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PARTIAL METHYLATION OF METHYL α-D-MANNOPYRANOSIDE. PREPARATION AND DISTRIBUTION OF MONO-, DI-, AND TRI-METHYL ETHERS OF D-MANNOSE*

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ABSTRACT

The partial methylation of methyl α-D-mannopyranoside produced a mixture of methyl ethers that was fractionated by extraction with chloroform, followed by t l c. on a column of hydrocellulose. Ethers that are difficult to separate by this procedure were converted into their *O*-trityl or *O*-isopropylidene derivatives, or one component was removed by oxidation with periodate to aid the isolation of pure material By combinations of these procedures, it has been possible to isolate the 2,3,4,6-tetra-, 3,4,6-, 2,4,6-, 2,3,6-, and 2,3,4-tri-; 2,6-, 3,6-, and 3,4-di-; and 2-, 3-, and 6-methyl ethers of D-mannose The partial methylation of methyl α-D-mannopyranoside by the procedures of either Haworth, Kuhn, or Hakomori gave the same methyl ethers but the degree of substitution at each position differed markedly. Methyl sulfate and sodium hydroxide gave a relative substitution in the order 6-OH>2-OH>3-OH>4-OH, whereas methyl iodide and silver oxide in *N,N*-dimethylformamide gave the order 2-OH>3-OH>4-OH≥6-OH and methyl iodide and methylsulfinyl carbanion in methyl sulfoxide gave 2-OH>6-OH>4-OH≥3-OH The reactions were carried out with limiting amounts of methylating reagents and were not time controlled

INTRODUCTION

The application of methylation techniques to the structural elucidation of oligosaccharides and polysaccharides has been applied widely in spite of its limitations In early work, the isolation of unknown methyl ethers as cleavage fragments from the hydrolysis of methylated polysaccharides frequently gave enough material to establish the positions of the methoxyl groups by chemical means, so that the proof of structure of these ethers by synthesis was not critical With the introduction of more delicate microanalytical procedures, such as g l.c , for the analysis of such

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mixtures, it has become necessary to prepare known compounds in order to standardize the method both qualitatively and quantitatively.

D-Mannose is commonly found in the carbohydrate groups of glycoproteins, glycolipids, and many polysaccharides Reference samples of the methyl ethers required for structural studies may be synthesized unambiguously by the methylation of known derivatives of D-mannose, from which the protecting groups can be removed finally to give the corresponding free methyl ethers. Certain methyl ethers are difficult to prepare by this route because derivatives having proper protecting groups cannot be prepared easily[1] It seemed reasonable therefore to prepare a mixture of the methyl ethers of D-mannose, such as might be obtained from a methylated highly branched D-mannan, by partial methylation of methyl α-D-mannopyranoside and to apply chromatographic procedures for their separation

Of the several methods of methylation in the field of carbohydrate chemistry, probably those most generally used involve methyl sulfate and sodium hydroxide (Haworth method)[2], methyl iodide and silver oxide in *N,N*-dimethylformamide (Kuhn method)[3], and the more recent method of Hakomori with methyl iodide and methylsulfinyl carbanion in methyl sulfoxide[4]. In these methods, it is usual to use a large excess of the reagents to ensure as complete methylation as possible in each reaction The present study approached the question of the partial methylation of methyl α-D-mannopyranoside by using less of the methylating reagents than usual and, in some cases, an amount insufficient to complete the etherification of the four hydroxyl groups in the glycoside For clarity the results of the work are presented in two parts, first, the application of partial methylation to the preparation of methyl ethers of D-mannose and second, the relative distribution of these ethers in the reaction mixtures as it reflects the reactivity of the hydroxyl groups in methyl α-D-mannopyranoside

PREPARATION OF METHYL ETHERS OF D-MANNOSE

A mixture of methyl ethers of methyl α-D-mannopyranoside, prepared in this case by the Haworth method, was fractionated first by the batch extraction of an aqueous solution with chloroform to give a concentration of the tetra- and trimethyl ethers in the organic phase. A more extensive fractionation could have been achieved by the countercurrent extraction procedure of Bell[5] or by chromatography on a column of hydrocellulose[6], but the loading of sample on these columns does not permit a convenient separation of large quantities It is true, nevertheless, that some of the finer separations, such as that of 2,4,6- from 2,3,6-tri-*O*-methyl-D-mannose, can be most expeditiously achieved by t l c in spite of these limitations However, the attempt was made in this study to avoid such limitations wherever possible and to separate the components by preparing specific chemical derivatives, such as the 6-trityl ethers or the 1,2-*O*-isopropylidene derivatives, or by oxidizing away some of the products with periodate Depending upon the particular methyl ether of D-mannose required, so the procedures described here were modified slightly.

TABLE I

RELATIVE RETENTION TIME OF PARTIALLY METHYLATED MANNOSES AS TMS DERIVATIVES

Methyl ethers of methyl α-D-mannoside	*Relative retention times*[a]	
	Column B	*Column* C
2,3,4,6-tetra-	0 654	0 665
3,4,6-tri-	0 691	0 700
2,3,4-tri-	0 729	0 731
2,4,6-tri-	0 743	0 757
2,3,6-tri-	0 748	0 769
3,4-di-	0 767	0 790
3,6-di-	0 798	0 814
2 4-di-	0 817	0 828
2,6-di-	0 870	0 877
4-mono-	0 859[b]	0 859[b]
3-mono-	0 869	0 883
6-mono-	0 909	0 919
2-mono-	0 902	0 934
methyl α-D-mannoside	1 000	1 000

[a]Relative to the TMS derivative of methyl α-D-mannopyranoside [b]Determined by shoulder of the peak

The sugars extracted by chloroform, CE-F*, separated on t l c into methyl 2,3,4,6-tetra-*O*-, methyl 2,4,6-tri-*O*-, and a mixture of the other methyl tri-*O*-methyl-α-D-mannosides Alternatively, hydrolysis of CE-F to the reducing sugars and treatment with acetone–sulfuric acid gave specifically 1,2-*O*-isopropylidene-3,4,6-tri-*O*-methyl-D-mannose, which was extracted from an aqueous solution of the mixture by ether From the remaining trimethyl ethers, reconverted into the methyl glycosides, the 2,3,4-isomer was separated as the 6-trityl ether, and the unreacted sugars were separated by t l c to give methyl 2,3,4,6-tetra-, methyl 2,3,6-tri-, and methyl 2,4,6-tri-*O*-methyl-α-D-mannoside It was noted occasionally that the first acetalation step to isolate the 3,4,6-trimethyl ether was not quantitative, so this isomer, remaining in the mixture after tritylation, was removed by periodate oxidation of the reducing sugar forms.

A similar preliminary separation was achieved with the methyl ether fraction (CN-F*) not extracted by chloroform from the original total mixture of methyl ethers The CN-F was treated with chlorotriphenylmethane to give the 6-trityl ethers of methyl 2-*O*-, 3-*O*-, 3,4-di-*O*-, and 2,4-di-*O*-methyl-α-D-mannoside, which after hydrolysis to remove the trityl and methyl glycosidic groups, were separated by chromatography

*CE-F refers to the fractions of methyl ethers that are extracted by chloroform from an aqueous solution, which then contains the residual non-extractable (CN-F) methyl ethers

on hydrocellulose to give all except the 2-methyl ether as pure components (Table II) 2-*O*-Methyl-D-mannose was obtained by treating the monomethyl ether fraction with benzaldehyde and oxidizing the crude mixture of 4,6-*O*-benzylidene derivatives of the 2- and 3-methyl ethers with periodate, thus degrading the 3-methyl ether to the corresponding D-arabinose derivative After removal of the benzylidene group, the methylated sugars were chromatographed on hydrocellulose to give pure 2-*O*-methyl-D-mannose 3-*O*-Methyl-D-mannose was also isolated from the methyl mono-*O*-methyl mannosides after periodate oxidation and rechromatography

TABLE II

CHROMATOGRAPHIC FRACTIONATION OF FRACTION CN-F(T)

Fractions	*Tube*[a]	*Weight (mg)*	*Analysis by g l c.*
1	19–25	15	3,4-di-*O*-methyl-D-mannose
2	26–42	71	mixture of two di-*O*-methyl-D-mannoses
3	43–57	23	2,4-di-*O*-methyl-D-mannose
4	67–84	21	3-*O*-methyl-D-mannose
5	85–99	99	mixture of mono-*O*-methyl-D-mannoses
6	100–169	224	mixture of mono-*O*-methyl-D-mannoses
7	170[b]	647	D-mannose
	Total	1100 mg[c]	

[a]Volume of each fraction was 17 6 ml [b]After tube No 169, 1850 ml of the eluate were collected as one fraction (No. 170) [c]Recovery of methyl D-mannoside ethers was 91%

6-*O*-, 2,6-Di-*O*-, and 3,6-di-*O*-methyl-D-mannose were obtained pure after chromatography on hydrocellulose of those ethers in CN-F that did not form an insoluble 6-trityl ether (Table III).

TABLE III

CHROMATOGRAPHIC FRACTIONATION OF FRACTION CN-F(NT)

Fractions	*Tube*	*Weight (mg)*	*Analysis by g l c.*
1	26–50	290	3,6-di-*O*-methyl-D-mannose
2	51–65	250	3,6- and 2,6-di-*O*-methyl-D-mannose
3	66–75	150	2,6-di-*O*-methyl-D-mannose
4	76–80	60	2,6-di-*O*-methyl-D-mannose and 6-*O*-methyl-D-mannose
5	81–105	730	6-*O*-methyl-D-mannose
	Total	1480[a]	

[a]The recovery was 98 0%.

Many alternative schemes were tried to effect a simple separation of the products from the partial methylation of methyl α-D-mannopyranoside by methods that could

be applied to larger scale operations. Those outlined above require relatively few steps to produce gram quantities of all of the methyl ethers (except the 4-, 2,3-, and 4,6-isomers) all of which are well characterized compounds and for which authentic samples are available[1-7].

DISTRIBUTION OF THE METHOXYL GROUPS FROM PARTIAL METHYLATION

The three methods of methylation studied differ from each other in several ways The Haworth procedure is effected in an aqueous medium, whereas the other two are carried out in non-aqueous, polar solvents Because of the reaction of excess base with the methyl sulfate, the reagents are usually added in aliquot fractions, and in this study aliquot eighths were used Since the total amounts of alkali added were close to the theoretical amount for complete alkoxide formation (see Table IV), it was expected that the hydroxyl groups would be etherified in the order of their relative reactivities and, with the exception of the preponderance of the 2,4,6-trimethyl ether over the 2,3,6-trimethyl ether, this is found to be the case (Table V)

Although when striving for complete methylation by the Purdie or Kuhn procedures, with silver oxide, it is best to add the base in portions over a period of time, the purpose of the present study was best served by a single addition at the start of the reaction, which is heterogeneous from the standpoint of the alkoxide formation Alkoxide formation and alkylation take place concomitantly, which complicates an explanation of the relative ratios of the products (Table VI) and the overall preponderance of substitution at each position on the sugar ring (Table VII) If each hydroxyl group reacts independently, then from the results in Table VII, one might expect the preponderating monomethyl ether to be 2-methyl, and the di- and trimethyl ethers to be 2,3- and 2,3,6- or 2,3,4- As in the case of the products from the Haworth reaction, substitution at position 4 is present in the principal trimethyl ether (2,4,6-), and for the Kuhn method, the 2,4-dimethyl fraction was also not predicted.

The Hakomori procedure is different in several respects from the two discussed above Alkoxide formation is driven to equilibrium before any alkylating reagent is added Since the conditions of the present reaction involved only about 90% of the theoretical amount of base, the least reactive hydroxyl group(s) would appear after alkylation in the lowest amount, with the others in proportion to the rate of reaction. This was generally the case (see Tables VI and VII)

The different proportions of the reagents used are summarized in Table IV, where it will be noted that the largest excess over that required for complete methylation was 2 4 times (Kuhn, Expt 2) and that several ratios down to equimolar amounts (Haworth, Expt 1) were analyzed It is clear that the amount of more highly substituted ethers (CE-F) (Tables IV and V) increased as the proportion of the methylating reagents was raised but that even in the experiments where more than theoretical amounts were employed, the CE-F fraction does not predominate.

Regardless of the relative amounts of base and alkylating reagent, the distribution of the methyl ethers in each method remained essentially the same. The

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TABLE IV

REACTION CONDITION AND YIELD OF METHYLATED PRODUCTS IN HAWORTH, KUHN, AND HAKOMORI METHODS

Haworth method	*Methyl α-D-mannoside, mmoles*	*Me_2SO_4 mmoles*	*NaOH (30%) mmoles*	*CE-F g*	*CN-F g*	*Total g*	*Yield[a] %*
Expt. 1	51.5	51 5	187.5	0 35	8.92	9.27	92.7
2	20 0	40.0	62 6	0 53	2.64	3 17	94 0
3	51 5	154.5	187 5	2 17	7 10	9 27	92.7
4	51 5	309 0	237.5	4 75	5 10	9 85	98.5
Kuhn method	*Methyl α-D-mannoside mmoles*	*MeI mmoles*	*Ag_2O g*	*CE-F g*	*CN-F g*	*Total g*	*Yield %*
Expt. 1	18 8	94	18	0 32	3 00	3 32	90.5
2	18.8	184	22	0.72	2 85	3 57	97.0
Hakomori method	*Methyl α-D-mannoside mmoles*	*MeI mmoles*	*Methylsulfinyl carbanion mmoles*	*CE-F g*	*CN-F g*	*Total g*	*Yield %*
Expt. 1	5 2	17.7	18	0.14	0.78	0.92	92 0
2	5 2	35.4	18	0 36	0 58	0 94	94.0

[a]See text.

TABLE V

YIELDS AND RELATIVE RATIO OF METHYL α-D-MANNOSIDE AND ITS MONO-, DI-, TRI-, AND TETRA-SUBSTITUTED FRACTIONS

Methyl substituted methyl α-D-*mannoside*	*Haworth method*								*Kuhn method*				*Hakomori method*			
	Expt. 1		*Expt. 2*		*Expt. 3*		*Expt. 4*		*Expt. 1*		*Expt. 2*		*Expt. 1*		*Expt 2*	
	g	%	*g*	%	*g*	%	*g*	%	*g*	%	*g*	%	*g*	%	*g*	%
Mono-	3 12	33.7	1.23	38 4	3 39	36 5	1 24	12 6	1.27	38 4	0.86	24.3	0.26	28 2	0.20	21.0
Di-	1.56	16.8	0.46	14 5	2.19	23 6	2.74	27 8	1 25	37 8	1 31	37.0	0.17	18 5	0 23	24.0
Tri-	0.28	3.0	0.41	12.8	1 12	12 1	2.59	26 3	0 28	8.5	0.51	14 4	0.10	11 0	0 20	21.6
Tetra-	0 06	0 7	0 12	3 8	1.05	11.3	2 16	22 0	0 04	1 1	0.21	5.9	0 04	3.4	0.16	16.7
Methyl α-D-mannoside	4.24	45 9	0 96	30 4	1 53	16 3	1 14	11 5	0 45	13 6	0.65	18 4	0 35	38 0	0.15	16.4
Total recovery	9 26		3 18		9 27		9 87		3.31		3 54		0 92		0.94	
Average degree of substitution[a]	0 79	1.21		1 65		2.35		1.44		1.65		1 12		2 00		

[a]The percentage of each fraction was multiplied by the degree of substitution for that fraction and the four values were added together For complete methylation the value would be 4 00

TABLE VI

RELATIVE RATIO OF ISOMERS IN MONO-, DI- AND TRI-METHYL ETHERS

	Haworth method			*Kuhn method*		*Hakomori method*	
	Expt 1 %	*Expt 3* %	*Expt 4* %	*Expt 1* %	*Expt 2* %	*Expt 1* %	*Expt 2* %
Mono-							
2-	29 4	28 4	30 6	31 4	31.3	35 4	35 0
3-[a]	20 5	18 7	17 7	26 0	26 2	22 2	21 2
4-[a]	4 1	3 7	3 5	26 9	27 1	7 7	7 3
6-	46 2	49 0	47 9	15 5	15 3	34 7	36 5
Di-							
2,4-	24 8	27 7	27 6	43 6	45 6	55 4	55 5
2,6-	35 5	36 5	37 0	18 3	21 0	21 4	22 2
3,4-	6 6	6 3	4 0	9 9	8 7	1 9	3 3
3,6-	33 5	31 1	31 1	28 0	28 8	21 4	18 9
Tri-							
2,3,4-	22 3	20 0	21 4	26 4	26 3	36 2	34 2
2,3,6-[b]	26 3	27 0	27 4	9 7	9 0	32 7	34 0
2,4,6-[b]	30 5	31.1	32 2	35 4	36 2	25 8	25 0
3,4,6-	20 9	21 7	18 8	28 2	28 5	5 0	6 6

[a]These ratios were calculated from the results obtained from periodate oxidation of a mixture of 3- and 4-isomers (see Table IV) [b]These ratios were obtained by g l c for the Kuhn and Hakomori method and by t l c for the Haworth method

absence of certain isomers, such as 2,3- or 4,6-di-*O*-methyl-D-mannose, although carefully sought in the reaction products, may indicate these groups were never introduced in this combination, or that once formed, the subsequent alkylation to a trimethyl ether was very rapid The choice between these types of alternatives will best be made by studying the alkylation of partially methylated methyl α-D-mannosides or by rate studies, which are quite feasible with the analytical facilities now at hand.

The differences in reactivity between the hydroxyl groups in monosaccharides and polysaccharides has been studied principally for D-glucose[8] and D-glucans[9–11], where the reaction rates are in the order 6-OH > 2-OH > 4-OH > 3-OH for the Haworth method[8]. No similar study has been reported for D-mannose or by other methylation procedures. The differences in reactivity will depend upon such factors as electronic and steric effects, but the distribution of the individual methyl ethers in the reaction mixture will reflect other conditions, including (*1*) the extent to which each of the hydroxyl groups is converted into the alkoxide by reaction with the base, (*2*) the relative reactivity of each alkoxide group with the alkylating agent, (*3*) the excess of free base that can compete with the alkoxide for the alkylating agent, (*4*) the total amount of alkylating reagent added, (*5*) the change in reactivity of the hydroxyl and alkoxide groups as methoxy groups are introduced into the molecule, and (*6*) the nature and order of addition of the methylating reagents.

To the first order of approximation, the relative reactivities of the four hydroxyl groups in methyl α-D-mannopyranoside will be proportionate to their overall relative substitution, considering all of the methyl ethers. Such an analysis of the position of methylation for each method and condition in the present study is summarized in Table VII, with the method of calculation given in Table VIII for one experiment.

TABLE VII

OVERALL RELATIVE SUBSTITUTION OF HYDROXYL GROUPS AT C-2, C-3, C-4, AND C-6

Methylation method	*2-OH*	*3-OH*	*4-OH*	*6-OH*
Haworth				
Expt 1	1 00	0.72	0 40	1 32
Expt 3	1 00	0 78	0 64	1 23
Expt 4	1 00	0 79	0 73	1 05
Kuhn				
Expt 1	1 00	0 94	0 74	0 74
Expt 2	1 00	0 87	0 79	0 67
Hakomori				
Expt 1	1 00	0 59	0 62	0 75
Expt 2	1 00	0 67	0 70	0 79

Methyl sulfate and sodium hydroxide gave a relative substitution in the order 6-OH > 2-OH > 3-OH > 4-OH, whereas methyl iodide and silver oxide in *N,N*-dimethylformamide gave the corresponding order 2-OH > 3-OH > 4-OH ≥ 6-OH, and methyl iodide and methylsulfinyl carbanion in methyl sulfoxide gave 2-OH > 6-OH > 4-OH ≥ 3-OH. The reactions were effected with limiting amounts of methylating reagents and were not time controlled. These orders of substitution were also those present in the monomethyl ethers for the corresponding methylation methods and, particularly where the di- and tri-methyl fractions were least, such as Haworth experiment 1, the initial reaction rate-constants for each hydroxyl group were reflected by the proportions of these monomethyl ethers. The system is complicated, however, by the effect that one substitution or a combination of substitutions has upon the reactivity of another hydroxyl group. Spurlin has calculated the relative rate-constants for etherification from the substitution pattern in homogeneous reactions[12]. A more precise derivation would require 32 rate constants for each method of methylation, but it is probable that some of the eight constants for the overall conversion of hydroxyl to methoxyl groups for each position would differ only slightly within a single procedure Some exceptions were noted in the present study. Whereas the relative amount of the 4-methyl ether in the Haworth method is low, 25% of the dimethyl fraction is the 2,4-isomer and the 2,4,6-trimethyl ether is the principal isomer in that fraction Similar exceptions are noted for the Hakomori method. The 2- and 3-monomethyl ethers amount to about 50% of that fraction in the Haworth and Hakomori methods, yet the 2,3-dimethyl ether was not detected, but the 2,3,4-

TABLE VIII

METHOD OF CALCULATION OF RELATIVE SUBSTITUTION OF HYDROXYL GROUPS (HAWORTH METHOD, EXPT 1)

Isomer (% of fraction)		*mmoles*	*mMoles of methoxyl residue for each isomer* ($\times 10^2$)			
			2-OH	*3-OH*	*4-OH*	*6-OH*
Mono-*O*-methyl fraction (3 12 g, 15 mmoles)						
2-	29 4	4 4	4 4			
3-	20 5	3 1		3 1		
4-	4 1	0 6			0 6	
6-	46 2	6 9				6 9
Di-*O*-methyl fraction (1 56 g, 7 mmoles)						
2,4-	24 8	1 7	1 7		1 7	
2,6-	35 5	2 4	2 5			2 5
3,4-	6 6	0 5		0 5	0 5	
3,6-	33 5	2 4		2 4		2 4
Tri-*O*-methyl fraction (0 28 g, 1 1 mmoles)						
2,3,6-	26 3	0 3	0 3	0 3		0 3
2,4,6-	30 5	0 5	0 4		0 4	0 4
2,3,4-	22 3	0 3	0 3	0 3	0 3	
3,4,6-	20 9	0 2		0 2	0 2	0 2
Tetra-*O*-methyl fraction (0 06 g, 0 2 mmoles)						
2,3,4,6-		0 2	0 2	0 2	0 2	0 2
Total substitution			9 8	7 0	3 9	12 6
Relative substitution	6-OH (12 6)> 2-OH (9 8)> 3-OH (7 0)> 4-OH (3 9)					
or	6-OH (13 2)> 2-OH (10 0)> 3-OH (7 2)> 4-OH (4 0)					

and 2,3,6-isomers were present in significant amounts One explanation would be that the reactivity of the 4-OH group is influenced by substitution at the 2-OH position, and that the activity of the 3-OH group is enhanced by substitution at positions 2 and 4, or 2 and 6 The most reactive group in the Kuhn and Hakomori procedures is the 2-OH, and the Kuhn methylation has the lowest degree of substitution at the 6-position, quite the opposite of the Haworth method. The explanation of these results call for a more detailed study of actual rates of methylation of not only the methyl α-D-mannopyranoside but also the various methyl ethers From a practical standpoint, however, it is seen that some hydroxyl groups are more difficult to methylate than others and that these groups are not the same for the different methylation methods or for the various sugars A combination of methylation procedures has been commonly employed to achieve complete methylation, without which the structural studies of carbohydrates by this approach is misleading

EXPERIMENTAL

General. — Reagents were used without further purification Solutions were concentrated *in vacuo* with bath temperatures no higher than 50° Melting points (Fisher–Johns apparatus) are uncorrected. T l c was effected on plates (20 × 20 cm) of Silica gel G (E Merck, Darmstadt, Germany) with the organic layer of the mixture benzene–ethanol–water–ammonia (200 47 15 1, v/v) as developing solvent Multiple ascents were used, twice for CE-F and five times for CN-F. The zones were detected by exposure of the plate to iodine vapor, the mobilities of the mono-, di-, tri-, and tetra-methyl ethers relative to methyl α-D-mannopyranoside being 1 5 5 10 18, respectively. Each zone was carefully scraped from the plate and the sugar extracted three times with methanol (20 ml). The combined methanol extracts were evaporated *in vacuo* The residue was weighed and dissolved in water, and the sugar content determined by the phenol–sulfuric acid procedure. Colorimetric determinations by the phenol–sulfuric acid procedure were standardized by using D-mannose or the methyl ethers of methyl α-D-mannopyranoside The absorbancies for 10 μg of methyl α-D-mannopyranoside, and its mono-, di-, tri-, and tetra-methyl ethers were 0.153, 0.153, 0 086, 0 080, and 0 067, respectively. G l c was carried out on an F and M model 1609 instrument with a hydrogen flame detector Helium was used as the carrier gas Three types of column and chromatographic procedures were used *A*, neopentylglycol succinate (1/4″ × 7 5′), 10% liquid phase on Anakrom at 160°, *B*, SE-52 (Applied Science Laboratories) stainless steel column (1/8″ × 5′), 3% liquid phase on 80–100 mesh dichlorodimethylsilane-treated Chromosorb W, with a linear temperature program from 60 to 150° at 1° per min; *C*, SE-52, similar to *B* but with 4% liquid phase and programing from 60 to 165°. The retention times, relative to methyl α-D-mannopyranoside, of the trimethylsilyl (TMS)-derivatives are summarized in Table I for columns *B* and *C*

Periodate oxidation — The sugar or mixture was dissolved in 0 2M periodic acid at room temperature At appropriate intervals of time the absorbancy at 222 5 nm of an aliquot, diluted 250 times, was determined When the oxidation was complete, as indicated by no further change in absorbancy, the excess periodic acid was reduced with an equivalent of ethylene glycol The solution was neutralized with barium hydroxide and filtered, and the filtrate evaporated to a syrup, from which the methyl ethers were extracted twice with absolute ethanol The combined extracts were evaporated to a syrup, and the procedure was repeated until the final syrup was free from inorganic salts

Methylation procedures — A Methylation with sodium hydroxide and methyl sulfate A solution of methyl α-D-mannoside in water (5 ml) was maintained at 50° and calculated amounts of methyl sulfate and sodium hydroxide (30%) were added dropwise with vigorous stirring in 1/8 portions every 10 min After addition of the reagents, the reaction mixture was maintained for 1 h at 50–60°, and the reaction completed by heating for 30 min at 100°. During all these procedures, the reaction mixture was stirred vigorously The reaction mixture was cooled and neutralized to

Methyl Red indicator with 10% sulfuric acid, and an equal volume of ethanol (95%) was added. The salt that precipitated was removed and the filtrate was evaporated to a syrup.

B. Methylation with methyl iodide and silver oxide in N,N-*dimethylformamide.* Dry methyl α-D-mannoside in dry *N,N*-dimethylformamide (70 ml) was stirred magnetically overnight at room temperature to dissolve the sugar and calculated amounts of silver oxide and methyl iodide were added successively with cooling in an ice bath. The mixture was stirred for 48 h at room temperature after which time the insoluble material was removed by centrifugation, and water (50 ml) was added to the supernatant, which was extracted directly with chloroform (25 ml × 3) as the first step in the analysis

C. Methylation with methyl iodide and methylsulfinyl carbanion in methyl sulfoxide. Sodium hydride in a three-necked round-bottomed flask was washed three times by stirring magnetically with *n*-pentane (30 ml) After the final wash, the residual was removed *in vacuo* and dry methyl sulfoxide (30 ml) was added The suspension of sodium hydride in methyl sulfoxide was stirred under nitrogen at 50° until the solution became clear and evolution of hydrogen gas ceased The concentration of methylsulfinyl carbanion was determined by titration with 0 1M hydrochloric acid in aqueous solution

For the preparation of methyl α-D-mannoside alkoxide, dry methyl α-D-mannoside (1 g) was stirred under nitrogen at 60° with methyl sulfoxide (25 ml) until it was dissolved (about 20 min) After cooling to room temperature, a solution of methylsulfinyl carbanion was added and the reaction mixture was stirred for 4 h at room temperature For the methylation reaction, the mixture was maintained first at 20° in a cold bath and methyl iodide was added dropwise with continued stirring The reaction mixture was kept for 15 min at room temperature To the solution was then added iced water (50 ml) and extraction with chloroform (50 ml × 3) removed the most volatile ethers The combined chloroform extracts were washed with water (50 ml × 3) and evaporated to a syrup under diminished pressure. The water phase was combined with washings of chloroform extracts and evaporated to a syrup These two syrups were each dissolved in 25 ml of water and combined The combined solutions were extracted with chloroform (25 ml × 3) to commence the analyses

Analytical procedures — A. Chloroform extraction. The products of each methylation reaction were fractionated by batchwise extraction of an aqueous solution with chloroform (50 ml × 3) The combined chloroform extracts were washed with water (25 ml × 3) and evaporated to a syrup (CE-F) under diminished pressure The water phase and the washings were combined and deionized with Amberlite IR-120 (H^+) and Duolite A-4 (OH^-) resins The resulting solution was evaporated to a syrup (CN-F) under diminished pressure

B Determination of relative ratios of isomers in the methyl substituted fractions CE-F and CN-F were separated by t l.c. into methyl α-D-mannoside and its mono-, di-, tri-, and tetramethyl ethers, which were determined by the phenol–sulfuric acid method. Each fraction (*ca* 10 mg), dissolved in 1 ml of dry pyridine, was treated

with hexamethyldisilazane (0.2 ml) (Eastman Organic Chemicals Co) and chlorotrimethylsilane (0.1 ml) (Aldrich Chemicals Co), the reaction mixture being shaken vigorously for 30 sec and then kept for 15 min at room temperature[13]. An aliquot of the reaction mixture (usually 10 μl) was injected into the gas chromatograph with trimethylsilylated methyl α-D-mannoside as an internal standard. Methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside also was used as an additional standard. Areas of the peaks in g.l.c. were measured by planimeter and the relative amounts of each sugar calculated.

C. Relative amounts of methyl 3-O- and methyl 4-O-methyl-α-D-mannoside. The ratio of 3- and 4-methyl ethers of methyl α-D-mannoside, which were not separated by g.l.c., was determined by periodate oxidation. The monomethyl ether fractions, isolated by t.l.c. as described previously, were weighed and each mixed with methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside (9.20 mg). Each mixture was submitted to periodate oxidation for 48 h as described above and the resulting sugar mixture was re-isolated. The sugar mixtures before and after oxidation were analyzed by g.l.c. and the peak areas corresponding to methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside and methyl 3-*O*-methyl-α-D-mannoside were measured. The difference in peak area of the latter compound and the mixture before and after oxidation was due to methyl 4-*O*-methyl-α-D-mannoside. All of the peak areas of methyl 3-*O*- and methyl 4-*O*-methyl-α-D-mannosides were related to the internal standard of methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside. The results are summarized in Table IX.

TABLE IX

RELATIVE RATIO OF 3- AND 4-METHYL ETHERS

Methylation method	*Before IO_4^- oxidation*		*After IO_4^- oxidation*		*Calculated ratio*		*Percentage*	
	Tetra-[a]	*3- and 4-*[b]	*Tetra-*	*3-*	*3-*	*4-*	*3-*	*4-*
Haworth (Expt. 4)	1.00	1.50	1.00	1.27	1.27	0.23	83.3	16.6
Kuhn (Expt. 2)	1.00	6.12	1.00	3.00	3.00	3.12	49.1	50.9
Hakomori (Expt. 2)	1.00	0.77	1.00	0.56	0.56	0.21	74.3	25.6

[a]Methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside. [b]Mixture of methyl 3-*O*- and 4-*O*-methyl-α-D-mannosides.

D. Relative amounts of 2,3,6- and 2,4,6-tri-O-methyl-D-mannose. The relative ratio of 2,3,6- and 2,4,6-trimethyl ethers was determined in the trimethyl ether fraction by treating first with chlorotriphenylmethane in the usual way to remove methyl 2,3,4-tri-*O*-methyl-α-D-mannoside. After hydrolysis of the remaining glycosides with 0.5M sulfuric acid, the sugar mixture was oxidized with periodate to remove 3,4,6-tri-*O*-methyl-D-mannose, and the final mixture of trimethyl ethers was separated by g.l.c. of the methyl glycosides. The methyl 2,3,6- and methyl 2,4,6-tri-*O*-methyl-α-D-mannosides thus separated were determined by the phenol–sulfuric acid procedure

(see Table X) Alternatively, the sugars could be analyzed by g l c on column *C* which, unlike column *B*, effected a separation of the two components (see Table VI) The t l c. and g l c. methods agree closely (see Tables VI and X).

TABLE X

RELATIVE RATIO OF 2,4,6- AND 2,3,6-METHYL ETHERS BY T L C ANALYSIS

Method	*Position of substitution*			
	2,4,6- *mg*	*2,3,6-* *mg*	*2,4,6-* *%*	*2,3,6-* *%*
Haworth (Expt 4)	25 6	20 8	53 3	46 7
Kuhn (Expt 2)	19 2	5 1	79 0	21 1
Hakomori (Expt 2)	10 5	14 1	42 7	57.3

E. Yield of methylated products. Fractions CN-F and CE-F were weighed after deionizing with ion-exchange resins and the total recovery of methyl ethers was taken as the combined weights of CN-F and CE-F The weight of the products expressed in terms of the weight of methyl α-D-mannoside ranged from 90 5 to 98 5% (Table IV)

Fractionation of methyl ethers of D-*mannose* — The experiments described for the separation and identification of the methyl ethers of D-mannose refer to the product from the partial methylation by the Haworth method, with reagents in the proportions given in experiment 2 (Table IV) Where larger quantities of material were fractioned, the preparation still followed the conditions used in experiment 2

*2,3,4,6-Tetra-*O*-methyl-α-*D*-mannose* Methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside was isolated from CE-F by t l c After hydrolysis of the glycoside with 0 5M sulfuric acid as usual, *O*-methyl-D-mannose (4 22 mmoles) was dissolved in ethanol (5 ml), to which was added aniline (43 mg, 0 46 mmoles) The resulting solution was heated for 3 h at 95° and the alcohol was evaporated off *in vacuo* The residue was triturated with petroleum ether and the 2,3,4,6-tetra-*O*-methyl-*N*-phenyl-D-mannosylamine crystallized, m p 135–138°, not depressed upon admixture with an authentic sample; $[\alpha]_D^{26}$ −82 2° → −6.7° (*c* 0 9, methanol, after 12 h)

*Tri-*O*-methyl-*D*-mannoses* The methyl tri-*O*-methyl-α-D-mannoside fraction, isolated from CE-F by t l c , showed three peaks on g l c. by column *A* More complete separation of the components was achieved by means of various chemical derivatives with the single exception of 2,4,6-tri-*O*-methyl D-mannose As each component was isolated, it was identified by g l c comparison with an authentic sample

*A 2,4,6-Tri-*O*-methyl-*D*-mannose* The methyl tri-*O*-methyl-α-D-mannosides were separated further by t l c to yield methyl 2,4,6-tri-*O*-methyl-α-D-mannoside as a single faster-moving band and a second band contained the remaining sugars After hydrolysis, 2,4,6-tri-*O*-methyl-D-mannose (28.6 mg, 0 13 mmole) was treated with aniline (20 mg, 0 21 mmole) in ethanol (1 ml) for 5 h at 100° The ethanol was removed by evaporation and the *N*-phenylglycosylamine was crystallized and recrys-

tallized from ether; yield 25.6 mg, m p. 123–125°, $[\alpha]_D^{27.8}$ −145 → +2 1° (*c* 2 56, methanol), $[\alpha]_D^{29}$ −152 5 → +6 2° (*c* 1.01, methanol) [lit.[14], m.p. 134°, $[\alpha]_D$ −150 → +8° (methanol)]

B 2,3,4-Tri-O-methyl-D-mannose The methyl tri-*O*-methyl-α-D-mannoside fraction (505 6 mg) was dissolved in pyridine (2 ml) and chlorotriphenylmethane (500 mg) was added After shaking until the reagents had dissolved, the reaction mixture was kept for 48 h at room temperature and water was added until the solution became turbid The mixture was stirred for 1 h and was then poured into 100 ml of water and stirred for a further 2 h The trityl derivatives were extracted with ethyl ether (50 ml × 5); the non-tritylated methyl ethers were isolated from the aqueous solution (see *C*) The combined ether extracts were washed with saturated sodium hydrogen sulfate (20 ml × 3), sodium hydrogen carbonate (20 ml × 3), and water (30 ml × 3) The extracts were dried (sodium sulfate) and evaporated to a syrup (50 mg) The syrup was dissolved in glacial acetic acid (2 ml) and 50% HBr (0 2 ml) was added After a few min, the precipitate that formed was filtered off and the filtrate was poured into about 100 ml of iced water containing sodium hydrogen carbonate The methylated sugar was extracted from the solution with chloroform (30 ml × 3) and the combined extracts were washed with sodium hydrogen carbonate solution and water, and evaporated to syrup. The syrup was dissolved in ethanol (1 ml), water (5 ml) added, the precipitate was removed by filtration, and the filtrate was evaporated to a syrup. This procedure was repeated until the ethanol solution was not turbid after the addition of water The final syrup was subjected to t l c to give 22 mg of methyl 2,3,4-tri-*O*-methyl-D-mannoside, $[\alpha]_D^{26}$ +44.3° (*c* 4 42, water) [lit.[15], $[\alpha]_D$ +47° (water)]

C 3,4,6-Tri-O-methyl-D-mannose The residual methyl tri-*O*-methyl-α-D-mannosides (389 mg) from (*B*) above were hydrolyzed for 15 h at 100° with 0 5M sulfuric acid (20 ml) in the usual way. The free sugars were dissolved in acetone (10 ml) containing concentrated sulfuric acid (0 25 ml) and the reaction mixture was stirred for 4 h at room temperature, after which time it was neutralized with anhydrous sodium carbonate The precipitate was removed and the filtrate was refluxed for 1 h in the presence of anhydrous sodium carbonate. The acetone solution was then evaporated to a syrup, which was purified by t l c to yield 1,2-*O*-isopropylidene-3,4,6-tri-*O*-methyl-D-mannose as a syrup (46 mg). The isopropylidene group was removed at 100° with 10 ml of 1% sulfuric acid and the resultant 3,4,6-tri-*O*-methyl-D-mannose (30 mg) showed $[\alpha]_D^{28.5}$ +10 1° (*c* 2 96, water), [lit[14], $[\alpha]_D$ +21 → +8.1° (water)]

The syrupy 3,4,6-tri-*O*-methyl-D-mannose was heated for 12 h under reflux with 3.75% methanolic hydrogen chloride After cooling, the solution was neutralized with silver carbonate, the suspension was filtered, and the filtrate was evaporated *in vacuo* to a syrup (10 mg) This was analyzed by gas chromatography and identified as methyl 3,4,6-tri-*O*-methyl-α-D-mannoside

D 2,3,6-Tri-O-methyl D-mannose Commencing with CE-F (4 5 g), the glycosides were hydrolyzed with 0 5M sulfuric acid, the free sugars in the mixture were treated

with acetone (120 ml) and concentrated sulfuric acid (2 8 ml) as usual, and the product was isolated and dissolved in water (100 ml), from which solution the isopropylidene acetals were extracted with ethyl ether The sugars remaining in the aqueous solution were converted into the methyl glycosides (3 14 g), which were treated with chlorotriphenylmethane (3 5 g) in pyridine (12 ml) as described under (*B*). The precipitated crude methyl 6-*O*-trityl-2,3,4-tri-*O*-methyl-α-D-mannoside was filtered off and the filtrate was evaporated to a syrup (2 45 g) from which the methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside was removed by t l c. and residual methyl tri-*O*-methyl-α-D-mannosides were isolated as a syrup (0 92 g) The syrup still contained some of the 3,4,6-trimethyl ether, which was removed by periodate oxidation after hydrolysis of the glycosides with 0.5M sulfuric acid for 16 h at 100°. The resulting syrup (0 43 g) was reconverted into the methyl glycosides and chromatographed by t l c to give two components, methyl 2,4,6-tri-*O*-methyl-α-D-mannoside (0 16 g), $[\alpha]_D^{29}$ +5 0° (*c* 2 83, methanol), and the slower moving methyl 2,3,6-tri-*O*-methyl-α-D-mannoside (0 14 g), $[\alpha]_D^{29}$ +5 0° (*c* 2 83, methanol).

The 2,4,6-trimethyl ether was hydrolyzed to the free sugar (0.14 g), $[\alpha]_D^{29}$ +14.3° (*c* 4.18, water)[14], which was converted into the *N*-phenylglycosylamine as described under (*A*), m p. 128–131°, $[\alpha]_D^{29}$ −149 → +4 2° (*c* 1 25, methanol)

The 2,3,6-trimethyl ether was similarly hydrolyzed to the free sugar, a syrup (0 11 g), $[\alpha]_D^{29}$ +7.4° (*c* 2 16, water), a portion (30 mg) of which was refluxed with aniline (43 mg) in ethanol (1 ml) for 4 h to give 2,3,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine, m p. 126–128°, $[\alpha]_D^{29}$ −158 → −31° (*c* 1.10, methanol, after 24 h) [lit [16] m p. 127–128°, $[\alpha]_D$ −155 → −39° (in methanol)]

Fractionation of CN-F fraction. — Tritylation To CN-F (10 g) in dry pyridine (20 ml) was added chlorotriphenylmethane (10 g). After 48 h at room temperature, the solution was cooled to 0° and water added with stirring until a turbidity was produced The turbid solution was stirred for 1 h and poured into ice–water (500 ml), and the syrupy precipitate was washed with water to remove the residual pyridine. During this time the syrup crystallized and the crude trityl ethers were isolated by filtration and dried over phosphorus pentaoxide; yield 13.5 g. The aqueous mother liquors and washings were concentrated together to about 100 ml, extracted with chloroform to remove the triphenylcarbinol, and evaporated *in vacuo* to a syrup (2.9 g), designated CN-F(NT).

The tritylated methyl mannoside ethers (13 g) were dissolved in glacial acetic acid (25 ml) by gentle warming To the solution, previously cooled to room temperature, was added HBr (50%, 4 ml) and after 3 min the precipitate formed was filtered off The filtrate was poured into iced water (50 ml) containing a slight excess of sodium hydrogen carbonate and passed through Amberlite IR-120 (H^+) followed by Duolite A-4 (OH^-), to remove sodium acetate. The eluate was concentrated under diminished pressure to a syrup (5.8 g), designated CN-F(T).

Separation of CN-F(T) by column chromatography on hydrocellulose — Fraction CN-F(T) was hydrolyzed with 0.5M sulfuric acid as usual to give the mixture of free sugars as a syrup (5.56), which was dissolved in water (10 ml) A portion (2 2 ml,

equivalent to 1 21 g of methyl sugars) was applied to a column of hydrocellulose[6] at 30.5° and methylated sugars were developed with butanone–water azeotrope The fractions were examined by g l c. and combined as shown in Table II or were collected as dimethyl and monomethyl ethers from a knowledge of the chromatographic behavior of the column

3,4-Di-O-methyl-D-mannose Fraction 1, Table III, $[\alpha]_D^{27.3}$ +1.1° (*c* 4 32, water) [lit [14], $[\alpha]_D^{27.3}$ +3° (water)] was converted into the methyl glycoside, $[\alpha]_D^{27.3}$ +66 9° (*c* 3 8, water) [lit [14], $[\alpha]_D$ +67° (water)], which co-chromatographed with an authentic sample of methyl 3,4-di-*O*-methyl-α-D-mannoside on g l c

2,4-Di-O-methyl-D-mannose. Fraction 3, Table III, $[\alpha]_D^{30.5}$ +11.0° (*c* 2 52, water) [lit [7], $[\alpha]_D^{25}$ +13 5° (*c* 0 6, water)] did not crystallize It was converted into the methyl glycoside, $[\alpha]_D^{29}$ +41.7° (*c* 1.26, chloroform), $[\alpha]_D^{27}$ +32 0° (*c* 1 01, methanol) [lit.[7], $[\alpha]_D^{25}$ +47.6° (*c* 1.0, ethanol)]

3-O-Methyl-D-mannose. A combined monomethyl ether fraction (160 mg) from a column chromatogram was converted into the methyl glycosides (134 mg), which were oxidized with periodic acid as described above. The product was hydrolyzed with 0 5M sulfuric acid as usual and rechromatographed on a column of cellulose to give 3-*O*-methyl-D-mannose as a syrup, $[\alpha]_D^{25}$ +2 7° (*c* 2 50, water) [lit [17], $[\alpha]_D^{19}$ +3° (*c* 0 60, water)]

2-O-Methyl-D-mannose. A combined fraction of monomethyl ethers (0.7 g) was stirred with freshly distilled benzaldehyde (20 ml) in the presence of powdered zinc chloride (2 g) for 4 h at room temperature The mixture was then cooled to 0° and diluted with water (50 ml), and after 30 min, the solid precipitate was filtered and washed successively with cold water (10 ml × 2) and cold *n*-pentane (10 ml × 2) The dried mixture of benzylidene derivatives (200 mg) was dissolved in aqueous *p*-dioxane and oxidized with 0 2M ammonium periodate for 15 days at room temperature The oxidation was terminated by adding ethylene glycol and the solution was evaporated to dryness The residue was extracted with ethanol (10 ml × 2) and the extract was evaporated to a syrup, which was deionized by passing an aqueous solution through Amberlite IR-120 (H^+) and Duolite A-4 (OH^-). The resulting solution was brought to 0 1M with respect to sulfuric acid and kept for 1 h at 60° Neutralization with barium hydroxide, and filtration and evaporation of the filtrate gave a syrup that was re-chromatographed on a column of hydrocellulose to give 2-*O*-methyl-D-mannose (68 mg), $[\alpha]_D^{25}$ +4.2° (*c* 1.21, water) [lit.[17], $[\alpha]_D^{19}$ +5° (*c* 1 3, water)]

The 2-methyl ether (50 mg) in water (1 ml) containing phenylhydrazine hydrochloride (0 1 g) and sodium acetate (0 2 g) was refluxed for 1 h D-*arabino*-Hexulose phenylosazone crystallized, m p. 198–200°, not depressed upon admixture with an authentic sample, $[\alpha]_D^{27.5}$ −55 → −35° [*c* 1.01, 6 4 (v/v) ethanol–pyridine] [lit [18], m p. 204–206°, $[\alpha]_D$ −65 → −34° (in ethanol–pyridine)]

Separation of CN-F(NT) by cellulose column chromatography — Fraction CN-F(NT) (1 76 g) was hydrolyzed with 0 5M sulfuric acid as usual to give the free sugars (1 52 g), which were separated on a column of hydrocellulose[6]. The results are summarized in Table III

3,6-Di-O-methyl-D-mannose Fraction 1, Table III, was chromatographically pure and the 3,6-dimethyl ether in fraction 2 could be isolated from the 2,6-dimethyl isomer by periodate oxidation of the methyl glycosides followed by rechromatography of the free sugars. 3,6-Di-*O*-methyl-D-mannose was isolated from fraction 2 in this way as a syrup (59 mg), $[\alpha]_D^{29.5}$ +22.4° (*c* 1.82, water); $[\alpha]_D^{29.5}$ +32.4° (*c* 1.86, methanol).

2,6-Di-O-methyl-D-mannose Fraction 3, Table III, gave a methyl glycoside $[\alpha]_D^{27.5}$ +50.3° (*c* 0.64, methanol). A sample (70.6 mg) was converted into 6-*O*-methyl-D-*arabino*-hexulose phenylosazone m.p. 169–172°, not depressed upon admixture with an authentic sample, $[\alpha]_D^{26}$ −70.5 → −39.6° (*c* 1.10, ethanol, after 15 h), [lit.[19], m.p. 172 $[\alpha]_D$ −68.6 → −48.0° (ethanol)]

6-O-Methyl-D-mannose Fraction 5 gave a syrup, $[\alpha]_D^{28}$ +14.0° (*c* 2.25, chloroform) [lit.[19], $[\alpha]_D$ +15.3° (chloroform)] which was converted in part into the methyl glycoside, $[\alpha]_D^{27.5}$ +83.5° (*c* 0.58, methanol) and is part into the 6-*O*-methyl-D-*arabino*-hexulose phenylosazone m.p. 172–175°, not depressed upon admixture with an authentic sample, $[\alpha]_D^{27.5}$ −65.2 → −45.0° (*c* 1.25, ethanol, after 15 h)

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