Structural Studies on Fucogalactan Produced by *Rhodotorula glutinis* K-24: Characterization of Acetyl Ester and Application of Sequential Periodate Oxidation

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The presence of O-acetyl ester on fucogalactan was suggested by IR spectrum and confirmed by gas-liquid chromatographic analysis. The determination of acetyl ester revealed the complete molar composition of fucogalactan to be fucose: galactose: acetic acid = 1:1:0.6. Sequential Smith degradation of the deacetylated fucogalactan gave a polysaccharide resistant to periodate oxidation, except for the terminal residues, suggesting the presence of a 1→3-linked core galactose-chain. Gas-liquid chromatographic analyses of hydroxy- and oxo-compounds produced by Smith degradation showed that all fucose residues were located in the side chains with some of galactoses. Further, every side chain was shown to be mainly composed of 1→2- and/or 1→6-linked sugars and terminated with fucose residue. These structural features were consistent with the results of linkage analyses of the partial acetolysis products from fucogalactan. The deacetylation of fucogalactan reduced greatly the intrinsic viscosity and increased the sedimentation coefficient.

The previous paper¹⁾ showed that the polysaccharide produced by *Rhodotorula glutinis* strain K-24 was composed of L-fucose and D-galactose in the approximate molar ratio of 1:1 and liberated a core galactan in partial acid hydrolysis. The present paper describes sequential Smith degradation and partial acetolysis studies applied to the fucogalactan. We also report about some physical properties of the biopolymer.

MATERIALS AND METHODS

Materials. Fucogalactan was prepared and purified as described previously.¹⁾ 1,2-Propanediol was of commercial origin, and 1,2,3-butanetriol was prepared by way of the epoxide from crotyl alcohol.²⁾

Sugar analysis. Reducing sugars in the acid hydrolysates of polysaccharides or polyalcohol derivatives were determined as follows: a polysaccharide (or polyalcohol, 50~100 µg) was placed in a small test tube (0.3×8 cm) and 0.1 ml of 1 N sulfuric acid was added. The glass tube containing the solution was sealed after evacuation and allowed to stand for 10 hr at 100°C. After cooling, 0.2 ml of an internal standard solution

containing 50 µg of xylose was added to the reaction mixture. The mixture was passed through a small column of Dowex 1×8 (0.2 ml of acetate form, 200~400 mesh) to remove sulfate ion, and the resin was washed with 1 ml of water. After evaporation of the effluent, the residue was dissolved in 50 µl of water and treated with 30 µl of 0.37 M sodium borohydride for 2 hr at room temperature. The excess borohydride was decomposed by the addition of 50 µl of acetic acid, and the solution was evaporated to dryness. Boric acid was removed by repeating dissolution of the residue in methanol and evaporation of the solution under reduced pressure. Then the residue was subjected to trimethylsilylation in 0.1 ml of dry pyridine with hexamethyldisilazane (50 µl) and trimethylchlorosilane (30 µl).³⁾ Gasliquid chromatography of the trimethylsilyl-derivatives was carried out with a Shimadzu Gas-chromatograph GC-4APF, equipped with a hydrogen-flame ionization detector. A glass column (0.4×200 cm) was packed with 3% OV-17 on Chromosorb W (60~80 mesh). The column temperature was 165°C and the flow rate of carrier nitrogen was 50 ml per min.

Measurement of IR spectra. Infrared spectra of polysaccharides were measured on KBr disks with a Hitachi Infrared Spectrophotometer EPI-S2. One mg of polysaccharide and 250 mg of potassium bromide were dissolved in 5 ml of water, and the solution was

lyophilized. The resulting powder was dried over phosphorus pentoxide and shaped to a disk.

Sedimentation analysis. The solution of 0.5% polysaccharide in 1% NaCl aq. was subjected to ultracentrifugation, as described in the previous paper.¹⁾

Measurement of viscosity. Viscosity of polysaccharide solutions was measured in a Ubbelohde-type viscometer at 30°C.

Determination of acetyl group on fucogalactan. Eighteen mg of the polysaccharide were dissolved in 3 ml of water, and an aliquot of the solution was used for the analysis of sugar content. Another aliquot (0.8 ml) was evaporated to dryness and methanolized with 1 ml of 3% methanolic hydrogen chloride at 100°C for 1 hr in a sealed tube. After cooling in ice-water, 0.2 ml of cold methanol containing 0.5 μ l of *n*-hexane as an internal standard was added to the reaction mixture, and 2 μ l of the resulting solution was immediately injected into a Gaschromatograph (Shimadzu GC-4APF). A steel column (0.3 $\times$ 300 cm) packed with 15% diethylene glycol succinate on Shimalite (60~80 mesh) was programmed from 40°C to 65°C at the rate of 2°C increment per minute, and the flow rate of carrier nitrogen was 19 ml per min. Various amounts of pentaacetyl- β -D-glucose corresponding to 100~500 μ g of acetic acid were treated exactly as described above to obtain a calibration line.

The acetyl ester on the polysaccharide was also determined by a titration method. One hundred mg of the polysaccharide were dissolved in 5 ml of water, and an equal volume of standardized 0.2 M sodium hydroxide was added to the solution. The mixture was stirred for 3 hr at room temperature and then titrated with 0.1 N hydrochloric acid using phenolphthalein as an indicator. The amount of acetic acid on the polysaccharide was calculated from the decrease of alkali in the solution.

Determination of degree of fucogalactan polymerization. The $\overline{DP}$ of fucogalactan was calculated from the result of the determination of formaldehyde produced by periodate oxidation of the reduced polysaccharide.⁴⁾

Deacetylation of fucogalactan. Native fucogalactan (500 mg) was treated with 0.1 N sodium hydroxide (100 ml) for 3 hr at room temperature. After neutralization of the reaction mixture with 0.5 N hydrochloric acid, the polysaccharide was precipitated with ethanol (70%, v/v) and separated by centrifugation. The precipitate was dissolved in water, dialyzed against distilled water, and lyophilized (450 mg). A small portion of the alkali-treated polysaccharide was supplied for measurement of IR spectrum to ascertain the deacetylation of fucogalactan.

Smith degradation

Sequential degradation of the deacetylated fucogalactan. The first step of the Smith degradation of fucogalactan was performed as follows: the deacetylated polysaccharide (400 mg) was oxidized with 0.1 M sodium periodate (100 ml) at 5°C for 2 days in the dark. The consumption of periodate was followed by measuring the absorbance at 290 nm.⁵⁾ The excess periodate was decomposed by the addition of ethylene glycol, and the mixture was subsequently treated with 1.5 g of sodium borohydride at 5°C overnight. After the excess borohydride was decomposed with acetic acid (pH 5), the solution was dialyzed against distilled water and lyophilized (DI-polyalcohol, 320 mg).

DI-polyalcohol (200 mg) was partially hydrolyzed with 0.5 N hydrochloric acid (40 ml) at 30°C for 3 hr. After neutralization of the controlled hydrolysis solution with sodium hydroxide, the reaction mixture was concentrated to a small volume, and applied on a column of Sephadex G-25 (3 $\times$ 150 cm). The elution of the products was carried out with 0.1 M acetic acid at a flow rate of 15 ml per hour, and carbohydrates in fractions were detected by the orcinolsulfuric acid reaction.⁶⁾ Fractions containing polysaccharides were combined and lyophilized (80 mg). The partially degraded polysaccharide (DI-polysaccharide) thus obtained was subjected to the second step of the Smith degradation.

DI-polysaccharide (55 mg) was treated as described in the first step of the Smith degradation, except that gel filtration with a column of Sephadex G-15 (3 $\times$ 150 cm) was adopted instead of dialysis to remove the low molecular weight substances. The second polyalcohol derivative (D2-polyalcohol, 40 mg) obtained was partially hydrolyzed as described above with the release of D2-polysaccharide.

Degradation of native fucogalactan. Native fucogalactan was also subjected to periodate oxidation followed by borohydride reduction to the corresponding polyalcohol derivative in the same way as described above.

Determination of hydroxy- and oxo-compounds in the polyalcohol derivatives. The hydroxy- and oxo-compounds produced by Smith degradation of the polysaccharides were determined according to the method of Yamaguchi *et al.*⁷⁾ To the aqueous solution (0.5 ml) containing 500 μ g of polyalcohol derivative were added 0.3 ml of 0.2 N hydrochloric acid and 0.2 ml of 2.5% hydroxylamine hydrochloride aqueous solution. The solution was heated at 80°C for 30 min in a sealed tube and evaporated to dryness under reduced pressure. The residue was dissolved in 0.1 ml of anhydrous pyridine (containing 100 μ g of trimethylolpropane as an internal standard), trimethylsilylated in the usual way,³⁾ and then analyzed by ^1_2glc .⁷⁾

Determination of the cleavage fragment situated at the aldehyde terminal of D1-polysaccharide. D1-polysaccharide (4 mg) was dissolved in water (2 ml), and an aliquot (50 μ l) of the solution was subjected to sugar analysis. Another aliquot (1 ml) was treated with sodium borohydride at room temperature for 2 hr. After the excess borohydride was decomposed with acetic acid, the solution was passed through a column of Dowex 50 $\times$ 8 (H^+ type). The effluent was evaporated to dryness under reduced pressure, and boric acid was removed as described under "Sugar analysis." The residue was methanolized with 1 ml of 3% methanolic hydrogen chloride at 80°C for 8 hr. The reaction mixture was evaporated to dryness, and the residue was trimethylsilylated.³⁾ The hydroxy-compound, glycerol or ethylene glycol derived from the aldehyde terminal of D1-polysaccharide was analyzed by glc as described under "Determination of hydroxy- and oxo-compounds."

Partial acetolysis of fucogalactan. The polysaccharide (255 mg) was dispersed in a mixture of 10 ml each of acetic acid and acetic anhydride and 1 ml of conc. sulfuric acid.⁸⁾ The mixture was stirred for 8 hr at 30°C, and then diluted with 100 ml of cold water. After the pH of the solution was adjusted to 5.0 by the addition of sodium carbonate, the acetylated products were extracted with five 20 ml portions of chloroform. The combined extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in 1 ml of methanol and evaporated again to remove the last traces of acetic anhydride.⁹⁾ Deacetylation of the products thus obtained was carried out by dissolving the sample in 0.5 ml of absolute methanol and adding a catalytic amount of sodium methoxide in methanol. The precipitate formed was dissolved by the addition of 1 ml of water and the solution was neutralized with diluted hydrochloric acid.

Fractionation and characterization of the partial acetolysis products. Gel filtration of the deacetylated products was carried out through a column of Sephadex G-15 (3 $\times$ 150 cm) equilibrated and eluted with 0.1 M acetic acid. The fractionated products were detected by the orcinol-sulfuric acid reaction,⁶⁾ and the appropriate fractions were lyophilized. Aliquots of the fractionated sugars were treated with sodium borohydride and desalted as described under "Sugar analysis." The reduced sugars obtained were treated with 1 N methanolic hydrogen chloride for 8 hr at 80°C in sealed tubes, evaporated to dryness, and then converted to trimethylsilyl derivatives in 0.1 ml of pyridine³⁾ containing 100 μ g of methyl α -D-glucopyranoside as an internal standard. Analyses by gas-liquid chromatography were performed using a stainless-steel column (0.3 $\times$ 200 cm) packed with 4% SE-52 on Chromosorb

W (80–100 mesh, treated with dichlorodimethylsilane). The column temperature was 175°C and the flow rate of carrier nitrogen was 30 ml per min.

The disaccharide fraction obtained by gel filtration was further fractionated by preparative paper chromatography (Whatman 3 MM) using *n*-butanol-ethanol-water (40:11:19)¹⁰⁾ as solvent. The location of the fractionated disaccharides was found by spraying alkaline silver nitrate reagent on strips cut away from both sides of the paper. Each disaccharide fraction was extracted with water from the paper corresponding to the locator zone, and an aliquot of the aqueous solution obtained was treated with borohydride, methanolized, and analyzed by glc as described above.

Gas-liquid chromatographic analysis of the linkage types of the disaccharides was performed according to the method of Yamaguchi *et al.*¹¹⁾

RESULTS AND DISCUSSION

1. Detection and determination of acetyl ester on fucogalactan

Infrared spectrum of fucogalactan was measured. As Fig. 1 shows, two absorption

FIG. 1. Infrared Absorption Spectra of Fucogalactan.

—, native polysaccharide; ----, alkali-treated polysaccharide.

peaks which could probably be assigned to O-acetylene were observed at 1725 cm^{-1} and 1260 cm^{-1} . To assure the existence of acetyl group, gas-liquid chromatographic analysis of the methanolysate of fucogalactan was tried. The liberated methyl ester was identified as methyl acetate (Fig. 2), and the molar ratio of acetic acid:sugar residue was found to be 1:3.2. This ratio was very close to that obtained by alkali-treatment of fucogalactan followed by titration with acid.

FIG. 2. Gas Chromatogram of the Methanolysate of Fucogalactan.

a, *n*-hexane (internal standard); b, methyl acetate; c, methanol.

Column, steel column packed with 15% diethyleneglycol succinate on Shimalite (0.3×300 cm); temperature, $40 \sim 65^\circ\text{C}$, $2^\circ/\text{min}$; carrier gas, nitrogen, 19 ml/min.

Thus, the molar composition of the polysaccharide was revealed to be fucose: galactose: acetic acid = 1: 1: 0.6.

2. Sequential Smith degradation

Fucogalactan was subjected to sequential Smith degradation to elucidate the structural features. The Smith degradation technique, which is based on periodate oxidation of a polysaccharide followed by reduction and analyses of the acid cleavage fragments of the resulting polyalcohol derivative, would provide detailed information about the structural features of the parent polysaccharide. However, the existence of O-acetyl esters on sugar residues may interfere with Malaprade oxidation of them. Therefore, the O-acetyl groups on fucogalactan were removed by mild alkali-treatment prior to periodate oxidation procedure. The infrared spectrum of the alkali-treated polysaccharide showed no absorption peaks due to an ester linkage (Fig. 1).

The polyalcohol derivative (D1-polyalcohol) was obtained by periodate oxidation of the deacetylated polysaccharide followed by borohydride reduction. This D1-polyalcohol was

partially hydrolyzed and subjected to gel filtration to obtain the smaller polysaccharide which was resistant to periodate oxidation in the first step of the Smith degradation (Fig. 3). Peak-1 indicates the elution of the

FIG. 3. Gel Filtration of the Controlled Hydrolysis Products of D1-polyalcohol.

Column, Sephadex G-25 (3×150 cm); solvent, 0.1 M acetic acid; flow rate, 15 ml/hr.

polysaccharide fraction (D1-polysaccharide) and peak-2 corresponded to the low molecular weight degradation products. D1-polysaccharide consisted of galactose and fucose in the molar ratio of 3.8:1 and gave a single peak in sedimentation analysis. No significant amount of either fucose or galactose was detected in the latter fraction.

Gas-liquid chromatographic analysis for the cleavage fragment situated at the aldehyde terminal of D1-polysaccharide detected only glycerol. Determination of the hydroxy compound showed that the mean degree of polymerization ($\overline{DP}$) of D1-polysaccharide was about 50. On the other hand, $\overline{DP}$ of the parent fucogalactan was determined to be about 120 in the reducing end analysis.⁴⁾ The difference between the two $\overline{DP}$ values is consistent with the fact that more than 50% of sugar residues of the parent fucogalactan were degraded by periodate in the first step of degradation (Table I).

D1-polysaccharide was subjected to the second step of degradation to prepare D2-polyalcohol. As shown in Table I, the

TABLE I. SMITH DEGRADATION PRODUCTS OBTAINED FROM POLYALCOHOL DERIVATIVES

The values are expressed as molar percentages of the products based on the total sugar residues in the native or deacetylated fucogalactan.

	Degradation products	Dl-Polyalcohol from native polysaccharide	Dl-Polyalcohol, D ₂ -Polyalcohol from deacetylated polysaccharide
From fucose unit	1, 2-Propanediol	34	36
	1, 2, 3-Butanetriol	1	2
From galactose unit	Glycerol	1	14
	Threitol	3	0
From fucose and galactose unit	Glycolaldehyde	23	36
	Glyceraldehyde	15	16
Residual sugars	Fucose	13	10
	Galactose	48	38

residual galactose in D1-polysaccharide was still mostly resistant to periodate oxidation in the second step of degradation. Partial hydrolysis of D2-polyalcohol gave a polysaccharide which contained solely galactose and reduced only a slight amount of periodate. These data suggest that D2-polysaccharide is a non-branched galactan containing mostly or entirely 1→3-linkage. In addition, D2-polysaccharide is no longer soluble in water, and this may be associated with a similar property of non-branched 1→3-linked glucans.¹²⁾ However, the fine structure of D2-polysaccharide which forms the inner part of fucogalactan will be elucidated by methylation technique.

Both D1- and D2-polyalcohol were submitted to gas chromatographic determination of the hydroxy- and oxo-compounds produced from degraded sugar residues (Fig. 4, Table I). As Table I shows, the residual fucose in D1-polysaccharide was completely degraded by periodate oxidation in the second step of degradation and gave 1,2-propanediol and glycolaldehyde with a trace amount of glyceraldehyde. These results indicate that those fucose residues were situated at the non-reducing terminals in D1-polysaccharide and attached directly to the core galactan forming branchings. On the basis of these results, we propose the following structure as the average repeating units of D1-polysaccharide:

FIG. 4. Gas Chromatogram of the Hydroxy- and Oxo-compounds Obtained by Smith Degradation of Deacetylated Fucogalactan.

a, 1,2-propanediol; b, glycolaldehyde (oximes); c, glycerol; d, 1,2,3-butanetriol; e, glyceraldehyde (oximes); s, trimethylolpropane (as an internal standard); x and y, impurities from reagents.

Column, steel column (0.3 × 300 cm) packed with 5% SE-30 on Chromosorb W; temperature, 100~180°C, 4°/min; carrier gas, nitrogen, 30 ml/min.

in which gal and fuc represent galactopyranosyl and fucopyranosyl residue, respectively. Thus, D1-polysaccharide has at least one side chain per 4 galactose residues.

In the first step of degradation (Table I), the predominant production of 1,2-propanediol suggests that most fucose residues are

situated at the non-reducing ends and/or linked at C₂, though the detection of 1,2,3-butanetriol showed the presence of a slight amount of 1→4-linked fucose and/or fucufuranosyl residue. On the other hand, galactose gave only glycerol as a hydroxy-compound, indicating that about 25% of galactose units are linked at C₂ and/or C₆ or located at the non-reducing end in the original polysaccharide. However, partial hydrolysis of the fucogalactan, as reported previously,¹⁾ provided evidence that galactose residues are situated in the inner part of the polysaccharide. Therefore, it is possible to deduce that all of the glycerols arose from galactose residues linked at positions C₂ and/or C₆, and that every side chain is terminated with fucose residue. Further, the analytical values of the oxo-compounds such as glycol aldehyde and glyceraldehyde, combined with those of the hydroxy-compounds, showed a high proportion of non-reducing terminal in this polysaccharide and a preponderance of 1→2-linked unit among the fucose residues.

The analytical data of the Smith degradation products of native fucogalactan gave interesting information about the positions of O-acetyl groups on the polysaccharide in comparison with those from the deacetylated fucogalactan. As shown in Table I, there were no significant differences between them in the oxidative degradation of fucose, whereas the presence of acetyl esters provided remarkable protection against the oxidation of galactose residues in native fucogalactan. The exact location of the acetic acid residues cannot be determined from the present results, but the above data indicate that most acetyl groups are linked to galactose residues. In addition, as Table I shows, a remarkable decrease in the production of glycerol and glycolaldehyde was observed in the case of native fucogalactan, while the glyceraldehyde production was almost unchanged. This indicates the preponderance of 1→6-linkage rather than 1→2-linkage with respect to the galactose residues in the side chains.

3. Partial acetolysis

In order to obtain additional information on the linkage types of sugar residues, the fucogalactan was subjected to partial acetolysis. The acetolysis products were fractionated by gel filtration on a column of Sephadex G-15 (Fig. 5). Gas-liquid chromatographic

FIG. 5. Gel Filtration of Acetolysis Products of Fucogalactan.

Column, Sephadex G-15 (3 × 150 cm); solvent, 0.1 M acetic acid; flow rate, 15 ml/hr.

analyses of the methanolysates of the borohydride-reduced fractions showed that F1, F2, and F3 were mixtures of monosaccharides, disaccharides, and trisaccharides, respectively. In this procedure, the total yield of these three fractions based on the original polysaccharide was about 60%. More than one-half this crop was comprised in the monosaccharide fraction (F1), in which fucose was detected predominantly. The disaccharide fraction (F2, amounting to about 15% of the original polysaccharide) was further fractionated by preparative paper chromatography with good separation. Three fractions, D-1, D-2, and D-3 were obtained. They were revealed to be fuc-fuc*, fuc-gal* (with a trace amount of gal-fuc*), and gal-gal* (asterisks represent the reducing ends), respectively, by gas-liquid chromatographic analyses. The amount of D-3 (gal-gal*) was very small compared with the others. A portion of each disaccharide fraction was analyzed for

elucidation of the linkage type by the method of Yamaguchi *et al.*¹¹⁾ Glyceric acid was obtained predominantly from all disaccharide fractions by bromine-oxidation followed by Smith degradation. This proves that not only most of fucose but a part of galactose are linked at position C₂. Judging from the results of the sequential Smith degradation studies, the 1→2-linkages in the disaccharides with reducing ends of galactose (D-2, D-3) may be assigned between the branching residues and the core galactose-chain. On the other hand, glycolic acid and ethylene glycol were detected from D-2 and D-3, supporting the presence of 1→6-linked galactose residues in the side chains.

4. Some physical properties of fucogalactan

Figure 6 shows the concentration dependence of viscosities of the native and the

FIG. 6. Concentration Dependence of Viscosity of Native and Deacetylated Fucogalactan.

○—○, native polysaccharide; ●---●, deacetylated polysaccharide.
Solvent, 1% NaCl aq.; temperature, 30°C.

deacetylated polysaccharides. The removal of acetyl groups reduced greatly the intrinsic viscosity (3.2 to 1.6 dl/g) and increased the sedimentation coefficient of fucogalactan (4.7S to 5.7S). This suggests that deacetylation caused a serious change in the conformation and/or intermolecular affinity of the

polysaccharide. However, no detailed studies have so far been reported on the effects of acetyl esters on the physical properties of polysaccharide.

On the basis of the results described above, we can give the following structural features of fucogalactan, leaving the exact linked positions uncertain at branching points. The polysaccharide contains a core galactose-chain linked mostly or entirely by 1→3-linkage. The core galactan has side chains, at least, in the proportion of one per 4 galactose residues. On the other hand, all fucose residues are located in the side chains, and most of them are situated at non-reducing ends or linked at position C₂. Furthermore, some galactose residues exist in the side chains, and they are linked mostly at C₆, though some of them may be linked at C₂. These structural features are quite different from fucogalactans of some fungus hitherto reported.^{13,14)}

Further investigations on the fine structural features of fucogalactan are being pursued by methylation technique.

REFERENCES

- 1) K. Fukagawa, H. Yamaguchi, D. Yonezawa and S. Murao, *Agr. Biol. Chem.*, **38**, 29 (1974).
- 2) D. Swern, J. T. Scanlan and G. B. Dickel, *Org. Syn.*, Coll. Vol. **4**, 317 (1963).
- 3) C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
- 4) A. M. Unrau and F. Smith, *Chem. Ind.*, London, **1957**, 330.
- 5) T. Ikenaka, *J. Biochem.*, **54**, 328 (1963).
- 6) C. François, R. D. Marchall and A. Neuberger, *Biochem. J.*, **83**, 335 (1962).
- 7) H. Yamaguchi, T. Ikenaka and Y. Matsushima, *J. Biochem.*, **68**, 253 (1970).
- 8) S. Peat, J. R. Turvey and D. Doyle, *J. Chem. Soc.*, **1961**, 3918.
- 9) H. Suzuki and E. J. Hehre, *Arch. Biochem. Biophys.*, **104**, 305 (1964).
- 10) P. A. J. Gorin and J. F. T. Spencer, *Can. J. Chem.*, **46**, 3407 (1968).
- 11) H. Yamaguchi and M. Kuriyama, *J. Biochem.*, **71**, 239 (1972).
- 12) T. Harada, A. Misaki and H. Saito, *Arch. Bio-*

- chem. Biophys.*, **124**, 292 (1968).
- 13) K. Axelsson and H. Björndal, *Acta Chem. Scand.*,
23, 1915 (1969).
- 14) A. Kardosova, K. Babor, J. Rosik and J. Kubala,
Chem. Zvesti., **23**, 454 and 462 (1969).
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