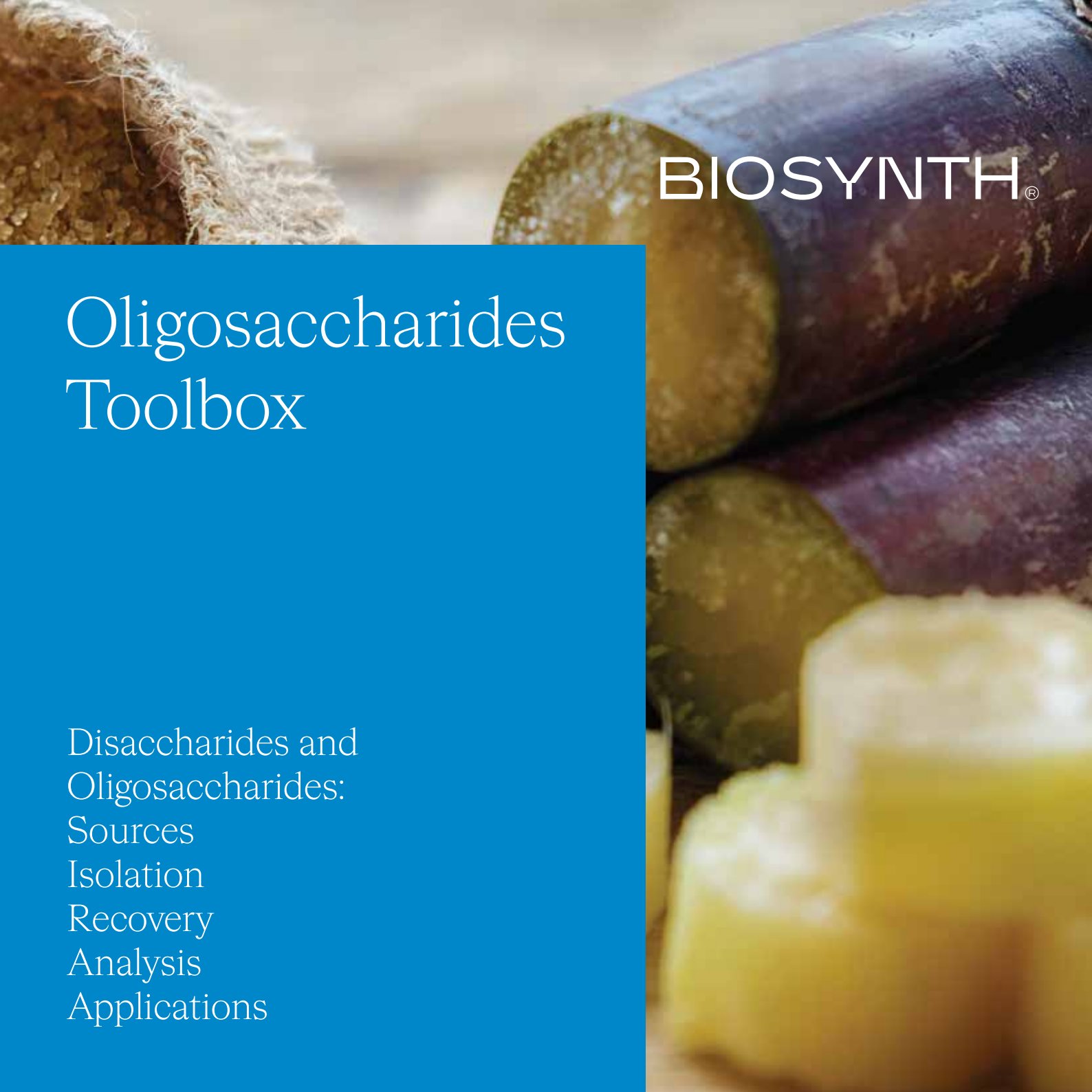
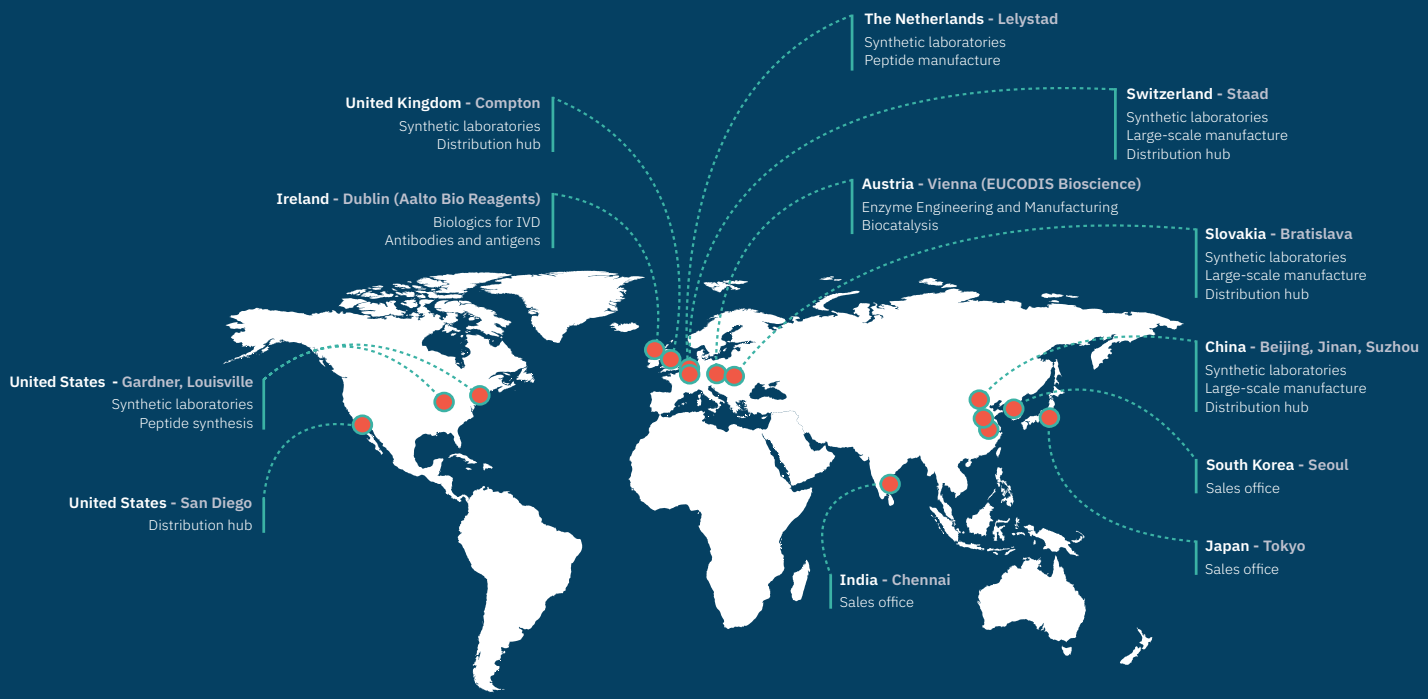


BIOSYNTH®

Oligosaccharides Toolbox

Disaccharides and Oligosaccharides:
Sources
Isolation
Recovery
Analysis
Applications

Global Reach



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About Biosynth

Biosynth is an innovative life sciences reagents, custom synthesis and manufacturing services company. We are by scientists, for scientists, securing supply chains with consistent quality, across the globe. We manufacture and source a vast range of chemical and biochemical products, and take pride in delivering products that others cannot. We are experts in complex chemistry, peptides and key biological raw materials. We provide a full range of products and services to support life science research and development, with more than half a million products in our research catalog and hundreds of complex manufacturing service projects. Our complex chemistry specialties include enzyme substrates, carbohydrate and nucleoside chemistry, with manufacturing services from the first idea to the finished product, from route scouting to GMP or ton scale production. For peptides, we also have a full end-to-end offering, from lead discovery and optimization, library production, through to GMP NCE or Neoantigen projects.

Biosynth's mission is to be the leading life sciences platform for scientists developing revolutionary medicines and diagnostics.

The trusted supplier, manufacturer and partner for the pharmaceutical, life science and diagnostic sectors, along with customers across food, agrochemistry and cosmetics, we have facilities across three continents and a rapid global distribution network. Our main chemical research and manufacturing laboratories are in Switzerland, the United Kingdom, Slovakia and China, with peptide production in the USA and the Netherlands. Enzyme projects are based in Austria and biological IVD reagents in Ireland. Our R&D resources and production facilities are modern and versatile, allowing us to produce chemicals on the milligram to ton scale, and at ISO 9001 and GMP, with peptides at mg to multikilogram scale.

Four Areas of Focus

Research Products	Complex Chemicals	Quality Peptides	Key Biologics
• Over 500,000 Products • Small Scale • Rapid Distribution • Retail Chemicals	• Custom Synthesis • Optimization • Large Scale Production • GMP Manufacture	• Custom Synthesis • Neoantigen Peptides • GMP Production • End to End Services	• Custom Antibodies • Reagents for IVD • Enzyme Development • Epitope Mapping

Development of Innovative New Products

Chemical Manufacturing Capabilities

- Custom Synthesis
- Large-Scale Manufacturing
- GMP Manufacturing
- Quality Control and Quality Assurance
- CDMO Services
- Custom Filling and Packaging
- Logistics and Warehousing
- Sourcing

Biosynth History

Biosynth was founded in 1966 by Hans Spitz, and has grown to a global products and services business. Biosynth merged with Carbosynth in 2019 to form Biosynth Carbosynth, creating a world leader in carbohydrates, nucleosides and enzyme substrates. In 2022 Biosynth Group acquired both vivitide and Pepscan, which strengthened the offering in peptide manufacturing services and catalog products. Also in 2022 Biosynth Group acquired Aalto Bio Reagents, expanding the range of biological products and key raw materials for clinical diagnotics including antigens and antibodies, as well as EUCODIS Bioscience, experts in Enzyme development. Following these acquisitons, the company returned to the Biosynth brand. Biosynth is owned by KKR, Ampersand and senior management.

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1 Introduction

Oligosaccharides are defined as short polymers of monosaccharides with three to ten sugar residues (IUPAC). In addition, we have also included in this review disaccharides, the result of two monosaccharide residues linked by a single glycosidic bond.

Oligosaccharides may be linear or branched and consist predominantly of hexoses or pentoses individually or in mixtures. Other monosaccharides may be present including uronic acids, sialic acids and anhydro sugars. The linkages found include 1,6-, 1,4-, 1,3-, 1,2- and 1,1-glycosidic linkages, and the resulting new stereocentre at the anomeric position leads to either an α- or a β-glycoside. In the case of the sialic acids, a family of acidic ketoses (including neuraminic acid, KDN and KDO), the linkages can also be 2,3-, 2,6- or 2,8. Moreover, a given oligosaccharide can be further individualised by carrying substituents such as sulfate, pyruvate, methyl and phosphate groups.

All these variables lead to a structural diversity of oligosaccharides that is much greater than that of oligopeptides or oligonucleotides. The large number of different sugar monomers aside, already for a single monomer such as the common D-glucose, these differences in anomeric configuration, ring size and the various attachment points give rise to a variety of possible disaccharides. Three different monosaccharides, pyranose forms only, can form 1056 different trisaccharides whilst only 6 different tripeptides can be formed from 3 different α-amino acids (sidechain reactions aside). Biologically relevant oligosaccharides are typically larger than trisaccharides; for instance, the number of possible hexasaccharides was calculated to be in the range of a trillion (Laine 1994).

Apart from the highly significant natural properties of mammalian oligosaccharides in both health and disease in what has been termed 'the sugar code' (embryonic evolution, cell-cell recognition, disease progression, defining blood groups etc) (Hennet 2009), oligosaccharides have emerged as the third alphabet of life (amino acids and the nucleotide bases are the first two alphabets of life) (Özdemir 2020).

Most oligosaccharides do not occur as free entities in nature; examples are blood group determinants, *N*- and *O*-linked glycans, which are covalently linked to proteins and lipids. However, there are important exceptions of free oligosaccharides including sucrose, lactose, naturally occurring glycosides and the oligosaccharides in human milk.

Polysaccharides are an important source of oligosaccharides via partial degradation with chemicals and enzymes. Finally, chemical, chemoenzymatic and enzymatic modifications and fermentation processes play a highly important role in the production of oligosaccharides.

Oligosaccharides are of great value in the food and pharmaceutical industries, for example as drug components and active fermentation substrates, surfactants, food ingredients, probiotics, excipients, emulsifiers and acidulants.

In this toolbox, oligosaccharides are examined in terms of sources, isolation, recovery and analysis. Their many applications in foods, pharmaceuticals, cosmetics and industry will be discussed together with their significance in biomedical research. Finally, the changes that occur to oligosaccharides in the diagnosis and progression of disease will be reviewed.

B

Section 2
Sources

2 Sources

2.1 Introduction

Oligosaccharides are obtained from a variety of sources as many occur naturally free and unattached such as native sugars sucrose and lactose, or occur as attached oligosaccharides, where aglycones such as steroids, saponins and terpenes are covalently attached to the carbohydrate structure.

Many oligosaccharides (glycans) are attached to proteins through *N*- or *O*- links via amino acid residues on the protein. Lipids can be functionalised with carbohydrates to form glycolipids. Polysaccharides are masked sources of oligosaccharides that can be released by selective degradation and other key sources are chemical, enzymatic or chemoenzymatic synthesis.

2.2 Free in nature, no aglycone

There are many di- and oligosaccharides that occur freely in nature.

Sucrose, (α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) (fig 1) is found in plants such as sugar cane and sugar beet (Nicol 1982), and in honey.

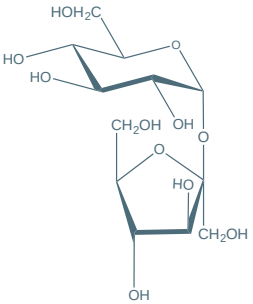


Fig 1 Sucrose

Fig 2 Granulated sugar (21st century)

Fig 3 Sugar cone (19th century)

Raffinose (fig 4) is a sucrose analogue that also occurs in sugar cane. Also **kestoses** (fig 5) (glucose-fructose oligosaccharides, e.g. 1-kestose) occur in sugar cane.

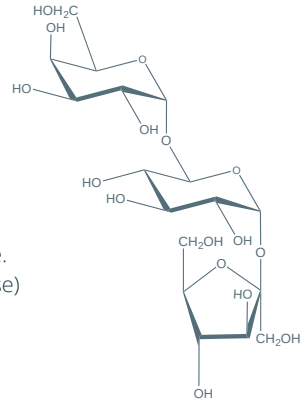


Fig 4 Raffinose

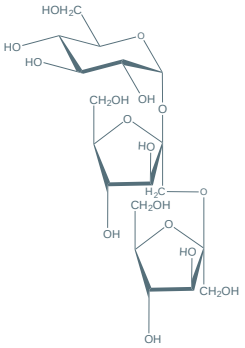


Fig 5 1-Kestose

In honey, while the major carbohydrate components are **glucose** and **fructose** (~70%) and **maltose** (4-O-α-D-glucopyranosyl-D-glucose) (7.5%) a variety of other oligosaccharides are found including **kojiobiose** (2-O-α-D-glucopyranosyl-D-glucose), **turanose** (α-D-glucopyranosyl (1,3)-α-D-fructofuranoside), **erlose** (α-maltoside-β-D-fructofuranoside), **panose** (O-α-D-glucopyranosyl (1,6)-O-α-D-glucopyranosyl (1,4)-D-glucose) and **nigerose** (3-O-α-D-glucopyranosyl-D-glucose) (~3%), (fig 6) (White 1975).

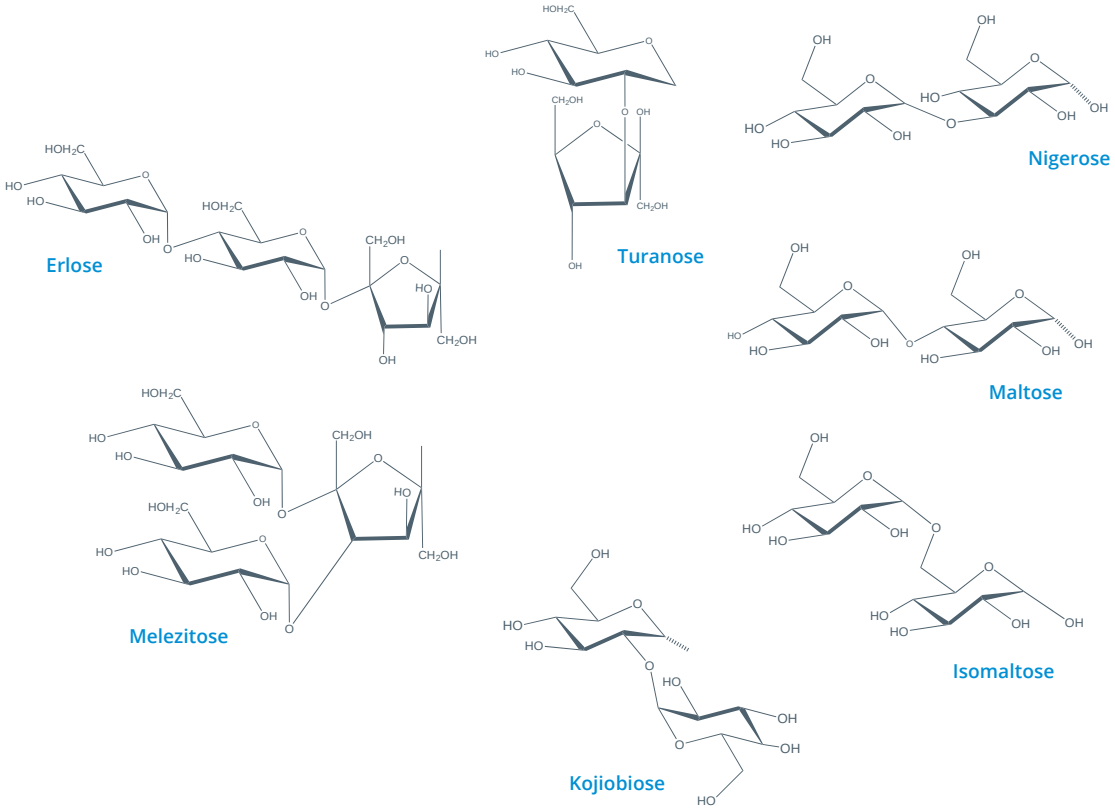


Fig 6 The oligosaccharides in honey

Trehalose (fig 7), (α-D-Glucopyranosyl-α-D-glucopyranoside) is another non-reducing disaccharide, similar to sucrose but with the fructose moiety replaced by glucose (Mathlouthi 1995). Trehalose occurs naturally in insects and plants and is used as a cryoprotectant for sensitive molecules such as enzymes against various environmental stress conditions, including heat, freezing, reactive oxygen species and drought (Elbein 2003).

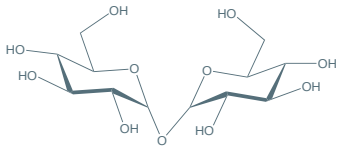


Fig 7 Trehalose

Another source of freely occurring oligosaccharides is human and animal milk. The major sugar in all milks of animal origin is the highly crystalline disaccharide **lactose** (fig 8 and fig 9).

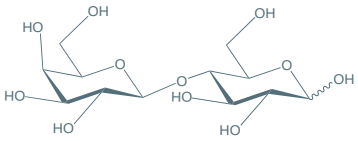


Fig 8 Lactose (4-O-β-D-Galactopyranosyl-D-glucopyranose)

Fig 9 Lactose crystal taken with birefringent light

Milks of animal origin contain approximately 7% of lactose by weight, which is a major source of nutrition. However, in human milk there are at least 200 complex di- and oligosaccharides that are regarded as conferring microbiome support and immunostimulatory properties to newborn infants. These oligosaccharides range from lactose plus a third sugar residue such as 2'fucosyllactose (fig 10) through tetrasaccharides incorporating sialic acid (for example sialylfucosyl-*N*-tetraose, fig 11), to highly complex oligosaccharides containing six or more sugar residues (Urashima 2011).

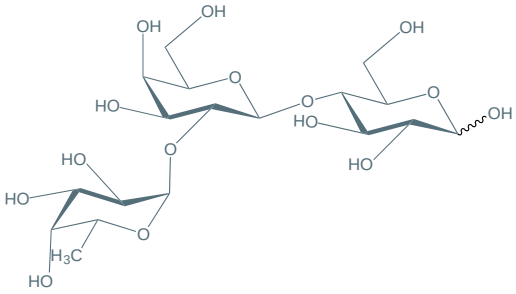


Fig 10 2'fucosyllactose

Fig 12 Human milk

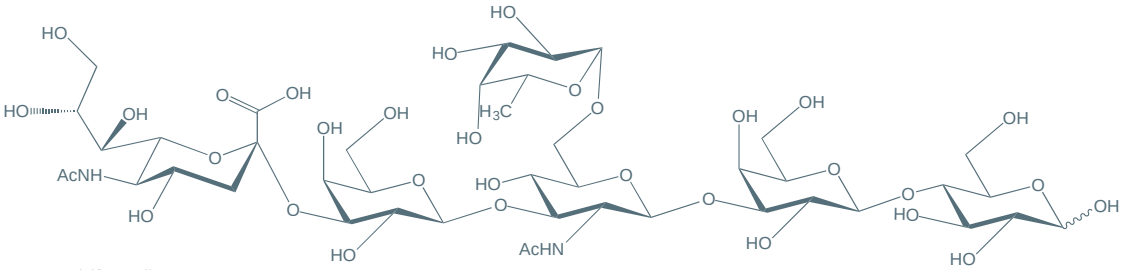


Fig 11 Sialylfucosyllacto-*N*-tetraose

Many plants contain natural products that include glycosides of terpenes, saponins and steroids. These include the high intensity sweeteners **stevioside** (fig 13) (Geuns 2003) and the sweetness inhibitor **ziziphin** (fig 14) found in *Ziziphus jujuba* (*Rhamnaceae*) (fig 15) (Mahajan 2009).

Fig 15 *Ziziphus jujuba (Rhamnaceae)*

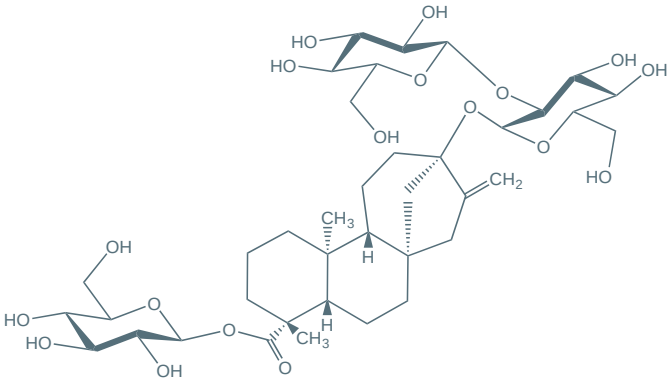


Fig 13 Stevia glycoside

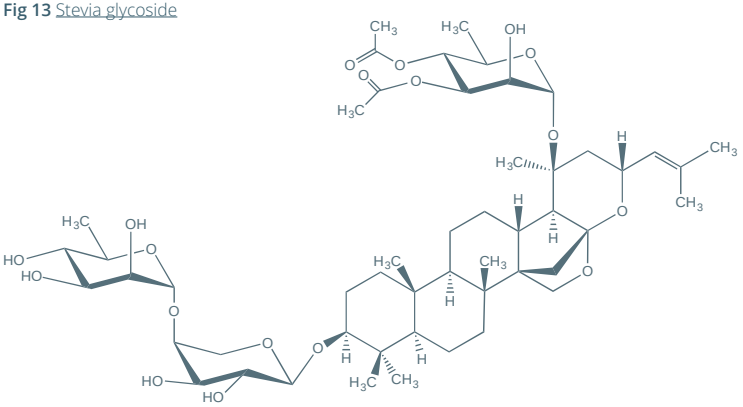


Fig 14 Ziziphin

Aescin (fig 16) is a mixture of saponin glycosides found in the seed of the horse chestnut tree, *Aesculus hipocastanum* (fig 17). Aescin is built up from the aglycon *protoescigenin* acetylated at C22 and a trisaccharide with glucuronic acid and two glucose moieties. The two aglycones differ in that one has the hydroxy group at C21 esterified with angelic acid and the other with tiglic acid. The therapeutic use is in the treatment of peripheral vascular disorders.

Fig 17 Horse chestnut

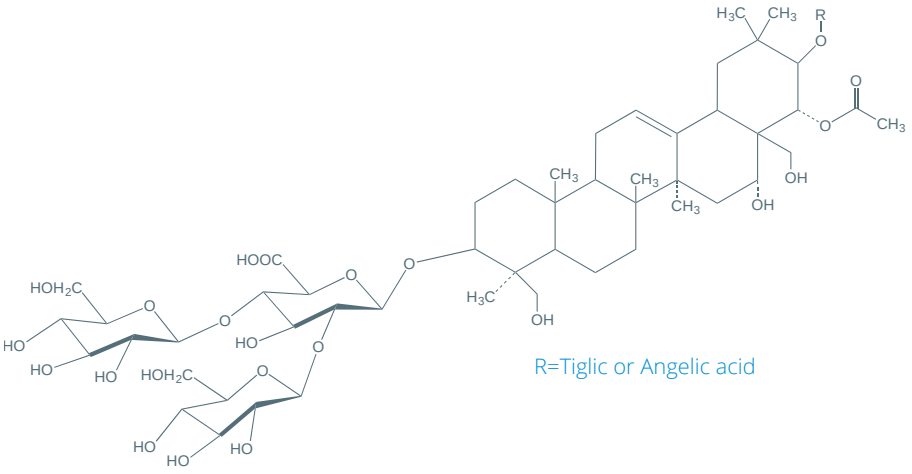


Fig 16 Aescin

Other members of this group include many other traditional remedies such as the memory loss therapeutic, **isoacteoside** (fig 18) (Young-Ji 2017). This product is found in a number of plant species including Ribwort Plantain (*Plantago lanceolate*, fig 19) and has been synthesised by chemical methods.

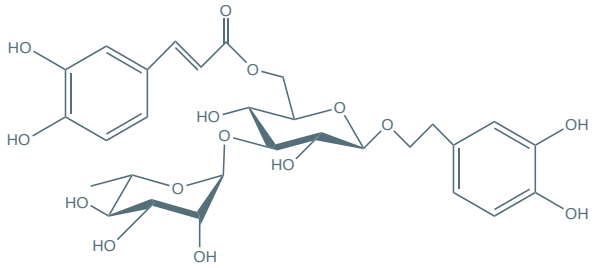


Fig 18 Isoacteoside

Fig 19 Ribwort plantain (*Plantago lanceolata*)

Neomycin, one of the many oligosaccharide containing antibiotics, is an aminoglycoside antibiotic that has activity against Gram-negative bacteria and is partially effective against Gram-positive bacteria.

Neomycin is a mixture of neomycin B (framycetin) and its epimer neomycin C, the latter component accounting for some 5–15% of the mixture. It is typically used as a topical preparation, such as Neosporin (neomycin/polymyxin B/bacitracin) but it can also be given orally, where it is usually combined with other antibiotics. Neomycin is not absorbed from the gastrointestinal tract and has been used as a preventive measure for hepatic encephalopathy and hypercholesterolemia. By killing bacteria in the intestinal tract, it keeps ammonia levels low and prevents hepatic encephalopathy, especially prior to GI surgery (Mouton 2017).

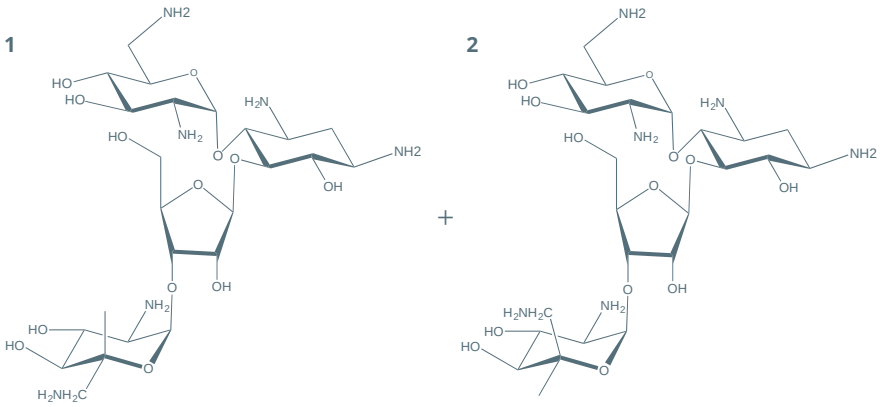


Fig 19a Structures of neomycin C **(1)** and neomycin B **(2)**

The glycoalkaloids **solamargine** and **solasonine** (fig 20 and fig 21) extracted from the fruit of *Solanum Sodomeaum* (The Devil's Apple, fig 22) have activity against squamous cell carcinomas (Friedman 1999, Jiang 2016).

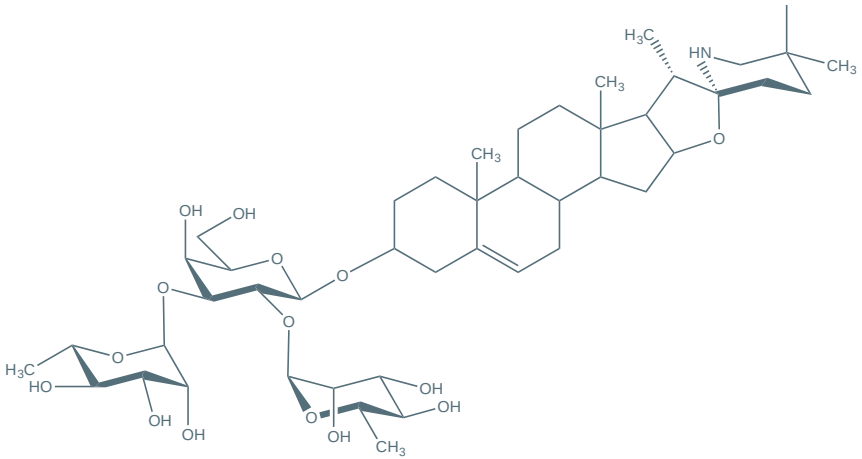


Fig 20 Solamargine

Fig 22 *Solanum Sodomeaum* (The Devil's Apple)

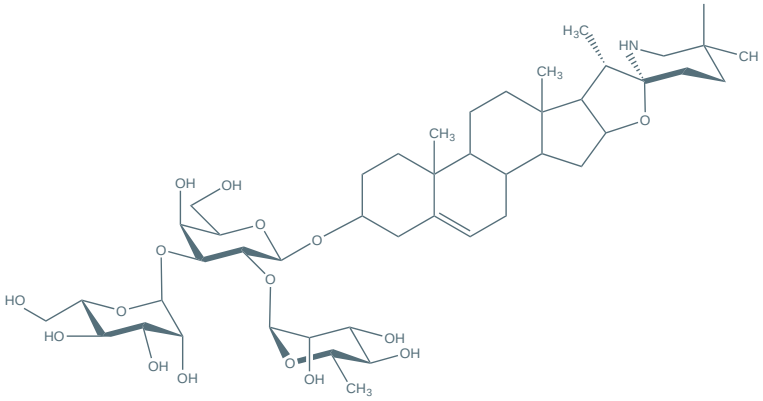


Fig 21 Solasonine

The last example is **digoxin** (fig 23), the heart drug from *Digitalis purpurea* (common foxglove, fig 24) (Virgadamo 2015).

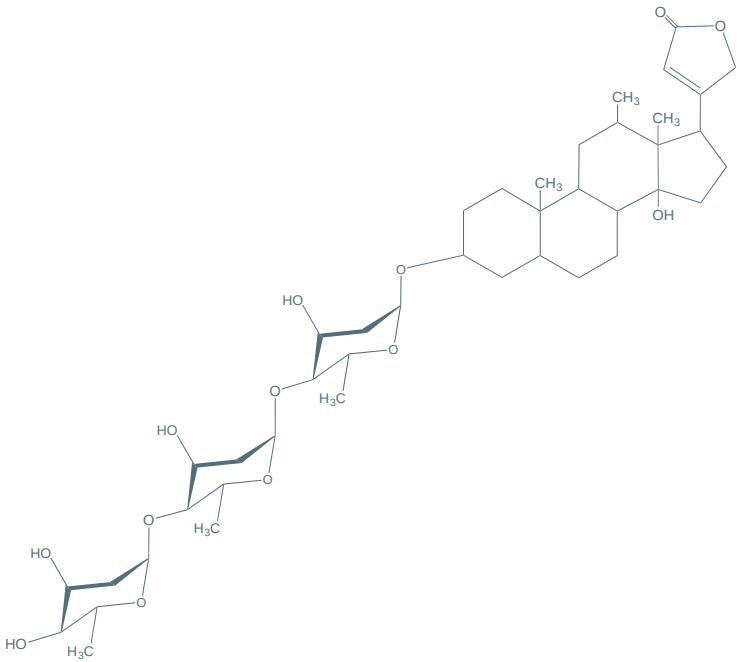


Fig 23 Digoxin

Fig 24 *Digitalis purpurea* (common foxglove)

2.4 Covalently attached to proteins (glycoproteins)

2.4.1 N-linked glycosylation

N-glycans are covalently attached to proteins by N-glycosidic bonds via an asparagine residue. Although there are five different N-glycan linkages known, N-acetylglucosamine to asparagine (GlcNAcβ1-Asn) is the most commonly occurring (Stanley 2009).

N-glycosylation influences properties of glycoproteins including their conformation, solubility, antigenicity and recognition by glycan-binding proteins. In analytical cell biology techniques, N-glycans are used as tags to detect localisation or movement of a glycoprotein through cellular compartments. Aberrant synthesis of N-glycans lead to a variety of diseases.

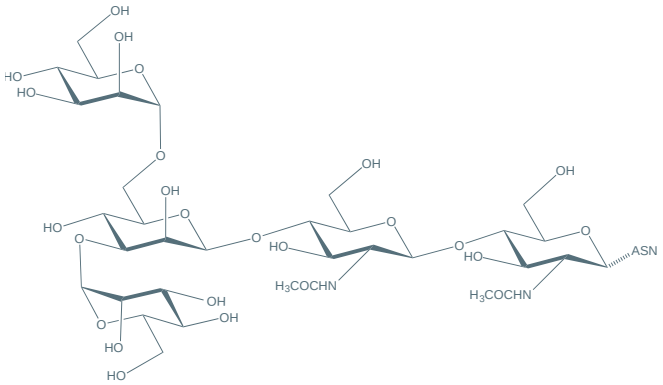


Fig 25 *N*-linked glycan core structure

As an example, the significance of protein glycosylation was illustrated by a recent report of the glycan structures on a recombinant SARS-CoV-2 S immunogen using site-specific mass spectral analysis. This analysis enabled mapping of the glycan processing states across the trimeric viral spike. It was shown how SARS-CoV-2 S glycans differ from typical host glycan processing, which may have implications in viral pathobiology and vaccine design. It was suggested that the SARS-CoV-2 S protein is less densely glycosylated and that the glycans form less of a shield compared with other viral glycoproteins, which may be beneficial for the elicitation of neutralising antibodies. The analysis also revealed high levels of fucosylation, with 98% of detected glycans bearing fucose residues (Watanabe 2020).

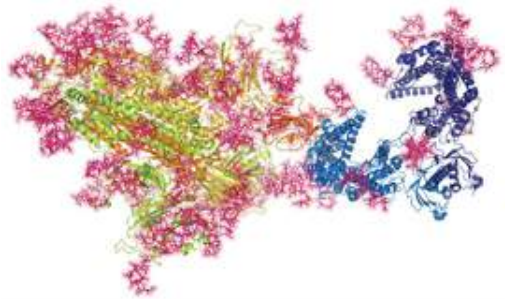


Fig 26 Glycosylated spike protein from SARS-CoV-2

All *N*-glycans share a common core oligosaccharide sequence:

Manα1,6(Manα1,3) Manβ1,4GlcNAcβ1,4GlcNAcβ1-Asn-X-Ser/Thr,

and have been classified into three types (fig 27):

- **Oligomannose**, in which only mannose residues are attached to the core; Man-5, Man-9, Man-5GlcNAc, Man6GlcNAc, NA3, NA4.
- **Complex**, in which "antennae" initiated by N-acetylglucosaminyltransferases (GlcNAcTs) are attached to the core; A2, A2F, A3.
- **Hybrid**, in which only mannose residues are attached to the Manα1–6 arm of the core and one or two antennae are on the Manα1,3 arm. A1**,** A1F.
- **Miscellaneous**, Man1-Manβ1,4GlcNAcβ1,4GlcNAcβ1 the core structure with a single mannose residue, Man3α-Manα1,6(Manα1,3) Manβ1,4GlcNAcβ1,4GlcNAcβ1, the complete trimannose core structure.

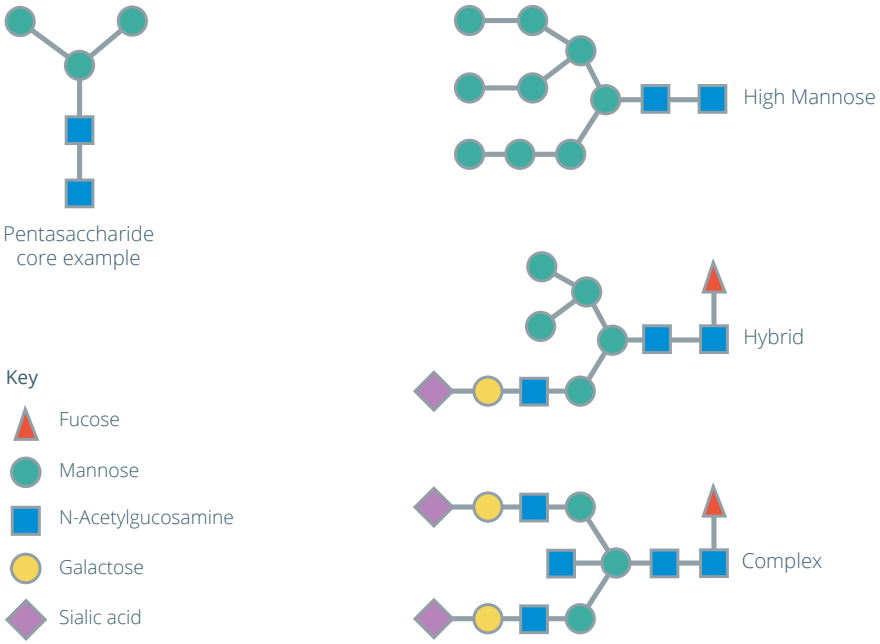


Fig 27 Types of N-glycans

2.4.2 O-linked glycosylation

The basis of *O*-linked glycosylation is the linkage of *N*-Acetylgalactosamine to serine or threonine. The best known of this type of glycosylation is found in mucins and the oligosaccharides vary in size from a single *N*-acetyl-D-galactosamine (GalNAc), through disaccharides to chains with 20 or more sugar residues (Corfield 2007).

Mucins are located in the gastrointestinal, urinary, respiratory and reproductive tracts and on the surface of the eye providing large surface area protection against the external environment (Galan 2010). Mucins are glycoproteins that are present both in the mucosal fluid of the epithelial tissue and are anchored to the surface of the tissue where they offer a plethora of potential binding sites for commensal and pathogenic microbes. They are also ligands for the targeting of leucocytes to endothelial cells (Hang 2005).

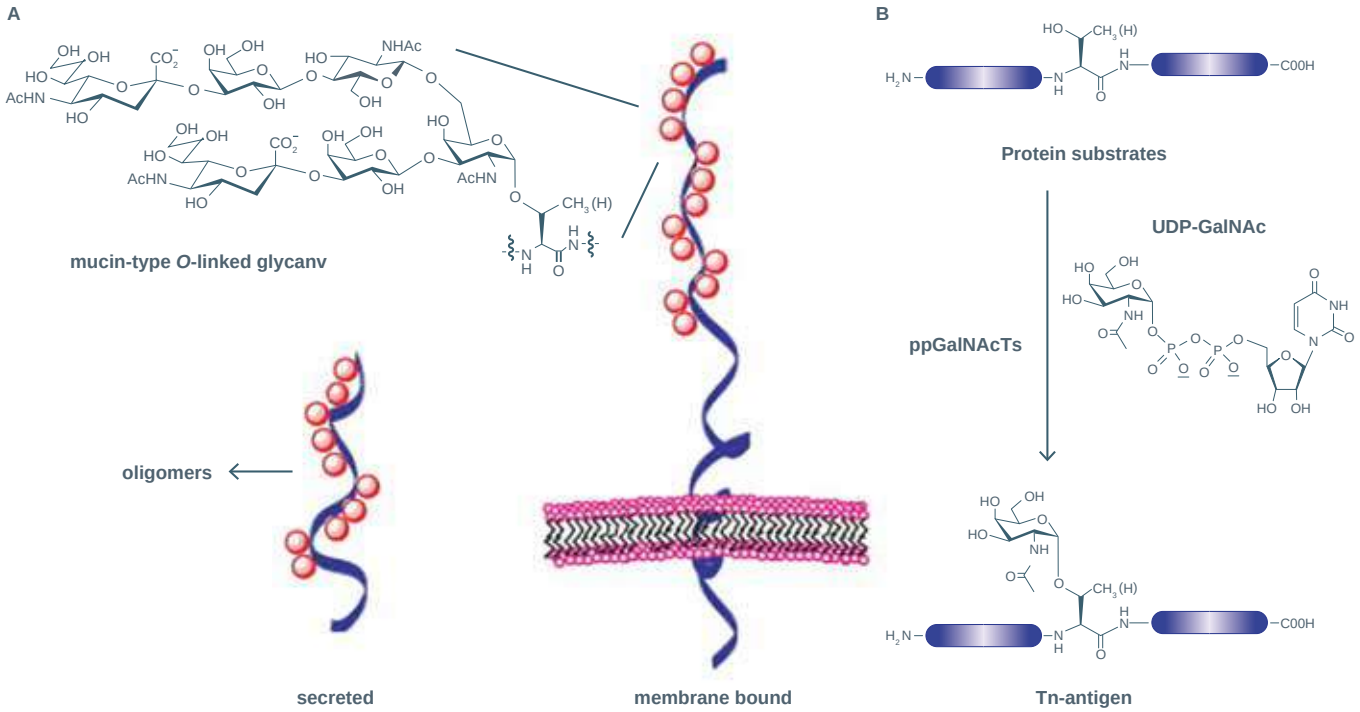


Fig 28 Attachment of O-glycans to mucins

Eight core structures have been identified (fig 28) with core 1 and core 2 being the most common. Different cores show a cell- and tissue-specific pattern which relates to their biological function (Van den Steen 1998, Brockhausen 2007, Corfield 2007).

Larger glycans are extended by Galβ1,3/4GlcNAc repeat units (poly N-acetyllactosamine, fig 29). The enormous variety of *O*-glycan oligosaccharide structures derives from the decoration of the core and backbone units. These modifications include fucosylation, sialylation, sulfation, acetylation and methylation.

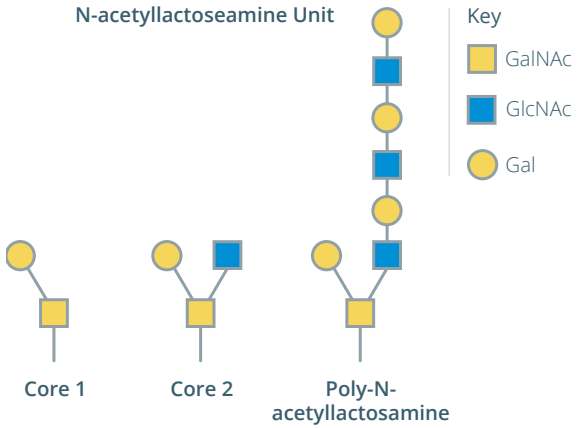


Fig 29 *O*-linked glycosylation core structures

2.4.3 Blood groups

There are 4 main blood types: A, B, O and AB and they represent contributions from both parents of the ABO and H genes that encode glycosyltransferase - an enzyme that will transfer monosaccharides to the oligosaccharide chains.

The 'O' blood group is the basic oligosaccharide structure for the different blood groups. It contains 2 galactose residues, a fucose and a *N*-acetylglucosamine. The 'A' blood group has the basic O structure but with the addition of a *N*-acetylgalactosamine residue and the 'B' blood group has the basic O structure with the addition of a further galactose residue. As humans with blood group AB have the genes for both type A and type B glycosyltransferases, they synthesise both blood antigens. Figure 30 shows the terminal structure of the A B and O antigens.

Blood-group ABO genotyping by DNA methods is now in widespread use (Morgan 2000). It should be noted that although best known as blood antigens, these antigens are expressed on many tissues including on epithelial and endothelial cells (Dean 2005).

The expression of the ABO blood group antigens in secretory tissues, such as salivary, gastrointestinal, respiratory and reproductive tracts, is governed by the secretor gene *Se*, which codes for the α1-2 fucosyltransferase, FUT2. People who have an inactive FUT2 gene, (*se* and not *Se*) do not have soluble forms of ABO determinants in saliva, milk oligosaccharides or in other secretory tissues. These individuals are called non-secretors and they make up about 20% of all populations worldwide. Non- secretors have a higher risk of type-1 diabetes; coeliac disease, inflammatory bowel disease; kidney disease, urinary tract disease and *candida* infection. They are susceptible to *Haemophilus influenza* and *Streptococcus pneumonia*, but resistant to norovirus and rotavirus diseases. In the case of human breast milk, the α1-2 fucosylated oligosaccharides are absent. This gives less protection against bacterial diseases at birth and also has an influence on the development of the infant microbiota in the first two years of life (Varki 2017).

One of the best known and important O-linked Antigens is Sialyl-Lewis ^{X} (fig 31), a tetrasaccharide usually attached to O-glycans on the surface of cells. It is known to play a vital role in cell-to-cell recognition processes, and is important in leukocyte tethering and rolling and plays a key role in human fertilisation (Feizi 1993).

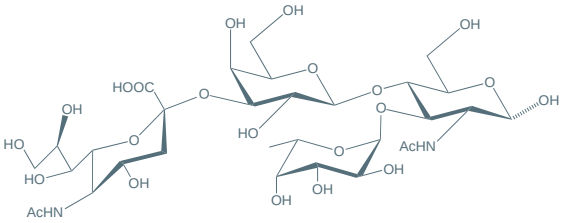


Fig 31 Sialyl-Lewis[x]

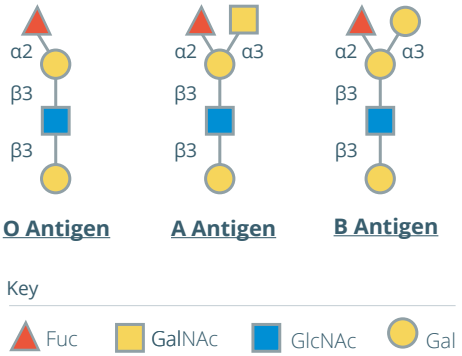


Fig 30 Terminal structures of blood groups

2.5 Covalently attached to lipids (glycolipids)

Glycolipids are glycoconjugates containing a carbohydrate moiety covalently linked to a lipid by a glycosidic bond. Glycolipids are integral parts of eukaryotic cell membranes, exposing their carbohydrate moiety in extracellular space extending from the phospholipid bilayer. Glycolipids play a role in maintaining stability of the cell membrane, are involved in cellular recognition and other cellular processes.

Some examples are:

Galactolipids differ from glycosphingolipids in that they do not contain nitrogen. They are the major lipids in plant membranes where they substitute phospholipids to conserve phosphate for other essential processes and contain a high quantity of monogalactosyldiacylglycerol (MGDG) and **digalactosyldiacylglycerol** (DGDG, fig 32). Galactolipids are thought to play a role in photosynthesis, as they have been found in the X-ray structures of photosynthetic complexes.

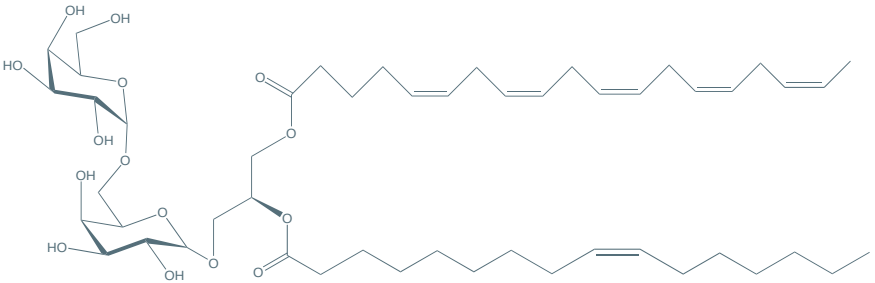


Fig 32 Digalactosyldiacylglycerol (DGDG)

Lipid A (fig 33) is a lipid component of the outer membrane of Gram-negative bacteria as well as a part of a bacterial endotoxin. The hydrophobic nature of lipid A allows it to anchor in the outer layer of the outer bacterial membrane and is the innermost component of the more complex lipopolysaccharide (LPS) (Raetz 2002). The detection of lipid A by immune system might play a role in immune response against infection with Gram-negative bacterial pathogens. (Tzeng 2002).

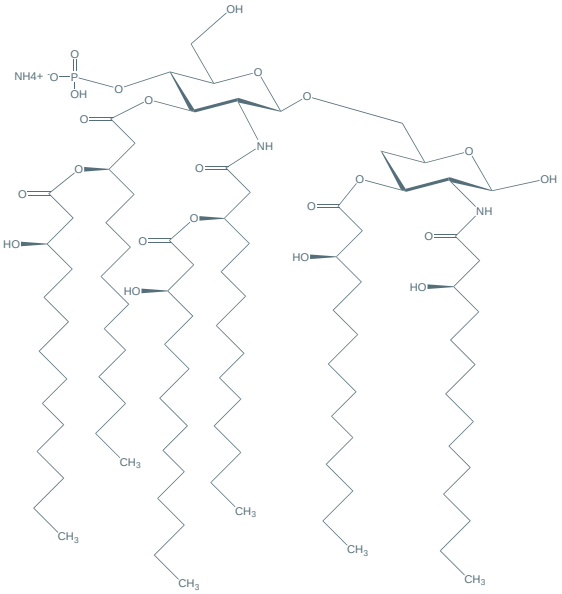


Fig 33 Lipid A

Sucrose tallowate esters (fig 34) are produced industrially by the reaction of sucrose with tallow (animal fat). The mono and diesters are excellent surfactants and have been used in many applications including skin cleaners, vinyl de-static products and fruit preservation coatings (Kollonitsch 1970).

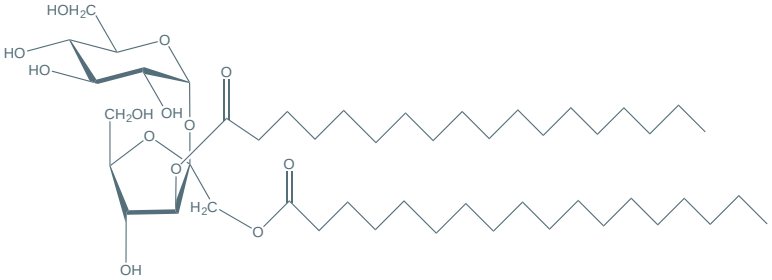


Fig 34 Sucrose stearate

Rhamnolipids (fig 35) are glycolipids produced by bacteria such as *Pseudomonas aeruginosa* and are well characterised as bacterial surfactants. In recent years, rhamnolipids have been commercialised into products including biodegradable household detergents and hand cleaners (Araujo 2018). They have a glycosyl head group and a 3-(hydroxyalkanoyloxy) alkanoic acid fatty acid tail, such as 3-hydroxydecanoic acid.

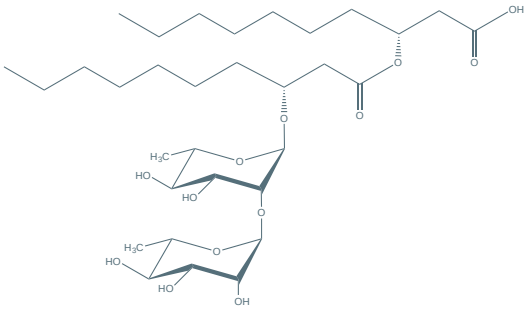


Fig 35 Rhamnolipid

GM1 (fig 36) is a ganglioside component of membranes in mammalian neurons and are widely represented in mammalian brains. Ganglioside GM1 is also present in intestinal epithelium where it acts as a receptor for the cholera toxin from *V. cholerae* and heat-labile toxin from *E. coli* (Aureli 2016).

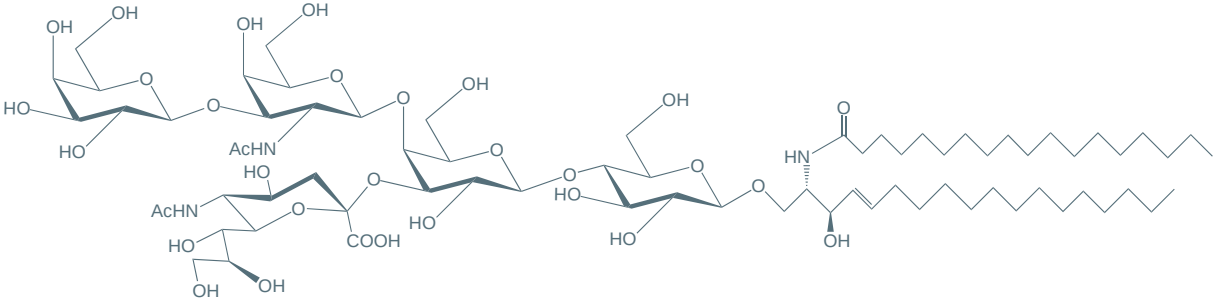
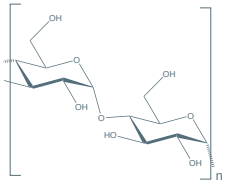
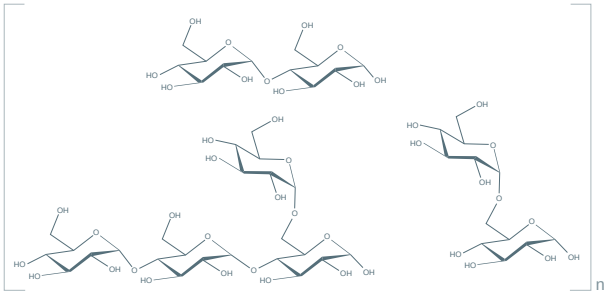


Fig 36 Ganglioside GM1

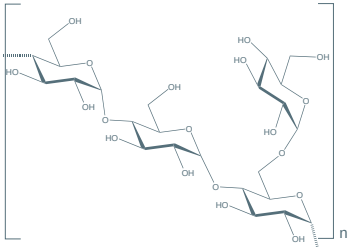
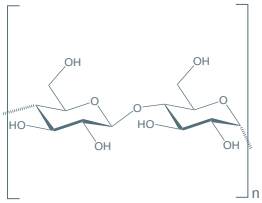
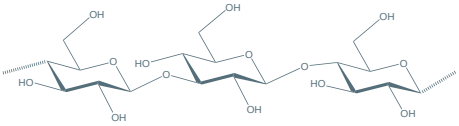
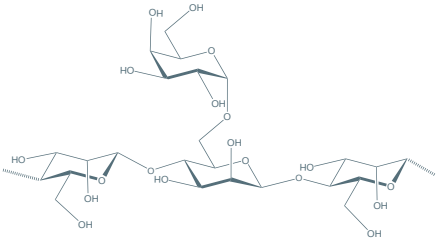
2.6 Derived from polysaccharides

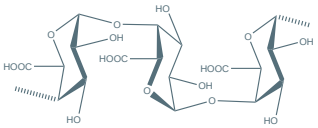
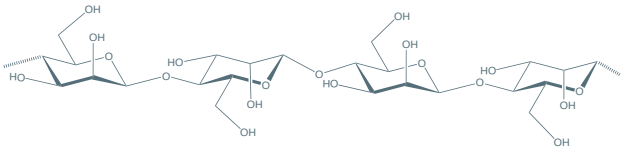
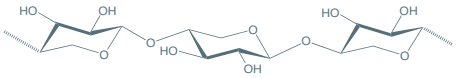
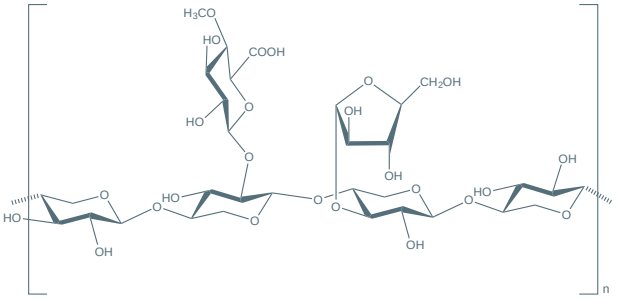
Polysaccharides are a key group of biopolymers and a source of a huge number of oligosaccharides. They contain many monosaccharide residues including glucose, galactose, mannose, arabinose, xylose and rhamnose, the uronic acids galacturonic, mannuronic and guluronic, and miscellaneous residues such as sialic acid, anhydro sugars, sulfates and phosphates. Oligosaccharides are released from polysaccharides by partial acid degradation, acetolysis, or enzymatic treatment.

Higher plants

Polysaccharide	Hydrolysis Product	Type of Reagent Used
Starch (amylose) 	Maltooligosaccharides (α1,4 glucose) 	Partial Acid and/or Enzymes (Amylase/Glucoamylase) (Sihui, 2017; Eggleston 2003)
Starch (amylose + amylopectin) 	Malto (α1,4 glucose) + Isomaltooligosaccharides (α1,6 glucose) 	Partial Acid and/or Enzymes (Amylase/Glucoamylase) (Sihui, 2017; Eggleston 2003)

Higher plants

Polysaccharide	Hydrolysis Product	Type of Reagent Used
Waxy maize starch (mainly amylopectin) 	Branched malto/Isomaltooligosaccharides 	Partial Acid and/or Enzymes (Amylase/Glucoamylase) ((Sihui, 2017; Eggleston 2003)
Cotton (Cellulose) 	Cellooligosaccharides (β1,4 glucooligosaccharides) 	Partial Acid and/or Cellulase (Vejdovszky 2015)
Oat Glucan 	β1,3 & β1,4 glucooligosaccharides 	Partial acid hydrolysis (Yan 2018)
Guar gum, Locust Bean Gum (Galactomannans) 	Mannose (β1,4) Oligosaccharides with Galactose (α1,6) branches 	Partial Acid (Kunz 2003)

Pectic Acid (Citrus fruits) 	Galacturonooligosaccharides (α1,4) 	Partial Acid or Pectinase (Gullón 2013)
Ivory Nut Mannan (custard apple, ebony and palm trees) 	Mannooligosaccharides (β1,4) 	Enzyme hydrolysis (Kusakabe 1983)
Linear Xylan (Beech-wood-hardwood), Hemicellulose 	Xylooligosaccharides (β-1,4 xylooligosaccharides) 	Xylanase (Amorim 2019)
Branched xylan (corn cob) 	L-Arabino (methyl-D-glucurono) xylooligosaccharides 	(Ebringerova, 2000) Partial acid and enzymatic hydrolysis.

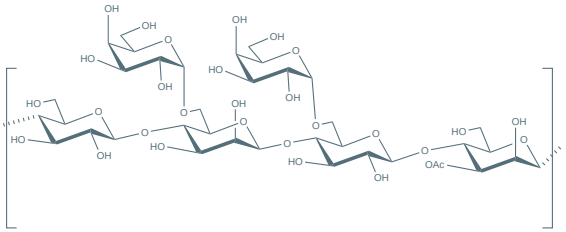
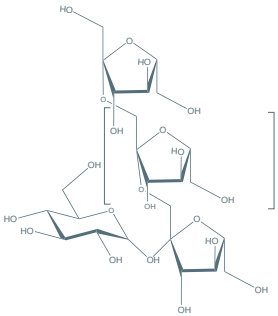
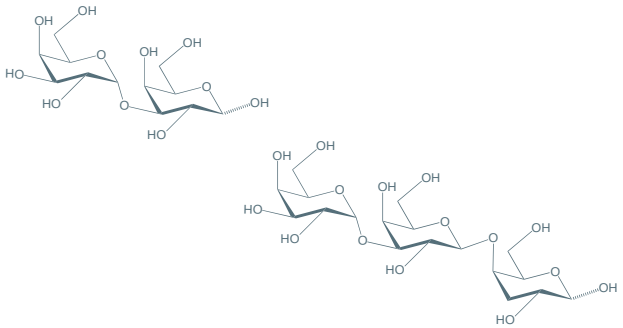
Higher plants Polysaccharide	Hydrolysis Product	Type of Reagent Used
Galactoglucomannan (Norway Spruce) 	Galactoglucomannan oligosaccharides 	(Lundqvist 2002) Partial acid hydrolysis
Inulin (Jerusalem artichoke) 	Fructooligosaccharides 	Mild Acid Hydrolysis (Ganaie 2014)

Table 1 Polysaccharide-derived oligosaccharides from higher plants

Algae Polysaccharide	Hydrolysis Product	Type of Reagent Used
Carrageenan (kappa, Iota) *(Gigartina stellata)* 	Polymer homologous series (α1,3 Gal, β1,4 Gal) (D-series) 	Partial Acid or Acetolysis (Lawson 1968)

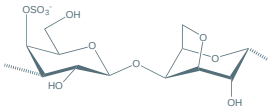
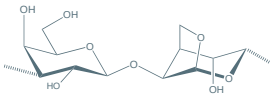
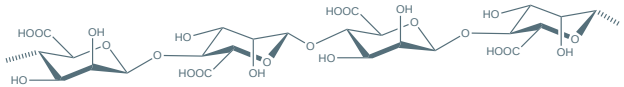
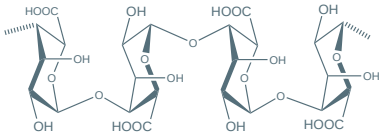
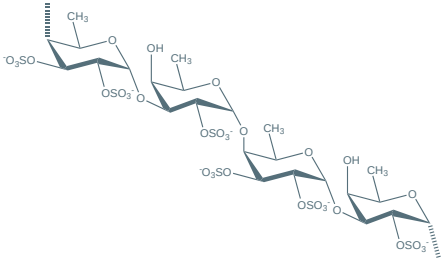
Carrageenan (Kappa) *(Chondrus crispus)* 	Sulfated carrabiose 	Enzyme (Carrageenase) (Jouanneau 2010)
Agar *(Gelidium spp)* 	Agarobiose (4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) 	Partial Acid or Acetolysis (Hirase 1958)
Alginate *(Macrocystis pyrifyra)* 	Mannuronooligosaccharides (β1,4-D), mannurono/ guluronooligosaccharides (β1,4-D & α1,4-L)  	Enzymatic hydrolysis (Heyraud 1998)
Fucoidan, *(Ascophyllum nodosum)* 	Sulfated fucooligosaccharides 	Partial acid hydrolysis (Daniel 2007)

Table 2 Polysaccharide-derived oligosaccharides from algae

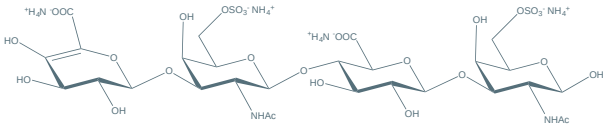
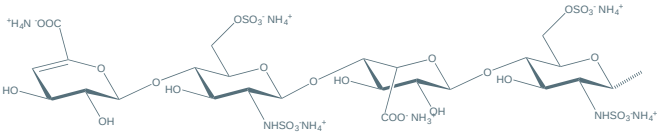
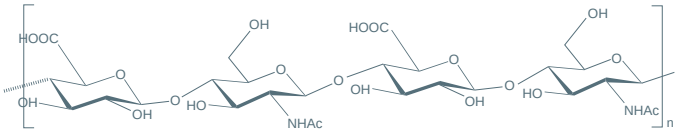
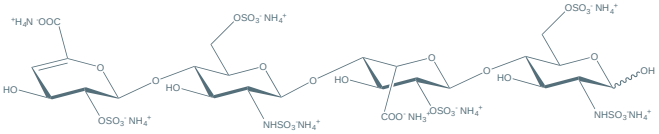
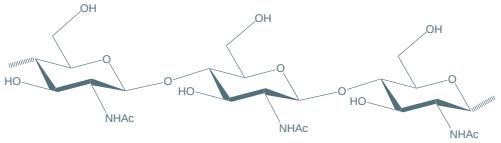
Animal tissues Polysaccharide	Hydrolysis Product	Type of Reagent Used
Chondroitin Sulfates (shark cartilage)  	Sulfated oligosaccharides, C4-C5 unsaturated at the non-reducing end.  	Enzyme hydrolysis (Kang 2018)
Hyaluronic acid-cocks comb 	β-1,3 glucuronic acid and β-1,4 N-acetyl glucosamine oligosaccharides 	(Tao 2017)
Heparin (intestinal mucosa) 	2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine, IdoA(2S)-GlcNS(6S) 	(Zhang 2019)
Chitin (Crustacians) 	Chitooligosaccharides 	Partial acid hydrolysis (Aam 2010)

Table 3 Polysaccharide-derived oligosaccharides from animal tissues

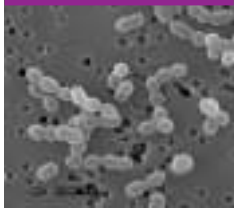
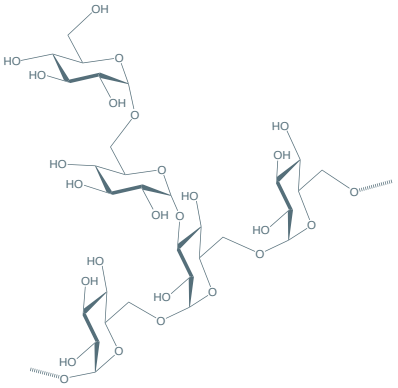
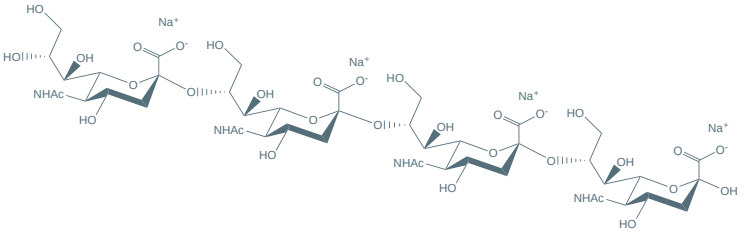
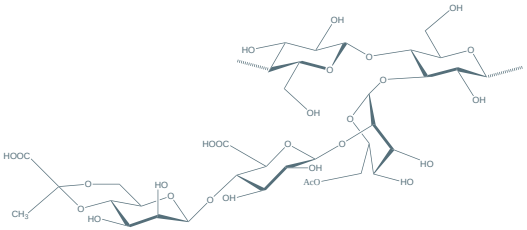
Bacteria Polysaccharide	Hydrolysis Product	Type of Reagent Used
Dextran *(Leuconostoc mesenteroides)* 	α1,6 glucooligosaccharides 	Dextranase (Kubik 2004)
Colominic acid (polysialic acid) *(E. Coli)* 	Sialic acid containing oligosaccharides 	Dilute acid, neuraminidase (Kakehi 1996, Chen 2015)
Xanthan gum *(Xanthomonas campestris)* 	β-1,4-linked glucooligosaccharides with trisaccharide side chains (mannose-glucuronic acid-mannose residues) 	(Rees, 1976; Lawson, 1977) Partial acid hydrolysis, acetolysis

Table 4 Polysaccharide-derived oligosaccharides from bacteria

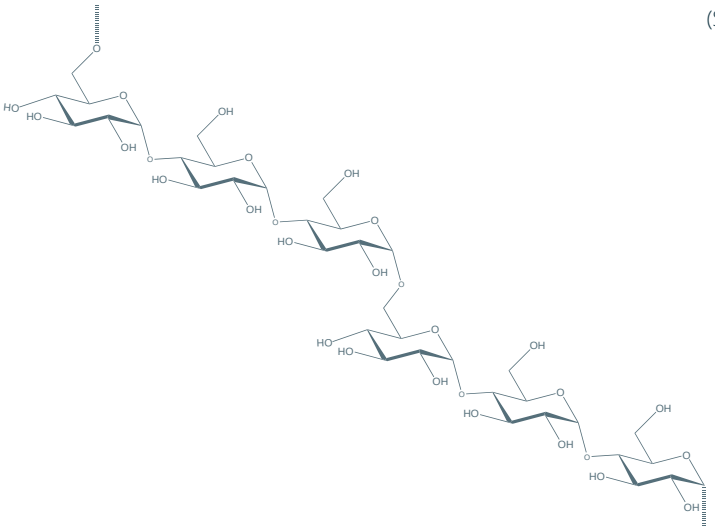
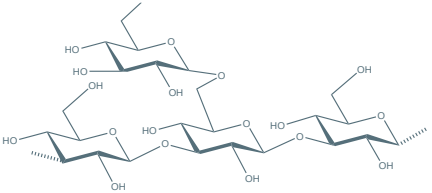
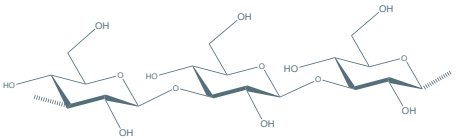
Yeast and fungi Polysaccharide	Hydrolysis Product	Type of Reagent Used
Pullulan *(Aureobasidium pullulans)* 	Maltotriose 	Pullulanase (Singh 2010)
Schizophyllan *(Schyizophyllum commune)* 	Linear β-1,3 glucooligosaccharides with β-1,6 branches 	(Saito, 1979), partial acid hydrolysis.
Curdlan *(Alcaligenes faecalis var. Myxogenes)* 	β1,3 glucooligosaccharides 	Partial acid hydrolysis (Wang 2017)

Table 5 Polysaccharide-derived oligosaccharides

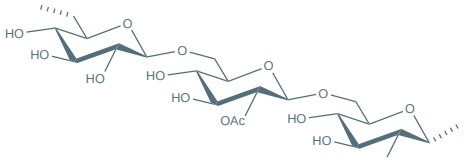
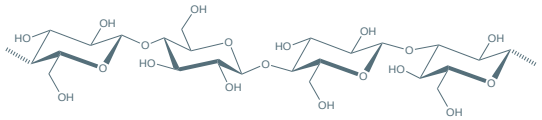
Yeast and fungi Polysaccharide	Hydrolysis Product	Type of Reagent Used
Pustulan *(Lasallia pustulata)* 	β-1,6-glucooligosaccharides 	(Karunaratne 2010) Partial acid hydrolysis
Lichenan *(Cetraria islandica)* (Iceland moss) 	Repeating β-1,3 and β-1,4 glucooligosaccharides 	(Perlin, 1962) Enzymatic degradation

Table 6 Polysaccharide-derived oligosaccharides from lichens

Note: Other sources of polysaccharide-derived oligosaccharides can be obtained from 'The Polysaccharide Toolbox' (Lawson 2019). This can be downloaded from the Biosynth website by visiting the Literature – Polysaccharides section.

2.7 By Enzymatic Transglycosylation

The only major group in this category are the cyclodextrins:

Cyclodextrins are α-1,4-linked cyclic oligosaccharides produced from starch by the action of cyclodextrin glucanotransferase, a transglycosidase that cleaves an α-1,4-linkage in amylose and forms a toroidal ring structure with a hydrophobic interior and a hydrophilic exterior giving them the ability to form inclusion complexes with hydrophobic molecules. The α-, β- and γ-cyclodextrins, having six, seven and eight D-glucose residues are the most extensively studied (Collins 2006).

The important property of cyclodetrins that have made them so interesting is that they are useful molecular chelating agents. They possess a cage-like supramolecular structure giving them a host–guest functionality that allows them to trap drugs, odours, and prevent degradation of sensitive molecules such as food fragrences. In fig 37, the structure of cyclodextrin is shown (B) with cholesterol (A) and a cartoon showing the cholesterol (C), the annular structure of cyclodextrin with an outer hydrophilic face and inner hydrophobic face (D). (E) is a representation of the cholesterol chelated within the hydrophobic annular structure of cyclodextrin.

Cyclodextrins have been used extensively in consumer products as for example in the odour freshener Febreze (Proctor and Gamble) which are claimed to entrap odour-producing lipophilic molecules. They are used for the improvement of water-solubility and bioavailability of drugs for example in tablets, aqueous parenteral solutions, nasal sprays and eye drop solutions.

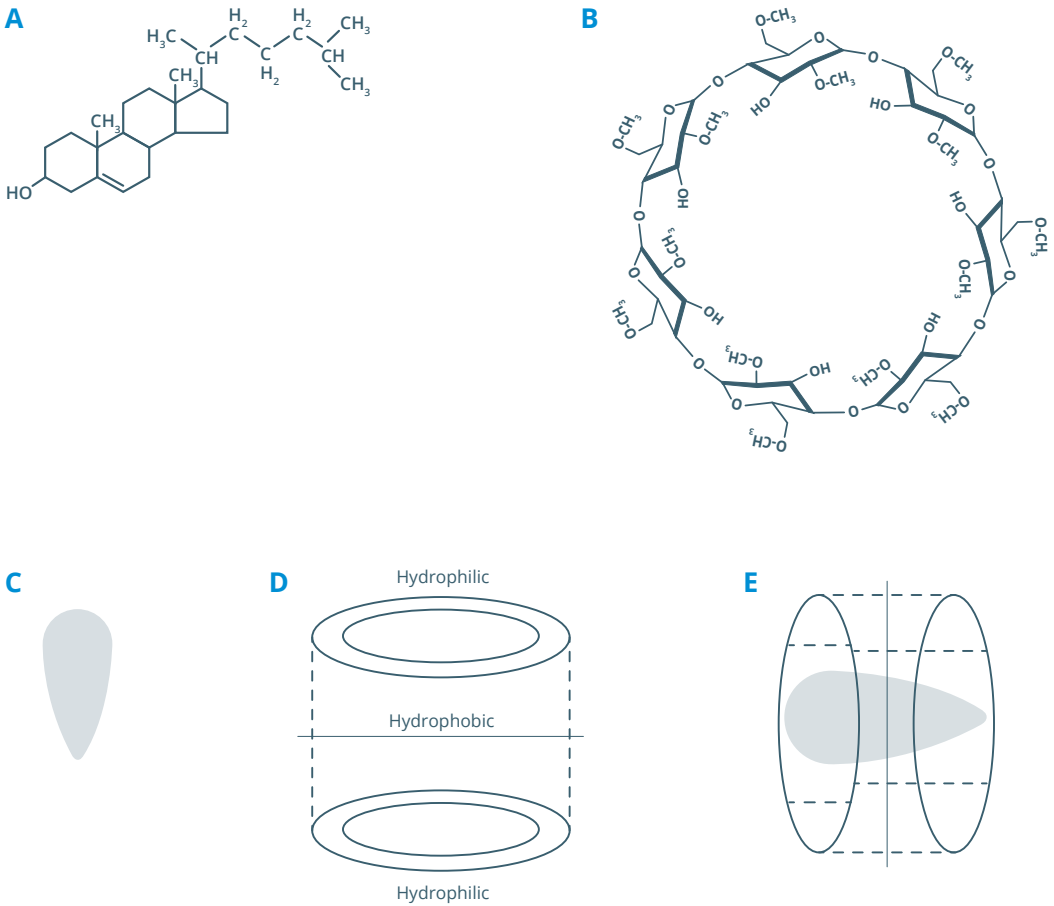


Fig 37 Representation of cyclodextrin chelating cholesterol. Figure adapted from Mahammad *et al,* (2015).

Examples of the use of cyclodextrins in medicines are β-Cyclodextrin in cetirizine tablets and cisapride suppositories, and γ-cyclodextrin in minoxidil solution. Recently, a pharmaceutical cocrystal of ibuprofen and a biocompatible metal–organic framework (CD-MOF) based upon a γ-cyclodextrin (γ-CD) tori, coordinated to alkali metal cations (e.g. K^+ ions) lead to an uptake of 23–26 wt % of ibuprofen within the CD-MOF. Bioavailability investigations on mice, and the ibuprofen/CD-MOF pharmaceutical cocrystal were compared with control samples of the potassium salt of ibuprofen and the same rapid uptake of ibuprofen as for the control sample was found (Hartlieb 2017).

α-Cyclodextrin has been approved in Europe as novel food ingredient (functional food) for use as a prebiotic (OJEU 2008), non-digestible carbohydrates, which support the gut flora in the large intestine (Roberfroid 2007). There is considerable interest in these types of food additives, since we consume only half the amount of non-digestible carbohydrates required to maintain the health of the gut flora. The properties of α-cyclodextrin make it ideally suited for this application, since it is readily water-soluble, does not affect the viscosity of the solution and is taste-neutral. α-Cyclodextrin is used to chelate a number of sensitive food ingredients such as flavours and fragrences as it helps to prevent oxidation, heat/light-induced degradation and loss by evaporation/sublimation (Kfoury 2016).

2.8 Synthesis

2.8.1 Challenges in oligosaccharide synthesis

Fig 38 Automated flash-chromatography

The structural diversity of oligosaccharides is much greater than those of oligopeptides or oligonucleotides. Apart from the large number of different monomers found in nature, already for a single monomer such as the common D-glucose, the differences in anomeric configuration, ring size and the various attachment points give rise to a variety of possible disaccharides. Three different monosaccharides, pyranose forms only, can form 1056 different trisaccharides whilst only 6 different tripeptides can be formed from 3 different α-amino acids (sidechain reactions aside). Biologically relevant oligosaccharides are typically larger, the number of possible hexasaccharides for instance, was calculated to be in the range of a trillion (Laine 1994).

To allow for the assembly of an oligosaccharide target from individual building blocks, a series of individual glycosylation reactions, i.e. the reaction of a glycosyl donor with a glycosyl acceptor to form a glycoside (a di-, tri-, tetrasaccharide etc.) have to be performed. This must happen as tightly controlled as possible, both with respect to the attachment point (regiocontrol), the absolute configuration at the resulting anomeric centre (stereocontrol) and the ring size in donor and acceptor (Levy 2006).

The way this is achieved in chemical oligosaccharide synthesis is quite different from oligosaccharide syntheses along the lines of the natural (bio-)synthesis of oligosaccharides, e.g. enzymatic syntheses or syntheses based on fermentation processes, although overlaps do exist (Qiu 2020, Rich 2012).

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2.8.2 Chemical oligosaccharide synthesis

Fig 39 Rotary evaporator

Fig 40 Liquid-chromatography/ mass spectrometry (LC-MS)

Classical: The aforementioned regioselectivity problem in oligosaccharide synthesis is addressed by the use of protecting groups, essentially for the various hydroxy groups but also for others such as amino groups (Wuts 2007, Ägoston 2016). The strategy is to selectively add or remove protecting groups during a multistep oligosaccharide synthesis in such a way as to allow only the desired hydroxy group to react in an individual glycosylation. Persistent protecting groups, such as benzyl ethers, are kept throughout most or all of the synthesis in contrast to temporary protecting groups such as silyl ethers, esters or carbonates that serve their purpose more intermittently. Careful choice of a protecting group strategy is crucial for the success of a synthesis. It should for instance be possible to introduce and remove a protecting group reliably and in high yield. Moreover, an individual group should not be reactive under the conditions of introduction or removal of another member of the set (or indeed glycosylation), meaning they ought to be 'orthogonal' (Ägoston 2016). Another important feature influencing the choice of protecting groups is the ability of some to directly influence the stereochemical outcome of a glycosylation.

The stereochemical outcome of each new glycosidic bond-formation during oligosaccharide synthesis is the second fundamental challenge to be discussed, as with an increasing number of glycosylation steps the individual yield, i.e. the yield of desired α- or β-glycoside, becomes ever more important. Participation of the ring oxygen and the relatively weak nucleophilicity of the alcohol acceptors drive glycosylation reactions often towards a S_N1-mechanism which is difficult to control. The vast majority of natural glycosides in oligosaccharides have a functional group in position 2 next to the anomeric centre, often a hydroxy group.

Depending on the stereochemical relationship between the two, we speak of 1,2-cis or 1,2-trans glycosides to be formed by glycosylation. For example, an α-glucoside and a β-mannoside would be 1,2-cis glycosides while their diastereomeric counterparts β-glucoside and α-mannoside are 1,2-trans glycosides (Levy 2006).

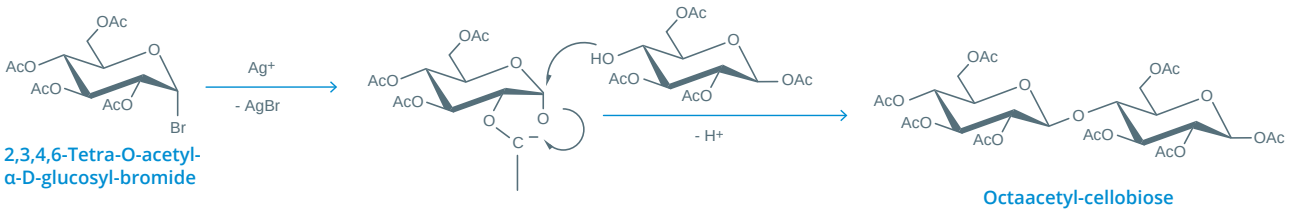
Fig 41 Walk-in fume cupboard with 20L reaction vessel and flash-chromatography

The most common methodology used for 1,2-trans glycosylation is taking advantage of neighbouring group participation (anchimeric assistance), provided by some types of protecting groups at position 2, such as esters, carbonates and carbamates (but not ethers for instance). Following activation of the glycosyl donor (e.g. a tetra-O-acetyl-α-D-glucopyranosyl bromide) by a suitable promoter (e g. a silver salt), a transitional oxocarbenium-ion intermediate is formed which is stabilised by the keto-oxygen of the neighbouring acetate via a 5-membered ring. The incoming nucleophile (e.g. another acetylated glucopyranoside with the hydroxy group in position 4 unprotected) will attack from the opposite site and thus a 1,2-trans glycoside is formed (in this case a peracetylated glucopyranosyl-β-1,4-glucopyranoside). Removal of the protecting groups would then lead to the natural product cellobiose.

A classical example of how anchimeric assistance can be exploited is the first chemical synthesis of sucrose by Lemieux and Huber in 1953 (Lemieux 1953).

For 1,2-cis glycosylation, an equally generic method has yet to be found. Apart from avoiding neighbouring group participation through the use of, e.g. benzyl ethers to protect position 2, conditions that favour the 1,2-cis product often have to be developed individually for a given glycosylation. An example would be the 'α-conditions' applied for galactosylation in the synthesis of blood group determinants (Schmidt 1991). The first more widely applicable method that tackled the problem was *in-situ* anomerisation, developed by Lemieux and colleagues and later extended by Paulsen (Lemieux 1975 Paulsen 1981). Halide ions are added as catalysts to a α-glycosyl halide which are in case of the common hexoses more stable than their β-counterparts. Nucleophilic substitution leads to the *in-situ* formation of the β-halide which in turn is substituted by the alcohol thus giving the α-(1,2-cis)-product, i.e. retention of the anomeric configuration. The driving factor is the lower energy barrier (and thus higher reaction rate) for the substitution of the β-halide, i.e. its higher 'reactivity'.

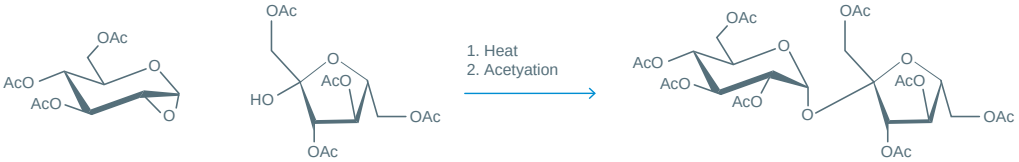
A



2,3,4,6-Tetra-O-acetyl-α-D-glucosyl-bromide

Octaacetyl-cellobiose

B



(α1,2)-(1,5)-3,4,6-Tri-O-acetyl-D-glucosan

1,3,4,6-Tetra-O-acetyl-D-fructose

Octaacetyl-sucrose

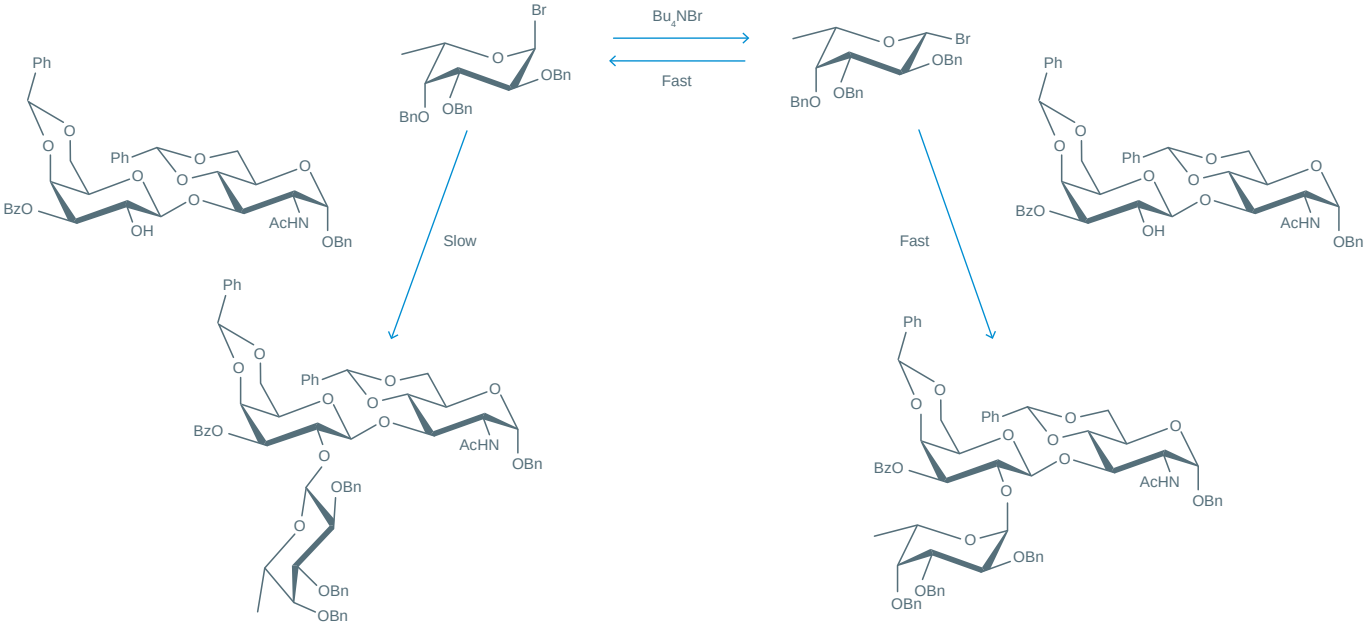
Scheme 1: Exploiting anchimeric assistance a synthesis of cellobiose **(A)** and the first chemical synthesis of sucrose **(B)**, in which case the anchimeric assistance is provided by the acetyl group in 6-position of the glucosan.

Intramolecular glycosylation, or intramolecular aglycon delivery, has been suggested and applied in some cases but usually requires more synthetic steps. More recently, conformationally restricted sulfoxide donors, introduced by Crich (Chandrasekera 2004), have helped solve at least part of the problem, in particular for the β-mannosidic linkages commonly found in biologically relevant oligosaccharide structures.

Naturally, development of novel glycosyl donors has always been another crucial aspect in the improvement of oligosaccharide synthesis. Glycosyl halides were the first widely used group and are still applied today, often for industrial scale reactions. Later glycosyl imidates, sulfoxides and sulfones, thioglycosides, glycosyl phosphites and phosphates, each coming with their individually suited promotor systems, have found more widespread use.

In summary, in the past 50 years, great strides forward have been made in oligosaccharide synthesis and the chemical synthesis of virtually any oligosaccharide structure has become possible, at least in theory. An example of rather high complexity would be the synthesis of Fondaparinux (Arixtra™, scheme 3), an antithrombin III binding pentasaccharide drug and replacement for heparin of animal-origin (Petitou 2004).

A

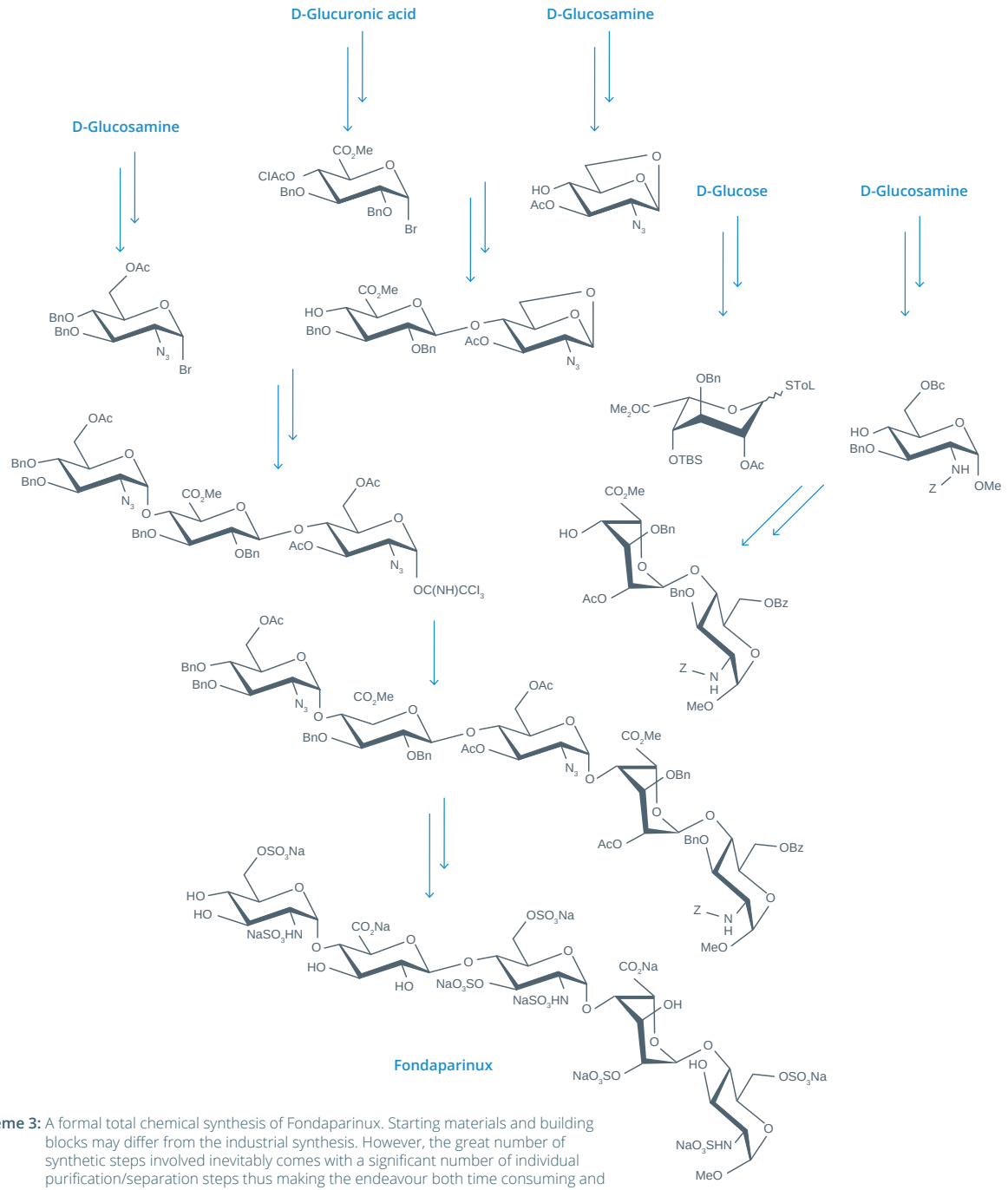


B

conformationally restricted mannosyl donor

β-mannoside, a 1,2-cis glycoside as major project

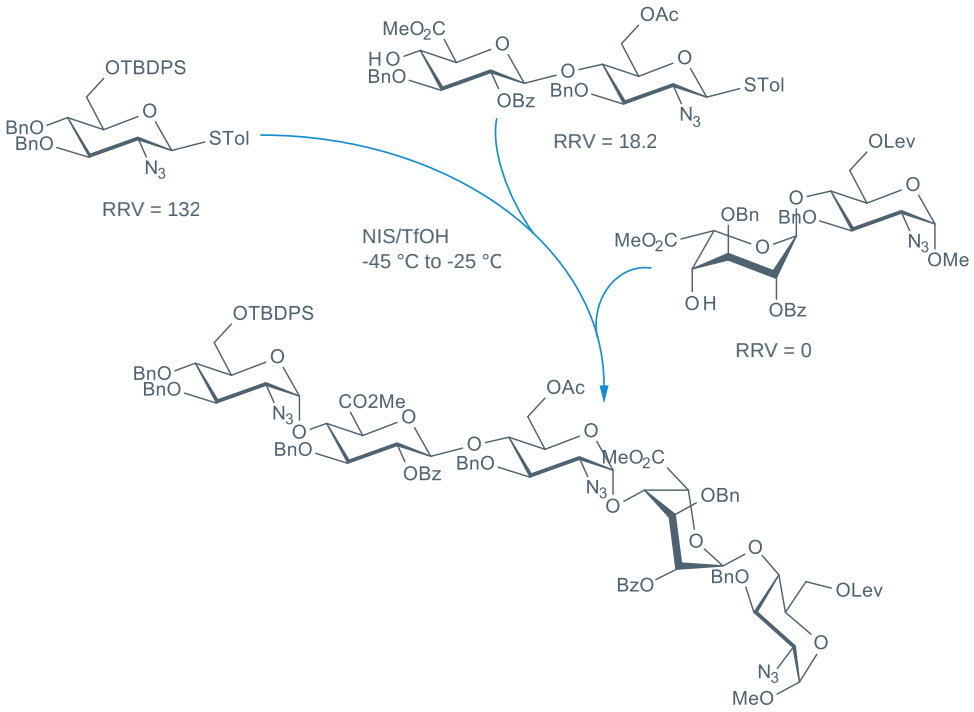
Scheme 2: A: In-situ anomerisation method for the generation of 1,2-cis glycosides.
B: Example of a conformationally restricted glycosyl donor allowing for the predominant formation of β-mannosides.



Scheme 3: A formal total chemical synthesis of Fondaparinux. Starting materials and building blocks may differ from the industrial synthesis. However, the great number of synthetic steps involved inevitably comes with a significant number of individual purification/separation steps thus making the endeavour both time consuming and expensive. For this reason, the past 20+ years have seen particular focus on ways to make chemical oligosaccharide synthesis more effective, two of which we shall mention here.

One-pot glycosylation sequences: The nature of the protecting groups impact strongly on the reactivity of a glycosyl donor with a given anomeric leaving group. For instance, a perbenzylated glycosyl bromide will be much more reactive, i.e. more easily activated by a promotor than its peracetylated counterpart, due to the difference in the electronic situation around the anomeric centre induced by the protecting groups.

This has been exploited systematically by Wong and coworkers by calculating the relative reactivity of a large library of thioglycoside donors (Cheng 2018). Based on this data, the Optimer program selects the most appropriate combination of building blocks and appropriate promotor for each individual glycosylation. Sequential addition of reagents thus allows for the synthesis of an oligosaccharide in one pot and renders most of the intermediate purification steps unnecessary.



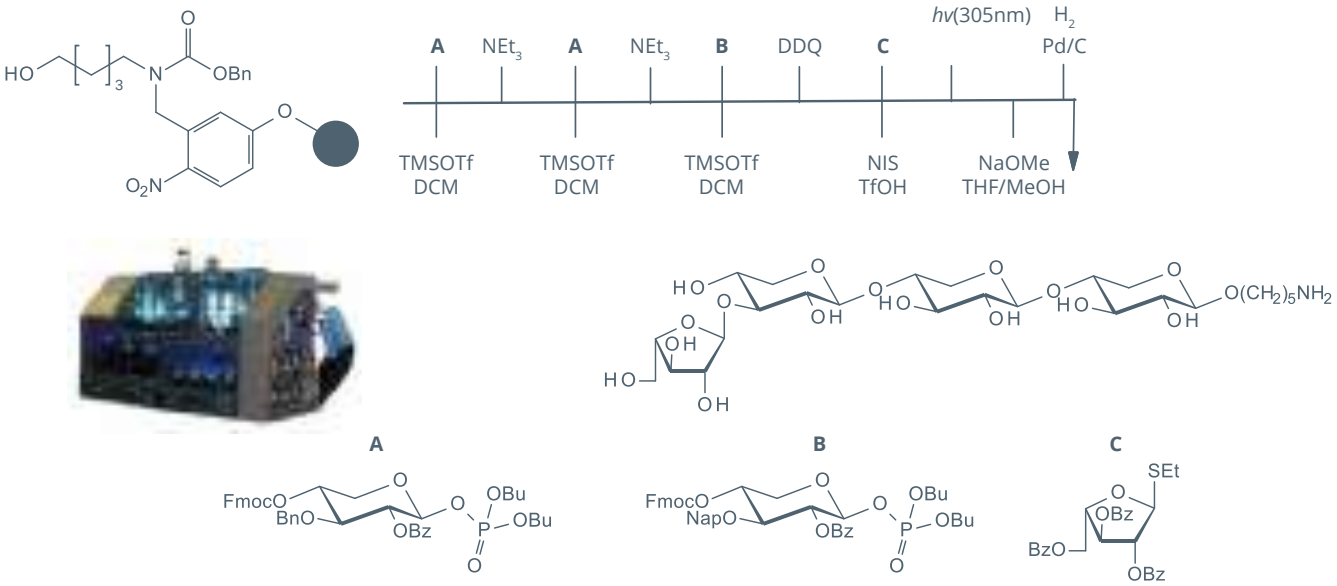
Scheme 4: Programmable one-pot synthesis of a heparin pentasaccharide.
The differential protection allows for the selective introduction of the sulfate groups.
RRV = Relative reactivity value.

Solid-phase oligosaccharide synthesis: Due to the higher complexity of oligosaccharide synthesis, solid-phase methodologies have evolved much slower than in the case of oligopeptides and oligonucleotides and automated synthesizers have only in recent years become available.

To avoid product heterogeneity and resulting purification problems, full stereocontrol in glycosylations is essential and protecting group sets as well as the linker system need to be fully orthogonal. Capping steps can be used to avoid if incomplete reactions are a problem.

With respect to glycosyl donors, acceptors, protecting group strategies and promotors, solid-phase oligosaccharide synthesis can rely on the full toolbox provided by its solution-phase predecessor but can target lengths of sequences where a strategy involving individual purification steps is no longer feasible. In addition, the synthesis of libraries of structurally related oligosaccharides have come within much easier reach.

It appears certain that in due course, access to complex oligosaccharides will therefore not be limited anymore to a small number of dedicated groups in industry and academia but will become possible much more broad to mainstream science as well (Seeberger 2015).



Scheme 5: Automated synthesis of a tetrasaccharide using the automated Glyconeer™ synthesizer.

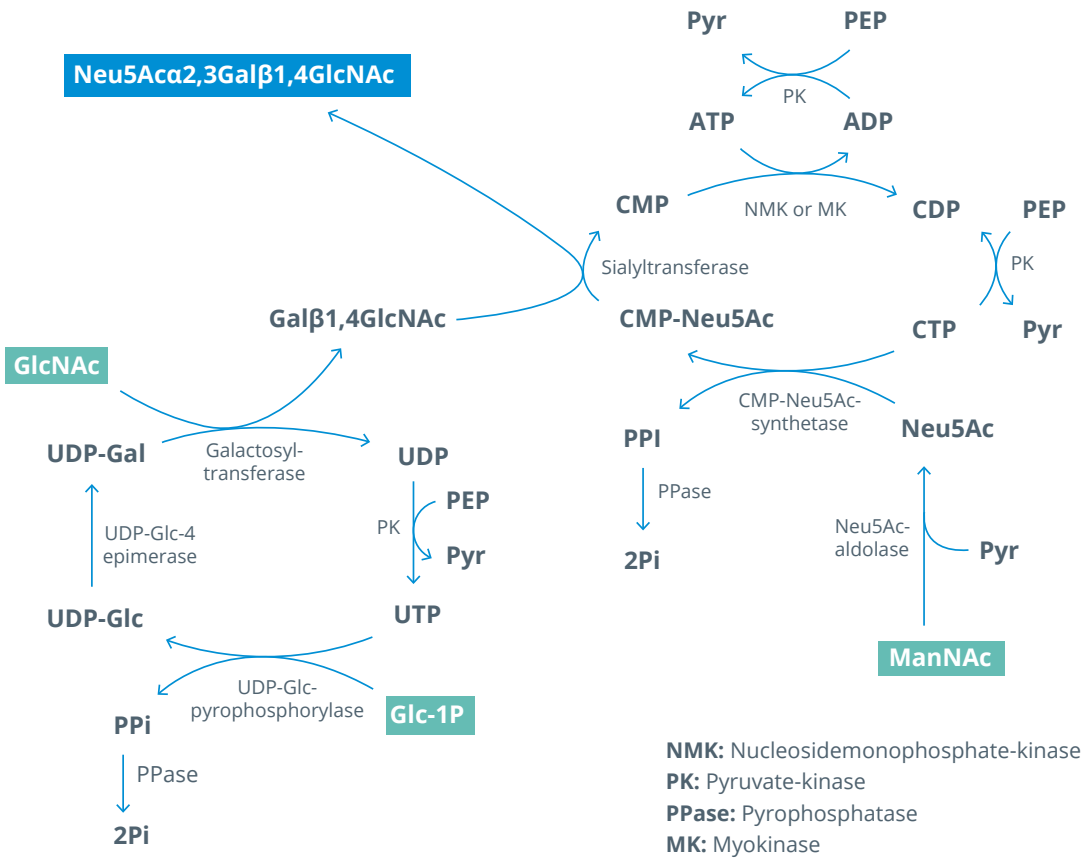
2.8.3 Enzymatic oligosaccharide synthesis

Glycoside formation and cleavage are essential biological processes for which evolution has produced sets of highly specific enzymes.

Glycosyltransferases catalyse the reaction of sugar-nucleotide donors such as several UDP-hexoses, GDP-fucose and -mannose or CMP-neuraminic acid with the respective carbohydrate (or other) acceptors to form glycosides.

They are powerful tools to construct oligosaccharides due to their high selectivity and activity. As they control all important factors such as regio- and stereochemistry during the glycosylation reaction, they render protecting groups, specific activators and leaving groups unnecessary.

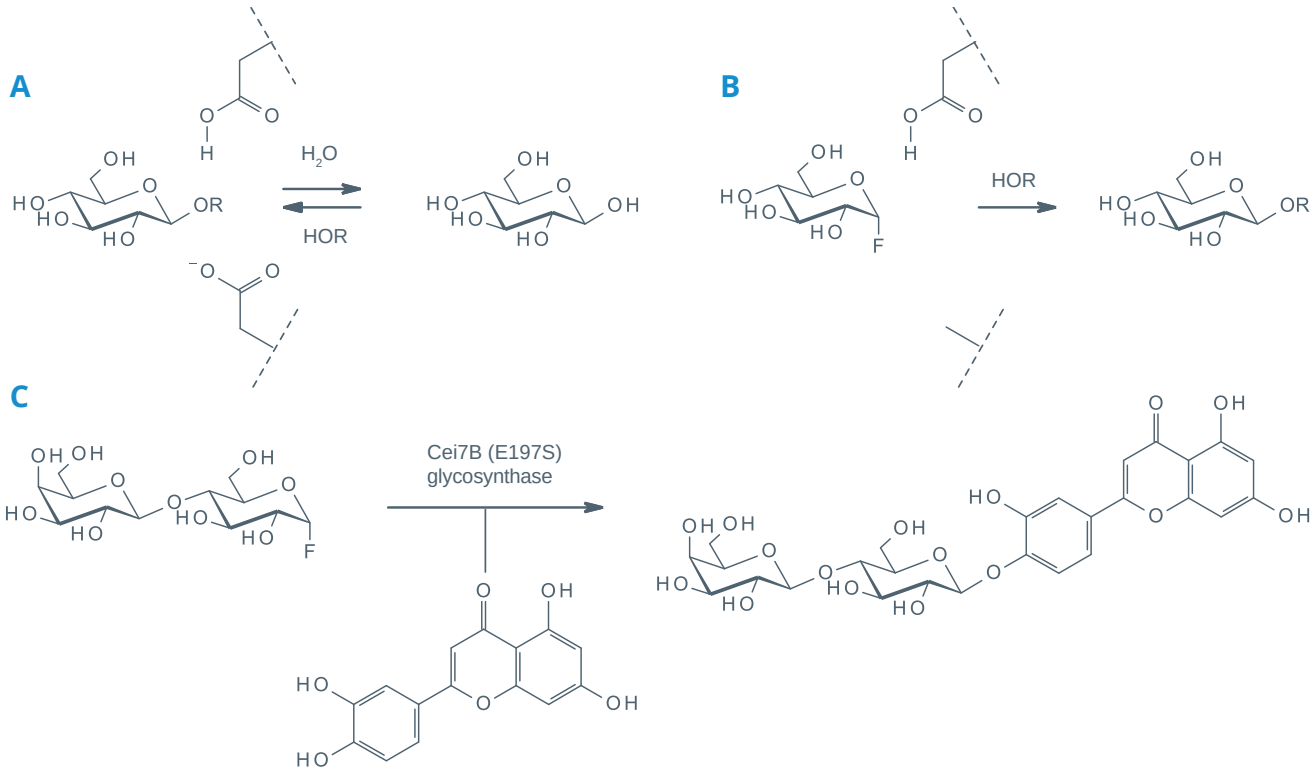
The drawback in many cases is, however, the fact that they are still not universally available. Their isolation is labourious and few have yet become widely commercially available as recombinant enzymes. In addition, the sugar-nucleotide donors have long been difficult and costly to obtain, thus making complicated regeneration reactions necessary, a problem that has eased in recent years (Ichikawa 1991a, Ichikawa 1991b) (see: Biosynth catalogue, section nucleosides and nucleotides).



Scheme 6: Synthesis of 6'-Sialyl-N-acetyllactosamine from building blocks GlcNAc, ManNAc and Glc-1-P in a multienzyme system including various cofactor regenerations.

Glycosidases, specifically hydrolyse glycosidic, bonds by formally transferring the glycosyl moiety to water as acceptor. Besides these two main groups employed in oligosaccharide synthesis, there are also trans-glycosidases that also cleave glycosidic bonds but transfer the glycosyl moiety to an acceptor other than water, e.g. another sugar or phosphate ions.

The finding with some glycosidases that, if deprived from their natural acceptor water and in presence of an excess of another acceptor (like a simple alcohol), the equilibrium can be shifted towards a transglycosylation to that acceptor has given rise to a fourth group, the glycosynthases. Whilst shifting the hydrolysis/transglycosylation equilibrium has remained difficult with very limited applications with the natural glycosidases themselves, recombinant DNA-technology has allowed the deletion of distinct amino acids involved in the catalytic process. These semi-synthetic (mutant) glycosynthases can still transfer the glycosyl residue from a synthetic glycoside (donor) to an acceptor but have largely lost the ability to hydrolyse the product glycoside. Thus, the equilibrium is shifted towards the product oligosaccharide and a number of glycans have meanwhile been synthesised using this relatively recent technology (Armstrong 2013, Rich 2012).



Scheme 7: A: Equilibrium in a retaining β-glucosidase.
B: Mutant retaining β-glucosidase where the catalytic nucleophile is substituted for a non-participating amino acid and thus allows for the synthesis of β-glucosides.
C: Glycosynthase-mediated synthesis of flavonoid glycosides (Yang 2007).

2.8.4 Chemo-enzymatic synthesis of oligosaccharides

The potential advantages of the combination of the individual strengths of chemical and enzymatic oligosaccharide synthesis offers are obvious. If a glycosyltransferase and its corresponding donor substrate are readily available, their application might remove the need for half a dozen synthetic steps due to the redundancy of protecting groups.

On the other hand, an oligosaccharide in question might have to be conjugated to a reporter group for diagnostics or might have to carry a modifiable linker at the reducing end to allow for immobilisation and so on, the possible applications are legion. In this case, the corresponding modified acceptor moiety will have to be chemically synthesised as it is likely that no enzyme exists for the step.

The continuation of the synthesis might then involve both chemical and/or enzymatic steps. Even the glycosynthase approach described in 2.5.3 is in fact a chemo-enzymatic one as most of the donors applied are non-natural glycosides (for instance carrying a para-nitrophenyl group as leaving group).

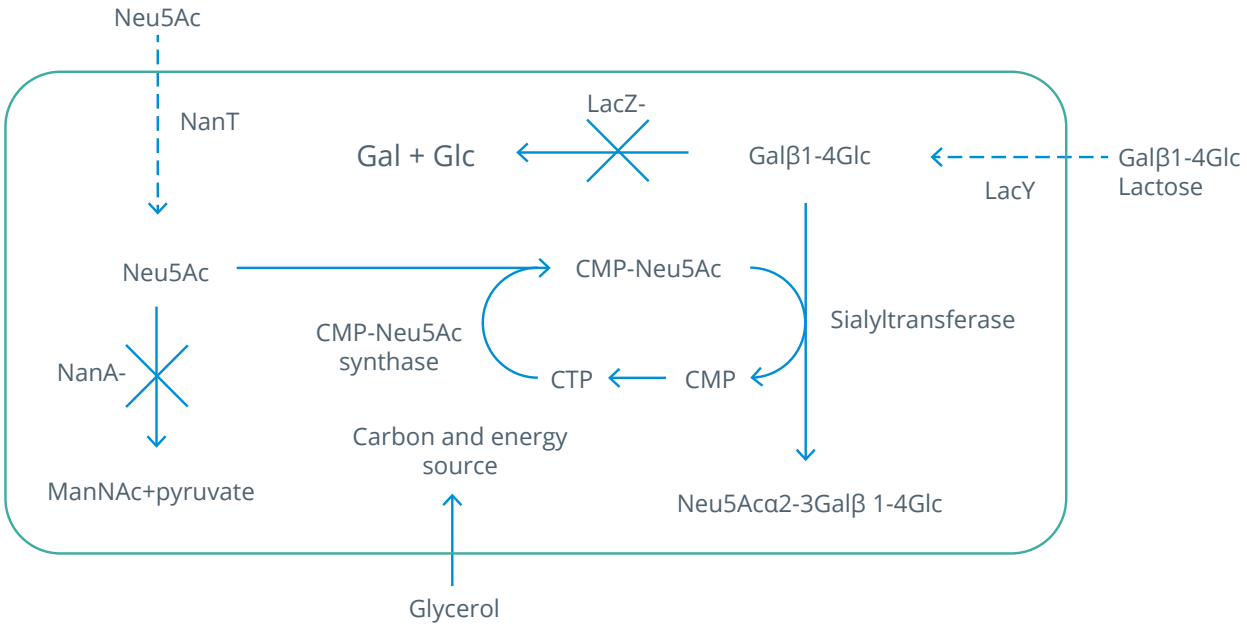
A relevant example, both from a medical and potentially commercial standpoint, would be the chemoenzymatic synthesis of structurally homogeneous ultra-low molecular weight heparins developed by Liu, Linhardt and colleagues (Xu 2011).

2.8.5 Oligosaccharide synthesis through fermentation processes

Recombinant DNA technology has already provided access to larger amounts of certain enzymes for oligosaccharide synthesis (see 2.5.3.) but it has also allowed for the synthesis of oligosaccharides in whole cells with only the need for feeding them certain basic substrates and a carbon and energy source. However, as the accumulation of one distinct molecule (that eventually will even become toxic for the cell) is not something cells normally have evolved for (some exceptions such as fat storage aside), some reactions within the cells sugar chemistry toolbox have to be interrupted in a targeted fashion. The following example describes the synthesis of the human milk trisaccharide 3'-sialyllactose (Neu5Acα2-3Galβ1-4Glc) in genetically modified *E. coli* K 12 bacteria (Priem 2002).

1. The building blocks lactose and N-acetylneuraminic acid (Neu5Ac) are both transported into the cell by respective permeases (the cell can live on Neu5Ac as sole carbon source for instance) but under normal conditions would then be degraded by a Neu5Ac aldolase (NanA) and β-galactosidase (LacZ), respectively. Therefore, both genes are knocked out and the engineered cells (NanA-, LacZ-) are now accumulating the building blocks for sialyllactose synthesis.

2. Neither the enzyme that synthesises the donor CMP-Neu5Ac from Neu5Ac (CMP-Neu5Ac synthase) nor the sialyltransferase (SiaT) that synthesizes 3'-sialyllactose from CMP-Neu5Ac and lactose are present in *E. coli* K12. Both genes are introduced into the cells through plasmids carrying the respective gene from N. *meningitidis* bacteria. The cells express both enzymes and thus 1.5g/L culture medium of the target sialyllactose is produced in less than a day, corresponding to a 49% yield. The carbon source used to feed the bacteria was cheap glycerol.

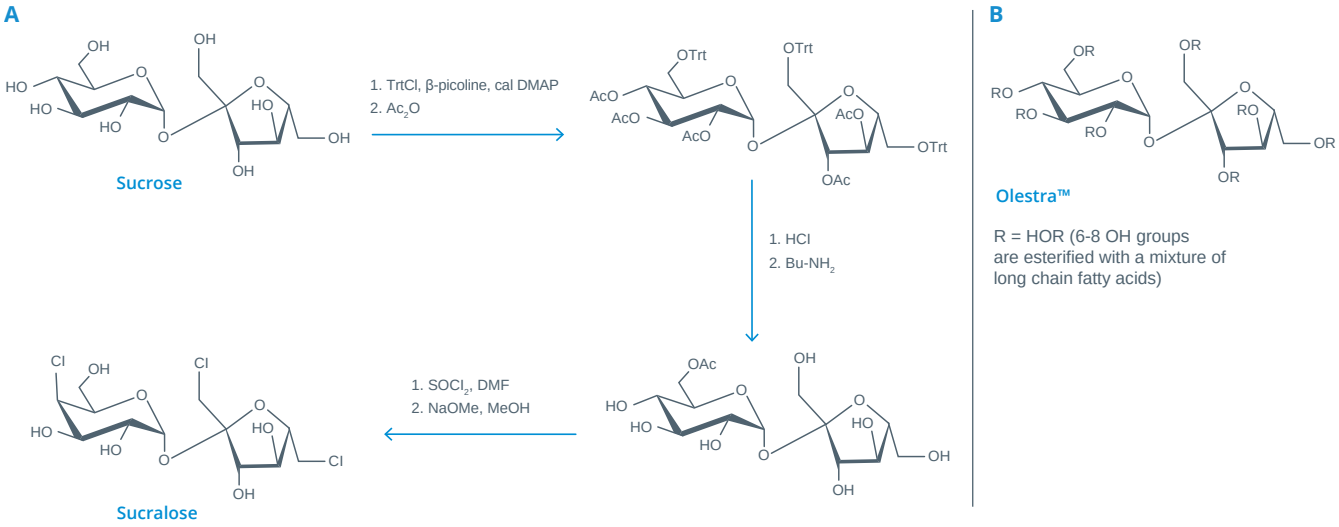


Scheme 8: Production of 3'-sialyllactose by genetically engineered *E. coli* K 12.

2.8.6 Chemical modifications of oligosaccharides

It may at times be desirable to chemically modify an existing oligosaccharide in order to enhance/decrease an existing property. This can range from interfering in biological recognition by changing lectin-binding to changing its ability to serve as substrate of a given enzyme to becoming an inhibitor of the respective metabolic reaction.

It may also be advantageous to confer entirely new properties to an oligosaccharide for uses in material science or the household and food industries. For instance, polyesterification of sucrose (Bernhardt 1988) has led to the fat substitute Olestra™, developed by Procter and Gamble whilst chlorination of some hydroxy groups in the same molecule leads to the Tate & Lyle's artificial sweetener sucralose (Splenda™). In 2006, the latter required amendment by the FDA of its regulations to allow for its inclusion as non-nutritive sweetener in food (Turner 2006).



Scheme 9: A: A chemical synthesis of sucralose from sucrose.
B: Structure of Olestra™, a mixture of long-chain fatty acid esters of sucrose.

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B

Section 3
Isolation and Recovery

3 Isolation and Recovery

As described in section 1, oligosaccharides occur in many environments. When they are free, they occur for example in the sap of plants, in milk and in blood. They can be attached to proteins, lipids and masked within polysaccharide structures. A recent review has addressed many of the issues relating to oligosaccharide production from matrices such as glycoproteins and glycolipids (Varki 2017). When produced synthetically, their environmental status will be dictated by the reaction sequence employed (reagents, solvents etc). In this section, their recovery and purification will be outlined.

3.1 Initial considerations

The environment of oligosaccharide containing material will dictate the initial procedures for product isolation. Most natural sources such as plant tissues, animal tissues or body fluids and microorganisms will contain unwanted components such as proteins, lipids and salts. Other potential hazards might include enzymes that could degrade the desired oligosaccharides.

Fig 42 Phenol-sulfuric acid test

Thus, it is important to pretreat the product-containing sources of oligosaccharides to ensure that they are released without damage and in as high a yield as possible. Methods for determining the carbohydrate content of extracts will be required and many of them are based on the use of sulfuric acid to form furfural derivatives which react with compounds such as phenols to form coloured products. Probably the best known of these methods is the phenol-sulfuric-acid method (Churms 1982 p178, Dubois 1956).

It is beyond the scope of this toolbox to review all the methods of preparation that have been employed, but a number of examples have been described (Churms 1982) and the nature of the plant cell wall has been discussed in a recent review (Burton 2010). Specifically, a process for treating plant material using ethanol (Holligan 1971) denatures unwanted enzymatic activity. Also, proteins and lipids can be removed from plant and bacterial preparations using appropriate protein and lipid cleaving enzymes although care must be taken in the isolation of oligosaccharides from glycoproteins and other sensitive molecules (Lawson 1969, Puri 2012).

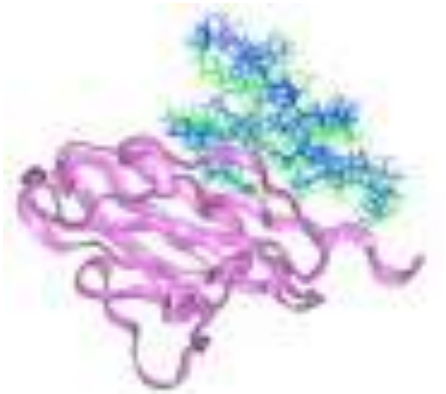


Fig 43 Typical glycoprotein showing glycosylation in blue/green (Courtesy Prof Liz Hounsell)

Other methods including ion exchange resins (fig 44) and dialysis (fig 44) are useful for removing unwanted inorganic contaminants (Wang 2016).

Fig 44 Ion exchange

Fig 45 Dialysis

3.2 Precipitation

For oligosaccharides, precipitation as a form of recovery is not in common use as the molecular weight of these molecules often precludes the use of solvents such as ethanol and isopropanol. However, high concentrations of ethanol (>70%) have been used to precipitate prebiotic galactosyl oligosaccharides (GOS) produced by β-galactosidase conversion of lactose, producing a mixture containing GOS, lactose, glucose and galactose. The addition of ethanol to aqueous solutions could be used to selectively precipitate and enrich GOS from this reaction mixture (Sen 2011).

Fig 46 Precipitate from solution

Fig 47 Oligosaccharide precipitate

3.3 Solvent extraction

The effect of extraction solvents and temperatures on the extraction yields of sucrose, and raffinose based oligosaccharides from plant materials was investigated (Johansen 1996). Toasted soybean meal, cotton seed meal, field peas, and a feed mixture were extracted in either water, 50% (v/v), or 80% (v/v) aqueous methanol or ethanol at 20 or 50 °C or at the boiling point of the solvent. Extraction in 80% (v/v) alcohol was strongly influenced by the extraction temperature and maximum extraction was only achieved at the boiling point.

3.4 Concentration and drying

Oligosaccharides in solution must be concentrated as part of the purification and isolation process. In the laboratory, this can be done in a number of ways including rotary evaporation (fig 48) in which the liquid is removed from a rotating film in a vacuum at reduced temperatures.

Another useful technique is lyophilisation (freeze drying, fig 49) where water is sublimed from the frozen state.

Fig 48 Rotary evaporation

Fig 49 Freeze drier

3.5 Oligosaccharide release

As mentioned in the introduction to this section, oligosaccharides are often bound by covalent bonds within structures such as glycoproteins, glycolipids and polysaccharides. Many methods have been reported for the release of oligosaccharides including the use of PNgaseF for the release of *N*-linked glycans from glycoproteins, for example (Goodarzi 1998) and for *O*-linked glycans using chemical methods including β-elimination and hydrazine (Karlsson 2017). For polysaccharides where oligosaccharides are contained within the covalently bound sugar chains, methods of release by chemical and enzymatic methods are mentioned in the table in Section 1 and references are given.

3.6 Separation

The liberation of the carbohydrate moieties of glycoproteins or glycopeptides can be achieved chemically or enzymatically. Chemically, the action of alkali in the presence of sodium borohydride liberates by β-elimination, the reduced glycans, which are *O*-glycosidically linked to serine or threonine residues *N*-glycosidically linked glycans can be removed by hydrazinolysis or by alkaline cleavage.

The enzymatic cleavage of the sugar-peptide linkage is carried out with specific endoglycosidases, such as peptide-*N*-glycosidase from *Flavobacterium meningosepticum* and *N*-glycanase F active on the glycoasparagines, glycopeptides, and glycoproteins, or the aspartyl-*N* -acetyl-glucosaminidase active on glycoasparagines only. Other enzymes cleave sugar-sugar glycosidic bonds, liberating part of the glycan as in the case of endo-A'-acetyl-β-D-glucosaminidases, which liberate *N*-glycans without the terminal *N*-acetylglucosamine residue, which remains linked to the asparagine residue (Montreuil 1996).

Fig 50 Thin layer chromatogram

Most of the released oligosaccharides will be in mixtures and a simple method of following reactions is by thin layer chromatography (fig 50). Plates coated with silica gel are used and 'spotted' with the oligosaccharide mixtures, eluted in an appropriate solvent (see Churms 1982), dried and visualised using typically a 5% solution of sulfuric acid in methanol that chars on drying as shown below.

Analytical techniques such as Dionex ion chromatography, HPLC and electrophoresis will also be discussed in section 4.

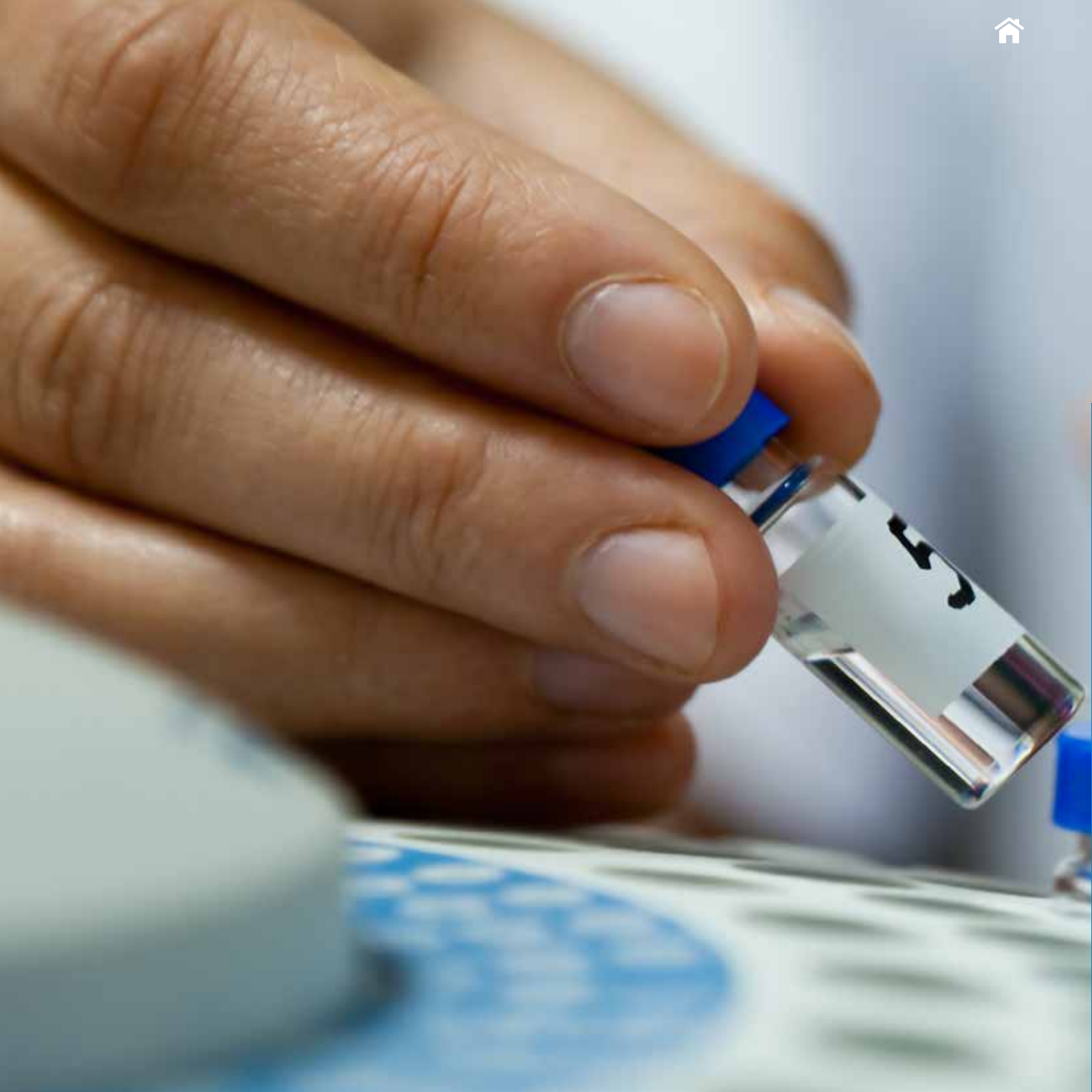
3.6.1 Preparative chromatography (silica gel, ion exchange, gel permeation)

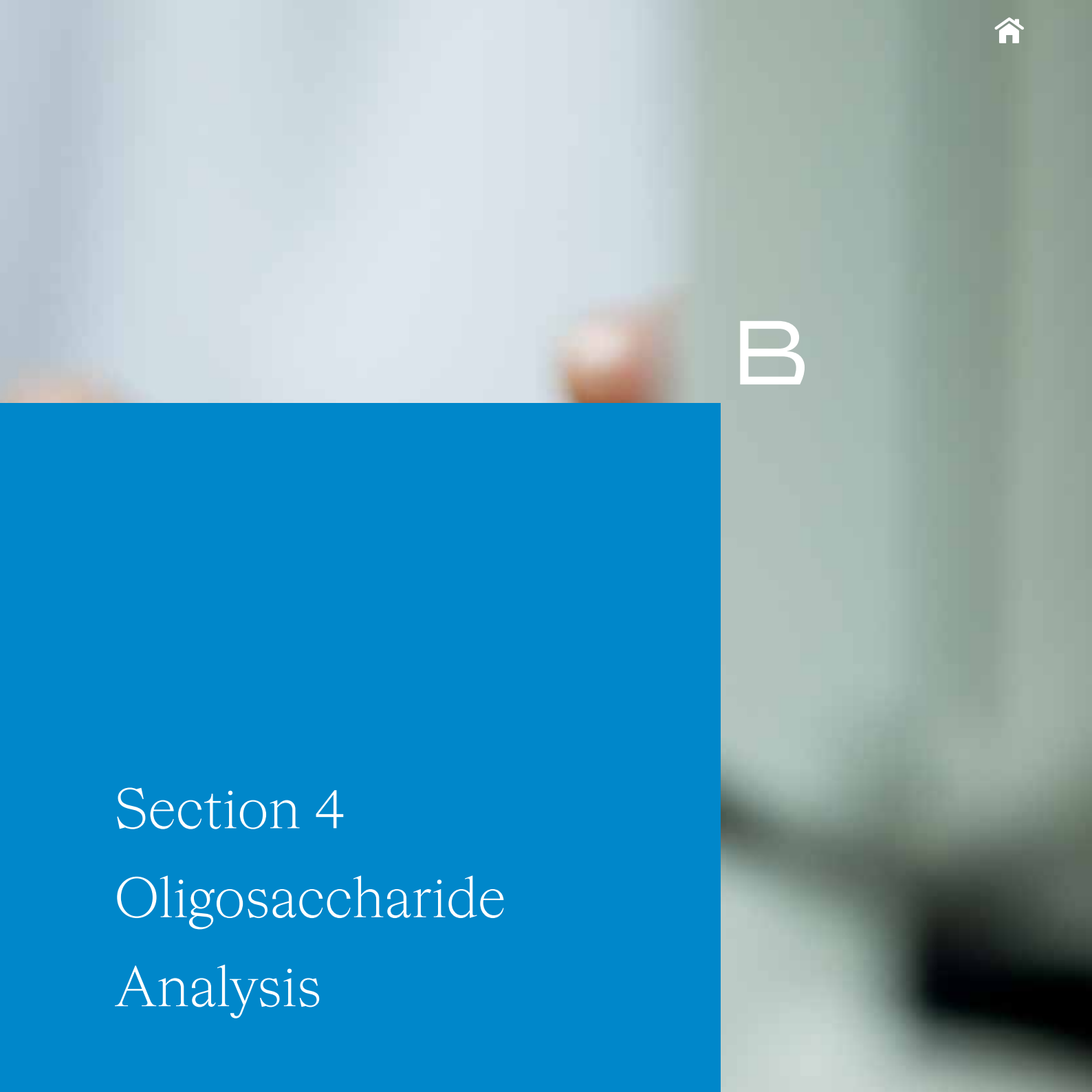
Many chromatographic techniques have been developed for separating both neutral and charged oligosaccharides from milligrams to multiple gram quantities. Packings employed include silica gel, porous graphitic carbon, TSK gel and cellulose (e.g. DEAE Sephadex). The methods of detection most used are ultraviolet, refractive index, pulsed Amperometric, mass, MALDI and fluorophore tagging, and the area has been comprehensively reviewed elsewhere (Hicks 2002, Wang 2016).

Fig 51 Preparative chromatography

An example of this methodology is the use of preparative electrophoresis to separate, recover and analyse (mass spectrometry) glycosaminoglycan oligosaccharides. This yielded a quantitative amount of sulfated oligosaccharides from excised gel bands. The compatibility of obtained oligosaccharides for subsequent MS analysis was ensured using a single, simple clean-up step on a mixed C18/graphite carbon solid-phase column that was fully effective for polymerization degrees ranging from di- to dodecasaccharides (Bodet 2017).

Fig 52 Preparative electrophoresis





B

Section 4
Oligosaccharide Analysis

4 Oligosaccharide Analysis

4.1 Introduction

Having worked up samples as described in section 3, they must be rigorously analysed. The strategies employed will be dictated by the amount of material available and this will depend to a large extent on the source.

For free sugars such as those found in human milk, urine and meconium, the amounts will range from grams to milligrams. From plants (seeds and fruits), bacterial, fungal (cell walls and exocellular) and animal tissues, the range will be in gram quantities.

When polysaccharides are used to provide the oligosaccharides by degradation, the amounts will depend on the starting quantities but may be tens of grams to kilos.

For glycoproteins, the quantities of released glycans will be much lower, often in microgram quantities.

The quantities of synthetic oligosaccharides available will depend on the starting amounts, the number of reaction steps and yields and the loss of weight from the blocking/deblocking strategies.

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4.2 Analytical strategies for milligrams or greater

For samples in this range it will be possible to use both spectroscopic and chemical methodology.

4.2.1 Chemical methods

4.2.1.1 Phenol-sulfuric acid analysis

As described in section 3, this is a quick and straightforward method for determining carbohydrate content and is sensitive to ~10mg/ml (Churms 1982 p 178, Dubois 1956).

4.2.1.2 Carbohydrate profile by chromatography

The initial requirement will be to identify and quantify the monosaccharide residues that make up the oligosaccharide. The usual way of determining this is by chemical or enzymatic degradation and the process of hydrolysis can be followed in a rapid and simple manner by thin layer chromatography (section 5.2.1.2.1). More comprehensive analysis is undertaken by quantitative chromatography with known internal standards (White, 1991, pp 293-333).

In order to release monosaccharides, it is necessary to employ reagents that can selectively attack (protonate) the glycosidic oxygen atom between each sugar residue. The selection of suitable reagents requires a degree of insight, trial and error too involved for discussion here, but useful candidates include acids (acetic, hydrochloric, trifluoroacetic, methanolic HCl) and enzymes (hydrolases).

A number of oligosaccharides will have glycosidic bonds which are resistant to hydrolysis e.g. polyuronides (containing carboxyl groups) when much stronger conditions will be required to break these but do not destroy the sugar residues themselves (Churms, 1982, pp 215-217).

It may also be necessary to undertake preparative chromatography in order to subject the recovered monosaccharides to more rigorous analysis (NMR, FTIR etc.) as outlined below. The oligosaccharide discussed above is likely to have been separated from a mixture such as maltooligosaccharides from starch or sulfated gycans from glycosaminoglycans and this is another process in which chromatography plays a crucial part.

4.2.1.2.1 Thin layer chromatography

In this method, pre-prepared silica gel plates are spotted with the oligosaccharide hydrolysate plus known standards of the monosaccharides that are thought to be present. The plates are then dried and then eluted in an appropriate solvent (Churms 1982), dried and dipped in a mixture of 5% conc. sulfuric acid in methanol. On drying, the plate then reveals spots as shown in fig 53.

Fig 53 Thin layer chromatogram

4.2.1.2.2 Paper chromatography

The earliest methods of chromatographic analysis were based on the elution of components on paper and visualisation by spraying or dipping the papers in a wide variety of reagents (Whistler 1962) (Fig 54).

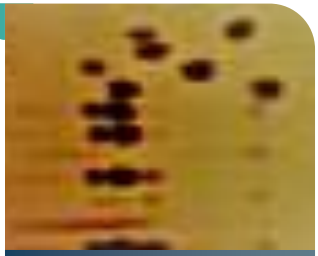


Fig 54 Paper chromatogram of monosaccharides from the hydrolysis of oligosaccharides

4.2.1.2.3 Gas chromatography

Both analytical and preparative methods were developed using this technique but were superseded by gas chromatography, a method that was difficult due to the need for volatile derivatives, not liked by carbohydrate analysts and not possible to do preparatively (Fig 55).

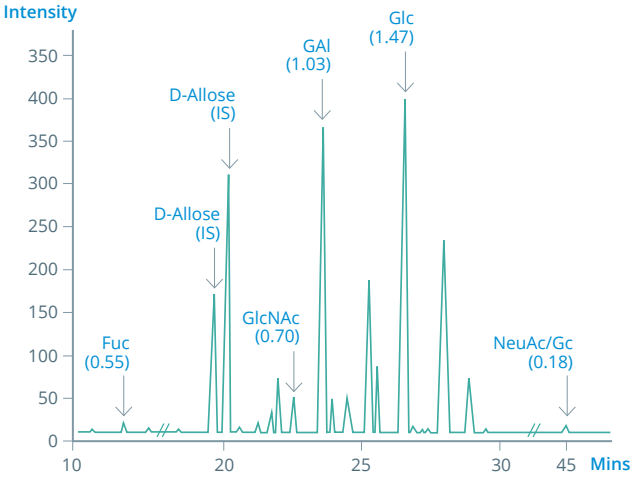


Fig 55 Gas chromatogram of monosaccharide trimethyl silyl ethers

4.2.1.2.4 High pressure/performance liquid chromatography

In the 1970s, high pressure/performance liquid chromatography (HPLC), a method that does not require derivatization, came into use and is now used in virtually all analytical laboratories worldwide. Early detectors such as refractive index are insensitive and were affected by solvents/temperature, and UV did not work very well as carbohydrates have no chromophore but these have improved and can now cope with gradient elution to enhance the separation of components.

Better column packings such as C18 reversed phase silica gel and porous graphitic carbon (Knox 1986) have emerged for carbohydrates and Dionex Ion Chromatography (fig 53) is followed by an inert PEEK splitter that transfers the effluent to the integrated pulsed amperometric detector (IPAD) and to an on-line single quadrupole mass spectrometer (MS) (Bruggink 2005).

Fig 56 Dionex chromatography of carbohydrates.

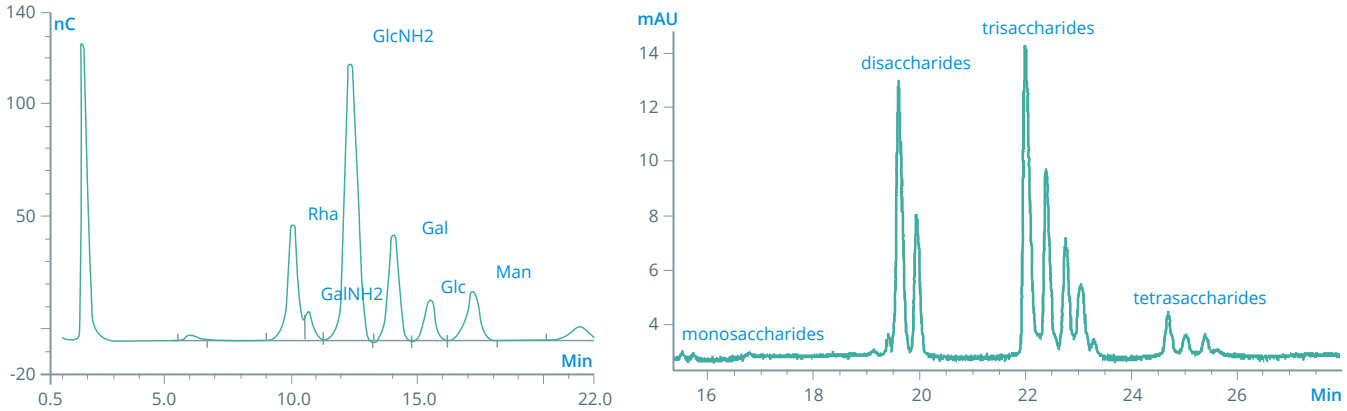


Fig 57 HPLC chromatograms

4.2.1.3 Carbohydrate profile by electrophoresis

4.2.1.3.1 Introduction

Electrophoresis is now a useful technique for the analysis of mono- and oligosaccharides. In past, the major problem was the lack of chromophores in most carbohydrates and this has required the development of precolumn derivatization with various tags.

The merits and drawbacks of the derivatizing agents, include 2-aminopyridine, 4-amino-benzoic acid and its analogues, which for the first time permitted the reproducible determination of aldoses, uronic acids and even ketoses in the low femtomole range by means of readily available UV detection, and other agents such as 8-aminonaphthalene-1,3,6-trisulphonic acid, 1-phenyl-3-methyl-5-pyrazolone and 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde. Based on the progress made, it can be concluded that capillary electrophoresis represents a powerful alternative and complement to existing methodology in the area of carbohydrate analysis.

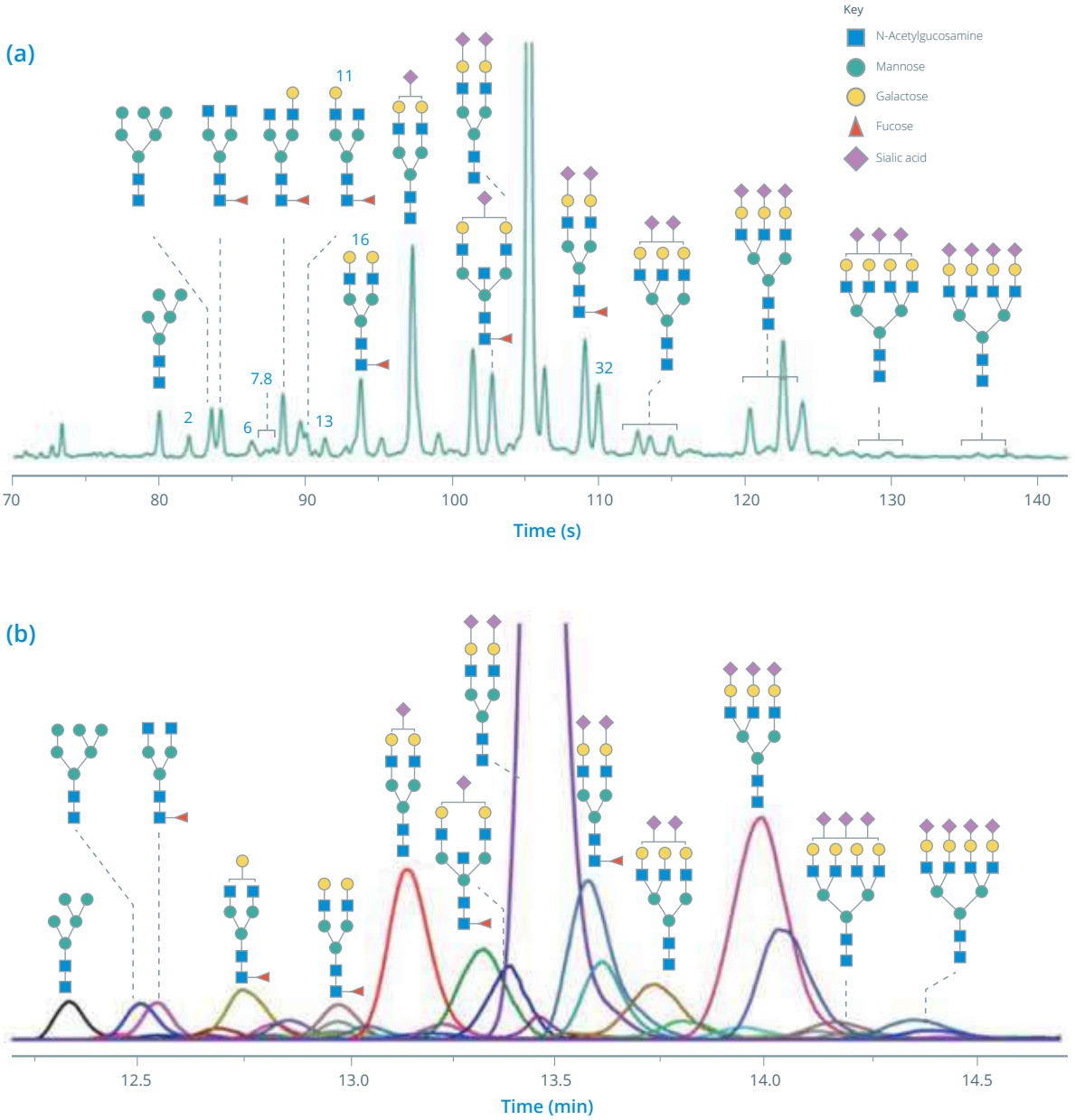


Fig 58a is an electropherogram of methylamidated, 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled N-glycans derived from human serum and analyzed by microchip electrophoresis (Snyder 2017).

Fig 58b is an extracted ion electropherogram of methylamidated, (APTS)-labeled N-glycans derived from human serum and analyzed by CE-MS (Snyder 2017).

4.2.1.3.3 Polyacrylamide-fluorophore assisted carbohydrate electrophoresis (FACE)

In the example shown below, the progress of a brewing process to produce lager beer (fig 59) is followed by FACE (fig 60). Lanes 1, 5 and 8 are standard ladders of maltooligosaccharides produced by the hydrolysis of starch. Lanes 2,3,4 6 and 7 show the progress of the fermentation from left to right as the glucose and oligosaccharides are consumed in the process (Lawson-unpublished).

Fig 59 Lager beer

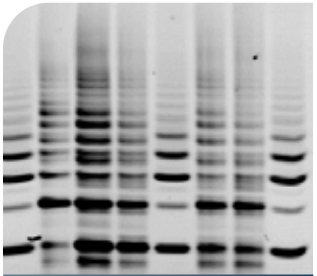


Fig 60 Electrogram of fermented lager

4.2.1.3 Smith periodate oxidation

The Smith periodate degradation aims at selectively degrading an oligosaccharide, from which structural information can be deduced. Hexose residues having free hydroxyl groups at C2 and C3 will give erythritol and glycolaldehyde when subjected to the Smith degradation, hexose residues with free hydroxyl groups at C3 and C4 will provide glycerol and glyceraldehyde, and nonreducing terminals with free hydroxyl groups at C2, C3, C4 and C6 will give glycerol, glycolaldehyde and formic acid. However, hexose residues without any vicinal hydroxyl groups will not be affected by periodate oxidation and remain as such after hydrolysis. The quantitative analysis of these cleavage products, erythritol, glycerol, glyceraldehyde and glycolaldehyde provides much information about the nature of glycosidic linkages in oligosaccharides (Abdel-Akher 1952).

4.2.1.4 Methylation

The oligosaccharide is treated with reagents that selectively convert the free hydroxyl groups to methyl ethers. Methyl ethers are very stable and the methylated oligosaccharide can then be hydrolysed to release monomers that are characteristic of the original oligomer, having free hydroxyl groups where the linkages had previously been.

In the experimental protocol, the released methylated monomers are reduced to alditols and acetylated, and these are characterised as partially methylated alditol acetates by a combination of gas chromatography and mass spectrometry (GC/MS).

This method has become the standard for linkage analysis in most laboratories throughout the world (Bouveng, 1960, Hakomori, 1964, Bjorndal, 1970). Two examples are given, namely a trisaccharide isolated from the acetolysis of xanthan gum (fig 62) (Lawson 1977). The mass spectrum shows evidence for 1,4-glucose linkages, terminal glucose and terminal mannose.

Fig 61 Mass spectroscopy

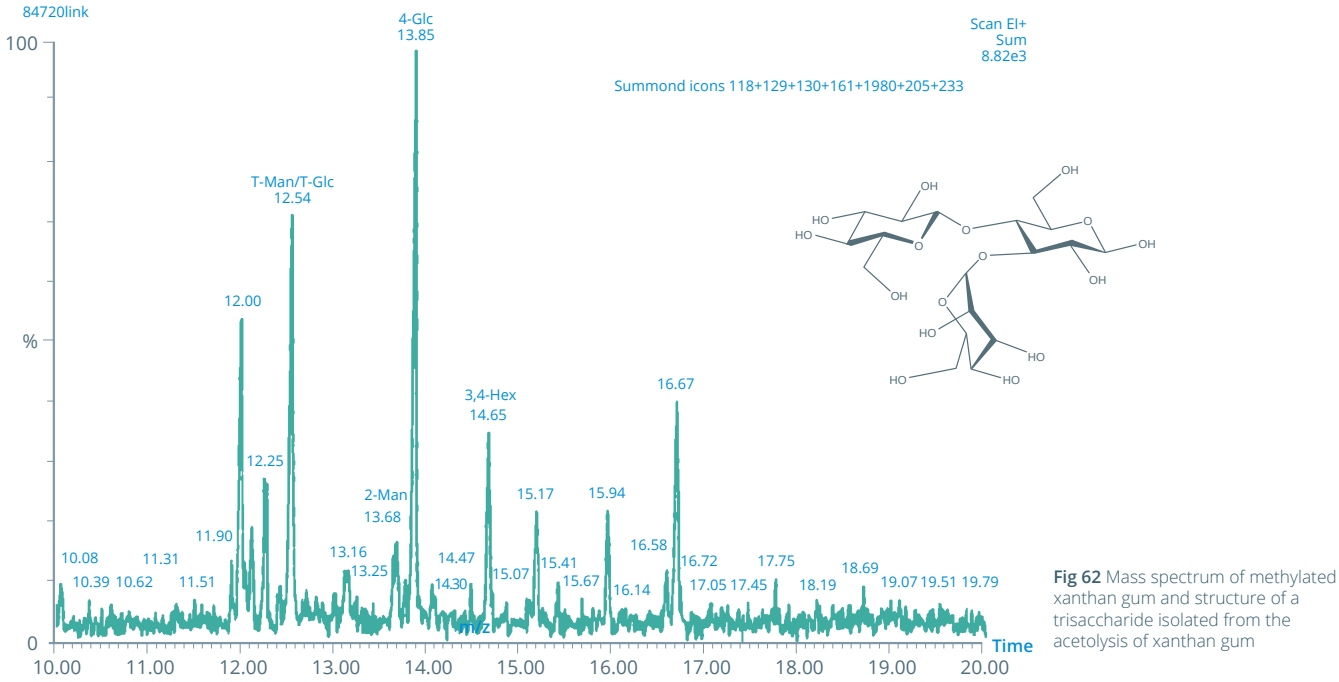
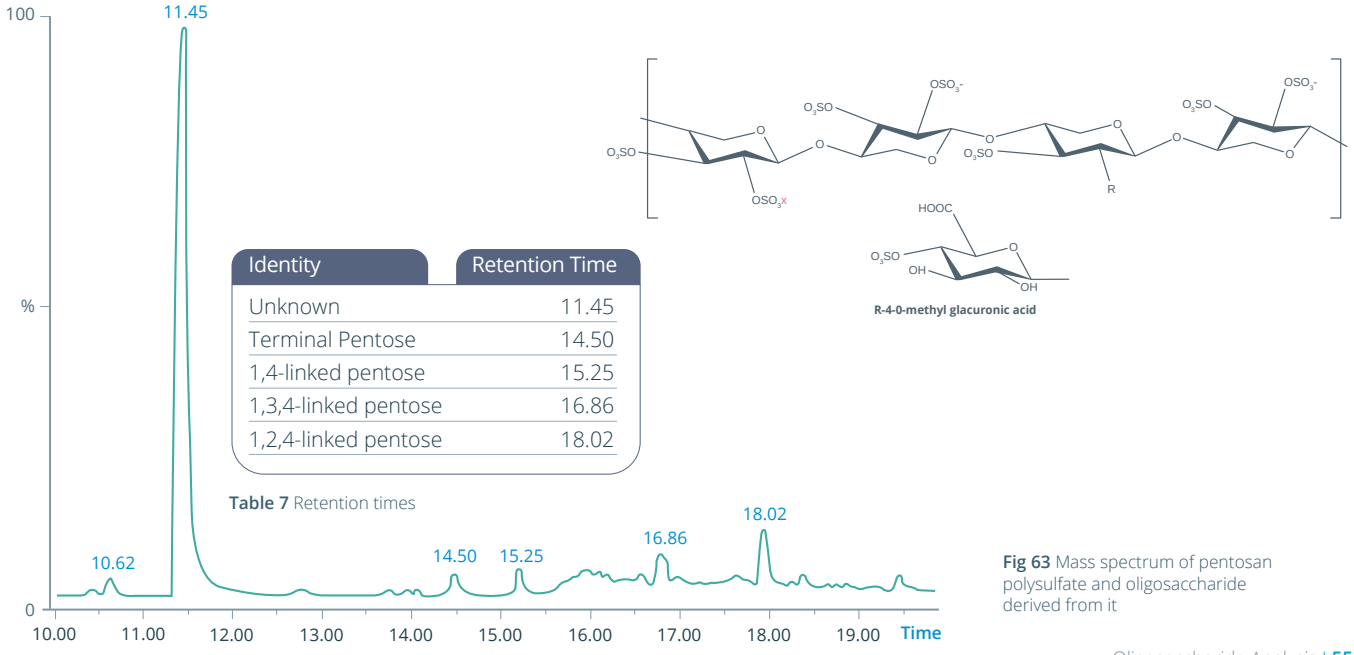


Fig 62 Mass spectrum of methylated xanthan gum and structure of a trisaccharide isolated from the acetolysis of xanthan gum

The second example is taken from a methylation study of the therapeutic oligosaccharide product pentosan polysulfate. As can be seen from the table 7, the spectrum (fig 63) shows evidence for terminal xylose residues and chain linkages (Lawson, unpublished results 2015).



Identity	Retention Time
Unknown	11.45
Terminal Pentose	14.50
1,4-linked pentose	15.25
1,3,4-linked pentose	16.86
1,2,4-linked pentose	18.02

Table 7 Retention times

Fig 63 Mass spectrum of pentosan polysulfate and oligosaccharide derived from it

4.2.2.1 NMR spectroscopy

NMR spectroscopy (fig 64) is one of the most important techniques in the elucidation of oligosaccharide structures (Hounsell 1995). Both proton ^{1}H and carbon ^{13}C spectra are of value as are 2D experiments such as NOESY for sequence and conformational analysis (Hounsell 1990 Donald 1986). The accepted value of NMR has long been regarded as best for pure samples >~1mg for ^{1}H spectra and >~10mg for ^{13}C spectra (Robyt 1998).

However, as discussed below, with the introduction of instruments operating at very high field strengths coupled with new experimental procedures and data manipulation, the method can produce acceptable information from much lower sample sizes.

Fig 64 Nuclear magnetic resonance (NMR)

4.2.2.1.1 ^{1}NMR spectroscopy

A useful attribute of the one dimensional proton spectrum is to fingerprint an oligosaccharide. A database of spectra of defined oligosaccharide structures is employed to match the unknown and if an exact match is found, the two structures must be identical. The technique is fast, requires about 50 nanomoles of material, and is very widely used. However, care must be taken to record spectra of the unknown compound under identical conditions (for example, pH, temperature, solvent, ionic strength) as those used in the spectral database.

A further use of the "fingerprint" method is to identify known structural units within a novel structure, but this must be done with care. As the NMR spectrum of any given nucleus depends on the local structure around a particular nucleus, a given structural unit is likely to make a similar contribution to the overall spectrum. For example, certain monosaccharides can be easily identified by the presence of well-resolved, characteristic signals (e.g. fucose gives a three-proton doublet at 1.2 ppm, while sialic acid gives two one-proton multiplets at 2.7 and 1.8 ppm). An extension of this concept is that a given oligosaccharide has a similar spectrum independent of the moiety to which it is linked.

In practice, it is not usually necessary to match the entire oligosaccharide spectrum, but only to concentrate on specific regions that contain particular diagnostic signals from "structural reporter groups" (Vliegenthart 1981, 1983). With the advent of much more sensitive instruments plus techniques such as computer averaging of transients (CAT), high field 1H and 13C-NMR was introduced for the determination of glycan primary structure based on the structural reporter group signals that contain the essential information for translation into primary structures. Techniques of this type are most useful for categories of glycans with a defined range of substructures, for example mammalian N-linked glycans of glycoproteins.

The reporter groups comprise:

- Anomeric protons,
- Protons attached to carbon atoms close to the substitution position in a monosaccharide constituent (so-called glycosylation shifts),
- Protons at desoxycarbon atoms,
- N-acetyl and N-glycosyl methylene protons,
- Protons shifted as a result of the presence of noncarbohydrate substituents.

In the proton spectrum of an oligosaccharide, the most important regions are the anomeric ($C^{1}H$) region from about 4.2 to 5.5 ppm (fig 65) ring protons from 3.2-4.0 ppm and the lowfield region from about 1.0 ppm to 3.0 ppm containing for example, methyl resonances.

Anomeric protons are in the region 4.2-5.2ppm, the ring protons 3.2-4.0 ppm, HOD at ~4.62 and the fucose methyl group at ~1.2ppm.

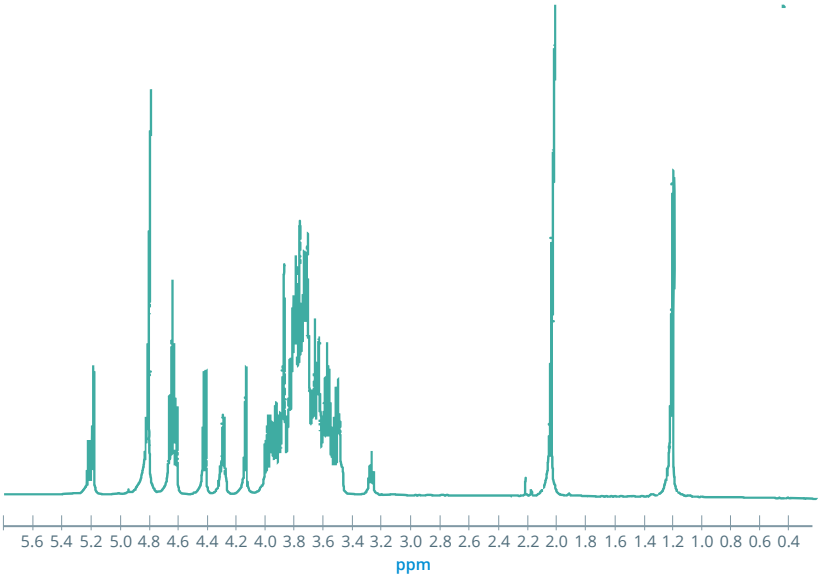
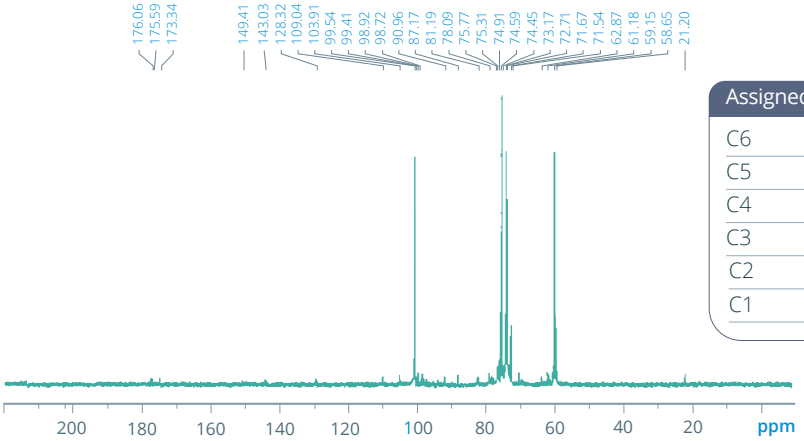


Fig 65 500MHz spectrum of LNFP1 in D_2O (Donald 1988). (Spectrum courtesy of Dr Jim Feeney)

4.2.2.1.2 ^{13}C-NMR spectroscopy

The proton-decoupled ^{13}C-NMR spectrum of oligosaccharides (fig 66) gives a signal for each of the specific types of carbons present. For highly asymmetric carbohydrate units, this usually means a signal for each of the carbons in the asymmetric unit. This has been demonstrated for many oligosaccharides (Gorin 1981). The ^{13}C spectrum shown is for the heptasaccharide pentosan polysulfate and shows the carbon assignments in the table.



Assigned Carbon	Chemical Shift	Type of Carbon
C6	58.40	$-OCH_3$
C5	58.91	$-CH_2$
C4	72.32	-CH
C3	72.81	-CH
C2	74.31	-CH
C1	99.38	-CH

Fig 66 ^{13}C NMR Spectrum of the heptasaccharide pentosan polysulfate

For oligosaccharides, each sugar residue can be regarded as an isolated spin system, so by irradiating a single proton the rest of the spin system can be revealed making it possible to differentiate all the protons of a specific sugar residue. For example, in the proton TOCSY (TOtal Correlated SpectroscopY) spectrum shown for sucrose, the red circles show the connections between proton 4 and all the other protons of the glucose ring. TOCSY is sometimes called "homonuclear Hartmann–Hahn spectroscopy" (HOHAHA) (fig 67).

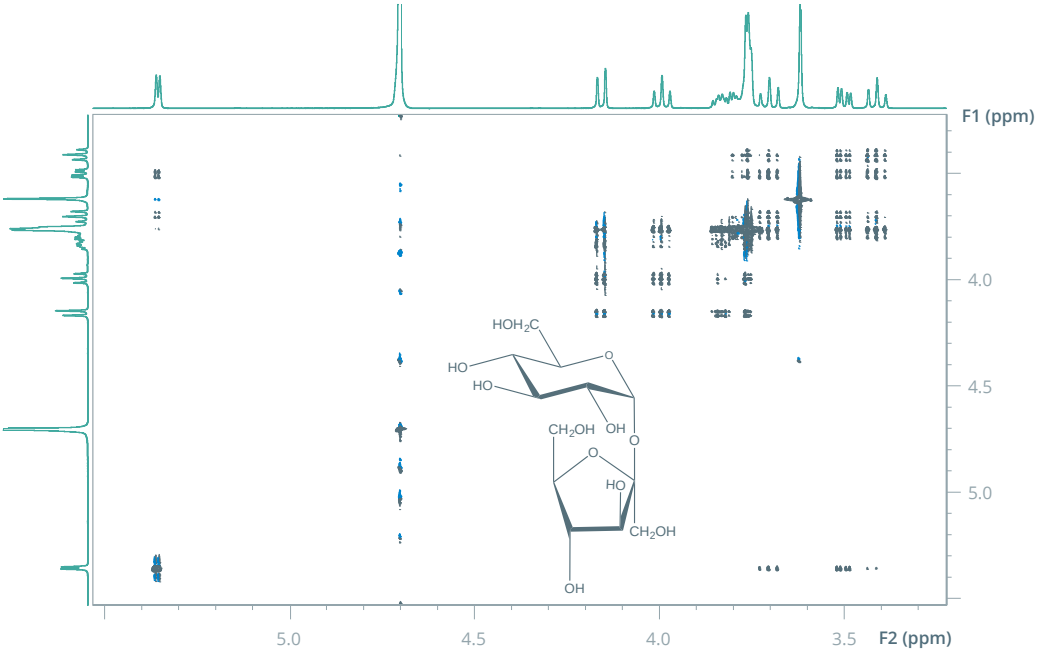


Fig 67 TOCSY spectrum for sucrose

In addition, two-dimensional NMR spectra such as correlation spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear multiple-quantum coherence spectroscopy (HMQC), and heteronuclear multiple bound correlation spectroscopy (HMBC), are used to obtain unambiguous assignments. The example given (Fig 68) is of an acetylated xylooligosaccharide having an average DP of 7.

Heteronuclear single quantum coherence spectroscopy (HSQC) is used to correlate the chemical shift of protons (displayed on the F2 axis) to the ^{13}C chemical shift (on the "indirect," F1 axis) of their directly attached carbons via the $^{1}J_{CH}$ coupling. Taking the natural abundance of ^{13}C into account, roughly ever y 100th molecule responds in the HSQC experiment (Foston 2016, Alekseeva 2020).

Besides these commonly used 1D and 2D techniques, a wide range of additional NMR experiments have been devised to address the conformation and dynamics of glycans, both free and bound to proteins. A concise account of recent developments is given by Gimeno *et al.* 2020.

Combinations of the various NMR techniques have been successfully applied to identify and quantify binding of biologically relevant oligosaccharides to their protein receptors. Clean TOCSY for instance, a spin-lock pulse sequence that reduces signals depending on the ROESY effect whilst keeping signals depending on scalar coupling (TOCSY effect) has been used to identify ligands binding to E-selectin, the receptor for the sialyl-Lewis^x tetrasaccharide (Egger 2013, Otting 1993). This recognition event is crucial for leucocyte recruitment to sites of inflammation. Addition of a known sialyl-Lewis^x mimetic, equipped with a nitroxyl spin-label, then allowed the evaluation of a library of antagonists binding to secondary, hitherto unknown, binding sites using paramagnetic relaxation enhancement (PRE NMR) experiments (Egger 2013).

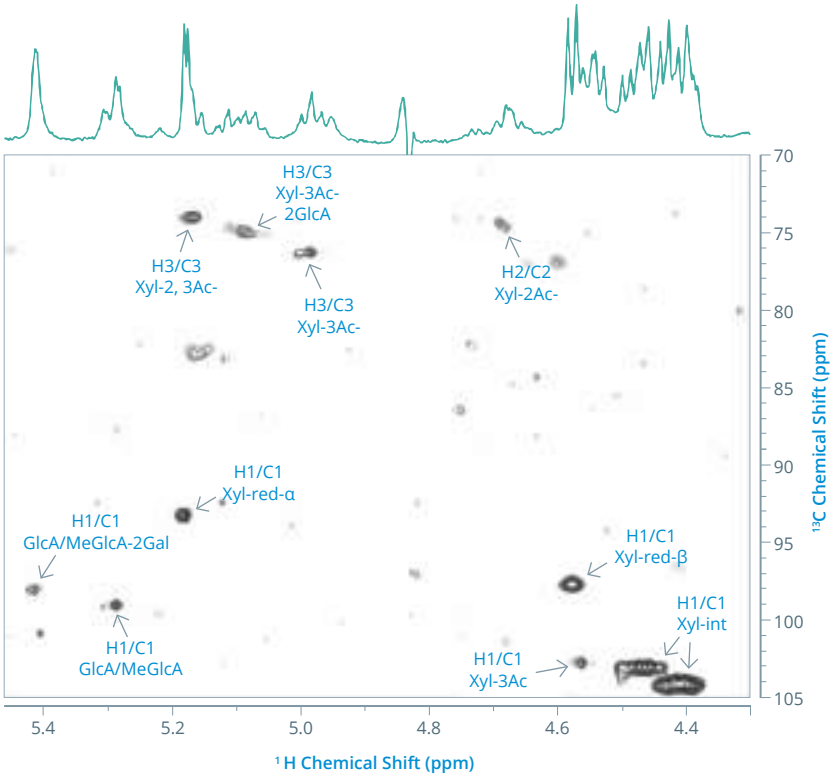


Fig 68 Acetylated pentosan HSQC NMR spectrum

A number of oligosaccharides are insoluble or sparingly soluble in solvents such as D_2O, the solvent commonly used for NMR experiments to suppress the water signal by replacing hydrogen with deuterium. Examples are oligosaccharides based on cellulose (cellotriose, cellotetraose, etc.) and Zymosan, a glucooligosaccharide from *Saccharomyces cerevisiae*. This technique has been attempted in following the removal of lignin in the purification of an acetylated xylan found in beechwood to produce pentosan polysulfate, a drug used in the treatment of interstitial cystitis (table 11 & Fig 69). In cases like this, the use of solid-state NMR would have been attractive except that until recently, the spectral resonances were too broad for the technique to be of great value.

However, recent publications have described methods of treatment that provide spectra that are much more acceptable. An example of this is the amination of Zymosan to produce acceptable spectra (Arumugam 2020).

Further, solid state NMR is now used for analysing solid-state forms of drugs. In the ^{13}C CP/MAS NMR spectra of the solid dosage forms, many of the signals originate from the excipients and can be distinguished from those of the active pharmaceutical ingredients. A recent report describes the successful analysis of the most common pharmaceutical excipients used in solid drug formulations such as anhydrous α-lactose, mannitol, sucrose, sorbitol, starches of different origin, microcrystalline cellulose, hypromellose and methylcellulose (Pisklak 2016).

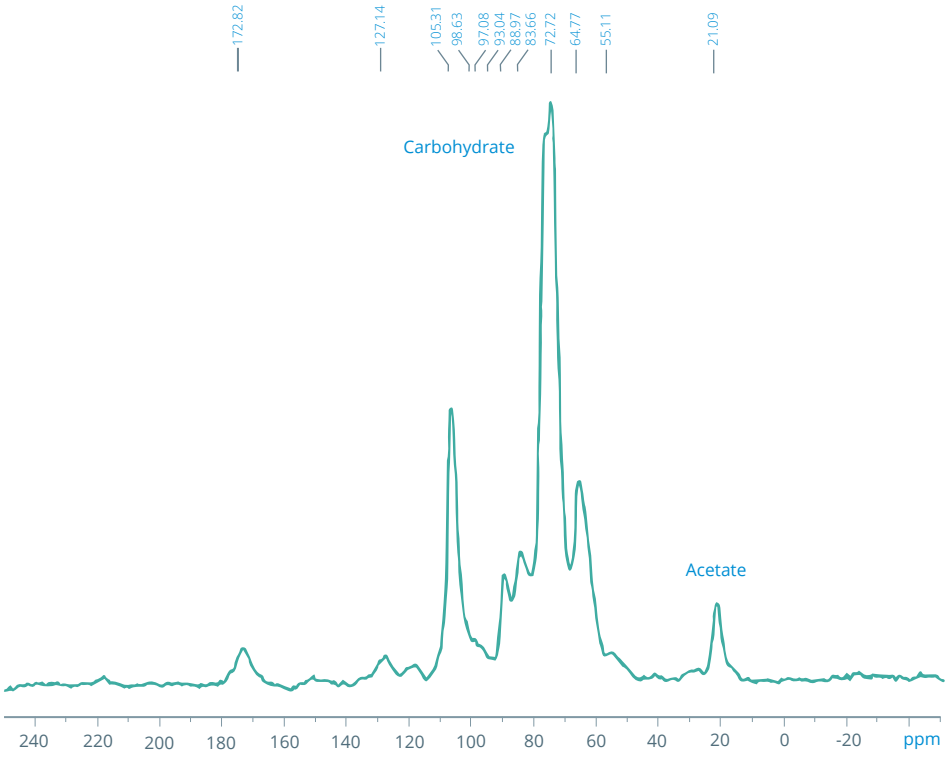


Fig 69 Solid state ^{13}C spectrum of pentosan (xylan) in the presence of lignin

4.2.2.2 Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a very useful analytical technique which can highlight structural features not shown up with other techniques (Kacurakova, 2001).

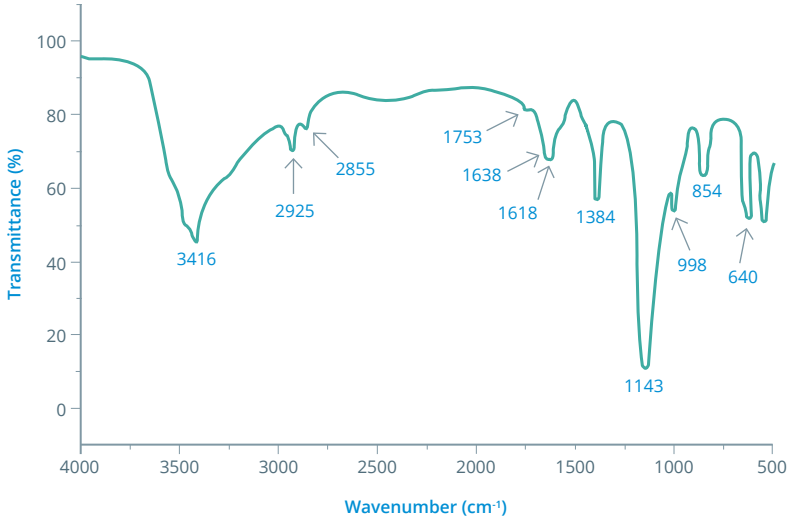


Fig 70 FTIR of oligosaccharides from starch

Two spectral regions are important for structural characterization of oligosaccharides. These are the "sugar region" (1,200-950 cm^{-1}) and the "anomeric region" (950-750 cm^{-1}). The highly overlapping intense bands of CO and CC stretching vibrations are in the former region. The latter region contains vibrations sensitive to the anomeric structure. Bands at 3,405 and 2,930 cm^{-1} are assigned to OH and CH bond stretching, respectively and at 1,420 and 1,366 from bending modes of CH_2, CH and OH.

4.3 Analytical strategies for sub milligram quantities

As mentioned earlier, in the 1970s the analytical techniques of HPLC coupled with sensitive pulsed amperometric (PAD) and mass detectors came into use. Also, NMR spectroscopy became much more powerful with the advent of supercooled magnets and increased computing power for data processing and Multi Angle Light Scattering coupled with Time of Flight Mass Spectroscopy was introduced. There were also many other innovations in the field of analytical chemistry with the result that oligosaccharides occurring in low abundance (< 1 mg) could be isolated and analysed.

This analytical capability has been applied, in particular to glycans from glycoproteins and glycolipids and many of the low abundance oligosaccharides in human milk, urine, body fluids and the surface of tumors. The role of oligosaccharides in disease states has been recognised and can now be addressed (Hennet 2009).

Similar to the analytical strategy described in section 4.2, the elucidation of the covalent structure of these glycans involves:

- The identification and determination of the constituent monosaccharides including the type, number, and location of noncarbohydrate substituents such as the methyl, pyruvyl and phosphoryl groups,
- The sequential arrangement of monosaccharide residues as well as the linkage between them and the definition of the anomeric configuration of the glycosidic bonds.

And in addition:

- The definition of the nature of the glycan-peptide linkage (Dwek 1993, Hounsell 1993, Verbert 1995).

4.3.1 Monosaccharide composition of glycans

For analysis of the monosaccharide composition, methods for this have been outlined in section 4.212 but in addition methanolysis, followed by a re-*N*-acetylation, trimethylsilylation, and gas-liquid chromatography (GLC) or gas-liquid chromatography-mass spectrometry (GLC-MS) can be employed for these glycans (Churms 1982).

4.3.2 Determination of glycan covalent structure

The huge advances in the determination of glycan covalent structure have been discussed in the introduction to this section and in summary are:

- The splitting of glycosidic bonds either by chemical cleavage-hydrolysis, acetolysis, hydrazinolysis, or by nitrous deamination,
- Methylation followed by hydrolysis or methanolysis and by GLC-MS of the partially methylated monosaccharides,
- The definition of the anomeric linkages between monosaccharides using exoglycosidases, which, in addition, can provide some insights into the glycan structure by sequential degradation.

The most important analytical advances have been in the fields of nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS).

4.3.2.1 Nuclear magnetic resonance spectroscopy

This method was described in the section 4.2.2.1.

Recent developments in mass spectrometry, particularly fast atom bombardment mass spectrometry (FAB-MS), electron-spray mass spectrometry (ES-MS) (Harvey 2008), mass spectrometry-mass spectrometry (MS-MS), and "matrix assisted laser desorption time of flight mass spectrometry" (MALDI-TOF-MS), have provided detailed structural information such as branching pattern number and length of branches and sequence.

A particularly useful aspect of MALDI-TOF has been in analysing the number of moles of an oligosaccharide conjugated to an unglycosylated protein such as Bovine Serum Albumin (BSA). The conjugate allows antibodies to be raised in laboratory animals and it is crucial to know how many moles of oligosaccharide are on the conjugate as this determines whether the experiment is likely to be successful. (Schocker 2015) (Fig 71).

Of the top 20 best-selling biopharmaceutical drugs up to 2013, 11 of them are glycoproteins, out of which eight are monoclonal antibodies (mAbs) and antibody Fc-based fusion proteins. It is crucial therefore that the quality control of these products is of a very high standard particularly as many of them are produced in mammalian cells and the glycosylation that they carry is influenced by the host cell. In the example shown, an important mAb, Herceptin (trastuzumab) is profiled against human serum IgG showing for example oligomannose structures, most notably Man5, are higher in the recombinant IgG samples. Their abundance in human serum IgG is much lower as a result of mannose-receptor-mediated clearance (Zhang 2016) (Fig 71).

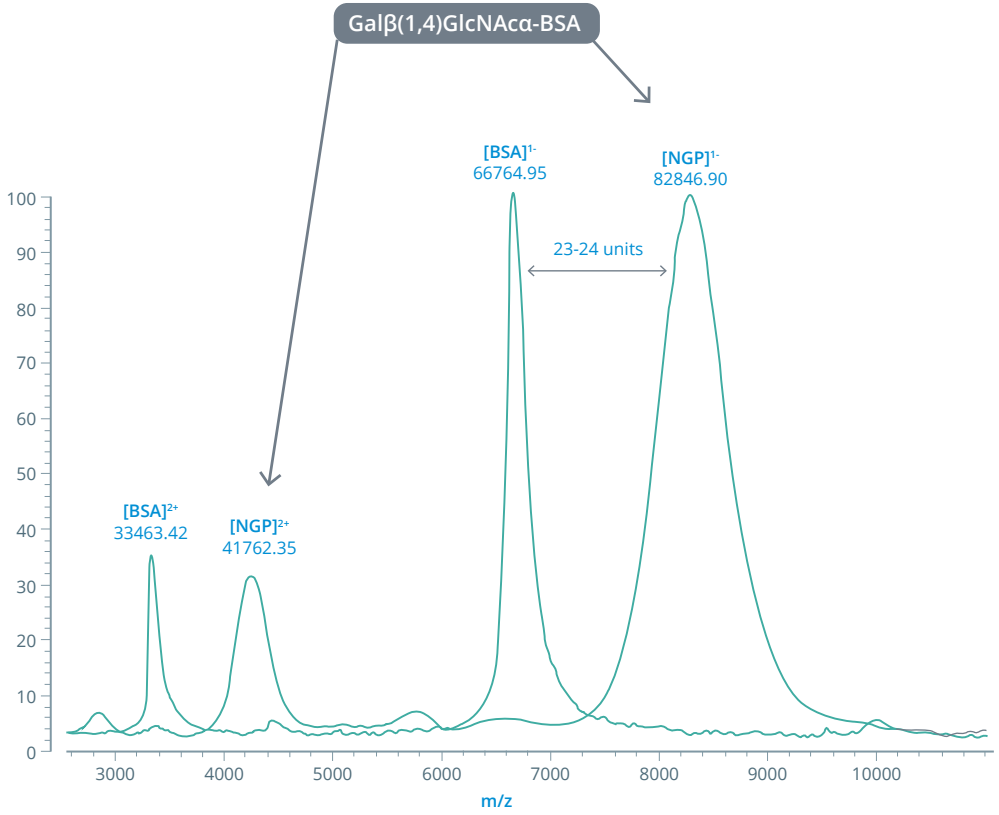


Fig 71 MALDI-TOF spectrum showing the increase in molecular weight of a NGP conjugate due to the addition of the disaccharide Galb(1,4)GlcNAc-BSA

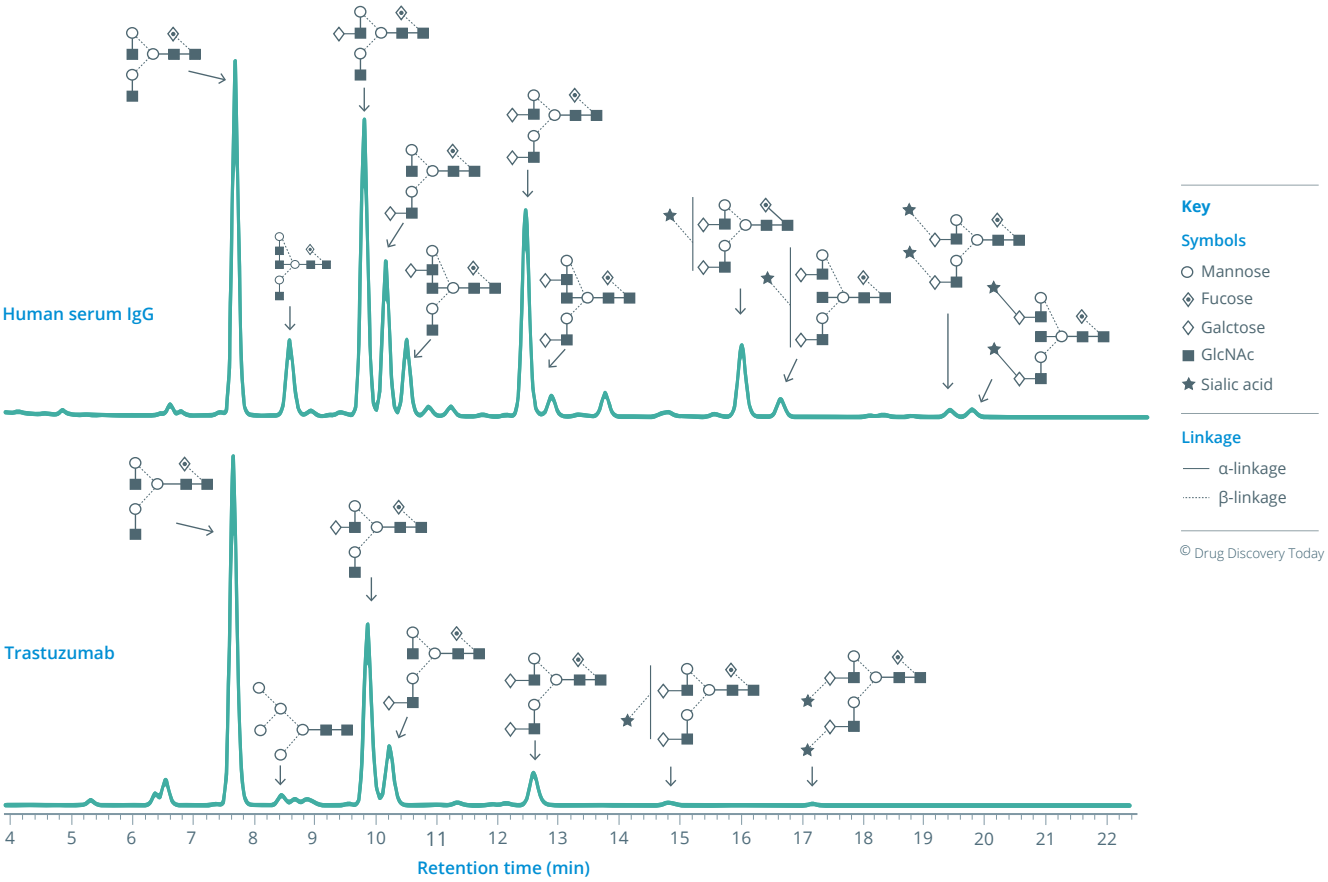


Fig 72 Mass spectrum of Herceptin (Trastuzumab) profiled against that of human serum IgG

A significant advantage of these techniques is that only low amounts of material are required (North 2009). However, a major defect of this method is the lack of information on linkage configuration (e.g. α- or β-) between sugar residues. A typical profile is shown overleaf (fig 73) illustrating the complexity of the glycan array that can be found on the surface of glycoproteins such as the spike protein of the COVID-19 virus.

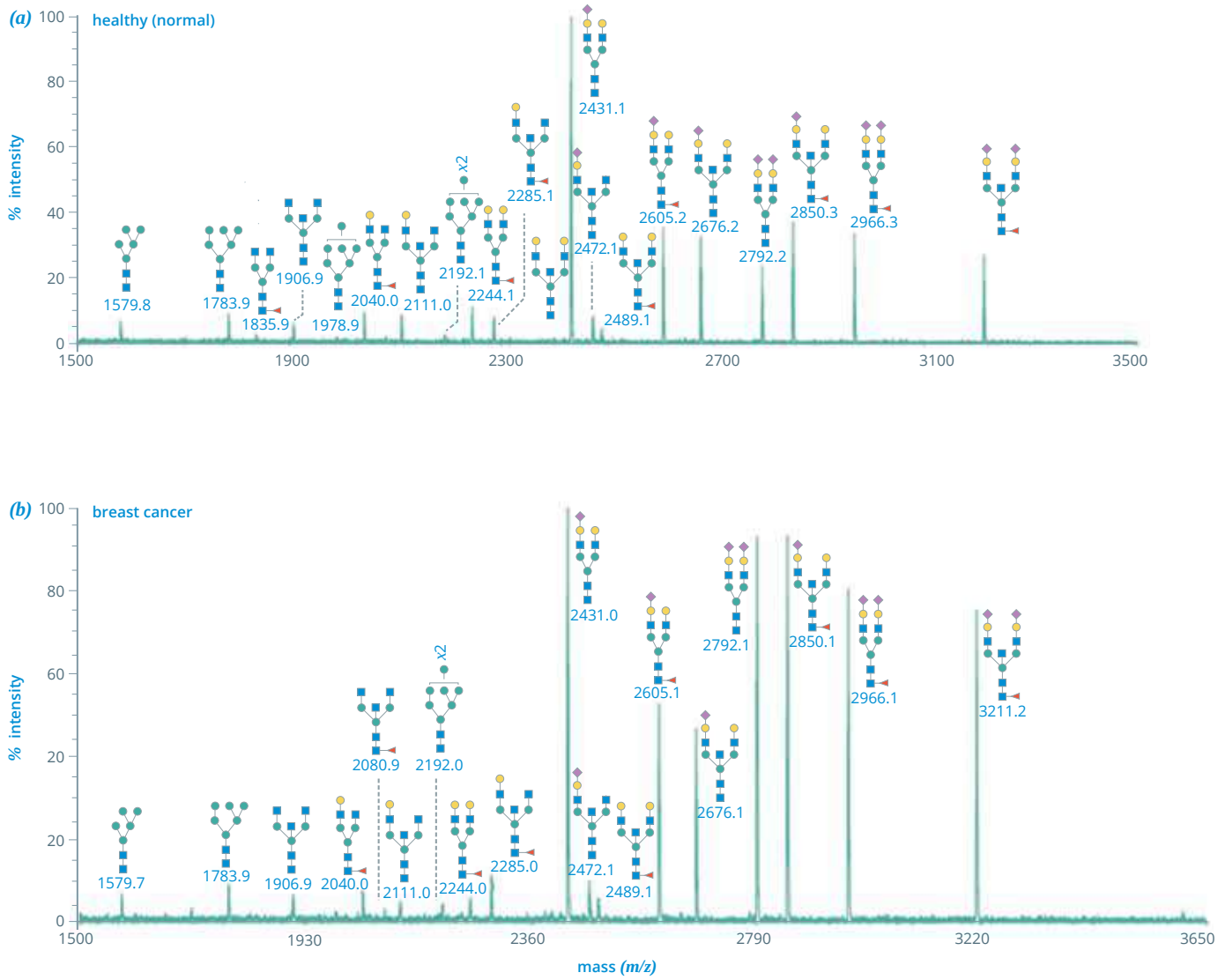


Fig 73 MALDI-TOF mass spectra of permethylated N-glycans of immunoglobulin (IgA1) purified from serum samples. N-glycan profiles of IgA1 from (a) normal healthy individuals (n = 4) and (b) breast cancer patients (n = 4) (Lomax-Browne 2018).

4.3.2.3 Chromatography and electrophoresis

The techniques of chromatography and electrophoresis are discussed in section 5.212, but particular mention is made below of FACE electrophoresis as it is a relatively simple and inexpensive technique that can supplement the other methods for low abundance glycans.

FACE is a useful method that was developed for the visualisation of glycan release from glycoproteins but can be used for other oligosaccharide mixtures, such as those released by degrading starch or dextran (see section 4.2.1.3.2). In this method, the oligosaccharides are tagged with charged fluorophores such as 8-aminonaphthalene-1,3,6- trisulphonate (ANTS) or the non-charged 2-aminoacridone (AMAC) (Jackson 1993, Robb 2017). The samples are run on highly crosslinked polyacrylamide gels and scanned using an ultraviolet scanner.

The figure 73 shows *N*-linked glycans released from a glycoprotein, lane 1 is a standard dextran hydrolysate ladder, released glycan samples are in lanes 2-6 and a G2 (disaccharide) quantification standard in lane 7 (Lawson unpublished).

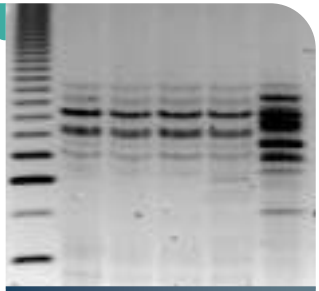


Fig 74 FACE electrogram

4.3.2.4 Structure-Focused Glycoproteomics, Future Methodology

As has been shown, the analysis of glycans that have been released from the surface of glycoproteins and glycolipids produces highly detailed structural information (monosaccharide identity, sequence and linkage information etc) for the analysis of both *N*- and *O*-linked glycans. There are now fully automated methods for releasing and analysing released glycans for example by coupled LC, MS and ion mobility. These methods are underpinned by linked data analysis programmes and data bases (Wongtrakul-Kish 2019, Walsh 2019). However, a significant problem is that until recently, available methods have not had sufficient resolution to analyse intact structures (glycoproteins, mucins etc) with the result that most glycoproteomics strategies, did not provide much information beyond the types of sugar residue present i.e. hexose (Hex), N-acetylhexosamine (HexNAc), deoxyhexose (dHex), and N-acetylneuraminic acid (NeuAc) or even, regarding glycans as undefined protein modifications based on their molecular mass. This situation has been particularly acute for *O*-linked glycans which has been labelled the 'dark' mucin *O*-glycoproteome (Chernykh 2021).

Thus, detailed profiling of the intact glycoproteome, now called 'structure- focused glycoproteomics', is now providing much more detail such as the difference between β-linked GlcNAc and α-linked GalNAc, and detailed linkage information including (α1,2/3/4-) versus core (α1,6-) fucosylation and triantennary (β1,4/6-) versus bisecting (β1,4-) GlcNAc-containing glycans. Glycoproteomics is a powerful quantitative technology that will enable the entire complement of glycoproteins expressed by cells, tissues or organisms to be studied, a key requirement in the study of human disease. Glycoproteomics studies undertaken between 2014–2016 typically reported between 100 and 2000 unique intact *N*- and *O*-linked glycopeptides (Thaysen-Andersen 2016). Studies published since 2018 are now routinely identifying and quantifying thousands of glycopeptides, some even beyond 10, 000 and 20, 000 intact glycopeptides (Shu 2020).

Some of the recently developed important methodologies such as SimpleCell technology, which manipulates cells to homogenously express truncated *O*-GalNAc glycans (Yang 2014), bacterial proteases such as OpeRATOR that cleaves mucin-type O-glycan motifs to aid the profiling of densely glycosylated mucin domains and non-mucin *O*-glycoproteins often left undigested are rapidly emerging (Shon 2020). Recent advances in mass spectrometry-based analysis of glycopeptides have involved improvements in dissociation and intelligent glycopeptide-centric LC–MS/MS data acquisition methods, both of which have led to a greater volume and quality of the analytical data. Glycopeptide-search algorithms and machine learning have enabled more efficient classification of *N*- and *O*-glycopeptides and their isomeric structural features (Abrahams 2020) and a rapid data analysis software for glycopeptides based on a predictive algorithm is also available. (Choo 2019). Finally, a new computational algorithm, *O*-GlycoProteome Analyzer was able to identify mucin-type *O*-glycosylation (e.g. core 1 versus core 2) in human urine and plasma (Park 2020).

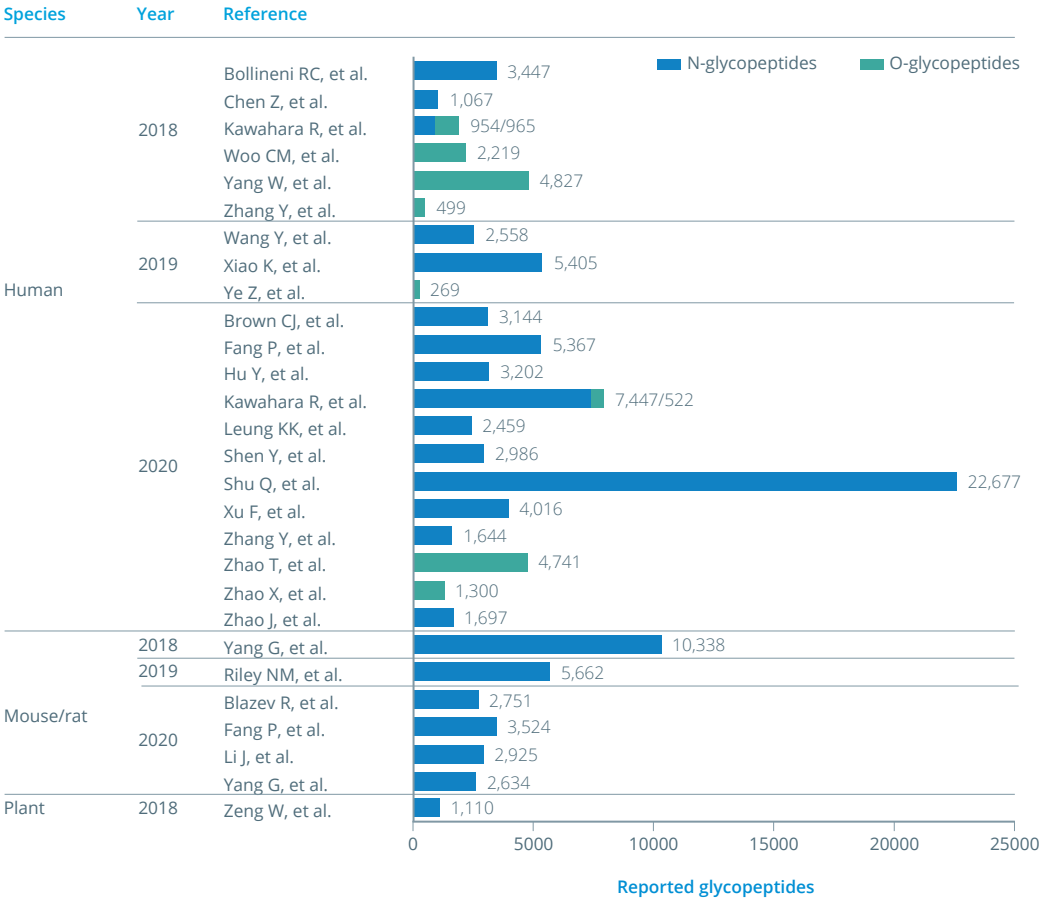
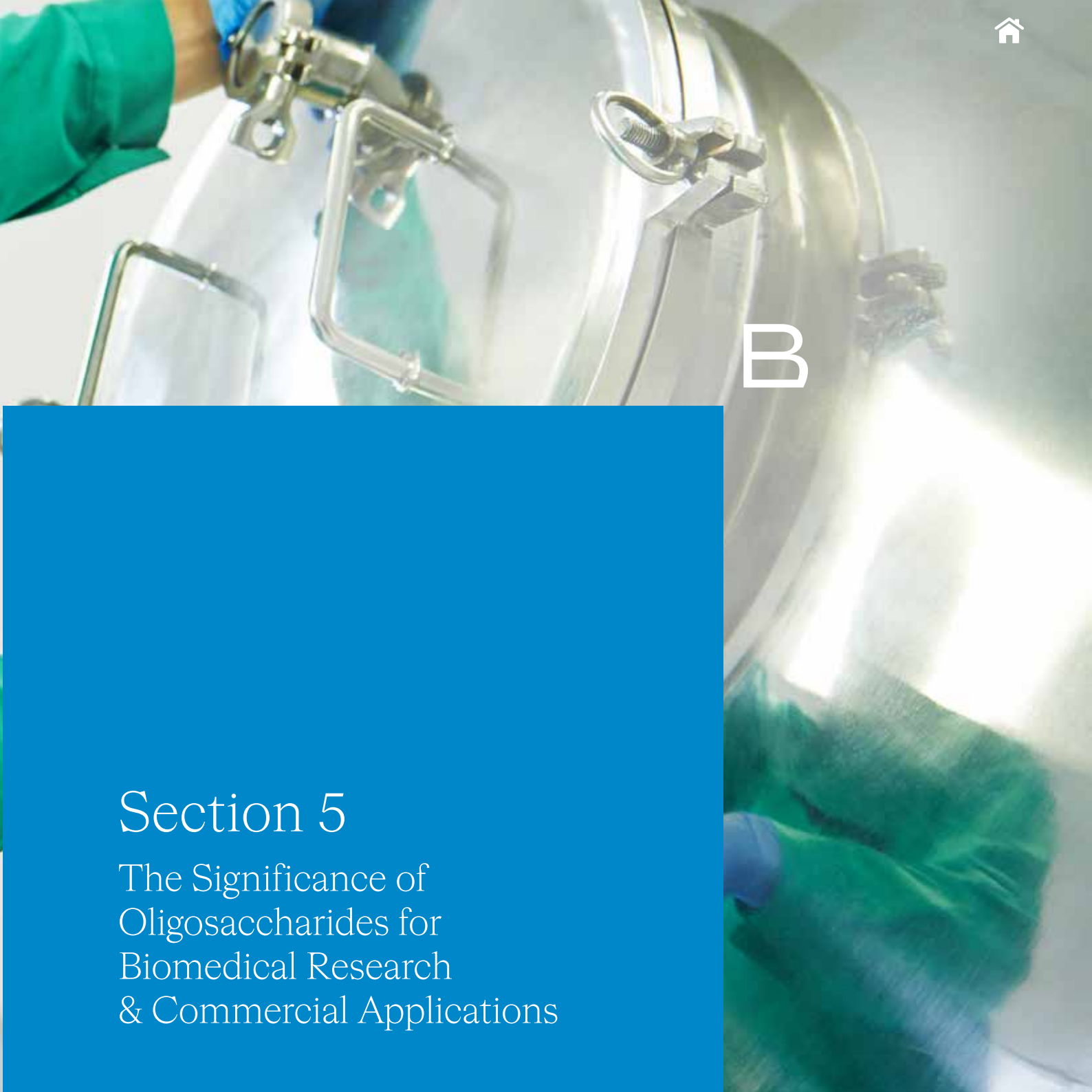


Fig 75 Glycoproteome coverage reported by recent glycoproteomics studies published in the period 2018–2020 arranged by the studied species and publication year. The studies included in this overview all performed large-scale glycoproteomics analysis of complex biological samples from unaltered or only mildly manipulated biological sources and reported a significant number of intact glycopeptides. Studies not matching these criteria and studies reanalysing existing glycoproteomics data were left out. We apologise for any omission of studies published in this period that we may have failed to identify in our survey of the literature. The coverage as measured by the reported unique (non-redundant) intact N-glycopeptides (yellow bars) and O-glycopeptides (blue bars) has been plotted for each study. Details of these 28 glycoproteomics studies including their full references are provided in Table 1.



B

Section 5

The Significance of Oligosaccharides for Biomedical Research & Commercial Applications

It is well established that structure and density/abundance of physiologically relevant oligosaccharides can become altered during disease. An example is the abnormal excretion of oligosaccharides in urine of patients with lysosomal storage or other metabolic disorders (Federico 1982 and lit. cited therein).

In another example, the apparent correlation between the higher risk of COVID-19 infection for analysed patient cohorts with blood group A and a protective effect for those with blood group O (Ellinghaus 2020) may prove, if confirmed, to be of great significance. O type blood is determined by the core H-glycoside, a bifurcated oligosaccharide terminated with galactose residues. The H glycoside becomes the A-glycoside by the addition of *N*-acetyl-D-galactosamine to C3 of the ends of the two branched chains (Morgan 2000).

Without the ambition of being comprehensive, table 8 provides some examples for diseases where alterations of oligosaccharides are involved. These observations have crucial importance for several reasons:

1. They may allow full investigation of their biological roles to give us a better understanding of their significance.
2. Access to novel oligosaccharide analogues and derivatives may allow manipulation of the diseased state by providing novel therapeutics.
3. Synthetic oligosaccharides may allow the design and development of vaccines to fight the diseases with which they are associated.

Table 8

Condition	Abnormal/Significant Glycosylation Affected Protein	Structural Change Observed Reaction	Reference
Acquired disease			
Alcohol Abuse	Transferrin	Removal of N-glycan chain	(Stibler 1976)
Reperfusion injury	Selectins	Mucin PSGL-1, sialyl-Lewis^X	(Lefer 1995)
Atopic and contact dermatitis	E-selectin	Mucin PSGL-1	(Schön 2005)
Infectious disease			
Viral infections:			
- HIV	GP120 glycoprotein	Changes in HIV envelope leading to stronger binding and fusion	(Scanlan 2007)
- Influenza (human)	Virus binds to terminal α2,6-linked sialic acids on cell surface glycans	Desialylation by viral neuramini-dase allows for spread of viral progeny	(Wagner 2002)
Bacterial infections:			
- Typhoid fever - Bacterial meningitis - Bacterial pneumonia - Cholera	Polysaccharide capsule shields pathogen from host immune system	Different structures in different serotype strains allow repeated infection by same pathogen	(Avci 2010)
- Gonorrhea	Lipopolysaccharide (LPS) on Gram-negative pathogens	Immune evasion through switching types of LPS (*N. gonorrhoeae*)	(Merrell 2004)

Condition	Abnormal/Significant Glycosylation Affected Protein	Structural Change Observed Reaction	Reference
Infectious disease (cont)			
Parasitic infections: - Malaria - Chagas' disease - African sleeping sickness - Leishmaniasis	*P. falciparum* EBA-175 protein *T. cruzi* trans-sialidase	Neu5Acα2-3Gal motif/ glycophorin A Increased protection from host defense by transfer of α2,3-linked sialic acids from host to parasite surface	(Koch 2017) (Schenkman 1994)
Urinary tract infections	Colonisation by *E. coli* and other microbes		(Weiss 2020)
Genetic disorders/Lysosomal Storage Diseases			
Defects in lysosomal degradation of glycoproteins: - α-Mannosidosis (various types) Glycolipids - Gaucher's disease Glycosaminoglycans - Hunter syndrome	α-Mannosidase β-Glucoceramidase Iduronidase and iduronate-sulfatase	High mannose oligosaccharides in urine Accumulation of glycosphingolipids Accumulation of dermatan and keratan sulfate	(Mashima 2020)
Muscular Distrophy	α-dystroglycan	Defects in *O*-mannosylation	(Van Reeuwijk 2005)
Cancers			
Cancers (general)	Tumor associated antigen	Increased sialylation	(Feizi 1993)
Prostate cancer	Prostate Specfic Antigen (PSA)	Upregulation of SLe^x, core fucosylation, O-GlcNAc, (potential biomarkers)	(Scott 2019)
Breast cancer	IGA1 antibody	Increase in disialo-biantennary *N*-linked glycosylation Increase in asialo & disialo TF *O*-linked glycosylation	(Lomax-Browne 2019)
Pancreatic cancer	MUC4 mucin	Overexpression of mucin & glycosylation	(Chaturvedi 2007)
Hepatic cancer	Mac-2-binding protein glycosylation isomer	High Mac-2 levels predictive of malignancy	(Murata 2020)
Ovarian cancer	Total serum glycome	Increases in core fucosylated, agalactosyl biantennary glycans (FA2) and sialyl LewisX(SLe^X)	(Saldova 2007)
Lung cancer	Transforming growth factor beta (TGFB) pathway	Aberrant *N*-linked glycan fucosylation	(Park 2020)

As discussed in the introduction, glycans are involved in a great variety of biological processes such as cell adhesion and recognition, cell development and many more. They can occur unattached such as the oligosaccharides in human milk but often they are conjugated to other biomolecules to form the respective glycoconjugates (glycoproteins, glycolipids etc.). This allows anchoring of the relatively polar glycan chains in the cell surface to serve as cell-specific 'antennae' in the extracellular matrix.

In the past, several factors have hampered the investigation and exploitation of such interactions. Firstly, glycan structures are not directly encoded in the genome and even if their biosynthesis and the respective enzyme expression levels are known, prediction of the precise structure of an individual glycan was difficult. Secondly, their large variety combined with difficult chemical synthesis and limited availability through isolation made the investigation of an individual recognition process with a glycan-binding protein (GBP) such as a lectin, a challenge (Taylor 2017). This was often exacerbated by rather weak monomeric interactions (affinity) with their respective GBPs- strong binding e.g. between cells involving glycans is typically achieved through multivalent binding (avidity).

As a consequence, new technologies that allow for the presentation of oligosaccharide mixtures or GBPs in a cell surface-like manner and analysis of binding to their counterparts in a high-throughput fashion have been developed through intense research over the past 30 or so years (Li and Feizi 2018, Wang and Cheng 2015, Gao 2019). This technology is now well established and a good example are the neoglycolipid based glycan arrays (Fukui 2002).

In a first step, the reducing glycans, e.g. designed libraries or isolated mixtures, were chemically conjugated to a lipid and the resulting neoglycolipid (NGL) mixtures were immobilised on nitrocellulose. Thus, glycan arrays could be displayed that retained some of the flexibility glycans show on a cell surface. The arrays were then reacted with GBP solutions, in this case of antibodies and interferons and, following detection and separation of the reactive NGLs, structure elucidation by mass spectrometry, several complex glycan receptors could be identified.

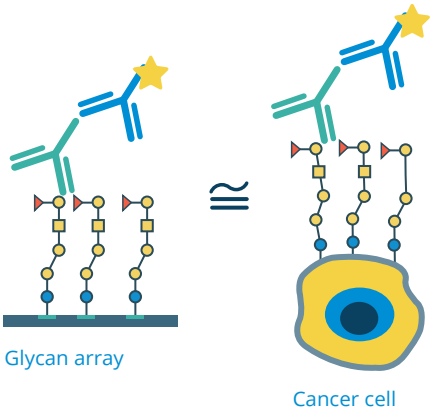


Fig 76 Glycan array on gold nanoparticles

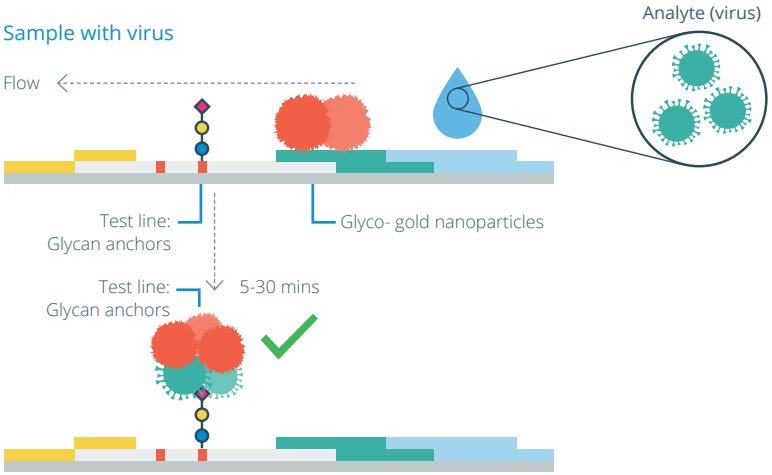


Fig 77 Design concept for glyco-lateral flow devices. Lateral flow assay for virus, using glycan capture units. Figure adapted from Baker *et al*, 2020

Glycan microarray technology today includes high-throughput robotic arraying and data analysis and have been successfully applied to various oligosaccharide ligand discoveries in the recent past, three examples from the field of biomedicine are given in table 9 (overleaf).

A recent example of glycan array technology has been host-pathogen glycan recognition (HPGR) technology that harnesses this principle through the use of gold nanoparticles coated with host carbohydrate structures. The carbohydrate molecules selected are recognized specifically by the target pathogen, say, SARS-CoV-2, or a human influenza virus, and will bind only to that pathogen if it is present in a sample. A glycan-based lateral flow detection system that can detect the spike glycoprotein from the SARS-COV-2 virus in under 30 min is now under development (Baker 2020).

Area	Carbohydrate binding protein/organism	Interaction	Reference
Microbial infection	*Salmonella enterica*	Glycan: Antibody	Blixt 2008
Cancer (prostate)	Anti-glycan Ab F77	Glycan: Antibody	Gao 2014
Innate immune system	Siglecs involved in cell signaling, adhesion and immune cell regulation	Glycan: Siglec	Gao 2019

Table 9 Examples of glycan microarrays.

5.3 Industrial applications

Oligosaccharides have many uses in foods, cosmetics, pharmaceuticals and other industrial areas. In foods they act as texturising agents, emulsifiers, acidulants, energy sources, adhesives, high intensity sweeteners and substrates in fermentation processes for the production of alcoholic beverages.

In cosmetics they have a role as emulsifiers in skin creams and lotions, and in pharmaceuticals as actual or potential drugs for cancer treatment, cystitis, as blood anticoagulants and for the treatment of heart disease.

Industrially, oligosaccharides are used to make surfactants, as polyols in the manufacture of polyurethanes, as adhesives and to produce acrylates. The tables below give examples of the many applications on sale worldwide.

5.3.1 Pharmaceutical applications

Oligosaccharide Name	Function	Reference
Solamargine/Solasonine	Skin cancer (squamous cell carcinoma)	(Jiang 2016)
α-Galactose/Galili antigen	Xenotransplantation	(Galili 2004)
Heparin fragments	Thrombosis treatment	(Spadarella 2020)
Carbohydrate antigens (Sialyl Leˣ)	Cancer	(Feizi 1993)
Hyaluronan Oligosaccharides	Cancer therapy	(Caon 2020)
Ganglioside GD2	Tumor marker	(Vantaku 2017)
Trehalose	Enzyme preservation	(Colaço 1992)
Acarbose	Diabetic drug	(Balfour 1993)
Chitooligosaccharides	Topical anti-inflammatory agent	(Fernandes 2010)
ABO epitope oligosaccharides	ABO blood determinants	(Morgan 2000)
White starch dextrins	Pharma binder	(Alvani 2011)
Pentosan polysulfate	Interstitial cystitis	(Moldwin 2018)
Digoxin	Atrial fibrillation/flutter, heart failure	(Sethi 2018)
Cyclodextrin	Solubilizing agents for lipophilic drugs	(Conceicao 2018)
Lactulose	Laxative	(Pranami 2017)
Aescin	Peripheral vascular disorders	(Hu 2016)

Table 10 Examples of commercially available oligosaccharides for pharmaceutical applications

5.3.2 Food applications

Oligosaccharide Name	Function	Reference
Fructooligosaccharides	Dietary fibre	(Fuller 2016)
Stevia glycosides	High intensity sweetener	(Shaifali 2017)
Galactooligosaccharides	probiotic	(Fuller 2016)
Maltooligosaccharides	food ingredient/fermentation substrate	(Eggleston 2003)
Fucosyllactose/sialyllactose	Infant milks	(Sprenger 2017)
Sucrose Esters	Fruit Preservation/Surfactant	(Plat 2001)
Sucralose (trichloro-galactosucrose)	High intensity sweetener	(Hough 1993)
Lactose	Sweetener	(Chattopadhyay 2014)
White starch dextrins	thickener, food additive.	(Sajilata 2005)
Galactooligosaccharides	probiotic	(Rabiu 2001)

Table 11 Examples of commercially available oligosaccharides for food applications

5.3.3 Cosmetic applications

Oligosaccharide Name	Function	Reference
Cyclodextrins	Cosmetic carrier	(Ammala 2013)
Hyaluronate oligosaccharides	Cosmetic skin hydration/barrier	(Kawada 2014)
Fructooligosaccharides	Shampoo & body wash	(Nizioł-Łukaszewska 2019)
Human milk oligosaccharides	Skin care	(Kialab.it, Sugarderm brochure)

Table 12 Examples of commercially available oligosaccharides for cosmetic applications

5.3.4 Other industrial applications

Name	Function	Reference
Canary starch dextrins	Adhesives	(Kennedy 1989)
Lactose, Sucrose, Maltose (polyols)	Polyurethane foams	(Savelyev 2015)
Sucrose Tallowate Esters	Surfactants (hand cleaners, antistatic agents, fruit preservation)	(Kollonitsch 1970) (Lee 1996)
Glucose syrups	Acrylic polymer production	(Hughes 1998)
Starch oligosaccharides	Biodegradable surfactants	(Throckmorton 1974)
Rhamnolipids	Biodegradable surfactants	(Araujo 2018)

Table 13 Examples of commercially available oligosaccharides for industrial applications

About the authors

Chris Lawson, Ph.D.

Ph.D. in Carbohydrate Chemistry & Enzymology (Edinburgh, 1969) with Professor Sir Dai Rees who demonstrated how polysaccharides form double helical networks in plant and animal tissues with profound effects on structure and texture.

Has published over 35 papers, patents and book chapters.

Fellow of the Royal Society of Chemistry, member of The Biochemistry Society, The American Chemical Society and the Society of Glycobiology.

In 1969 joined Tate & Lyle to manage a research team developing microbial polysaccharides. Helped to bring a number of products to market including Xanthan Gum, sucrose ester surfactants and Sucralose. In 1989, founded Dextra Laboratories with a colleague and developed it into a successful glycobiology company with a catalogue of key research carbohydrates and a specialist custom synthesis business. In 2006 set up Glycomix Ltd to focus on polysaccharide structure and function. Sold Glycomix to Carbosynth in 2016 and joined the company as a Scientific Advisor to help develop the carbohydrate business.

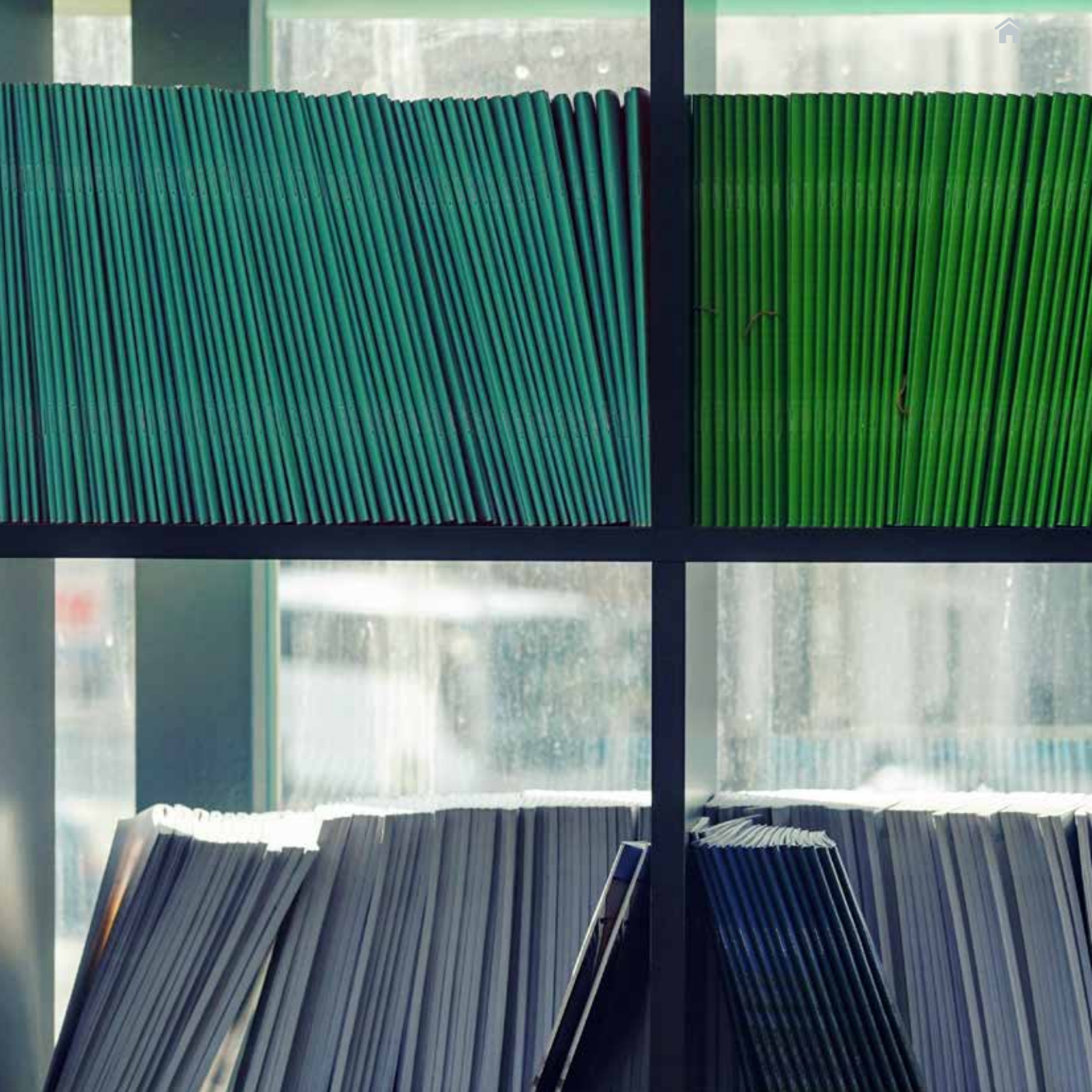
Hansjorg Streicher, Ph.D.

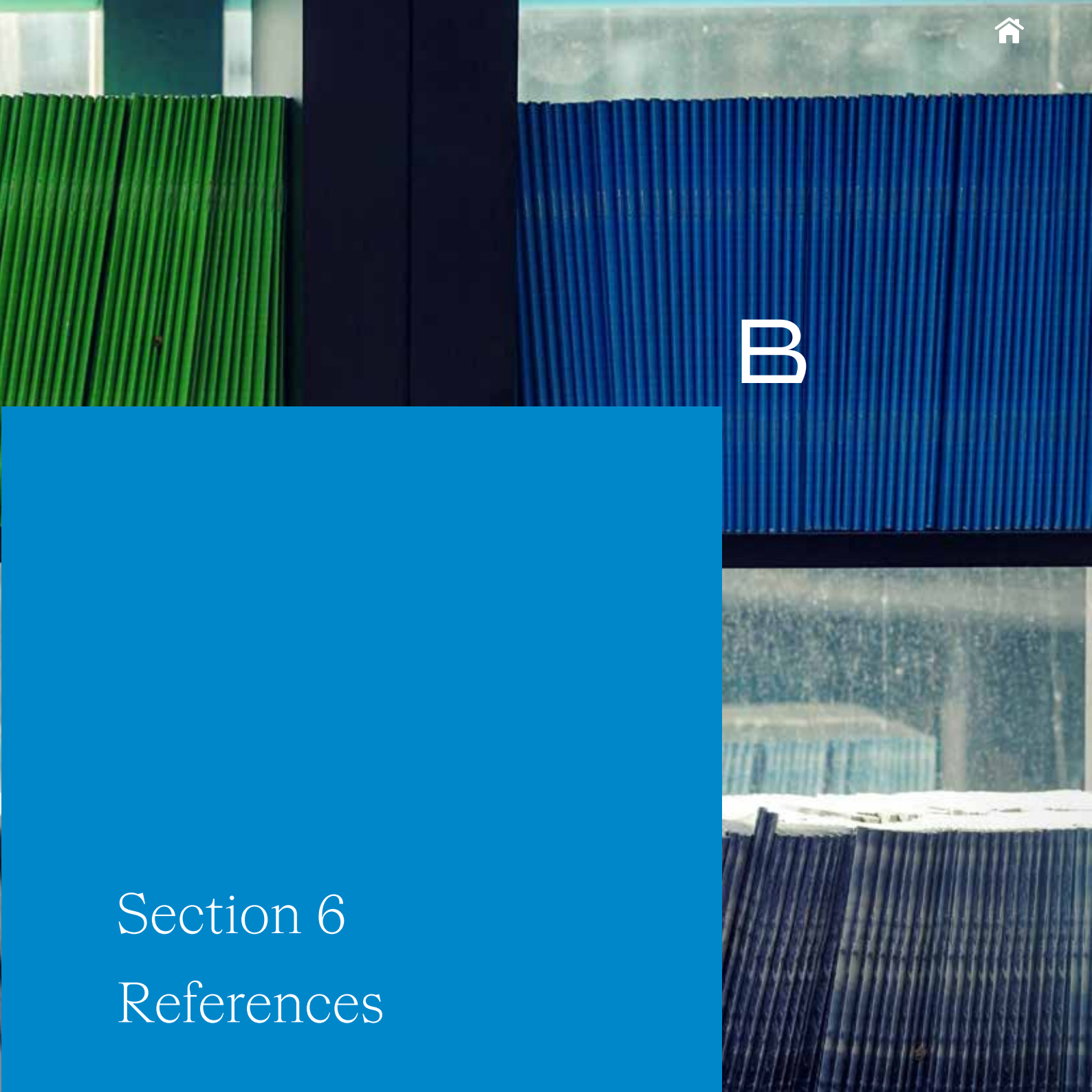
Ph.D. in Chemistry in Konstanz, Germany (1995) with Prof. R. R. Schmidt on 'Transition-State Analogues for the Antibody-Catalysed Glycosyltransfer'.

Holds a habiltation in organic chemistry and has published around 30 papers and book chapters.

Held various academic positions at the Weizmann Institute of Science (Rehovot, Israel), University of Konstanz and University of Sussex (Brighton, UK) with a scientific focus on lectins and carbohydrate mimetics.

Established as a consultant in the life science sector since 2014 and has worked with Biosynth as a Scientific Advisor.





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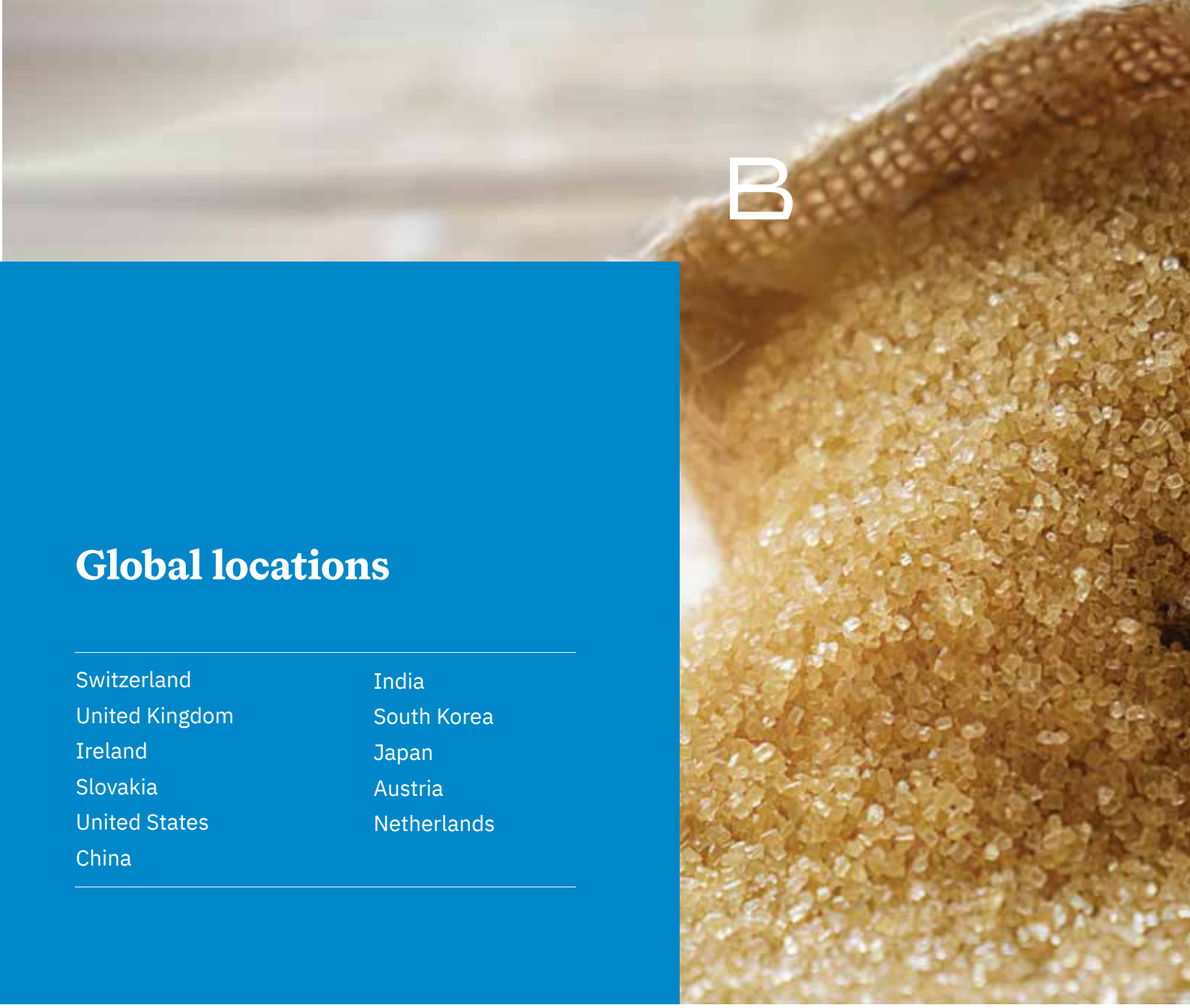

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