent was evaporated to dryness on a rotary evaporator. The colorless material obtained was dissolved in dimethylformamide (20 ml) and an ethereal solution of diazomethane was added to give a permanent yellow coloration. After 3 min the excess diazomethane was decomposed with glacial acetic acid and the solution was evaporated. This methyl ester was dissolved in water (10 ml) and an excess of sodium borohydride was added (300 mg) at 0°. The reduction mixture was stirred at 0° for 2 hr and allowed to warm up to room temperature, and stirring was continued for a further 17 hr. The excess sodium borohydride was decomposed with a few drops of glacial acetic acid and the solution was deionized with Amberlite MB-3 ion-exchange resin. The column eluent was evaporated, methanol (10 ml) was added, and the solution was evaporated. This procedure was repeated four more times.

A sample (25 mg) of the nonepimerized glucuronidoinositol was similarly esterified and reduced with sodium borohydride. Both products were analyzed by gas–liquid partition chromatography and the extent of epimerization found to be 27%.

Preparation of N-Acetyl Phytoglycolipid. Flax sodium phytoglycolipid tri- and tetrasaccharides (1.03 g) were suspended in 10% aqueous pyridine (50 ml) with stirring. NaOH (400 mg) in 50 ml of 10% aqueous pyridine was added and the suspension was stirred at room temperature for 30 min. The solution was then chilled in an ice bath while acetic anhydride (0.45 ml) was added dropwise over a period of 4 min. The mixture was allowed to stir for a further 24 hr and then percolated through a cation-exchange column of IR-120 (H^+). The water eluate was lyophilized to afford *N*-acetyl phytoglycolipid (1.01 g).

Methyl Ester of N-Acetyl Phytoglycolipid. N-Acetyl phytoglycolipid (500 mg) was suspended in dimethylformamide and an ethereal solution of diazomethane added until the solution turned turbid at which time the ether was removed with a stream of nitrogen. More diazomethane was added in this manner until a persistent yellow color was obtained. The solution was then evaporated and the product lyophilized from water to yield a buff colored solid (592 mg). The infrared spectrum showed an ester band at 1720 cm^{-1}.

Reduction of Methyl Ester of N-Acetyl Phytoglycolipid. The methyl ester (580 mg) was dissolved in water

(25 ml) to which a few drops of pyridine had been added. Sodium borohydride (300 mg) dissolved in water (2.5 ml) was added to the chilled phytoglycolipid solution. This solution was stirred at 0° for 2 hr and then at room temperature for 22 hr. Acetic acid was added to decompose the excess sodium borohydride and the solution was passed over a column of IR-120 (H^+) cation-exchange resin. The eluent was evaporated to dryness. This procedure with methanol was repeated four more times. The product was lyophilized to afford 542 mg. A Dische test indicated the presence of some hexuronic acid so the above esterification and reduction was repeated to yield material that contained 2% hexuronic acid.

Hydrolysis of N-Acetylcarboxyl-Reduced Phytoglycolipid. *N*-Acetylcarboxyl-reduced phytoglycolipid (796 mg) was refluxed with a saturated solution of $Ba(OH)_2$ (150 ml) for 16 hr. The hydrolysate was filtered hot and the filtrate, when cool, passed over IRC-50 (H^+) cation-exchange resin. The water eluent was adjusted to pH 4 and then passed over a large column of Dowex 2 (HCO_3^-) anion-exchange resin. The carboxyl-reduced nonphosphorylated oligosaccharide (85 mg) was eluted with the first 200 ml of water.

This material (85 mg) was dissolved in 3 ml of water and passed over a 2 × 66 cm column of Sephadex G-25; 3-ml fractions were collected. The fractions corresponding to the tri- and tetrasaccharide were combined and lyophilized (57 mg).

Nitrous Acid Degradation of Reduced Oligosaccharide. The tri- and tetrasaccharide fraction (56 mg) was dissolved in water (5 ml) to which sodium nitrite (50 mg) was added. The solution was adjusted to pH 4 with glacial acetic acid and the mixture was left stirring overnight (17 hr). The solution was passed down a column of IR-120 (H^+) cation-exchange resin and then down a column of Amberlite MB-3. The eluent was concentrated and passed down a 2 × 60 cm column of Sephadex G-10 from which 2.4-ml fractions were collected. The disaccharide fractions were run on gas–liquid partition chromatography as their trimethylsilyl derivatives; glucosylinositol was present but not idosylinositol. Hydrolysis of the di- and trisaccharide fractions from nitrite-degraded tri- and tetrasaccharides from the Sephadex column with 1 N HCl for 75 min at 100° was done. The resulting monosaccharides were examined by gas–liquid partition chromatography of their trimethylsilyl derivatives, showing peaks corresponding to inositol, glucose, and mannose in the case of the trisaccharide. Idosan could not be detected.

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