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A CONVENIENT METHOD FOR METHYLATION OF GLYCOPROTEIN GLYCANS IN SMALL AMOUNTS BY USING LITHIUM METHYLSULFINYL CARBANION*

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ABSTRACT

Treatment of dimethyl sulfoxide with butyllithium leads to rapid formation of lithium methylsulfinyl carbanion. The reaction products tend to be significantly freer from impurities when lithium methylsulfinyl carbanion is used rather than sodium or potassium methylsulfinyl carbanion. This reagent gives less background in g.l.c. and thus may be used to methylate micro-quantities of glycoprotein glycans (down to 10 μg) without the necessity of identifying methyl ethers by mass spectrometry.

INTRODUCTION

Methylation analysis combined with proton nuclear magnetic resonance spectroscopy is the most powerful method for structural analysis of the oligosaccharidic parts of glycoproteins[1–4]. During the past twenty years, the Hakomori procedure[5] using sodium hydride and dimethyl sulfoxide to produce a more powerful nucleophile than the bases previously used[6] has been applied to achieve rapid and complete methylation of all free hydroxyl groups as well as *N*-methylation of the acetamido group of hexosamine residues, without any loss of *N*-acetyl groups[7] in complex carbohydrates. Because the reagent contains impurities (sodium hydride is dispersed in oil), methods have been developed to increase the sensitivity in the chromatographic analysis of methylated sugars. In this way, gas–liquid chromatography coupled to mass spectrometry allows the identification of small amounts of partially methylated sugars; furthermore, chemical-ionization mass spectrometry

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has been used to greatly enhance the sensitivity of detection of methylated sugars by removing background from gas chromatograms[8–11]

In order to obtain a cleaner reagent to perform the methylation of small amounts of sample, Finne, Krusius, and Rauvala[12] reported a new methylation reagent prepared from potassium *tert*-butoxide and demonstrated that *tert*-butoxide in dimethyl sulfoxide tended to give less background in g.l.c., was more rapidly, conveniently and safely prepared, and gave fewer interfering impurities in the analysis of methylated carbohydrates.

In 1981, Phillips and Fraser[13] developed a method for methylation of carbohydrates with dimsyl potassium in dimethyl sulfoxide and noted significant improvements in the preparation of the reagent and in the purity of the product.

Butyllithium was prepared originally by Jones and Gilman[14] from butyl bromide and lithium wire in ether at an initial temperature of $-10°$ and then at 0–10° during a total time of ~3 h. This reagent may also be prepared by using butyl chloride[15]. By reaction with butyllithium in Me_2SO followed by treatment with an alkyl halide, Chaykovsky[16] described a method for the N^8-alkylation of 2,4-diamino-7,8-dihydropteridine. In his paper, the author specifies that lithium methylsulfinyl carbanion is the actual nucleophile that causes deprotonation of the 7,8-dihydropteridine. Butyllithium is a carbanion widely used in organic chemistry[17–25]. In the field of carbohydrate chemistry, butyllithium has been used to prepare carbohydrate phosphates[26]. Hoppe and Schöllkopf[27] demonstrated that methylmagnesium bromide, phenylmagnesium bromide, and also butyllithium react with 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose to give diastereomeric adducts.

MATERIALS AND METHODS

Materials. — The following reagents were obtained from the companies indicated: butyllithium, 1.6M in hexane, from Janssen Chimica (Beerse, Belgium); methyl iodide for synthesis from Merck (Darmstadt, G.F.R.); acetic anhydride R.P. Normapur, pyridine R.P. Normapur and sodium thiosulfate R.P. Normapur from Prolabo Rhône–Poulenc (Paris, France). The solvents were: chloroform for chromatography, methanol for spectroscopy and dimethyl sulfoxide p.a. and dried from Merck (Darmstadt, G.F.R.). Dimethyl sulfoxide was re-dried by using 3 Å molecular sieve, beads about 2 mm (LAB), from Merck (Darmstadt, G.F.R.) and was distilled under diminished pressure ~12 mm Hg under nitrogen. The water used was type milli Q (Millipore, Mass). All glassware was stored in chromium trioxide/conc. sulfuric acid, washed, and dried.

Methyl α-D-mannoside was obtained from K and K Labs (Plainview, NY). Oligosaccharide-alditol 11 was prepared from hen ovomucoid by hydrazinolysis in combination with liquid chromatography on bonded primary-amine packings as described by Paz Parente *et al.*[28,29]

Preparation of lithium methylsulfinyl carbanion. — The apparatus for preparation of lithium methylsulfinyl carbanion consists of a 500-mL, two-necked flask

equipped with a magnetic stirrer, fitted with a 250-mL dropping funnel and a distillation system fitted with a drying tube packed with calcium chloride, and with a manometer and a 1-L one-necked trap-flask. The entire apparatus is connected to a water pump. Before the reaction procedure, the system is purged with a stream of helium.

Under helium, dimethyl sulfoxide (freshly dried and distilled, 120 mL) is placed in the two-necked flask and butyllithium (1.6M in hexane, 250 mL) is placed in the dropping funnel. At room temperature and under diminished pressure (30 mm Hg), butyllithium in hexane is gradually added with effective stirring. The butane produced during the reaction of butyllithium with dimethyl sulfoxide and hexane is eliminated by water pump. The addition is made during ~2 h, after which time the color of the mixture changes to dark green. After all of the 1.6M butyllithium in hexane has been added, the mixture is kept for 2 h at room temperature. After 4 h, the system is brought back to atmospheric pressure by introduction of a stream of helium. The reagent was stored under helium in Teflon-lined screw-cap tubes (13 × 100 mm, Sovirel, France) at 4°.

Methylation procedure. — (*a*) *Assay.* In order to assay the method, 1 mg of methyl α-D-mannoside was dissolved in dimethyl sulfoxide (200 μL) in a Teflon-lined screw-cap tube. Lithium methylsulfinyl carbanion (200 μL) was added under an inert atmosphere and the mixture was sonicated for 60 min. After cooling to −4°, cold methyl iodide (400 μL) was added. Sonication was conducted in a sonication bath (20°) for 45 min.

The methylation was terminated by addition of water (4 mL) containing sodium thiosulfate, and the permethylated product extracted with chloroform (3 × 2 mL). The chloroform phase was washed with water (6 × 4 mL), dried (sodium sulfate), and evaporated.

(*b*) *Micromethylation procedure.* Oligosaccharide alditol 11 isolated from hen ovomucoid (10 μg) was methylated in a small glass tube (6 × 80 mm) placed in Teflon-lined screw-cap tubes under the previous experimental conditions, except that the quantities of solvents and reagents were dimethyl sulfoxide, 20 μL; lithium sulfinyl carbanion, 20 μL; and methyl iodide, 40 μL. The alkylation must be performed at room temperature to avoid the decomposition of methyl iodide to iodine. The methylation is stopped by addition of water (0.4 mL) containing thiosulfate, and the permethylated oligosaccharide-alditol is extracted with chloroform (3 × 0.4 mL). After washing of the chloroform phase with water (5 × 0.5 mL), it was dried, filtered, concentrated, and freeze dried at 10^{-3} mmHg at 25°.

Analysis of methyl ethers. — The permethylated oligosaccharide alditol was treated with 0.5M methanolic HCl (150 μL) for 20 h at 80°. The methyl ethers were analyzed after peracetylation in 1:1 pyridine–acetic anhydride (40 μL, 24 h, 37°) according to Fournet *et al.*[9].

RESULTS AND DISCUSSION

Reaction of butyllithium with dimethyl sulfoxide gives lithium methylsulfinyl

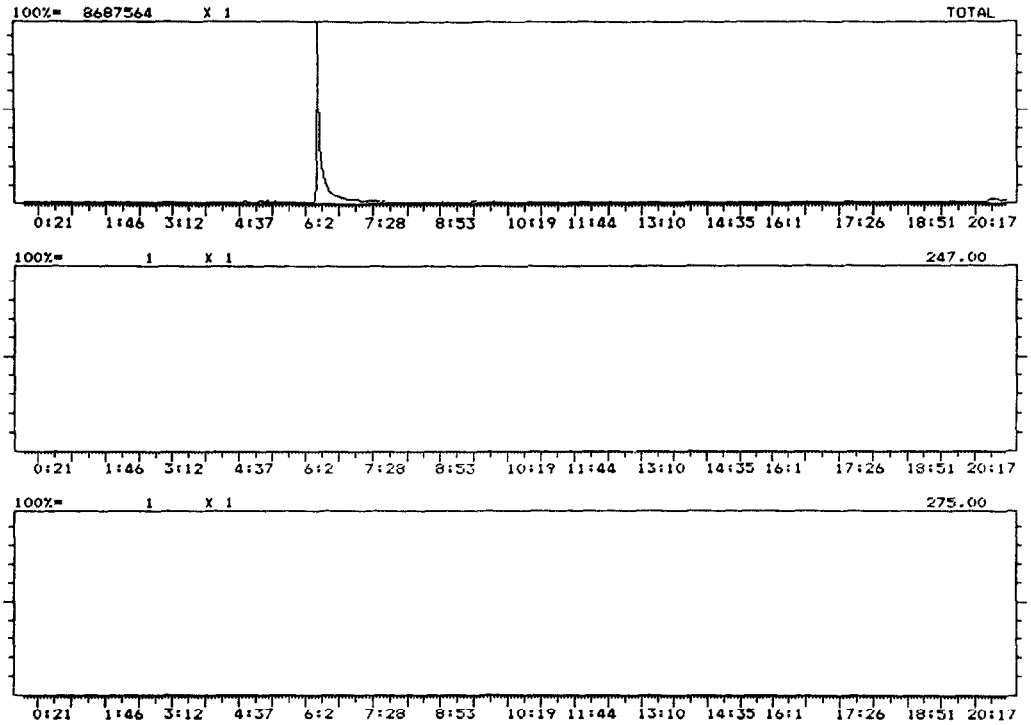

Fig. 1. Total ion-current recording for methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside, showing absence of undermethylation products, recording at *m/z* 247 (A_1 fragment of trimethylated derivatives) and *m/z* 275 (A_1 fragment of dimethylated derivatives). G.l.c.–m.s. (Ribermag R 10-10 mass spectrometer apparatus) on a glass capillary column; walls coated with OV-101.

carbanion quantitatively. The base concentration in the reagent was 3.33M. This base was stable during several months when stored at 4° under helium.

The results of methylation of methyl α-D-mannoside are presented in Fig. 1. In a chromatogram based on total ionizing current, a single peak is observed having the retention time of methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside. In order to detect the possible presence of undermethylation products, this compound was acetylated and analysed by g.l.c.–m.s. using the fragmentometry technique at *m/z* 247 (A_1 fragment of trimethylated derivatives) and at *m/z* 275 (A_1 fragment of dimethylated derivatives)[9]. No traces of undermethylated products from methyl α-D-mannoside could be observed.

Fig. 2 records results of permethylation of 10 μg of oligosaccharide alditol 11 isolated from hen ovomucoid[28,29]. The analysis was performed by g.l.c. on a silicone OV-101 capillary column for methyl ethers, using an injected quantity of 0.15–0.8 μg/peak. It may be observed that g.l.c. on a silicone OV-101 capillary column with flame-ionization detection permits the identification of all methyl ethers without the use of m.s., showing that the reagent gives less background in g.l.c. By using chemical-ionization (ammonia) mass spectrometry with specific ions: $(M + 18)^+$ for neutral methyl ethers (*m/z* 268 for permethyl derivatives, *m/z* 296 for mono-*O*-

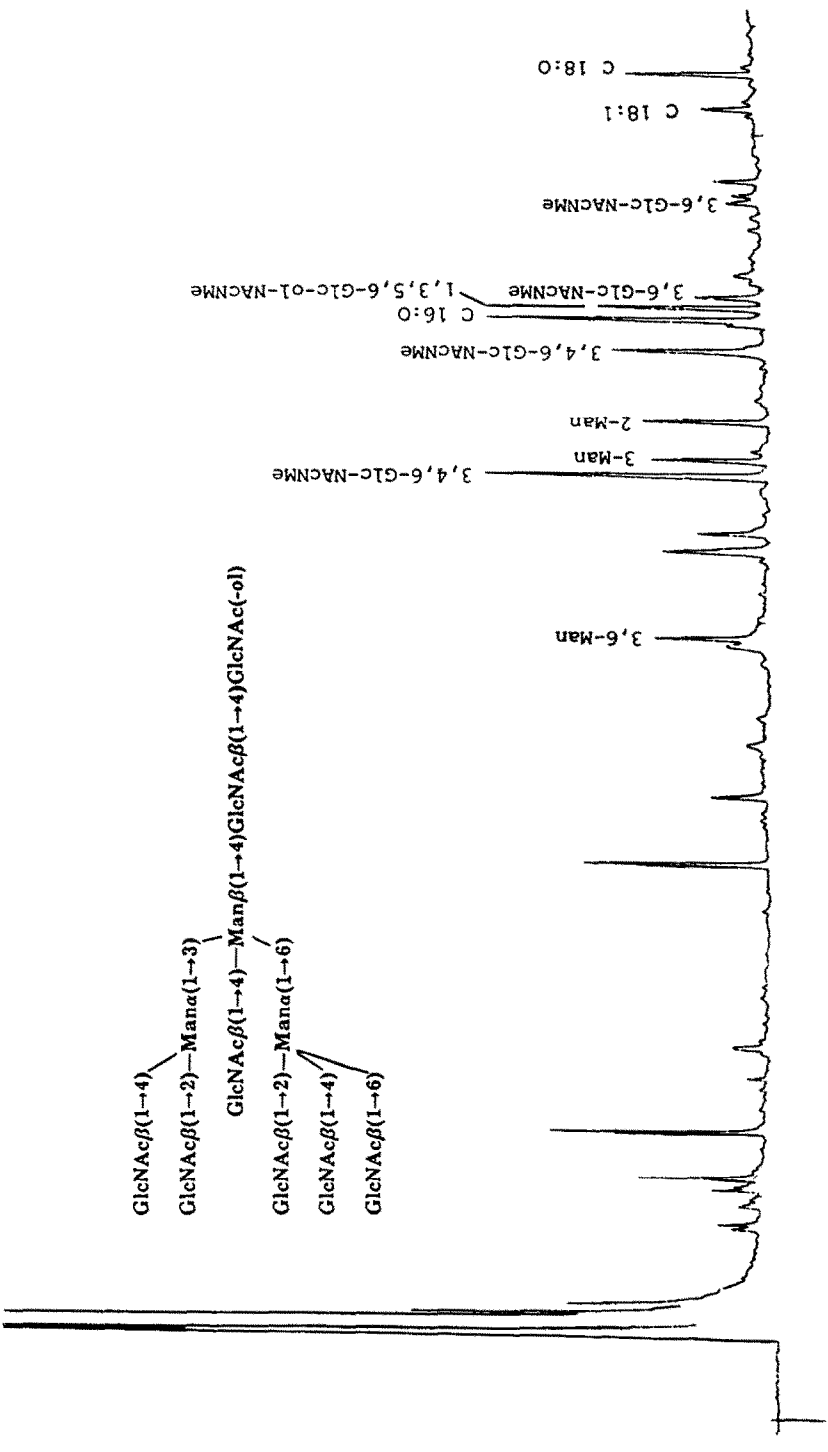

Fig. 2. Gas chromatogram (glass capillary column; walls coated with OV-101; f.i.d. detection) of partially methylated and acetylated methyl glycosides obtained by methylation and subsequent methanolysis of 10 μg of oligosaccharide-alditol 11 from hen ovomucoid (Injection of one-sixth of the initial sample).

acetyl-tri-*O*-methyl derivatives, *m/z* 324 for di-*O*-acetyl-di-*O*-methyl derivatives, *m/z* 352 for tri-*O*-acetyl-mono-*O*-methyl derivatives); $(M + 1)^+$ for hexosamine methyl ethers (*m/z* 292 for permethyl derivatives, *m/z* 320 for mono-*O*-acetyl-di-*O*-methyl derivatives, *m/z* 348 for di-*O*-acetyl-mono-*O*-methyl derivatives); $(M + 1)^+$ for the methyl ether of *N*-acetylneuraminic acid (*m/z* 408), we are able to analyse complex carbohydrate with samples containing less than 1 μg of total sugars.

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