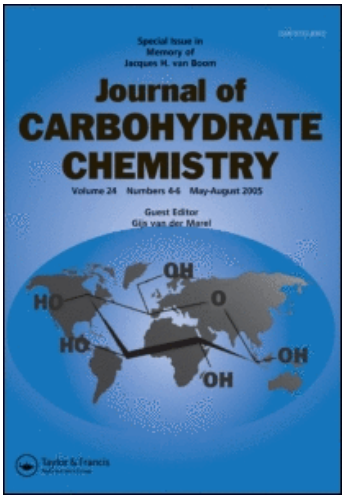
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

The Methylation Reaction in Carbohydrate Analysis

Andrew Jay[a]
[a] Institute of Food Research, Norwich Laboratory, Norwich Research Park, UK

To cite this Article Jay, Andrew(1996) 'The Methylation Reaction in Carbohydrate Analysis', Journal of Carbohydrate Chemistry, 15: 8, 897 — 923
To link to this Article: DOI: 10.1080/07328309608005698
URL: http://dx.doi.org/10.1080/07328309608005698

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. CARBOHYDRATE CHEMISTRY, 15(8), 897-923 (1996)

REVIEW

THE METHYLATION REACTION IN CARBOHYDRATE ANALYSIS

Andrew Jay

Institute of Food Research, Norwich Laboratory, Norwich Research Park, Colney, Norwich NR4 7UA, UK

Table of Contents

Copyright © 1996 by Marcel Dekker, Inc.

Downloaded At: 08:25 23 January 2011

1. INTRODUCTION

Over the last 30 years, the technique of linkage analysis of carbohydrates, commonly called "methylation analysis", has become one of the standard methods of elucidating their chemical structure. Used in conjunction with NMR spectroscopy, the data can yield the linkage positions and sequence of sugar residues in oligosaccharides and polysaccharides. The complete procedure of linkage analysis has recently been reviewed.[1-5] However, the special problems involved in the methylation step and the variety of procedures used deserve a special overview in the light of several recent developments. Thus the methylation of model carbohydrates[6-8] and naturally occurring polysaccharides[9] has been studied using comparative methods. This has led to improvements in technique, especially in the use of NaOH as a base.[6,8,10,11] Improvements are also being sought for the mild methylation of polysaccharides,[12-14] and important new reactions for the cleavage of permethylated polysaccharides have been successfully established.[15,16] These developments will be discussed in the relevant sections below.

Downloaded At: 08:25 23 January 2011

Methylation of all free hydroxyl (and other proton-donating groups) on a polysaccharide is usually the first step in derivatization for GC-MS analysis, in which information about the linkage pattern of each type of sugar residue is preserved. The methylated polymer is then further derivatized prior to GC-MS. It may be preferable to chemically modify functional groups, before or after methylation. Glycosidic links in the methylated polymer are then cleaved by acid hydrolysis or other means (see later). Total cleavage produces monosaccharides and therefore information only about linkage positions; partial cleavage gives oligosaccharides and information about sequence also. Hydrolysis products are reduced with sodium borodeuteride and the final derivatization step is most commonly acetylation.[17-19] This yields partially methylated acetylated alditols (or 1,5-anhydroalditols or methyl glycosides, depending on type of glycosidic cleavage used) which can be separated by GC and identified by MS.

Although widely used by carbohydrate chemists and biochemists, it is clear from the above that there is no single, "standard" version of linkage analysis. The same is true of the methylation step; opinions probably differ over the best variation. This depends on the application; what works well in particular circumstances is not readily abandoned, while other workers are constantly seeking to develop improvements. As it stands, the method is still only

semi-quantitative due to factors such as undermethylation, non-dispersal of polymer in nonaqueous solvents, and selective degradation and losses of residues at this and later stages in derivatization.

This article provides an overview of the background of non-anomeric *O*-methylation and its application to microanalysis of polysaccharides, particularly the advantages and disadvantages of methods currently in use. A résumé of the other chemical steps in methylation analysis is also given; appropriate choice of these is just as relevant to the analytical strategy as at the methylation step. This review is intended to bring together all the key developments to date, including some illustrative examples from modern research; it should therefore form an instructive guide to chemists new to the field who have an interest in methylation analysis and related preparative work.

Downloaded At: 08:25 23 January 2011

2. PREPARATIVE METHYLATION

Methylation analysis has its roots in synthetic chemistry from the turn of the century. Its use as an analytical tool has necessitated the synthesis of authentic standards[20-25] or model compounds for the study of new derivatization reactions.[26] In these cases, preparative scale methylation must be compatible with protecting groups present as well as giving good yields. Until the 1960s the methods described below were used for preparative and analytical methylation of carbohydrates.[27]

The first completely methylated sugars were prepared by Purdie by treating carbohydrate dissolved in methanol with iodomethane and silver oxide.[28] This method can only be used for non-reducing derivatives (e.g., glycosides) since Ag_2O oxidizes reducing sugars to carboxylic esters. Methanol is an unsatisfactory solvent because reagents are wasted in methylation of the solvent.[29] For example, the methylation of methyl-α-D-glucopyranoside[30] involved adding 5 mol of Ag_2O (dry, freshly prepared) over 5 h to 1 mol methyl glucoside and 10 mL CH_3I dissolved in CH_3OH at 45 °C with further reflux. After extraction with chloroform of the Ag salts the recovered material required remethylation 3 times.

The Haworth methylation[31,32] more conveniently uses an aqueous solution of carbohydrate, to which dimethyl sulphate and 30% aqueous NaOH are added (under N_2) at 0 °C with vigorous stirring. Further additions are made at 40, 60 and 70 °C followed by reflux

for 1 h. *O*-Acylated carbons are also converted to methyl ethers because acyl esters are cleaved by strong bases.[33] The product is extracted from the neutralized mixture with $CHCl_3$. Unfortunately Me_2SO_4 is highly toxic and makes this synthetically efficient method hazardous for routine work.

Variations of the Haworth methylation include: Me_2SO_4 and solid NaOH in acetone solution, suitable for isopropylidene and other hydrophobic derivatives;[34] and in tetrahydrofuran (THF), for partially methylated material.[35] Methylated products are recovered on partitioning in $CHCl_3/H_2O$.

Sodium metal has also been used to complete the methylation of partially methylated material.[36] The material is dissolved in dry ether and shaken with Na for 12 h. The decanted liquid is then reacted with CH_3I.

The Kuhn modification[37] employs dimethylformamide (DMF) as the solvent and gives complete methylation in one reaction, provided sufficient excess of Ag_2O is used. DMF is a much better solvent for carbohydrates, especially polysaccharides, than methanol and this method is a less hazardous option for synthetic work than others. Excess CH_3I is required due to a slow side reaction between DMF and CH_3I.[38] Free sugars give α and β anomers and pyranose and furanose forms. A variation involves the use of thallium (I) hydroxide and CH_3I.[39,40] Generally the Kuhn method has been shown to be superior to the Purdie and Haworth methylations for mono- and oligosaccharides.[41]

Downloaded At: 08:25 23 January 2011

Methylation in liquid ammonia involves generation of the alkali metal derivative of the carbohydrate in liquid NH_3,[42] followed by addition of CH_3I.[43,44] Free sugars and 1-*O*-acyl sugars are converted to their glycosylamines by liquid NH_3 but other *O*-acyl groups are only lost on addition of alkali metal.[45] However, the product of carbohydrate and metal must be NH_3-soluble and reactive or else incomplete methylation results due to reaction of CH_3I with NH_3.[46,47] Removal of NH_3 before addition is best,[44] with another solvent (e.g., 1,2-dimethoxyethane) substituted at room temperature.[48] The difficulties of handling liquid NH_3 and metal amide solutions make this method unsatisfactory for routine work but it has been applied successfully to polysaccharides (e.g., amylose)[49] and adapted for an early micro-scale permethylation.[50]

In a forerunner to modern methylation techniques, sodium hydride was used with iodomethane in THF to methylate a tertiary hydroxyl group on a sugar precursor.[51]

3. PRE-METHYLATION MODIFICATION OF POLYSACCHARIDES

Chemical modification of polysaccharides prior to methylation may be necessary and so is discussed here before covering methylation of polysaccharides in detail. It is used in order to assist solubilization in the methylation solvent, to improve recovery of base-labile residues, and to label the position of functional groups.

Charged polysaccharides are rendered more soluble in dipolar aprotic solvents by replacing metal counter-ions with bulky organic cations. Sulphated polysaccharides such as carboxyl-reduced heparin[52] and carrageenans[16] can be converted to the pyridinium or triethylammonium forms to solubilize them in DMSO. The latter form is more stable[16] probably due to the higher pK_a of Et_3NH^+ and the conversion is best done either by ion-exchange with the Et_3NH^+ form of Dowex resin or by dialysis against Et_3NHCl solution. Uronic acid-bearing bacterial lipopolysaccharides can be similarly converted to the Et_3NH^+ salts, which disperse in Me_3PO_4 for mild methylation studies.[53] Bacterial polysaccharides such as xanthan and acetan,[54,55] when converted to the tetrabutylammonium salts, dissolve relatively rapidly in DMSO, and reasonably well in Me_3PO_4.[16]

Uronic acids and their esters may also be modified by carboxyl reduction. The free acids require activation by a carbodiimide such as CMC and are treated with $NaBH_4$ (or $NaBD_4$ if to be labelled) at pH 4 in imidazole buffer.[56] This is suitable for polyuronides such as alginates, chondroitin sulphate, heparin and hyaluronic acid, where dispersion in methylation solvents is impossible and glycosyluronic acid bonds resist subsequent acid hydrolysis.[57] Unfortunately the activated polysaccharides are alkali-labile and $NaBH_4$ is acid-labile, so the pH requires careful control, and reduction is incomplete. However, it has been applied with >90% extent of reduction to grass cell wall polyuronides.[58] Native methyl esters are directly reduced with $NaBH_4$ or $NaBD_4$ and labelling can be used to distinguish them from unesterified residues.[58,59]

Desulphation of polysaccharides is not essential for successful methylation,[16] but may help with structural studies,[22] and is achieved by solvolytic heating. Hot alkali will convert galactosyl 6-sulphate in carrageenans to 3,6-anhydrogalactosyl residues.[60]

Trifluoroacetylation using trifluoroacetic acid (TFA) and its anhydride (TFAA) has been applied to hexosamine-containing oligosaccharide alditols.[61] Treatment with TFA/TFAA (100 °C, 48 h) derivatizes all the OH groups (hence stabilising the glycosyl linkages to

Downloaded At: 08:25 23 January 2011

cleavage) and then the amine groups (replacing any *N*-acyl substituents). The *O*-trifluoroacetyl groups are removed simply with MeOH and permethylation produces permethylated NTF-oligsaccharide alditols which can be analysed by GC-MS.

Partial degradation of polysaccharides prior to methylation can aid structural elucidation. Mild acid hydrolysis is used to generate mixtures of oligosaccharides, which, when separated, reduced and methylated, yield information on the sequence of the polysaccharide, using FAB-MS.[62,63] Typical conditions involve 0.1-0.5M H_2SO_4 or TFA at 100 °C for 1-4 h; such conditions leave uronic acids intact. Oligosaccharides are then reduced to oligoalditols using $NaBH_4$ or $NaBD_4$ prior to methylation, since they are susceptible to base-catalysed end-peeling reactions, especially when NaOH is used as the catalyst (here methylation is the last step in derivatization). Another form of mild acid hydrolysis is that using 48-60% HF, which selectively cleaves phosphoric esters of teichoic acids[64] and bacterial polysaccharides.[65]

Downloaded At: 08:25 23 January 2011

Nitrous acid deamination of polyglycosamines[66] is a way of specifically cleaving glycosaminic linkages to produce oligosaccharides. Glycosamines must first be *N*-deacylated with hydrazine/hydrazine sulphate,[67,68] or NaOH/sodium thiophenolate[69] and are usually subsequently reduced with $NaBH_4$ prior to methylation. Nitrous acid is used to convert the free amine to a diazonium salt that internally rearranges to a 2,5-anhydrosugar. The partially methylated alditol acetate of this sugar confirms the presence and linkage sites of the glycosamine.[70]

Periodate oxidation of adjacent OH groups[66] can be used to selectively eliminate certain residues prior to methylation, since not all linkage residues contain these. In the Smith degradation, oxidation is followed by $NaBH_4$ reduction,[71] and has been used before methylation analysis.[72] This degradation may be used in steps to reveal multiple branching, while methylation and trideuteriomethylation used before and after the mild hydrolysis, respectively, reveal sites of linkage of labile residues.[73] Smith degradation studies are otherwise used as a complementary technique of structural analysis to methylation studies, and can provide an alternative chemical means of estimating the ratio of linkage residues in complex interrupted-type[1] polysaccharides such as in cell walls.[74]

Enzymatic hydrolysis is very specific to types of glycosidic linkage but is limited to hydrolases that are commercially and naturally available.[75] Examples of their use relevant to linkage analysis include the digestion of starch glucans by amylases,[76] and the use of

hemicellulases in studying non-cellulosic plant cell wall polysaccharides.[77] A branched, interrupted[1] polymer such as pectin may be modified by paring back arabinose and galactose side-chains,[78] or further characterized as oligomers by degradation with rhamnogalcturonase.[79] Although NMR was used directly in the last case, these examples indicate the potential for using enzymatic degradation with methylation analysis.

Finally, a method developed to ensure subsequent methylation of only the positions bearing native acyl groups is the methyl replacement technique.[80] This involves the protection of all free OH groups using methyl vinyl ether in DMSO and *p*-toluenesulphonic acid catalyst. The derivatized material must be purified by nonaqueous gel filtration (e.g. on Sephadex LH-20), to avoid hydrolysis of the *O*-(1-methoxyethyl) protecting groups which are removed after methylation.

Downloaded At: 08:25 23 January 2011

4. METHYLATION OF POLYSACCHARIDES IN DIMETHYL SULPHOXIDE

The earlier uses of dimethyl sulphoxide (DMSO) were really variations of the Haworth and Kuhn methylations, and were also used on a preparative scale. These methods employ either DMSO/DMF or DMSO alone, with nonoxidizing bases such as BaO or $Ba(OH)_2$, and MeI or Me_2SO_4.[81,82] Good yields with a high degree of methylation in one step were reported, especially for DMSO/NaOH/Me_2SO_4.[83] Polysaccharides such as starch and pullulan were methylated and the methyl glycosides analyzed by GC.[82]

Addition of strong bases (such as NaH, KH, *n*-butyllithium and even potassium *tert*-butoxide) to DMSO generates the methylsulphinylmethanide or "dimsyl" anion.[84] Hakomori[85] famously used this reagent with CH_3I to methylate sugars and polysaccharides, for which it is a superior methylating catalyst to the bases described so far. It is also superior for complete methylation of polyuronates,[86] and the method has been refined[87] and is now in widespread use for the linkage analysis of polysaccharides.[3]

The preparation of the dimsyl reagent is laborious and hazardous since air and moisture must be rigorously excluded during its preparation (from highly flammable NaH or KH) and in storage. Alkali metal hydrides must be carefully washed free of oil with hexane and the presence of O_2 and/or H_2O in the DMSO causes formation of yellow-green by-products, so technical expertise is required to minimize the appearance of impurities in analytical work. Detailed practical procedures have been described elsewhere.[2,3,88,89] The K salt is easier to

prepare and more reactive,[1] with less contamination than Na salt.[17,90,91] Solutions of dimsyl reagent can be stored under argon at -20 °C for at least two years.[5] Preparation from LiH requires excessive heating while *n*-butyllithium is easier to handle and gives a cleaner product;[84,91] it can even be added directly to a suspension of polymer in DMSO, followed by hexane evaporation.[92,93]

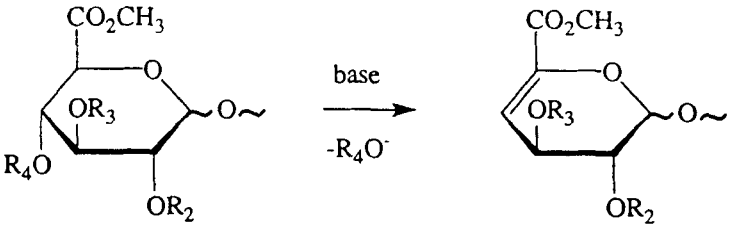
Triphenylmethane is used to indicate the presence of dimsyl anion by formation of a deep red adduct[94] which can be titrated with methanol. It is suggested that 3 to 6 equivalents of dimsyl per exchangeable H^+ are used with Na or K salt in solution.[95] A clear solution of the reagent is injected directly into a solution or suspension of carbohydrate in DMSO under argon, using 1 mL of 2M dimsyl/mL of carbohydrate solution. Incubation for 4 h[87] is followed by freezing and addition of 0.5 mL of CH_3I/mL of dimsyl. The reagent catalyses stoichiometric methylation of monosaccharides and can be used on a preparative scale. On an analytical scale, Na dimsyl has been used on as little as 15 μg of glycolipid pentasaccharides.[89]

Downloaded At: 08:25 23 January 2011

However, polysaccharides present complications. Undermethylation caused by insolubility or inadequate dispersion in DMSO may be overcome by lyophilizing or cryogenic milling of the starting material, followed by degassing, heating and sonicating the suspension in DMSO. Even so, non-solubilization of polysaccharide regions leads to variable amounts of unmethylated products in the preparation of PMAAs,[87,96,97] as demonstrated by remethylation.[17] It has been suggested that residual H_2O tightly bound to the carbohydrate preserves intramolecular hydrogen-bonded regions.[9] However, one plant cell-wall rhamnogalacturonan was dispersed and methylated successfully by first stirring it (60 mg) in H_2O (110 μL), 'diluting' with DMSO (11 mL) and adding excess Li dimsyl then MeI after 4 h.[98]

Another deficiency noted is the variable undermethylation of terminal glycosyl residues, possibly related to steric crowding of "ionized" hydroxyls. Finally, once added, CH_3I reacts rapidly with the base, competing with further activation of remaining free hydroxyls. Needs and Selvendran[9] found that a 25-fold excess of base to hydroxyl works well, and the rate of addition of CH_3I is "less critical".

Esterified uronic acid residues that are 4-*O*-substituted or that have been 4-*O*-methylated are susceptible to β-elimination by base (Scheme 1, R_4 = glycosyl or CH_3).[66,99,100] This is not normally a problem in methylation with dimsyl since the base disappears on addition of CH_3I, but it will happen if prolonged contact with base or remethylation is necessary. This



Scheme 1

$$(CH_3)_3CO^-K^+ + (CH_3)_2SO \rightleftharpoons (CH_3)_3COH + CH_3S(O)CH_2^-K^+$$

Scheme 2

Downloaded At: 08:25 23 January 2011

can be used deliberately to cleave a polymer into oligomers[101] and to label the site of attachment of the uronic acid (see below).

Opinions vary as to the efficacy of different metal salts of dimsyl. In spite of the advantages of the K salt, it sometimes gives levels of undermethylation which are too high for detailed linkage analysis of complex carbohydrates (e.g. from plant cell walls).[9] The Li salt *N*-methylates amino-sugars[92] and has been used to permethylate pectin and other non-cellulosic polysaccharides of the plant cell wall.[3]

Potassium *tert*-butoxide also forms the dimsyl ion in DMSO[84,102] and has also been used to methylate polysaccharides[103] but it is not as reactive as hydride preparations[104] and often undermethylates.[17,95] *tert*-Butyl alcohol formation may also interfere with methylation by solvating the base and reducing its basicity (Scheme 2).[104]

Although *O*-acyl groups are lost in the presence of these strong bases, *N*-acyl groups are retained, which can be vital to elucidating linkage structure.[105]

5. METHYLATION IN DMSO USING HYDROXIDE AS BASE

Both hydrides and *tert*-butoxide give low yields (about 30%) of permethylated material from the starting material. Addition of NaOH to *t*-BuOK to improve its performance led to the

discovery of the effectiveness of solid hydroxides alone.[95] Powdered dry NaOH or KOH are added to a solution or suspension of carbohydrate in DMSO, eliminating the preparation of dimsyl reagent, but requiring a good grinder. Between 2.4 and 24% w/w of NaOH/DMSO gives about 99% yield of permethylated mono-, di- and trisaccharides,[95] but there is significant degradation of uronic acids at NaOH concentrations >2 mmol per mL of DMSO; 1 mmol/mL appears sufficient for permethylation, without β-eliminative cleavage.[6]

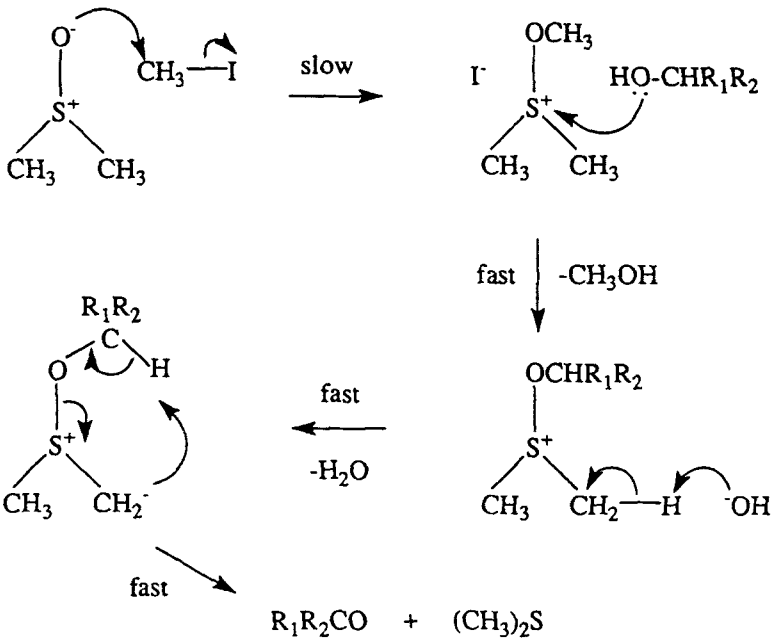
Ciucanu and Kerek[95] suggested that OH^- is the active base in deprotonation of carbohydrate OH groups rather than methanide ion. Alkoxide ions are very strong bases in DMSO,[104] and OH^- probably behaves similarly. However, it is less basic than dimsyl anion,[106,107] so NaOH does not dissolve in DMSO to produce dimsyl (in fact NaOH is only sparingly soluble). Needs & Selvendran suggest[9] that a key property of solid powdered NaOH is its ability to scavenge residual H_2O from "dry" DMSO and from carbohydrates. This is one reason why it is effective at catalysing methylation, and why adding a trace of water to Na dimsyl reagent (to give dispersed NaOH) does not produce any improvement.

Downloaded At: 08:25 23 January 2011

Application of NaOH-catalysed methylation to oligoglycosyl alditols results in significant numbers and amounts of oxidation products.[7] Oxidation occurs via reaction between CH_3I and DMSO[108] to produce a sulphonium ion which then converts alcohols to carbonyl compounds (Scheme 3).[109] This is possible because the concentration of carbohydrate in DMSO is greater than NaOH, whereas in Na dimsyl, the base is sufficiently dissolved to attack the sulphonium first.

Sequential addition of NaOH and then CH_3I immediately after NaOH minimizes this oxidation and gives the least undermethylation of polysaccharides, as shown by comparative studies on the methylation of cellobiose.[8] The hydroxide-polysaccharide-DMSO mixture is degassed, purged with argon and sonicated for up to 3 h, and then cooled to below 10 °C before addition of CH_3I.[10] Cooling slows the reaction between CH_3I and base, so NaOH continues catalysing methylation to near completion. The prolonged treatment with NaOH causes loss of *O*-substituents such as pyruvate acetal,[12] which is reported to be stable to prolonged methylation with K dimsyl.[101]

Recently a finely divided suspension of NaOH in DMSO has been prepared by diluting 50% NaOH with DMSO/MeOH and washing the precipitate with DMSO.[11] It is essentially anhydrous and can be used for the methylation and linkage analysis of as little as 10 μg of carbohydrate.



Scheme 3

Downloaded At: 08:25 23 January 2011

It should be noted that the procedure using NaOH catalyst is less suitable than that using "dimsyl" salts in the presence of certain functional groups. Thus the 6-phosphate of 2-acetamido-2-deoxy-D-glucose could be directly methylated with MeI/Na dimsyl/DMSO, but the free acid had to be first methylated with CH_2N_2 before methylating with MeI/NaOH/DMSO.[110] Also, the NaOH-catalysed methylation of sulphated oligosaccharide alditols gave a product that was too "salty" for FAB-MS studies, unlike that produced using Na dimsyl.[111]

6. METHYLATION OF POLYSACCHARIDES IN OTHER SOLVENTS

Solubility of polymers in DMSO varies considerably, and while charged polymers can usually be rendered more soluble by conversion to salts of organic cations (or anions), e.g. tetrabutylammonium, the degree of hydrogen-bonding in others such as cellulose and konjac mannan renders them very insoluble. For water-insoluble polysaccharides which cannot be lyophilized to a fibrous form, dry-milling to a fine powder aids swelling and dispersion in

DMSO[112] but is rarely completely satisfactory. Acetylation prior to methylation aids solubility for some polymers (e.g., cellulose) but not others (e.g., pectin).

A number of other solvent systems have been tried. 1,1,3,3-Tetramethylurea disrupts hydrogen bonds and 1:1 mixtures with DMSO give good permethylation of polysaccharides when used with dimsyl reagent.[113] Cellulose can be dispersed and permethylated in SO_2/DMSO/Et_2NH:[114] 40 µL of dry SO_2/DMSO (15 g/50 mL) and 20 µL of Et_2NH are injected into 1 mL of DMSO suspension and methylated with NaOH/CH_3I. Others investigated include LiCl/*N,N*-dimethylacetamide, 4-methylmorpholine *N*-oxide, *N*-methyl-2-pyrrolidinone and NO_2-DMSO[115,116] and show varying degrees of solubilization of cellulose but depolymerization is common[114,116] and methylation yields can be poor.[17,117]

7. METHYLATION WITHOUT STRONGLY BASIC CATALYSTS

Downloaded At: 08:25 23 January 2011

Carbohydrates which are labile to bases or which possess alkali-labile substituents, such as acetate or pyruvate, cannot be methylated under basic conditions without loss of some or all of those groups. There are two main ways of methylating carbohydrates at non-anomeric OH groups without the use of strongly basic catalysts.

In the absence of a suitable catalyst, a solution of diazomethane in ether or methanol causes esterification of carboxylic and other acidic groups, including the anomeric OH on mono- and oligosaccharides.[118,119] Some partial methylation of non-anomeric OH occurs but even after six treatments only 83% substitution of the latter results. Boron trifluoride etherate catalyses methylation by CH_2N_2[120] but is still considered to be unsatisfactory for permethylation, and requires the carbohydrate to be soluble in non-polar organic solvents. The inconvenience of keeping and handling labile CH_2N_2 does not make it the preferred option.

Methyl trifluoromethanesulphonate (methyl "triflate") is a good methylating agent in nonpolar solvents[121,122] but polar solvents such as DMSO, DMF and hexamethyl phosphoric triamide decrease its reactivity.[123] However, a compromise solvent, trimethyl phosphate has been successfully used for the mild permethylation of several bacterial lipopolysaccharides as Et_3NH^+ salts, and oligosaccharides.[53] As with DMSO, a small amount (1-5 mg) of carbohydrate is dried then dissolved by sonication in 1-2 mL of Me_3PO_4 and 150 or 300 µL[124] of 2,6-di-*tert*-butylpyridine (DTBP, a proton scavenger) and 100 µL of methyl triflate are added. The mixture is incubated at 50 °C for 2 h or may be left at room temperature for several

days[12] if the polysaccharide is labile to heating in this reagent. The methylated material is recovered using C_{18} reverse-phase cartridges (see below) or partitioning with $CHCl_3/H_2O$; a brief wash with 2M HCl in either case removes the weak base (which can be recovered). Trimethyl phosphate is not such a good solvent for some neutral polysaccharides, which cannot be converted to salts of bulky organic cations. However, the method has been applied to cellulose acetates.[13] A second mild methylating agent, trimethyloxonium tetrafluoroborate, was used in CH_2Cl_2 with DTBP, to check completion of methylation with the first. An improved Prehm procedure has been developed using a combination of methyl triflate methylation and methylation using CD_3I under basic conditions.[14]

8. RECOVERY OF METHYLATED CARBOHYDRATES

Downloaded At: 08:25 23 January 2011

Methylated mono- and oligosaccharides can be recovered on analytical and preparative scales by diluting with $CHCl_3$, filtering any solid off and washing with water several times. Syrups obtained on evaporation of the solvent are best purified by flash column chromatography on silica gel, and this can be done after deglycosidation. The presence of free OH groups in undermethylated sugars will ensure their separation from the bulk of permethylated material.

Suspensions of methylated polysaccharides usually require evaporation of excess CH_3I followed by dilution with water and exhaustive dialysis against water. This is time-consuming and much water must be evaporated off afterwards. The use of mini-columns of C_{18} reverse-phase material (e.g., Waters Sep-Paks^R) facilitate easy recovery in high yield and a short time in a small quantity of organic solvent.[125,126] The crude mixture is diluted with 5 vol of H_2O and loaded onto a primed cartridge. The saccharides are adsorbed and the reagents etc. are washed off with water. Mono- and oligosaccharides begin to elute with 15% MeOH and polysaccharides at >40% MeOH, though MeOH then MeOH/$CHCl_3$ ensure complete recovery. Alternatively, elutions with water, 1:4 MeCN-H_2O, MeCN then EtOH are used, the last two containing the permethylated polysaccharide.[2] Methylated sulphated polymers must be isolated by dialysis since they are too polar to be retained by C_{18} columns.

9. DETERMINATION OF DEGREE OF METHYLATION

It is important to check the completeness of methylation when characterizing a synthetic standard and for effective structural determination of a polysaccharide. In the latter

case undermethylation gives rise to products indicative of multiply-branched sugar residues and reduces the yield of genuine products. A first indication of undermethylation is the presence of finely suspended material in DMSO solutions: permethylated polysaccharides are usually soluble.[3] Infrared spectroscopy can be used to detect R-OH bonds and thus to estimate the degree of undermethylation,[85,113] and it should be possible using modern techniques to apply this to small samples. Conversion of the methylated polysaccharide to derivatives for GC should reveal an equimolar ratio of terminally linked sugars to branch points; an excess of the latter indicates undermethylation, although sometimes it happens only at the sterically crowded branch points, such as in the mild methylation of xanthan and acetan.[12] Remethylation of the methylated polymer with CD_3I, followed by derivatization and GC-MS should reveal sites of initial undermethylation by the fragmentation patterns.

A more direct method of quantifying degree of methylation is to determine methoxyl by colorimetry.[127] This involves hydrolysis with hot concentrated H_2SO_4, dilution, distillation of the liberated methanol, oxidation to formaldehyde with permanganate, then reaction with chromotropic acid to produce a chromophore which is measured spectrophotometrically. The process is laborious and was designed for preparative scale samples (1 g) but can probably be easily adapted for mg scale analysis.

Downloaded At: 08:25 23 January 2011

10. POST-METHYLATION MODIFICATION

The subsequent steps used in polysaccharide linkage analysis also affect the yield of products and must be considered when choosing the most appropriate method. Many of these reactions have been reviewed in detail elsewhere, so their main features and problems will be summarized here, in connection with methylation analysis.

Ethylation with iodoethane and trideuteriomethylation with CD_3I are performed similarly to methylation. They can be used as a way of labelling OH groups freed during partial hydrolysis of methylated polysaccharides,[128] e.g., to distinguish 4-linked pyranosyl from 5-linked furanosyl residues.[129] Partially methylated oligosaccharide alditols can be ethylated and separated by HPLC as part of the strategy in structural analysis of complex carbohydrates.[130] The di- and trisaccharides are volatile enough to characterise by GC-MS, whilst higher oligomers can be analysed by HPLC-MS or hydrolysed and converted to partially alkylated alditol acetates and submitted to GC-MS. Trideuteriomethylation is used instead of methylation

when native methyl groups are present, to determine their location from MS fragmentation patterns.[131]

Carboxyl reduction can be used after methylation as well as before. Uronic acid containing polymers that are amenable to permethylation, such as bacterial polysaccharides, can readily be carboxyl-reduced (and labelled) by $LiAlD_4$ in THF at 64 °C in 4 h.[87] The work-up is laborious and a superior method is $LiBDEt_3$ in THF for 1 hour,[2,98] which is quenched with dilute acetic acid, dried, then desalted on Dowex resin. Carboxyl reduction following methylation is thus more convenient but cannot be applied when glycosyluronates are labile to methylation, e.g., in pectic polysaccharides.[2]

11. CLEAVAGE AND REDUCTION OF METHYLATED POLYSACCHARIDES

Downloaded At: 08:25 23 January 2011

Following methylation (and modifications), glycosidic links in the methylated polymer can be broken by a number of means: acid hydrolysis,[17,132,133] formolysis,[134] methanolysis,[135] HF solvolysis,[136] reductive hydrolysis[137,16] and reductive cleavage.[15,22] β-Eliminative cleavage[66] is specific to uronic acid-containing polysaccharides in this context. Unmethylated carbohydrates have been studied in greatest detail concerning most of these techniques, but all can be applied to methylated polysaccharides prior to derivatization for GC-MS analysis.

Acid hydrolysis is the most widely used and potentially most destructive means of deglycosidation. Permethylated polysaccharides are generally insoluble in water, so 90% formic acid (100 °C, 2 h; i.e., formolysis)[134] followed by 0.25M H_2SO_4 (100 °C, 12 h) was originally used. Formic acid (90%) alone can also be used (100 °C, 4 h).[138] However, cleavage of neutral hexoses with 2M (~25%v/v) TFA is essentially complete at 121 °C in 1 h,[132,139] and the acid is removed simply by evaporation at 30 °C. Unfortunately, not only are rates of hydrolysis variable for different residues, so are the rates of decomposition of the liberated monosaccharides: aldopentoses, aldohexofuranoses, deoxysugars, and aldonic and uronic acids are all labile under the conditions described. Milder conditions can be used, e.g. permethylated fructans hydrolyse in 2M TFA at 60 °C in 30 min,[140] and methylated fructose is selectively released from heteropolymers with 50% acetic acid at 100 °C in 1 h.[105] *N*-Methylated aminosugars are resistant to acid hydrolysis, particularly once deacylated by the acid,[68,141] though they are stable once released. Also, >30% *O*-demethylation at C-1 of methylated oligohexosamine alditols has been observed after acid-catalysed cleavage.[142]

Methanolysis with 1M HCl in MeOH (80-100 °C, 3-24 h) cleaves most glycosidic linkages with minimal decomposition,[133] including glycosaminic linkages in glycoproteins[135] and glycuronidic linkages in chondroitin sulphate and dermatan sulphate.[143] Each monosaccharide produces several methyl glycosides and conditions must be anhydrous to prevent equilibration with free sugars. However, liberated methyl glycosides are stable, with the exception of partial de-*N*-acylation of glycosamines. The HCl is removed by evaporation or neutralization with ion-exchange resin. Recently, methanolysis followed by TFA hydroysis has been shown to improve recovery of monosaccharides from pectic, fungal and animal polysaccharides,[144] and could be potentially useful in methylation analysis. In spite of the advantages, methanolysis has not found wide application in this area.

Hydrofluorinolysis in anhydrous HF also cleaves most glycosidic linkages with no decomposition, leaving *N*-acyl groups and, under certain conditions, *O*-acyl groups intact.[145] The rate of cleavage depends on the sugar, anomer, neighbouring residues, temperature and time. Thus at 20 °C it liberates most neutral sugars and glycosamines,[136] whereas at -40 °C it selectively cleaves to produce oligosaccharides,[146] e.g., the solvolysis of α-L-rhamnopyranosyl in gellan,[147] so it could be useful in sequencing. Sugars are converted to α-glycosyl fluorides and the HF is removed by coevaporation with methanol (producing methyl glycosides) or by quenching with water (producing glycoses). Handling of anhydrous HF requires special apparatus and safe techniques due to its hazardous properties, and this appears to have limited its use. But it has been applied to the methylation analysis of bacterial polysaccharides where retention of *N*-acyl groups was required,[148,149] including polysaccharides linked *via* *N*-glyceroyl substituents.[105]

Reduction with sodium borodeuteride[17] is used after hydrolysis to convert glycoses to alditols, prior to acetylation for GC-MS analysis. The deuterium labels the reducing ends of the partially methylated sugars to differentiate products which would become otherwise stereoisomerically identical when reduced. Reduction in 1-3M NH_3 is quantitative for partially methylated pentoses and 2,3,4,6-tetra-*O*-methylglucose in 60 min at 60 °C, and >98% complete for other hexoses; it is less efficient in non-aqueous solvents.[17] To overcome incomplete reduction, solubilization in 0.03M NaOH in 30% MeOH has been suggested[11] (reduction done at 37 °C for 4 h in this case). Excess $NaBD_4$ is subsequently destroyed with acetone or glacial acetic acid, but if borate must be removed completely, acidification with Dowex 50 (H^+) resin is preferred, followed by coevaporation with MeOH three times.[19]

Downloaded At: 08:25 23 January 2011

Reductive hydrolysis conveniently combines acid hydrolysis and reduction to alditols in one reaction, but in a 3-step procedure.[15,20] This uses 4-methylmorpholine-borane in 2M TFA (120 °C, 1 h) for the main step, and was developed for recovery of acid-labile 3,6-anhydrogalactose from algal galactans. Preliminary hydrolysis in 0.5M TFA was found necessary to apply this method successfully to methylated polysaccharides.

Reductive cleavage[150,10] provides an alternative to conventional methylation analysis since it does not rely on acid hydrolysis at all. Instead it cleaves glycosidic linkages by Lewis acid-catalysed organosilane reduction of the glycosidic carbon, to form partially methylated 1,5-anhydroalditols from pyranosides or 1,4-anhydroalditols from furanosides. These are characterized by GC-MS as their acetates or by ^{1}H NMR as their benzoates, yielding ring size in addition to identity and linkage positions (conventional methylation analysis gives the first detail only with extra steps[129]). Reduction times are long (8-24 h) and water must be rigorously excluded from the reaction otherwise ring contraction (pyranose to furanose) and hydrolysis can occur.[151] For neutral sugars, trimethylsilyl (TMS) 'triflate' or TMS 'mesylate'/$BF_3.Et_2O$ catalyse total cleavage, while glycosaminic[152] and glycuronidic linkages[153] can be resistant or undergo side reactions. Selectivity is exhibited in other ways. 1,6-Linked glucopyranoside is resistant when $BF_3.Et_2O$ is the sole catalyst,[153] TMS mesylate selectively catalyses cleavage of fructofuranosides.[154] *O*-Acyl groups are stable under certain conditions,[155,156] so this may be useful in combination with mild methylation.[124]

Degradation by β-elimination of uronic acid-containing carbohydrates by base can be used deliberately to aid structural studies.[99,66,100] The requirements of the reaction are that the carboxyl groups are esterified (to make it sufficiently electron-withdrawing) and that *O*-4 is substituted (Scheme 1); methylation with Na or K dimsyl in DMSO achieves this (selective degradation of uronic acids already linked at *O*-4 can be done without methylation, provided they are esterified). The methylated polysaccharide is treated either with NaOMe/MeOH or a further aliquot of Na or K dimsyl in DMSO, or the methylation is simply extended;[101] water must be excluded. The unsaturated product is acid-labile and so is lost when the poly- or oligosaccharides are further cleaved by acid hydrolysis. If mild acid treatment is initially used, the freed OH groups can be trideuteriomethylated to label the sites of glycuronidic linkage before proceeding to complete cleavage (any reducing ends produced by the mild hydrolysis should be reduced with $NaBH_4$).[66] This has been applied to branched bacterial and plant polysaccharides.[157,92,68]

Downloaded At: 08:25 23 January 2011

12. ACETYLATION

Acetylation with acetic anhydride is the usual final step in derivatization of cleaved, permethylated polysaccharides. Acetates of partially methylated monosaccharides are volatile enough to separate by GC and are stable enough for cleanup procedures and storage. Six catalysts have been used under the following conditions:

(i) Ac_2O/pyridine (0.1 mL each), 120 °C, 20 min, cool, coevaporate with toluene.[157,158]

(ii) Ac_2O/sodium acetate, 120 °C.[159,126]

(iii) Ac_2O/4-dimethylaminopyridine (DMAP), 20 °C, 2-4 h, quench H_2O, extract into $CHCl_3$.[11,5]

(iv) Ac_2O/*N*-methylimidazole (NMIM) (2-3 mL:0.2-0.5 mL), 30 °C, 30 min.[160-162,17,18]

(v) Ac_2O/EtOAc/AcOH (0.2:1:3 mL) then $HClO_4$ (0.1 mL), 5 min, then H_2O/NMIM (10:0.2 mL), extract into CH_2Cl_2.[17,163]

(vi) As (iv), but using CF_3SO_3H instead of $HClO_4$.[19]

Pyridine and DMAP only work in the absence of borate, so if $NaBH(D)_4$ reduction precedes acetylation, borate is first removed as the trimethyl ester by repeated coevaporation with MeOH. This risks loss of more volatile alditols; the toluene coevaporation in (i) similarly risks loss of PMAAs. Pyridine also involves excessive heating and formation of byproducts with Ac_2O, while DMAP is a solid and must be worked into mixtures.[11] However, DMAP reduces artefact formation better than NMIM.[11] NMIM is useful because it is a solvent as well as an efficient, low-temperature catalyst[160] and does not require removal of H_2O or borate, except when analysing for 1,3-linked glucopyranoside (the alditol strongly complexes to borate).[17] NMIM gives reduced sensitivity for deoxyhexoses and heptoses, and is unsuitable for certain aminosugars (e.g., muramic acid): NaOAc gives good yields for all these and less background noise,[159] but requires stronger heating.[126] Acid catalysis does not require borate removal either,[17] and CF_3SO_3H was found to work as well as $HClO_4$,[19] without the explosive hazard of $HClO_4/Ac_2O$ mixtures. Unfortunately, side reaction are possible in strong acids,[164] producing artefacts, and significant loss of terminally linked sugars is reported, especially from oligoalditols.[163] The final evaporative step to remove water-washed CH_2Cl_2 extracts in (ii)-(vi) is unlikely to cause significant losses of some PMAAs if done at <40 °C for <1 h.[19]

Downloaded At: 08:25 23 January 2011

13. SUMMARY: CHEMICAL STRATEGY

Given the breadth of chemical techniques covered, it is perhaps useful to provide a guide indicating the appropriate strategy when considering methylation analysis of a polysaccharide of unknown structure. It is assumed that some idea of its composition has been inferred from its source and preliminary chemical analysis (colorimetric tests, neutral sugars by GLC). Table 1 lists carbohydrate polymers according to broad classes of chemical feature, and suggests the preferred chemical sequences that can be used for linkage analysis. It should give a general starting point based on existing knowledge, rather than a rigid set of methods. Some carbohydrates belong to more than one class and appropriately combined sequences of steps are required. For many, it can be seen that several possibilities exist and should even be tried. Variations in reaction conditions discussed may also be optimised by experimentation, for example the duration of treatment necessary with NaOH and MeI in sequential base-catalysed methylation.

The linkage analysis of all these polymers should be complemented by spectroscopic studies of the studies of them or their oligomers. This is usually NMR of solutions in D_2O or sometimes deuterated DMSO, but FAB-MS of acetylated oligosaccharides may be applicable if the sample is very small or simply insoluble.

Downloaded At: 08:25 23 January 2011

14. THE FUTURE

The constant advances in NMR technology are increasingly placing it at centre stage in complex carbohydrate analysis. Linkage analysis in its various forms is now often preliminary to NMR. However, the expertise in one is usually practised alongside the other, and one advantage of linkage analysis lies in its applicability to much smaller quantities of sample (as little as 10 μg versus 10 mg for NMR). So it is still clearly worth seeking improvements to the technique, in particular the methylation step. A number of variations are in use and several can be applied successfully to glycoconjugate oligosaccharides and readily dispersed regular bacterial polysaccharides. Special problems arise with non-dispersible polymers, in particular irregular heteropolysaccharides, such as pectins. These are less amenable to NMR spectroscopy, and quantitative determination of linkage ratios depends crucially on successful methylation and derivatization. Recoveries of sugar residues are sensitive to the method used[19] and further comparative methylation studies are currently under way to assess these.

Table 1. Suggested sequences of steps for the structural analysis of carbohydrates, involving methylation.

Carbohydrate type (polysaccharide unless stated)	Pre-methylation step(s)	Methylation step	Cleavage/ reduction and intermediate steps	Other post-methylation steps
Neutral oligosaccharide	7	1 or 2	-	27
	7	1 or 2	20	26
Neutral, DMSO-soluble ps	(14,6)	1 or 2	20, 6a	8a,26
	(14,6)	1 or 2	21a	8a,26
Neutral DMSO-insoluble ps	1 or 8b or 14,6	3 (or 1)	20, 6a	8a,26
	1 or 8b or 14,6	3	21a	8a,26
Pentose, furanose		1 or 2	25,6a,5a,12,6a	8a,26
		1 or 2	21	8a,26
Deoxysugar		1 or 2	23b(or 13);5;20.. or 13;6a	8a or d,26
Uronic acid, periodic	(9)	1 or 2	10b or 17;20,6a	8a,26
		1 or 2	21	8a,26; or 28
Uronic acid, interrupted	10a, (14,6)	1 or 2	20,6a	8a,26
	(14,6)	1	21	8a,26; or 28
Esterified uronic acid	7	1	20,6a	8a,26
			21	8a,26; or 28
Glycosamine	(16,6b)	1	24,(13,6a)	8a or c or d;26
Oligoglycosamine	(20),12	1	-	26
Glycoprotein		1 or 2b	24,(13,6a)	8a or c;26
Sulphated	11	1	20 or 22 or 24	6a,8a,26
	14	1a	-	26 or 27
Phosphorylated		1	23b(or... ...13),5,20,6a	8a or c,26
O-Acyl substituents	(9)	4	20,6a	8a,26
	(9)	4	21	8a,26; or 28
	(9),19	1	20,6a	8a,26

Downloaded At: 08:25 23 January 2011

Table 1. (continued)

Carbohydrate type (polysaccharide unless stated)	Pre-methylation step(s)	Methylation step	Cleavage/ reduction and intermediate steps	Other post-methylation steps
N-Acyl substituents		1	23a	8a,26
Highly branched	18,6b	1 (or 2)	(20,6a)	8a or c;26
	(13)	1 (or 2)	13,6a,5	8a or c;26

Key to chemical steps:
1. Methylation ("dimsyl"): DMSO and a) NaH or b) KH or c) *n*-BuLi, then MeI.
2. Methylation: DMSO/NaOH then MeI; a) "sequentially" b) micro-scale.
3. Methylation: DMSO/$(Me_2N)_2CO$/NaH then MeI.
4. Mild methylation: Me_3PO_4/MeOTf/DTBP.
5. Ethylation or trideuteriomethylation: DMSO/NaH or NaOH then a) EtI or b)CD_3I.
6. Reduction: a)$NaBD_4$ or b)$NaBH_4$ in 2-3M NH_3.
7. Reduction: $NaBD_4$ or $NaBH_4$ in imidazole pH 5-7.
8. Acetylation: Ac_2O and a) NMIM, 30 °C or b) pyridine, 100 °C or c) DMAP, 20 °C or d) NaOAc, 120 °C.
9. Convert UA to Et_3NH^+ or Bu_4N^+ salt.
10. Reduce carboxyl: a) CMC then $NaBH_4$ or $NaBD_4$ in imidazole pH 5-7; b) $LiBDEt_3$/THF.
11. Desulphation: Solvolytic heating or NaOH/heat.
12. *N*-Trifluoroacetylation: TFA/TFAA, 100 °C.
13. Mild acid hydrolysis: 0.1M H_2SO_4 or 0.5M TFA or 50% AcOH, 100 °C.
14. Enzyme digestion.
15. *N*-Deacetylation: $N_2H_4/N_2H_6SO_4$, 105 °C (or NaOH/PhSH/DMSO/H_2O).
16. Deamination: $NaNO_2$/HCl.
17. Base-catalysed elimination: NaH or KH or NaOH and DMSO.
18. Periodate oxidation: $NaIO_4$, pH 3-3.5.
19. Methyl replacement: MeOCH=CH_2/TsOH/DMSO.
20. Acid hydrolysis: a) 2M TFA, 120 °C or b) 90% HCO_2H then 0.5M H_2SO_4, 100 °C.
21. Reductive cleavage: Et_3SiH and a) TMSOTf or TMSOMs/$BF_3.Et_2O$; or b) $BF_3.Et_2O$ or c) TMSOMs.
22. Reductive hydrolysis: MMB/2M TFA, 120 °C.
23. Hydrofluorinolysis: HF, a) 20 °C; b) -40 to -20 °C.
24. Methanolysis: 2M HCl/MeOH (anhydrous), 80-100 °C.
25. Formolysis: 90% HCO_2H, 90 °C.
26. GLC-EI-MS.
27. FAB-MS.
28. Benzoylation, HPLC, ^1H NMR.

Downloaded At: 08:25 23 January 2011

Perhaps eventually an efficient aqueous methylation will be developed for organically-insoluble polysaccharides, based on the Haworth reaction. Perhaps a procedure with fewer steps will be developed - for example, the reductive cleavage and reductive hydrolysis reactions already combine two of the subsequent steps into one (hydrolysis and reduction). Meanwhile routine derivatization of polysaccharides will become possible, with care, on smaller scales. The

limit of detection of FID for an individual PMAA in a sample with a "clean" GC baseline is about 1-2 ng; with 100% recovery, there is theoretically room for miniaturization of sample size to ten times this, using appropriate GC sample vials and injection volumes.

Presumably the ultimate goal of any "wet" biopolymer analysis is automated chemical sequencing, as is done for proteins and nucleic acids. In its present form the methylation step seems unlikely to be incorporated into such a process. Already an automated alternative has appeared that can be applied to ng samples of antigen oligosaccharides.[165] The technique combines enzymatic degradation with chromatography and computer analysis of fragments. Whether it can be practically and logically extended to polysaccharides, thus superseding current chemical analysis, may be a matter of time.

15. ACKNOWLEDGEMENTS

This work was funded by the UK Office of Science and Technology. The author would like to thank Dr. Paul Needs and Mr. Neil Rigby for helpful discussions.

Downloaded At: 08:25 23 January 2011

16. REFERENCES

1. G.O. Aspinall, *The Polysaccharides*, Vol. 1; Academic Press: New York, 1982, p 35.
2. W.S. York, A.G. Darvill, M. McNeil, T.T. Stevenson and P.Albersheim, *Methods Enzymol.*, **118**, 3 (1985).
3. N.C. Carpita and E.M. Shea in *Analysis of Carbohydrates by GC and MS;* C.J. Biermann and G.D. McGinnis, Eds.; CRC Press: Boca Raton, 1989, p157.
4. R.R. Selvendran and P. Ryden, in *Methods in Plant Biochemistry.* Vol. 2: *Carbohydrates;* P.M. Dey and J.B. Harbourne, Eds.; Academic Press: San Diego, 1990, p 549.
5. F-G. Hanisch, *Biol. Mass Spec.*, **23**, 309 (1994).
6. I. Ciucanu and C. Luca, *Carbohydr. Res.*, **206**, 71 (1990).
7. W.S. York, L.L. Kiefer, P. Albersheim and A.G. Darvill, *Carbohydr. Res.*, **208**, 175 (1990).
8. P.W. Needs and R.R. Selvendran, *Carbohydr. Res.*, **245**, 1 (1993).
9. P.W. Needs and R.R. Selvendran, *Phytochem. Anal.*, **4**, 210 (1993).
10. C.A. MacCormick, J.E. Harris, A.P. Gunning and V.J. Morris, *J. Appl. Bacteriol.*, **74**, 196 (1993).
11. K.R. Anamula and P.B. Taylor, *Anal. Biochem.*, **203**, 101 (1992).
12. C. Ojinnaka, A.J. Jay, I.J. Colquhoun, G.J. Brownsey, E.R. Morris and V.J. Morris, *Int. J. Biol. Macromol.*, (in press).
13. P. Mischnick, *J. Carbohydr. Chem.*, **10** (4), 711 (1991).
14. I.M. Sims *et al.*, *Carbohydr. Res.*, (in press).

15. G.R. Gray, *Methods Enzymol.*, **138**, 26 (1987).
16. T.T. Stevenson and R.H. Furneaux, *Carbohydr. Res.*, **210**, 277 (1991).
17. P.J. Harris, R.J. Henry, A.B. Blakeney and B.A. Stone, *Carbohydr. Res.*, **127**, 59 (1984).
18. A.B. Blakeney, P.J. Harris, R.J. Henry and B.A. Stone, *Carbohydr. Res.*, **113**, 291 (1983).
19. P.W. Needs and R.R. Selvendran, *Carbohydr. Res.*, **254**, 229 (1994).
20. D. Rolf, J.A. Bennek and G.R. Gray, *Carbohydr. Res.*, **137**, 183 (1985).
21. S.H. Doares, P. Albersheim and A.G. Darvill, *Carbohydr. Res.*, **210**, 311 (1991).
22. R. Falshaw and R.H. Furneaux, *Carbohydr. Res.*, **252**, 171 (1994).
23. D.P. Sweet, R.H. Shapiro and P. Albersheim, *Carbohydr. Res.*, **40**, 217 (1975).
24. H. Björndal, B. Lindberg, Å. Pilotti and S. Svensson, *Carbohydr. Res.*, **15**, 339 (1970).
25. L.E. Elvebak II, C. Abbott, S. Wall and G.R. Gray, *Carbohydr. Res.*, **269**, 1 (1995).
26. S.G. Zeller and G.R. Gray, *Carbohydr. Res.*, **198**, 285 (1990).
27. H.O. Bouveng and B. Lindberg, *Adv. Carbohydr. Chem.*, **15**, 53 (1960).
28. T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).
29. E.L. Hirst and E. Percival, in *Methods in Carbohydrate Chemistry,* Vol II; R. Whistler and M.L. Wolfrom, Eds; Academic Press: NY & London, 1963, p145.
30. T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **85**, 1049 (1904).
31. W.S. Denham and H. Woodhouse, *J. Chem. Soc.*, **103**, 1735 (1913).
32. W.N. Haworth, *J. Chem. Soc.*, **107**, 8 (1915).
33. W.N. Haworth and E.L. Hirst, *J. Chem. Soc.*, **119**, 193 (1921).
34. W.L. Glen, G.S. Myers and G.A. Grant, *J. Chem. Soc.*, 2568 (1951).
35. E.L. Falcover and G.A. Adams, *Can. J. Chem.*, **34**, 338 (1956).
36. E. Pascan and S.M. Trister, *J. Am. Chem. Soc.*, **61**, 2442 (1939).
37. R. Kuhn, H. Trischmann and I. Löw, *Angew. Chem.*, **67**, 32 (1955).
38. N. Kornblum and R.K. Blackwood, *J. Am. Chem. Soc.*, **78**, 4037 (1956).
39. R.C. Menzies and C.M. Fear, *J. Chem. Soc.*, 937 (1926).
40. C.C. Barker, E.L. Hirst and J.K.N. Jones, *J. Chem. Soc.*, 783 (1946).
41. H.G. Walker Jr., M. Gee and R.M. McCready, *J. Org. Chem.*, **21**, 2100 (1962).
42. L. Schmid and B. Becker, *Chem. Ber.*, **58**, 1966 (1925).
43. I.E. Muskat, *J. Am. Chem. Soc.*, **56**, 693 (1934).
44. I.E. Muskat, *J. Am Chem. Soc.*, **56**, 2449 (1934).
45. R.S. Tipson, in *Methods in Carbohydrate Chemistry, Vol II;* R. Whistler and M.L. Wolfrom Eds.; Academic Press: NY & London, 1963.
46. K. Freudenberg and W. Rapp, *Chem. Ber.*, **69**, 2041 (1936).
47. K. Freudenberg, E. Plankenhorn and H. Boppel, *Chem. Ber.*, **71**, 2435 (1938).
48. K. Freudenberg and R.M. Hixon, *Chem. Ber.*, **56**, 2119 (1923).
49. J.E. Hodge, S.A. Karjala and G.E. Hilbert, *J. Am. Chem. Soc.*, **73**, 3312 (1951).
50. H.S. Isbell, H.L. Frush, B.H. Bruckner, G.N. Kowkabany and G. Wampler, *Anal. Chem.*, **29**, 1523 (1957).
51. D.M. Lemal, P.D. Pacht and R.B. Woodward, *Tetrahedron*, **18**, 1275 (1962)
52. S.A. Barker, R.E. Hurst, J. Settine, F.P. Fish and R.L. Settine, *Carbohydr. Res.*, **125**, 291 (1984).
53. P. Prehm, *Carbohydr. Res.*, **78**, 372 (1980).

Downloaded At: 08:25 23 January 2011

54. R.O. Cuoso, L. Ielpi and M.A. Dankert, *J. Gen. Microbiol.*, **133**, 2123 (1987).
55. P.-E. Jannson, J. Lindberg, M. Spellman, T. Hofstad and N. Skaug, *Carbohydr. Res.*, **137**, 197 (1985).
56. R.L. Taylor and H.E. Conrad, *Biochem.*, 11, 1383 (1972).
57. J.N. BeMiller, *Adv. Carbohydr. Chem. Biochem.*, **22**, 25 (1967).
58. J.-B. Kim and N.C. Carpita, *Plant Physiol.*, **98**, 646 (1992).
59. I.M. Sims and A. Bacic, *Phytochem.*, **38**, 1397 (1995).
60. D.A. Rees, *J. Chem. Soc.*, 5168 (1961).
61. B. Nillson and D. Zopf, *Methods Enzymol.*, **83**, 46 (1982)
62. B. Lindberg, J. Lönngren, J.L Thompson and W. Nimmich, *Carbohydr. Res.*, **25**, 49 (1972).
63. M.A. O'Neill, R.R Selvendran, V.J. Morris and J. Eagles, *Carbohydr. Res.*, **147**, 295 (1986).
64. A.J. Mort and D.T.A Lamport, *Anal. Biochem.*, **82**, 289 (1977).
65. C.A. Abeygunawardana, C.A. Bush, S.S. Tjoa, P.V. Fennessey and M.A. McNeil, *Carbohydr. Res.*, **191**, 279 (1989).
66. B. Lindberg, J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, **31**, 185 (1975).
67. Z. Yosizawa, T. Sato and K. Schmid, *Biochim. Biophys. Acta*, **121**, 417 (1966).
68. B. Lindberg and J. Lönngren, *Methods Enzymol.*, **50**, 3 (1978).
69. C. Erbing, K. Granath, L. Kenne and B. Lindberg, *Carbohydr. Res.*, **47**, c5 (1976).
70. T. Doco, J-M. Wieruszeski and B. Fournet, *Carbohydr. Res.*, **198**, 313 (1990).
71. I.J. Goldstein, G.W. Hay, B.A. Lewis and F. Smith, *Methods Carbohydr. Chem.*, **5**, 361 (1965).
72. B. Lindberg, J. Lönngren and W. Nimmich, *Acta Chem. Scand.*, **26**, 2231 (1972).
73. B. Lindberg, J. Lönngren, U. Rudén and W. Nimmich, *Acta Chem. Scand.*, **27**, 3787 (1973).
74. K.C.B. Wilkie and S.-L. Woo, *Carbohydr. Res.*, **4**, 399 (1966).
75. R.J. Sturgeon in *Methods in Plant Biochemistry.* Vol 2: *Carbohydrates;* P.M. Dey and J.B. Harbourne Eds.; Academic Press: San Diego, 1990, p 5.
76. P. Cairns, V.J. Morris, R.L. Botham and S.G. Ring, *J. Cereal Sci.*, **23**, 265 (1996).
77. R.F.H. Dekker and G.N. Richards, *Adv. Carbohydr. Chem. Biochem.*, **32**, 277 (1976).
78. F. Guillon, J.-F. Thibault, F.M. Rombouts, A.G.J. Voraga and W. Pilnik, *Carbohydr. Res.*, **190**, 97 (1989).
79. H.A. Schols, A.G.J. Voragen and I.J. Colquhoun, *Carbohydr. Res.*, **256**, 97 (1994).
80. A.N. de Belder and B. Norrman, *Carbohydr. Res.*, **8**, 1 (1968).
81. R. Kuhn and H. Trischmann, *Chem. Ber.*, **96**, 284 (1963).
82. K. Wallenfals, G. Bechtler, R. Kuhn, H. Trischmann and H. Egge, *Angew. Chem.*, **75**, 1014 (1963).
83. H.C. Srivastava, P.P. Singh, S.N. Harshe and K. Virk, *Tetrahedron Lett.*, 493 (1964).
84. J.E. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, **84**, 866 (1962).
85. S-H. Hakomori, *J. Biochem. (Tokyo)*, **55**, 205 (1964).
86. D.M.W Anderson and G.M. Cree, *Carbohydr. Res.*, **2**, 162 (1966).
87. P.A. Sandford and H.E. Conrad, *Biochem.*, **5**, 1508 (1966).

Downloaded At: 08:25 23 January 2011

88. A. Dell, A.J. Reason, K-H. Khoo, M. Panico, R.A. McDowell, and H.R. Morris, *Methods Enzymol.*, **230**, 108 (1994).
89. S.B. Levery and S-I. Hakomori, *Methods Enzymol.*, **138**, 13 (1987).
90. L.R. Phillip and B.A. Frazer, *Carbohydr. Res.*, **90**, 149 (1981).
91. A.B. Blakeney, and B.A. Stone, *Carbohydr. Res.*, **140**, 319 (1985).
92. J.P. Parente, P. Cardon, Y. Leroy, J. Montreuil, B. Fornet, and G. Ricart, *Carbohydr. Res.*, **141**, 41 (1985).
93. A.L. Kvernheim, *Acta Chem. Scand. Ser. B*, **41**, 150 (1987).
94. H. Rauvala, *Carbohydr. Res.*, **72**, 257 (1979).
95. I. Ciucanu and F. Kerek, *Carbohydr. Res.*, **131**, 209 (1984).
96. D.P. Sweet, P. Albersheim and R.H. Shapiro, *Carbohydr. Res.*, **40**, 199 (1975).
97. R.R. Selvendran and S.E. King, *Carbohydr. Res.*, **195**, 87 (1989).
98. T.T. Stevenson, A.G. Darvill and P. Albersheim, *Carbohydr. Res.*, **179**, 269 (1988).
99. B. Lindberg, J. Lonngren and J.L. Thompson, *Carbohydr. Res.*, **28**, 351 (1973).
100. G.O. Aspinall and K.-G. Rosell, *Carbohydr. Res.*, **57**, c23 (1977).
101. R. Somme, *Carbohydr. Res.*, **152**, 237 (1986).
102. J.I. Brauman, J.A. Bryson, D.C. Kahl and N.J. Nelson, *J. Am. Chem. Soc.*, **92**, 6679 (1970).
103. J. Finne, T. Krusius and H. Rauvala, *Carbohydr. Res.*, **80**, 336 (1980).
104. J.H. Exner and E.C. Steiner, *J. Am. Chem. Soc.*, **96**, 1782 (1974).
105. P.-E. Jannson, J. Lindberg, K.M.S. Wimalasiri and M.A. Dankert, *Carbohydr. Res.*, **245**, 303 (1993).
106. E.C. Steiner and J.M. Gilbert, *J. Am. Chem. Soc.*, **85**, 3054 (1963).
107. D.R. Burfield and R.H. Smithers, R.H. *J. Org. Chem.*, **43**, 3966 (1978).
108. A.P. Johnson, and A. Pelter, *J. Chem. Soc.*, 520 (1964).
109. C.R. Johnson, and W.G. Phillips, *J. Org. Chem.*, **32**, 1926 (1967).
110. U. Zahringer, H. Moll, E.Th. Rietschel, B. Kraska, M. Imoto and S. Kusumoto, *Carbohydr. Res.*, **196**, 147 (1990).
111. A. Dell, M.E. Rogers, J.E. Thomas-Oates, T.N. Huckerby, P.N. Sanderson and I.A. Nieduszynski, *Carbohydr. Res.*, **179**, 7 (1988).
112. J.A. Lomax, A.H. Gordon and A. Cheeson, *Carbohydr. Res.*, **122**, 11 (1983).
113. T. Narui, K. Takahashi, M. Kobayashi and S. Shibata, *Carbohydr. Res.*, **103**, 293 (1982).
114. A. Isogai, A. Ishizu, J. Nakauo, S. Eda, and K. Kato, *Carbohydr. Res.*, **138**, 99 (1985).
115. A. El-Kafrawy, *J. Appl. Polym. Sci.*, **27**, 2435 (1982).
116. J-P. Joseleau, G. Chambat and B. Chumpitazi-Hermoza, *Carbohydr. Res.*, **90**, 339 (1981).
117. S.M. Hudson and J.A. Cuculo, *J. Macromol. Sci., Rev. Macromol. Chem.*, **18**, 1 (1980).
118. R.J. Ferrier and P.M. Collins, *Monosaccharide Chemistry*; Penguin: London, 1972.
119. L. Hough and R.S. Theobald, in *Methods in Carbohydrate Chemistry*, Vol II; R. Whistler and M.L. Wolfrom, Eds; Academic Press: NY & London, 1963 p162.
120. I.O. Mastroni, S.M. Flematti, J.O. Deferrari and E.G. Gros, *Carbohydr. Res.*, **3** 177 (1966).

Downloaded At: 08:25 23 January 2011

121. J. Arnap, L. Kenne, B. Lindberg and J. Lönngren, *Carbohydr. Res.*, **44**, c5 (1975).
122. J.M. Berry and L.D. Hall, *Carbohydr. Res.*, **47**, 307 (1976).
123. C.R. Wong, L.M. Jackman and R.G. Portman, *Tetrahedron Lett.*, 921 (1974).
124. J.D. Stankowski and S.G. Zeller, *Carbohydr. Res.*, **224**, 337 (1992).
125. A.J. Mort, S. Parker and M-S. Kuo, *Anal. Biochem.*, **133**, 380 (1983).
126. T.J. Waege, A.G. Darvill, M. McNeil and P. Albersheim, *Carbohydr. Res.*, **123**, 281 (1983).
127. A.P. Mathers and M.J. Pro, *Anal. Chem.*, **27**, 1662 (1955).
128. B.S. Valent, A.G. Darvill, M. McNeill, B.K. Robertson and P. Albersheim, *Carbohydr. Res.*, **79**, 165 (1980).
129. A.G. Darvill, M. McNeill and P. Albersheim, *Carbohydr. Res.*, **86**, 309 (1980).
130. M. McNeil, A.G. Darvill, P. Åman, L.K. Franzén and P. Albersheim, *Methods Enzymol.*, **83**, 3 (1982)
131. N.C. Carpita and D. Whittern, *Carbohydr. Res.*, **146**, 129 (1986).
132. P. Albersheim, D.J. Nevins, P.D. English and A. Karr, *Carbohydr. Res.*, **5**, 340 (1967).
133. C.J. Biermann, *Adv. Carbohydr. Chem. Biochem.*, **46**, 251 (1989).
134. I. Croon, G. Herrstrom, G. Kull and B. Lindberg, *Acta Chem. Scand.*, **14**, 1338 (1960).
135. R.E. Chambers and J.R. Clamp, *Biochem. J.*, **125**, 1009 (1971).
136. G.L. Rorrer, M.C. Hawley, S.M. Selke, D.T.A. Lamport and P.M. Dey, in *Methods in Plant Biochemistry. Vol. 2: Carbohydrates;* P.M. Dey and J.B. Harbourne, Eds.; Academic Press: San Diego, 1990, p 581.
137. P.J. Garregg, B. Lindberg, P. Konradson and I. Kvarnström, *Carbohydr. Res.*, **176**, 145 (1988).
138. M.H. Saier Jr. and C.E. Ballou, *J. Biol. Chem.*, **243**, 4319 (1968).
139. H.E. Conrad, J.R. Bamburg, J.D. Epley and T.J. Kindt, *Biochemistry*, **5**, 2808 (1975).
140. C.J. Pollock, M.A. Hall and D.P. Roberts, *J. Chromatogr.*, **171**, 411 (1979).
141. H. Rauvala, J. Finne, T. Krusius, J. Karkainen and J. Jarnfelt, *Adv. Carbohydr. Chem, Biochem.*, **38**, 389 (1981).
142. B. Nillson, *Glycoconjugate J.*, **2**, 335 (1985).
143. S. Inoue and M. Miyawaki, *Anal. Biochem.*, **65**, 164 (1975).
144. G.A.De Ruiter, H.A. Schols, A.G.J. Voragen and F.M. Rombouts, *Anal. Biochem.*, **207**, 176.
145. Yu.A. Knirel, E.V. Vinogradpv amd A.J. Mort, , *Adv. Carbohydr. Chem, Biochem.*, **47**, 167 (1989).
146. A.J. Mort, J.P. Utille, G. Torri and A.S. Perlin, *Carbohydr. Res.*, **121**, 221 (1983).
147. M.-S. Kuo and A.J. Mort, *Carbohydr. Res.*, **156**, 173 (1986).
148. M. Caroff, D.R. Bundle and M.B. Perry, *Eur. J. Biochem.*, **139**, 195 (1984).
149. L. Kenne, B. Lindberg, C. Lugowski and S. Svennson, *Carbohydr. Res.*, **151**, 349 (1986).
150. D. Rolf and G.R.Gray, *J. Am. Chem. Soc.*, **104**, 3539 (1982).
151. J.A. Bennek, D. Rolf and G.R.Gray, *J. Carbohydr. Chem.*, **2**, 385 (1983).
152. J.A. Bennek, M.J. Rice and G.R.Gray, *Carbohydr. Res.*, **157**, 125 (1986).
153. S.A. Vodonik and G.R.Gray, *Carbohydr. Res.*, **172**, 255 (1988).

Downloaded At: 08:25 23 January 2011

154. J.-G. Jun and G.R. Gray, *Carbohydr. Res.*, **163**, 247 (1987).
155. S.G. Zeller and G.R.Gray, *Carbohydr. Res.*, **211**, 309 (1991).
156. J.S. Sherman and G.R.Gray, *Carbohydr. Res.*, **231**, 221 (1992).
157. B. Lindberg, *Methods Enzymol.*, **28**, 178 (1972).
158. R.J. Redgwell, M.A. O'Neill, R.R. Selvendran and K.J. Parsley, *Carbohydr. Res.*, **153**, 97 (1986).
159. R.S. Whiton, P. Lau, S.L. Morgan, J. Gilbart and A. Fox, *J. Chromatogr.*, **347**, 109 (1985).
160. K.A. Connors and N.K. Pandit, *Anal. Chem.*, **50**, 1542 (1978).
161. G.D. McGinnis, *Carbohydr. Res.*, **108**, 284 (1982).
162. H.N. Englyst and J.H. Cummings, *Analyst*, **103**, 937 (1984).
163. M. Abdel-Akher, J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **73**, 4691 (1951).
164. P.L. Durette, P. Köll, H. Meyborg and H. Paulson, *Chem. Ber.*, **106**, 2333 (1973).
165. A. Coghlan, *New Scientist*, 10 Sept., 17 (1994).

Downloaded At: 08:25 23 January 2011