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Elimination of Oxidative Degradation during the per-*O*-Methylation of Carbohydrates

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Abstract: The possible oxidative degradation mechanism occurring during the per-*O*-methylation of carbohydrates in dimethyl sulfoxide with methyl iodide in the presence of base is described. Evidence is presented that this process occurs only under anhydrous conditions when there is a long reaction time between the carbohydrate dissolved in dimethyl sulfoxide and methyl iodide, followed by reaction with the base. Under these specific conditions, the oxidative degradation of alditols, and cyclic carbohydrates, with and without a free hemiacetal group, is observed. The reaction between carbohydrate and methyl iodide in dimethyl sulfoxide can result in a complete oxidative degradation depending on the type of carbohydrate and the time of reaction. The oxidative degradation can be accelerated by replacing methyl iodide with dimethyl sulfate. Mass spectrometric identification of the degradation products of D-glucitol indicates simultaneous oxidation processes at all the hydroxyl groups, with site dependent rates of their reactivity. This oxidative process is not a characteristic of the methylation of carbohydrates in dimethyl sulfoxide with methyl iodide in the presence of solid sodium hydroxide and can be totally avoided by treating the carbohydrate with powdered sodium hydroxide before introduction of methyl iodide under nonanhydrous conditions, or by adding a trace of water in dimethyl sulfoxide before methyl iodide, or by using *N,N*-dimethylacetamide as the solvent. The degradation of the per-*O*-methylated carbohydrates in the liquid−liquid extraction process is also taken into account, and it is found that the degradation process can be avoid by neutralization of the base before extraction, by using benzene or tetrachloromethane as extraction solvent, and by drying the final organic extract.

Introduction

The structural analysis of polysaccharides is performed by sophisticated instrumentation such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry. Among the instrumental methods, MS has become an indispensable tool, and although it can be performed on many underivatized oligosaccharides and glycoconjugates, the analysis of methylated derivatives is still preferred, because the per-*O*-methylation of polysaccharides confers to the structural analysis several advantages in the determination of the molecular mass, of the monosaccharide composition, of the sequence of monosaccharide residues, of the position of branches, of the interglycosidic linkages, and of the presence of configurational and conformational isomers.

It has been 100 years since the first *O*-methylated sugar was prepared by Prudie and Irvine[1] by treating carbohydrates dissolved in methanol with methyl iodide (MeI) in the presence of silver oxide. In 1915, Haworth[2] added a solution of sodium hydroxide (NaOH) and dimethyl sulfate (Me₂SO₄) to an aqueous solution of carbohydrates. Both methods gave partially *O*-methylated carbohydrate. per-*O*-Methylation was obtained by

several remethylations of the material recovered by extraction. Until the 1960s, these two methylation methods, with some modifications, were used for preparative and analytical *O*-methylation of carbohydrates. In 1964, Hakomori[3] performed the per-*O*-methylation of polysaccharides in one step, by adding a solution of sodium methylsulfinyl carbanion (Na dimsyl) and methyl iodide to the carbohydrate dissolved in dimethyl sulfoxide (Me₂SO). This method was improved,[4−6] especially by using potassium dimsyl and lithium dimsyl, but the preparation of the dimsyl reagent is still laborious and hazardous because moisture, air, and carbon dioxide must be rigorously avoided during its preparation and storage, to minimize side-reactions and to enhance the purity of the final analytical product. These considerations are most important when small amounts of polysaccharides are to be analyzed. To obtain unequivocal results, full *O*-methylation is a prerequisite condition. However, a single treatment of complex carbohydrates with Na dimsyl often results in incomplete methylation.[7−9] These undermethylated materials are extracted and remethylated, but

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small amounts of undermethylated complex carbohydrates can still exist even after very considerable effort. [7]

Some of these disadvantages could be overcome by avoiding the presence of Na dimsyl in the reaction mixture by directly adding powdered sodium hydride (NaH) and methyl iodide[10] into the carbohydrate solution in dimethyl sulfoxide. It was found that per-*O*-methylation of carbohydrates can also be performed in *N,N*-dimethylacetamide[10] and *N,N*-dimethyl-formamide[11] solution where the Na dimsyl does not exist. The per-*O*-methylation yields were higher and the products were cleaner. However, this methylation method was not widely used because of the dangers of working with alkali metal hydrides that are flammable and moisture-sensitive, and thus must be handled only in small quantities and with extreme care. Another attempt was the adding of solid sodium hydride in small portions, with gentle stirring during 1 h, into the carbohydrate dimethyl sulfoxide solution, followed by dropwise adding of methyl iodide over 2h,[12,13] but the formation of Na dimsyl could not be avoided.

Methylation of carbohydrates in dimethyl sulfoxide by treating with powdered sodium hydroxide and methyl iodide was introduced by Ciucanu et al. for per-*O*-methylation of carbohydrates,[10,14,15] polyols,[16] fatty acids, and hydroxyl fatty acids,[17] and has had its most widespread application in the permethylation of complex polysaccharides, glycolipids and glycoproteins, because *O*- and N-methylation are both possible.[18] This methylation method avoids all the above disadvantages. The methylation can be performed without special care for the prevention of the contact with moisture or air, the yield is high, the final product is cleaner and the reaction time is up to 20 times shorter. However, York et al.[19] found, without giving exact details of the experimental procedure used, that Ciucanu method of per-*O*-methylation can give certain artifacts in the glycosyl-linkage analysis of oligoglycosyl alditols obtained from complex carbohydrates with reducing end residues. This oxidation was not detected during Hakomori methylation. The authors[19] suggested that the results were consistent with oxidation of the alditol hydroxyl groups by the reagent, and proposed a mechanism for this. Later, Needs and Selvendran[20] noted a confusion of some authors over the conditions used in Ciucanu method. They observed this oxidative degradation for the alditol residue when the per-*O*-methylation was performed under argon and anhydrous conditions, as in the Hakomori method, but adding the methyl iodide first and finely powdered sodium hydroxide after a while. They suggested that this oxidation can be reduced under anhydrous conditions by adding the base before the methylating agent. Oxidative degradation was also observed even with Hakomori method when the base was added first. However, no definite conclusions about the effect of varying the duration of the treatment with either base or methylating agent could be drawn.

This paper investigates the mechanism of the oxidation process and ways to avoid the oxidation of the carbohydrate, with an accent on the alditols, during per-*O*-methylation with methyl iodide, and powdered sodium hydroxide in dimethyl sulfoxide.

Experimental Section

Reagents. Raffinose, trehalose, cellobiitol, cellobiose, maltose, maltitol, D-glucitol, D-glucose, D-fructose, naphthalene, anthracene, sodium hydroxide (pellets), sodium hydride (dry, 95%), methyl iodide (stabilized by copper), anhydrous methyl alcohol (99.9%), dimethyl sulfoxide (99.9%), and *N,N*-dimethylacetamide (99.9%) were from Sigma-Aldrich (Milwaukee, WI) and were of the highest grade available.

Instrumentation. A TRACE GC Series 2000 gas chromatograph coupled to a quadrupole ion trap GCQ[plus] mass spectrometer (Thermo-Quest, Austin, TX) was used. High purity helium was used as carrier gas at a constant flow rate of 1 mL/min. All gas chromatographic (GC) separations were performed on a DB-5MS fused silica capillary column (30 m × 0.25 mm i.d.) with 0.25 μm poly(dimethylsiloxane) cross-bonded film (J&W Scientific).The GC oven temperature was maintained at 70 °C for 5 min after sample injection, increased to 100 °C at 30 °C/min, then increased to 240 °C at 6 °C/min, and maintained at 240 °C. The mass spectra were recorded in the positive-ion electron ionization (EI) mode over the scan range *m/z* 50 to 600. The filament emission current was 20 μA at 200 °C; it was turned off for the first 3 min. The transfer line temperature was 270 °C. The electron multiplier voltage and automatic gain control target were set automatically. Positive-ion chemical ionization (CI) was performed with methane as reagent gas at 0.3 mL/min. Other parameters were the same as were used to the EI mode. Identification of the peaks was performed by interpretation of the fragmentation patterns for EI mass spectra as well as by the comparison of the spectra with mass spectra of standards, of the MS library, and from the literature. Quantitation of permethylated carbohydrates was carried out by integration of the peak area and using naphthalene and anthracene as internal standards.

The methylation reactions were performed at 22 °C using 2 mL-glass-vials having a V shape and with silicone septa lined screw-caps and a magnetic stirrer (VWR Scientific, Marlboro, MA).

Analytical Methods

Micro-Solid-Phase Extraction with Helical Sorbent.[21] The dimethyl sulfide and other volatile organic compounds were extracted at room temperature from the headspace of the vial with a poly (dimethylsiloxane) helical sorbent. The volatile organic compounds generated after 30 min of reaction between D-glucitol and methyl iodide in dimethyl sulfoxide, followed by 10 min reaction in the presence of sodium hydroxide under anhydrous conditions were extracted for 30 s with the helical sorbent and were analyzed by GC–MS.

Hakomori Methylation Method with Sodium Dimsyl and Methyl Iodide in Dimethyl Sulfoxide.[22] Sodium dimsyl was prepared in a dry 500-mL three-necked round-bottom flask by adding dry sodium hydride (8 g) to anhydrous dimethyl sulfoxide (100 mL) under a dry nitrogen atmosphere. The magnetically stirred reaction mixture was heated slowly to 50 °C and maintained at this temperature until hydrogen evolution had ceased. The mixture was centrifuged to produce a clear, gray-green solution. The solution was then aliquoted in 1-mL portions under nitrogen into Teflon-lined screw-capped vials and frozen at −20 °C. For permethylation, the carbohydrates (5–7 mg) were introduced into a conical vial, which was dried overnight under vacuum, and then with dry nitrogen, and was sealed. Dimethyl sulfoxide (1 mL) was added by dry syringe, followed by Na dimsyl (3 equiv per mol of replaceable H) (0.3 mL Na dimsyl solution), and the reaction mixture

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was stirred at room temperature for 1 h. Methyl iodide (3 equiv per mol of replaceable H) (~90 μL) was added after cooling the reaction mixture in an ice bath. After flushing with dry nitrogen, stirring was continued at room temperature for 2 h.

Ciucanu Methylation Method with Sodium Hydroxide and Methyl Iodide in Dimethyl Sulfoxide.[10] To a carbohydrate sample (5−7 mg) introduced into a conical-glass vial, dimethyl sulfoxide (1 mL) was added without using special drying conditions or inert gas atmosphere. The sodium hydroxide pellets were ground in a dry mortar and a pestle to obtain a fine powder. A weighed amount of powdered sodium hydroxide (3 equiv per mol of replaceable H) (~30 mg) was added to the sample solution and was stirred at room temperature for a while to get a suspension. Methyl iodide (3 equiv per mol of replaceable H) (~60 μL) was added with a syringe, and the mixture was stirred vigorously for 10 min.
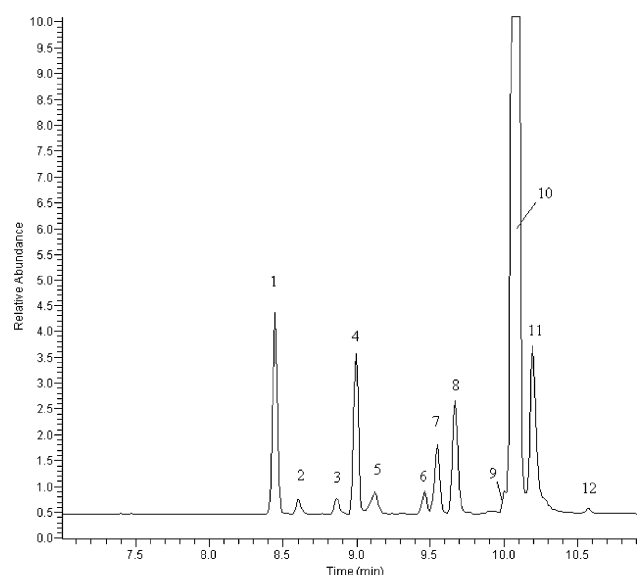
Methylation with Methyl Iodide and Sodium Hydroxide in Dimethyl Sulfoxide under Anhydrous Conditions. The carbohydrate sample (5−7 mg) was dried overnight under vacuum in a conical-glass vial at 50 °C. The vial was flushed with dry nitrogen at the room temperature. Dimethyl sulfoxide (1 mL) and then methyl iodide (3 equiv per mol of replaceable H) (~60 μL) were introduced with dry syringes, and the solution was stirred for 30 min under anhydrous nitrogen atmosphere. The sodium hydroxide pellets, were ground using a mortar and a pestle, and then dried overnight at 80 °C under vacuum; the fine powder (3 equiv per mol of replaceable H) (~30 mg) was introduced into the sample vial under the anhydrous conditions of a drybox. The manipulation of sodium hydroxide was done as quickly as possible to minimize the absorption of the trace moisture from the atmosphere. The mixture was stirred for 10 min at room temperature.

Methylation with Sodium Hydroxide and Methyl Iodide in Dimethyl Sulfoxide with the Presence of Water. To a carbohydrate sample (5−7 mg) introduced into a conical-glass vial, dimethyl sulfoxide (1 mL) and a small amount of water or alcohol (10−100 μL) was added with a syringe, under stirring, to dissolve the sample. Finely powdered sodium hydroxide (4 equiv per mol of replaceable H) (~40 mg) was added to the solution. The contents of the vial were stirred vigorously at room temperature to get a suspension and then methyl iodide (4 equiv per mol of replaceable H) (~80 μL) was added. The mixture was stirred at room temperature for 10 min. No inert gas atmosphere or drying condition was employed.

Isolation of Permethylated Products. In each case, the methylation reaction was quenched with water and carefully neutralized with 1 M hydrochloric acid solution. The permethylated products were extracted many times by the addition of the organic solvent (benzene, dichloromethane, trichloromethane, or tertrachloromethane) (1.0 mL), shaking the mixture, and separating the layers by centrifugation. The combined organic layers were washed at least three times with water and dried with dry nitrogen and under vacuum at 40 °C. The residue containing the per-*O*-methylated sample was redissolved in benzene, dichloromethane, pentane, or a mixture of these solvents, and an aliquot was used for GC−MS analyses.

Results and Discussion

The oxidation reaction[19,20] involving the alditol moiety present at the reducing end of the oligosaccharides in the per-*O*-methylation process has been studied using low-molecular-weight carbohydrates such as glucitol, maltitol, and cellobitol using the Ciucanu method[10] and the Hakomori method.[3] Both methods gave approximately the same amount of oxidation products when the reaction was performed under anhydrous conditions by adding first the methyl iodide and then the base (sodium hydroxide and Na dimsyl, respectively). An understanding of the mechanism of carbohydrate oxidation requires a complete inventory of the reaction products. The oxidation products of D-glucitol generated in the *O*-methylation process

Figure 1. GC−MS total ion chromatogram of the oxidative degradation products generated by the per-*O*-methylation of D-glucitol in dimethyl sulfoxide under anhydrous conditions by adding first the methyl iodide and, after 90 min, the sodium hydroxide (3 equiv of methyl iodide per mol replaceable H; 3 equiv of NaOH per mol of replaceable H). The numbered peaks are identified in Table 1. The GC−MS conditions can be found in the Experimental Section.

were separated by GC, and analyzed by MS. The compounds present in the peaks 1−12 in the GC−MS total ion chromatogram (Figure 1) were identified primarily from the EI mass spectra that are summarized in the Table 1. No molecular ion peak was identified in any of the EI mass spectra. CI mass spectra had very low abundance $M - 1$ ions for GC peaks 1−9 and 11−12, and low abundance $M + 1$ ion for GC peak 10. The interpretation of mass spectra was based on the known fragmentation of methylated neutral monosaccharides.[23−26] The components in peaks 1, 7, and 8 gave very similar mass spectra. The most abundant fragment ions are usually those at m/z 73, 75, 88, and 101. Peaks above m/z 101 have rather low abundance. The comparison of these mass spectra with literature data and GC retention time of the standards indicated the presence of β and α anomers of the per-*O*-methylated-D-glucopyranose and per-*O*-methylated-D-glucofuranose, respectively. The components in peaks 2, 4, 6, and 12 gave similar fragment ions with an abundant fragment ion at m/z 205. These mass spectra corroborate with the GC retention time of the standards indicate the components to be β and α anomers of the per-*O*-methylated-D-fructofuranose and per-*O*-methylated-D-fructopyranose, respectively. The mass spectrum of the component in peak 3 with an abundant fragment ion at m/z 71 and characteristic ions at m/z 161 and 129 is compatible with the structure methyl (2,3,6-tri-*O*-methyl-α(β)-D-*gluco*-hexos-5-ulo-1,4-furanose). The component in peak 5 has in its mass spectrum an abundant fragment ion at m/z 101, and characteristic ions at m/z 205, 173, and 141, which indicate that it is methyl (2,3,6-tri-*O*-methyl-α(β)-D-*gluco*-hexos-2-ulo-2,5-furanose). The mass spectrum of the component in peak 9 had an abundant

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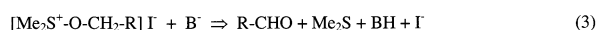(26) Lonngren, J.; Svensson, S. *Adv. Carbohydr. Chem. Biochem.* **1979**, *29*, 41−105

Table 1. Partial EI Mass Spectral Data for the Numbered Peaks from Figures 1 and Figure 3

peak no.	normalized area, %	m/z (relative abundance, %)
1	3.24	73(41.6), 75(99.1), 85(5.6), 88(100), 89(6.8), 101(96.7), 102(5.4), 111(3,2), 127(4.7), 131(3.1), 145(2.3), 146(1.9), 155(0.9), 187(0.9), 205(0.08)
2	0.25	73(7.9), 75(24.4), 85(9.3), 101(100), 119(21.0), 131(4.1), 141(72.8), 145(9.3), 173(30.8), 187(6.4), 205(82.4), 206(8.0), 218(1.7), 219(0.4)
3	0.28	71(100), 72(29.7), 75(55.0), 85(24.6), 101(34.9), 103(9.0), 115(2.6), 129(22.1), 131(2.5), 149(1.3), 161(56.1), 162(3.5), 187(0.9)
4	2.67	73(5.1), 75(14.7), 85(6.3), 101(100), 113(7.6), 119(14.2), 131(1.8), 141(65.6), 145(6.1), 155(1.1), 173(29.9), 187(3.5), 205(66.6), 206(5.7), 218(0.4), 219(0.3)
5	0.39	73(10.6), 75(34.9), 85(19.5), 88(11.2), 89(1.3), 101(100), 102(5.6), 119(15.4), 131(2.1), 141(26.5), 145(4.7), 173(11.0), 205(10.9)
6	0.35	73(32.9), 75(26.7), 85(12.8), 88(66.0), 89(3.5), 101(48.0), 102(1.4), 113(8.5), 119(52.9), 131(3.4), 141(90.9), 155(1.6), 173(50.2), 174(4.5), 187(5.7), 205(100), 206(10), 218(0.7), 219(0.5)
7	1.27	73(35.8), 75(93.7), 85(4.8), 88(100), 89(7.2), 101(71.6), 102(3.7), 111(11.4), 127(3.7), 131(2.3), 145(1.4), 155(1.7), 187(2.5), 219(0.1)
8	1.97	73(3.1), 75(66.5), 85(9.6), 88(5.3), 89(5.0), 101(100), 102(5.1), 117(5.6), 127(2.6), 131(1.2), 145(1.0), 155(3.1), 187(0.6), 205(0.2)
9	0.33	73(28.5), 75(34.2), 85(14.7), 88(100), 89(7.5), 101(20.4), 102(1.7), 119(15.3), 141(21.9), 155(0.9), 173(5.3), 187(1.1), 205(31.5), 206(4.3)
10	86.48	73(20.7), 75(26.5), 85(12.8), 88(100), 89(7.5), 101(91.1), 102(5.1), 113(7.0), 131(6.8), !33(4.6), 145(100), 146(7.3), 157(13.1), 177(8.8), 189(4.6), 221(2.9)
11	2.70	73(24.7), 75(65.8), 85(6.8), 88(100), 89(7.1), 101(38.2), 102(2.4), 111(2.9), 127(1.3), 131(1.3), 145(1.5), 155(0.4), 187(1.3)
12	0.07	73(35.7), 75(25.3), 85(12.8), 88(71.4), 89(3.2), 101(58.3), 113(6.1), 115(8.6), 119(53.1), 120(3.8), 141(72.7), 145(8.0), 155(2.1), 173(47.6), 187(6.9), 205(100), 206(7.5), 218(2.7)

Table 2. Identification of the Oxidation Products Generated in the per-O-Methylation of D-Glucitol under Anhydrous Conditions in Dimethyl Sulfoxide by Adding First the Methyl Iodide and, after 90 min, the Sodium Hydroxide. Peak Number Corresponds to the Numbered Peaks in Figures 1 and Figure 3

per-O-methylated compound	M.W.	1	2	3	4	5	6	7	8	9	10	11	12
D-glucose	250	+						+	+				
D-fructose	250		+		+		+						+
D-gluco-hexos-5-ulose	234			+									
D-gluco-hexos-2-ulose	234					+							
D-gluco-hexodialdose	234									+		+	
D-glucitol	266										+		

Scheme 1. Suggested Mechanism for the Oxidation of the Carbohydrate in the Methylation Process Adding First the Methyl Iodide and then the Base, under Anhydrous Conditions

$$Me_2SO + MeI \Leftrightarrow [Me_2S^+\text{-}O\text{-}Me]\ I^- \tag{1}$$

$$[Me_2S^+\text{-}O\text{-}Me]\ I^- + R\text{-}CH_2\text{-}OH \Leftrightarrow [Me_2S^+\text{-}O\text{-}CH_2\text{-}R]\ I^- + MeOH \tag{2}$$

$$[Me_2S^+\text{-}O\text{-}CH_2\text{-}R]\ I^- + B^- \Rightarrow R\text{-}CHO + Me_2S + BH + I^- \tag{3}$$

unsaturated aldehyo or keto sugar, which is readily subject to further degradation.[27]

By adding the base first and later the methyl iodide, the oxidation products were significantly reduced in both methods, but they could not be avoided. These experiments emphasized, as was suggested,[19,20] that the oxidation occurs as the result of the interaction between dimethyl sulfoxide and methyl iodide, which can produce methoxydimethylsulfonium iodide.[28,29] The stepwise reactions of a possible mechanism involved in the oxidation of carbohydrates are presented in Scheme 1.

The generation of the sulfonium ion in reaction 1, as the result of the electron-acceptor character of dimethyl sulfoxide, is an equilibrium process at room temperature.[28,30][30] Once formed, the sulfonium ion will suffer a nucleophilic attack on sulfur upon introduction of an appropriate reagent such as carbohydrate hydroxyl ($R\text{-}CH_2\text{-}OH$), and the methoxy group will be replaced with a deprotonated carbohydrate hydroxyl (carbohydrate alkoxide), in reaction 2. The addition of anhydrous methanol (10 μL) to the carbohydrate solution after 30 min of reaction with methyl iodide reduced the oxidation process by 65%. This suggested that the alkoxide exchange reaction is also an equilibrium process. In the absence of alkoxides or other catalysts, the exchange is quite slow.[29] In the presence of a base (B^-), the alkoxy-sulfonium salt will be irreversibly converted in reaction 3 to dimethyl sulfide and an aldehyde. Dimethyl sulfide was identified by GC−(EI)MS after extraction from the headspace of the reaction vial by micro-solid-phase extraction with helical sorbent. It would seem that direct nucleophilic attack of the carbohydrate hydroxyl on the sulfur atom is not possible, despite the locating of a partial positive charge and vacant d orbital of the sulfur atom, because no oxidation occurs by mixing dimethyl sulfoxide with alditols. It is not surprising that in almost all of the reactions in dimethyl sulfoxide that proceed with a nucleophilic attack on sulfur, sulfonium salt formation seems to be the initial critical step,[28] and this process can be aided by an electrophilic attack of the methylating agent on the

fragment ion at m/z 88, characteristic ions at m/z 205, 173, and 141 and lacked a fragment ion at m/z 101. These data indicate the component to be methyl (2,3,5-tri-O-methyl-α(β)-D-*gluco*-hexoaldo-1,4-furanose). The mass spectrum of the component in peak 10 has intense fragment ions at m/z 101 and 145 and characteristic ions at m/z 113, 177, 189, and 221, which matches the mass spectrum of 1,2,3,4,5,6-hex-O-methy-D-glucitol.The mass spectrum of the component in peak 11 with the most abundant fragment ions at m/z 73, 75, 88, and 101, and characteristic ions at m/z 111, and 127 is compatible with the structure methyl (2,3,4-tri-O-methyl-α(β)-D-gluco-hexoaldo-1,5-pyranose). The parent monosaccharides corresponding to the methylated derivatives in peaks 1−12 are presented in Table 2. The presence of the cyclic forms in the GC chromatogram is the result of the high tendency in aldehyo and keto carbohydrate to cyclize into pyranoside and furanoside forms, a process that can reduce the degradation by β-eliminative cleavage. However, this cyclization can only delay the immediate elimination of a methoxy or hydroxyl residue in the β-position of the aldehyo or keto group that leads to the formation of an

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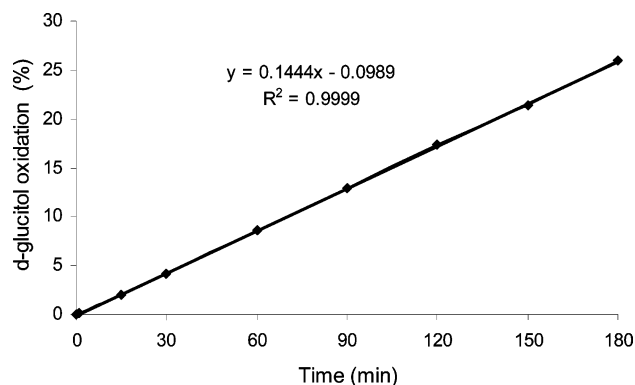
Figure 2. Degree of D-glucitol oxidation in dimethyl sulfoxide under anhydrous conditions at different reaction times with methyl iodide, followed by 10-min reaction in the presence of sodium hydroxide (3 equiv of methyl iodide per mol replaceable H; 4 equiv of NaOH per mol of replaceable H).

oxygen atom. Replacing methyl iodide with dimethyl sulfate significantly increased the degradation process. After a 30-min reaction with dimethyl sulfate followed by 10-min reaction with powdered sodium hydroxide, the peak of per-*O*-methylated D-glucitol disappeared from the chromatogram. This result was an indication that the dimethyl sulfate pushed the equilibrium to sulfonium salt faster than methyl iodide.

As can be seen in Table 1, more than 10% of the alditol was converted to derivatives with one or two aldehyde functional groups, and only a little more than 3.5% to derivatives with keto group in position C-2 and C-5. No derivative oxidized at C-3 and C-4 was found, probably due to steric hindrance or to their low stability in the presence of base. Lesser oxidation of the other hydroxyl groups of alditol suggested that the primary hydroxyl groups in alditols have the highest reactivity for a nucleophilic attack. This indicates that the initial oxidation occurred at the primary hydroxyl groups (C-1 and C-6) of the alditol. This is in accordance with the reactivity of the hydroxyl groups in alcohols following their acidity in the sequence primary > secondary > tertiary. However, the reactivity of the hydroxyl groups in oligosaccharides can be modified by specific interaction with the solvent, by intramolecular hydrogen bonding and steric hindrance that can complicate the understanding of the process.

Figure 2 shows the degree of D-glucitol oxidation at different reaction times with methyl iodide under anhydrous conditions at room temperature, followed by 10-min reaction in the presence of sodium hydroxide. The reproducibility of the results depended on the reproducibility of the anhydrous conditions. A small trace of water significantly reduced the oxidation process. It can be seen that after 1 min of mixing D-glucitol and methyl iodide in dimethyl sulfoxide, the degradation of D-glucitol was very low The increase of the mixing time increased the degradation reactions associated with yellow color formation and, after 12 h, no peak for permethylated D-glucitol or oxidative degradation products appeared in the chromatogram This result could be explained by a simultaneous interaction of more hydroxyl groups from carbohydrate with the methoxy-dimethylsulfonium iodide, generating in the presence of the base, derivatives with two functional groups such as dialdoses, and ketoaldoses, and even with more functional groups. In a basic and anhydrous medium, these carbohydrate derivatives suffer β-eliminations and cleavages into smaller saturated and unsaturated molecules. These molecules have a high volatility, and

probably eluted in the peak of the solvent. However, by micro-solid-phase extraction with helical sorbent[21] from the headspace of the reaction vial, formaldehyde, dimethyl ether, methyl glyoxal, 2-propenal, and 3-methoxy-2-propenal were identified by GC−(EI)MS. Cyclic carbohydrates with free hemiacetal groups such as D-glucose, maltose, and cellobiose had a similar behavior, but in their case, the degradation process was faster. The derivatives detected by the oxidation of the maltose and cellobiose with methyl iodide in dimethyl sulfoxide for 60 min were glucose and a very tiny amount of other derivatives. The presence of hemiacetal group in these molecules accelerated the decomposition to small derivatives, which were not detected in chromatogram. It is interesting, but not surprising, that even nonreducing oligosaccharides such as trehalose and raffinose were decomposed by oxidation to corresponding monosaccharides and small amounts of other oxidation compounds.

The only solution to eliminate this undesired oxidation process is to avoid the formation of sulfonium salts. As any methylating agents can generate the sulfonium salt with dimethyl sulfoxide, the easiest solution would be the use of a different dipolar aprotic solvent. Reactions carried out in *N,N*-dimethylformamide and *N,N*-dimethylacetamide gave no oxidation of the alditols, when the base was added after 30 min from the interaction of the aprotic solvent with methyl iodide and alditol, but the yield in the per-*O*-methylated alditol was low. However, with an excess of 5−6 equiv powdered sodium hydroxide per mol of replaceable H of the alditol, the yield of per-*O*-methylation in *N,N*-dimethylacetamide was the same as in dimethyl sulfoxide, although a longer reaction time was required.

Because dimethyl sulfoxide and methyl iodide gave the best yields of per-*O*-methylated carbohydrates in a very short time, we have tried to find ways that can avoid the oxidation with this system. Upon sequential addition to the carbohydrate dissolved in dimethyl sulfoxide of sodium hydroxide and later the methyl iodide, under anhydrous conditions, the methoxy-dimethylsulfonium salt is rapidly transformed by the base to formaldehyde and dimethyl sulfide,[29] and the oxidation process of alditols was significantly reduced. The oxidation process under anhydrous conditions could not be totally avoided in either the Ciucanu or Hakomori method, even if the base was added first, especially when the reaction time with methyl iodide was longer than 10 min. However, a long reaction time between alditol and sodium hydroxide in dimethyl sulfoxide, before the addition of methyl iodide, did not generate oxidative degradation. A reaction time up to 10 min between carbohydrate and solid sodium hydroxide before the addition of the methyl iodide can be especially useful to avoid the undermethylation of the complex carbohydrate with low reactivity hydroxyl groups. At a longer reaction time, the viscosity of the reaction mass increased significantly. Moreover, a prolonged contact of some nonneutral carbohydrates with base can generate degradation by β-elimination reaction.[14]

It is very well-known that the per-*O*-methylation of alditols in dimethyl sulfoxide by adding first the sodium hydroxide and then the methyl iodide, under non anhydrous conditions,[10] can give a very good yield in the per-*O*-methylated carbohydrate. Also, the mechanism of oxidation from Scheme 1 suggests that the oxidation could be reduced by a competing nucleophilic attack on the sulfur atom of the sulfonium ion, using a reagent with a higher reactivity than the primary hydroxyl group in
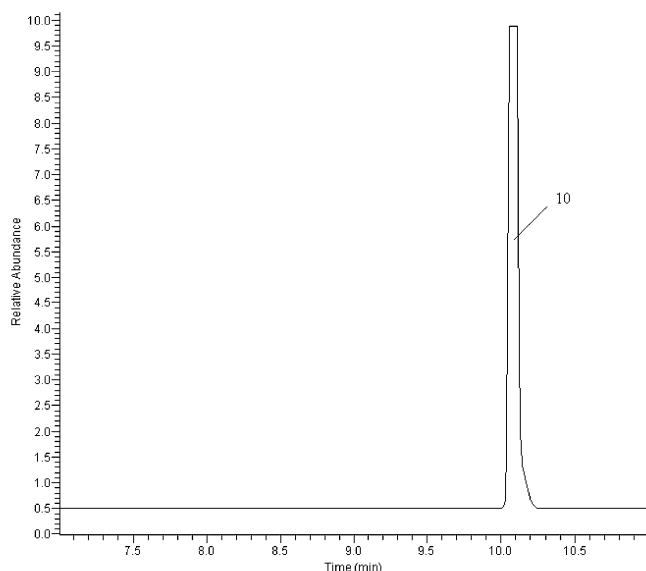
Figure 3. GC−MS total ion chromatogram of per-*O*-methylated D-glucitol generated in dimethyl sulfoxide with 1% water by adding first methyl iodide and after 1 min sodium hydroxide (3 equiv of methyl iodide per mol of replaceable H of the alditol; 6 equiv of sodium hydroxide per mol of replaceable H; 3 mol of NaOH/L of Me₂SO). GC−MS conditions as in Figure 1.

alditol. Such reagents could be methanol or water, as suggested by our experiments. Their acidity in dimethyl sulfoxide follows the sequence water > methanol > primary carbohydrate hydroxyl. Adding anhydrous methanol before methyl iodide reduced the oxidation process by 75%, but very small amounts of water (10 μL) in dimethyl sulfoxide totally eliminated the oxidation products of alditols. Figure 3 shows the chromatogram of D-glucitol per-*O*-methylated by first adding methyl iodide and then sodium hydroxide in dimethyl sulfoxide containing trace of water. Similar results were obtained for cellobitol and maltitol. The per-*O*-methylation was performed by mixing, at room temperature for 1 min, a solution of alditol in dimethyl sulfoxide with 1% water, 3 equiv of methyl iodide per mol of replaceable H of the alditol, followed by 10 min reaction with 6 equiv of sodium hydroxide per mol of replaceable H (3 mol of NaOH/L of Me₂SO). An explanation of the total elimination of the oxidation products could be the reaction of the water with methoxydimethylsulfonium salt, resulting in the formation of methanol and dimethyl sulfoxide. Methanol generated in this way could also push the equilibrium from sulfonium salt to alditol in reaction 2. However, after the consumption of the small trace of water in the reaction with the sulfonium salt, the oxidative degradation of carbohydrate was started again. Consequently, the oxidative degradation could not be avoided for a long reaction time between dimethyl sulfoxide, methyl iodide and carbohydrate.

The introduction of the water into the system has an opposite effect on the methylation yield because, in accordance with the mechanism of the methylation of carbohydrates,[10] the first step of the methylation involves the deprotonation of carbohydrate hydroxyl groups in an equilibrium reaction with the formation of the alkoxides and water. Before addition of methyl iodide, this equilibrium process is depending on the strength and concentration of the base, the reactivity of the carbohydrate hydroxyl groups, the solvent reaction medium, the temperature, and the elimination of water from the system. Solid sodium
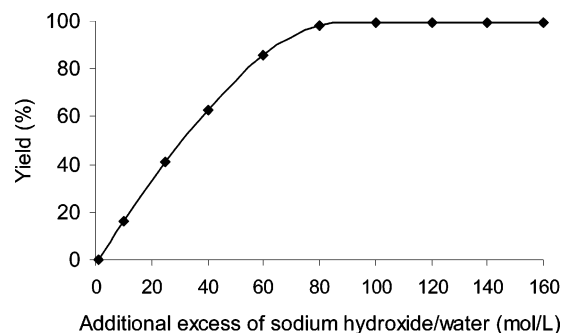
Figure 4. Yield of per-*O*-methylated D-glucitol as a function of the additional excess sodium hydroxide/water ratio (mol/L) (3 mol of NaOH/L of Me₂SO; 3 equiv of methyl iodide per mol replaceable H; 3 equiv of NaOH per mol of replaceable H).

hydroxide is only sparingly soluble in dimethyl sulfoxide, but it is very hygroscopic. It acts as a desiccant, removing the water from the system and pushing the equilibrium decisively over to the alkoxide side. For this reason, the solid sodium hydroxide must be used in at least one excess of 3 mol of sodium hydroxide per mol of replaceable H. The amount of water that can be accepted by the methylation system in order to avoid the oxidation without affecting in the per-*O*-methylation yield is strong correlated with an additional amount of solid sodium hydroxide, which should be added to the reaction vial for scavenging this water. The effect of the ratio between the additional excess of sodium hydroxide, and the water added into the system on the per-*O*-methylation yield of D-glucitol is shown in Figure 4. When the amount of water was high, the solid sodium hydroxide became a paste on the wall of the vial, and the yield in per-*O*-methylated D-glucitol was low. Increasing the additional excess of solid sodium hydroxide, the per-*O*-methylation yield was increased and complete per-*O*-methylation was achieved with an additional excess of sodium hydroxide/water ratio higher than 100 mol NaOH/1 L H₂O.

The presence of a small amount of water in the system can have other advantages. For example, the water improves the solubility of some carbohydrates with low solubility in dipolar aprotic solvent such as dimethyl sulfoxide and *N*, *N*-dimethyl-acetamide, because the low solubility of the carbohydrate in the reaction solvent was the main cause of the undermethylation. However, the presence of too much water can be the source of undesirable reaction for chemically labile carbohydrates. This will be the subject of the following study.

Degradation of the carbohydrates was also observed in the liquid−liquid extraction step, as a function of the extraction conditions. The choice of the organic solvent was also important for avoidance of degradation in the extraction process. Quenching the methylation reaction by adding water followed by extraction with chloroform generated up to 3% of degradation products. Extraction of the per-*O*-methylated sample with dichloromethane reduced the degradation below 0.5%, but tetrachloromethane and benzene eliminated these byproducts. It is worth noting that the degradation products generated in the extraction step with chloroform and dichloromethane were significantly reduced by the neutralization of the base by addition of 1 N hydrochloric acid before the extraction. The solubility of the per-*O*-methylated carbohydrates in benzene and tetrachloromethane is lower than in dichloromethane and chloroform. Consequently, for 99.5% recovery, 10 extractions with benzene or three extractions with dichloromethane were

done. Keeping the sample in chloroform with trace of dimethyl sulfoxide, water, and unreacted methyl iodide can increase the amount of degradation products in time. For this reason, the extracted sample must be washed at least three times with water and dried with a current of dry nitrogen or under vacuum. For injection into GC−MS system, the sample should be redissolved in hexane, benzene, or dichloromethane.

Conclusion

The data presented show that the oxidation of alditols in the powdered sodium hydroxide/methyl iodide/dimethyl sulfoxide methylation process represents an artifact produced by treating, under anhydrous conditions, the alditol solution in dimethyl sulfoxide first with methyl iodide and after a long time (10−90 min) with solid sodium hydroxide or sodium dimsyl. A similar behavior was also observed for reducing and nonreducing carbohydrates. These results described above indicate that oxidative side reaction can significantly alter the structural analysis. On the basis of this evidence for the mechanism of oxidative degradation during per-*O*-methylation of carbohydrates, a new simple protocol has been designed. This oxidative process is not a characteristic of the original Ciucanu methylation method,[10] which was performed in nonanhydrous conditions, upon addition to the carbohydrate dissolved in dimethyl sulfoxide first the sodium hydroxide and then the methyl iodide. This method can be considered successfully even in the presence of a small trace of water, if a small additional excess of sodium hydroxide is added. The addition first of the methyl iodide followed after a short time by powdered sodium hydroxide in the presence of dimethyl sulfoxide with small trace of water made possible the realization of the per-*O*-methylation process without oxidative degradation of neutral carbohydrate. The extraction of the per-*O*-methylated sample can also be a source of degradation, but was eliminated by the neutralization of the base before extraction, using benzene or tetrachloromethane as extraction solvent and removing the remaining methyl iodide, dimethyl sulfoxide, and water in the final per-*O*-methylated sample. Because the extraction with dichloromethane was very efficient, dichloromethane can be used if the reaction mass is neutralized before extraction.