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Review

Derivatization of carbohydrates for analysis by chromatography; electrophoresis and mass spectrometry☆,☆☆

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ABSTRACT

Carbohydrates display a large diversity of structures and their analysis presents many obstacles as the result of properties such as isomeric diversity, existence of branched structures and the lack of chromophores or fluorophores. Consequently, many analytical approaches depend on the application of chemical modifications such as hydrolysis or derivative formation. This review covers various aspects of derivatization that are used for such approaches as improving thermal stability and volatility for gas-phase analyses, introduction of fluorophores for optical detectors, introduction of charge for mass spectral analyses and attachment of bioaffinity tags for bioactivity studies. Reducing carbohydrates contain, in addition to multiple hydroxyl groups, several other sites for derivatization such as the single anomeric site that has been used in numerous methods for attaching various property-enhancing tags. Other sites are restricted to specific carbohydrates but include carboxy groups in sialic acids and amino groups in glycosylamines. All of these groups have been the targets of derivatization and this review attempts to summarise the main methods used for these various functional groups.

Abbreviations: 2-AA, 2-aminobenzoic acid; AA-Ac, 3-(acetylamino)-6-aminoacridine; 2-AB, 2-aminobenzamide; ABBE, butyl-*p*-aminobenzoate (*p*-aminobenzoic acid butyl ester); ABDEAE, 4-aminobenzoic acid 2-(diethylamino)ethyl ester; ABEE, ethyl-*p*-aminobenzoate (*p*-aminobenzoic acid ethyl ester); ABG, *N*-(4-aminobenzoyl)-L-glutamic acid; ABGly, *N*-(2-aminobenzoyl)glycine; ABGlyAmide, *N*-(2-aminobenzoyl)-glycinamide; ABGlyDIMED, *N*,*N*-dimethyl-*N*′-(2-aminobenzoyl)-ethylenediamine; ABN, 4-aminobenzonitrile; ABS, aminosulfonic acid and *Arthrobacter ureafaciens* sialidase; AEAB, 2-amino-*N*-(2-aminoethyl)-benzamide; AMAC, 2-aminoacridone; AMC, 7-amino-4-methylcoumarin; 3-ANDA, 3-aminonaphthalene-2,7-disulfonic acid; ANDS, 7-aminonaphthalene-1,3-disulfonic acid; ANSA, aminonaphthalene-1-sulfonic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; 2-AP, 2-aminopyridine; APT, aminoterephthalic acid; APTS, 1-aminopyrene-3,6,8-trisulfonic acid; 3-AQ, 3-aminoquinoline; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; ATT, 6-aza-2-thiothymine; BACH, biotinamidocaproyl hydrazide; BAP, 2-amino-(6-amidobiotinyl)pyridine; BNAH, biotinyl-L-3-(2-naphthyl)-alanine hydrazide; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; BTFA, *bis*-trifluoroacetamide; BTG, bovine testis β-galactosidase; CBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; CHCA, α-cyano-4-hydroxycinnamic acid; CE, capillary electrophoresis; CI, chemical ionization; CID, collision-induced dissociation; CPH, (4-cyanophenyl)-4-piperidinecarbohydrazide; DAAB, 4′-*N*,*N*-dimethylamino-4-aminoazobenzene; DBD-F, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DDB, 1,2-diamino-4,5-dimethoxybenzene; DEAEAB, *N*,*N*-dimethylaminoethyl-4-aminobenzoate (Procainamide); DHB, dihydroxybenzoic acid; DHPE, 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine; DIEA, *N*,*N*-diisopropylethylamine; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DMBA, dimethylbenzylamine; DMEQ-COCl, 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(*1H*)-quinoxalinone; DMSO, dimethylsulfoxide; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride; DNPO, 2,4-dinitrophenyl)octylamine; DNS-Cl, dansylchloride; EDTA, ethylenediaminetetraacetic acid; EI, electron-impact; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FAB, fast-atom bombardment; FACE, fluorophore-assisted carbohydrate electrophoresis; FITC, fluorescein-5-isothiocyanate; Fmoc, fluorenylmethyloxycarbonyl; Fuc, fucose; GAGS, glycosaminoglycans; GC/MS, combined gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; GlcNAc, *N*-acetylglucosamine; GRIL, glycan reductive isotope labelling; GU, glucose unit; GUH, *Streptococcus pneumoniae* hexosaminidase; h, hours; HILIC, hydrophobic interaction liquid chromatography; HMDS, hexamethyldisilazane; HPAEC, high performance anion exchange chromatography; HPLC, high-performance liquid chromatography; IR, infrared; IRMPD, infrared multi-photon dissociation; LC/MS, liquid chromatography/mass spectrometry; LED, light-emitting diode; LIF, laser-induced fluorescence; MALDI, matrix-assisted laser desorption/ionization; Man, mannose; MBTFA, *N*-methyl-*bis*-trifluoroacetamide; MEKC, micellar electrokinetic capillary chromatography; MMT, 3-methyl-1-*p*-tolyltriazene; MS, mass spectrometry; NAIM, naphthimidazole; NBD, 7-nitro-2,1,3-benzoxadiazole; NBD-F, 7-nitro-4-fluoro-2,1,3-benzoxadiazole; NDA, 2,3-naphthalenedialdehyde; NMP, 1-(2-naphthyl)-3-methyl-5-pyrazolone; NMR, nuclear magnetic resonance; NP, normal-phase; ODS, octadecylsilane; OPA, *o*-phthaldialdehyde; PAAN, peracetyl-aldononitrile; PAD, pulsed amperometric detection; PAGE, polyacrylamide gel electrophoresis; PDAM, 1-pyrenyldiazomethane; PFBAB, pentafluoro-*p*-aminobenzoate; PMAA, partially methylated alditol acetate; PMP, 1-phenyl-3-methyl-5-pyrazolone; PMPA, 4-(3-methyl-5-oxo-2-pyrazon-1-yl) benzoic acid; PMPMP, 1-(4-methoxyphenyl)-3-methyl-5-pyrazolone; PPMP, 1-(4-isopropyl)-phenyl-3-methyl-5-pyrazolone; PSC, 1-pyrenesulfonyl chloride; PTC, phenylisothiocarbamyl; PyAOP, (7-azabenzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate; RP, reversed-phase; SDS, polyacrylamide gel electrophoresis; SPR, solid-phase extraction; TFA, trifluoroacetyl; TFAI, trifluoroacetylimidazole; TFAN, 4-trifluoro-acetamidoaniline; TLC, thin-layer chromatography; TMAPA, *p*-aminophenyl ammonium chloride; TMCS, trimethylchlorosilane; TMPP, tris(2,4,6-trimethoxyphenyl)phosphonium amides; TMS, trimethylsilyl; TOF, time-of-flight; UV, ultra violet.

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Contents

1. Introduction

Carbohydrates comprise one of the largest group of compounds found in nature and range in size from simple monosaccharides to very large molecules with molecular weights exceeding 1 mDa. Their analysis is challenging for several reasons: many compounds are isomeric, monosaccharides can adopt different structures (ring opened or closed, different ring sizes and conformations), structures of oligosaccharides are often branched, unlike the other common biopolymers (proteins and nucleic acids) and most carbohydrates lack chromophores or fluorophores, a property that makes detection difficult. But it is perhaps the large number of isomers that discourages investigators from analysing these compounds. For example, it has been estimated that there are 1.05×10^{12} possible isomers for a reducing hexasaccharide [1] which, in terms of the average size of a carbohydrate molecule, is quite small. However, in reality, the situation simplifies considerably when it is realised that, in nature, these compounds are synthesised by very specific enzymatic reactions and within many types of carbohydrate only a very few, often only one, of the possible isomers actually exists. Nevertheless, even in these cases, structural determination can still present major problems.

Nuclear magnetic resonance (NMR) spectrometry is possibly the most powerful technique for structural analysis and has been applied extensively in the analysis of carbohydrates. However, in many cases, such as the analysis of *N*- and *O*-linked glycans from glycoproteins, there is seldom enough material available and the preferred techniques are chromatography and mass spectrometry (MS). Derivatization plays a major role in these analyses, both for the analysis of the intact molecules and for products obtained from various methods of depolymerization. For example, lipophilicity can be increased for studies by gas-phase techniques by derivatizing all hydroxy groups and, in addition, the unique reducing group in reducing sugars provides a convenient attachment point for fluorescent tags. This review will attempt to outline the major uses of derivatization in this field but, because of the enormous amount of reported work, by no means claims to be comprehensive. For other earlier reviews on carbohydrate derivatization, see [2–10]

and the more general book on derivatization for mass spectrometry by Zaikin and Halket [11].

2. Derivatization of hydroxyl groups

2.1. Permethylation

Permethylation of carbohydrates is the most widely used technique for reducing polarity and for increasing thermal stability for analysis by techniques such as combined gas chromatography/mass spectrometry (GC/MS) and fast-atom bombardment (FAB) MS. Permethylation is used today with the newer mass spectral techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) MS for improving detection limits and providing linkage information from mass spectral fragmentation. One of the main advantages over other alkylation and acylation techniques is the relatively small (14 mass units) mass increment attending derivatization. This property is important because of the large number of hydroxyl groups in these compounds.

Unfortunately, quantitative preparation of per-methyl ethers is more difficult than that for most other derivatives and reactions usually need at least one purification step before analysis can be attempted. Early methods for the preparation of these derivatives were reviewed by Levery in 1997 [12] and consisted mainly of relatively inefficient reactions with methyl iodide catalysed by silver oxide under a variety of conditions. A simpler procedure was introduced by Hakomori in 1964 [13] and has been widely adopted. The carbohydrate, in dimethylsulfoxide (DMSO), is reacted with methyl iodide with the reaction catalysed by the methylsulfinyl carbanion which, in turn, is prepared from sodium hydride. This reaction converts hydroxyl groups into methyl ethers, carboxylic acids into methyl esters and NH groups, such as those in *N*-acetylamino sugars, into *N*-Me. Because *O*-Acyl groups are replaced by *O*-Me, information on the presence of native *O*-Me groups can be lost unless [$^2H_3$]-methyl iodide is used as the methylating reagent. Alternatively, ethyl iodide can be used [14] to produce per-ethyl derivatives but at the expense of considerably increasing the molecular weight. Several variations of the Hakomori method have been published, notably the use of methylsulfinyl carbanion made with bases such as potassium hydride [15,16] and butyllithium [17–19]. The latter reagent has the advantage of purity; the hydrides are usually supplied in oil that is difficult to remove completely.

In 1984, Ciucanu and Kerek [20] published a simple, rapid and quantitative procedure that used finely divided sodium hydroxide in place of the methylsulfinyl carbanion, with DMSO as the solvent and use of this reaction has generally surpassed that of the original Hakomori procedure. Some oxidation of alkoxides to carbonyls has been noted under certain conditions [21] but this reaction can be avoided by a number of procedures. Among these is treatment of the carbohydrate with powdered sodium hydroxide before introduction of methyl iodide, addition of methyl iodide some time after the sodium hydroxide [22], adding a trace of water in dimethyl sulfoxide before methyl iodide, or by using *N*,*N*-dimethylacetamide as the solvent [23]. However, there are still problems associated with ester hydrolysis and "peeling" (base catalysed removal of monosaccharide residues from the reducing terminus) during the work-up stage because of the basic nature of the reaction mixture. These problems can be minimized by addition of acetic acid to the final reaction mixture and by keeping the mixture at 0 °C [24]. A recent investigation of the optimum conditions necessary for this reaction, using D-galactose and L-fucose as substrates, has shown that the reaction is essentially complete within 15 min [25]. However, it was found that the furanose:pyranose ratio of monosaccharides was dependent on the reaction conditions; reactions carried out at low (10 °C) temperatures or short reaction times favoured the formation of furanose structures whereas higher temperatures and longer reaction times produced more pyranoses. Several modifications of this method and of the Hakomori and other procedures have been published and are described in reviews by Jay [26] and Levery [12].

Another problem often associated with this reaction is the appearance in mass spectra of a series of ions 30 Da larger than those from the fully methylated carbohydrate. These "overmethylation" ions appear to arise following a side reaction that occurs between the permethylation reagents with the formation of small amounts of iodomethyl methyl ether. This reagent can then compete with methyl iodide for reaction with the carbohydrate hydroxy groups resulting in partial incorporation of a methoxymethyl moiety instead of a methyl group, detected as '+30' artefact ions. [27].

A recent modification of the sodium hydroxide method that is useful for derivatization of very small amounts of carbohydrate is the use small microspin columns or fused-silica capillaries (500 mm i.d.) packed with sodium hydroxide powder to which are added the analytes, mixed with methyl iodide in dimethyl sulfoxide solution containing traces of water. Reactions were said to take less than one minute and the procedure minimized oxidative degradation and peeling reactions and avoided the need of excessive clean-up. Picomole amounts of linear and branched, sialylated and neutral glycan samples were rapidly and efficiently permethylated by this approach [28,29] and a high-throughput extension of the method utilizing spin columns packed with sodium hydroxide beads has recently been described [30].

Much of the early gas-liquid chromatography (GLC) and GC/MS work on permethylated polysaccharides used packed columns and it was difficult to handle compounds larger than tetrasaccharides [31,32]. Bonded-phase capillary columns gave better results but dedicated thermostable, bonded phase columns were needed in order to handle larger compounds. Even so, the methods were restricted to carbohydrates with a maximum of about 11 residues. Larger carbohydrates needed temperatures in excess of 400 °C [33–38] although this caused extensive decomposition. In addition, few GC/MS instruments are capable of operation at these temperatures.

Sulfated glycans generally eliminate sulfate under MALDI MS conditions but an extension of the original permethylation method allows these compounds to be analysed [39]. Samples were first permethylated and then subjected to methanolytic cleavage of the sulfate groups (which do not methylate) to reveal the linking hydroxyl group. The desulfated, permethylated glycans were then subjected to another permethylation step using deuteromethyl iodide to label the newly exposed hydroxyl groups. The number of attached sulfate groups could be calculated from the mass-shift caused by the presence of the deuterium label and the position of the sulfate substitution could be determined by collision-induced dissociation (CID). The method was validated with linear standard glycans and used to identify sulfated *N*-glycans released from bovine thyroid-stimulating hormone.

Permethylation has been used extensively for structural studies of carbohydrates by FAB MS [40–44] where it is necessary to make the analyte molecules non-polar so that they migrate to the surface of the FAB matrix. Much work on *N*-linked sugars was performed with these derivatives in the 1980s and 1990s but this ionization technique has now largely been superseded by MALDI MS. Nevertheless, some investigators still examine sugars as their permethyl ethers because they tend to increase sensitivity and have been reported to be better than native carbohydrates for quantitative studies [45]. In addition, the fragmentation spectra of permethylated carbohydrates provides considerable information on detailed structure relating to linkage and branching position because gly-

cosidic cleavages leave underivatized hydroxy groups at the site of the original linkage [46–48].

2.1.1. Linkage analysis

An important application of the permethylation reaction, developed in the late 1960s by Lindberg in Stockholm [49,50], is linkage or "methylation analysis" as it is sometimes known, for determination of the linkage positions between sugar rings in carbohydrate oligomers. Analysis is by GC/MS and the method has been the subject of many articles and reviews [12,51–57]. Basically, the procedure is one in which all hydroxyl groups in a polysaccharide are first permethylated (Scheme 1) after which the molecule is hydrolysed to generate additional free hydroxyl groups at the sites of linkage. The resulting monosaccharides are then reduced in order to avoid the production of two peaks on chromatographic analysis due to α- and β-anomers and, finally, the resulting alditols are derivatized with a different reagent such as acetic anhydride to produce partially methylated alditol acetates, known as PMAAs. The positions of the various substituents are subsequently located by GC/MS in order to determine which of the hydroxyl groups (those derivatized as OAc in this example) were originally involved in bonding.

Reduction of the hydrolysed permethylated carbohydrate also generates a free hydroxyl group at positions that reflect the original ring size. For hexoses, these will be generated at C4 and C5 for furanoses and pyranoses respectively. Consequently discrimination between certain sugars such as 4-linked hexopyranoses and 5-linked hexofuranoses, both of which will produce 1,4,5-triacetoxy products, is problematical. However, for carbohydrates such as those derived from mammalian glycoproteins where only pyranose structures appear to exist, this difficulty should not present problems. The situation is different for carbohydrates from sources such as bacteria where furanose and pyranose rings can occur. Procedures to overcome this problem, such as mixed methylation and ethylation [58] and the "reductive cleavage" method, introduced by Rolf and Gray in 1982 [59–61] also identify hydroxyl groups arising from ring cleavage. Although the above procedures will identify which hydroxyl groups on a given sugar ring are involved in linkage, information as to which sugar is attached at that site is not available and must be obtained by other means.

Many other modifications of the original method have been developed. Thus, for example, methanolysis to produce methyl glycosides has been used to replace the borohydride reduction step and trimethylsilylation has been used in place of acetylation [62]. Acetolysis rather than hydrolysis has been employed by several investigators but it has been noted that both reactions tend to cause some *O*-demethylation, particularly from 2-acetamido-2-deoxy-sugars [63,64] under harsh conditions. The released monosaccharides have been reacted with hydroxylamine to produce oximes which were converted into aldonitriles on acetylation (see below) [65,66]. Lowe and Nilsson [67] have introduced a chromatographic step to separate PMAAs derived from hexosamines from those originating from hexoses obtained from protein-bound glycans. One of the most sensitive procedures appears to be that devised by Geyer and Geyer [19]. Micromethylation was performed with the lithium methylsulfinyl carbanion [17,18] and the mass spectrometric step was performed using ammonia chemical (CI) rather than electron-impact (EI) ionization with selected ion monitoring to increase sensitivity. Results were obtained from as little as 10 ng of carbohydrate.

Several reviews have presented data on the retention times and mass spectra of these derivatives [12,55,56]. Fragmentation of PMAAs occurs primarily along the carbon chain following charge localization on a nitrogen (amino sugar) or oxygen atom. Secondary fragments are the result of the further elimination of neutral fragments such as acetic acid (60 u) ketene (42 u), formaldehyde (30 u) or methanol (32 u). The base peak is usually at m/z 43 (CH_3CO^+). The preferred site for bond cleavage is between carbons carrying an *N*-methyl-acetylamino- and a methoxy group with charge retention on the nitrogen atom. From 2-acetamido-2-deoxy-sugars, this cleavage produces a characteristic primary fragment containing both C1 and C2 at m/z 158 and a secondary ion at m/z 116 as the result of ketene loss. If the 1-position is methylated, these ions appear at m/z 130 and 88, respectively. The next most likely bond to cleave is that between two carbon atoms where each carries a methoxy group. The charge can reside on either fragment but appears most stable on the smaller one. The next most labile bonds are those between carbon atoms that carry methoxy and acetoxy groups; the charge usually resides on the methoxylated fragment. Finally, bonds between carbon atoms that both carry acetoxy groups are the most difficult to fragment.

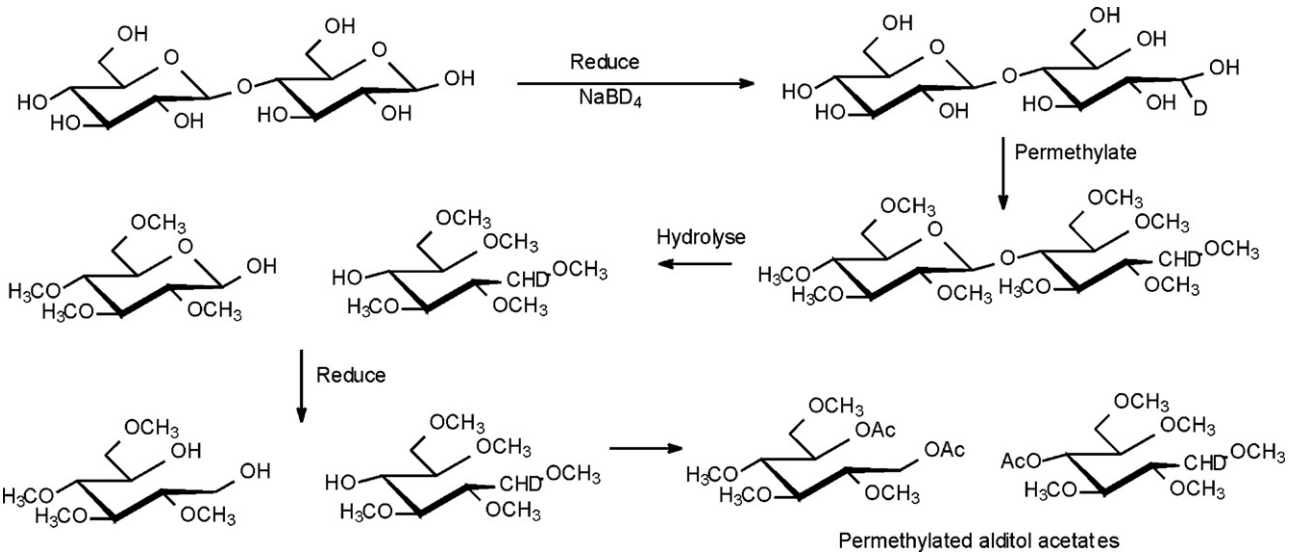
2.2. Acetates

Acetylation is much less frequently used than methylation, partly because of the higher mass increment (42 units per OH group) attending derivatization. Acetates, first used by Gunner et al. in 1961 [68], may be prepared by reaction of the sugar with acetic anhydride and either a basic solvent such as pyridine or sodium acetate as catalyst. However, it is necessary to remove any boric acid that may be present as the result of previous reductions with borohydride whereas this is not the case with acetylations catalysed with 1-methylimidazole [69,70]. Pyridine and acetic anhydride are volatile and can easily be evaporated before the sample is analysed; sodium acetate requires a solvent extraction with, typically, water:chloroform. Analysis by GLC gives two peaks from each monosaccharide as the result of anomeric separation [71] but this problem can be resolved by prior reduction to alditols [72,73] a procedure that forms the basis of the alditol acetate technique [74] for identification of monosaccharides. Experimental details for formation of acetates have been described in several reviews [75–77]. Many stationary GLC phases have been used to analyse these compounds; polar phases such as SP-2330 appear to produce better separations than non-polar phases. The EI spectra of these derivatives have been discussed in several reviews [51,78] and outlined above.

2.3. Trifluoroacetates

Trifluoroacetates, first reported for carbohydrates in the early 1960s [79,80], are much more volatile than acetates and give good resolution on polar GLC columns such as OV-225, OV-275 or SP-2330. Like the acetates, they can be prepared from the corresponding acid anhydride in pyridine but other reagents such as *N*-methyl-*bis*-trifluoroacetamide (MBTFA), *bis*-trifluoroacetamide (BTFA) and trifluoroacetylimidazole (TFAI) can also be used. The reagents are highly corrosive because of the liberation of TFA and must be handled with care. It is necessary to remove residual reagent before chromatographic analysis to prevent excessive tailing. The acyl amide reagents, introduced by Donike [81], are easier to use as they work in the presence of borate ions (often present from earlier reduction reactions) and are very volatile; thus, no reagent-removal step is necessary. The acylimidazoles are much less reactive but can be used for selective reactions. Sugars in the form of their TMS derivatives are stable, both in the presence of TFAI and the trifluoroacetamides, thus enabling amino groups to be trifluoroacylated. To achieve this derivatization, the sample is trimethylsilylated with reagents such as *N*,*O*-bis(trimethylsilyl)acetamide (BSA) in pyridine, followed by the addition of the trifluoroacylating reagent and heating for 15 min at 75 °C.

Mass spectra are generally simple and are characterized by the elimination of TFA. Molecular ions are usually present in the EI spec-



Scheme 1. Linkage analysis by formation of partially methylated alditol acetates.

tra [82] but molecular weights tend to be fairly high because the mass increment attending derivative formation 96 mass units per derivatized function. Further details on these derivatives can be found in the article by Englmaier [83].

2.4. Aldononitrile acetates

These early derivatives of monosaccharides were prepared by heating the sugar with hydroxylamine hydrochloride in pyridine at 90 °C for 30 min to form the oxime followed, after cooling, by treatment with acetic anhydride under similar conditions to dehydrate the oxime and form acetyl derivatives. Analysis by GLC gave single peaks [84–86]. The acetylation reaction can also be catalysed with 1-methylimidazole [69,70] although this catalyst is not suitable for use with amino sugars such as muramic acid [87].

2.5. Trimethylsilyl (TMS) derivatives

Trimethylsilylation of carbohydrates is a much simpler reaction than permethylation but the mass increment added by each group is greater (72 rather than 14 units). Uses are mainly for GLC and GC/MS. Derivatization of carbohydrates was first reported by Sweeley et al. in 1963 [88] for studies by GLC and early work with these derivatives was reviewed by Petersson in 1975 [89]. The original reaction, as performed by Sweeley et al., was performed in pyridine with hexamethyldisilazane (HMDS) catalysed by trimethylchlorosilane (TMCS) and gave excellent yields of the fully trimethylsilylated products. More recent methods tend to use reagents such as BSA or *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with the addition of small amounts of trimethylsilylimidazole (TMSI) if catalysis is required. These latter reagents lead to trimethylsilylation of amino groups whereas HMDS/TMCS does not [90]. Normally, no non-volatile reagents or by-products are involved and the complete reaction mixture can be injected directly into the gas chromatograph, thus avoiding any clean-up stages.

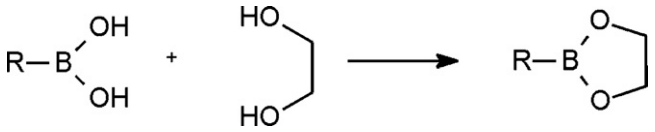
The EI-induced mass spectra of the TMS derivatives of carbohydrates was studied by Chizhov in 1967 [91] and more extensively by DeJongh et al. [92] in 1969. Molecular ions from non-reduced compounds are normally very weak or absent and the high-mass end of the spectra is usually dominated by ions formed by loss of methyl groups and/or trimethylsilanol (90 mass units). The spectra of isomeric compounds such as hexoses generally contain ions at common *m*/*z* values although the relative abundance of these ions can be considerably different. All spectra contain the trimethylsilylcation ($(CH_3)_3Si^+$, *m*/*z* 73), another, weaker ion at *m*/*z* 75 ($(CH_3)_2OHSi^+$) and a rearrangement ion at *m*/*z* 147 ($[(CH_3)_3Si\text{-}O\text{-}Si(CH_3)_2]^+$). Ions such as *m*/*z* 103 ($[(CH_3)_3Si\text{-}O{=}CH_2]^+$), 117 ($[(CH_3)_3Si\text{-}O{=}CH\text{-}CH_3]^+$) and 129 ($[(CH_3)_3Si\text{-}O{=}CH\text{-}CH{=}CH_2]^+$) are usually also present. A triplet of ions containing two silicon atoms appears at *m*/*z* 191 ($[(CH_3)_3Si\text{-}O{=}CH\text{-}O\text{-}Si(CH_3)_3]^+$), 204 ($[(CH_3)_3Si\text{-}O\text{-}CH{=}CH\text{-}O\text{-}Si(CH_3)_3)]^+$, *m*/*z* 203 from derivatized amino-sugars) and 217 ($[(CH_3)_3Si\text{-}OCH(CH)CH\text{-}O\text{-}Si(CH_3)_3]^+$) and confirms the occurrence of OTMS groups on adjacent carbon atoms. Ions containing three TMS groups are often present although usually weak.

TMS derivatization is not normally used for techniques such as MALDI MS but, in a recent publication [93] small oligosaccharides were reacted with TMSI and examined by MALDI-time-of-flight (TOF) MS from 2,5-dihydroxybenzoic acid (DHB). The spectra contained the molecular ion of the per-trimethylsilylated carbohydrate and a series of ions separated by 72 mass units produced either by incomplete reaction or partial hydrolysis enabling the number of hydroxyl groups in the carbohydrate to be enumerated.

2.5.1. Sugar phosphates

Monosaccharide phosphates may be derivatized as TMS or methyl ester-TMS derivatives and generally give good GC/MS performance. Derivatives of sugars containing a phosphate group in the 6-position are generally stable but those containing phosphate in the 1-position are less so and tend to lose phosphate. Consequently, they are best prepared by reaction with TMSI in order to avoid the presence of the acidic TMCS which tends to catalyse phosphate loss. It is important to use GLC columns that are not contaminated with acidic residues because the latter also can catalyse TMS loss. Retention values of most common sugar phosphates on SE-30 and OV-17 stationary phases have been published [94].

The EI mass spectra of these compounds are dominated by ions formed by cleavages around the derivatized phosphate group and are characterized by extensive migrations of the TMS moieties [94–96]. Major ions are derived from tri- and tetra-(trimethylsilyl)-phosphate and fragments derived by losses of $CH_3^\bullet$ The ion at *m*/*z* 229 ($[((CH_3)_3Si\text{-}O)_2\text{-}P(OH)\text{-}O\text{-}Si(CH_3)_2]^+$) is often the base peak. The losses of $CH_3^\bullet$ are from TMS groups as shown by the spectrum of the corresponding [$^2H_9$]-TMS derivative [97]. Other major ions in the spectra tend to be the same as those in the spectra of the TMS derivatives of unphosphorylated compounds. Another interesting feature of the spectra is the appearance of ions produced



Scheme 2. Formation of cyclic alkane boronates.

by ion-molecule reactions involving the parent molecule and fragments containing siliconium groups [98,99]. Reaction with the TMS cation (Me_3Si^+) itself is particularly favourable.

2.6. Cyclic alkane boronates

These derivatives are prepared by reaction of the sugar, usually a monosaccharide, with an alkylboronic acid, a reagent that forms pentacyclic boronate esters with suitably placed *cis*-diols (Scheme 2) and hexacyclic esters with some other diols, depending on their stereochemistry [100,101]. Many monosaccharides form two such rings leaving only one free hydroxyl group that can subsequently be converted into a TMS ether [102]. As ring-formation requires suitably positioned hydroxyl groups, it is sensitive to stereochemistry. Methyl (**1**, Scheme 3), butyl (**2**) or phenylboronic acids (**3**) are most commonly used [103] and the resulting derivatives have the advantage of a greatly reduced molecular weight, compared with derivatives such as acetates and TMS ethers. Although single GLC peaks can be obtained from many monosaccharides [100], the situation becomes more complicated with oligosaccharides where multiple derivatives are possible. EI spectra are characterized by prominent ions at *m*/*z* 160, 140 and 98 from phenyl-, butyl- and methyboronates respectively when 6-membered rings are present and *m*/*z* 146, 126 and 84 from pentacyclic-containing compounds [104].

Pikulski [105] have derivatized small oligosaccharides with the IR-active boronic acid, **4** and shown rapid formation (less than 1 min) of predominantly one derivative involving the reducing-terminal hydroxyl and the hydroxyl group at C2. The derivatives were used for infrared multi-photon dissociation (IRMPD) fragmentation studies with the IR-active phosphonate group of the derivative facilitating the photon absorption process. Fragmentation was almost exclusively by Y-type cleavages (Domon and Costello [106] fragmentation nomenclature, Scheme 4). Monosaccharides, disaccharides and larger carbohydrates have been derivatized with 3-aminophenylboronic acid (**5**, 3-APBA) at low pH (2.7–3.0) and the products examined by positive ion mode electrospray. Protonation of the amino group gave signals that were more intense than those obtained by lithium ion cationization. Compounds containing the boronic acid derivative could easily be identified by the presence of the $^{10}B/^{11}B$ isotopes in the ratio 1:4. The derivatives were applied to a complex carbohydrate mixture from honey [107]. Ferrocenyl boronates (**6**) of *N*-acetylaminohexoses have been differentiated by mass spectral fragmentation after they were formed within the spray of an electrospray instrument [108] and differentiation of isomeric mono- and -disaccharides has been achieved by the same technique [109]. It was believed that both five- and six-membered rings were formed.

Several fluorescent alkylboronate derivatives have been synthesised. Thus, Suenaga et al. [110] compared eight aromatic boronic acids and concluded that the biphenyl-3-boronic acid (**7**) and naphthalene-2-boronic acids (**8**) gave the most intense signals with D-fructose as the test sugar.

Another recent use of boronate formation is extraction of glycoproteins from protein and glycoprotein mixtures [111–114]. Thus, boronates have been coupled to various matrices such as magnetic nanoparticles and their reversible reaction with carbohydrates used to bind the glycoproteins.

3. Derivatization of the reducing terminal

The single reducing terminus of carbohydrates has been exploited in many ways to attach groups such as fluorescent labels or charged moieties in order to render the molecules amenable to various analytical techniques. Predominant among these is the attachment of chromophores or fluorophores, mainly for detection following high-performance liquid chromatography (HPLC).

3.1. Schiff bases

Reducing carbohydrates in their open-chain form contain a carbonyl group at their reducing terminus that readily reacts with amines to form a Schiff base. Although most of these derivatives are not particularly stable, they have, nevertheless been used for carbohydrate derivatization. Thus, derivatives of monosaccharides and *N*-linked glycans have been separated as derivatives of the highly fluorescent Rhodamine 110 (**9**, Scheme 5) [115] and Rhodamine B (**10**) [116] by HPLC with detection limits in the low fmole region. Caesar et al. [117] have prepared derivatives from pentafluoro-*p*-aminobenzoate (PFBAB, **11**), a reagent than not only imparts fluorescence to the carbohydrate but also contains a pentafluorophenyl ring that acts as a good electron-capturing group for negative ion CI MS.

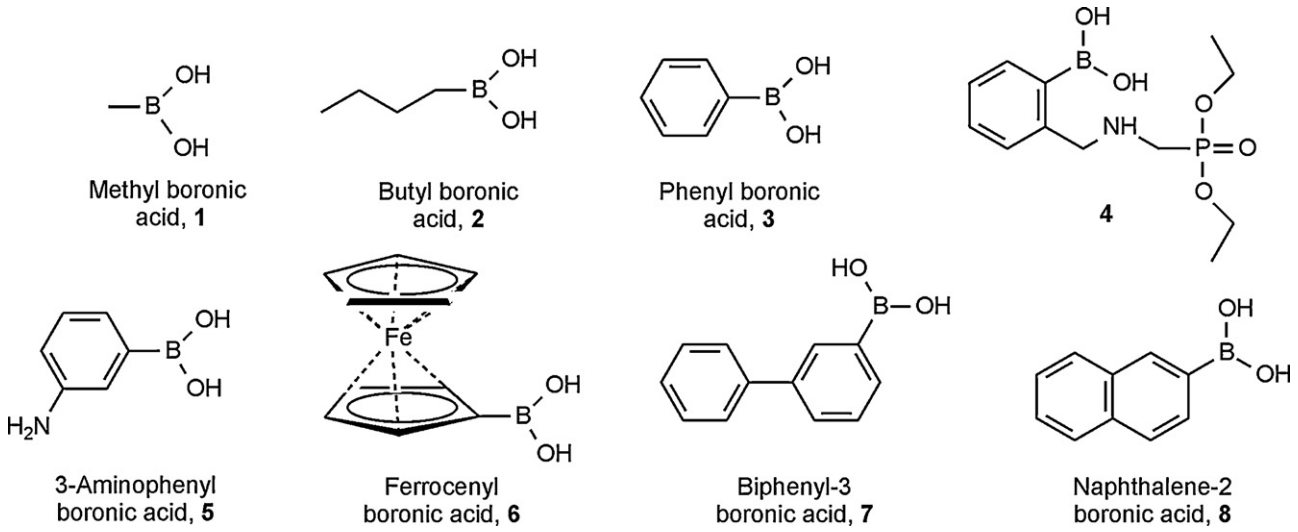
MALDI analysis of carbohydrates from a 2,5-DHB/aniline matrix has given improved sensitivity of detection than analysis in the absence of aniline (**12**). The aniline was found to form a Schiff base derivative with the carbohydrate even in the solid phase [118]. Increases in sensitivity were seen with the residual native glycan and derivative and have also been observed with a DHB/*N*,*N*-dimethylaniline matrix [119,120] as the result of a more homogeneous sample spot. Both native and derivatized glycans ionized as sodium adducts and had similar MS/MS fragmentation patterns consisting mainly of Y/B-cleavage ions.

Schiff bases can exist in equilibrium with the closed ring form of the sugar and have been assumed to be in this form in studies by Cheng et al. [121,122]. The derivatives with ethyl-*p*-aminobenzoate (ABEE, **13**) gave good HPLC characteristics and structural information was obtained by negative ion electrospray MS.

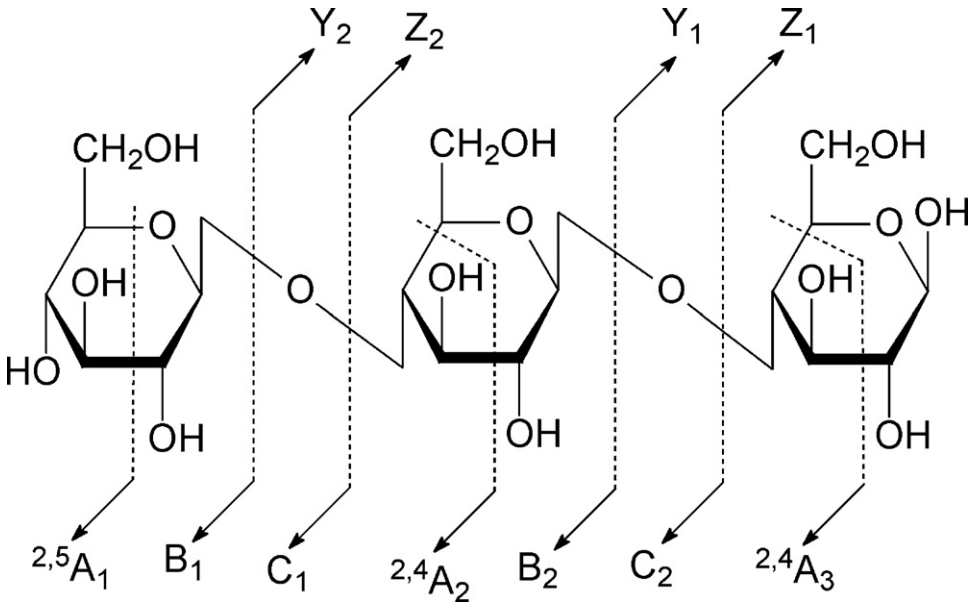
3.2. Derivatives prepared by reductive amination

As mentioned above, Schiff bases are not particularly stable and are usually reduced to the corresponding secondary amine in a process known as reductive amination (Scheme 6). The sugar, usually dissolved in DMSO containing acetic acid to promote ring opening, is treated with the amine and a reducing agent, commonly sodium cyanoborohydride and heated at temperatures up to about 100 °C for several hours. Optimum conditions depend to a large extent on the amine [123]. Dimethylaminoborane [124], $NaBH(OAc)_3$ [125] and pyridine-borane [126] have also been used as a reducing agents and, recently, 2-picoline-borane, a reagent that is active in aqueous or non-aqueous environments, has been proposed as a non-toxic replacement for sodium cyanoborohydride [127]. Many amines have been used (Table 1), the most common being 2-aminobenzamide (2-AB, **14**, Scheme 7) [128], favoured by Western investigators and 2-aminopyridine (2-AP or PA, **15**, [129] that is used extensively in Japan.

The reaction usually requires the removal of a large excess of reagent. Several methods are available. Paper chromatography has been popular for 2-AB-labelled glycans [128] but has largely been superseded by hydrophobic interaction liquid chromatography



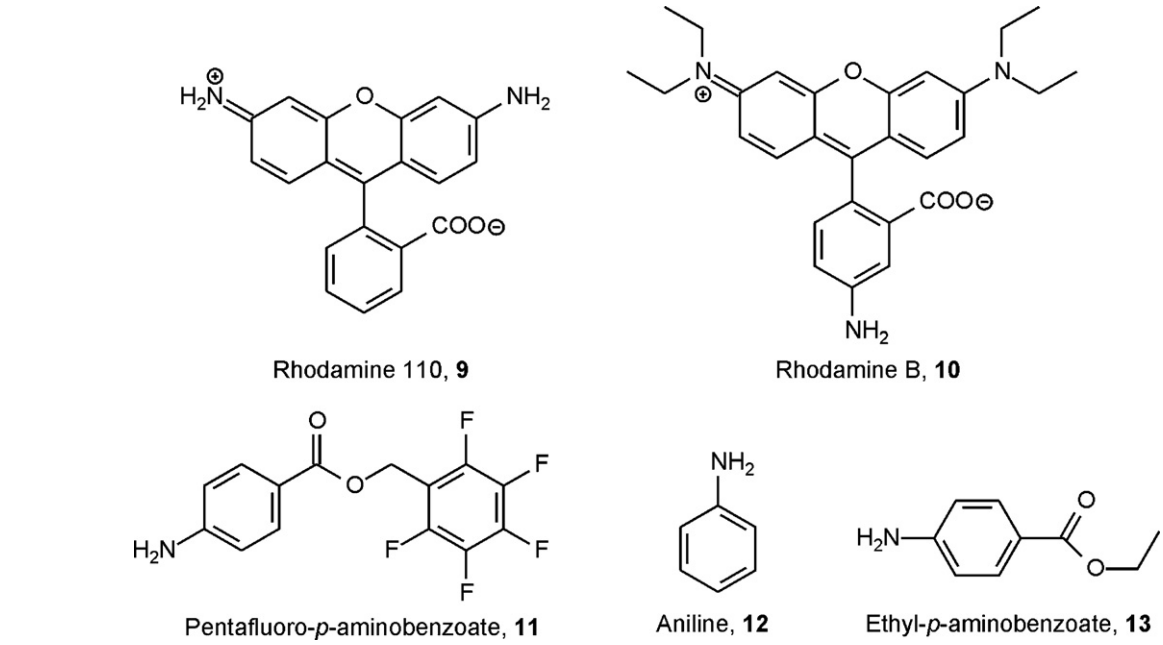
Scheme 3. Common boronic acids used to derivatize carbohydrates.



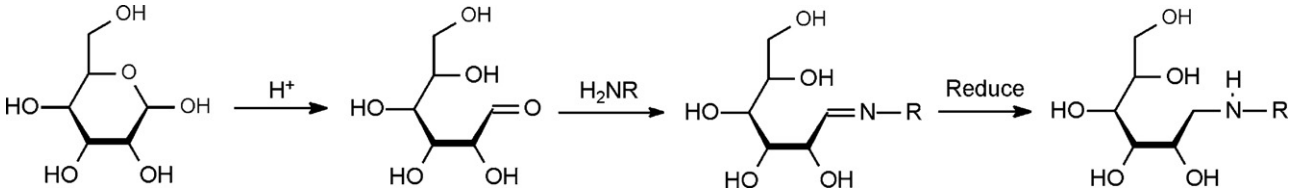
Scheme 4. Nomenclature for the fragmentation of carbohydrates as proposed by Domon and Costello [106]. Subscript numbering is from the reducing terminus when the charge is located at this end of the molecule and *vice versa*. Superscript numbers are used to denote the bonds cleaved in formation of cross-ring cleavages.

(HILIC) on glass, cellulose, or hydrophilic solid-phase extraction (SPE) material such as cyano-silica [130–132]. Polyamide S6 and nylon filter disks have been used for 2-aminobenzoic acid (2-AA, **16**) derivatized carbohydrates [133], Oasis HLB cartridges from Waters have been employed for glycans derivatized with 3-(acetylamino)-6-aminoacridine (AA-Ac, **17**) or 2-aminoacridone (AMAC, **18**) and graphitized carbon has been used for carbohydrates labelled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS, **19**) [134] (further discussion of these specific derivatives is presented below). Gel filtration has been favoured by Hase and co-workers, [135] and phenol/chloroform extraction is one of the more unusual methods [136]. All of these procedures can be time-consuming and can lead to sample loss. To overcome these problems, Pabst et al. [10] have developed a method that is applicable to a wide range of labelled sugars (derivatives from 14 amines were examined). The amine was dissolved in 0.2 ml of acetic acid, and 10 μl of this coupling reagent was added to a dry sample. After incubation at 90 °C for 1 h, the sample was allowed to cool and 0.5 ml of acetone (water free) was added. The sample was vortexed for 30 s and centrifuging at 13,000 rpm for 5 min, following which, the solvent was decanted and the procedure was repeated twice. After drying for several seconds, reduction was accomplished by adding 5 μl of a dimethylborane complex solution (5 mg plus 8 μl of water and 15 μl of acetic acid) and incubating at 90 °C for 20 min. The reaction was stopped by cooling to room temperature and adding starting buffer for NP-HPLC. Removal of the reagent was virtually complete.

Suzuki et al. [137] have developed a method for recovering free carbohydrates from derivatized carbohydrates (ABEE (**13**), 2- (**20**) and 4-aminobenzonitrile (ABN, **21**), 7-amino-4-methylcoumarin (AMC, **22**) 2-AA (**16**), 2-AB (**14**), 2-AP (**15**), ANTS, (**24**)) prepared by reductive amination, involving heating the derivatives



Scheme 5. Amines used to form Schiff bases of carbohydrates.



Scheme 6. Scheme for the synthesis of derivatives by reductive amination.

at 30 °C with an aqueous solution of hydrogen peroxide/acetic acid. Recoveries of maltose from ABEE, 4-ABN, and AMC derivatives gave approximately 90% recovery but recoveries from the *o*-substituted derivatives (**14**, **16**, **20**) were only 5–40%; maltose was unrecoverable from 2-AP and ANTS derivatives. Nevertheless, prior treatment of a 2-AP derivative of maltose with cyanogen bromide enabled the regeneration of the free sugar in high yields.

3.2.1. 4-Aminobenzoic acid alkyl esters

4-Aminobenzoic acid esterified with various alcohols have found considerable use for carbohydrate derivatization, particularly for introducing a UV absorbing group and for increasing lipophilicity for techniques such as FAB MS. The ethyl ester (**13**) was used by Wang et al. [138] and by Webb et al. [139] to provide a chromophore for sensitive detection following HPLC and to increase sensitivity in FAB MS and Poulter et al. [140,141] have investigated the effect of several alkyl-4-aminobenzoates of linear and *N*-linked glycans, ionized FAB, and found that sensitivity increased with chain length with optimal results being obtained with the *n*-octyl ester. Since their introduction, these derivatives, particularly the ethyl (**13**) and butyl (ABBE, **23**) esters have found considerable use in carbohydrate analysis by techniques such as ESI and MALDI MS and HPLC. For example, Schmid et al. [142] have compared the methyl, ethyl and butyl esters of maltodextrins and oligosaccharides from human milk in separations by HPLC using four C18 phases and found that the butyl esters gave the best performance. Similar results were obtained by Kratchmar et al. [143] for separations by micellar electrokinetic capillary chromatography (MEKC).

Derivatization with 4-aminobenzoic acid 2-(diethylamino)ethyl ester (ABDEAE, 24) has been used by Takao et al. [144] to improve detection limits by MALDI MS by virtue of the high proton affinity of the tertiary amine; increases of 1000-fold over that of the free sugar were claimed for maltoheptaose whereas a more modest 50-fold increase was reported by Harvey [145] for the derivatized high-mannose *N*-glycan, $Man_5GlcNAc_2$. Increases in sensitivity produced by the more stable *N*-(2-diethylamino)ethyl-4-aminobenzamide (procainamide, 25) derivative have also been reported [146] and fragmentation of these derivatives ionized by electrospray as various alkali metal adducts has been studied [146].

3.2.2. Aniline and other substituted anilines

Several other simple *p*-substituted anilines have been investigated but have not found as much use as the esterified amino benzoic acids. These are listed in Table 1. *p*-Nitroaniline (**26**) was said to have three advantages as a derivative for capillary electrophoresis (CE); it has excellent water solubility; second, it has high molar absorptivity; and third, it is possible to obtain sensitive detection using a UV or blue light-emitting diode (LED) as the light source [147]. Nine monosaccharides were separated by a CE system within 16 min using a 0.17 M boric acid buffer at pH 9.7. Although the initial report stated that the formation of the derivative took only 30 min, later work employing 600 W microwave radiation reduced this to 5 min [148].

A method using aniline in reductive amination, termed glycan reductive isotope labelling (GRIL) has been introduced for glycan quantitation. Free glycans or those released from glycoproteins were derivatized by reductive amination with either [$^{12}C_6$]aniline (**12**) or [$^{13}C_6$]aniline. These dual-labeled aniline-tagged glycans

Table 1
Amines used for derivatization of carbohydrates by reductive amination.

Amine	Mass added to carbohydrate[a]	Optical properties (nm)	Use	Structure number	Early or representative reference
3-Acetamido-6-aminoacridine (AA-Ac)	235.1109	λ_{exc}: 442, λ_{em}: 525	NP-, RP-HPLC, CE	17	[187]
2-Amino-(6-amidobiotinyl)pyridine (BAP)	318.1515	–	Antibody binding	56	[218,219]
4-Aminoacetophenone	119.0735	UV	LC-FAB	57	[138]
2-Aminoacridone (AMAC)	194.0844	UV: 250, 276, 421 λ_{exc}: 428, λ_{em}: 525	NP-, RP-HPLC, CE	18	[180,308]
4-Aminoazobenzene	181.1004	UV: λ: 377 λ: 318	HPLC	58	[309]
4-Amino-1,1′-azobenzene-3,4′-disulfonic acid	341.0140	UV: 489	SDS-PAGE	59	[310]
2-Aminobenzamide (2-AB)	120.0687	λ_{exc}: 330, λ_{em}: 419	NP-, RP-HPLC, CE	14	[128,311]
3-Aminobenzamide (3-AB)	120.0687	λ_{exc}: 306, λ_{em}: 452	HPLC	34	[179]
2-Aminobenzoic acid (2-AA)	121.0528	λ_{exc}: 230, 245, 360, λ_{em}: 425	NP-, RP-HPLC, CE, TLC	16	[162,170,172,173]
3-Aminobenzoic acid (3-AA)	121.0528	–	Fluorescence	33	[312]
4-Aminobenzoic acid (4-AA)	121.0528	UV: λ: 303 λ_{ex}: 313, λ_{em}: 358	CE, HPLC	38	[309,312–314]
4-Aminobenzoic acid ethyl ester (ethyl-*p*-aminobenzoate) (ABEE)	149.0841	UV	HPLC, FAB, ESI	13	[138,139,141–143]
4-Aminobenzoic acid butyl ester (ABBE)	177.1154	λ_{exc}: 285, λ_{em}: 350		23	[141–143]
4-Aminobenzoic-2-(diethylamino)ethyl ester (ABDEAE)	220.1576		MALDI, ESI	24	[144,145]
4-Aminobenzonitrile (ABN)	102.0582	λ_{exc}: 387, λ_{em}: 343	CZE, MEKC	21	[315]
N-(2-Aminobenzoyl)-glycinamide (ABGlyAmide)	177.0902	λ_{exc}: 325, λ_{em}: 415	RP-HPLC, HPAEC-PAD	28	[169]
N-(2-Aminobenzoyl)glycine (ABGly)	178.0742	λ_{exc}: 325, λ_{em}: 410	RP-HPLC, HPAEC-PAD	29	[169]
2-Amino-5-bromo-benzoic acid	198.9633	–	ESI, MALDI	32	[178]
2-Amino-4-chloro-benzoic acid	155.0138	–	ESI, MALDI	31	[178]
3-Amino-9-ethylcarbazole	194.1208		HPLC, MALDI	60	[316,317]
9-Aminofluorene	165.0942	UV: 267	HPLC (PGC)	61	[318]
5-Aminofluorescein	331.0845	λ_{exc}: 490	CE	62	[319]
O-2-[Aminoethyl]fluorescein	342.1130	λ_{exc}: 484, λ_{em}: 512	CE	63	[320]
7-Amino-4-methylcoumarin (AMC)	159.0684	λ_{exc}: 360, λ_{em}: 430	TLC, HPLC, ESI	22	[126,321]
2-Amino-*N*-(2-aminoethyl)-benzamide (AEAB)	163.1109	–	Preparation of arrays	52	[214]
2-Amino-5-bromopyridine	155.9687	–	HPLC, ESI	27	[161]
3-Aminonaphthalene-2,7-disulfonic acid (3-ANDA)	286.9922	UV: 235	CE	43	[199]
7-Aminonaphthalene-1,3-disulfonic acid (2-amino-6,8- disulfonic acid) (2-ANDS)	286.9922	UV: 247 λ_{exc}: 315, λ_{em}: 420	CE	44	[123,199]
2-Aminonaphthalene-1-sulfonic acid (2-ANSA)	207.0354	UV: 235	CE	41	[199]
5-Aminonaphthalene-2-sulfonic acid (5-ANSA)	207.0354	UV: 235	CE	42	[199]
8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS)	366.9490	UV: 220, 370, 360 λ_{exc}: 353, λ_{em}: 535	CE, FACE	19	[201–203,322]
2-(2-Aminophenyl)-indole	192.1051	UV: 223 λ_{exc}: 223, λ_{em}: 397	HPLC	64	[309]
2-Amino-3-phenylpyrazine	155.0847	λ_{exc}: 331	HPLC, CE	65	[323]
1-Aminopyrene	201.0942	UV: 241 λ_{exc}: 241, λ_{em}: 462	HPLC	66	[309]
1-Aminopyrene-3,6,8-trisulfonic acid (APTS)	440.9647	λ_{exc}: 455, λ_{em}: 512	CE	45	[204,206,211]
Aminopyrazine	79.05344	λ_{exc}: 246, λ_{em}: 410	RP-HPLC	67	[124,324]
2-Aminopyridine (2-AP)	78.05818	UV: 240 λ_{exc}: 310–320, λ_{em}: 375	NP- RP-HPLC, CE, FAB, ESI	15	[129,151,154,325]
3-Aminoquinoline (3-AQ)	128.0738	–	MALDI	35	[190]
6-Aminoquinoline (6-AQ)	128.0738	UV: 245 λ_{exc}: 375, λ_{em}:445	CE	36	[193,194,326]
5-Aminosalicylic acid (5-AS)	137.0477	–	Fluorescence	46	[312]
2-Aminosulfonic acid (2-ABS)	157.0197	–	Fluorescence	47	[312]
3-Aminosulfonic acid (3-ABS)	157.0197	–	Fluorescence	48	[312]
4-Aminosulfonic acid (4-ABS)	157.0197	–	Fluorescence	49	[312]
2-Aminoterephthalic acid (2-APT)	165.0426	–	Fluorescence	73	[312]
N-(4-Aminobenzoyl)-L-glutamic acid (ABG)	249.1001	UV: 237	CE	68	[327]
Aniline	77.06293	UV	HPLC, FAB	12	[138,149]
Benzylamine	91.07858	–	ESI	39	[328]
1,2-Dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE)	647.5618	–	Array	54	[216]

Table 1 (*Continued*)

Amine	Mass added to carbohydrate[a]	Optical properties (nm)	Use	Structure number	Early or representative reference
N,*N*-Dimethyl-*N*'-(2-aminobenzoyl)-ethylenediamine (ABGlyDIMED)	191.1422	λ_{exc}: 325, λ_{em}: 420	RP-HPLC, HPAEC-PAD	30	[169]
N,*N*-dimethylaminoethyl-4-aminobenzoate (DEAEAB, Procainamide)	219.1735	–	ESI	25	[145,146]
1-(5-(1',2'-Dithiocyclopentyl)pentamido)-5-aminopyridine (f-mono)	281.1021	λ_{ex} 335, λ_{em} 380	ESI, HPLC, array	53	[215,329]
4′-*N*,*N*-dimethylamino-4-aminoazobenzene (DAAB)	224.1426	–	TLC, HPLC	69	[330]
p-Nitroaniline	122.0480	λ_{max}: 380	CE, HPLC	26	[147,148]
N-Octyl-4-aminobenzamide	232.1939	–	Array	71	[329]
5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid 4-aminomethyl-benzylamide	346.1827	–	HPLC, MALDI, ESI	40	[198]
Pentafluoro-p-aminobenzoate	301.0526	–	CI-MS	11	[117]
Rhodamine 110	314.1055	λ_{exc}: 499	CE	9	[115]
Rhodamine B	441.2416	λ_{ex} 552, λ_{em} 573	RP-HPLC	10	[116]
4-Trifluoro-acetamidoaniline (TFAN)	187.0609	–	HPLC	70	[331]
4-Trimethyl-aminoaniline (TMAPA)	135.1286	–	FAB	37	[41]
Tryptamine	144.1051	UV: 240	MEKC	72	[332]

[a] Monoisotopic mass, after reductive amination.

could be recovered by reversed-phase chromatography and could be quantified by UV absorbance or MS. For quantification, one labelled variety was used as the reference standard against which the test glycan, labelled with the other isotope was measured by MALDI-TOF MS. Unlike previously reported isotopically coded reagents for glycans, the derivatives do not contain deuterium, which can be chromatographically resolved. The technique allowed linear relative quantitation of glycans over a 10-fold concentration range and could accurately quantify sub-picomole levels of released glycans [149].

3.2.3. 2-Aminopyridine (2-AP)

2-AP (or PA) derivatives (**15**) were first described in 1978 by Hase [129,150] and applied to *N*-glycans from glycoproteins in 1981 [151]. Detection limits were reported to be as little as 0.1 pmoles. The reagents, in water were mixed in a sealed tube and then the reaction mixture was heated at 100 °C for 3 h. About 3 ml of Dowex 50 (H^+ form) was then added to bring the pH to 3, the resin was washed with water, and the pyridylamino derivatives were eluted with aqueous ammonia. Separation and product identification were carried out by HPLC with a C18 reversed phase column or by gel permeation chromatography. The reaction was later modified to increase the yield [152]. A solution of 2-AP and concentrated hydrochloric acid was prepared at pH 6.2, or 8.3 if the carbohydrate contained sialic acids in order to minimize sialic acid loss. The coupling reaction was performed in a sealed tube at 100 °C for 13 min and then the sodium cyanoborohydride reducing agent was added and the mixture was heated for a further 15 h at 90 °C. Yields were in the region of 70%.

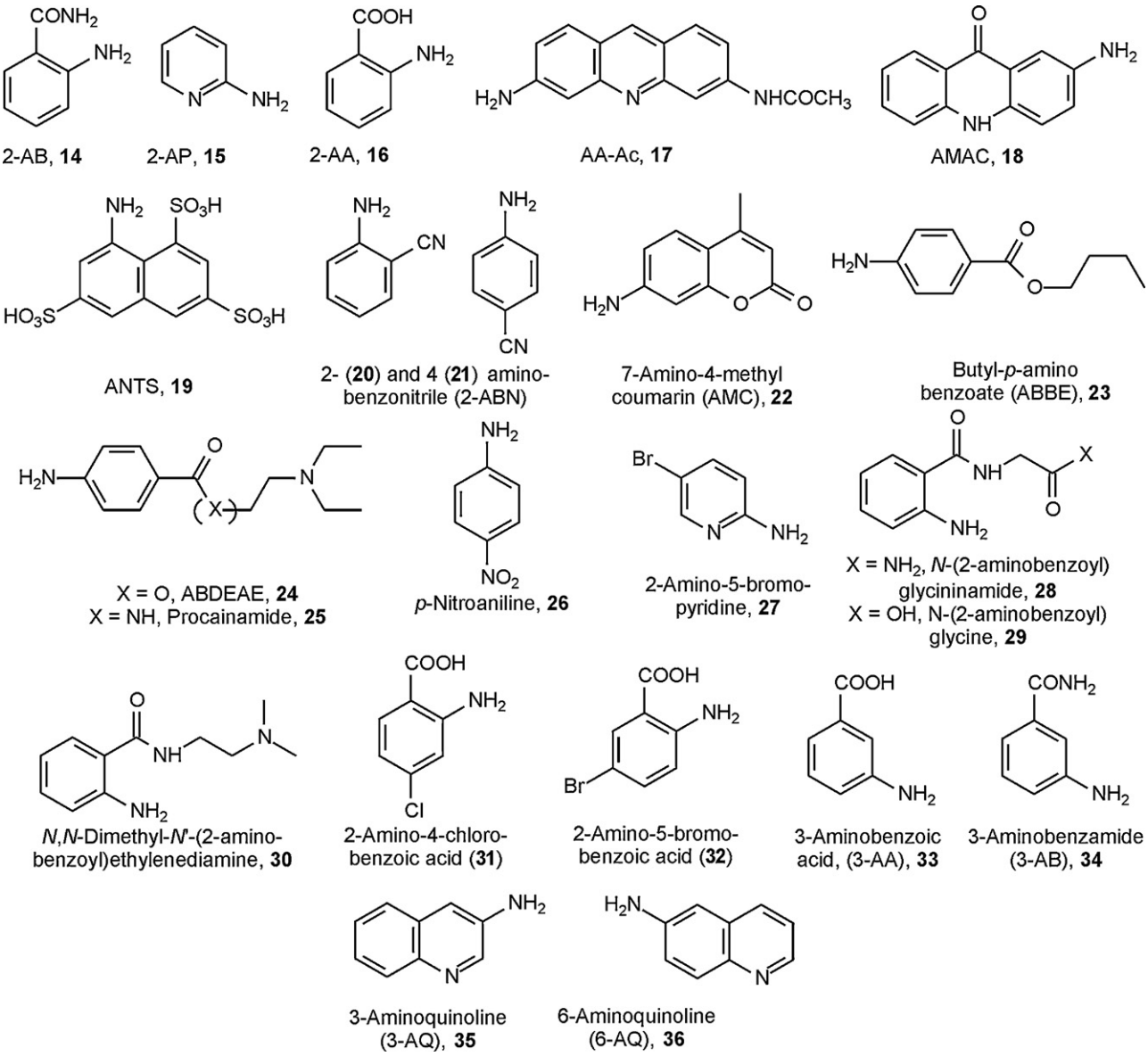
Retention times for derivatized high mannose glycans on reversed-phase HPLC columns were calculated by addition of the partial retention times for each sugar residue although some deviations were observed. To overcome this problem, Yanagida et al. [153] have devised a new scale, termed the R scale, based on the curve obtained from eight reference 2-AP-labelled glycans. The new scale reduced variations caused by minor variations in the elution conditions.

The reaction has since been modified and used in numerous studies, both to add a UV/fluorescent tag and for detection by MS (e.g. [151,154–156]. A relationship between structure and retention time for *N*-linked glycans on reversed-phase columns has been found allowing the retention times of unavailable glycans to be calculated [157,158]. Preferential protonation in MS gives spectra containing mainly B- and Y-type cleavage ions that are easier to interpret than those of $[M+Na]^+$ ions that are formed by underivatized carbohydrates and which tend to be more complicated. One reported problem following ionization from 2,5-DHB in MALDI MS analysis is the formation of $[M+2]^+$ ions, a reaction traced to reduction of the pyridine ring [159]. The 2-AP group can be removed quantitatively by catalytic reduction followed by mild treatment with hydrazine [160].

A modified derivative, useful for mass spectrometric analysis, contains a bromine atom (**27**). This atom produces doublet ions in the mass spectrum because of the naturally-occurring bromine isotopes [161]. This property allows derivatized glycans to be identified in mixtures and labels the reducing end fragments for MS/MS studies.

3.2.4. 2-Aminobenzamide (2-AB)

2-Aminobenzamide (2-AB, **14**) derivatives were introduced by Bigge et al. in 1995 [128] and have been used extensively for structural and quantitative work. Normally a relatively high concentration of the 2-AB reagent is used necessitating a clean-up stage before analysis. However, recently Maury et al. [162] have used a much reduced concentration (10^{-4} molar) and have been able to examine the reaction directly by HPLC after 6 h. Chromatography on normal phase, amide columns produces retention times that reflect glycan size and molecular weight of the glycans and the technique is used extensively, coupled with exoglycosidase digestion for structural studies of *N*- and *O*-linked glycans (Fig. 1). Retention times are compared with those of a dextran ladder to produce glucose units (GU) in the same way as hydrocarbons are used to generate methylene units in GLC. A database of available glucose units has recently been published [131]. Reversed-phase columns are not as useful for separation of these derivatives and do not generate glucose units; however comparisons with arabinose oligomers has been reported (to yield corresponding arabinose units) but these have not been widely adopted. The label has been shown to affect the relative abundance of fragment ions in ESI and MALDI MS and comparisons with other derivatized glycans have been published by Lattová et al. [163]. In positive [164] and negative ion MS [165–168], the presence of the derivative reduces the number of diagnostic fragments because the reducing-terminal ring is opened as the result of the reductive step.



Scheme 7. Common amines used to prepare derivatives by reductive amination.

A series of substituted 2-AB derivatives in which the amide group has been substituted with further groups containing a carboxyamide (**28**, neutral), carboxylic acid (**29**, acidic) and *N*,*N*-dimethylamine (**30**, basic) that enable the charge to be modified have been synthesised and found to be useful for reversed-phase (RP)-HPLC and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) [169].

3.2.5. 2-Aminobenzoic acid (2-AA)

Derivatives with 2-aminobenzoic acid (2-AA, **16**) [170,171] have been reported to give twice the fluorescence of the 2-AB derivatives and are favoured by some investigators. Quantitative labelling has been achieved in acetate-borate buffered methanol (~pH 5.0) at 80 °C for I hr and appeared to cause negligible or no desialylation from sialylated glycans [170]. The labelling reaction has also been performed on hydrazine-released *O*-linked glycans following re-acetylation without any clean-up of the re-acetylation reagents [172]. Both normal-phase polymeric-NH_2 bonded (Astec) column- HPLC and anion exchange (NP-HPAEC) were used to monitor the products. However, it has been reported that the reductive amination reaction can cause up to 15% epimerization of the reducing-terminal GlcNAc residue to mannosamine [3]. Sato et al. [173] have optimized conditions for the reaction and have obtained the best results with 0.2 M 2-AA and 1.0 M sodium cyanoborohydride in water. The optimal reaction for the derivatization of neutral-sugars, aminosugars, and uronic acids was performed at 40 °C for 16 h. Monosaccharides could be derivatized at 65 °C for 4 h. The greater sensitivity imparted by this derivative to 2-*N*-acetylamino-sugars has been partly accounted for by stabilization caused by hydrogen bond formation between the carboxyl and *N*-acetylamino groups leading to high yields of the derivative [174].

On normal-phase columns, there is significant overlap between sialylated and neutral oligosaccharides but relative retention times measured as GU cannot be calculated across the entire gradient. Neville et al. [175] have overcome this difficulty by use of a Dionex AS11 column that combines both hydrophilic interaction and anion-exchange chromatography. The method can also be used for 2-AB and other fluorophore-labelled oligosaccharides. Among recent uses of this derivative is the monitoring of free high-mannose oligosaccharides found in cytosol as the result of cleavage from glycoproteins [176].

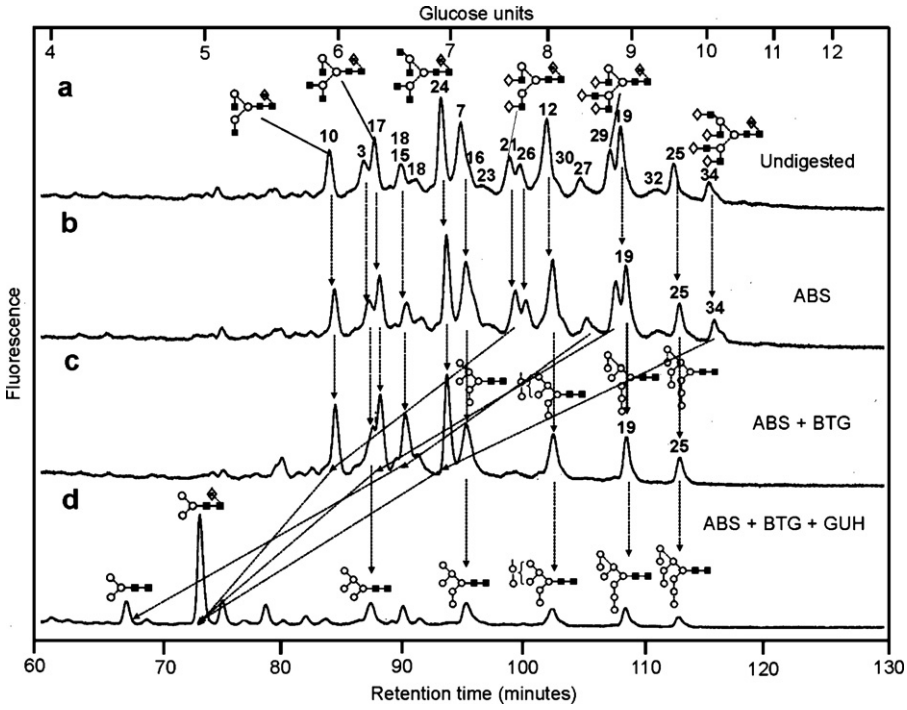


Fig. 1. Exoglycosidase sequencing of 2-AB-labelled *N*-linked glycans from SARS virus spike glycoprotein monitored by normal-phase HPLC. Panel a, Profile of the undigested glycans. Panel b, Following digestion with *Arthrobacter ureafaciens* sialidase (ABS, EC 3.2.1.18) (negligible digestion). Panel c, After digestion with ABS and bovine testis β-galactosidase (BTG, EC 3.2.1.23). Panel D, After digestion with ABS, BTG and *Streptococcus pneumoniae* hexosaminidase (GUH, EC 3.2.1.30). Arrows show the movement of the peaks after each digestion. All complex and hybrid glycans digest to the two core structures $Man_3GlcNAc_2$ and $Man_3GlcNAc_2Fuc_1$. High-mannose glycans are not digested and show up in Panel d. The numbers on the peaks refer to the structures given in the original publication [306]. Symbols used for the structural formulae: ■ = GlcNAc, ○ = mannose, ◇ = galactose, ◈ = fucose. The angle of the lines connecting the symbols shows the linkage with full and broken lines specifying β- and α-linkages, respectively. Further details are given in the paper by Harvey et al. [307]. Reproduced with permission from Elsevier Inc.

The derivatives have the advantage for MS that they readily form $[M-H]^-$ ions, leading to good sensitivity. Unfortunately though, the localization of the charge on the derivative suppresses formation of many of the diagnostic ions [177] that have made negative ion MS/MS such a valuable method for structural studies of carbohydrate such as *N*-linked glycans [165–168]. The negative ion fragmentation spectra of 4-chloro- (**31**) and 5-brom-2-aminobenzoic acids (**32**) have also been studied; the advantage of the halogeno-substituents being that they label fragments containing the derivative by virtue of their characteristic isotopic pattern [178].

3.2.6. 3-Aminobenzoic acid (3-AA) and 3-aminobenzamide (3-AB)

3-Aminobenzoic acid (3-AA, **33**) and 3-aminobenzamide (3-AB, **34**) show higher reactivity with carbohydrates than the corresponding 2-AA and 2-AB reagents but the fluorescence intensities and molar absorptivities of the resulting derivatives are not as high [179] and they have not found general use. Nevertheless, they have been shown to provide excellent CE separation of sialic acid-containing *N*-linked carbohydrates derived from fetuin and thyroglobulin as well as from high mannose-type and hybrid-type carbohydrates.

3.2.7. 2-Aminoacridone (AMAC)

2-Aminoacridone (AMAC, **18**) was introduced as a carbohydrate fluorophore by Jackson in 1991 [180] and was soon adopted by Lamari et al. [181] for analysis of disaccharides from chondroitin and dermatan sulfates by CE. The derivatives have been used extensively for examination of *N*-linked carbohydrates by CE and polyacrylamide gel electrophoresis (PAGE) and their hydrophobic nature allows analysis by reversed- and normal-phase HPLC and by CE/ESI MS and MALDI MS [182–186]. Much of the early work is included in the review by Lamari et al. [6].

3.2.8. 3-(Acetylamino)-6-aminoacridine (AA-Ac)

3-(Acetylamino)-6-aminoacridine (AA-Ac, **17**) was introduced as a label for the sensitive detection of *N*-glycans by Charlwood et al. [187] and was found to have at least twice the fluorescence intensity as its predecessor, AMAC. Product analysis was by both normal and reversed-phase HPLC and the derivative is also compatible with MALDI MS, free capillary electrophoresis, and capillary electrophoresis/electrospray ionization MS. The derivatives contain an ionisable group making them useful for mass spectrometric analysis and they have been used to study *N*-glycans released from within sodium dodecyl sulfate (SDS)-PAGE gels with MALDI or HPLC monitoring [188]. More recently, the derivative has been used to study recombinant monoclonal antibodies by liquid chromatography/mass spectrometry (LC/MS) [189] and the reagent is sold by Ludger Ltd. under the LudgerTag™ brand.

3.2.9. 3-Aminoquinoline (3-AQ)

3-Aminoquinoline (3-AQ, **35**) was used as a MALDI matrix 1994 by Metzger et al. [190] for the analysis of plant inulins and was said to produce better sensitivity and resolution than the most popular matrix, DHB. However, it did not gain popularity for the analysis of reducing carbohydrates because of its tendency to form Schiff bases [191]. However this property has been turned to advantage by Rohmer et al. [192] who have developed conditions for ensuring quantitative conversion of the sugars to their 3-AQ derivatives on the MALDI target where the reagent also acts as the matrix. Optimal conditions employed a 3-AQ solution containing 20 mg/ml of reagent in a MeCN-water mixture (1:2, v/v) with 0.07% inorganic acid to give a pH of 5. The use of hydrochloric

acid enabled negative ion spectra to be obtained due to formation of $[M+Cl]^-$ ions. An advantage of this method for derivative formation is that the high concentration of reagent ensures that the equilibrium position of the Schiff base reaction lies strongly towards the derivative. PSD fragmentation in positive and negative ion mode was enhanced, providing information on oligosaccharide sequence, linkage, and branching. The method was applied to trifucosyllacto-*N*-hexaose and trifucosyl-para-lacto-*N*-hexaose, two isomers occurring in human breast milk, which were easily identified. Enhanced formation of cross-ring cleavage ions compared with those in the CID spectra of several other derivatives was also noted following ionization by ESI [145]. The related 6-AQ (**36**) has been used for CE separations [193] and has been reported to give eight times the signal strength of 2-AP-labelled glycans [194].

3.2.10. Derivatives containing a cationic charge

Several investigators have attempted to increase detection limits for MS by derivatizing carbohydrates by reductive amination with reagents containing a cationic charge. Thus, Dell et al. [41] used *p*-aminophenyl ammonium chloride (TMAPA, **37**, Scheme 8) for FAB MS and Okamoto et al. [195,196] compared results from this derivative with the detection limits obtained with derivatives from 4-aminobenzoic acid (**38**) and 2-AP (**15**). Results were variable. Under ESI conditions, the TMAPA derivatives were claimed to give a 5000-fold increase in sensitivity [195] whereas only a ten-fold increase was reported following MALDI analysis from DHB [196]. In this second study, the uncharged 2-AP derivatives appeared to be the most sensitive, increasing the detection limit by some 100-fold.

Broberg et al. [197] have derivatized oligosaccharides by reductive amination with benzylamine (**39**) and then reacted the product with methyl iodide in ethanol in the presence of an ion exchange resin that had been equilibrated with sodium hydroxide. This latter reaction quaternized the secondary amine linking the derivative to the sugar to form dimethylbenzylamine (DMBA) derivatives. In a comparative analysis by MALDI MS, the derivatives appeared to be twice as sensitive as those prepared from Girard's *T* reagent (also possessing a cationic charge, see below) and ten times as sensitive as the underivatized glycans.

A label that combines bioaffinity with a cationic charge has recently been constructed by Hsu et al. [198] and used for sensitivity increases in MALDI and ESI MS. The compound, 5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid 4-aminomethyl-benzylamide (**40**), contains a biotin function and the cationic charge was introduced by quaternization of the linking secondary amine after linkage to the target sugars by reductive amination. As little as 50 pmol of starting material could be efficiently labeled with minimal loss to side reactions and 100 fmol of high-mannose *N*-glycans released from ribonuclease B could be detected by MALDI-TOF MS. The label was reported to be compatible with HPLC allowing analysis by LC/MS.

Phosphonium salts have also been used to introduce a cationic charge and are covered in Section 3.3.

3.2.11. Derivatives containing anionic charges

Several sulfonated aromatic amines have been deployed to introduce charges for separations based on techniques such as CE, MEKC and SDS-PAGE.

3.2.11.1. Sulfonated aromatic amines. Sulfonated aminonaphthalenes are popular derivatives for studies by CE and related electrophoretic techniques. Chiesa and O'Neil, [199] compared five mono-, di- and tri-sulfated aminonaphthalenes (**19**, **41–44**) and noted greater electrophoretic mobility and resolution with the more highly sulfated compounds such as ANTS (**19**) which has become well used as a derivatization agent in this area [200–203].

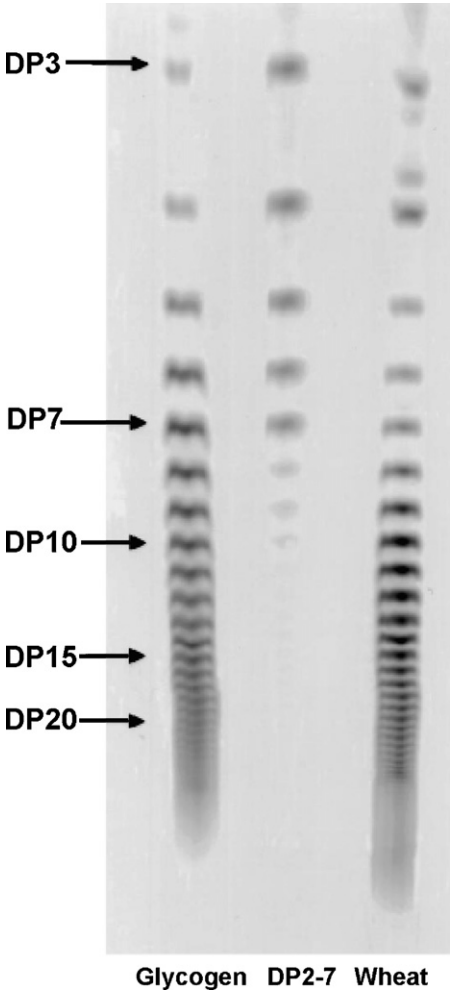
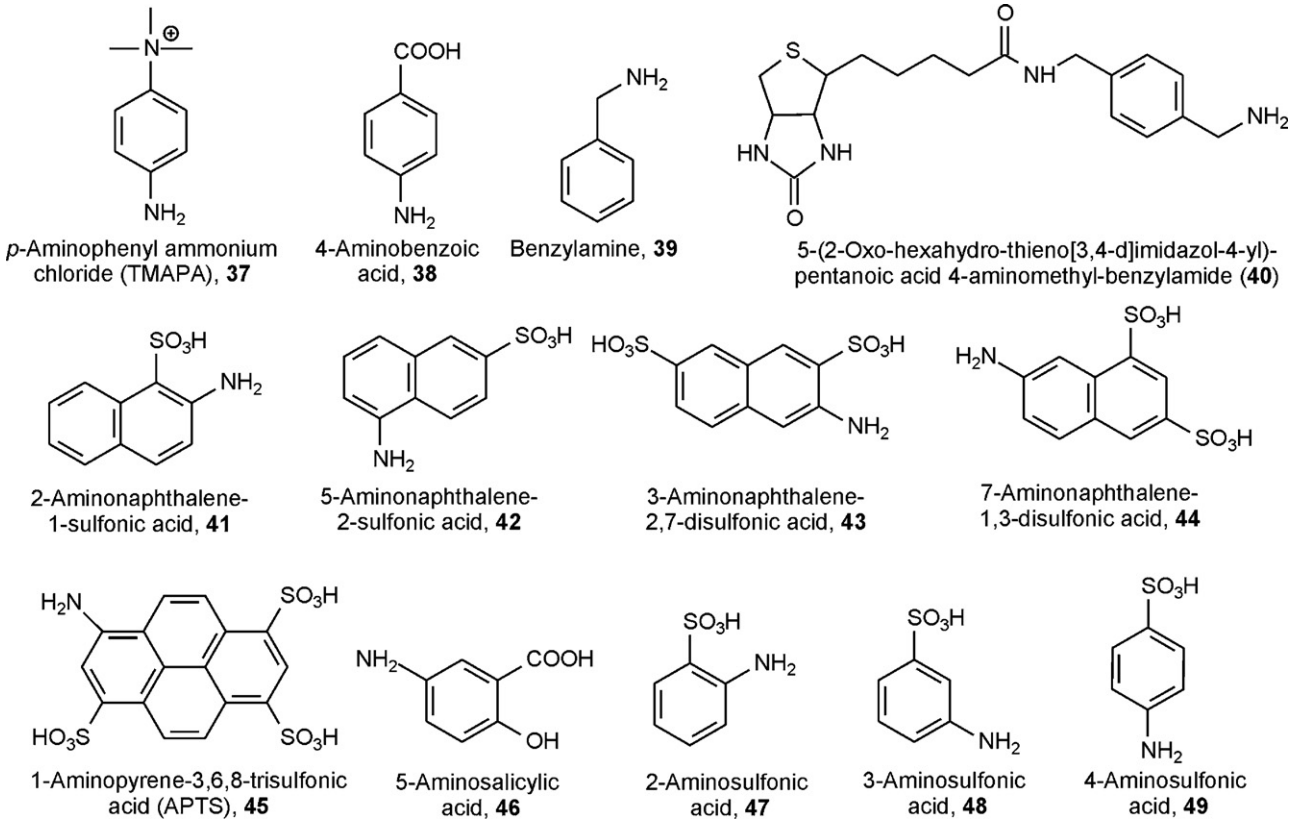


Fig. 2. FACE profile of APTS-labelled debranched glycans from glycogen and wheat starch on a 20% polyacrylamide gel in 1× TBE buffer for 4 h at 8 °C and a constant power of 75 W. From reference [211] with permission from Elsevier Inc.

Another trisulfonated aromatic amine is 1-aminopyrene-3,6,8-trisulfonic acid (APTS, **45**) which was used by Evangelista et al. [204] to derivatize mono and oligosaccharides for analysis by CE/laser-induced fluorescence (LIF). Use of an argon-ion laser for excitation at 488-nm and a narrow band filter at 520 nm for fluorescence emission enabled the derivatized sugars to be selectively detected in the presence of the reagent. APTS-derivatized monosaccharides were readily separated in borate buffer (pH 10.2), while the oligosaccharide ladders were analyzed using either an acidic phosphate buffer pH 2.2) or the alkaline borate buffer for separation. The yield of the derivative was shown to be dependent on the pH of the acid, normally acetic, used to catalyse ring opening prior to Schiff base formation but citric and malic acids have been shown to increase the yield [205]. The effect was particularly noticeable with *N*-acetylamino-sugars [206]. The best yields were obtained with citric acid at 75 °C with heating for about 1 hour. A detailed study of the reaction conditions was later made by Guttman et al. [207] who suggested lowering the reaction temperature to 55 °C to minimize losses of sialic acid. An improved preparative method for APTS derivatives using citric rather than acetic acid has recently been reported [208]; sialic acid loss from sialylated carbohydrates was said to be negligible.

These tri-sulfonic acids derivatives have been separated on polyacrylamide slab gels in a method termed fluorophore-assisted carbohydrate electrophoresis (FACE, Fig. 2) [209–211]. Imaging was with a Molecular Dynamics Fluorimager that collects fluores-



Scheme 8. Amines used to prepare charged derivatives of carbohydrates by reductive amination.

cent data while scanning the gel with a low intensity argon-ion laser (488 nm). Polymers with up to 30 residues were observed. *N*-Glycans have been similarly analysed using ANTS as the fluorescent derivative [202]. Using DNA sequencer technology, O'Shea and Morel [212] have used this derivative to resolve sugar chains with up to 80 residues.

Di-sulfonated-naphthalenes such as 7-aminonaphthalene-1,3-disulfonic acid (ANDS, **44**) have also been used for CE separations as exemplified by Pfaff et al. [123] who showed significant differences between CE and HPAEC-PAD in the behaviour of maltooligosaccharides derivatized with ANDS. The proportion of lower molecular weight compounds was higher in CE and the differences were attributed to degradation of the higher molecular weight compounds. Further details on sulfonated aromatics can be found in the review by Lamari et al. [6] and some other reported compounds are listed in Table 1 (structures in Scheme 8).

3.2.12. (2,4-Dinitrophenyl)octylamine (DNPO) derivatives

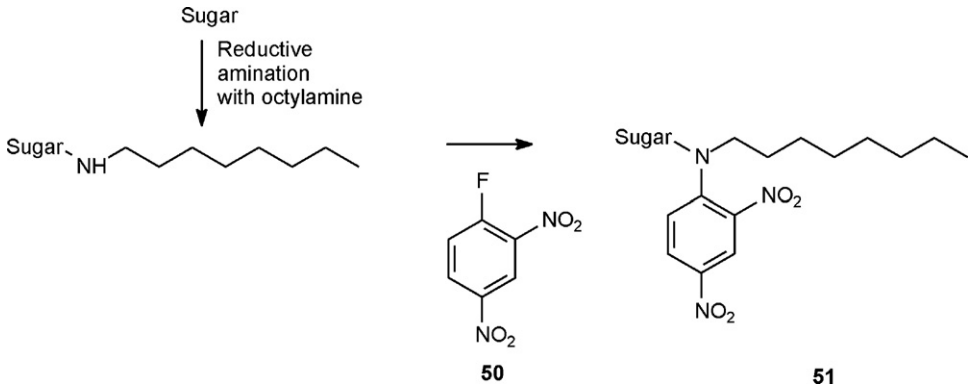
2,4-Dinitrophenyl)octylamine (DNPO, **51**) derivatives (Scheme 9) illustrate the preparation of derivatives where reductive amination is used to derivatize the carbohydrate and then a further reaction is carried out to form the final derivative. In this example [213], the sugar is first derivatized with *n*-octylamine by reductive amination and then heated with 2,4-dinitrofluorobenzene (**50**) overnight at 45 °C in the presence of triethylamine. The derivatives were claimed to be more sensitive than those prepared from aminobenzoic acid alkyl esters and the *n*-octyl group provided high lipophilicity for analysis by FAB MS.

3.2.13. Derivatives for preparation of arrays

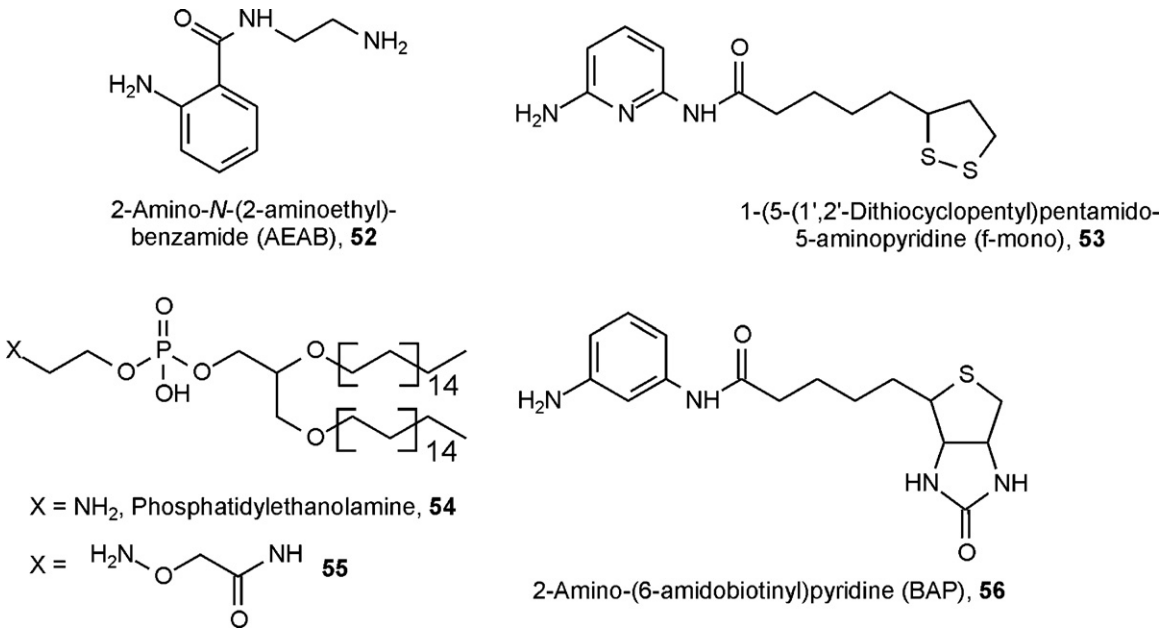
Glycan arrays are becoming increasingly popular for binding studies and are frequently prepared *via* reducing terminal derivatization of a range of carbohydrates. Thus, for example, Song et al. [214] have used the bifunctional amine 2-amino-*N*-(2-aminoethyl)-benzamide (AEAB, **52**, Scheme 10) first to label the glycans by reductive amination *via* the aromatic amine and then to couple the sugar to *N*-hydroxysuccinimide-activated glass slides *via* the second amine. By use of such arrays and fluorescence-based screening it was demonstrated that galectin-1 recognizes a wide variety of complex *N*-glycans, whereas galectin-3 primarily recognizes poly-*N*-acetyllactosamine-containing glycans independent of *N*-glycan presentation.

Another derivative prepared by reductive amination from 1-(5-(1′,2′-dithiocyclopentyl)-pentamido)-5-aminopyridine (**53**), named "f-mono" is suitable for coupling to gold coated chips and can also be used for HPLC/fluorescence analysis. The derivative has the useful property of being partially reduced during mass spectral analysis (ESI), the normal $[M+H]^+$ ions are accompanied by a second ion ($[M+H+2H]^+$) appearing two mass units higher. The doublet peaks enable derivatized glycans in a mixture to be easily distinguished from other compounds [215]. Lipids such as 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE, **54**) have also been coupled *via* reductive amination and, in order to avoid using a carbohydrate with an open reducing terminal ring, the related compound (**55**) has been coupled *via* oxime formation [216,217].

Carbohydrates labelled with biotin-containing groups can be coupled to streptavidin-coated plates to form arrays. Several investigators have developed suitable reagents than can be attached *via* reductive amination or by hydrazide-coupling for array construction or for analysis by HPLC. Thus, for example, Rothenberg et al. [218] have synthesised 2-amino-(6-amidobiotinyl)pyridine (BAP, **56**) from diaminopyridine and coupled it to glycans by reductive amination for binding studies. The method has been extended to



Scheme 9. Scheme for the preparation of (2,4-dinitrophenyl)octylamine (DNPO) derivatives.



Scheme 10. Amines used for the preparation of glycan arrays by reductive amination.

include HPLC analysis and formation of the related derivative without reduction [219].

3.2.14. Other amines used for reductive amination

Some other amines (**57**–**73**) that have been used for reductive amination are listed in Table 1 and structures are shown in Scheme 11.

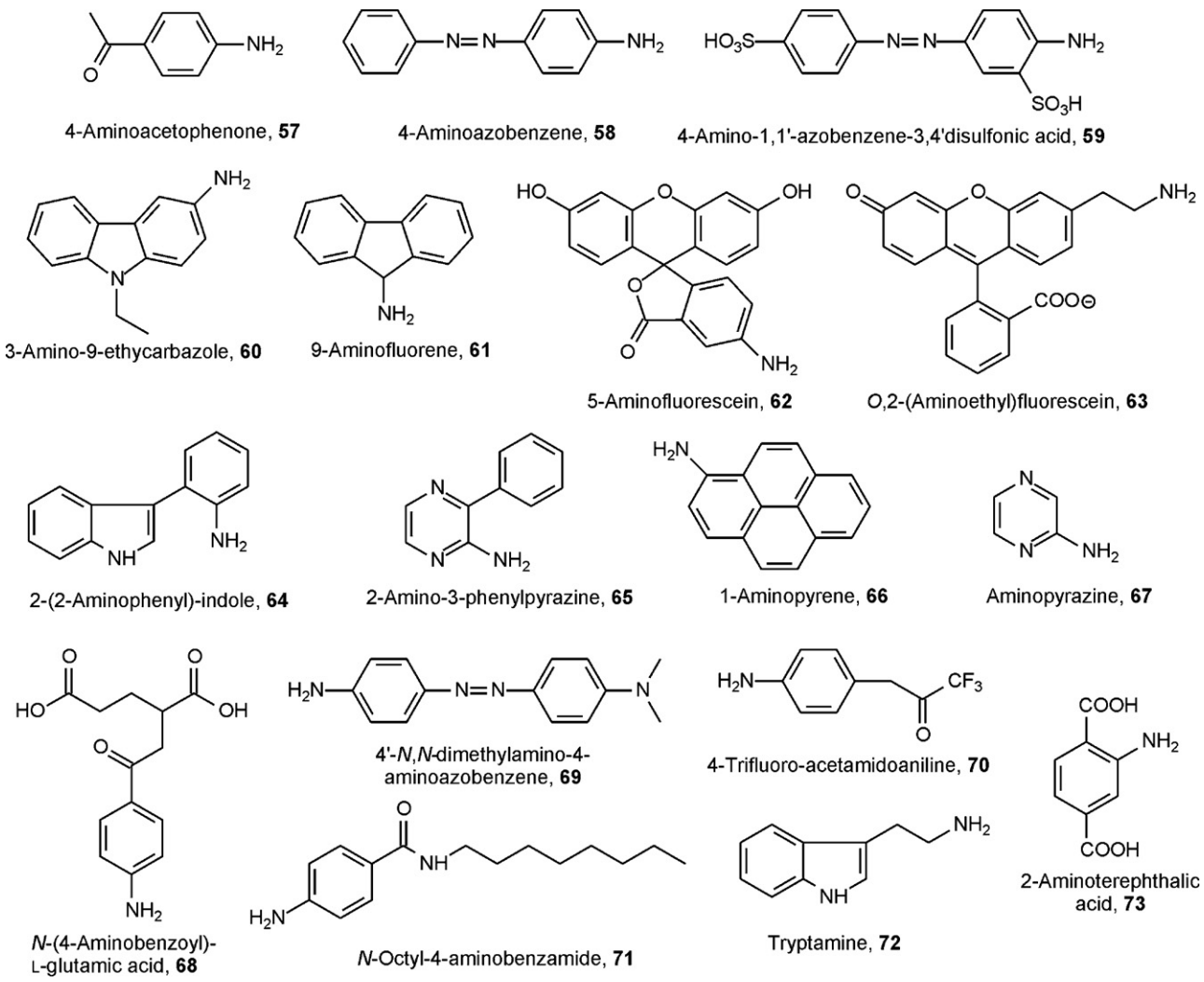
3.3. Derivatives from glycosylamines

PNGase F is an amidase and releases *N*-glycans from glycoproteins as the glycosylamines. Normally, these compounds rapidly hydrolyse to the free glycans, particularly at low pH. However, it is possible to capture these intermediate glycosylamines directly by reaction with suitable reagents. Thus, for example, Kamoda et al. [220] have captured released *N*-glycans as 9-fluorenylmethyloxycarbonyl (Fmoc) derivatives by reaction with fluorenylmethyloxycarbonyl chloride (Fmoc-Cl, **74**, Scheme 12) and examined them by HPLC. Sensitivities exceeded by 5-fold those obtained from 2-AA derivatives prepared in the conventional manner and, furthermore, the free glycans could be released by incubation with morpholine in dimethylformamide solution. The method has recently been extended with capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI MS) detection enabling the authors to analyse the *N*-glycans on a glycoprotein in only 5 h [221].

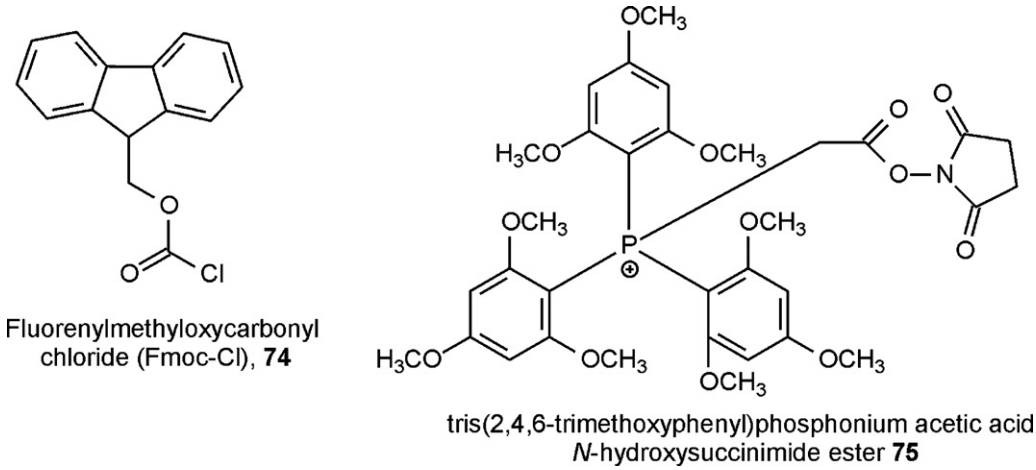
Reducing sugars can easily be converted into glycosylamines by reaction with ammonium bicarbonate and, again, this enables reactions to be carried out with suitable carbonyl-containing and other amine-reactive reagents. As an example, Kallin et al. [222] also prepared Fmoc derivatives and used them to examine derived carbohydrates from human milk. The reaction could be reversed by a brief treatment with 15% aqueous ammonia. Liu et al. [223] have used tris(2,4,6-trimethoxyphenyl)phosphonium acetic acid *N*-hydroxysuccinimide ester (**75**) to derivatize glycosylamines as TMPP amides. Basic buffer conditions are generally used for this condensation reaction but aqueous buffer systems were found not to be suitable for the coupling of this reagent to glycosylamines because of the instability of the glycosylamines in aqueous solution. Thus, an organic base, anhydrous *N*,*N*-diisopropylethylamine (DIEA), was used. The glycosylamines were prepared by a microwave-assisted Kochetkov reaction using only a small excess of ammonium carbonate.

3.4. Derivatives prepared by carbonyl condensation reactions

Several other carbonyl-reactive reagents can be used to derivatize the reducing-terminus of carbohydrates; many of these



Scheme 11. Additional amines used to prepare derivatives of carbohydrates by reductive amination.

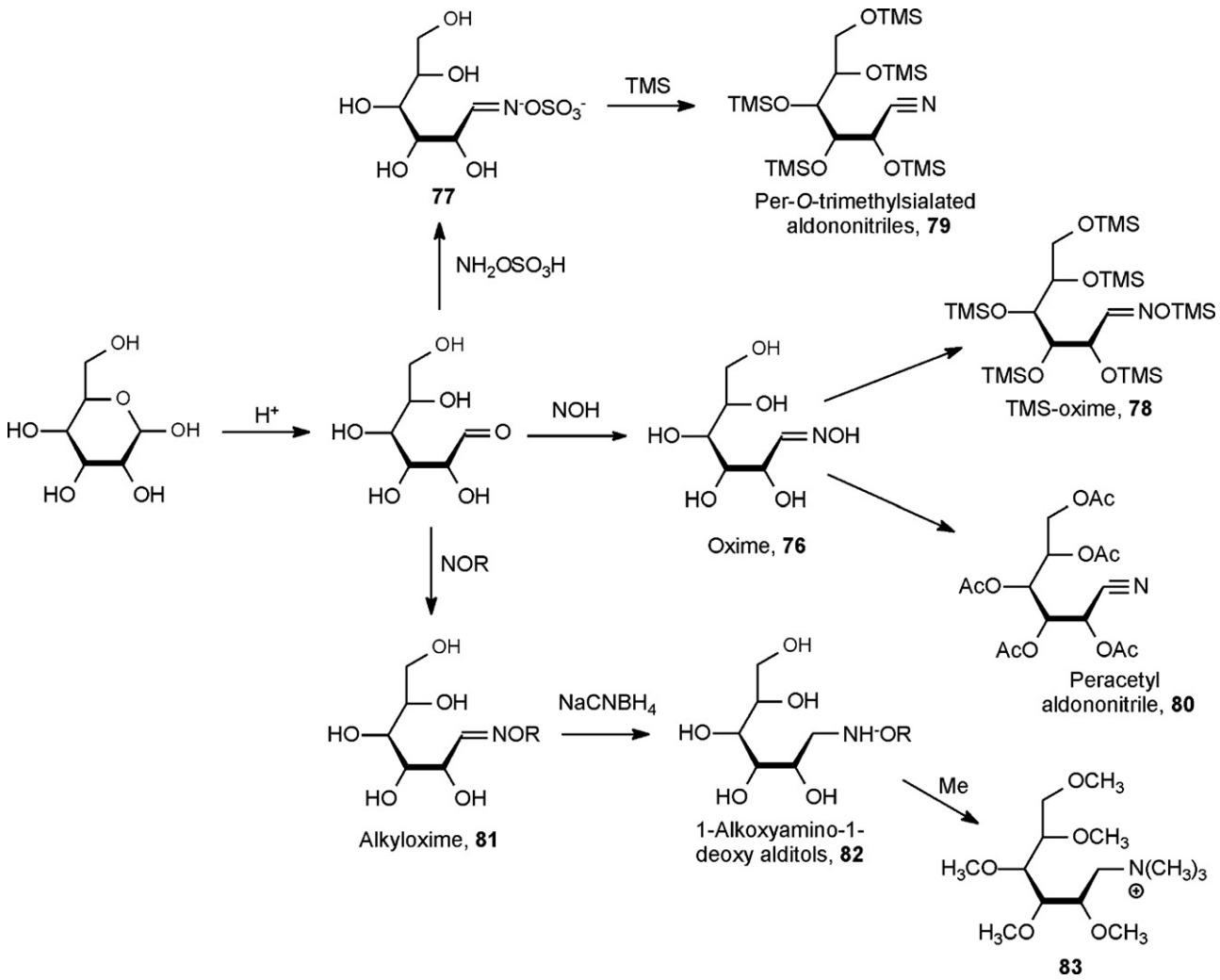


Scheme 12. Structure of fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) and the TMPP reagent.

produce stable products that do not require reduction. Such reagents are hydroxylamines, giving oximes and hydrazines or hydrazides yielding hydrazones.

3.4.1. Oximes and derived compounds

Reaction of sugars with hydroxylamine hydrochloride yields the corresponding unsubstituted oxime (**76**, Scheme 13). Its subsequent behaviour depends on further derivatization steps. If the oximated sugar is converted into its TMS derivative, the hydroxyl group of the oxime is simply derivatized to give the TMS oxime (**78**) whose behaviour during GC/MS analysis is similar to that of the alkyloximes [88,224]. If the sugar is acetylated with acetic anhydride catalysed by either pyridine or 1-methylimidazole, however, the resulting acetyloxime is dehydrated to the corresponding



Scheme 13. Scheme for the preparation of carbohydrate oximes and their derivatives.

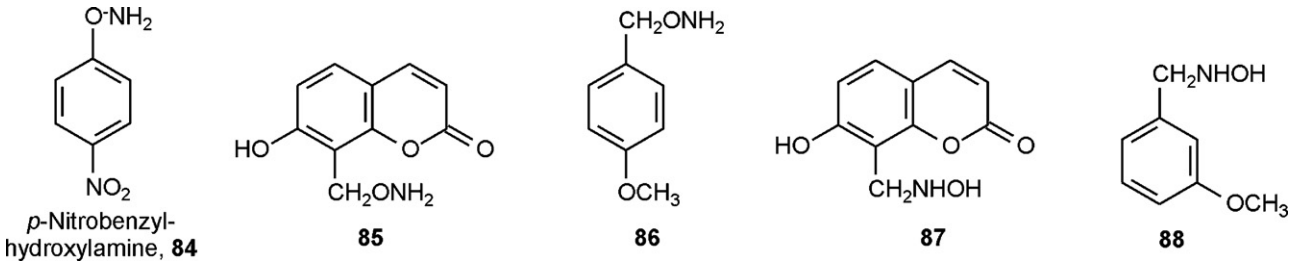
peracetyl-aldononitrile (PAAN, **80**) derivative [65,69,70,225]. Per-*O*-trimethylsialated aldononitriles (**79**) have also been prepared by the reaction of BSTFA on the substituted oxime formed by reaction of sugars with hydroxylamino-*O*-sulfonic acid [226,227]. These aldononitrile derivatives have the advantage over peracetyl or per-TMS derivatized glycans of only producing a single peak in the GLC profile. Prominent fragment ions in their EI mass spectra contain the nitrile group [228] and problems due to weak molecular ions in their EI spectra have been addressed with isobutane CI which produces prominent $[M+1]^+$ ions [229]. Alkyl oximes (**81**) are prepared by use of the appropriately substituted oximes.

3.4.1.1. Derivatives formed by reduction of oximes. von Deyn et al. [230] have formed 1-alkoxyamino-1-deoxy alditols (**82**) by condensation of substituted hydroxylamines and reduction of the resulting oximes with sodium cyanoborohydride. Permethylation resulted in elimination of an aldehyde moiety from benzyl and *p*-nitrobenzyl oximes to give a quaternary amine (**83**) that gave strong signals by FAB MS. The 1-deoxy-1-[(4-*p*-nitrophenyl)butoxyamino] alditol derivatives, however, were stable. *p*-Nitrobenzyl derivatives have been prepared, as above, by reaction of the sugar with *p*-nitrobenzylhydroxylamine (**84**, Scheme 14) followed by reduction. In a later study, the permethylation step was not performed and the derivatives were examined directly by HPAEC and HILIC chromatography [231]. However, because yields on reduction were not quantitative, Ramsay et al. [232] have investigated alternative derivatives and have prepared *O*- and *N*-substituted 7-hydroxycoumaryl- and 3-methoxybenzyl-hydroxylamines (**85**–**88**) which were shown to give quantitative yields of oximes with model carbohydrates and to give quantitative oximation under very mild, aqueous conditions. The derivatives were fluorescent, gave good HPLC properties on reversed-phase columns and were compatible with LC/MS analysis.

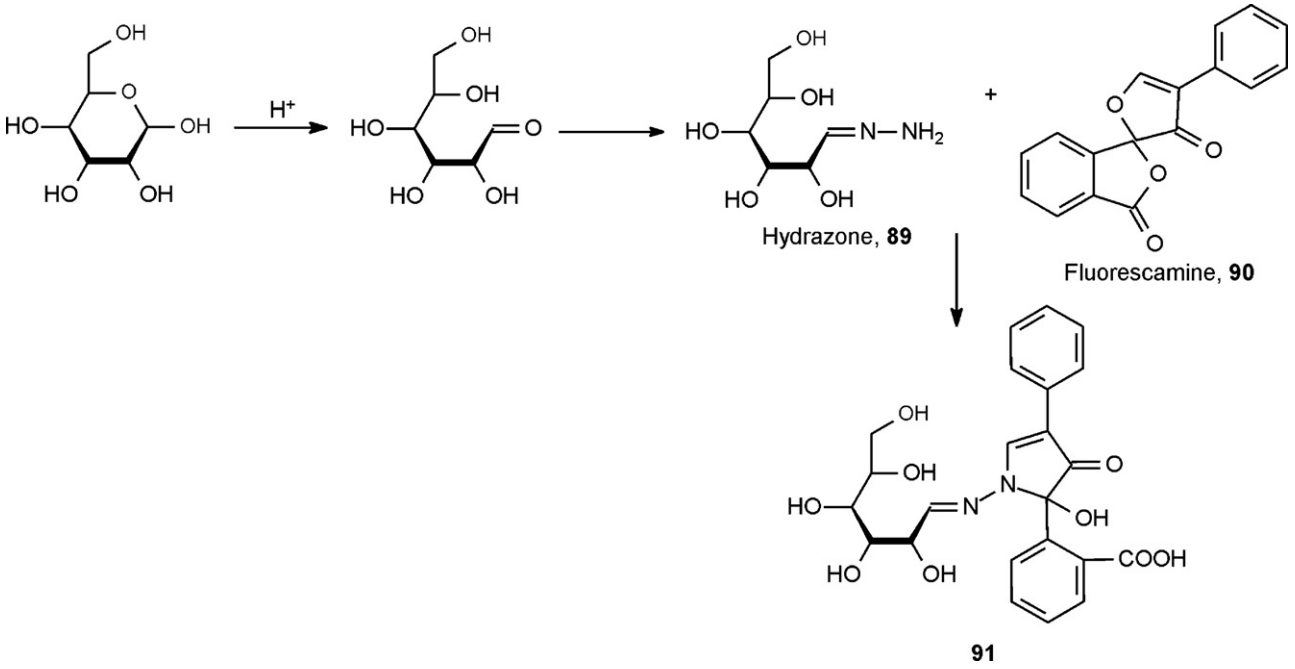
3.4.2. Hydrazones

Hydrazine reacts with reducing sugars to give a hydrazone (**89**, Scheme 15). Whereas this reaction does not introduce any analytical advantage, hydrazones can be reacted further with, for example, fluorescamine (**90**) to give the fluorescent product (**91**) (λ_{ex} = 400 nm, λ_{em} = 490 nm [233]. Most other reactions have employed the reaction of substituted hydrazines or hydrazides to give hydrazones with sometimes an additional reductive step to stabilize the product.

Phenylhydrazine (**92**, Scheme 16) has been used to form phenylhydrazones of carbohydrates and the derivatives have been reported to increase the sensitivity of detection for MS and to provide ultraviolet detection for HPLC [234,235]. The derivatization reaction was complete in 1 h at 70 °C and, after removal of the excess of reagent, analysis was by direct injection or on-line HPLC/electrospray ionization or by MALDI MS. An advantage of this type of derivatization reaction is that no salts are used or produced in the reaction which can, thus, be used for direct on-target



Scheme 14. Substituted hydroxylamines used to prepare carbohydrate oximes.



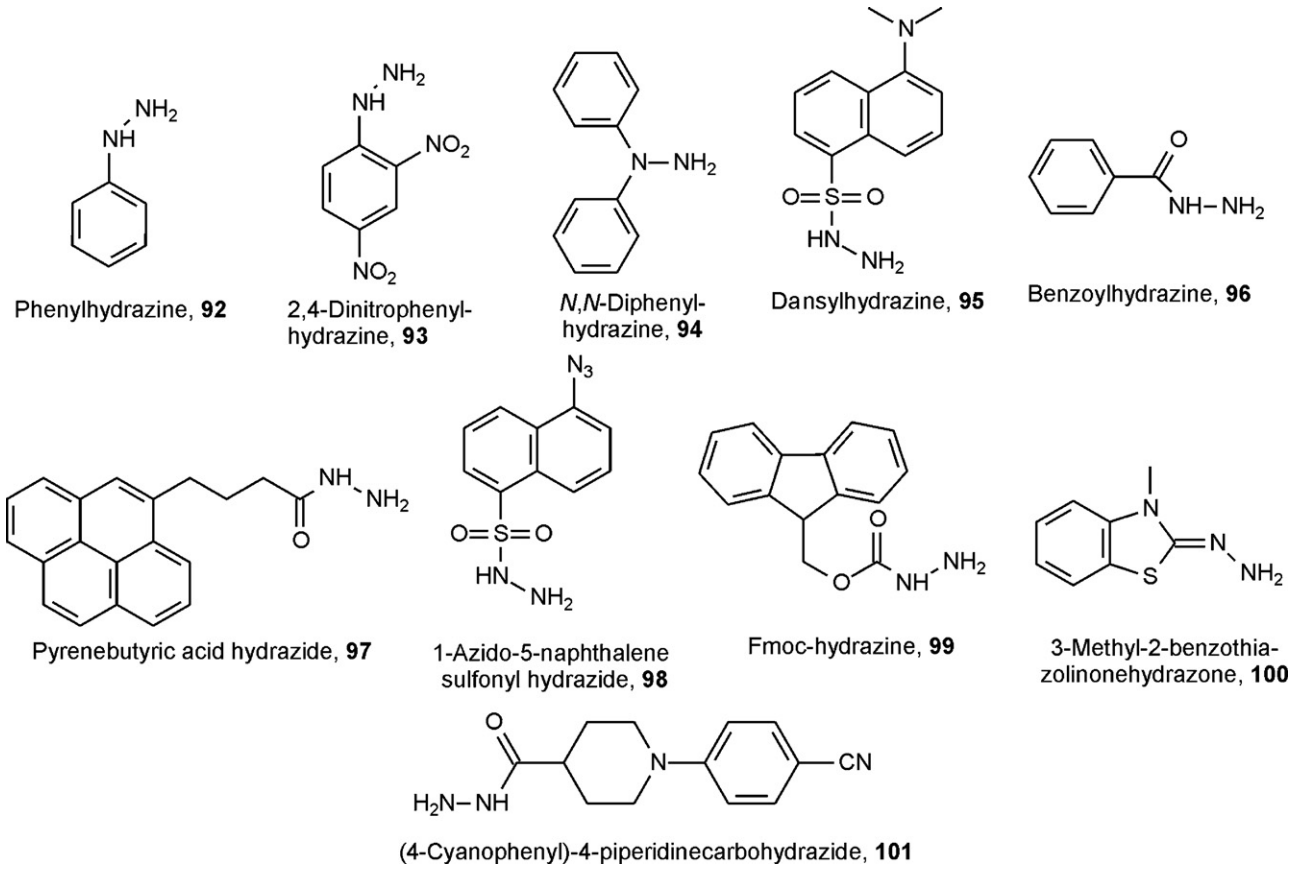
Scheme 15. Scheme for the preparation of fluorescamine derivatives *via* hydrazone formation.

derivatization for MALDI-MS. The simplicity of the reaction has also led to the development of a post-column derivatization technique catalysed by acetic and phosphoric acid at 150 °C [236].

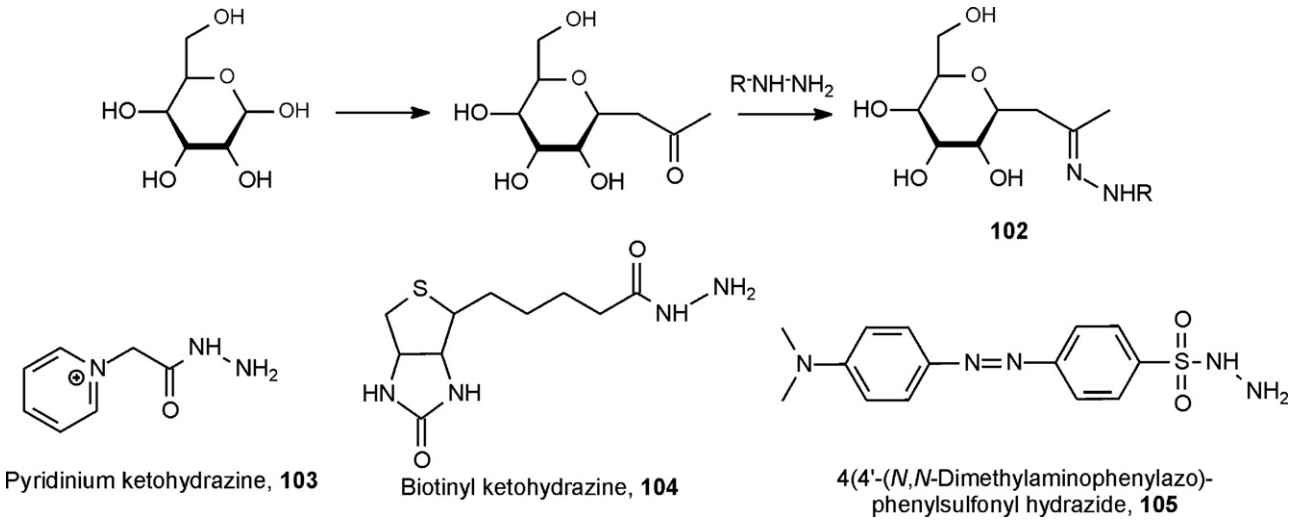
Among other substituted hydrazones that have been reported but not widely used for chromatographic or mass spectrometric analysis are those prepared from 2,4-dinitrophenylhydrazine (**93**) of monosaccharides (HPLC analysis with UV detection at 352 nm [237]). Mono- and *bis*-hydrazones have been formed from *N*,*N*-diphenylhydrazine (**94**) and di-carbonyl carbohydrates [238] and hydrazones have been prepared from monosaccharides with dansylhydrazine (**95)** and analysed by thin-layer chromatography (TLC) [239]. The same reagent has been used to derivatize malto-oligosaccharides and *N*-linked glycans from chicken ovalbumin for analysis by liquid chromatography on silica gel modified with 1,4-diaminobutane [240]). Pyrenebutyric acid hydrazide (**97**) was used by Sugahara et al. [241] to label oligosaccharides from human milk; they were detected by fluorescence polarization λ_{ex} = 341 nm, λ_{em} = 376 nm). Amounts of carbohydrate as low as 10 pmol could be derivatized and the derivatives were also suitable for enzyme-linked immunosorbent assay (ELISA). In an extension to the method, Zhang et al. [242] have developed an on-MALDI target method for derivatization of keratan sulfates and examined their negative ion MS/MS spectra with a TOF/TOF instrument to give mainly B, C and Y-type glycosidic fragment ions with some cross-ring cleavages that provided information on the location of the sulfate groups.

3.4.2.1. C-glycoside ketohydrazones. Most derivatives described above differ from the native carbohydrate in having an open reducing-terminal ring. In order to overcome this disadvantage Price et al. [243] have prepared C-glycoside ketohydrazones by reaction of the sugar with a β-diketone (4 h at 80 °C) to give the mono-ketone derivative (**102**, Scheme 17) followed by reaction with several substituted hydrazines included phenylhydrazine (**92**), pyridinium ketohydrazine (**103**), biotinyl ketohydrazine (**104**), dansyl hydrazine (**95**) and the azo-compound (**105**) thus introducing various properties such as colour, fluorescence, cationic charge and bioaffinity.

3.4.2.2. Hydrazones imparting a cationic charge. The substituted hydrazone, 2-hydrazino-*N*,*N*,*N*-trimethyl-2-oxo-ethanaminium chloride (Girard's *T* reagent, **106**, Scheme 18) has been used to introduce a fixed cationic charge into carbohydrates for sensitive ESI and MALDI MS detection [244] and to increase sensitivity of detection by about 10-fold. This derivative has also been used [245,246] for quantitative studies of *N*-glycans by MALDI MS, the argument being that, because the cationic charge is already present in the molecule, ionization efficiencies of the different glycans should be equivalent. Improvements in sensitivity of about 10-fold over those obtained from underivatized glycans were reported. Gouw et al. [247] also report sensitivity increases with the use of this derivative and have also introduced the use of the related glycidyltrimethylammonium chloride (**107**). Whereas Girard's *T* reagent is specific for aldehyde and ketone groups,



Scheme 16. Substituted hydrazines and related compounds used for the preparation of hydrazones.

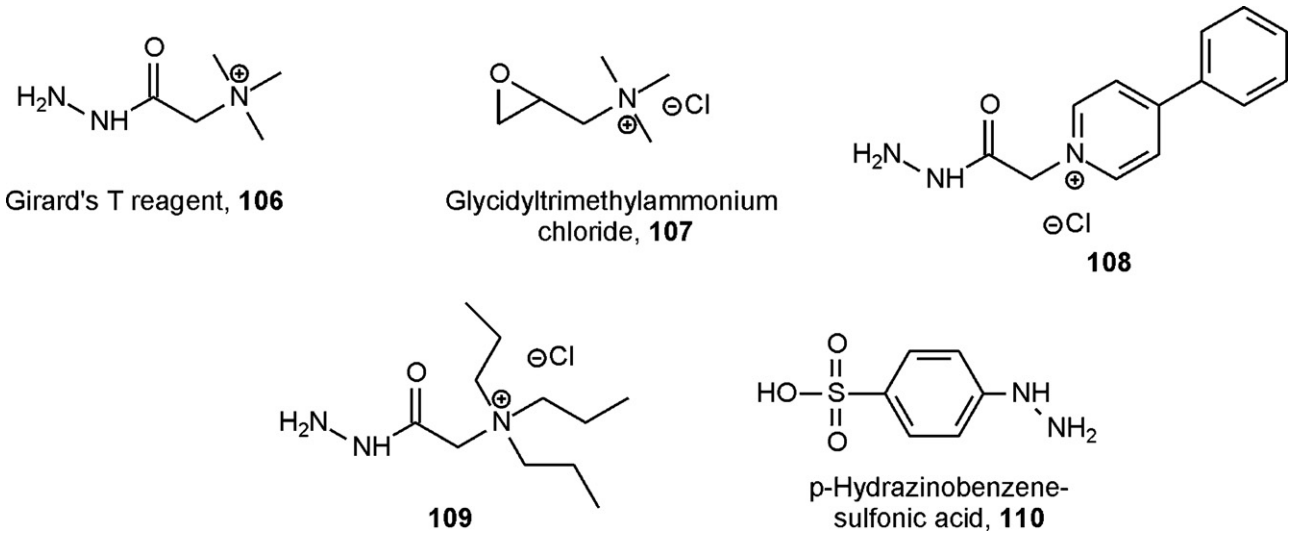


Scheme 17. Scheme for the preparation of ketohydrazone derivatives and some of the hydrazines and related compounds used.

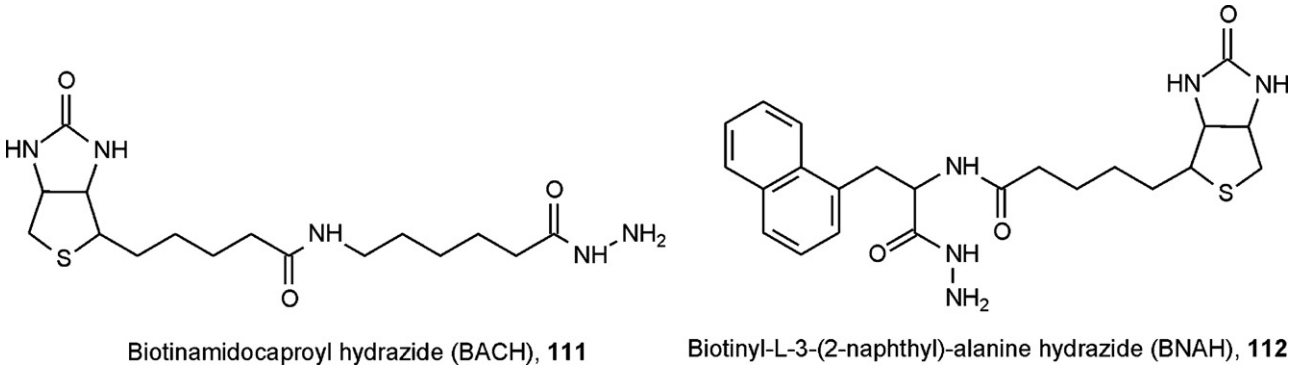
glycidyltrimethylammonium chloride reacts with sugar alcohols such as sorbitol. Again, considerable increases in sensitivity were recorded by MALDI and electrospray MS by use of this reagent.

Two related derivatives prepared from the 4-phenyl pyridine (**108**) and the tripropylamino-analogue of Girard's *T* reagent (**109**) have been prepared and examined by HILIC nano-LC/MS [248]. Both derivatives but particularly the tripropylamino-analogue, produced an increase in sensitivity but, unlike the earlier reports, the sensitivity of the Girard's *T* derivative of maltoheptaose was lower that that from the unlabelled carbohydrate. The authors attributed this lack of sensitivity to the compound's relatively high hydrophobicity. *p*-Hydrazinobenzenesulfonic acid (**110**) has been used as an ultraviolet labeling reagent for capillary electrophoresis of mono-, di- and trisaccharides. The labeling reaction which used 100 mM borate buffer at pH 10.24 was rapid (about 10 min) and introduced both a chromophore and a charged group and gave a detection limit in the low fmol region [249].

3.4.2.3. Hydrazone derivatives for preparation of arrays. The methods used to prepare arrays involving coupling groups to the sugar



Scheme 18. Hydrazines and related compounds used to prepare charged hydrazones.



Scheme 19. Hydrazides used to prepare glycan arrays.

via reductive amination leave the reducing sugar in the open-ring form, a condition that could affect affinity in subsequent binding studies. To preserve the reducing end of the carbohydrate, Shinohara et al. [250] used hydrazide coupling with biotinamidocaproyl hydrazide (BACH, **111**, Scheme 19) and Leteux et al. [251] have introduced a fluorescent label with the production of biotinyl-L-3-(2-naphthyl)-alanine hydrazide (BNAH, **112**). Grün et al. [252] and Ridley et al. [253] have formed hydrazone derivatives from **111** and reduced them with sodium cyanoborohydride in a one-step process similar to reductive amination. Derivatives were amenable to analysis by both ESI and MALDI-TOF MS.

In a related reaction to the above, glycosylamines, prepared by reaction with ammonium bicarbonate were condensed with acyloyl chloride, the product reacted with ozone and the resulting ketone reacted with AEAB (**52**) for glycan array synthesis [254]. This technique, unlike reductive amination, retains the cyclic structure of the reducing-terminal ring.

Other hydrazines and hydrazides used for carbohydrate derivative formation are shown in Table 2 and Scheme 16 (**98**–**101**).

3.5. 1-phenyl-3-methyl-5-pyrazolone (PMP) and related derivatives

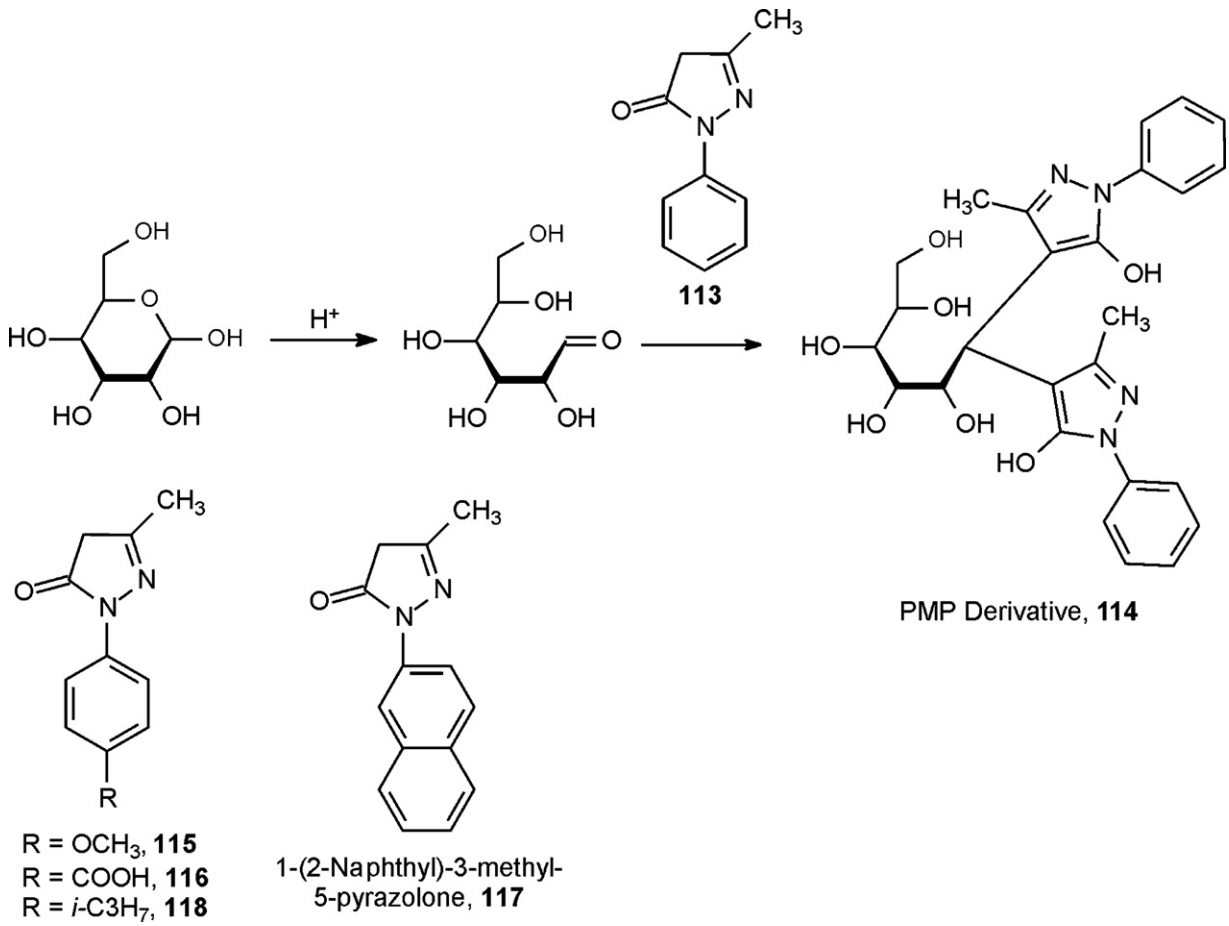
Reaction of reducing carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP, **113**, Scheme 20) has been found to give an almost quantitative yield of a *bis*-derivative (**114**) which strongly absorbs the UV light at 245 nm and which can be detected electrochemically giving detection limits of 1 pmol or 100 fmol, respectively [255]. These popular derivatives give good performance on C18 stationary phases and are suitable for analysis by MALDI MS [256] and ESI [257,258] MS but quantification is said to be less reliable [259]. To prepare the derivative, the sugar was dissolved in 0.3 M aqueous sodium hydroxide, a 0.5 M methanolic solution of PMP was added and the mixture was heated at 70 °C for 2 h. More recently, an "in-capillary" method has been described with a reaction taking 35 min at 57 °C [260]. The reaction has recently been modified by using liquid ammonia instead of sodium hydroxide as the base [261]. The derivatives could be analysed directly by MALDI-TOF without a desalting procedure. Both CE and capillary electrochromatography [262] have been used to analyse these derivatives. In CE, it has been noted that they can be used to separate epimers having the 2,3-*trans* hydroxyl groups from those with 2,3-*cis* hydroxyl groups. The *trans* epimers migrate faster because of intramolecular ring formation by hydrogen bonding between the carbonyl group in a pyrazolone ring and the hydroxyl groups at C-2 and C-3 in the carbohydrate moiety [263]. Resolution in CE has been enhanced by formation of anionic borate complexes in borate buffer. These complexes moved to the cathode in an uncoated fused-silica capillary by the combined effects of electroosmosis and electrophoresis [264].

Related derivatives have been formed with 1-(4-methoxyphenyl)-3-methyl-5-pyrazolone (PMPMP, **115**) [265] and 4-(3-methyl-5-oxo-2-pyrazon-1-yl) benzoic acid (PMPA, **116**) [266]. The methoxy reagent showed higher reactivity towards carbohydrates and both derivatives had a higher UV absorbance

Table 2
Hydrazines and hydrazides used for derivatization of carbohydrates.

Hydrazine/hydrazide	Mass added to carbohydrate[a]	Structure number	Early or representative reference
1-Azido-5-naphthalene sulfonyl hydrazide	245.0371	98	[333]
Benzoylhydrazine		96	[334]
Biotinamidocaproyl hydrazide (BACH)	353.1886	111	[250,252,253]
Biotinylketohydrazine	240.1045	104	[243]
Biotinyl-L-3-(2-naphthyl)-alanine hydrazide (BNAH)	437.1886	112	[251]
4(4'-(*N*,*N*-Dimethylaminophenylazo)-phenylsulfonyl hydrazide	301.0997	105	[335]
(4-Cyanophenyl)-4-piperidinecarbohydrazide	226.1218	101	[336]
Dansylhydrazine	247.0779	95	[239,240,337]
2,4-Dinitrophenylhydrazine	180.0284	93	[237]
N,*N*-Diphenylhydrazine	166.0895	94	[238]
Fmoc-hydrazine	236.0950	99	[338]
p-Hydrazinobenzenesulfonic acid	170.015	110	[249]
2-Hydrazino-(4-phenylpyridyl)-2-oxo-ethanaminium chloride	210.1031	108	[248]
2-Hydrazino-*N*,*N*,*N*-trimethyl-2-oxo-ethanaminium chloride (Girard's T reagent)	114.1031	106	[244]
2-Hydrazino-*N*,*N*,*N*-tripropyl-2-oxo-ethanaminium chloride	198.197	109	[248]
3-Methyl-2-benzothiazolinonehydrazone	161.0412	100	[339,340]
Phenylhydrazine	90.0582	93	[234–236]
Pyrenebutyric acid hydrazide	284.1313	97	[241,242]
Pyridinium ketohydrazine	134.0718	103	[243]

[a] Monoisotopic mass, after hydrazone formation.



Scheme 20. Scheme for the preparation of PMP derivatives and some of the reagents used.

than PMP derivatives. Because all of these derivatives were prepared under relatively mild conditions, they were suitable for the analysis of glycans containing sialic acid. 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP, **117**) is another recently-introduced reagent with high UV absorption and has been used for HPLC [267], LC/MS [268], LC/MS with additional diode-array detection [269] and CE studies [270] of carbohydrates. To improve lipophilicity and recovery of the derivatives, Zhang et al. [271] have synthesised 1-(4-isopropyl)-3-methyl-5-pyrazolone (PPMP, **118**) and used it to analyse 12 monosaccharides by LC/MS.

The separation of PMP, PMPMP and NMP derivatized monosaccharides has been shown to be pH-dependent under reversed phase conditions and acceptable separations can be obtained at pH < 4.5. A recent comparative study [272] of the three derivatives has shown that the elution orders can be rationalized by geometric factors involving the hydroxyl groups at the C2 and

C3 positions of the saccharide moiety. When PMP or PMPMP were used as labeling agents glucose and galactose could be completely separated, while arabinose and xylose were observed to co-elute. On the other hand, NMP could be used to separate arabinose and xylose while glucose and galactose were co-eluted. MS-MS spectra contained characteristic fragment ions resulting from cleavage between the C2–C3 bond (*m*/*z* 373 for PMP derivatives, *m*/*z* 433 for PMPMP derivatives and *m*/*z* 473 for NMP derivatives). Cleavage of the C1–C2 bond to give *m*/*z* 359, 419 and 459 that were characteristic of PMP, PMPMP and NMP derivatives respectively.

3.6. 7-nitro-2,1,3-benzoxadiazole (NBD)-tagged N-methylglycamines

In order to overcome problems with quantification of PMP derivatives and to develop a more sensitive label that would be compatible with retention of sialic acids, Honda et al. [259] have produced 7-nitro-2,1,3-benzoxadiazole (NBD)-tagged *N*-methylglycamines (**120**, Scheme 21). Although the derivatization involved a two-step reaction (reductive *N*-methylamination followed by condensation with 7-nitro-4-fluoro-2,1,3-benzoxadiazole (NBD-F, **119**), they can be performed in a one-pot fashion and gave a quantitative yield within about 50 min. The derivatives exhibited good CE properties and were sensitively detected (attomolar amounts). They were successfully applied to analysis of *N*-glycans from microgram quantities of glycoprotein. Alternatively, the sugar has been reduced with a dimethylamine-borane complex prior to complexation with NBD-F [273].

D-Glucosamine, D-galactosamine and their reduced forms have also been labelled with NBD-F (**119**) at pH 6.0 to produce fluorescent derivatives. After clean-up, the derivatives were purified on a C18 gel plate and analyzed by electrophoresis and argon laser-induced fluorescence detection. Amino sugars could be detected at the 0.5 fmol level with a signal-to-noise (S/N) ratio of 3 [274].

For earlier work on fluorescent derivatives prepared by reactions with amides and guanidine compounds, see the review by Gao et al. [7].

3.7. Naphthimidazole (NAIM) derivatives

A new type of fluorescent derivative (**122**, Scheme 22) that claims to be capable of detecting carbohydrates at less than 1 pmol per sample by MALDI-TOF MS involves the iodine-promoted oxidative condensation reaction of the reducing terminal group with 2,3-diaminonaphthalene (**121**) to form aldo-naphthimidazoles [275]. The reaction proceeds in high yield under mild conditions over six hours and requires no purification other than evaporation of the solvent and residual iodine. It causes little or no decomposition of the sugar and sensitivity was claimed to be higher than that achieved with 2-AB labelling.

4. Derivatives of sugars with naturally-occurring amino groups

The presence of amino groups in sugars, often at the 2-position as, for example, following deacylation of acetylamino-sugars, provides the opportunity to use other chemistries as well as carbonyl condensation reactions to prepare derivatives. Acylation, as for acetylation (above) is possibly the most commonly used method but other methods are available.

4.1. 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) derivatives

Fluorescent derivatives (**124**, Scheme 23) were made from 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA, **123**) in the early 1990s for analysis by capillary electrophoresis/laser-induced fluorescence [276] using the reagent that had been synthesised earlier that year [277]. To prepare the derivatives, the sugars were first reductively aminated and then reacted with the reagent at room temperature in the presence of potassium cyanide. However, although the derivatives were claimed to achieve attomole sensitivity they have not been extensively used for carbohydrate analysis probably because of the use of potassium cyanide.

4.2. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatives

A sensitive and reliable HPLC method with fluorescence detection based on the derivatization of glucosamine with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC, **125**, Scheme 24) has been developed for the quantitative determination of glucosamine in rat plasma with a limit of detection of 30 ng/ml [278]. The derivative (**126**) was prepared by first adding 70 μl of 0.2 M borate buffer (pH 8.8) containing 5 mM disodium EDTA and 15 μl of reconstituted AQC (10 M in acetonitrile) to an aqueous solution of the sugar after protein precipitation from plasma. The samples were vortex mixed and allowed to react for 20 min at 45 °C before analysis by HPLC.

4.3. o-Phthaldialdehyde (OPA) derivatives

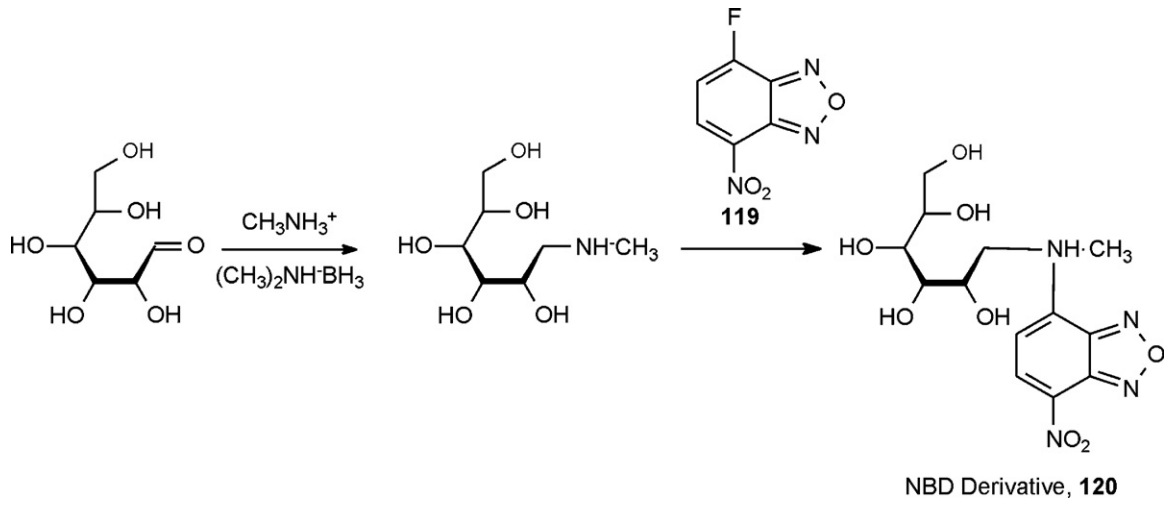
Fluorescent derivatives (**129**) of primary amines are formed by the reaction with phthaldialdehyde (OPA, **127**, Scheme 25) and a thiol such as ethane thiol. The reaction has, for example, been used to derivatize glycosaminoglycans using the thiol 3-mercaptopropionic acid (**128**) [279]. The derivatives gave a quantitative response from glucosamine- and galactosamine-containing glycosaminoglycans (GAGs) with reversed-phase columns and with a sensitivity of 40 fmol. Altmann [280] has used the reaction to analyse glycosylamines derived from *N*-linked glycans by acid hydrolysis. For more information on this reaction, see the review by Kutlán et al. [281].

4.4. Phenylisothiocarbamyl (PTC) derivatives

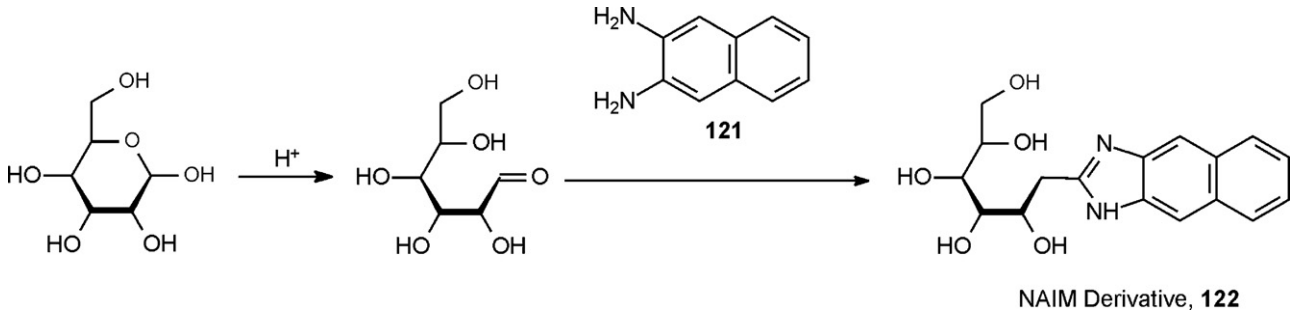
Glycosylamines and sugar alcohols can be converted into phenylisothiocarbamyl (PTC) derivatives (**131**, Scheme 26) by reaction with phenylisothiocyanate (**130**) and the reaction is frequently used to analyse these compounds by HPLC. The reaction is rapid (20 min) and is achieved by mixing the reactants and methanol:triethylamine and allowing them to stand at room temperature. The excess of reagents can then be remove by chloroform extraction [282]. The reaction has been used, for example, to examine glycosylamines obtained from *N*-glycans by alkaline borohydride treatment [280].

5. Derivatives for stabilization of sialic acids in MALDI MS

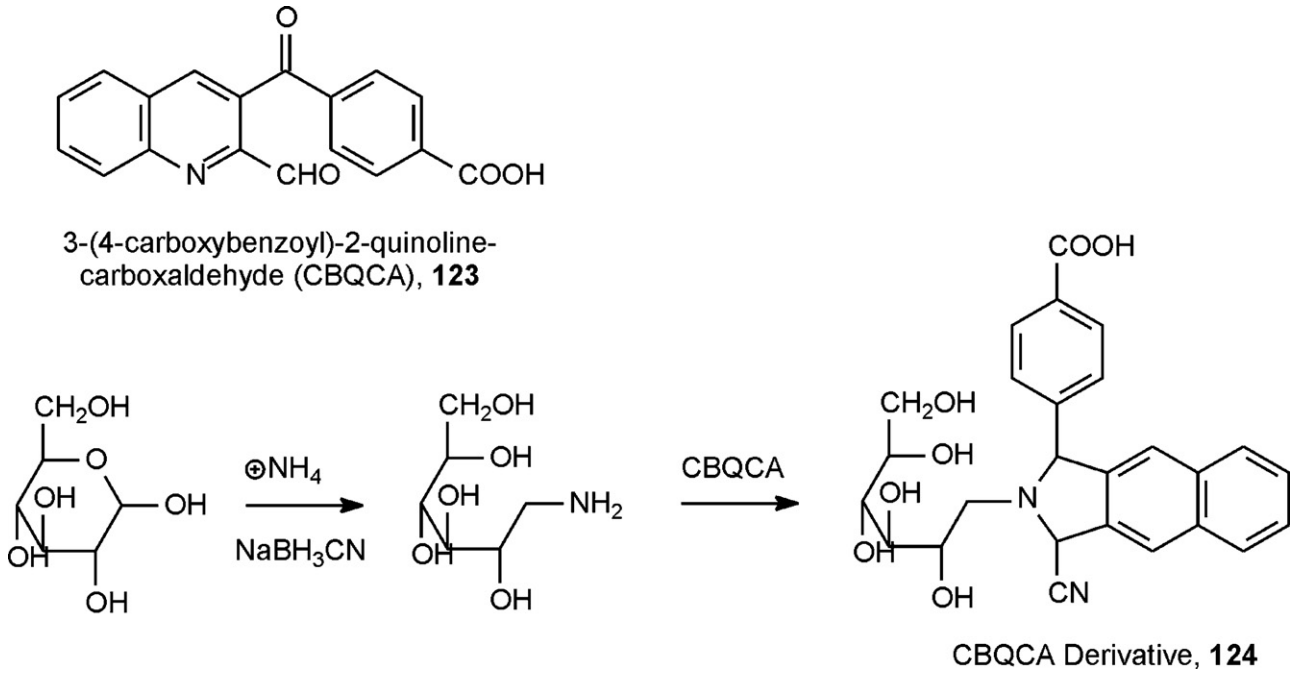
Sialic acids are notoriously unstable under MALDI MS conditions and readily eliminate the sialic acid moiety. This reaction is the result of the labile acidic proton and thus, blocking this reaction by salt formation or derivatization of the acid stabilizes the compounds. Methyl ester formation is a common technique for achieving this stabilization and was introduced by Powell



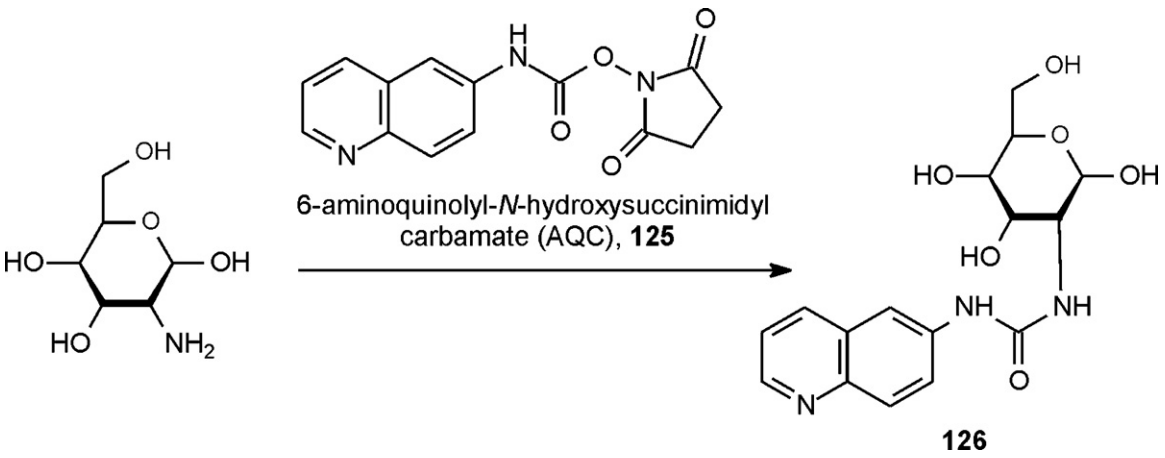
Scheme 21. Scheme for the preparation of NBD derivatives.



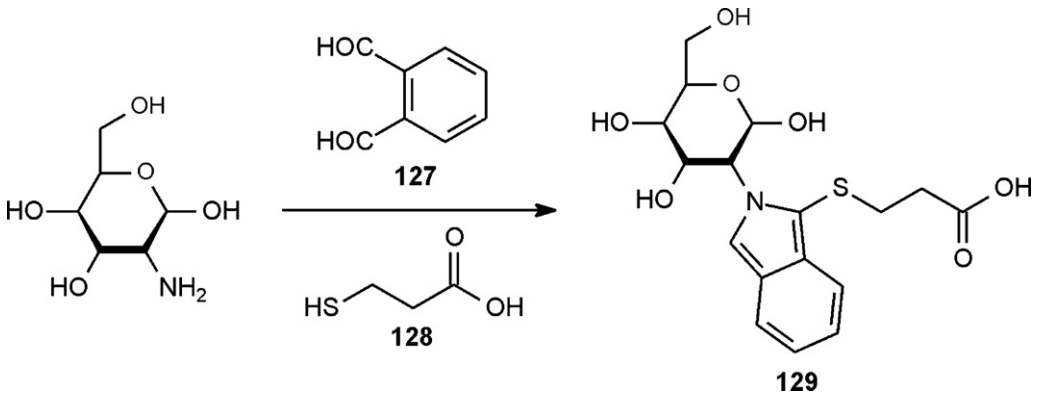
Scheme 22. Scheme for the preparation of NAIM derivatives.



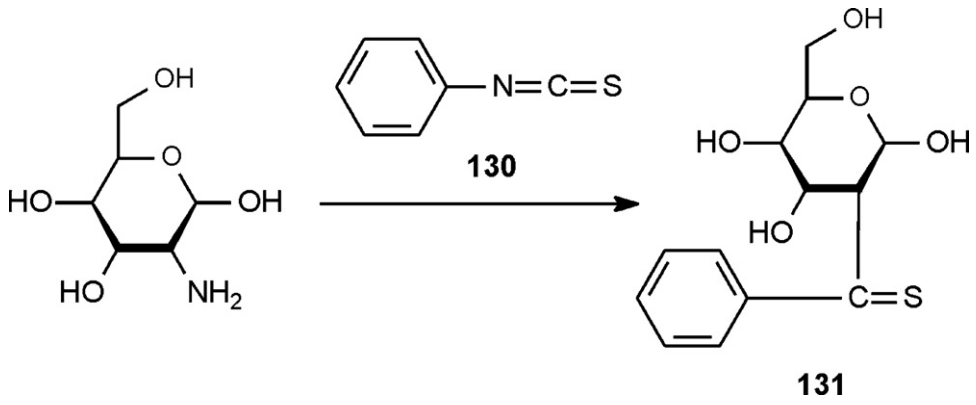
Scheme 23. Scheme for the preparation of CBQCA derivatives.



Scheme 24. Scheme for the preparation of AQCderivatives.



Scheme 25. Scheme for the preparation of *o*-phthaldehyde derivatives.



Scheme 26. Scheme for the preparation of PTC derivatives.

and Harvey for *N*-glycans and gangliosides in 1996 [283]. Reaction was achieved by conversion of the acid to its sodium salt with an AG50 column that had been equilibrated with sodium hydroxide, followed by reaction with methyl iodide. The reaction produced a high yield of methyl esters that were stable under MALDI conditions (Fig. 3a) and has subsequently been used by several investigators [284,285]. Liu et al. [284] have methylated sialylated glycans obtained by pronase E digestion of glycoproteins. This proteolysis reaction leaves asparagine attached to the carbohydrate, which, in the presence of the methyl iodide is converted into its trimethyl quaternary ammonium salt. The acid of the asparagine residue is simultaneously converted into its methyl ester. The reaction was reported to increase the detection limit by ten-fold and was applied to glycans from ribonuclease B, ovalbumin and transferrin.

Attempted formation of methyl esters of acidic carbohydrates with diazomethane in the presence of water has generally been unsuccessful. However, glycolipids and glycoproteins are sufficiently lipophilic to allow organic solvents to be used with success. Thus, MacDonald et al. [286] successfully formed methyl esters of gangliosides by reaction with diazomethane in methanol:ether. To achieve a quantitative reaction, the gangliosides were first passed through a Dowex AG-50 (H^+ form) column after which the reaction was rapid and could be completed in under an hour. Amano et al.

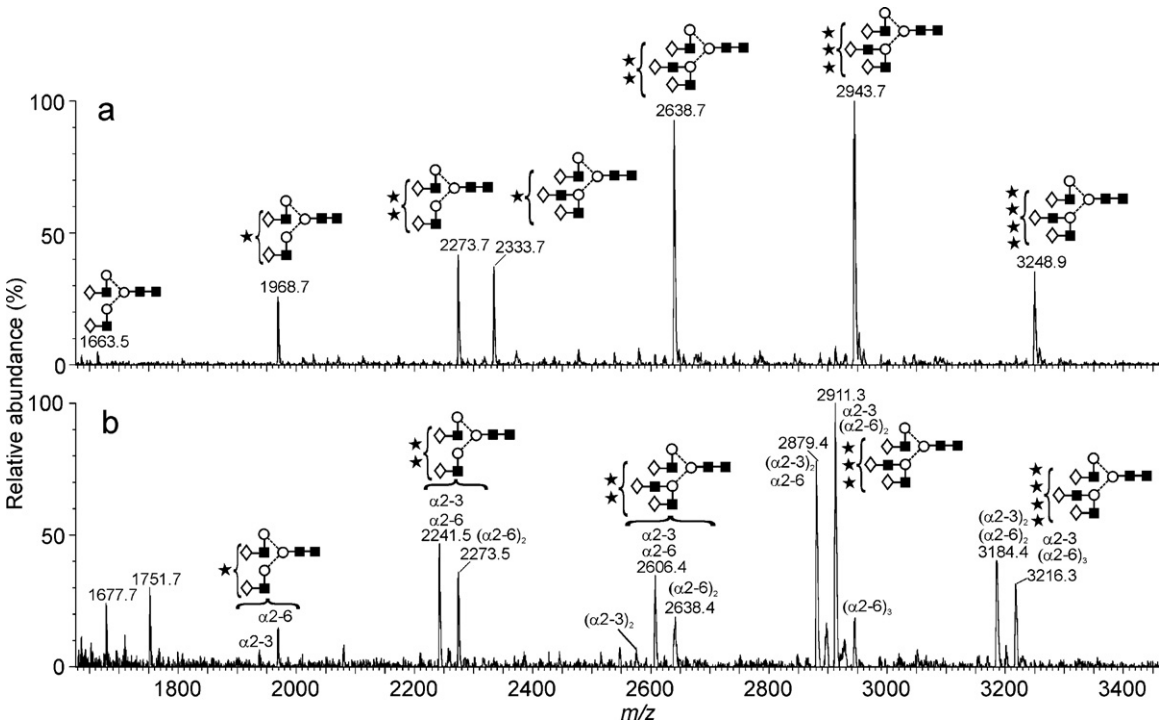


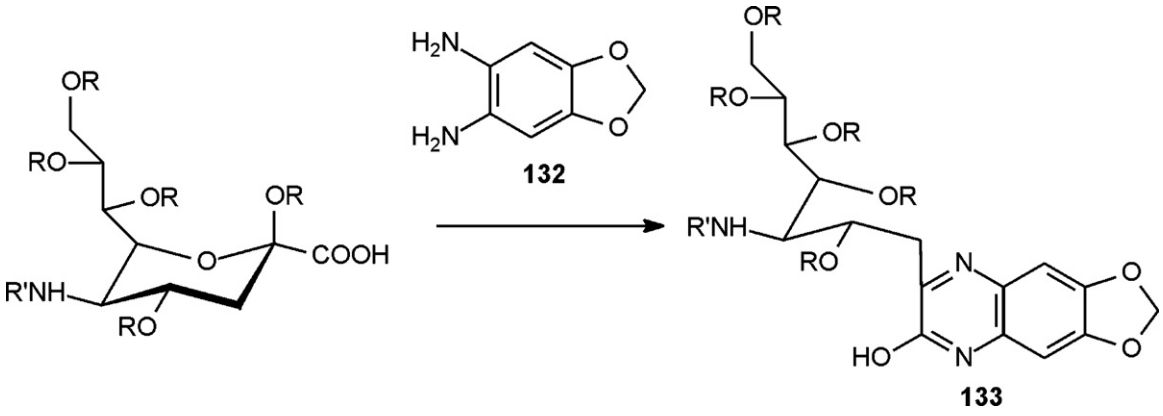
Fig. 3. Positive ion MALDI-TOF mass spectra of *N*-linked glycans from bovine fetuin after derivatization with (a) methyl iodide and (b) methanol/DMT-MM. The symbols for the structural formulae are defined in the legend to Fig. 1.

[287] have recently reacted glycoproteins containing sialic acid with 1-pyrenyldiazomethane (PDAM) and found greatly improved detection limits of the products in the presence of peptides. Use of DHB caused loss of the ester group allowing the free sialic acid to be observed whereas the intact ester was ionized with the ionic liquid matrix 3AQ/α-cyano-4-hydroxycinnamic acid (CHCA).

The triazene derivative, 3-methyl-1-*p*-tolyltriazene (MMT) has recently been used to prepare methyl esters from *N*-glycans. The reaction was originally performed in solution (DMSO:acetonitrile) but then migrated to a solid-phase platform [288]. Methyl esters have also been prepared by reaction with methanol catalysed by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) [289] but, in this case, only α-(2→6)-linked sialic acids react in this way. The α-(2→3)-linked acids formed lactones and, because the mass of these compounds is 32 units below that of the α-(2→6)-linked acids, the linkage can easily be determined by MALDI MS as shown in Fig. 3b. Negative ion fragmentation of the methyl esters is dominated by ions produced by elimination of methanol but the negative ion spectra of *N*-glycans still provide much structural information. This catalyst has also been used to convert the COOH group of sialic acids to amides by use of ammonium hydroxide [290]. Although the sialic acid is stabilised, the amide only produces a mass shift of one unit.

A recent preparation of methyl amides can overcome this problem. Thus Liu et al. [291] have reacted sialylated glycans with methylamine in the presence of (7-azabenzotriazol-1-yloxy) trispyrrolidinophosphonium hexafluorophosphate (PyAOP) and reported quantitative yields of the methyl amides from both α2→3- and α2→6-linked sialic acids. The derivatives were compatible with both ESI and MALDI-TOF MS.

Lactone formation was also used by Galuska et al. [292] to stabilize polysialic acids for analysis by MALDI-TOF MS. Lactonization was achieved with TFA and *o*-phosphoric acid and, of several matrices tested, 6-aza-2-thiothymine (ATT) proved to be the most satisfactory with DHB being nearly as good. α-(2→8)-linked sialic acids reacted readily under these conditions, but α-(2→9)-linked



Scheme 27. Scheme for the preparation of DMB derivatives of sialic acids.

acids were much more reluctant to form lactones, thus providing a method for differentiating the two linkages. When sialic acids were α-(2→8)-linked, cyclisation occurred with the hydroxyl group at C-9 of an adjoining sialic acid residue, whereas with α-(2→9)-linked poly-sialic acids, cyclisation was with the hydroxyl group at C-8. Good signal-to noise ratios were obtained with masses as high as 10,000 Da and polymers with up to 100 residues could be examined.

Because of difficulties that have been noted in methyl ester or amide formation from the acid group of α2→3-linked sialic acids, Toyoda et al. [293] have investigated alternative methods of derivatizing these groups and have produced a method using aceto-hydrazide derivatization. The reaction appeared to be quantitative with both α2→3- and α2→6-linked sialic acids as demonstrated with *N*-glycans released from bovine fetuin, a glycoprotein containing sialic acids in both linkages. The method was, however, not appropriate for glycans with an intact reducing terminus because the reagent also reacts with aldehydes. Thus, the glycans had first to be derivatized at this terminus, in this case with 2-AP, after which the reaction worked well.

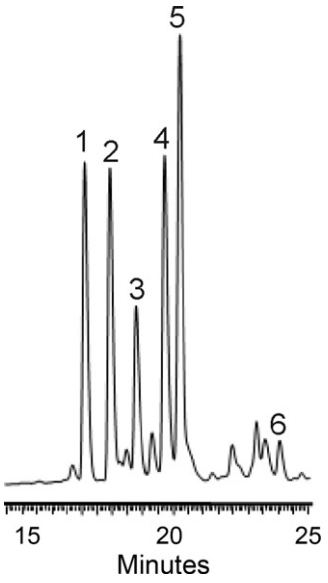
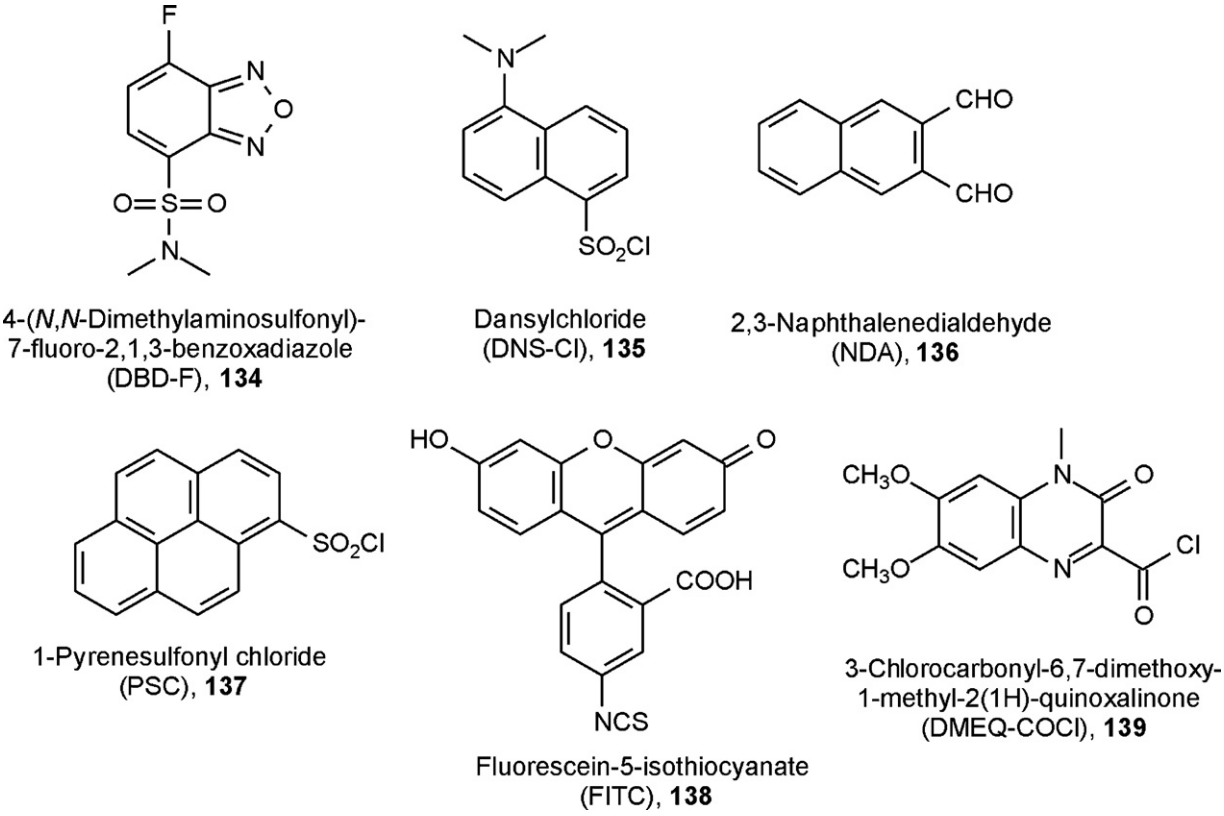


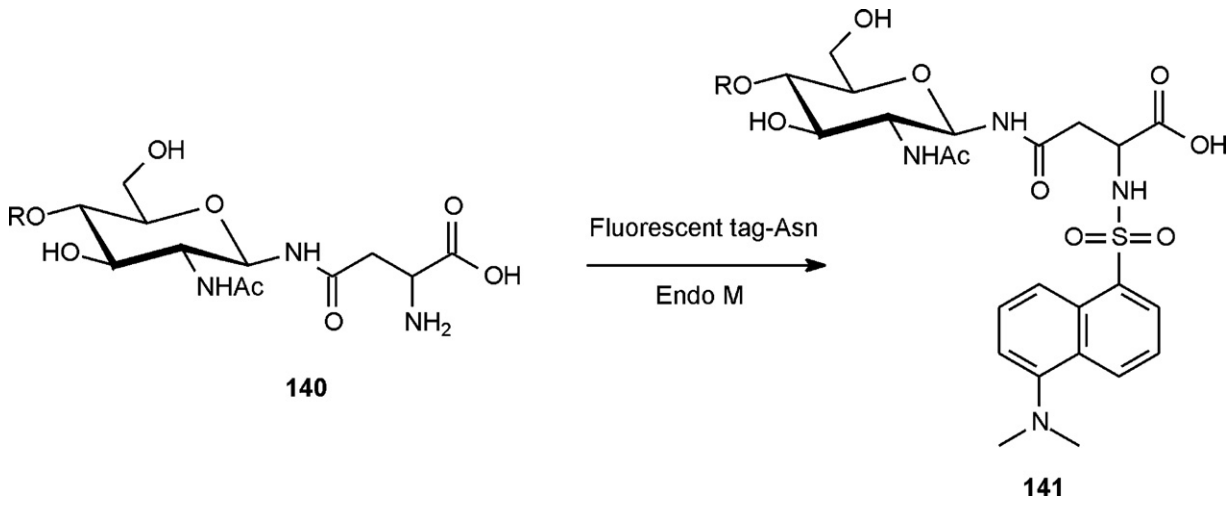
Fig. 4. HPLC separation of PMP derivatives of neuraminic acids. Peak identification—**1** *N*-glycolylneuraminic acid, **2** *N*-acetylneuraminic acid, **3** 5,7-diacetylneuraminic acid, **4** 5-glycolyl-9-acetylneuraminic acid, **5** 5,9-diacetylneuraminic acid and **6** 5-7/8-9-triacetyl neuraminic acid.

6. 1,2-Diamino-4,5-methylenedioxybenzene (DMB) derivatives of sialic acids

The α-keto-carboxy group of sialic acids has been reacted with 1,2-diamino-4,5-methylenedioxybenzene (DMB, **132**, Scheme 27) in dilute acetic acid to form fluorescent (λ_{exc}: 373 nm, λ_{em}: 448 nm) quinoxaline derivatives **133** [294] that have proved very useful for separations of different sialic acids by HPLC, ESI [295] and MALDI [296] MS. Although sialic acids are known to migrate under certain conditions, the reaction did not appear to be susceptible to this problem. The reagent is sold in kit form by Ludger Ltd. under the LudgerTag™ brand. Using these derivatives Klein et al. [297] have identified as many as 28 sialic acids by LC/MS from the glycolipids of the sea urchin *Lovenia cordiformis*). An example of the separations that can be achieved is shown in Fig. 4. The method replaced an earlier one using the fluorogenic reagent 1,2-diamino-4,5-dimethoxybenzene (DDB) which, although also forming a derivative from α-keto acids, produced two peaks. The standard procedure for DMB labeling at 50 °C for 2 h results in significant hydrolysis of oligomers of α2→8-linked-Neu5Ac [298]. Changing the conditions to 10 °C for 48 h in 0.02 M TFA produced a



Scheme 28. Compounds used for the preparation of derivatives of asparaginyl carbohydrates.



Scheme 29. Synthesis of carbohydrate derivatives by transglycosylation reactions from Asn-tagged molecules.

compromise between the minimum degradation of the oligomers and a rapid rate of derivatization [299]. Oligomers with a degree of polymerization of 19–28 have been recovered with a yield of 80% after prolonged treatment at 10 °C.

7. Derivatives of asparaginyl carbohydrates

N-glycans are sometimes examined as asparaginyl derivatives after hydrolysis of the protein chain of their parent glycoproteins with pronase. Asparagine contains both carboxy and amino groups that can be targets for further derivatization to impart chromophores or fluorophores. Eight fluorescence reagents, (4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F, **134**, Scheme 28), NBD-F (**119**), dansylchloride (DNS-Cl, **135**), 2,3-naphthalenedialdehyde (NDA, **136**), 1-pyrenesulfonyl chloride (PSC, **137**), fluorescein-5-isothiocyanate (FITC, **137**), Fmoc-Cl (**76**), and 3-chlorocarbonyl-6,7-dimethoxy-1-methyl-2(*1H*)-quinoxalinone (DMEQ-COCl), **139**), which are reactive to amino functional groups, have been examined by Kurihara et al. [300] and have given fmolar sensitivity. To explore the utility of the derivatives, glycans released from chicken ovalbumin were labelled with PSC and examined by UPLC-ESITOF-MS; Fifteen *N*-glycans were detected. This derivative was later used by the same group to extend the analysis to other *N*-linked glycans [301,302].

An enzyme-catalysed transglycosylation reaction has been developed to label *N*-linked glycans with a series of fluorescent dyes such as DNS, DBD, NBD and FITC (see above). The dyes were attached to the amino group of GlcNAc-Asn and the transglycosylation reaction was performed using Endo M isolated from *Mucor hiemalis* to produce the target tagged asparaginyl-glycan (**141**, Scheme 29). Analysis was by C18 HPLS and LC/MS [303,304]. In further work, the fluorescent oligosaccharides were isolated from the non-fluorescent oligosaccharides with fluorescence detection by 2D-HPLC. The first dimension involved an amide-80 column and the second was an octadecylsilane (ODS) column. Detection was by ESI-TOF-MS [305].

8. Conclusions

It is not possible, in a review of this size, to cover all aspects of carbohydrate derivatization but it is hoped that examples of most of the major methods are covered. Although a large number of techniques have been described, only a few may be regarded as standard in this area. Many of the older methods for monosaccharides have given way to newer methods that are more appropriate for the new instrumental techniques that have been introduced over the last two decades. However, it should be remembered that techniques such as the soft ionization methods (ESI, MALDI) in MS may not necessarily be better than the older, mature techniques such as GC/MS. Indeed, GC/MS is still a vital method for aspects such as linkage determination and for identification of constituent monosaccharides in poly- and oligo-meric compounds. Some aspects of derivatization, such as permethylation, originally developed for gas phase techniques, have found application in newer methods such as FAB and MALDI-TOF MS whereas some others are now rarely used. Emphasis at present is largely in areas such as liquid chromatography and LC/MS where reducing-terminal derivatization is most appropriate. Of the various derivatives in this area the 2-AB, 2-AA and 2-AP fluorescent tags appear to be the most widely used. Development of new derivatization methods shows no sign of slowing and it is expected that many more novel techniques will be developed over the coming years.

Acknowledgements

I thank Professor Raymond A. Dwek, Director of the Oxford Glycobiology Institute for his help and encouragement.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.11.010.

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