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Review

A guide into glycosciences: How chemistry, biochemistry and biology cooperate to crack the sugar code

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ABSTRACT

Background: The most demanding challenge in research on molecular aspects within the flow of biological information is posed by the complex carbohydrates (glycan part of cellular glycoconjugates). How the 'message' encoded in carbohydrate 'letters' is 'read' and 'translated' can only be unraveled by interdisciplinary efforts.
Scope of review: This review provides a didactic step-by-step survey of the concept of the sugar code and the way strategic combination of experimental approaches characterizes structure–function relationships, with resources for teaching.
Major conclusions: The unsurpassed coding capacity of glycans is an ideal platform for generating a broad range of molecular 'messages'. Structural and functional analyses of complex carbohydrates have been made possible by advances in chemical synthesis, rendering production of oligosaccharides, glycoclusters and neoglycoconjugates possible. This availability facilitates to test the glycans as ligands for natural sugar receptors (lectins). Their interaction is a means to turn sugar-encoded information into cellular effects. Glycan/lectin structures and their spatial modes of presentation underlie the exquisite specificity of the endogenous lectins in counterreceptor selection, that is, to home in on certain cellular glycoproteins or glycolipids.
General significance: Understanding how sugar-encoded 'messages' are 'read' and 'translated' by lectins provides insights into fundamental mechanisms of life, with potential for medical applications.

1. Introduction

The alphabet of nucleotides is the basis for storing genetic information in the germ line, starting its downstream flow to effector molecules by transcription and ensuring replication. Its size is confined to four characters. Intuitively, adding letters to this alphabet would make more sequence combinations of nucleotides possible, hereby increasing the informational contents of oligomers. The downside of this at first sight attractive perspective likely is impairing copying fidelity. Summing up thorough theoretical investigations based on structural, energetic and information-theoretic studies, "a certain alphabet size (probably four) seems to be optimal as a compromise between stability and evolvability, between fidelity and catalytic efficiency, and between information density and error resistance" [1]. The 5′,3′-phosphodiester linkage between nucleotides gives the nucleic acid chain its direction, for copying and for translation. The genetic code, relating two sequence types (i.e. nucleic acids and proteins) in a linear (one-dimensional)

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manner, underlies the conversion of triplet coding to proteins, to sequences which are arranged in bioactive shapes [2]. Considering the high degree of flexibility of peptides, proteins can, in part, be considered as a framework to let the embedded functionally relevant peptides adopt a distinct (bioactive) conformation and to enable conformational switches. Thus, it is essential that proteins reach their optimal conformation, suited for activity. To minimize detrimental deviations, every assistance possible to folding (or removal of misfolded waste) is welcome, and this is where the functionality of glycans already sets in. As follows, we introduce the concept of sugar coding by a case study of protein glycosylation, a ubiquitous process which has formerly been mostly interpreted to alter physicochemical properties of the protein.

N-glycosylation (the formation of an *N*-glycosidic linkage between the amide nitrogen of asparagine and reducing-end *N*-acetylglucosamine (β-Glc*N*Ac) of a glycan) is a common process in the three urkingdoms of life (eukaryotes and prokaryotes divided into archae- and eubacteria). It is mostly started co-translationally by the transfer of a lipid-linked 14mer oligosaccharide (i.e. GlcNAc_2 Man_9 Glc_3, with Man for mannose and Glc for glucose) to the protein's acceptor sequence (N-X-S/T-X; X≠P), in eukaryotes at the entry side of the endoplasmic reticulum (ER) [3–13]. Of note, at this stage all glycoproteins share the same sugar part. As a consequence of the glycan addition when leaving the cytoplasm, eukaryotic proteins on the lumenal side of the ER, in the Golgi regions, transport vesicles or lysosomes and on the plasma membrane as well as in the extracellular space carry this modification, and at high frequency in spatially accessible β-turns as examining mature glycoproteins delineated [6,14–18]. The thorough profiling also uncovered that, despite the phylogenetically ubiquitous occurrence, a fine-tuning of this type of protein modification has developed. In interspecies comparison, presence of potential *N*-glycosylation sites can differ to a notable degree so that, focusing on an endothelial intercellular adhesion molecule (ICAM-1), "examining the role of *N*-glycosylation in murine ICAM-1 and extrapolating the results to the human protein would seem imprudent" [19]. Turning back to the just mentioned possibility for co-translational additions providing assistance in folding, it would mean missing a splendid opportunity if the *N*-glycans were not recruited to this task. Fittingly, they are, and – due to the common origin – the sequence regions both in the core, that is in direct vicinity to the protein surface [20–22], and in outer branches [6,23,24] are at this stage of *N*-glycan functionality strictly constant at every site of glycosylation, a factor favoring a general role. Later, branch ends are subject to the same route of processing by stepwise truncations of the preformed glycan block [5,6,25–27]. As a consequence of the rather bulky nature of the added glycan and its structural uniformity, every *N*-glycan is capable of aiding the folding process by directly interacting with the protein via its core and by facilitating recognition of molecular chaperones via branch ends [23,24,28,29].

At this stage, Glc, which is not a part of mature glycoproteins, plays a prominent role in quality control, and at every stage of the stepwise removal of the three Glc moieties from the *N*-glycan. They are presented spatially accessible on a branch end. After the first of the three Glc moieties (in α1,3-linkage) had been cleaved from the *N*-glycan right after protein *N*-glycosylation by oligosaccharyltransferase ($t_{1/2}$ < 2 min) (in eukaryotes a multi-subunit enzyme, in prokaryotes a single-subunit protein [30]), this done so rapidly to preclude reversibility of the transfer by hydrolytic cleavage of glycan from the protein by this enzyme, the diglucosylated glycan becomes a ligand for malectin, an ER-resident protein [31] (please also see Table 1, comment on [368] in entry 20). Combined with ribophorin I and its capacity to interact with misfolded proteins, this complex may retain dysfunctional products in the ER, to start the process of quality control via glycan recognition of *N*-glycans [32]. The next step of trimming of the glycan chain (now at the Glcα1,2Glc linkage) makes the monoglucosylated contact point to two molecular chaperones (calnexin and calreticulin) available, and taking away the third Glc moiety (cleavage of Glcα1,2Man linkage) abrogates their binding. A sensor surveying conformations of glycoproteins in the ER (a bifunctional protein, which will restore presence of this Glc residue crucial for chaperone contact by its enzymatic UDP-Glc:glycoprotein transferase activity) will ensure signal presence for the association with these chaperones based on Glc recognition by reglucosylation if necessary [6,33–36]. Glucosylation of *N*-glycans thus is a transient phenomenon, an investment to make *N*-glycosylation an irreversible process and to help in ER-based quality control via chaperones.

As to the core, it is engaged in interaction with the protein (please see above). Its loss of contact to the protein is even later a sign for unfolding, 'read' by a receptor targeting the innermost GlcNAc_2 structure, and therefore a diagnostic signal for the need to direct these dysfunctional glycoproteins to proteasomal degradation by ubiquitinylation [37,38]. The indicated importance of the *N*-glycan implies the potential of defects in glycan assembly/processing to cause diseases. Indeed, careful analysis of *N*-glycan profiles in serum samples of patients using isoelectric focusing (started on the glycoprotein transferrin by the Swedish neurologist H. Stibler as diagnostic test for alcohol abuse in 1976) led to the discovery of anomalies (false-positives in the highly specific and sensitive assay to confirm abstinence), as class referred to as congenital diseases of glycosylation (CDG) [39–41]. For example, the absence of the α-glucosidase-I initiating the trimming cascade causes lethality in humans. The thorough delineation of causes for these diseases thus teaches intriguing lessons on functional aspects of *N*-glycans, reveals differences in comparison to animal models and allows therapeutic options to be devised [40,42–44] (for information on animal models, please see Table 1, entry 37).

Following this involvement in folding and quality control, *N*-glycans then have a second phase in their 'social' life. They undergo a substantial remodeling in the Golgi regions to prepare (equip) them for new functions (for details, please see Section 8). The stepwise size reduction to the pentasaccharide stage (Man_3 GlcNAc_2) and subsequent neosynthesis open the way to the known complexity of the cellular *N*-glycome, in terms of branching, the type and length of the branches, the terminal structures and the patterns of their substitution [3,5,6,45,46]. A fingerprint-like profile thereby takes the place of the initial sequence identity, an unmistakable hint to more than a random variation [47]. Based on total cellular glycomics, a high degree of glycome dependence on the cell type was found, prompting these authors to describe glycomes to have "utility as unique cellular descriptors" [48]. The same applies to the glycan chains of glycolipids, a key chemical platform of glycan presentation especially in the nervous system [49,50], and of proteoglycans [51,52]. Using glycan-binding laboratory tools, glycophenotyping of cells and tissues can conveniently be accomplished (for respective information, please see Table 1, entries 32 and 33).

Beyond such descriptive aspects, already structurally rather small changes can make their presence felt for protein properties such as (enzyme or receptor) activity [53]. Status of oligomerization or stability is other affected parameter, as exemplarily shown for a mammalian growth factor [54–57]. Considering that *N*-glycosylation (with the Asn-Glc*N*Ac linkage in β-configuration first described in 1961 [58]) is just one from at least 41 bonds between carbohydrates (13 different sugars known to be a part of the conjugate) and amino acids (eight different acceptor sites detected; hydroxylysine and hydroxyproline, acceptors of Glcα1,2Galβ1,*O* in collagen or arabinogalactan chains in plant glycoproteins [59–61], are produced by distinct hydroxylases, three in man, at least two in plants [62,63]) within proteins [64,65] (for further information on mucin-type *O*-glycosylation and mucins, the other frequently occurring type of protein glycosylation, please see [66]), carbohydrates (attached to proteins and lipids) have so broad a distribution profile that postulating a truly fundamental role is reasonable. Having started off with a look at the strictly ordered processes during *N*-glycosylation and then broadening the scope to sugars as part of cellular glycoconjugates, this assumption prompts to examine more closely the properties of carbohydrates, in comparison to nucleotides and amino acids.

Table 1
Experimental approaches to characterize protein/glycan features and protein–carbohydrate interaction (each entry provides basic information on the method and its applications including limitations as well as notes on practical examples, in this category (suggested reading) giving preference to studies on a selected protein or protein group (here galectins) and its ligands studied by different techniques to let readers most conveniently analyze merits and limits of applicability).

	***IN SILICO* METHODS**
1.	**Computational techniques**
Principle	Concepts of theoretical chemistry are incorporated into efficient computer programs for calculating the structure and properties of molecules. Ab initio methods are based entirely on theory from first principles. Empirical or semi-empirical methods also employ experimental results, e.g. knowledge-based modeling.
Information provided	Prediction/modeling of the structure of molecules (from a monosaccharide to complex glycans/from peptides to proteins) and receptor–ligand complexes, estimation of absolute and relative (interaction) energies, electronic charge distributions, dipoles and higher multipole moments, vibrational frequencies, and other spectroscopic parameters.
Applications	Exploration of glycan/protein structure and dynamics and of recognition mechanisms. Assistance in the design of target-directed compounds, in conjunction with X-ray and/or NMR information, and in conformational analysis of sugars in solution.
Limitations	Demands for computer time and other resources (such as memory and disk space) increase rapidly with the size of the system being studied, e.g. by including water molecules and introducing flexibility in a docking process
Example	Molecular mechanics and dynamics simulations describe the conformational space with grading according to energy level and in time for glycans. Rigid-body and flexible docking protocols characterize the recognition area of human galectins for physiological oligosaccharides and the contributions of Coulomb/van der Waals energy to binding.
Suggested reading	Siebert et al. 2003 [130] • Computational docking of a set of ligands, i.e. the ganglioside GM1's pentasaccharide and its two building blocks (Neu5Acα2,3Galβ1,4Glc and Galβ1,3Gal*N*Ac), with human galectin-1 and interaction analysis of the resulting complexes afford a detailed view on the way the branched and linear glycans make contact with this lectin, the starting point for growth regulation in vitro. NMR-spectroscopic analyses (NOESY, TrNOESY and STD; for further information on these techniques, please see entries 14, 26, and 27) provide experimental information on the free- and bound-state conformations of the ligands. The lectin part of the cholera toxin selects a conformer different from that bound by the human lectin, a case of *differential conformer selection*. André et al. 2005 [269] • Using the way human galectin-1 interacts with ganglioside GM1 as template, affinity for GM1 is predicted for other galectins using computational methods and experimental studies. Krzeminski et al. 2011 [125] • Solid-phase binding assays combined with in silico work reveal that branching in *N*-glycans and clustering of core 1 *O*-glycans are positive modulators for human galectin-3, whereas α2,6/α2,3 sialylation of *N*-/core 1 *O*-glycans, respectively, along with core 2 branching, are unfavorable factors. Computational interaction analysis by flexible docking shows no major conformational changes in the binding site or deviations from the low-energy conformation of the carbohydrate ligand upon complex formation, but a mutual adaptation. Martín-Santamaría et al. 2013 [316] • The interaction of a variety of saccharides and mimetics with human galectin-1 is studied using a combination of molecular modeling and NMR spectroscopy techniques, illustrating the complementarity of both methods to obtain a detailed perspective of the conformational and structural features of the recognition process.
	SOLID-STATE STRUCTURAL ANALYSIS
2.	**X-ray crystallography**
Principle	Analysis of the reflections of different intensity produced by interference of X-ray waves by the electrons of a molecule (or pairs of molecules, e.g., protein–ligand complexes) that has been crystallized. The diffraction pattern is the fingerprint of the periodic arrangement of atoms in the crystal.
Information provided	The diffraction pattern of suitable crystals yields an electron density map from which the three-dimensional structure of the molecule(s) is built.
Applications	Determination of the three-dimensional structure of glycans, lectins and lectin–ligand complexes. Obtaining detailed information at atomic level of lectin–ligand contacts.
Limitations	Crystallization may require non-physiological conditions, and crystal packing may cause artifacts. Allows no insights into dynamics to infer level of structural entropy (please, see NMR spectroscopy toward this aim) and its changes upon complex formation. Soaking of lectin crystals in ligand-containing solution may yield structures of complexes different from real co-crystals and structures in solution.
Example	The crystal structure of human galectin-1 reveals its β-sandwich topology, with the intricate network of hydrogen bonding/hydrophobic interactions at the interface that sustains the homodimer status discerned by gel filtration and analytical ultracentrifugation; explains the functionality of the sequence signature, with the central Trp residue (for C – H/π-interactions to the B-face of Gal; please see also fluorescence techniques) and His/Asp residues for hydrogen bonding to the 4′- and 6′-OH groups of Gal; and provides information on the bound-state conformation of the ligand (please, see NMR spectroscopy/trNOESY for investigation of conformer selection in solution).
Suggested reading	Sumner, 1919 [317] • First crystallization of a globulin from jack bean, concanavalin A, later categorized as lectin. Edelman et al. 1972 [318] • Determination of the tentative amino acid sequence of concanavalin A and identification of two anti-parallel pleated sheets, i.e. the β-sandwich arrangement, as the predominant structural element. The first crystal structure of a lectin. López-Lucendo et al. 2004 [319] • Crystallographic characterization of human galectin-1. Two single-site mutations at some distance from the carbohydrate-binding site are shown to induce a series of displacements of loops connecting the β-strands and to alter the position of key residues in the binding site, with a concomitant decrease in binding affinity unveiled by ITC. Ruiz et al. 2013 [320] • X-ray crystallographic analysis is completed for homodimeric chicken galectins. In combination with CD and ITC measurements (entries 4 and 22), it identifies distinctive features for each proto-type chicken galectin, the presence of lactose having a different impact on thermal stability depending on the protein.
	SOLUTION ANALYSIS OF LECTIN FEATURES **(including ligand-induced changes; described methods are often also applicable for glycans)**
3.	**Analytical ultracentrifugation**
Principle	Application of a gravitational force to a macromolecule in solution leads to the movement of the macromolecule toward the bottom of the centrifuge cell. In a sedimentation velocity (SV) experiment, a sufficiently large centrifugal force leads to the formation of a concentration gradient (boundary), and the rate of movement and broadening of the boundary as a function of time is registered. Sedimentation equilibrium (SE) experiments are performed at lower speeds, at which the process of diffusion significantly opposes sedimentation, reaching eventually an equilibrium concentration distribution throughout the cell.
Information provided	SV is sensitive to the mass and shape of the macromolecule, providing information on its homogeneity, molecular mass and hydrodynamic parameters. SE is only sensitive to the mass, allowing the determination of the average molecular mass.

Table 1 (*continued*)

Applications	Evaluation of sample purity and homogeneity (size distributions). Determination of molecular mass, degree of association (quaternary structure), hydrodynamic shape and conformational changes. Measurement of equilibrium constants and thermodynamic parameters for self- and hetero-associating systems.
Limitations	Data analysis is quite complex. The system may change during the course of the experiment, as long scan times are required. Some buffer components and additives may cause interferences depending on the detection system.
Example	Determination of the weight-average molecular weight by SE and analysis of the hydrodynamic properties in SV experiments evidence that galectin-1 in solution is a stable dimer, both in the absence and presence of a bound ligand. No sign of further oligomerization is observed under conditions proven to detect self-association of a galactose-binding plant lectin with trefoil structure, i.e., the toxic agglutinin from *Viscum album* (VAA).
Suggested reading	Morris et al. 2004 [321] • Analytical sedimentation velocity and equilibrium data shows that bovine galectin-1 and human galectin-7 are dimeric, whereas murine galectin-3, as well as its proteolytically derived C-terminal domain, is predominantly monomeric, the presence of Lac*N*Ac having no significant effect on the oligomerization state of the three proteins. Jiménez et al. 2005 [322] • The plant toxin VAA is found to occur as monomer at submicrogram per ml concentrations, and to form dimers at concentrations above this level, with evidence of nonideality, self-association, and polydispersity in sedimentation equilibrium and velocity experiments, caused by aggregation at high concentrations. Citraconylation of lysine residues destabilizes the dimer, shifting the equilibrium to the monomer. Kaltner et al. 2008 [323] • Cloning and expression of the prototype chicken galectin CG-2 allow the detection of an unusual hydrodynamic behavior for this homodimeric (proto-type) galectin in gel filtration and sedimentation velocity experiments, not observed for human galectin-1 or the two other homodimeric chicken galectins.
4.	**Circular dichroism (CD)**
Principle	Optically active molecules may absorb right- and left-handed circularly polarized light to different extents, giving rise to circular dichroism spectra. In proteins, the main chromophores are the peptide bond (below 240 nm) and aromatic amino acids (in the 260–320 nm range).
Information provided	The different types of secondary-structure elements in proteins account for characteristic CD spectra in the far UV, so the CD spectrum of a protein in this region reflects its secondary-structure composition. In the near UV, the spectrum depends on the type, number, mobility and local environment of aromatic amino acids; it is a reflection of the tertiary/quaternary protein structure.
Applications	Estimation of lectin's secondary-structure content. Detection of conformational changes due e.g. to protein–ligand interactions. Determination of protein stability against chemical or thermal denaturation and evaluation of ligand-induced changes in stability. Determination of binding constants by e.g. monitoring involvement of aromatic residues.
Limitations	Certain buffer systems and ligands may interfere because of high absorption at low wavelengths, limiting measurements in the far-UV region and therefore precluding a reliable estimation of the secondary-structure content.
Example	CD analysis of human galectin-1 provides evidence for β-structure in solution, as observed in the crystal structure and other galectins. It identifies a characteristic fingerprint in the near-UV region that is sensitive to the presence of lactose, evidencing ligand-induced conformational rearrangements that extend beyond the binding site, as also detected by NMR spectroscopy. Monitoring of ellipticity during thermal denaturation as sensor of thermal stability reveals a ligand-induced stabilization.
Suggested reading	Jirgensons, 1979 [324] • The diagnostic power of CD spectroscopy to detect conformational transitions for lectins is illustrated by exposing the β-sandwich kidney bean leukoagglutinin (PHA-L) to alkali, acid and SDS. Solís et al. 2010 [325] • A combination of biophysical techniques, including CD, is used to characterize the structural organization, stability and topological aspects of ligand binding of the *N*-domain of galectin-9, taking also this information to the level of tissues by cell/histochemical assays. Using cell mutants with defects in glycosylation, this lectin domain is shown to preferentially bind *N*-glycans without α2,3-sialylation, as also seen by galectin histochemistry in situ [305]. Ruiz et al. 2013 [320] (please see comment in entry 2)
5.	**Electrospray ionization mass spectrometry (ESI MS)**
Principle	Electrospray ionization (ESI) is a 'soft ionization' technique that uses electrical energy to assist the transfer of ions from solution into the gas phase before they are subjected to mass spectrometric analysis. Bringing ionic species into the gas phase starts with the dispersal of a fine spray of charged droplets, followed by solvent evaporation and ion ejection from the highly charged droplets. ESI overcomes the propensity of macromolecules to fragment when ionized and may produce multiply charged ions, effectively extending the mass range of the analyzer, even to protein association and protein–ligand complexes, which may not be accessible to matrix-assisted laser desorption ionization (MALDI) mass spectrometry.
Information provided	The exact mass of the molecular ion helps in the identification of unknown species. Isotope patterns can be used to flag the presence and number of elements within the molecule. Ionization profile informs about the possibility of presence of conformers in solution. Fragmentation patterns obtained from MS/MS experiments provide information about the structure of the molecule.
Applications	Mass elucidation of molecules from oligosaccharides/peptides to biomacromolecules. Identification and quantification of protein variants, e.g. detection of mutants due to single-nucleotide polymorphisms and of posttranslational modifications. Investigation of their quaternary structure using conditions approaching a pseudo-physiological milieu. Clinical application in e.g. therapeutic drug monitoring, screening for inborn errors of metabolism, and diagnosis of galactosemia and peroxisomal disorders.
Limitations	Quantification requires compound standards. Limited structural information can be gained from the simple mass spectrum obtained.
Example	Nano-ESI MS analysis of the quaternary structure of galectins-1 and -7, using an aqueous solution buffered at pH 6.8, successfully detects the occurrence of dimers (also confirmed e.g. by gel filtration and analytical ultracentrifugation), whereas under the same conditions galectin-3 is found to be monomeric. Occurrence of a bimodal charge distribution for galectin-3 is indicative of presence of two distinct conformers in solution (compact *vs* extended). The gentleness of the conditions for maintaining properties of the native proteins is further underscored by detection of complexes of the galectin-7 homodimer with di- and tetravalent carbohydrate ligands.
Suggested reading	Ho et al. 2003 [326] • General description of ESI-MS, explaining the electrospray ionization process, design of mass spectrometers with separation capability, characteristics of the mass spectrum, and practical considerations in quantitative analysis, finally focusing on some clinical applications. Kopitz et al. 2003 [327] • Solid-phase and cell assays show that the sugar chain of ganglioside GM1 is a ligand for galectin-7. In serum-supplemented proliferation assays, galectin-7 reduces neuroblastoma cell growth, mechanistically resembling galectin-1 despite their structural differences. The ESI MS spectra reveal homodimeric status for galectins-1 and -7 and indicate two conformational states for galectin-3. Wang et al. 2003 [328] • Following a historical survey of application of ES-MS in glycobiology, starting with measuring binding of a Glc*N*Ac hexamer to lysozyme in 1991, the method is described for two examples, i.e. trisaccharide binding to single-chain variable fragment of a monoclonal antibody and P^k trisaccharide binding to the homopentamer (lectin part) of Shiga-like toxin 1.

(*continued on next page*)

Table 1 (*continued*)

6.	**Fluorescence spectroscopy**
Principle	Measurement of the wavelength and intensity of light emitted by molecules whose electronic state has been elevated from a ground (low-energy) level, by absorbing light at an excitation wavelength, to a higher-energy electronic state, when they drop down back to the ground state. Trp and Tyr are the most important fluorophores in proteins, with a smaller contribution of Phe.
Information provided	The intrinsic fluorescence of a folded protein reflects the microenvironment of aromatic residues. Complex formation may result in changes of protein/ligand fluorescence due to alterations in microenvironment/surface presentation and/or direct contact.
Applications	Quick information on the conformational state of a protein. Quantitative fluorescence titration curves can be used for estimation of ligand-binding parameters.
Limitations	Fluorescence intensity is very sensitive to fluctuations in pH and temperature. Fluorescent impurities may cause significant interferences. Photobleaching, i.e. the photochemical destruction of the fluorophore, may occur.
Example	The presence of a Trp moiety in the binding site of galectin-1 can be used as reporter for ligand binding. In particular, shift/quenching of its fluorescence emission indicate changes in the local environment of the Trp sensor, from the free to the bound state (please see also entries 7 and 8 on fluorescence anisotropy and fluorescence correlation spectroscopy).
Suggested reading	Teichberg et al. 1975 [329] • First purification of a galectin (electrolectin), with detection of specificity to β-galactosides by hemagglutination and a blue shift of fluorescence emission by lactose loading, with 20% enhancement of emission intensity. Levi and Teichberg, 1981 [330] • The involvement in ligand binding of the single Trp residue per monomer of the electric eel galectin is inferred from the enhancement by lactose of the protein fluorescence and prevention of Trp oxidation (and associated loss of binding activity) in the presence of this ligand. Iglesias et al. 1998 [331] • Conformational changes of galectin-1 induced by ligand binding are examined using different techniques. Quenching of tryptophan fluorescence emission upon addition of lactose is exploited for quantitating the affinity for this ligand. To characterize the binding specificity of the lectin, the minimal concentration for inhibition of its hemagglutinating activity is determined for various sugars. Ke et al. 2012 [212] • The recognition of different mono- and disaccharides by a synthetic bis-anthracenyl system is investigated by NMR, ITC, and fluorescence titrations, revealing remarkable selectivity for glucose (e.g. ~50:1 *vs* galactose). The strong anthracene fluorescence is sensitive to ligand binding, the addition of glucose causing an up to 2.5-fold increase in emission intensity. As inferred from the NMR-based structure of the complex with methyl β-*D*-glucoside (please see Fig. 11b), the increase in fluorescence may derive from a loss of receptor mobility and the change from angled to parallel anthracene units.
	Fluorescence applications
7.	a) **Fluorescence anisotropy**
Principle	Fluorescence anisotropy occurs when the light emitted by a fluorophore has unequal intensities along different axes of polarization. When polarized light is applied to a motionless fluorophore, the emitted light is polarized within a range of angles (intrinsic anisotropy). If the fluorophore changes its orientation before re-emitting the photons, the degree of polarization of the emitted light will be reduced in direct and inverse proportion, respectively, to its rotational and fluorescence lifetimes.
Information provided	If the fluorophore is conjugated to a small molecule, the rate at which it tumbles can decrease significantly upon binding to a large protein, this providing evidence for interaction. In proteins, Trp fluorescence anisotropy can serve as reporter for ligand binding and/or conformational changes affecting its motional freedom.
Applications	Measurement of binding constants and kinetics of reactions causing a change in the rotational time of the molecules. Intrinsic fluorophores in proteins can serve as sensors to monitor folding dynamics; a fluorescent ligand can serve as probe for binding. In microscopy, it can be used to study the local viscosity of the cytosol or of membranes, the latter giving information on membrane microstructure, a regulatory parameter of glycan/lectin presentation.
Limitations	The scrambling of orientations can occur by tumbling of the whole molecule or by rotation of only the fluorescent part, in that case not providing information on the rotation of the molecule it is attached to.
Example	The fluorescence anisotropy of human galectin-1 is altered by about 5% in the presence of lactose, enabling calculations of the binding stoichiometry and dissociation constant ($n = 1.2 \pm 0.1$/$K_d = 156 \pm 11$ μM, for 300/350 nm excitation/emission wavelengths), in accord with FCS-derived estimates, and of association and dissociation rate constants ($0.5 \pm 0.1\ s^{-1}\ M^{-1}$ and $1.3 \pm 0.3 \times 10^{-4}\ s^{-1}$, respectively) by measuring the decay in anisotropy with time.
Suggested reading	Göhler et al. 2011 [332] • The tryptophan residue present in the binding site of human galectin-1 is used as reporter for ligand contact by measuring polarized fluorescence emission, enabling the calculation of the equilibrium binding and kinetic rate constants. Detailed analysis of the depolarization process indicates fast conformational dynamics within the binding site. Göhler et al. 2012 [333] • Tryptophan fluorescence anisotropy and fluorescence correlation spectroscopy are employed for detecting structural alterations in homodimeric galectins and determining equilibrium binding and kinetic rate constants, revealing galectin-specific responses to oxidation and lactose binding.
8.	b) **Fluorescence correlation spectroscopy (FCS)**
Principle	Spontaneous fluctuations in fluorescence intensity due to minute deviations of the system from equilibrium are continuously occurring. The statistical analysis of these fluctuations by temporal autocorrelation of the fluorescence intensity yields essential information about processes governing molecular dynamics.
Information provided	Data on all physical parameters that give rise to fluctuations in fluorescence, such as equilibrium concentrations, reaction kinetics and diffusion rates, can be obtained.
Applications	Determination of local concentration, molecular interactions, intramolecular dynamics, mobility, and rate constants of inter-/intramolecular reactions. Detection of ligand-induced changes.
Limitations	FCS experiments are restricted to nanomolar concentrations, leading to a series of disadvantages, e.g., a meaningful weight of photophysical damage or of the adherence of protein molecules to surfaces on the number of detected molecules, and the need of long data acquisition times. Fluorescent labeling may have a bearing on protein parameters.
Example	Monitoring of the translational diffusion constant of human galectin-1 using FCS reveals a lactose-induced increase in this parameter, as also detected by small angle neutron scattering (please see comment on [348] in entry 15). A similar behavior is observed for one of its chicken ortholog CG-1B, whereas the diffusion constant of CG-1A is not affected by the presence of the ligand, clearly evidencing intergalectin differences in paralog pairs.
Suggested reading	Göhler et al. 2010 [334] • The influence of ligand binding and presence of the linker peptide in tandem-repeat-type proteins on the hydrodynamic properties of members of the three groups of galectins is investigated. The effect of lactose binding on the diffusion constant differs depending on the type of galectin. Binding kinetics is estimated from repeated measurements over time. Göhler et al. 2012 [333] (please see comment in entry 7)

Table 1 (*continued*)

9.	c) **Fluorescence resonance energy transfer (FRET)**
Principle	A donor chromophore in its electronic excited state may transfer energy to an acceptor chromophore, located at a proper distance, through nonradiative dipole–dipole coupling.
Information provided	The efficiency of the energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor, making FRET extremely sensitive to small distances. Thus, FRET measurements are a molecular ruler for measuring the distance between two fluorophores.
Applications	Investigation of a variety of biological phenomena that occur with changes in molecular proximity. Measurement of distances between domains of a single protein, providing information on protein conformation, and branches in complex-type *N*-glycans. Detection of interaction between proteins and of protein aggregation, e.g. in response to signaling, in cells and interactions of proteins with other molecules and cellular structures, including ligands or membranes.
Limitations	External illumination is required to initiate the fluorescence transfer, which can lead to background noise from direct excitation of the acceptor, or to photobleaching. Labeling can alter glycan or protein properties.
Example	Monitoring of the fluorescence lifetime of lactose-containing FRET donor and acceptor glycopolymers after insertion into viable cell membranes reveals a lactose-inhibitable decrease in fluorescence lifetime of the donor in the presence of galectin-1, indicative of cross-linking of donor and acceptor, what is not observed for analogous cellobiosylated glycopolymers used as control (cross-linking of ligands is also the basis of precipitation analysis described in entry 29).
Suggested reading	Rice et al. 1991 [335] • By establishing donor–acceptor pairs in complex-type triantennary *N*-glycopeptides (naphthyl-2-acetyl as donor on the Asn-Ala *N*-terminus, dansylethylenediamine as acceptor), the Manα1,6Man branch is found to have notable flexibility, as indicated by accessing two distances (18.2 Å and 11.7 Å), as also seen for the α1,3-branch (21.7 Å and 9.7 Å). Only a single population is seen for the branch initiated by *N*-acetylglucosaminyltransferase-IV at an average donor–acceptor distance of 18.4 Å. Nieminen et al. 2007 [336] • Using galectin-3 labeled at the C-terminus with Alexa 488 (FRET donor) or Alexa 555 (FRET acceptor), oligomerization of galectin-3 on cell surfaces in different biological settings is monitored using FRET. Belardi et al. 2012 [337] • A new approach for probing galectin-induced multimerization of glycoconjugates on cultured cells, using synthetic glycopolymers functionalized with lactose (or cellobiose as control) along the backbone, a lipid group on one end and a FRET donor or acceptor fluorophore at the other end, is described. After insertion of the glycopolymers into cell membranes in vitro, their fluorescence lifetime and diffusion time are measured in the presence and absence of cross-linking galectin-1.
10.	**Gel filtration (chromatography)**
Principle	Particles of different sizes and shapes elute (filter) through a porous stationary phase at different rates, as they diffuse into the beads to greater or lesser degrees. Both molecular weight and shape contribute to the extent of retention.
Information provided	The apparent molecular mass and hydrodynamic shape of the protein can be estimated using columns calibrated with appropriate protein standards.
Applications	Analysis of the hydrodynamic behavior as a hint of quaternary structure and of a respective influence of the ligand. Analysis of purity.
Limitations	Interactions of the protein with the stationary phase may delay elution and must be prevented. Concentration-dependent effects need to be considered. Independent study of the quaternary structure by analytical ultracentrifugation is recommended.
Example	Elution of human galectin-1 in gel filtration reveals a homodimer status (also confirmed by analytical ultracentrifugation) with no evidence for dissociation, as actually observed for human galectin-7 at low concentrations (apparent $K_d \sim 1.7 \times 10^{-6}$ M). Deviation from the proto-typical chromatographic behavior is observed for other homodimeric galectins, as e.g. CG-2, the chicken ortholog of human galectin-2, or an oxidized form of CG-1B, a galectin-1 ortholog.
Suggested reading	Giudicelli et al. 1997 [338] • Size-exclusion chromatography analysis of human galectin-1, using a dextran-based (Sephacryl) gel, gives no evidence for dimer dissociation at loading concentrations down to 2 μM, questioning the previously reported existence of a monomer–dimer equilibrium for hamster galectin-1 to be a general phenomenon. Kaltner et al. 2008 [323] (please see comment in entry 3) López-Lucendo et al. 2009 [339] • An intersubunit disulfide bridge between Cys7 residues is found in the crystal structure of the CG-1B homodimer. In solution, the additional occurrence of a compacted form with an intrasubunit Cys2–Cys7 disulfide is detected by gel filtration and mass fingerprint analyses. Ermakova et al. 2013 [340] • Dissociation of the galectin-7 homodimer at low protein concentrations is observed by gel filtration and supported by hetero-FRET spectroscopy. Ligand binding-induced stabilization of the dimer through long-range effects across the protein is substantiated by NMR, CD and molecular dynamics simulations. Indications for positive cooperativity are obtained from Monte Carlo fits of HSQC titrations with lactose, using a two-site model.
11.	**Nuclear Magnetic Resonance (NMR) spectroscopy**
Principle	Quantum mechanical magnetic properties of an atom's nucleus. The chemical shift, i.e. the frequency at which a nucleus resonates when a magnetic field is applied, differs among NMR-active nuclei (^{1}H, ^{13}C, ^{15}N, etc.) and is also related to the particular chemical environment of each individual nucleus.
Information provided	Constitution, conformation, dynamic and binding features of small, medium-size and large molecules, both in solution and in the solid state. Powerful tool for structure elucidation (for glycans and proteins).
Applications	Determination of three-dimensional structure and details of molecular recognition. Analysis of structural dynamics at different timescales.
Limitations	The access to high magnetic fields is rather expensive. For protein structure determination, milligrams of isotopically enriched material may be required. The assignment of the NMR spectra of large proteins (>40 kDa) may be difficult.
Example	The solution structure and internal dynamics of galectin-3, elucidated by heteronuclear NMR spectroscopy and molecular modeling, reveal that the β-strands and loops around the carbohydrate-binding site can partially cover the cleft for Gal binding (with the NGWR motif for bcl-2 interaction). Loading this site dampens internal motions at the binding site while increasing motions elsewhere throughout the protein.
Suggested reading	Jiménez-Barbero and Peters, 2002 [341] • An overview of applications of NMR methods to study structure, conformation, dynamics and interactions of carbohydrates. Umemoto et al. 2003 [342] • Based on residual dipolar couplings (^{15}N – ^{1}H for the protein, ^{13}C – ^{1}H for ^{13}C-labeled Lac*NAc*) the solution structures of the lectin domain of human galectin-3, free of ligand and loaded with ligand, are defined. Evidence for a concerted change around the binding site is collected. Diehl et al. 2009 [343] • Using ^{15}N spin relaxation experiments (on amide backbone, Trp's indole and Arg's guanidine group) and MD simulations on the lectin domain of human galectin-3 without/with lactose an increase in conformational entropy is detected. This change appears to occur throughout the protein without triggering a structural alteration, contributing markedly to the entropic factor in the overall thermodynamics of binding.
	NMR applications
12.	a) **Heteronuclear single-quantum coherence (HSQC)**
Principle	NMR pulse sequence that allows the identification of the chemical shifts of directly connected NMR-active pairs of nuclei.

(*continued on next page*)

Table 1 (*continued*)

Information provided	Identification of the connectivity (chemical bond) between a particular heteronucleus (^{15}N, ^{13}C, etc.) and its corresponding attached proton, allowing the detection of different chemical environments within a molecule, from small to large entities. For a protein, the $^{1}H-{}^{13}C$ HSQC easily identifies backbone and side chain regions of aromatic residues. For an oligosaccharide molecule, it discriminates the anomeric $^{1}H-{}^{13}C$ pairs from the other in the ring and from the exocyclic hydroxymethyl group. The region or nature of additional chemical modifications in a sugar ring can also be distinguished.
Applications	Identification of the chemical environments (heteronuclear chemical shifts) in a molecule. It can be employed as a fingerprint for a given molecule. For molecular recognition purposes, the HSQC of a protein in the presence of a given ligand will show specific chemical shift perturbations of the HSQC signals that are located at the ligand-binding region. Both $^{1}H-{}^{13}C$ or $^{1}H-{}^{15}N$ methods can be employed.
Limitations	For ^{13}C applications, mM concentrations of the molecule at natural-abundance level for ^{13}C are required. For ^{15}N applications, isotope labeling with this particular nucleus is required. High-field NMR spectrometers are usually necessary.
Example	$^{1}H-{}^{15}N$ HSQC data of human galectin-1 in the absence and presence of Galα1,3Gal, Galα1,4Gal, and Galα1,6Glc, as well as lactose for comparison, show similar but non-identical chemical shift maps that reflect sugar-specific involvement of protein groups in recognition (please see entries 19 and 26 for information on chemical mapping and STD for elucidation of involvement of sugar determinants). Binding constants are obtained from titration curves of chemical shift changes *vs* sugar concentration, evidencing a lower affinity of the α-linked disaccharides compared to lactose.
Suggested reading	Meyer and Peters, 2003 [344] • An authoritative review of the applications of different NMR methods to monitor receptor–ligand interactions from both the perspective of the ligand and of the receptor. Miller et al. 2011 [345] • A combination of $^{15}N-{}^{1}H$ HSQC chemical shift monitoring and STD NMR epitope mapping with docking analysis reveals binding of the low-energy conformers of α1,3/4-digalactosides, preferentially via the non-reducing-end galactose moiety, and also of Galα1,6Glc. For the α1,4-linked disaccharide, the typical CH–π interaction is significantly diminished, yet the reduction appears to be partially compensated for by hydrogen bonding. Ermakova et al. 2013 [340] (please see comment in entry 10)
13.	b) **Laser photo CIDNP (chemically induced dynamic nuclear polarization)**
Principle	The CIDNP effect (nuclear spin polarization) arises from unpaired electrons (radicals) generated from dye–protein radical pairs in a back reaction. In proteins, the side chains of Tyr, Trp and His are able to produce CIDNP signals after laser irradiation in the presence of a suitable radical pair-generating dye (flavin I mononucleotide).
Information provided	Elicitation of a response in proteins implies surface accessibility of the respective groups to the light-absorbing dye enabling measurement of this parameter.
Applications	Characterization of transient free radicals and their reaction mechanisms. Examination of the surface structure of proteins, both in native and partially folded states, and of the possible impact of site-directed mutagenesis on the accessibility of reactive side chains, reflecting conformational alterations (please see entry 35 on mutant design). Monitoring of the effect of ligand binding to a receptor, when CIDNP-reactive amino acids are involved.
Limitations	A minimum area of surface accessibility is required for generation of a CIDNP signal (estimated values of 70 Å for Trp, and around 50 Å for Tyr). The comparatively reduced reactivity of the His side chain as flavin triplet quencher can translate into a decreased level of sensitivity.
Example	Measurement of CIDNP signals in the absence and presence of lactose confirms the involvement in sugar binding of the central Trp residue at the carbohydrate-binding site of galectin-1, observed in the crystal structure and assessed in solution using different fluorescence-based techniques.
Suggested reading	Siebert et al. 1997 [346] • The laser photo CIDNP technique is used as a measure for surface accessibility of His, Tyr and Trp in different lectins, and the results are compared with available crystallographic data. Accessibility to Trp is impeded by lactose for galectins, underscoring the contact between Trp/Lac. Long-range effects are detected in a plant lectin after single-site mutation. Jiménez et al. 2006 [347] • By systematically covering a broad range of lactose/lectin ratios in isothermal titration calorimetry, evidence for dissimilar binding affinities for the two galactoside-binding Trp/Tyr-sites per monomer of the plant toxin VAA is obtained. Laser photo CIDNP measurements of Trp and Tyr accessibility help to attribute lectin activity in the VAA dimer primarily to the Tyr-sites, full access to the Trp-sites being gained on dimer dissociation.
14.	c) **Nuclear Overhauser effect spectroscopy (NOESY)**
Principle	Through-space dipole–dipole interaction (cross-relaxation) based on mutually sensing each other's magnetic dipole, between two spins that transfers magnetization. The signal intensity of one spin is altered by saturation of the neighboring spin (with r^{-6}). This alteration is measured.
Information provided	The measured intensity change of a signal assigned to a particular nucleus serves as a molecular ruler for distances between nuclei. Information on spatial vicinity helps delineate conformational properties in solution, for a lectin and its ligands.
Applications	Structural elucidation of lectins and their ligands, free in solution. Combination with computational techniques (please see entry 1) will help to relate signal to a model of conformation.
Limitations	Complete assignment is often required, and signal overlap can compromise respective assessment. Spin diffusion (three-spin processes) must be excluded.
Example	NMR parameters such as NOE-based information are non-linear ensemble averages so that combining these data into a single conformation yields a 'virtual' structure, whose actual relevance is uncertain. Combined with modeling and measuring other experimental constraints makes structure elucidation feasible, that is characterizing the conformations accessed by a glycan and its receptor.
Suggested reading	Carver, 1991 [147] • The review highlights that dynamics of carbohydrates is significant and deals with the application of NOESY to generate a realistic structural representation as an ensemble. Asensio et al. 1995 [253] • This seminal report, the first NMR-based structure definition of a lectin with its ligand in water, uses NOE-based sequential information (120 constraints) together with 221 medium to long-range constraints. The collective evidence is translated into distances to build models for the 43-amino-acid plant lectin hevein (from *Hevea brasiliensis*) (please see Fig. 12 and Section 7 of the text for further information on hevein–ligand complexes) Ferrand et al. 2009 [198] • ^{1}H NMR titrations of a synthetic carbohydrate receptor with monosaccharides are used to measure the binding affinity, while NOESY information serves to assign intermolecular contacts and to estimate approximate distances, from which the lowest energy structure of the complex is derived (please see Fig. 10c). The synthetic lectin shows similar affinity for β-Glc*N*Ac as the wheat germ agglutinin (WGA) and increased selectivity for glycopeptides.
15.	**Small angle neutron/X-ray scattering (SANS/SAXS)**
Principle	Measurement of the reflections of different intensity produced by scattering of neutron/X-ray waves by atoms within a single molecule, averaged for all the molecules in solution. The Fourier transformation of the scattering profile yields the interatomic distance distribution function, which is sensitive to the molecular shape and to symmetry and domain structure within the molecule.

Table 1 (*continued*)

Information provided	High-precision information on size and shape of proteins under close-to physiological buffer conditions. The molecular envelope can be modeled from scattering data using ab initio algorithms and/or rigid-body refinement based on available high-resolution structures. Self-association and molecular flexibility can be analyzed.
Applications	Determination of the size and shape of proteins in solution, and of ligand-induced alterations.
Limitations	The sample must be highly pure and monodisperse; sample aggregation must be carefully avoided. Radiation damage may arise and induce aggregation. Perfectly matched solvent blanks are important for data analysis. A critical evaluation of sample and data quality and understanding of modeling assumptions are essential.
Example	Data obtained for human galectin-1 are in agreement with a similar conformation in solution and in the crystal, and support the homodimeric state deduced by gel filtration and ultracentrifugation analyses. Comparison of the scattering intensities in the absence and presence of Lac*NAc* reveals a significant decrease of its gyration radius (from 19.1 ± 0.1 Å to 18.2 ± 0.1 Å), as also noticed by FCS.
Suggested reading	He et al. 2003 [348] • The radius of gyration of human galectin-1 confirms a homodimeric status and is found to decrease upon ligand binding. When shifted from aqueous solution to dimethyl sulfoxide, which does not impair binding capacity, galectin-1 forms tetramers (dimer of dimers) with a cylindrical shape. He et al. 2010 [349] • SAXS and SANS are combined to obtain structural information on a PEG-hGal-2 conjugate (modification exclusively at the Cys75 residue), as potential means for improving galectin stability, solubility, and bioavailability (for further information, please see comment on [410] in entry 34). SAXS signals derive mainly from the protein, while SANS data obtain information from both the protein and PEG moieties. Jacques and Trewhella, 2010 [350] • This review provides a roadmap through the small-angle scattering experiment and a set of guidelines for critical evaluation of scattering data.
	INTERACTION ANALYSIS
16.	**Affinity chromatography**
Principle	The chromatographic behavior of an analyte on a column containing a matrix, typically functionalized with a potential counterreceptor, is examined. Retardation of elution or retention of the analyte within the column evidences interaction.
Information provided	Elution of the bound analyte by subsequent addition of a solution containing a selective hapten proves specific binding. Immobilized lectin/ligand will lead to purification of respective counterreceptor(s) from solution. Quantitative information on association constants can be obtained by frontal affinity chromatography (FAC), where an excess volume of the analyte's solution is continuously applied and the delay of the elution front allows the calculation of the association constant.
Applications	Purification of binding partners from complex mixtures. Quantitative assessment of protein–ligand interactions.
Limitations	Non-specific interactions with the matrix may disguise the behavior of the analyte. Low-affinity interactions may not be detected. Interaction can depend on the extent of bead functionalization.
Example	Affinity chromatography on lactosylated Sepharose is routinely exploited for the purification of galectins, bead-immobilized galectins for counterreceptor (glycoconjugate) isolation. Similarly, purification of anti-galectin antibodies (of use for detection of galectins in e.g. microarray binding assays, cytofluorometry or lectin cyto-/histochemistry), with possibility for removal of cross-reactive material by serial affinity chromatography, is accomplished using immobilized galectins, also helpful to detect autoantibodies. Moreover, this set-up allows the semi-quantitative analysis of the galectin's binding specificity. For example, using FAC, human galectin-1 is found to bind branched *N*-glycans containing non-extended terminal Lac*NAc* units with ~10-fold higher affinity than linear Lac*NAc* di- to pentamers, and also to show significantly lower affinity for α2,6-sialylated complex-type *N*-glycans than for the non-sialylated ones, a behavior also confirmed using the microarray technology. Binding of lectin to resin-presented ligand facilitates to obtain carbohydrate-binding peptides after in situ proteolysis and purification.
Suggested reading	Agrawal and Goldstein, 1965 [351] • The pioneering report describes a simple method for the isolation of the lectin from crude extracts of jack beans by specific adsorption on a column of commercially available cross-linked dextran (Sephadex) and elution with D-glucose. Cummings and Kornfeld, 1982 [352] • Description of a general technique for fractionating asparagine-linked oligosaccharides on the basis of oligosaccharide structure, using strategically selected plant lectins. Powell and Whitney, 1984 [353] • Covalently coupled rat lung galaptin (galectin-1) to Sepharose (with binding capacity of 125 μg asialoorosomucoid/ml resin) is applied to identify membrane glycoproteins (M_r 160–200,000) as interaction partners in extracts using 1% Triton X-100. Ohannesian et al. 1995 [354] • Galectin-3 is isolated from human colon carcinoma KM12 cells by affinity chromatography and the purified lectin is used to identify galectin-3 ligands by lectin blotting, co-immunoprecipitation and affinity chromatography on immobilized galectin-3, proving the lectins's interaction with several adhesion molecules and suggesting a role in human colon carcinoma cell adhesion. Hirabayashi et al. 2002 [355] • FAC analysis of the binding specificity of a total of 13 galectins from six species (two mammals, bird, nematode, sponge and mushroom), using pyridylaminated oligosaccharides, confirms the key groups involved in the recognition and also reveals galectin-type-specific features in terms of branching of *N*-glycans, repeating Lac*NAc* units, or substitutions at positions 2 and 3 of the non-reducing terminal Gal, as α1,2Fuc, α1,3Gal, α1,3Gal*NAc* or α2,3Neu5Ac. Moise et al. 2011 [227] • The generation of tryptic peptides from human galectins-1 and -3 when in contact to a resin-presented ligand (lactose) or free in solution (and then processed by affinity chromatography) leads to sequence stretches of the binding site. Synthesized peptides prove reactivity in competition and binding assays to lactose. Sarter et al. 2013 [356] • Monitoring of anti-galectin IgG autoantibodies in the sera of patients with chronic inflammatory rheumatic diseases, using ELISA tests with a series of galectins immobilized onto the surface of microplate wells, discloses a disease-associated occurrence of autoantibodies against particular galectins, the presence of anti-galectin autoantibodies possibly contributing to immune dysregulation.
17.	**Affinity cross-linking (please see also Photoaffinity labeling)**
Principle	A receptor–ligand pair is covalently connected by a homo- or heterobifunctional reagent, which can be cleavable during preparation for analysis to separate the two components, employing disulfide bridges in the reagent. One functionality can be photoactivatable (please see entry 28).
Information provided	Binding partners of a sugar receptor can become attached to the lectin, enabling their identification, with controls ascertaining lack of cross-link formation if glycan recognition is blocked.
Application	Spatial vicinity of binding partners facilitates stable conjugate formation and partner identification, e.g. by Western blotting. Also, conjugate formation of a glycan with a scaffold or a (neo)glycoprotein with a marker enzyme generates tools for interaction analysis.
Limitations	Rigorous controls must exclude fortuitous vicinity, recommending immunoprecipitation as parallel approach for independent experimental confirmation.
Example	The hepatic asialoglycoprotein receptor can be biochemically identified on hepatocytes by cross-link formation with a glycoprotein equipped with *N*-hydroxysuccinimide ester-based homobifunctional cross-linkers. Homo- and heterobifunctional cross-linkers are instrumental for probe

(*continued on next page*)

Table 1 (*continued*)

Example	generation ((neo)glycoprotein-enzyme and glycan (deacylated ganglioside)-presenting neoglycoprotein (for application of these probes, please see cyto- and histochemistry in entry 33)).
Suggested reading	Gabius et al. 1987 [357] • The heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithio) propionate forms conjugates of an indicator enzyme with a neoglycoprotein. Such a conjugate is applied analytically for detection of lectins in solid-phase assays and in tissue sections. Herzig and Weigel, 1989 [358] • Five *N*-hydroxylsuccinimide ester-based homobifunctional cross-linkers are used to prepare asialoorosomucoid conjugates ready for forming complexes with the counterreceptor of this asialoglycoprotein on rat hepatocytes. This reaction depend on Ca^{2+}, typical for a C-type lectin, and the molecular masses of the three bands are congruent with those of subunits of the asialoglycoprotein receptor. Immunoprecipitation with antibodies specific for this C-type lectin confirm this result. Gabius et al. 1990 [359] • The homobifunctional cross-linker bis(sulfosuccinimidyl)suberate forms conjugates of a lysoganglioside to a carrier protein. Such a conjugate is applied analytically for detection of glycan binding on cells and in tissue sections and also for affinity chromatography.
18.	**Atomic force microscopy (AFM)**
Principle	A surface is imaged at high resolution by rastering a sharp tip in close, but not direct, contact over the surface.
Information provided	Determination of binding strength under force of surface- and tip-presented receptor/ligand pairs; rupture force and density effects are indicative for the potential of the receptor to engage in transient or firm contacts.
Applications	Imaging the three-dimensional structure of biological specimens in a physiological environment. Real-time monitoring of biochemical and physiological processes at electron-microscope resolution. Investigation of molecular interactions based on the frequency of complex formation, the magnitude of rupture forces, and the shape of the force–distance curve.
Limitations	The samples must be firmly attached to an appropriate surface, e.g. resin beads. Water condensation or contaminants covering the probe and/or sample may severely reduce the resolution and cause molecular damage or displacement. Application of a high contact force may result in nonspecific ruptures that are indistinguishable from those of specific interactions.
Example	AFM examination of the binding of bovine galectin-1, presented on the cantilever, to lactose and asialofetuin immobilized on Sepharose beads (a typical set-up for affinity chromatography) yields similar rupture forces (34 ± 6 pN and 37 ± 3 pN, respectively, at a loading rate of 3 nN/s), the variation of the pulling velocity revealing a linear dependence between the rupture force and the natural logarithm of the loading rate (please see SPR for analysis of dissociation kinetics at zero force in entry 31).
Suggested reading	Dettmann et al. 2000 [360] • Analysis of the forces required to break the association between lactose and asialofetuin *N*-glycans from *Ricinus communis* and *Viscum album* agglutinins, bovine heart galectin-1, and a human β-galactoside-binding IgG fraction by AFM, with variations of the external force, shows sensitivity to inter-protein/-ligand differences. Measurement at zero force (by SPR) reveals that the dissociation kinetics at zero force cannot predict the behavior in force-driven experiments. Bowers et al. 2013 [361] • Increased contact forces (>250 pN) result in increased probabilities of binding and decreased blocking efficiencies for the ligand–receptor pair lactose/galectin-3, but also give rise to non-specific ruptures of control systems with no known affinity, as mannose/galectin-3.
19.	**Chemical mapping**
Principle	Evaluation of the effect on a receptor's affinity of single-site replacement of a hydroxyl group in a carbohydrate ligand by a different group or an atom (mostly hydrogen, fluorine or an O-methyl group) with different hydrogen-bonding potential and/or steric requirements; the equivalent of site-directed receptor mutagenesis.
Information provided	Relative importance on the binding of hydrogen bonding and steric aspects at the position of the hydroxyl group that is chemically altered.
Applications	Delineation of topological features of lectin–ligand interactions, useful for comparative analysis and in the design of selective inhibitors for a particular member of a given lectin family.
Limitations	Requires a substantial synthetic work. Repulsion between the electronegative fluoro substituent and a proximal negatively charged group within the binding site may complicate the interpretation of the results.
Example	Chemical mapping of bovine heart galectin-1 using monodeoxy, O-methyl and fluorodeoxy derivatives of methyl β-lactoside confirms the key involvement of hydroxyl groups at positions 4′ and 6′ of Gal and at position 3 of Glc, common to all galectins, and reveals differential contribution of other hydroxyls to the binding. Comparison with chicken galectins CG-1A and CG-2 unveils galectin-type specific features, such as the capability of CG-2 to accommodate an axial hydroxyl at position 3 (crystallographic analysis later providing a rationale for this potential) or the enhancement of galectin-1's binding affinity upon introduction of a methyl group at position 2′, of relevance to the design of ligands with improved affinity and selectivity.
Suggested reading	Solís et al. 1994 [362] • Chemical-mapping studies of bovine heart galectin-1 using methyl β-lactoside derivatives assign key hydrogen-bonding interactions to one side of the disaccharide molecule, *O*-methylation at the other side causing in general an enhancement of the binding affinity. Solís et al. 1996 [363] • Detailed comparison of the mode of binding of chicken galectins CG-1A and CG-2 reveals minor differences, besides preservation of key interactions, suggesting overlapping but distinct ranges of potential ligands. Solís et al. 2009 [364] • Description of the basic structural and thermodynamic features of protein–carbohydrate interactions revealed by different methodological approaches, including insights into atomic features gained by X-ray crystallography and by chemical mapping, related to information on the bound-state conformation of the carbohydrate ligand obtained by TrNOESY, and analysis of the different factors governing the thermodynamics of binding, which can be examined using titrations in NMR experiments or ITC. Solís and Díaz-Mauriño, 1997 [365] • Principles of the chemical-mapping approach and examples of applications to a variety of carbohydrate-binding proteins, including lectins, carbohydrate-active enzymes and anti-carbohydrate antibodies.
20.	**Equilibrium dialysis/ultrafiltration**
Principle	When two solutions are separated by a semipermeable membrane, the concentration of a diffusible substance becomes equal on both sides of the membrane after a certain time. In protein–ligand binding experiments, the protein solution is placed at one side of a membrane that does not permit diffusion of the protein, while a diffusible ligand is placed at the other side. At equilibrium, the concentration of free ligand is the same at both sides. The presence of receptor-bound ligand, however, increases the overall concentration in the receptor side in relation to the affinity. Separation of bound and free ligand under pressure by an ultrafiltration membrane is a technically easy alternative.
Information provided	By performing measurements using different ligand concentrations, the relationship between ligand concentration and binding serves to determine the number of binding sites on the receptor and the binding affinity using a Scatchard plot analysis.
Applications	A classical method for the determination of association constants of sugar–protein interactions.
Limitations	Large quantities of test compounds are required. Protein leakage through the membrane and fluid volume shifts within the system may be a source of significant errors. Due to the need of a long time to reach equilibrium, it is not appropriate for testing unstable compounds/systems.

Table 1 (*continued*)

Example	Equilibrium dialysis experiments provide information on the stoichiometry (1 ligand molecule per protein) and affinity ($K_a = 2.07 \times 10^4\ M^{-1}$) in the binding of lactose to murine galectin-3.
Suggested reading	Zentz et al. 1978 [366] • The interaction of the plant toxin ricin with galactose and lactose is studied by equilibrium dialysis, analytical ultracentrifugation, fluorescence polarization, microcalorimetry and hemagglutination, providing evidence for the presence of two distinct saccharide-binding sites per molecule. Knibbs et al. 1993 [367] • The carbohydrate-binding specificity of murine galectin-3 (previously known as carbohydrate-binding protein 35) is investigated by quantitative precipitation using a series of six glycoproteins and four BSA-based neoglycoproteins conjugates and by inhibition of precipitation by well-defined carbohydrate haptens. Quantitative determination of affinity/stoichiometry for lactose binding is achieved by equilibrium dialysis experiments (affinity constant of $2.04 \times 10^4\ M^{-1}$ and $n = 0.84$). Takeda et al. 2013 [368] • Affinity measurements for high-mannose-type *N*-glycans and two lectins of the quality control/ER-to-Golgi transfer network (malectin, VIP36) reveal separation of bound and free ligand by ultrafiltration to be feasible, an alternative to equilibrium dialysis.
21.	**Hemagglutination**
Principle	Bivalent (or multivalent) lectins can bind simultaneously to different cells resulting in cell bridging.
Information provided	A classical method for determining lectin activity by adding serially diluted lectin-containing solutions to aliquots of erythrocyte suspensions. Lectin-mediated cell association is measured (please see entries 29 and 30 (precipitation analysis and quantum dots (QDs)) for analogous approaches using glycoproteins and sugar-functionalized QDs).
Applications	Classical assay for lectin activity. Determination of inhibitory potential by saccharides.
Limitations	Selection of erythrocytes may be critical, as their glycomic profile varies between species.
Example	Agglutination of trypsin-treated rabbit erythrocytes by electrolectin, the first galectin studied. The presence of ligands in solution, such as e.g. Lac*NA*c, inhibits galectin-dependent cell bridging, whereas non-cognate sugars, such as Glc, have no inhibitory effect.
Suggested reading	Mitchell, 1860 [369] • Observation of blood "coagulation" by rattlesnake venom, attributed to Ca^{2+}-dependent lectins present in *Crotalidae* venoms more than 100 years afterwards. Stillmark, 1888 [370] • Detection of erythrocyte agglutination by protein fractions from castor beans and other plant seeds, the pioneering report on plant lectins and also the most detailed compilation of in vivo toxicity of ricin. Watkins and Morgan, 1952 [229] • Detection of α-linked *L*-fucose as integral part of the histo-blood group H (O) by blocking agglutination of respective erythrocytes with eel serum (containing a lectin specific for this blood group) by fucose and α-fucoside. Teichberg et al. 1975 [329] (please see comment in entry 6)
22.	**Isothermal titration calorimetry**
Principle	Almost any chemical reaction, interaction or physical change is accompanied by heat absorption (endothermic processes) or release (exothermic processes).
Information provided	Measurement of this heat as a function of the molar ratio of the involved molecules allows the determination of the association constant (K_a), stoichiometry (n) and the enthalpy of binding ($\Delta H°$), hereby facilitating calculations of entropy ($\Delta S°$) and also of the change in heat capacity (ΔCp) when measurements are carried out at different temperatures.
Applications	Most often used to study the binding of small ligands to proteins. Determination of the thermodynamic parameters allows further optimization of compounds in an iterative drug design process. Enthalpy/entropy determinants for a lectin with two binding sites facilitates structure–function considerations (e.g. on the role of Trp/Tyr for contact to Gal).
Limitations	It may require substantial lectin/ligand quantities. Other possible events associated to the binding process, e.g. a conformational change or a variation in the pKa of ionizable groups of the protein or/and ligand, may contribute to the apparent binding enthalpy (titrations at different pH values or using buffers with dissimilar ionization enthalpies facilitate the determination of intrinsic binding parameters).
Example	Analysis of the binding of lactose and Lac*NA*c to human galectin-1 reveals enthalpy–entropy compensation, as typically observed for lectin–sugar interactions. When binding to a multivalent ligand (the glycoprotein ASF with nine Lac*NA*c sites), a gradient of decreasing binding constants with a 6000-fold difference between the first and last epitopes is measured. The thermodynamic parameters for the wild-type protein and single-site mutants differ, as also found for galectin-1 from different mammalian species, hinting at differences in the architecture of the binding site (described at atomic level by X-ray crystallography), in the relative contribution of the sugar groups to the binding (information achievable through chemical mapping studies; please see entry 19), and/or in the entropic contribution at the level of the protein (SANS and FCS reveal a more compact structure for human galectin-1 after ligand loading).
Suggested reading	López-Lucendo et al. 2004 [319] (please see comment in entry 2) Velázquez-Campoy et al. 2004 [371] • Principles, applications and basic protocols for direct measurement of protein binding energetics by ITC. Dam et al. 2005 [372] • ITC measurements of human galectins reveal negative cooperativity of binding to a nonavalent ligand (the glycoprotein asialofetuin with nine Lac*NA*c termini). Whereas the microscopic binding constant for binding the first epitope is in the nM range, the affinity decrease follows a gradient with increasing level of saturation, independent of the galectin's quaternary structure. These data are relevant for lectin interaction with polyvalent ligands on cell surfaces. Jiménez et al. 2006 [347] (please see comment in entry 13) Martín-Santamaría et al. 2011 [144] • Analysis of the recognition of dithiodigalactoside by VAA and five human galectins, in comparison to thiodigalactoside and lactose, using solid-phase inhibition assays, STD, ITC, FACScan, and computational docking, reveals binding to VAA and considerably less reactivity, with intrafamily grading, for galectins, proving selectivity and showing the potential of glycosyldisulfides as chemical platform for inhibitor design. Sookcharoenpinyo et al. 2012 [206] • The recognition of disaccharides by two tricyclic synthetic receptors is studied by NMR, fluorescence titrations and ITC, the results derived from the three methods being consistent with 1:1 complexation. ITC measurements reveal that binding is driven by both negative ΔH and positive ΔS, in contrast to lectins for which negative binding entropies are common.
23.	**Lectin-affinity electrophoresis**
Principle	The electrophoretic mobility of a glycoprotein is reduced due to its interaction with a lectin present in the electrophoretic support, immobilized by entrapment or by covalent linkage (similar principle as for lectin affinity chromatography).
Information provided	Reduction of electrophoretic mobility evidences interaction. When applied to mixtures, it discriminates between interacting and non-interacting species, also separating glycoforms. Dependence of the retardation on the lectin concentration, using lectin-gradient affinity electrophoresis, provides information on binding affinity. Further information can be obtained by exploiting two-dimensional combinations with other electrophoretic approaches (e.g. immunoelectrophoresis) or by using two different lectins for the first and second dimensions, also run as capillary affinophoresis.

(*continued on next page*)

Table 1 (*continued*)

Applications	Detection, structural analysis, and quantitation of glycans in glycopeptides and glycoproteins. Separation of glycopeptides and glycoproteins in complex mixtures. Detection and characterization of glycoforms (of potential diagnostic use). Electrophoretic separation of lectin isoforms in the first dimension, followed by affinity electrophoresis against the separated lectin isoforms, allows evaluation of their binding capabilities. Estimation of binding affinities.
Limitations	Non-specific interactions may occur. Lectin immobilization and/or electrophoretic conditions may affect binding capability, the detection limit being also dependent on the glycoprotein (as for lectin blots and lectin affinity chromatography). Weak complexes may not be detected.
Example	Historically, affinity constants of the interaction of human serum glycoproteins (α_1-antitrypsin, α_1-acid glycoprotein and others) with concanavalin A were determined in the order of 10^{-5} M.
Suggested reading	Bøg-Hansen and Takeo, 1980 [373] • Establishing lectin affinity electrophoresis as quantitative assay for determination of affinity constants of the interaction of concanavalin A with a glycoprotein (mixture). Linhardt et al. 1995 [374] • Description of a two-dimensional lectin affinity electrophoresis protocol that affords separation of fluorescently labeled disaccharides, also applicable to the separation and analysis of glycopeptides and glycoproteins. Bøg-Hansen, 1998 [375] • Detailed presentation of experimental protocol for application. Taketa, 1998 [376] • Description of different modalities of lectin affinity electrophoresis using several two-dimensional combinations: two different lectins for the first and second dimension, lectin-gradient affinity electrophoresis and electrophoretic separation of lectin isoforms in the first dimension, followed by affinity electrophoresis against the separated lectin isoforms. Shimura and Kasai, 2003 [377] • Following description of synthesis of a monovalent affinophore, application is presented for two human galectins and the pea lectin, to illustrate affinity measurements. Kakehi and Kinoshita, 2009 [378] • Development of a method for glycan analysis based on high-resolution separation of fluorescent carbohydrates by capillary electrophoresis with laser-induced fluorescent detection, in the presence of carbohydrate-binding proteins at different concentrations. The technique affords simultaneous determination of glycans having similar structures even in complex mixtures.
24.	**Lectin blots**
Principle	Detection and characterization of glycans on proteins (or lipids) immobilized onto an adsorbent membrane (e.g., nitrocellulose or polyvinylidene difluoride), via direct spotting or electrotransfer from a gel after electrophoresis, by exploiting the carbohydrate-binding specificity of lectins (the same principle underlying lectin cyto- and histochemistry).
Information provided	Structural features of the glycans can be inferred based on the fine specificity of the lectins. When combined with two-dimensional SDS-PAGE (and mass spectrometric fingerprinting), it serves to identify glycoproteins of interest by comparison with a reference protein map.
Applications	Monitoring of the glycosylation pattern of samples with different levels of complexity, from purified proteins to complex cell lysates or human fluid samples. Characterization of alterations in glycosylation associated to development or disease, or after manipulation of glycosylation pathways (please see glycome engineering in entry 38).
Limitations	The detection limit depends both on the glycoprotein and the lectin. False-positive (non-specific) binding may occur, inhibition of binding by appropriate haptens being decisive to confirm specific lectin–sugar binding.
Example	Lactose-inhibitable binding of human galectins-1 and -3 to the glycoproteins laminin, carcinoembryonic antigen (CEA), lamp-1 and lamp-2, electroblotted onto nitrocellulose membranes after electrophoresis, is observed by lectin blotting and detection of bound galectin with anti-galectin antibody. Carbohydrate-mediated binding to CEA is also detected in a dot blot set-up, using the glycoprotein immobilized onto nitrocellulose and biotinylated galectin-3 as probe, revealing dose-responsiveness of lectin binding to increasing glycoprotein amounts.
Suggested reading	Gabius et al.1991 [379] • Detection of galectin-reactive glycoproteins in human sialic nerve by lectin blotting, flanked by lectin affinity chromatography and lectin histochemistry detecting reactivity primarily in epineurium and nerve fibers. Ohannesian et al. 1994 [380] • Human galectin-1 purified from placenta is used as probe for testing glycoprotein reactivity in blots identifying the glycoproteins CEA and lamp1/2 as ligands. Ohannesian et al. 1995 [354] (please see comment in entry 16)
25.	**Microarrays**
Principle	A series of molecules, fragments or even cells (probes) attached in a regular pattern onto a supporting material (typically a microscope glass-slide) serve as platform for assaying biological molecules (targets) using high-throughput screening methods.
Information provided	Probe-target reactivity is detected and quantified using labeled (usually fluorescent) targets or anti-target antibodies in an ELISA-type arrangement. The information derived from the assay depends on the system, for lectins/glycans providing data on mutual reactivity.
Applications	Gene/protein/glycan profiling. Detection of disease biomarkers. Identification of protein–protein and protein–ligand interactions. Elucidation of ligand-binding specificity of a given receptor.
Limitations	A large collection of probes is usually required. Selection of surface and method of attachment may be critical for preservation of the biological activity of the probes and shelf-life of the arrays.
Example	Testing of galectin-1 using glycan microarrays reveals low avidity of galectin-1 for fucose-containing A and B histo-blood group antigens (in comparison to galectins-2 and -3) and binding to α2,3- but not α2,6-sialylated glycans, as similarly found by FAC and predicted from chemical mapping studies and inspection of the crystal structure. Screening bacterial polysaccharides for galectin binding can be medically relevant.
Suggested reading	Campanero-Rhodes et al. 2007 [381] • Using carbohydrate microarrays, the high specificity of simian virus 40 for ganglioside GM1 is confirmed, also revealing that *N*-glycolyl GM1 is a better ligand than the *N*-acetyl analog and confers better virus binding to cells and increased infectivity. Stowell et al. 2008 [382] • Glycan microarrays using synthetic oligosaccharides are used to examine the binding specificity of galectins-1, -2, and -3, in parallel to binding studies to promyelocytic HL60 cells and to human erythrocytes. Leppänen and Cummings, 2010 [383] • Description of a simple fluorescence-based solid-phase assay for the study of protein–glycoconjugate interactions, including examples of the study of P- and L-selectins and galectin-1 binding to immobilized glycopeptides, oligosaccharides, and cells, thereby extending the information achievable using glycan microarrays to more "natural" glycoconjugate arrays. The method uses commercial streptavidin-coated microplates, in which biotinylated ligands are immobilized at a defined density. Hirabayashi et al. 2013 [384] • Lectin microarrays have emerged as a novel platform for rapid and high-sensitivity profiling of glycans, targeting various forms of glycans and even cells. Here, the essence of lectin microarrays is described, with focus on evanescent-field-activated fluorescence detection. Knirel et al. 2014 [385]

Table 1 (*continued*)

Suggested reading	• Presentation of a wide range of bacterial polysaccharides (up to 147) reveals specific binding properties of certain glycans to human tandem-repeat-type galectins, defining targets for assays on growth inhibition. Presence of canonical epitopes does not necessarily lead to reactivity, and an α-rhamnan (*E. coli* O19ab) is a binder.
	NMR applications
26.	a) **Saturation transfer difference (STD) spectroscopy**
Principle	For a given receptor–ligand mixture and following the selective saturation of the resonance signals of the receptor, there is a transfer of this excitation from the macromolecule to the ligand, due to spatial proximity during chemical exchange between the free and bound states. The signals are assigned to distinct ligand atoms, revealing a contact pattern.
Information provided	Allows monitoring the association of a particular ligand (or mixture of ligands) to a large receptor, providing also information on the ligand epitope in contact to the receptor. No isotope labeling of the receptor is required.
Applications	Screening and identification of ligands in mixtures for a given receptor. Delineation of the ligand's binding epitope and estimation of binding affinities and dissociation kinetics. Using ^{19}F-based sensors (e.g. a fluoro derivative of a sugar, taking advantage of 100% natural abundance, large chemical shift dispersion and sharp signals), screening in the inhibitory setting is also feasible. Ideally, it is complemented by chemical shift perturbation analysis of protein resonances (please see HSQC in entry 12).
Limitations	The dissociation rate of the exchange process must be fast in the relaxation timescale. It is therefore valid for interaction processes of weak to moderate binding affinities. A molar excess of the ligand over the receptor is required.
Example	STD analysis of the binding of Galα1,3Gal and Galα1,4Gal to human galectin-1, in comparison with lactose, reveals that the non-reducing Gal moiety is the major epitope for recognition, main STD intensities being observed for H4′–H6′, in agreement with the information derived from chemical mapping studies (information on protein groups involved in the binding is accessible by HSQC). The interaction also involves the ligands' reducing ends, what is not observed when testing melibiose as control. The STD pattern differs from that measured when testing a plant toxin (VAA), and is in accord with the differential tolerance of the lectins to the two types of sialylation (α2,6 for VAA, α2,3 for galectin-1).
Suggested reading	Mayer and Meyer, 1999 [386] • Presentation of the STD concept and the first application in the carbohydrate–protein interaction field. Siebert et al. 2003 [130] (please see comment in entry 1) Miller et al. 2011 [345] (please see comment in entry 12) André et al. 2012 [387] • Extension of the STD method using ^{19}F NMR spectroscopy, this special nucleus presented on the carbohydrate ligand. The applicability of the technique for inhibitor screening is documented with two leguminous lectins and a plant toxin as target proteins.
27.	b) **Transferred nuclear Overhauser effect spectroscopy (TrNOESY)/pseudo contact shifts**
Principle	Resonance phenomenon arising from a through-space dipolar interaction between two ligand protons in chemical exchange between the free and bound states in the presence of a receptor.
Information provided	The alteration of intensity of the assigned signals serves as molecular ruler for distance assessment and, therefore, provides information on the bound-state conformation of the ligand, in complex with a particular receptor.
Applications	Determination of the bioactive conformation of a ligand for a given receptor. Discrimination between interacting and non-interacting molecules within a mixture in the presence of a receptor. Combination with molecular modeling techniques and/or other NMR methods (paramagnetic lanthanide-binding tags to induce pseudo contact shifts from dipolar interactions between unpaired electrons of a lanthanide ion and nuclei in the vicinity; monitoring residual dipolar couplings to measure orientational constraints) is also useful to deduce the key structural features of the ligand.
Limitations	The dissociation rate of the exchange process must be fast in the relaxation timescale. It is therefore valid for interaction processes of weak to moderate binding affinities. A molar excess of the ligand over the receptor is required, spin diffusion must be excluded.
Example	TrNOESY experiments demonstrate that, in solution, bovine heart galectin-1 selects the *syn* conformation of lactose (as observed in the crystal structure), typified by the H1′–H4 contact. The same *syn* conformation of *C*-lactose (the nonhydrolyzable *C*-glycoside), but not the conformer at the global minimum for this analog, the *anti* conformation, is recognized by the lectin, what represents a case of conformer selection by a lectin's binding site.
Suggested reading	Asensio et al. 1999 [137] • The bound conformation of *C*-lactose to galectin-1 in solution is determined by TrNOESY. Docking of the analog within the galectin's binding site furnishes explanations, in structural terms, for the exclusive recognition of the *syn* conformer. Umemoto et al. 2003 [342] (please see comment in entry 11) Siebert et al. 2003 [130] (please see comment in entry 1) Zhuang et al. 2008 [388] • By covalent engineering of the lectin domain of human galectin-3, which is extended by a 17-amino-acid lanthanide-binding tag, Dy^{3+}/Tb^{3+} (with large magnetic susceptibility anisotropy) is placed in immediate vicinity to the lectin to monitor pseudo contact shifts and field-induced residual dipolar couplings. Distance constraints up to 35 Å become detectable, applied for the Glc moiety of the ligand. Mallagaray et al. 2011 [156], Canales et al. 2013 [389] • Presentation of a novel concept, taken from the protein field, to derive conformational aspects of saccharides by using paramagnetic ions in spatial vicinity, this accomplished by glycan derivatization with a lanthanide-binding tag including a biphenyl unit as part of the rigid linker and a chelating (EDTA-like) terminus. The monitoring of biofunctional glycans provides conformational information on branched (symmetric) oligosaccharides and defines the contact sites for lectins. Roldos et al. 2011 [390] • A comprehensive review describing different applications of NMR methods to monitor protein–carbohydrate interactions, especially from the perspective of the ligand. Martín-Santamaría et al. 2012 [316] (please see comment in entry 1)
28.	**Photoaffinity labeling**
Principle	A bifunctional reagent (ligand derivative) is converted by photolysis to an extremely reactive intermediate capable to covalently attach the bound ligand part to a receptor. Alternatively, the lectin can be derivatized so that a cognate glycoconjugate will be conjugated after photoactivation.
Information provided	Reagent molecules reversibly in contact with the respective counterreceptor become covalently linked upon photolysis, thereby allowing complex formation of binding partners with high specificity.
Applications	Labeling identifies counterreceptors for the ligand part of the probe, as single protein and in aggregates such as the hepatic asialoglycoprotein receptor. When using derivatized lectin, useful for the detection of physiological, and as yet unidentified, ligands for proteins that cannot be detected by other techniques because of their relatively weak interaction.
Limitations	The derivatization of the ligand/protein may alter the expected mechanism and give rise to non-specific labeling: enormous reactivity of the derivative after photoactivation can generate random labeling.
Example	A covalent complex between the model glycoproteins asialofetuin and laminin and a human galectin-1 mutant obtained by introducing a Cys residue at position 28 (K28C) in a special form of human galectin-1 (i.e., with C to S replacement of accessible Cys residues 2, 16, 88 and 130) (please see mutant design) is obtained by chemical cross-linking using a photoactivatable sulfhydryl reagent (benzophenone-4-maleimide). The complex is not formed in the presence of a competing sugar, or when β-galactosidase-treated asialofetuin is used, proving the dependence on carbohydrate binding of the labeling.

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Table 1 (*continued*)

Suggested reading	Oda and Kasai, 2004 [391] • The radioactive, heterobifunctional, photoactivatable and cleavable cross-linking reagent [^{35}S]*N*-succinimidyl-3[(2-nitro-4-azidophenyl)-2-aminoethyldithio] propionate is first reacted with a chicken galectin (CG-16) to generate modified receptor. The aryl azide group then makes cross-linking to cognate glycoconjugates possible by UV irradiation, which become radioactively labeled. The lectin part can be separated by disulfide cleavage, hereby producing radioactively labeled counterreceptor. Proteoglycans with Lac*NA*c repeats appear as counterreceptors, sensitive to endo-β-galactosidase treatment. Rice et al. 1990 [392] • Affinity labeling of the asialoglycoprotein receptor on rat hepatocytes with triantennary complex-type glycopeptides using *N*-5-azido-2-nitrobenzoyloxy succinamide is crucial to determine the precise binding geometry between the trivalent ligand and the hetero-oligomeric RHL1 and RHL2/3 subunits. Muramoto et al. 1994 [393] • A photoactivatable 1-azido-5-naphthalene sulfonyl (ANS)-lactose derivative, with the same activity as lactose in hemagglutinating assays, is used for photoaffinity labeling of a conger eel galectin. Isolation of fluorescent chymotryptic peptides by reverse-phase HPLC and characterization by amino acid analysis and *N*-terminal sequencing identifies peptide 31–45 as the major labeling position. Tamura et al. 2009 [394] • A combination of cysteine mutagenesis with chemical cross-linking using a photoactivatable sulfhydryl reagent is used to obtain a covalent complex between human galectin-1 and the model glycoprotein ligands asialofetuin and laminin. The cross-linked product is only obtained when the galectin-1 mutant binds these glycoproteins via their carbohydrate moiety.
29.	**Precipitation analysis**
Principle	Multivalent lectins may bind and precipitate polysaccharides, multiantennary oligosaccharides, glycopeptides, and glycoproteins, due to the formation of homogeneous cross-linked lattices.
Information provided	Precipitation evidences binding. Quantitative precipitation studies using a series of lectin and ligand concentrations provide information on the stoichiometry of the complexes, which depends on the sugar–receptor pair involved.
Applications	Detection of carbohydrate-binding and cross-linking activity. Determination of inhibitory potential by saccharides (please see also hemagglutination in entry 21).
Limitations	Relatively large amounts of lectin and ligand are required. Not valid for monomeric lectins.
Example	Galectin-1 forms stoichiometric cross-linked complexes with asialofetuin (ASF). At low ASF concentrations (up to 15 μM), 1:9 ASF/lectin (monomer) complexes are formed, whereas at higher ASF concentrations (up to 60 μM) the ratio decreases to 1:3, after which it remains constant, indicating the formation of two different cross-linked complexes by occupying with lectin either the three or only one branch termini of the three triantennary N-glycans. Inhibition of precipitation (e.g. of laminin by murine galectin-3) is a measure of affinity for oligosaccharides, the extent of precipitation of affinity for (neo)glycoproteins.
Suggested reading	Sumner and Howell 2009 [395] • Concanavalin A is identified as jack bean hemagglutinin. Precipitation of starch, glycogen and mucins by concanavalin A defines carbohydrate as ligands and points to "a carbohydrate group in a protein" as binding partner on erythrocytes. Knibbs et al. 1993 [367] (please see comment in entry 20) Gupta et al. 1996 [396] • Quantitative precipitation studies, measuring stoichiometry of glycoprotein/lectin presence in the formed complexes, of the Lac-specific plant lectins from *Viscum album* and *Ricinus communis*, and the homodimeric galectin from chicken liver (CG-1A) with the glycoprotein asialofetuin with its up to nine glycan antennae, yielding glycoprotein-lectin lattices. Nakagawa et al. 2011 [220] • Combination of the co-precipitation analysis based on the aggregation of pradimicin A (PRM-A) in the presence of Ca^{2+} with the study of the binding of mannose using solid-state NMR. ITC and precipitation experiments show that PRM-A harbors two binding sites for mannose with significantly different affinities, facilitating the preparation of solid 1:1 PRM-A:Man-OMe complexes for subsequent NMR analysis. A model for the primary mannose–PRM-A interaction is proposed, the architecture of the binding site resembling those of synthetic tripodal receptors.
30.	**Quantum dots (QDs)**
Principle	Quantum dots (QDs) are nanotechnology crystals that absorb light and then re-emit longer-wavelength photons for a period of time. The wavelength with which they emit light depends on the size of the crystals so that the control of their size allows a very precise control over the wavelength of the re-emitted photon.
Information provided	The broad range of applications of QDs described below generates very diverse information.
Applications	Use as physical platforms to develop biosensors and bioprobes. Detection of protein–ligand interactions using QDs functionalized with appropriate ligands. Suitable for high-sensitivity applications like fluorescent tagging and live-cell imaging. QDs can be used in various forms, e.g. as small crystals in liquid solutions, as quantum dust, and in bead form.
Limitations	Surface defects result in blinking of the QDs and deteriorate the quantum yield. Difficulties for using QDs within cells include their delivery into the cells (without killing the cells in the process), aggregation inside the cells, and dose-dependent toxicity of both the coating shell and the core. In vivo, QDs have been found to accumulate in kidney, spleen, and liver, clearance from the body being unknown, possibly a biohazard.
Example	Mimicking hemagglutination and cross-linking of natural ligands, sugar-functionalized QDs are used for the sensitive detection of lectins using an agglutination assay, the selective aggregate dissociation by specific haptens proving that the process is reversible and specific.
Suggested reading	Babu et al. 2007 [397] • Three different sizes of QDs functionalized with lactose, melibiose, and maltotriose are used for lectin detection through agglutination assays. Agglutination of sugar-QDs occurs through specific multivalent carbohydrate–lectin interactions. Reichardt et al. 2013 [398] • Survey of potential applications of glyconanomaterials as multivalent scaffolds for drug delivery, enzyme inhibition and vaccine development, of glycan-functionalized QDs and nanoparticles in molecular imaging or as biosensors for lectin/glycan detection based on nanomaterials, and introduction to emerging concepts for the affinity separation and analysis using nanomaterials or nanotools.
31.	**Surface plasmon resonance (SPR)**
Principle	Determination of the refractive index near the planar surface of a sensor chip, presenting immobilized lectin/ligand, upon passing an analyte solution over the chip. Binding is measured by real-time changes in the refractive index and is recorded in resonance units (RU; 1000 RUs ≈ 1 ng of mass per mm^2).
Information provided	Determination of association and kinetic constants of ligand–receptor pairs.
Applications	Study of biomolecular interactions of label-free compounds in real-time. Automated evaluation of binding assays on chips using appropriate binding models.
Limitations	It uses expensive sensor chips of limited re-use capacity. It may require complex chemistry for ligand or protein immobilization. It cannot predict the behavior in force-driven experiments (please see entry 18).
Example	SPR analysis of galectin-1 injected over a sensor presenting potential/cognate ligands allows detection of binding, estimation of affinity and kinetic parameters and evaluation of the inhibitory potential of new compounds (e.g. synthetic glycopeptides), also revealing inter-galectin differences when compared with its chicken ortholog CG-1A or with galectin-3 (complementing information from FAC or ITC). The low k_{off} value from a

Table 1 (*continued*)

Example	surface presenting abundant β-galactosides points to the suitability of galectin-1 for being engaged in *cis*-interactions on the cell surface (please see entry 32 for measurement of binding to cells).
Suggested reading	Dettmann et al. 2000 [360] (please see comment in entry 18) Maljaars et al. 2008 [399] • The study of the inhibitory potency of synthetic (glyco)peptides toward human galectins-1 and -3 reveals beneficial effects of presenting the carbohydrate ligand in distinct hexapeptide contexts and evidences differences in fine-specificity and affinity for the two lectins. Muñoz et al. 2010 [400] • The bioactivity of functionalized fluorescent lactose is tested with galectins-1, and -3 and CG-1A, confirming binding affinity and enabling kinetic analysis of the interaction by SPR. No major contacts to the aglyconic part are detected, supporting the potential of this approach for the detection and characterization of the binding specificity of these lectins.
	CELL- AND TISSUE-BASED METHODS
32.	**Fluorescence-activated cell scanning (cytofluorometry) (FACScan)**
Principle	Following incubation with a fluorescent probe (glycan-presenting scaffolds/lectins), cells suspended in a stream of fluid pass in single file by an excitation light source. When an excitation light strikes the moving particles, light is scattered and fluorescence is emitted.
Information provided	Light scattering is directly related to morphological cell features, e.g. size and internal complexity, while fluorescence occurs if there is a fluorescent probe within the cells or bound to their surface. As the extent of fluorescence emitted is proportional to the amount of bound fluorescent probe, by measuring mean fluorescence intensity and percentage of positive cells, qualitative and quantitative information is obtained. Scintillation counting of radioactivity or OD-measurements provides equal information, if radiolabelled probes or neoglycoenzymes are used.
Applications	Analysis of DNA/RNA content, intracellular events or membrane dynamics. Characterization of the glycophenotype/lectin presence and detection of differences with cell-status changes (e.g. differentiation) or upon defined changes in glycan processing (please see entry 38 on glycome engineering) or in the proteomic profile, e.g. by transfection. Detection of ligands on the cell surface for endogenous lectins. Also applicable for profiling of lectin specificity using e.g. fluorescent neoglycoconjugates and lectin-loaded cells (affinity chromatography and microarrays also providing information on lectin specificity) and for screening of compounds competing with cell surface glycans for the lectin's site.
Limitations	A suitable fluorescent probe, which is incubated with the cells before the analysis, is required. When used for cell culture, serum constituents may impair binding, whereas serum-free culture may affect cell properties. Cell-aggregate formation is a problem that impedes the one-cell-at-a-time analysis scheme of flow cytometry.
Example	FACScan analysis reveals binding of human galectin-1 to cells, dependent on their physiological parameters or on the presence of glycomimetics in screening of compound libraries. Probing galectin-loaded cells with fluorescent neoglycoconjugates reveals inter-galectin specificity differences. Scatchard analysis provides K_d-values and number of bound probe molecules at saturation, e.g. depending on clustering in microdomains. A simple alternative to monitoring fluorescence (or radioactivity) using enzyme-conjugated probes facilitates quantitation by OD-measurements.
Suggested reading	Gabius et al. 1993 [401] • Neoglycoenzymes with four different sugar parts characterize receptor expression of three human B-cell-lineage lines, with K_d-values in the range of 3–292 nM. Binding studies to a sugar-exposing matrix reveal especially strong adhesion for the myeloma line. André et al. 1999 [402] • FACScan analysis of the cell-surface expression of galectins-1 and -3 and accessible galectin-binding sites on various tumor cells reveals different ligand accessibility for the two galectins. Differences are also apparent in the reduction by galectins of the binding of tumor cells to laminin and plasma/placental fibronectin. Biodistribution of radioiodinated galectins is quantitated in vivo. André et al. 2007 [302] • Combined microarray for selected glycosyltransferase genes with 2D chromatographic glycan profiling and FACScan analysis of binding of a plant lectin panel reveals major differences between tumor suppressor $p16^{INK4a}$-positive and control pancreatic carcinoma cells concerning expression of β1,4-galactosyltransferases as well as decreased *O*-glycan α2,3-sialylation and *N*-glycan α2,6-sialylation, resulting in enhanced binding of galectin-1 to the cells. The level of transcription of the galectin-1 gene also increased noticeably in $p16^{INK4a}$-positive cells. Rapoport et al. 2008 [103] • A cell-based assay using fluorescent glycoconjugates as flow cytometry probes is used for examining the binding specificity of human galectins-1 and -3 loaded onto the surface of Raji cells, disclosing characteristic profiles of glycan reactivity. Kopitz et al. 2010 [403] • Cholesterol depletion of membranes in human neuroblastoma cells drastically reduces cell binding of galectins-1 and -3. Binding affinity is also significantly lowered by blocking ganglioside synthesis, besides disruption of microdomain integrity, showing that target specificity and topology of ligand presentation act together, using iodinated galectins as probes for Scatchard analysis. Giguère et al. 2011 [127] • Screening of a galactoside library covering different anomeric extensions and substitutions, including also three bi- to trivalent galactopyranoside and lactoside clusters, as inhibitors of the binding of VAA and galectins-1, -3, -8, and -9 in solid-phase and cell binding assays shows that, within the tested panel, the increase in valency is more suited to generate enhanced inhibitory capacity than derivatization.
33.	**Lectin cyto- and histochemistry/reverse lectin cyto- and histochemistry**
Principle	Detection of glycans/lectins in cell preparations and frozen/fixed tissue sections by exploiting the superior carbohydrate-binding specificity of lectins, as compared with conventional histochemical stains for carbohydrates, and the binding capacity of lectins to their natural counterreceptors.
Information provided	Localization of glycan/lectin-reactive sites. Specificity of tissue lectins can be delineated by testing a panel of neoglycoconjugates and (semi) quantitative assessment of staining intensity. Structural features of the reactive glycans in the biological/clinical material can be inferred based on the oligosaccharide specificity of the lectin.
Applications	Characterization of the glycophenotype and detection of changes at different physiological and pathological stages, with diagnostic implications. Determination of presence and distribution of ligands for endogenous lectins, both extra- and intracellularly, and of receptors for the glycan part of neoglycoconjugates in reverse lectin cyto- and histochemistry (please see also FACScan analysis for detection of lectins/ligands on the cell surface in entry 32).
Limitations	Use of organic solvents for cell/tissue fixation and embedding can extract glycoconjugates such as glycolipids. Controls to demonstrate the specificity of the staining by lectins/neoglycoconjugates are required. Labeling of the lectin can affect its properties.
Example	Staining profiles of cells/tissues with neoglycoconjugates/lectins differ, with potential for functional implications and diagnostic application. One- or two-step protocols for processing and specificity controls are readily applicable. Testing homologous lectins reveals non-overlapping profiles as indication for development of protein-type-specific functions.
Suggested reading	Gabius et al. 1988 [181] • The presence and distribution of endogenous sugar receptors in sections from benign and malignant breast lesions, in comparison to normal breast, are examined using a panel of biotinylated (neo)glycoproteins, revealing differential patterns of staining localization and intensity, even between various types of breast cancer. Gabius et al. 1989 [404] • Chemical glycosylation of an enzyme (here bacterial β-galactosidase), to produce a neoglycoenzyme, combines the installed ligand property for lectins with the natural label of enzyme activity. Receptors for the conjugated sugars are detected in various settings of assays such as ELISA-type tests and in tissue sections.

(*continued on next page*)

Table 1 (*continued*)

Suggested reading	Purkrábková et al. 2003 [405] • Following the analysis of biotin incorporation into galectins by two-dimensional gel electrophoresis, a protocol is described for testing human lectins as probes. Nuclear binding of galectin-1 is detected in stromal cells of human bone marrow and keratinocytes, along with nuclear presence of the lectin. Habermann et al. 2011 [406] • The potential of galectins as cyto/histochemical tools for glycophenotyping is demonstrated by using confocal laser scanning microscopy and bovine germinal vesicle oocytes as test system, plant lectins being employed in parallel to map the sialylation status. Detailed comparison of the staining properties reveals galectin-specific and non-uniform staining. Schlötzer-Schrehardt et al. 2012 [407] • The presence of galectins in the human eye is examined by immunodetection using a panel of non-cross-reactive anti-galectin antibodies, revealing the presence of all the galectins examined with different regional distribution and characteristic galectin-specific profiles. Localization of galectin-binding sites by employing biotinylated galectins as tools leads to distinct staining characteristics, pointing to potential for diagnostic application. Dawson et al. 2013 [408] • Applications of galectin-specific antibodies and labeled galectins define distinct localization profiles in routinely processed sections of colon cancer. The immunohistochemical part extends network analysis from previous work on galectins-1, -2, -3, -4, and -8 to analyzing presence of galectins-7 and -9. Tumor positivity for labeled galectin-3 is associated with lymphatic invasion.
	***IN VIVO* METHODS**
34.	**Biodistribution**
Principle	A labeled compound of interest (lectin or (neo)glycoprotein) is tracked in circulation in vivo. Time-resolved monitoring with a high degree of sensitivity and specificity is facilitated by imaging technologies. Measurements of clearance from serum are possible.
Information provided	Quantitative assessment of the fate of administered compounds, including organ uptake, tissue metabolism, and excretion, and their biochemical and physiologic effects.
Applications	Tracking of compounds of relevance in healthy controls and in disease states. Assessment of biological targeting and competitive binding. Evaluation of pharmacokinetics and pharmacodynamics.
Limitations	Data from animal studies with iodinated probes cannot simply be extrapolated (due to e.g. marked specificity differences among mammalian asialoglycoprotein receptors).
Example	Biodistribution analysis in vivo of radioiodinated galectin-1 after intravenous injection in mice reveals differences in retention depending on the organ, the highest content being found in kidney, salivary glands and stomach, whereas the lowest uptake is found for heart. Galectin PEGylation (for details on respective procedure, please see [349] in entry 15) is a means to prolong serum presence. Neoglycoproteins with systematically varied glycan structures teach lessons on their impact on serum clearance.
Suggested reading	Morell et al. 1968 [161] • Injection of radioactive desialylated ceruloplasmin into rabbits results in a rapid disappearance of the protein from serum (more than 90% 15 min after injection, as opposed to less than 10% for native, sialylated ceruloplasmin) and its appearance in parenchymal cells, but not in Kupffer cells, of the liver. The rapidity of the transfer depends on the presence of intact galactose residues at the nonreducing ends of the glycan. Kojima et al. 1990 [409] • Quantitation of tissue distribution of a panel of radioiodinated neoglycoproteins with variations in the carbohydrate determinant, its density and linkage to the carrier, after intravenous injection into mice, reveals individual responses on the organ level, the increase in sugar density causing a decrease in tissue retention. Measurement of uptake using mammalian lectins as radiotracers also shows differences in retention depending on the organ. André et al. 1999 [402] (please see comment in entry 32) Unverzagt et al. 2002 [89] • Core fucosylation of complex-type biantennary *N*-glycans is shown to result in an up to five-fold alteration of affinity for lectins in solid-phase assays and marked differences in cell surface binding of cultured tumor cells and in organ distribution. In vivo, α2,6-sialylated neoglycoproteins show a reduced serum half-life in mice relative to the α2,3-sialylated isomer and the non-fucosylated congener. Kopitz et al. 2013 [410] • Serum clearance in rats of the Cys57Met mutant of galectin-2 is markedly retarded by PEGylation. In addition, biochemical, cell biological, and histochemical analyses also reveal a concomitant decrease in affinity/signal intensity, with context dependence.
	MISCELLANEOUS
	Design of mutants
35.	a) **Lectins**
Principle	Substitution of a given amino acid (or a set of amino acids), naturally by single nucleotide polymorphism or in recombinant proteins by any other residue, here using site-directed mutagenesis, is tested to record an impact on the protein's properties.
Information provided	The behavior of the mutant in comparison to the wild-type protein may provide information on the role of the original amino acid.
Applications	Testing hypothesis on the role of specific residues, in e.g. ligand binding or self-association. When occurring in natural variants, deriving information on potential correlations with disease states. Engineering of ligand-binding specificity. Generation of protein variants with superior properties, as improved stability, enhanced binding activity or tailored pH-profiles.
Limitations	Single-site replacements may induce long-range effects on the protein, obscuring interpretation of the local effect at the site of mutation. Complementary structural information is essential for establishing structure–function relationships. Yields in protein expression may decrease dramatically.
Example	Substitution at the binding site of human galectin-1 of the central Trp residue by Leu results in an inactive protein, whereas on replacement by Phe or Tyr the lactose-binding activity is reduced but maintained, demonstrating the potential of these residues to be engaged in C–H/π-interactions to the B-face of galactose. A Cys2Ser substitution alters thermodynamics of binding; a single-site mutation in a fungal galectin at Asn46 shifts binding from β-galactosides to a distinct α-linked epitope.
Suggested reading	Abbott and Feizi, 1991 [411] • Several mutants of bovine galectin-1 are expressed for gaining insight into the polypeptide length required to form the carbohydrate recognition domain and on the functional importance of some of the highly conserved amino acids. Hirabayashi and Kasai, 1991 [412] • The role of selected amino acid residues of human galectin-1 is studied by site-directed mutagenesis. Ten mutant proteins are produced by single-site substitution of Cys residues (potentially related to the stability of the lectin) or of residues highly conserved in the galectin family (Asn46, Trp68, Glu71, and Arg73). Differential sensitivity to the position of the Cys-to-Ser substitution is disclosed, and the assumed importance of signature amino acids underlined. López-Lucendo et al. 1984 [319] (please see comment in entry 2) Hu et al. 2013 [413] • Substitution of Asn46 in the *Agrocybe cylindracea* galectin by site-directed mutagenesis increases the binding to Gal*N*Acα1,3Galβ-containing

Table 1 (*continued*)

Suggested reading	glycans, while eliminating binding to any tested β-galactoside, as shown by glycoconjugate microarray analysis and frontal affinity chromatography (please see entries 16 and 25 for details on these techniques).
36.	b) **Glycogenes**
Principle	Inactivation or overexpression of glycogenes, i.e. genes encoding glycan-processing enzymes (glycosyltransferases, glycosidases and enzymes for modifications such as sulfotransferases) or transporters for activated sugars, in mutant/transfected cells leads to altered glycosylation profiles. Cell models are also obtained by screening for spontaneous mutations (e.g. testing resistance to lectin toxicity) or by chemical mutagenesis followed by screening, as done for the Chinese hamster ovary (CHO) panel of glycosylation mutants.
Information provided	As above, wild type *vs* mutant comparison delineates functional aspects.
Applications	Binding studies to delineate structure–function correlations. Production of suitably engineered pharmaglycoproteins.
Limitations	Cellular glycophenotype may be subject to compensations obscuring direct effect of a mutation. Interspecies differences should always be considered prior to extrapolations.
Example	Glycosylation mutants (from the CHO panel) are instrumental to define specificity differences on cell surfaces, e.g. between human galectins-8 and -9. X-ray crystallography and NMR spectroscopy provide a structural rationale for this behavior. Glycosylation also affects glycoprotein stability, as shown for the epidermal growth factor receptor.
Suggested reading	Patnaik and Stanley, 2006 [414] • Survey of generation of lectin-resistant CHO glycosylation mutants, the characterization of the molecular defects and their representation on the level of protein glycosylation. Solís et al. 2010 [325] (please see comment in entry 4) Gabius et al. 2012 [57] • Four glycosylation mutants of CHO cells with defects in *N*-glycan processing and branch-end maturation are tested for EGFR expression, production, functionality and routing after transfection with a vector encoding for human EGFR, revealing dependence of EGFR expression on the presence of β1,4-galactosyltransferases-I–VI.
37.	c) **Animal models**
Principle	Introduction of a null mutation into a lectin gene (or glycogene) by homologous recombination in embryonic stem cells for gene knock-out (KO) in both alleles.
Information provided	Effects of absence of functional lectin/glycogene product in vivo.
Applications	Obtaining information on in vivo response in development, to physiological challenges and in disease states.
Limitations	Gene defect can be compensated by regulatory processes: animal model thus is not a wild type with a defined defect but a biological system dynamically responding to the defect; easily done only for single-copy genes. As for glycogene mutants, potential for interspecies differences must be considered.
Example	Normal development, viability and fertility are observed in KO mice deficient of galectin-1, but impaired axon guidance is noted in olfactory bulb. Defects in ganglioside production impair galectin-1-mediated immune regulation. Defects in glycan production have multiple associations to disease states.
Suggested reading	Poirier and Robertson, 1993 [415] • A strain of mice is generated by introducing a null mutation in the gene coding for galectin-1. Mutant animals develop normally and are viable and fertile, suggesting that other protein(s) compensate for the absence of galectin-1. Puche et al. 1996 [416] • The topographical organization of the olfactory pathway in mice carrying a null mutation for galectin-1 is examined by lectin histochemistry (please see entry 33), using *Dolichos biflorus* agglutinin as marker. An aberrant topography of olfactory axons is observed, unveiling a role of galectin-1 in neuronal pathfinding in the mammalian nervous system. Honke and Taniguchi, 2009 [43] • Survey of the impact on the phenotype of engineered deficiencies for glycogenes in mice, covering glycoproteins, proteoglycans and glycolipids. Wang et al. 2009 [267] • In vivo studies demonstrate suppressive effects of both B-subunit of cholera toxin and galectin-1 on murine experimental autoimmune encephalomyelitis and an enhanced susceptibility to the disease in mice lacking ganglioside GM1. Parallel in vitro studies suggest a role of galectin-1/GM1 interplay in the mechanism of autoimmune suppression via effector/regulatory T cell communication. Orr et al. 2013 [417] • A phenotype screening of 36 mutant mouse strains deficient in a gene coding for either a glycan-binding protein or glycosyltransferase, carried out by the mouse phenotype core of the Consortium for Functional Glycomics (CFG) during 10 years. In total, >300 phenotype changes are observed, but considering the over 100 assays performed on each strain, most of the phenotypes are unchanged.
38.	**Glycome engineering**
Principle	Blocking of steps in glycan biosynthesis/remodeling by addition of inhibitors of processing enzymes or feeding/perturbing glycan biosynthesis by addition of metabolic precursors/sugar derivatives result in cells with altered glycosylation profiles.
Information provided	Examination of the behavior of proteins/cells after glycan engineering provides information on the role of glycans. Comparative analysis of lectin binding to cells exhibiting different glycosylation patterns (e.g. by FACScan analysis, entry 32, or in the microarray setting, entry 25) serves to elucidate the oligosaccharide-binding specificity of lectins on cell surfaces.
Applications	Study of glycosylation pathways. Delineation of the functional roles of glycans/lectins in intracellular sorting and secretion of glycoproteins as well as in their cell surface presentation following routing.
Limitations	Glycome changes can alter cell biological parameters and expression profiles of the proteome, a caveat for functional studies.
Example	Glycan remodeling by increased sialylation, using the metabolic precursor *N*-acetylmannosamine to enhance sialic acid production, reveals this feature as molecular switch in galectin-1-dependent growth control of pancreatic carcinoma cells. Treatment of cells with peracetylated 4-fluoroglucosamine shifts the *N*-glycan profile to biantennary structures and reduces extent of presence of terminal Lac*N*Ac and sialyl Le^x on *N*-glycans and core 2 *O*-glycans, with associated abrogation of galectin-1 binding. Chlorate-mediated depletion of sulfatide impairs sorting of galectin-4 and its cargo (glycoprotein L1) to axonal membrane, as blocking *N*-glycan maturation by 1-deoxymannojirimycin precludes association of L1 to galectin-4.
Suggested reading	Barthel et al. 2011 [418] • Treatment of human sLe^x-positive T cells and acute myelogenous leukemia KG1a cells with 4-F-Glc*N*Ac is shown to reduce the content and structural diversity of tri- and tetra-antennary *N*-glycans and of *O*-glycans, in turn increasing biantennary *N*-glycans, and to reduce terminal Lac*N*Ac and sLe^x presentation on *N*- and *O*-glycans probably due to interference with UDP-Glc*N*Ac biosynthesis. Hereby, binding to the endogenous lectins galectin-1 and E-selectin is abolished. Amano et al. 2012 [117] • A combination of genetic and metabolic glycome engineering with cytofluorometric analysis of lectin binding reveals a tumor suppressor $p16^{INK4a}$-induced reduction in sialylation resulting from down-regulation of two enzymes in the sialic acid biosynthetic pathway, unveiling a second means of modulating cell reactivity to galectin-1, in addition to the common route of altering α2,6-sialyltransferase expression (please see also comment on [302] in entry 32). Velasco et al. 2013 [419] • Combination of knocking down galectin-4 expression and metabolically blocking of sulfatide biosynthesis with impairing *N*-glycan maturation

(*continued on next page*)

Table 1 (*continued*)

Suggested reading	reveals this bivalent lectin and its counterreceptor sulfatide, their interaction being crucial for polarized sorting, as cargo-transport vehicle for distinct *N*-glycosylated glycoproteins, such as the neural cell adhesion molecule L1, to let it take residence in discrete segments of the exonal membrane, required for proper axon growth.
39.	**Therapeutic applications**
Principle	Physiological protein–carbohydrate interaction is either taken advantage of to direct a therapeutic agent (e.g. a glycosylated enzyme from natural sources without or after altering glycan display or a neoglycoenzyme (please see comments on [401] in entry 32 and on [161] in entry 34)) to a cellular destination or is intentionally blocked when involved in a disease state.
Information provided	The assumption on the specificity of the recognition process/pathophysiological relevance is verified in vivo.
Applications	Deficiencies in presence of active enzymes in certain cell types can be corrected by replacement therapy with glycoenzymes using sugar sequences as postal codes for delivery and lectin-mediated uptake. Equally important, potentially fatal lectin-mediated cell adhesion can be precluded by an anti-adhesion therapy targeting the lectin, e.g. to protect patients with the inherited blood disorder sickle cell disease from vaso-occlusion.
Limitations	Biodistribution in vivo may lead to delivery to undesired places or will block physiological adhesion processes, hereby potentially causing collateral damage by impairing effector mechanisms.
Example	Insufficient enzymatic degradation of sphingolipids causes storage disorders. Accumulation of glucosylceramide in spleen and liver of patients with Gaucher disease, the most prevalent metabolic storage disorder of humans, can be prevented by targeting β-glucocerebrosidase to macrophages such as Kupffer cells, to let injected enzyme come to the rescue.
Suggested reading	Furbish et al. 1981 [420] • Lectin-mediated uptake of glycoproteins into cells can furnish supply of active proteins to correct enzyme deficiency in a lysosomal storage disease (please see also comment in entry 34). β-Glucocerebrosidase is a glycoprotein, which is responsible for Gaucher disease when impaired. Glycoprotein import into non-parenchymal liver cells is enhanced by enzymatic trimming of the N-glycans, most pronounced after removing the antennae from the trimannoside core. The authors conclude that "naturally occurring receptors can be utilized for targeting glucocerebrosidase to the non-parenchymal cell in liver". Barton et al. 1991 [421] • The clinical efficiency of human placental β-glucocerebrosidase remodeled for macrophage uptake is reported to prove the merit of enzyme replacement therapy for Gaucher disease, initially suggested in 1966 [422]. Clinical improvements in 12 patients "provide a strong foundation for further investigation", paving the way to the establishment of this treatment modality and its extension to therapy of other lysosomal storage disorders [423]. This work "led to a veritable revolution" in the management of Gaucher disease [424]. Chang et al. 2010 [425] • The pan-selectin antagonist GMI-1070, a glycomimetic rationally designed to interfere with selectin binding in inflammation (in vivo largely acting as E-selectin antagonist), is effective to inhibit interactions between leukocytes and sickle red blood cells in a mouse model, hereby improving the microcirculatory blood flow ($p < 0.001$) and animal survival ($p = 0.007$). These results are considered to "warrant further study in a clinical trial to treat acute vaso-occlusion".

2. Carbohydrates: the chemical platform of the sugar code

Theoretically, biochemical information coding, without the constraint of having to reach optimal fidelity in replication (and ribosomal production of linear products), can be envisioned to reach a high density. Necessarily, the space on cell surfaces for a multitude of signals being limited, such high-density coding will especially be expected at this site. The building blocks of oligomers ('letters of code words') must therefore facilitate a level of structural versatility, which rests on more than sequence permutations. In other words, isomer formation should be possible when linking two building blocks. Four structural characteristics of sugars account for making them suited: potential for variability in i) the anomeric status of the glycosidic linkage (α or β), ii) the positions of this bond (e.g. 1–2, 1–3, 1–4 or 1–6) between two pyranoses, iii) the ring size and iv) site-specific substitutions (akin to post-translational protein modifications such as phosphorylation or sulfation). In aggregate, these sources for producing many, many isomers make carbohydrates "ideal for generating compact units with explicit informational properties" [67]. In numbers, extent of isomer formation based on carbohydrates surpasses the equivalent ability of the nucleotide and amino acid alphabets by orders of magnitude [68]. To give an example, 8000 (20^3) trimers can be built by 20 'letters' by sequence permutation alone. Adding anomeric status, linkage points and ring size for unsubstituted trisaccharides drives the theoretically possible number up to 9×10^6 [68]. The cases of the chains of α- or β-linked Glc units (starch or cellulose) or the 2,3/6-linkages in sialylgalactosides frequently presented at terminal positions of glycan chains (please see below for a structural depiction; [6,69,70]) convincingly document how a rather minor alteration markedly affects the biochemical personalities of glycans, despite sharing the same sequence.

Equally intriguing, branching is common, a natural way to tailor compact structures with high local density of readily accessible termini [6,65,70], and occurrence of branching is associated with physiological parameters, as for example apparent in disease states of mucins where core 2 branching is reduced to shift the glycan profile to short linear sequences [66]. Another well-known example for branching is illustrated in Fig. 1 with the histo-blood group ABH epitopes. Here, the central galactose (Gal) unit brings in three linkage points (the strategy to solve the problem of chemical synthesis of these determinants and their prominent place in the history of lectinology will be outlined in Sections 3 and 7). By its strategic position, it is a hinge between the terminal sugar, the short branch and the rest of the chain. Of note, serum antibodies distinguish the seemingly minor structural difference between the A and the B epitopes, a capacity of crucial importance for compatibility of blood transfusions (Fig. 1). This ability of immunoglobulins to be selective in 'reading' glycans underscores that *N*-acetylgalactosamine (Gal*N*Ac) and Gal are really different 'letters' within the sugar vocabulary (the third alphabet of life). The structural resemblance together with the difference in 'meaning' intimates the comparison to the German umlaut.

To realize all these possibilities for structural variability, a highly refined enzymatic machinery for glycan synthesis is available. It works without a genetic blueprint. The interplay of a variety of parameters renders dynamic regulation feasible. Factors such as concentration of the activated sugars (substrates) in the Golgi as well as presence and the distinct localization of glycosyltransferases, together with glycosidases and enzymes for entering modifications during site-specific glycan remodeling, add up to determine the glycome, already likened to a phenotypical signature of a cell above. An intriguing sensitivity to genetic or environmental factors, also infections, characterizes the glycophenotype, and species-dependent profile differences are known to occur, of note as caveat when implying biomedical relevance of data on murine cell systems (for examples, please see [71–76]; functional case studies will be presented in Section 8). Special features such as core bi- and trifucosylation mark phylogenetically widely separated organisms such as fly or worm [5]. The underlying investment in genetic coding for the glycogenes (e.g. 20 different enzymes ensure proper sialylation [6,77–79] and eight enzymes facilitate β1,3-transfer of Glc*N*Ac to galactoside acceptors [80], the entire share of genomic coding for glycosyltransferases estimated to be at least about 1% of the

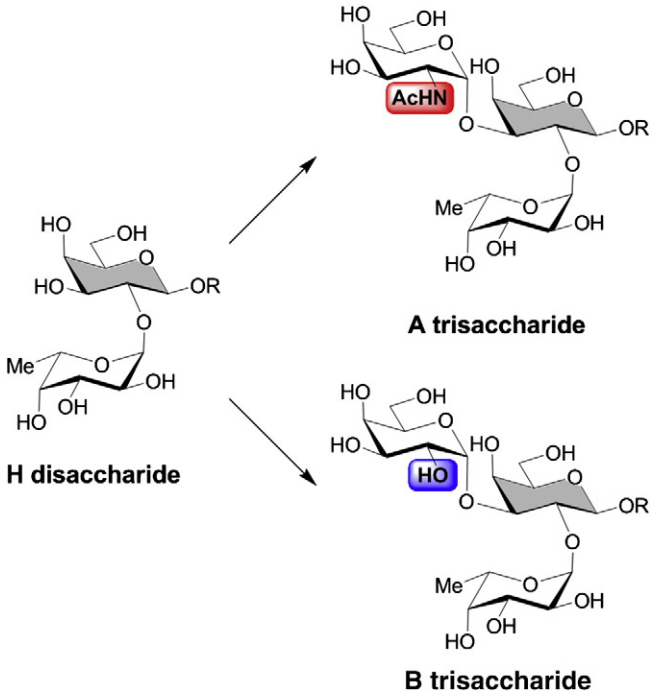


Fig. 1. The H-type disaccharide core (Fucα1,2Gal, the Gal moiety highlighted in gray and its extension at the anomeric center indicated by R) can be enzymatically extended to the A- or B-type trisaccharides via an α1,3-linkage, yielding a branched trisaccharide at glycan-chain termini. The sole structural difference resides in the highlighted 2′-position of the Gal/Gal*N*Ac extension, giving the two 'words' the different molecular 'meaning'.

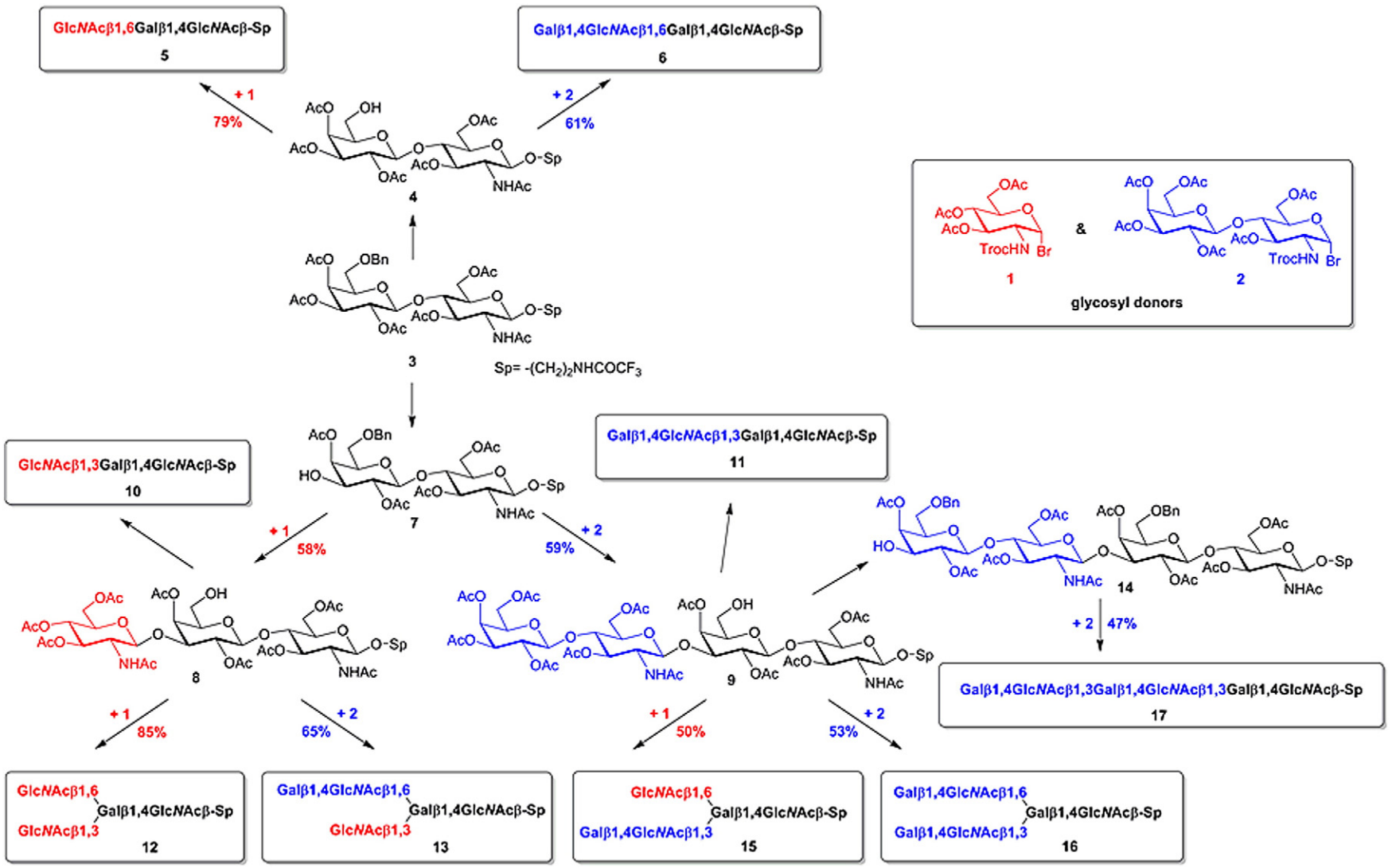
coding regions [81]) is a further strong argument for a broad impact of glycans on cell sociology, together with the ubiquitous presence of glycosylation noted above. To prove the assumed wide physiological relevance, testing glycans in respective assays is required. However, the exceptional structural diversity attained naturally poses a formidable challenge for synthetic and analytic chemistry, one reason to explain why work on the sugar code (functional glycomics) "has apparently lagged so far behind the other fields" (i.e. genomics, proteomics and functional proteomics) [82]. With ingenious strategies and tools developed [83], central problems of oligosaccharide synthesis are now solved, as described in the next section.

3. From carbohydrate 'letters' to 'words': glycan synthesis

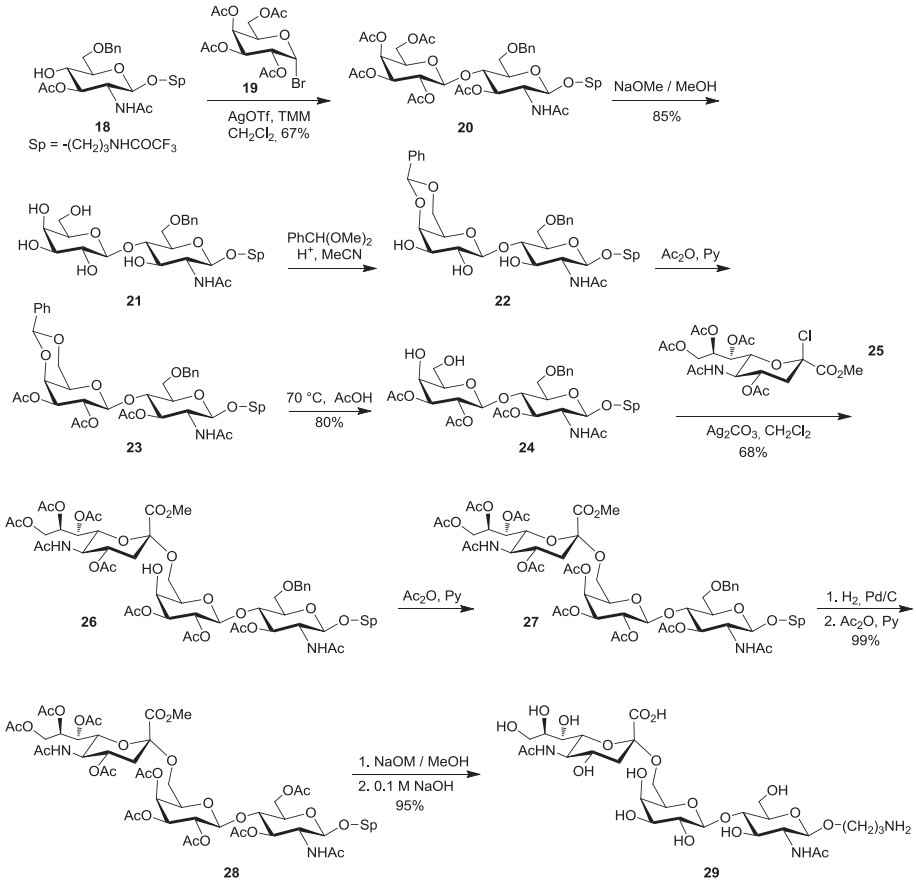
To connect to the astounding progress in characterizing the enzymatic machinery of glycosylation, described above, an obvious route to oligosaccharides would be to use glycosyltransferases. Their specificity to form a distinct glycosidic linkage (i.e. regio- and stereospecificity) has attracted the attention of chemists and prompted to recruit enzymes to glycan production [84–86] and *N*-glycosylation of proteins [87]. Indeed, this has worked well for tailoring branch ends of *N*-glycans, whose core is prepared chemically. Its subsequent extension by β1,4-galactosylation and α2,3/6-sialylation yielded complex-type bi- and triantennary glycan derivatives, which then enabled detailed binding and clearance studies in vitro and in vivo by application of the neoglycoproteins (synthetic conjugates of glycan derivative and its carrier proteins) presenting the chemoenzymatically produced glycans [88–92] (for information on biodistribution studies, please see Table 1, entry 34, and comment on [89]). Obviously, neoglycoproteins are versatile tools to study interactions of defined glycans presented by a natural scaffold with sugar receptors (for selected examples on their use in lectin research, please see Table 1, entries 17, 20 and 33). Their origin by chemical synthesis explains the prefix *neo*. Limits to the availability of the respective enzymes and activated substrates in quantities sufficient for preparative work account for the continued central role of chemical synthesis, when producing a certain oligosaccharide is an aim. Along this way, the status of refinement of this approach, albeit laborious by having to deal with protection groups and activated centers to guide the reaction to regio- and stereoselectivity, has reached a stage, which enables to take on any challenge. Instead of using a sugar activated to a mono- or diphosphonucleotide as donor for an enzymatic reaction, the alcoholic target hydroxyl group (as nucleophile, coined "glycosyl acceptor") is reacted e.g. with a glycosyl bromide (or thioglycoside or trichloroacetimidate, coined "glycosyl donors") [83, 93]. Their rather simple preparation and activation by promoters (mainly heavy atom salts) make forming the glycosidic linkage easy, in principle, albeit with no site specificity. As a consequence, all hydroxyl groups, already at the stage of the donor, must be protected to avoid self-reaction. The same requirement applies to the acceptor. Hereby, access to reactive groups, whose participation would lead to undesired side products, is blocked. This procedure takes the place of the specific selection of donor/acceptor pairs in the enzymatic process. The chemical reaction is commonly run in an apolar organic solvent (completely free of traces of water) and is followed by purification of the product to avoid contaminations. Their presence would rule out unambiguous interpretation of data from biotesting.

To develop directions for this approach, i.e. in chemical glycosylation, thorough strategic considerations of 'what to do when' are required to obtain the desired product in reasonable yield. Whether a natural assembly-line-like procedure (as in mucin-type *O*-glycosylation [65] or *O*-mannosylation [94–96]) or blockwise preparation is preferable should be decided prior to the outset. To address the issue of stereoselectivity on the level of the anomer, characteristics of the neighboring protecting group can be employed favorably. To give an example, an acyl group directs glycosylation to the β-anomer, alkyl (especially benzyl) presence results in the α-anomer, and also long-range effects are known [97]. The number of different groups for blocking should practically not be too large to achieve adequate deprotection at a distinct site, e.g. to allow chain elongation by next-step glycosylation. So-called orthogonal groups, whose removal requires particular conditions, can help at this stage. When the glycan is planned to be conjugated to a carrier, e.g. to produce neoglycoproteins as already referred to above, it is advantageous to insert a reactive functional group, often spacered, from early on. On the plus side, a suitably chosen spacer at the same time acts as a protecting group, helping to make progress in synthesis for the chemist easy.

Toward this end, as alluded to above, and following Nature's example, to look for and find building blocks in a multi-step procedure, so-called synthons, will reduce the number of otherwise necessary steps of glycosylation. At each step, the yield will inevitably be lowered by the generation of side products, although minimized by an optimal pairing of particular donor/acceptor reagents. This problem of stereoselectivity at the individual level of each glycosidic bond (i.e. obtaining both α- and β-anomers in a glycosylation reaction) makes oligosaccharide synthesis so fundamentally different from oligonucleotide preparation under identical standardized conditions and automated synthesis a formidable challenge. An elegant example for applying building-block methodology, hereby restricting the type of different protecting groups to acetyl/benzyl substitutions [98,99], is the preparation of *N*-acetyllactosamine (Lac*N*Ac) oligomers. Enzymatically, the intricate cooperation of a β1,4-galactosyltransferase with a β1,3-*N*-acetylglucosaminyltransferase for the stepwise elongation is under strict control, implying important functions in situ [80]. The Lac*N*Ac repeats are physiologically present in complex-type tri- and tetraantennary *N*-glycans and core 2/4 *O*-glycans [6,65,100]. In addition to making glycan antennae longer, these repeats are docking site of certain tissue lectins, i.e. galectins [101–104] (for an introduction to various techniques to measure interactions, please see Table 1, entries 6, 12, 21, 22, and 28; for structural depiction of the galectin fold, please see respective entry in Gallery of Lectins). Especially their just mentioned ligand properties make it attractive to probe into such repeats' signal capacity by having



Scheme 1. Convergent synthesis of nine related *N*-acetyllactosamines. Only two types of *O*-protection groups are used in this strategy.

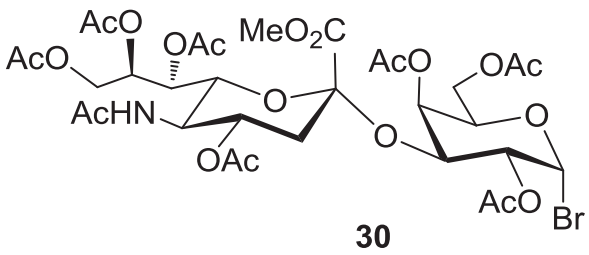


Scheme 2. Stereoselective synthesis of α2,6-sialylated oligosaccharides involving the sialyl donor **25** for glycosylation.

the compounds handy. The synthetic route, illustrated in Scheme 1, facilitates to obtain linear or branched oligosaccharides, either with Gal or Glc*N*Ac termini. The starting point of the synthetic procedures is the availability of the glycosyl donors **1** & **2** and the spacered disaccharide **3**.

Initial removal of the benzyl group, an example of an orthogonal leaving group, converts **3** to the mono-OH glycosyl acceptor **4**. The elongation at the 6′-position of the Gal moiety produces either trisaccharide **5** or tetrasaccharide **6**. As alternative for processing **3**, simple re-protection (not shown in detail) leads to a second mono-OH acceptor **7**, for elongation at the 3′-position. When the monosaccharide glycosyl donor **1** is used for the elongation as above, Glc*N*Ac-terminated trisaccharide **10** is obtained, whereas the disaccharide glycosyl donor **2** renders tetrasaccharide **11** (the equivalent to **6** as above) available. Protected trisaccharide **8** and tetrasaccharide **9**, obtained as indicated and both harboring a single free hydroxyl group for adding sugars, are convenient for the synthesis of branched glycans, i.e. the two pairs **12** and **13** & **15** and **16**. Routine re-protection of **9** results in another mono-OH glycosyl acceptor **14**, glycosylation of which with donor **2** leads, after simple deprotection, to the linear hexasaccharide **17**. As with *N*-glycome analysis, sophisticated structural assessment will ascertain the product characteristics; conversely, results obtained with such synthetic products can then become new entries to data banks of glycan analysis and hereby serve as valuable standards in glycome profiling [74,105].

Physiologically, termini of these Lac*N*Ac repeats (free or as part of a complex-type *N*-glycan) or a single unit can be capped by α2,3/6-sialylation. This enzymatic process has inherent specificity according to the type of the enzyme/branch pair, the Lac*N*Ac dimer being an about four-fold better substrate for α2,3-sialylation (in relative activity) than for α2,6-sialylation [106,107]. The presence and linkage type of sialic acid completely alter the "personality" of the oligosaccharide. This molecular capping acts as molecular switch for biomolecular interactions, e.g. for viral hemagglutinins at the heart of influenza infections [69,108–111] (for examples on the influence of α2,3/6-sialylation on recognition by tissue lectin (galectins), please see Table 1, entries 1, 4, 16 and 25 as well as Section 8, dealing with its role as molecular switch in tumor growth regulation). Fittingly, status and type of sialylation have become parameters of interest in the quest to define glycobiomarkers of diseases, attention interest given to malignancy [112–114]. In this research area, the association of expression of oncogenes/tumor suppressors with sialylation [72,115–117] is one reason to develop facile protocols for the synthesis of sialo-oligosaccharides also exploring how to implement the different substitutions [118], this work being essential for rigorously defining ligand properties and functionality. Chemically, the absence of a substituent in the immediate vicinity of the anomeric center at C-3 poses an additional challenge for the synthetic procedure. Connecting to the preparation of the group of Lac*N*Ac-based oligosaccharides shown in Scheme 1, examples for how to solve the conundrum of stereoselective chemical sialylation are presented next:



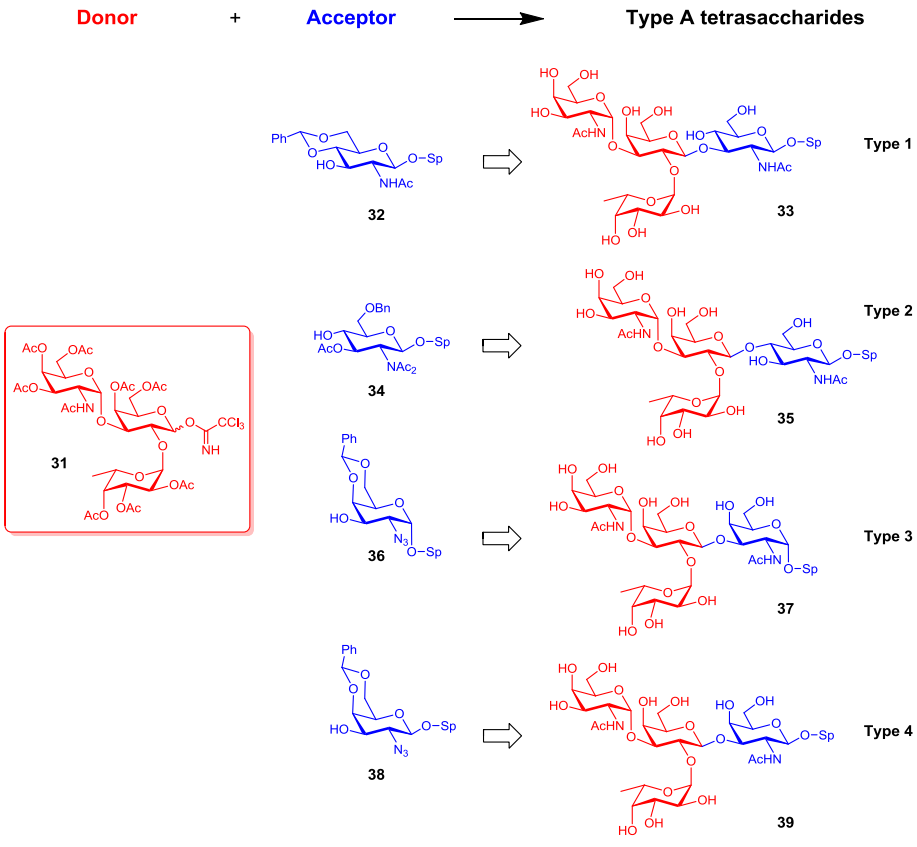
Scheme 3. The α2,3-sialylgalactoside (activated and protected) for convenient completion of synthesis of glycans sialylated at branch ends.

a.) α2,6-Sialooligosaccharides. The most convenient approach for sialylation of primary alcohols is based on glycosylation of a suitably prepared acceptor with a practically rather easily accessible sialyl donor. Scheme 2 outlines the principle of acceptor formation and its further processing to let the sialyl donor form the trisaccharide. The best results were obtained in the case of glycosyl acceptors possessing two neighboring hydroxyl groups such as 6-OH and 4-OH, in the disaccharide acceptor **24**, a Lac*NA*c derivative prepared from spacered and protected Glc*NA*c (**19**) and peracetylated α-galactosyl bromide (**18**). The required 4,6-diols are readily produced by a synthetic route via 4,6-benzylidene protection–deprotection (**21** ➔ **24**, Scheme 2). Routine processing of the protected trisaccharide **26** yields the α2,6-sialylated trisaccharide **29** in its spacered form.
Sialylation with donor **25** in the mentioned conditions proceeds stereoselectively (no β-anomers) and with high yields [119]. In quantitative terms, 6-sialylated Lac*NA*c was obtained in ~70% yield and without formation of β-anomer; the synthesis can be scaled up easily to produce 10 g of this biologically relevant trisaccharide.

b.) α2,3-Sialooligosaccharides. Formation of a glycosidic bond involving a secondary OH-group is known to be prone to more trouble than dealing with a primary group with respect to stereoselectivity and yields, especially in the case of oligosaccharides. In order to reach high yields, the protected disaccharide block Neu5Acα2,3Gal (**30**, Scheme 3) or similar derivatives are used as the glycosyl donor instead of the monosaccharide sialic acid [120]. Once synthesized, this building block enables convenient preparation of a wide set of glycans. Thus, in fact, the rather tedious sialylation is substituted by a more effective reaction with Neu5Acα2,3Gal (in protected and activated form, **30**). Capping of chains with this reagent is therefore feasible.

The ability of carbohydrates to be arranged not only in linear but also in branched structures is one of the characteristics underlying their exceptionally high coding capacity. The aims to compare properties of linear and branched oligosaccharides and to verify the assumed bioactivity of branched glycans explain the interest to synthesize such compact structures. Histo-blood group ABH determinants, a role model for turning a linear into a branched structure, graphically exemplify this feature. Referring to Fig. 1, the central Gal moiety is trisubstituted at positions 1, 2 and 3. The figure also highlights the difference between the A/B-determinants, residing in the 2′-*N*-acetyl vs 2′-hydroxyl groups in the terminal Gal unit and sufficient to explain the fatal complications by incompatible transfusions. In principle, the core trisaccharide, here the A-type trisaccharide, is found to be naturally extended in four different ways (please see Scheme 4, right panel). Type 1 and type 2 tetrasaccharides differ in the linkage positions of the terminal Glc*NA*c residue (β1,3 vs β1,4), type 3 and type 4 in the anomeric position of the terminal Gal*NA*c residue.

Highlighting the common trisaccharide **31** in red in Scheme 4 intimates that the strategy "trisaccharide donor + monosaccharide acceptor" may work best, despite the investment into trisaccharide synthesis. In fact, using the same donor in this case was strategically superior to any other option and proved its practical value [121]. A set of four different acceptors (please see Scheme 4, middle panel) will then produce the desired products, i.e. the type 1–4 A-tetrasaccharides. Performing the reaction with donor–acceptor pairs is graphically illustrated in Scheme 4. For example, glycosylation of acceptor **32** with trisaccharide donor **31** leads (after removal of protecting groups) to the type 1 tetrasaccharide **33**, letting **34** react with the same donor to the derivative of A (type 2) tetrasaccharide (**35**). Of note, the acceptor **34** has an unusual substituent at the 2′-position of the glucosamine moiety, namely $-NAc_2$. The second acetyl group (easily removable at the end of the synthesis) is introduced in order to avoid side glycosylation by the −*N*H group (i.e. *N*-glycosylation) known to take place in the case of glycosyl



Scheme 4. The building blocks (donor/acceptor pairs) and the [3 + 1] route to the four types of A-tetrasaccharide.

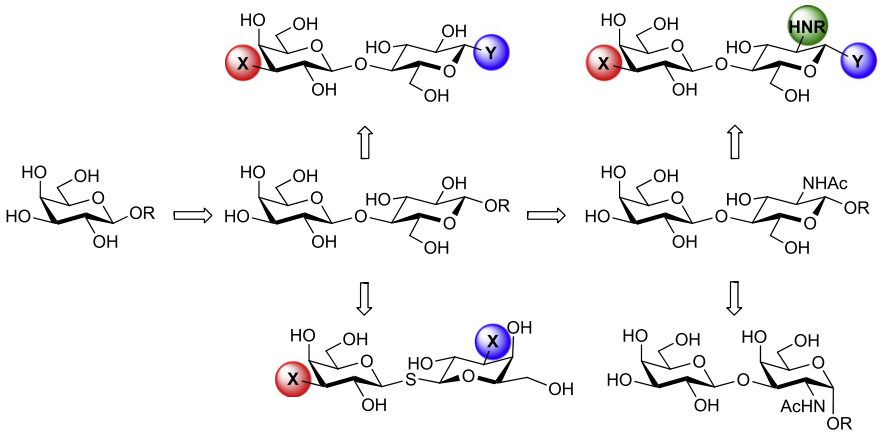


Fig. 2. Galactose, lactose and Lac*NA*c (middle panel, structures listed as given) can conveniently be derivatized at the indicated bioinspired positions (top panel, with α1,3-substitutions as in histo-blood A/B-epitopes (please see Fig. 1) in red, anomeric extension in blue and additions to the *N*-acetyl group in green) or used either as non-hydrolyzable thioglycosides or natural derivatives (here the core 1 *O*-glycan disaccharide: Galβ1,3Gal*NA*c, bottom panel) to improve specificity/selectivity as inhibitors of counterreceptor binding by sugar receptors.

acceptors with a constrained HO group. The problem of side *N*-glycosylation during the synthesis of type 3 (**37**) and type 4 (**39**) tetrasaccharides is solved using a different approach, i.e. transient conversion of −*N*HAc into $-\underline{N}_3$. Since the reactivity of the −OH group in the glycosyl acceptor **32** is the highest, protection of the *N*H part is not necessary in this case. The high nucleophilicity of this hydroxyl group gives the additional benefit of the best yield (of type 1 in this case) and the best, practically 100%, stereoselectivity of β-glycosylation. These products can then be analyzed for structural aspects, e.g. for intramolecular dynamics (please see Table 1, entry 14), and ligand properties, to test the hypothesis on their functional relevance.

As free compound (in solution or linked to a surface for display in microarrays) or conjugated to a carrier, the bioactivity of these compounds in interaction studies or receptor localization underscores their value in glycobiology [122–125] (for the example of a synthetic strategy to prepare even longer histo-blood group epitopes, i.e. the Le^b-based hexasaccharides, please see [83]). With respect to broadening the profile of bioactivity, further synthetic tailoring is possible by moving into three directions. With lactose/Lac*NA*c as example, i) substitutions can be introduced into each hexopyranose, including the *N*-2 position, ii) the *O*-glycosidic bond can be exchanged to a non-hydrolyzable thio-glycosidic (or *C*-glycosidic) linkage, and iii) an aglyconic substituent can be added (Fig. 2; for exemplary documentation of synthetic access to compound libraries by substitutions, please see [126,127]).

This section has thus paid tribute to what carbohydrate chemistry can do so elegantly: join 'letters' to 'words', in chemical terms to linear and branched structures (first and second dimensions of the sugar code). As briefly mentioned, their availability enables to gain new ground in the exploration of the conformational space of oligosaccharides, the third dimension of the sugar code. Questions on low-energy conformations of oligosaccharides, their flexibility and dynamics can be answered experimentally by using these synthetic products, teamed up with computational methods.

4. Glycan conformation: the third dimension of the sugar code

The main factors determining the conformational behavior of oligosaccharides are stereoelectronic effects and steric interactions across the glycosidic linkage. To familiarize readers with the terminology of the staggered conformations around the glycosidic linkage, the spatial constellations of α- and β-anomers are shown in Fig. 3. Regarding the aglyconic bond, the three constellations are denoted as *syn*-(+), *syn*-(−) and *anti*, their dihedral angle at values of 60°, −60° and 180°, respectively. Conformational denominations apply, too, to the exocyclic C5 – C6 bond. It adopts the three geometries called GG, GT, and TG (Gauche ±60°; Trans 180°). This terminology refers first to the torsional angle O5 – C5 – C6 – O6, followed by the C4 – C5 – C6 – O6 angle. Bringing the parameter "energy" into relation to angle positions of the glycosidic bond (in analogy to the "height above sea level" in topographical maps) and using a suitable force field for calculations result in a grading of the energy potential (Φ,Ψ,E) in maps (Fig. 4; for a comparison of force fields that encompass a detailed parameterization of all terms for the various physical contributions to the total energy of the molecular system, please see [128]). The two dihedral angles are defined as Φ (H1′-C1′-O-CX) and Ψ (C1′-O-CX-HX). Such relaxed energy maps can then be refined to take into account different orientations for the hydroxymethyl group [129]. As illustrated in Fig. 4, a hilly conformational landscape with few energetically privileged "valleys" is drawn. Low-energy constellations accessed by the disaccharide alternate with unfavorable geometries ("hills"), thus markedly limiting the theoretically possible conformational space (please also see entry 1 in Table 1). By its nature, a glycan would therefore not lose a large extent of entropy if engaged in intermolecular recognition in a low-energy form (when having introduced sugar receptors in Section 7, this topic is then further discussed). What takes the context of a protein to let a peptide adopt certain conformation(s) comes by Nature for glycans, and, again, seemingly small structural changes have drastic effects on this parameter. An intriguing example how changes in the linkage positions can drastically affect this characteristic has already been mentioned for the α2,3/6-sialylation of glycans, explicitly the pair of sialylgalactose disaccharides, whose synthesis has just been documented.

When involving the exocyclic C5 – C6 bond by α2,6-sialylation into the conformational degrees of freedom, this sialylgalactoside is turned into a rather flexible molecule, which accesses a much larger fraction of the conformational space than its 2,3-isomer, for which, as it turned out, only three energetically privileged conformers arise from the profiling [69,130]. Using molecular dynamics calculations, it has been revealed that intramolecular movements have sufficient energy to let the molecule cross barriers to interconvert among conformers. This principle of the possibility for limited flexibility does hold true not only on the level of disaccharides but also for bioactive oligosaccharides found at termini of glycan antennae [131–134]. Equally important, in view of the design of glycan mimetics, the behavior of *C*- (naturally present in flavonoids and in the form of *C*-mannosylation of tryptophan, first found in human RNase 2 [135,136]), *S*-, *S*–*S*- and *Se*-glycosides, for example for lactose or the ABH histo-blood group epitopes, has been examined this way. This work described the range of impact of the *O*/*X*-substitution of the oxygen atom by the listed alternatives, all yielding products resistant to glycosidases, on conformational equilibria and restructuring of the accessible conformational space [137–145]. The

chemical synthesis of variants for the canonical *O*-glycosidic bond thus helps broaden the data base on glycan behavior in terms of shape and dynamics in response to respective changes.

In essence, these computational calculations describe glycans as molecules with ability to adopt a rather small set of shapes, epitomizing what the third dimension of the sugar code means for glycans. The enormous structural variability in chains and branched structures (first and second dimensions) and the preference for certain shapes (third dimension; with interconversion in time) are all favorable factors for information coding (for further information on molecular mechanics and dynamics calculations, please see Table 1, entry 1). To solidify the concept of glycans having a well-structured conformational space with few energy minima, experimental data on the glycans in solution to back the results of the calculations need to be added, calling on NMR spectroscopy (please see Table 1, entries 11, 12, 14 and 27).

First, ring conformations are assessed, e.g. by analyzing *J* values for pyranose protons, to verify presence of the common $^4C_1$ chair (notably, other constellations can occur [146] and in certain cases, such as 2′-sulfated *L*-iduronic acid, have a physiological meaning [70]). A key approach to gain information on Φ/Ψ-angle combinations actually realized is exploiting the nuclear Overhauser effect (NOE) (for brief introduction and examples, please see Table 1, entry 14). The through-space dipolar interaction of an interresidual proton pair, in a transmitter–receiver relation, is clear evidence for spatial vicinity, the r^{-6} decrease of signal intensity with distance establishing a molecular ruler [147–150]. If a contact between a certain pair of protons can only occur in one of the conformers, this situation resulting in an exclusive NOE, detecting a respective signal ascertains the presence of this conformer (Fig. 5). Free in solution, other signals will likely arise from different conformers, which can dynamically interconvert if not arrested by a contact to a receptor. Evidently, what is measured will be decisive to answer the question how the conformational space is populated: only conformers leaving a footprint in NOE NMR spectroscopy are real. However, the experimental data should be cautiously interpreted, in view of what this technique records.

Since NMR measurements cannot record snapshots in the ps-scale, all data obtained inevitably are time- and ensemble-averaged [151], and here the combination of experimental and computational data proves its particular merit: the obtained information may not be attributable to a single ("virtual") conformer but to a set of energetically privileged structures, inferred by the force-field-based calculations. Experimental and theoretical data solidify the notion that carbohydrates can have an inherently low degree of conformational flexibility. Disaccharides such as lactose or the building block of chitin, i.e. N,N′-diacetylchitobiose, the second most abundant organic compound on Earth [152], have been processed in this strategic manner, with satisfactory results [137,141,153], and more recent technical advances exploiting long-range $^3J_{CH}$ coupling constants and residual dipolar contacts or recruiting paramagnetic ions to measure pseudo contact shifts and paramagnetic relaxation enhancements have extended the panel of experimental approaches toward the aim of characterizing spatial

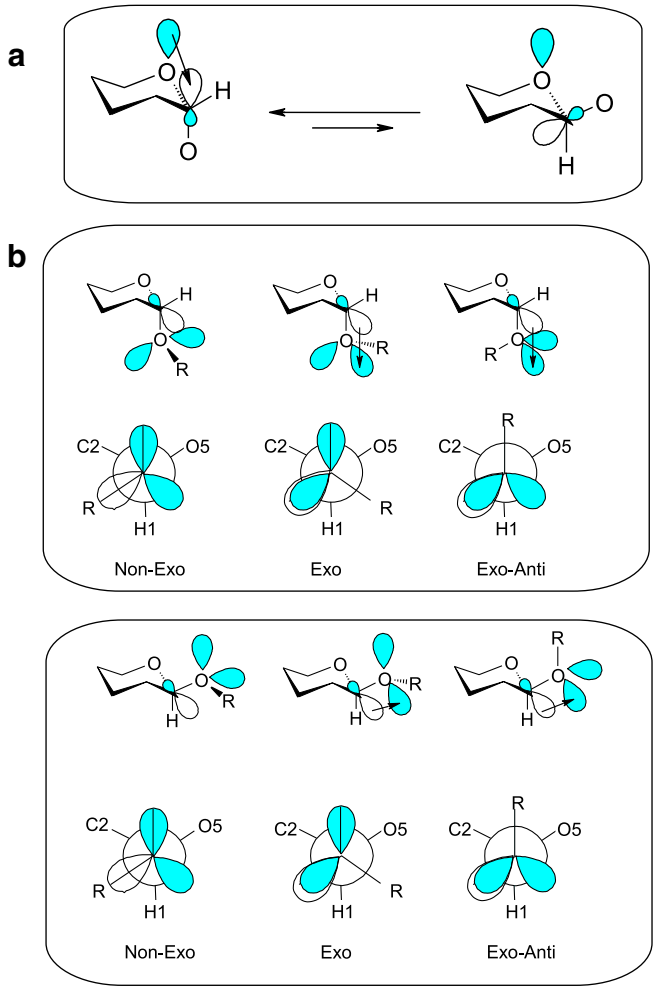


Fig. 3. Anomeric effects and conformation around the glycosidic linkage. The top scheme (a) describes the stereoelectronic stabilization of α-anomers in sugars provided by the endo-anomeric effect, with overlapping of the lone electron pair on the endocyclic oxygen atom with the s* orbital of the axial C – O bond (left). This stabilization cannot take place with equatorial C – O bonds (right). The three possible staggered conformations around the C – O glycosidic linkage are shown below (b): in the top panel, the possibilities for axial C – O bonds are illustrated. Two of these geometries ("exo" and "exo-anti") are stabilized by the exo-anomeric effect, with overlapping between the s* orbital of the endocyclic C – O bond and one of the lone electron pairs of the exocyclic oxygen atom. In the bottom panel, the analogous possibilities for equatorial C – O bonds are shown. In both cases, steric effects destabilize the "exo-anti" forms with respect to the "exo" conformers. Moreover, "non-exo" geometries do not yield any stereoelectronic stabilization.

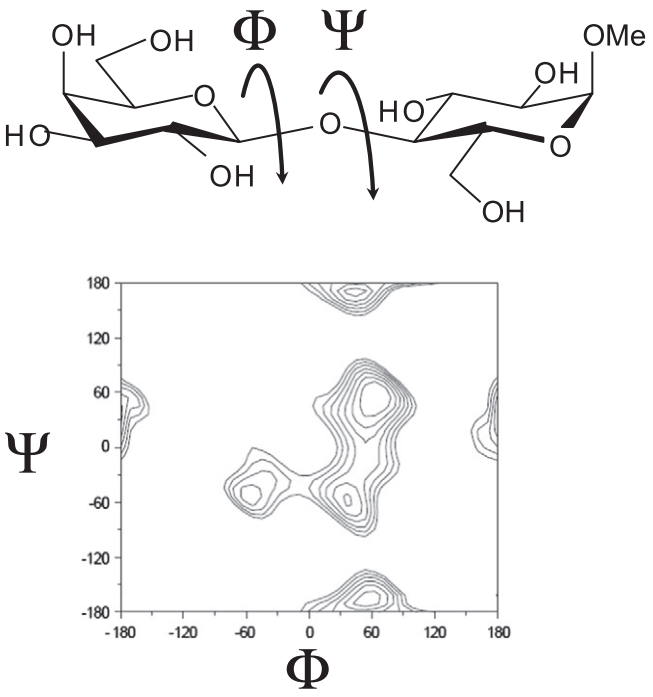


Fig. 4. Ramachandran-type representation of the potential energy map for a natural disaccharide (Galβ1,4Glc, lactose), whose conformations are defined by the Φ/Ψ angles of the glycosidic bond. The low-energy regions in the map identify energetically privileged conformations with distinct combinations of the torsion angles for this disaccharide. The most stable region has an "exo" conformation around Φ and may adopt either slightly positive (more favored) or negative values around Ψ. Additional minima are found for either anti-Φ or anti-Ψ geometries (please see edges of map).

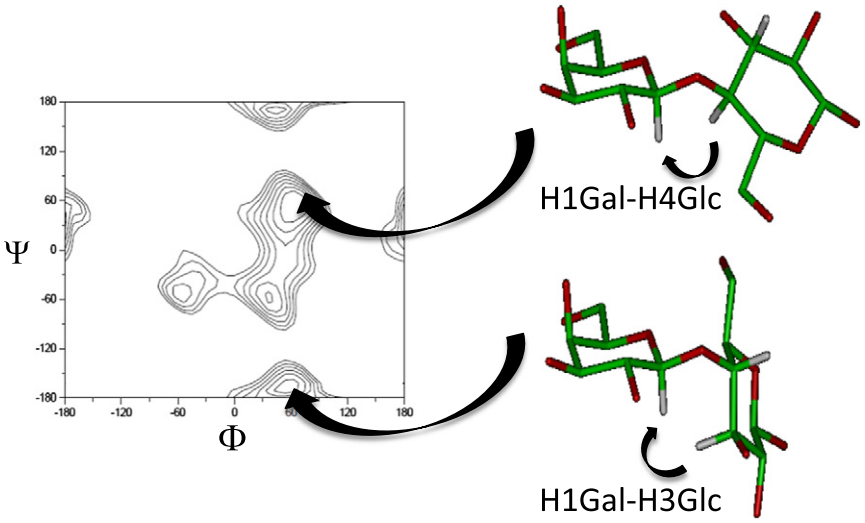


Fig. 5. The concept of the exclusive NOE, with lactose as example. For every low-energy region of the conformational map of lactose (please see Fig. 4), there is one specific (exclusive) proton–proton contact. Therefore, its experimental observation in NOESY-based experiments (please see respective entry in Table 1 for further information) is evidence for the existence of the corresponding conformer in solution. Regarding the central region of the map, which corresponds to the exo-Φ geometry with a slightly positive Ψ-value (*syn*-Ψ), the disaccharide's hydrogen atoms H1 Gal and H4 Glc are close in space, while H1 Gal and H3 Glc are far apart. On the other hand, when the Ψ-angle adopts a negative value (bottom part of the conformational map), H1 Gal and H3 Glc are close in space, whereas H1 Gal and H4 Glc are now separated by more than 3.5 Å. Therefore, if the H1 Gal/H3 Glc NOE contact is observed, the *anti*-Ψ conformation should exist. If the H1 Gal/H4 Glc NOE is detected, the *syn*-Ψ geometry necessarily occurs. If both NOEs are simultaneously measured, no single conformation can account for their presence: a conformational equilibrium between two conformers provides the explanation. Running experiments in the presence of a receptor will identify the characteristics of the bound-state conformer(s).

free-state properties of carbohydrates in solution [154–156] (for further information and also comment on [156], please see Table 1, entries 11, 12, 14 and 27). Chemistry is thus crucial to analyze glycan structure in terms of sequence and shape as well as to prepare glycans, and there is more. In addition to making copies of the local branching in natural sugar chains, as in histo-blood group determinants, complex-type *N*-glycans or core 2/4 mucin-type O-glycans, carbohydrate epitopes can be arranged in clusters of any design, another challenge for the chemist's creativity [157,158], the next section starting with the historical roots of preparing new glycoproteins.

5. From free glycans to synthetic conjugates: glycocluster design

Already back in 1929, the covalent attachment of carbohydrates (as diazonium salts derived from p-aminophenyl glycosides) to a carrier protein (horse serum globulin) was used with the intention to raise sugar-specific antibodies by using the neoglycoprotein as antigen [159,160]. Studies on the regulation of serum presence of ceruloplasmin, a transport protein relevant for copper homeostasis, nearly four decades later paved the way to a new application. Having delineated that, "in contradistinction to homologous, native ceruloplasmin, which survives for days in the plasma of rabbits, intravenously injected asialoceruloplasmin disappears from circulation within minutes and accumulates simultaneously in the parenchymal cells of the liver. The rapidity of this transfer of asialoceruloplasmin from plasma to liver has been shown to be dependent upon the integrity of the exposed, terminal galactosyl residues" [161] (please see Table 1, entry 34, for further information on biodistribution analysis and comment on [161]). With complex-type triantennary glycopeptides obtained from the glycoprotein fetuin and conjugated to protein carriers (albumin and lysozyme) by toluene-2,4-diisocyanate, a similar routing of the synthetic conjugate was achieved [162]. The authors concluded that "the coupling technique offers a feasible method for directing the hepatic uptake of various proteins that otherwise would not "home" to the liver. In addition, it may also be possible to use this technique to specifically induce the hepatic uptake of other substances such as drugs" [162]. Besides Gal, Gal*N*Ac and also α2,6-sialylated Gal/Gal*N*Ac are active determinants for transport, with interspecies differences in relative efficiency [163–165], and transport pathways by clathrin-mediated endocytosis have been thoroughly mapped [166]. In the given cases, work has been performed with the asialoglycoprotein (Ashwell) receptor, which is formed as hetero-oligomer of two type 2 transmembrane glycoprotein subunits (for information on further endocytic lectins, please see Table 2, for details on medical application of such an uptake route in enzyme replacement therapy, please see Table 1, entry 39). This sparked interest to explore aspects of sugar presentation on carriers other than proteins, with an eye on the emerging functionality of glycans by interactions with receptors.

Consequently, the panel of methods and scaffolds to prepare synthetic glycoconjugates (neoglycoconjugates) was systematically extended, from proteins to synthetic and biocompatible polymers, facilitating to produce a broad panel of products of varying glycan density and spatial constellation for testing their impact on bioactivity [157,158,167–173]. The spacing between individual glycans on a polymer is referred to as macroclustering, the equivalent of glycosylation of a protein at different sites. To let sugar presentation resemble the natural design of branched chains in the same oligosaccharide, with high local density of headgroups, glycoclusters (microclusters) can be tailored, too. The ease of preparation using a synthetic core with suited attachment points is illustrated for 1,3,5-triiodobenzene-based trivalent compound (Fig. 6a) [174]. Inter-residue distances (shown in Fig. 6b for this trivalent and also for a tetravalent glycocluster) reach values as encountered in complex-type biantennary *N*-glycans with backfolding [175]. The characteristics of the synthetic core, i.e. rigidity and size, can be systematically varied according to the needs, and branching additionally introduced, and this even in consecutive synthetic cycles, to create a dendrimeric constitution (Fig. 7) [176,177]. With an aromatic (cyclic) core, flexibility in the center is restricted. The given example is a representative of a family of cyclic glycoclusters including scaffolds such as cyclodextrin, peptide circles, calixarenes or glycophanes (for a recent review, please see [178]; for exemplary illustration of a persubstituted (Gal-, lactose- or Lac*N*Ac-bearing) cyclodextrin tested in binding assays [179], please see Fig. 8). In all such cases, the synthetic products are tools to relate spatial features to biofunctionality, in the quest to delineate the physiological significance of these aspects for natural glycans and reactivity profiles of tissue receptors. Moving on in size, with an eye on attaining a biomimetic for cell surfaces, covalent conjugation was substituted by self-assembly of

Table 2
Functions of animal and human lectins.[a]

Activity	Example of lectin
Recognition of stem region of *N*-glycans, a signal for ubiquitin conjugation when accessible in incorrectly folded glycoproteins	F-box proteins Fbs1 and Fbs2, which comprise the ligand-specific part of SCF[b] ubiquitin ligase complexes
Molecular chaperones with dual specificity for $Glc_2/Glc_1Man_9GlcNAc_2$ and protein part of nascent glycoproteins in the ER	Malectin/ribophorin I complex, calnexin, calreticulin
Targeting of misfolded glycoproteins with $Man_{8-5}GlcNAc_2$ as carbohydrate ligand to ER-associated degradation (ERAD)	EDEM1,2[c]/Mnl1 (Htm1) (lectins or glycosidases?), Yos9p (MRH[d] domain) in yeast, erlectin (XTP3-B[e]) and OS-9[f] in mammals
Intracellular routing of glycoproteins and vesicles and apical delivery	Comitin, ERGIC53[g] and VIP36[h] (probably also ERGL[i] and VIPL[j]), galectins-3, -4 and -9, P-type lectins
Intracellular transport and extracellular assembly	Non-integrin 67 kDa elastin/laminin-binding protein
Enamel formation and biomineralization	Amelogenin
Inducer of membrane superimposition and zippering (formation of Birbeck granules)	Langerin (CD207)
Cell type-specific endocytosis	Cysteine-rich domain (β-trefoil) of the dimeric form of mannose receptor for Gal*N*Ac-4-SO_4-bearing glycoprotein hormones in hepatic endothelial cells, dendritic cell and macrophage C-type lectins (mannose receptor family members (tandem-repeat type) and single-CRD[k] lectins such as trimeric langerin/CD207 or tetrameric DC-SIGN[l]/CD209), hepatic and macrophage asialoglycoprotein receptors, HARE[m], P-type lectins
Recognition of foreign glycans (β1,3-glucans, cell wall peptidoglycan, LOS[n] and LPS[o]), mycobacterial glycolipid or host-like epitopes	CR3[p] (CD11b/CD18, Mac-1 antigen), C-type lectins such as collectins, DC-SIGN, dectin-1, Mincle and RegIIIγ (murine)[q] or HIP/PAP (human), ficolins, galectins, immulectins, intelectins, *Limulus* coagulation factors C and G, siglecs, tachylectins
Recognition of foreign or aberrant glycosignatures on cells (including endocytosis or initiation of opsonization or complement activation) and of apoptotic/necrotic cells (glycans or peptide motifs)	Collectins, C-type macrophage and dendritic cell lectins, CR3 (CD11b/CD18, Mac-1 antigen), α/Θ-defensins, ficolins, galectins, pentraxins (CRP, limulin), RegIIIγ (HIP/PAP), siglecs, tachylectins
Targeting of enzymatic activity in multimodular proteins	Acrosin, *Limulus* coagulation factor C, laforin, β-trefoil fold ($(QxW)_3$ domain) of Gal*N*Ac-Ts[r] involved in mucin-type *O*-glycosylation, frequent in microbial glycosylhydrolases for plant cell wall polysaccharides, termed carbohydrate-binding modules
Bridging of molecules	Cerebellar soluble lectin, cytokines (e.g. IL-2[s]-IL-2R and CD3 of TCR), galectins
Induction or suppression of effector release (H_2O_2, cytokines etc.)	Chitinase-like YKL-40, galectins, I-type lectins (e.g. CD33 (siglec-3), siglecs-7 and -9), selectins and other C-type lectins such as CD23, BDCA2 and dectin-1, Toll-like receptor 4
Alteration of enzymatic activities in modular proteins/receptor endocytosis via lattice formation	Mannan-binding lectin (acting on meprins); galectins
Cell growth control, induction of apoptosis/anoikis and axonal regeneration	Amphoterin and other heparin-binding proteins, cerebellar soluble lectin, chitinase-like lectins, C-type lectins, galectins, hyaluronic acid-binding proteins, siglecs (e.g. CD22 and CD33)
Cell migration and routing	Galectins, hyaluronic acid-binding proteins (CD44, hyalectans/lecticans, RHAMM[t]), I-type lectins, selectins and other C-type lectins
Cell–cell interactions	Galectins, gliolectin, I-type lectins (e.g. siglecs, N-CAM[u], P_0 or L1), selectins and other C-type lectins such as DC-SIGN or macrophage mannose receptor
Cell–matrix interactions	Calreticulin, discoidin I, galectins, heparin- and hyaluronic acid-binding lectins including hyalectans/lecticans
Matrix network assembly	Galectins (e.g. galectin-3/hensin), non-integrin 67 kDa elastin/laminin-binding protein, proteoglycan core proteins (C-type CRD and G1 domain of hyalectans/lecticans)

[a] Adapted from [243], with permission, and extended.
[b] Skp-1-Cul1-F-box protein complex.
[c] ER degradation enhancing α-mannosidase-like protein.
[d] Mannose-6-phosphate receptor homology.
[e] XTP3-transactivated gene B precursor.
[f] Osteosarcoma 9.
[g] ER–Golgi intermediate compartment protein (lectin) (MW: 53 kDa).
[h] Vesicular-integral (membrane) protein (lectin) (MW: 36 kDa).
[i] ERGIC-53-like protein.
[j] VIP36-like protein.
[k] Carbohydrate recognition domain.
[l] Dendritic cell-specific ICAM-3-grabbing nonintegrin.
[m] Hyaluronan receptor for endocytosis.
[n] Lipooligosaccharide.
[o] Lipopolysaccharide.
[p] Complement receptor type 3.
[q] Member of regenerating (reg) gene family of secreted proteins.
[r] UDP-Gal*N*Ac: polypeptide *N*-acetylgalactosaminyltransferases.
[s] Interleukin-2.
[t] Receptor for hyaluronan-mediated motility.
[u] Neural cell adhesion molecule.

amphiphilic building blocks. Such a class of compounds, the Janus glycodendrimers (with its two "faces") (Fig. 9), can aggregate into vesicle- or rod-like structures called glycodendrimersomes or glycodendrimermicelles, a structural platform toward shaping tunable surfaces [180]. The shown 1,4-disubstituted 1,2,3-triazole connecting sugar headgroups and backbone are generated by Huisgens so-called click chemistry, the Cu(I)-catalyzed azide-alkyne cycloaddition, a very popular reaction to link carbohydrate derivatives to scaffolds.

As noted above for the glycan part of (neo)glycoproteins acting as routing signal for cellular (hepatic) uptake, the implied physiological significance of glycan attachment is "to impart a discrete recognitional role" on the carrier [67]. That means that carbohydrates are bioactive ligands for receptors, and, clearly, the neoglycoconjugates are versatile tools to prove this assumption. Indeed, specific binding of labeled neoglycoconjugates to cells and tissue sections underscores the presence of receptors for carbohydrate determinants in human and

animal tissues [122,170,181–183], beyond the hepatic receptor for asialoglycoproteins [184] (for further information on reverse lectin cyto- and histochemistry and comment on Ref. [181], please see Table 1, entry 33). Carbohydrate signals can thus be "read" by endogenous receptors in the same way as antibodies select their glycan targets, alluding to the common roots of early work on both classes of sugar receptors in blood-group typing [185–187]. Their specificity for distinct epitopes, which enables distinguishing even the structurally closely related Gal and Gal*N*Ac in histo-blood group A/B epitopes reliably, prompts the question on the modes of molecular recognition (for a survey of experimental approaches to address this issue, please see Table 1, especially entries 2, 12, 19, 22, and 31). This 'reading' of the sugar code by letting a glycan form a complex with a receptor is another highly fertile area for chemistry: in fact, the principles of carbohydrate binding can be studied by devising receptors of minimal structural complexity.

6. Synthetic carbohydrate receptors: chemical principles of recognition

As outlined above, carbohydrates have exceptional properties for information coding. To facilitate turning this information into functional consequences, they offer enormous versatility for intermolecular interactions. Both the topologically distinct display of hydroxyl groups, to be sensed by complementarity involving directional hydrogen (H) bonds (or Ca^{2+}-dependent coordination bonds [188]), and patches of two or three polarized C–H bonds suited for stacking to a π-electron system as that of an amino acid (Trp, Tyr, Phe) should make distinction between different sugar 'letters' possible (Fig. 10a) [189–191]. Herewith, the chemical principles of the molecular recognition are defined. To address the challenges to design synthetic receptors and get them to work, we enter the field of supramolecular chemistry. In this field, we have a toolbox at hand much larger in size

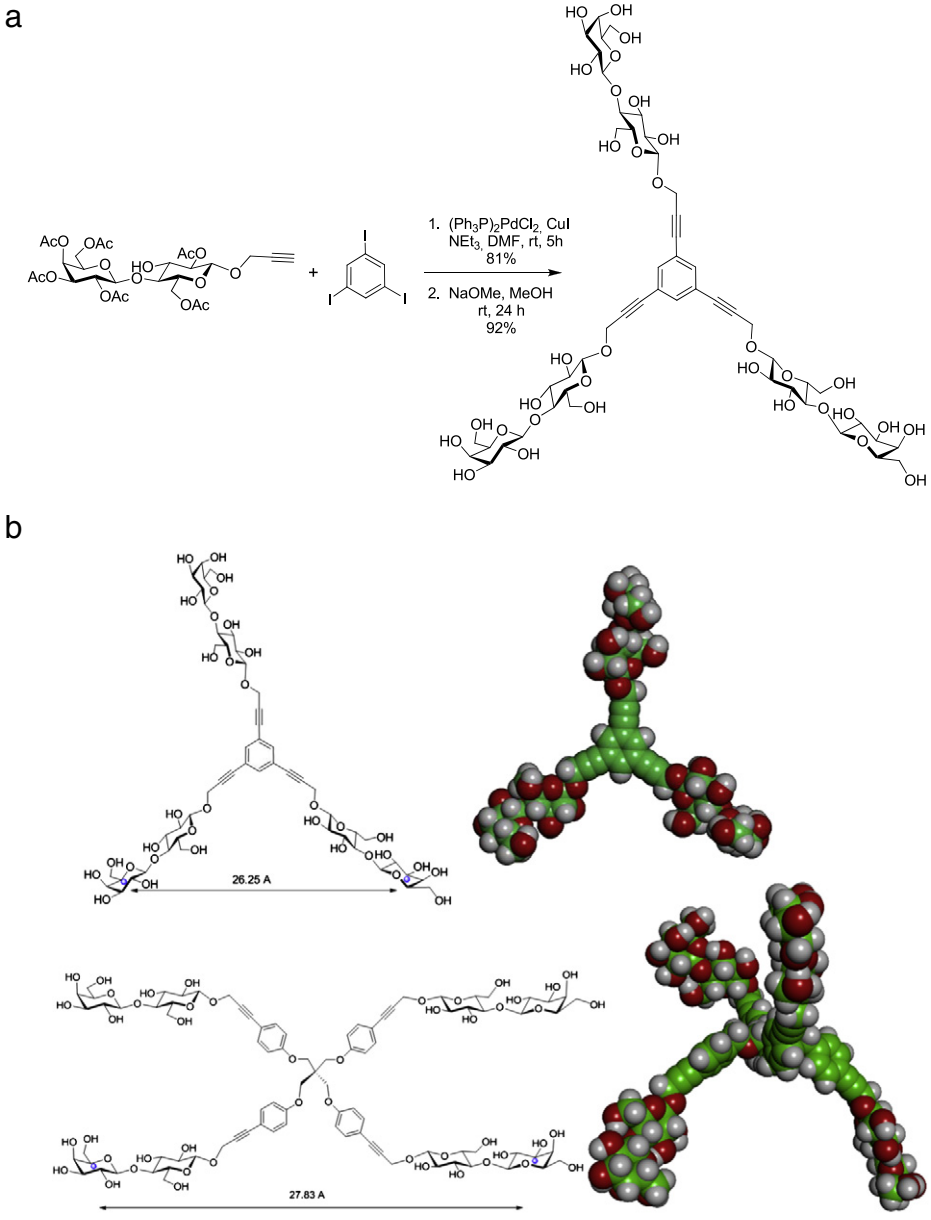


Fig. 6. Example for a synthetic route toward a glycocluster, starting from propargyl lactoside containing an alkyne functionality. Its conjugation to a 1,3,5-triiodobenzene core using the palladium-catalyzed Sonogashira reaction with dichlorobis(triphenylphosphine)palladium(II) ($(Ph_3P)_2PdCl_2$) and deprotection resulted in the shown product in an excellent yield (a). The trivalent glycocluster and a pentaerythritol-based tetravalent scaffold for lactose presentation, both shown in (b), exhibit rather similar headgroup distances and are potent inhibitors of cell binding of human galectin-3, compared to their activity against homodimeric galectins (for details, please see [174]).

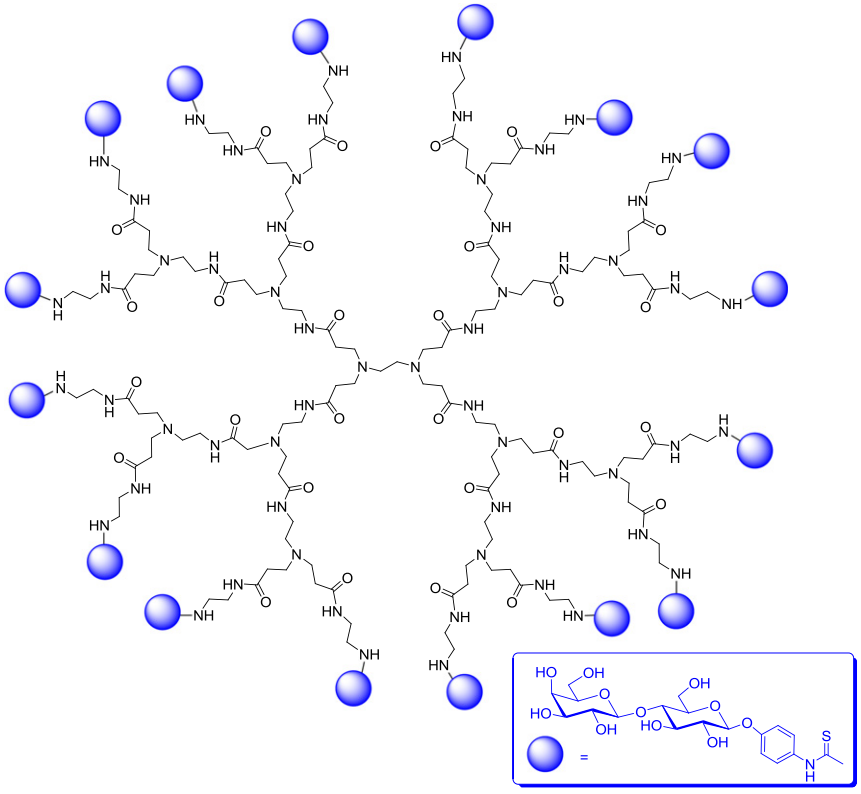


Fig. 7. Poly(amidoamine) dendrimer (PAMAM) scaffold of the G2 generation presenting 16 lactoside residues attached through thiourea linkages to the core, with potent activity to block binding of a plant toxin to a glycoprotein (asialofetuin) with *N*-glycans presenting Lac*N*Ac termini (for details, please see [176]).

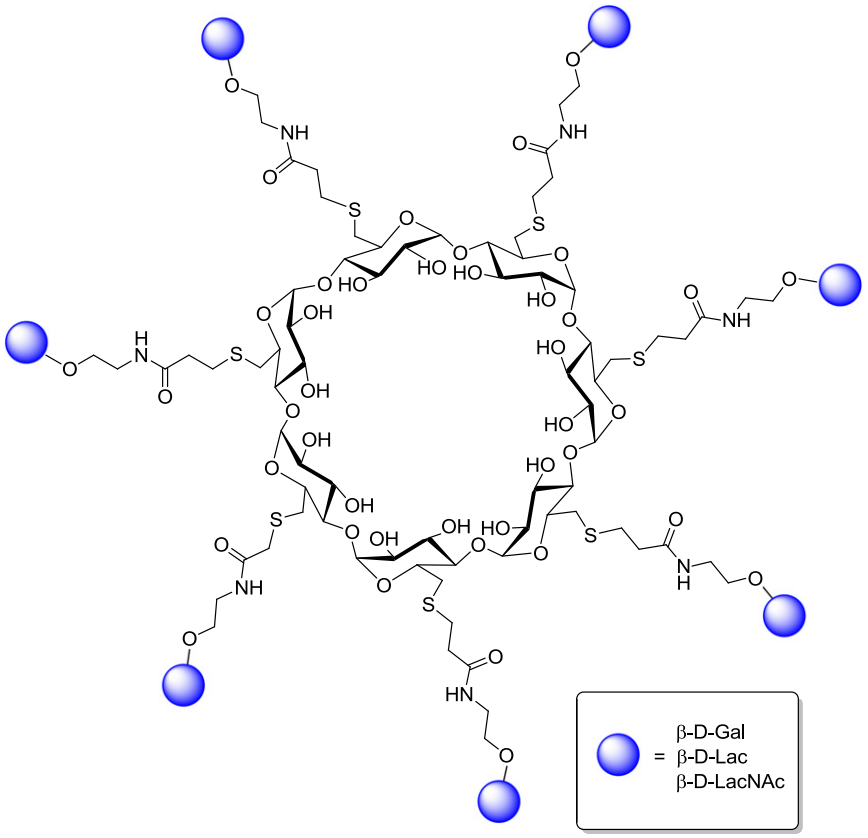


Fig. 8. Persubstituted β-cyclodextrin presenting seven galactoside headgroups, obtained by coupling 3-(3-thioacetyl propionamido)-propyl glycosides (after de-*O*,*S*-acetylation) via their sodium thiolate to the heptakis 6-deoxy-6-iodo-β-cyclodextrin (for details, please see [179]).

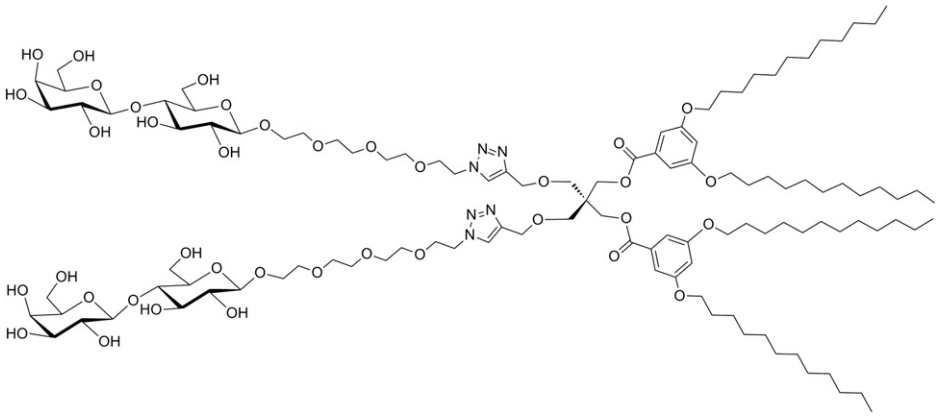


Fig. 9. Illustration of the structure of a bivalent Janus glycodendrimer, which can self-assemble into glycodendrimersomes presenting lactose as headgroup (for details, please see [180]).

than the panel of proteinogenic amino acids. This allows us to turn any idea for optimizing the contact into structural adaptations. Since water-solubility can become an issue with compounds rich in aromatic constituents, experiments were extended to use organic solvents, with different degrees of success [192,193]. Of course, the experience gained under these conditions cannot simply be extrapolated to mechanisms under physiological conditions. To give an explicit example: by running molecular dynamics simulations comparatively in an aprotic solvent and in water for binding of chitin's disaccharide unit to a receptor (the plant protein hevein; please see Section 7 for details on its structure and its recognition of sugar, and also see entry 14 in Table 1), a shift in contributions to complex stability from hydrophobic to polar interactions has been noted when exchanging water by dimethyl sulfoxide molecules [194]. With this context dependence kept in mind, we are prepared to enter the realm of how chemists devise sugar receptors.

The approach taken has been, simply, to design cavities which are as complementary as possible to the saccharide ligands. To do so ideally, hydrophobic patches formed by the carbohydrate's (polarized) C – H groups are topologically matched by apolar surfaces, while the polar substituents find complementary H-bond donors or acceptors. As noted, the apolar surfaces should be aromatic so that hydrophobic effects can be reinforced by C – H/π-interactions [195,196]. It is important to emphasize that both named types of interaction combine for optimal affinity and selectivity, remembering the natural complexity of the glycome. Even if one believes that hydrogen bonding can make little or no contribution to the binding energy in the physiological (aqueous) milieu, polar "valencies" of a receptor must be satisfied to reach complementarity. If the hydroxyl group of a carbohydrate will face a non-complementary structural context, the energy required for its desolvation cannot be re-gained by forming new contacts. Ultimately, the goal is to create a binding site, which is i) a perfect match for the ligand, and ii) as hostile as possible to water molecules, to preclude energetically unfavorable desolvation. In this case, replacement of water molecules by functionalities of the ligand will yield the maximum possible free energy of binding.

To date, this strategy has been executed mainly in the context of the "all-equatorial" β-glucosyl family of ligands. These ligands may be envisaged crudely as squat cylinders with apolar upper and lower surfaces (composed of axial C – H groups) and polar circumferences (Fig. 10b). To a first approximation, a complementary cavity may be constructed by separating apolar (aromatic) surfaces by polar spacers. The resulting "temple" architecture, shown in Fig. 10b, was first realized as a tricyclic receptor designated as compound **40** in Fig. 10c [197,198]. In chemical terms, its apolar surfaces are biphenyl units, the spacers are isophthalamides, and water solubility is ensured by the four tricarboxylate units. These latter are critical to the design, doing much more than ensuring solubility: not only is **40** soluble in water but it remains monomeric up to concentrations of ~1 mM, allowing studies by ^{1}H NMR spectroscopy. Binding constants could thus be measured by titrations, in addition to fluorescence spectroscopy, induced circular dichroism and (in some cases) calorimetry (for details on these techniques, please see Table 1, entries 4, 6, 11 and 22). The most outstanding feature of this receptor is remarkable affinity and selectivity for the β-*O*-Glc*N*Ac moiety, known as the only unit of a distinct type of *O*-glycosylation (i.e. O-Glc*N*Acylation) akin to protein phosphorylation [65,199,200]. The β-methyl form, i.e. β-Glc*N*Ac*O*Me (**46**, Fig. 10c), is bound with K_d ~ 1.6 mM and ≥300:1 selectivity over other common sugars such as Gal or Man and their *N*-acetyl derivatives verifying the hypothesis for steric hindrance by an axial hydroxyl group [201]. At this level of structural complexity, the synthetic receptor can compete with the monosaccharide-binding properties of some natural receptors [198,201] (please see comment on [198] in Table 1, entry 14), and introducing further substitutions, not as readily possible for the natural receptors, can tell us more about structure–activity correlations, even advancing the receptor's performance.

To do so, substituents were introduced to the tricyclic cage of **40** in the biphenyl 4 and 4′ positions, as shown in **41**–**45** in Fig. 10c. This presence revealed some surprising effects. For example, while C – H/π-dependent effects should decrease as the π-system becomes more electron-poor [202], the electron-withdrawing fluorine atom in **41** yields a small *increase* in affinity [203]. The OH/OR groups in **42**–**45** produce further increases (in line with the C – H/π effect), but deprotonation of the hydroxyl group is counterproductive. Among the OR substituents, propoxy (in **44**) gives higher affinities than ethyl- and butyloxy [204]. Although these results are at this moment difficult to rationalize completely, the practical outcome is that **44** is a model compound for a purely synthetic β-glucosyl receptor. To provide numbers, the unsubstituted Glc is bound with K_d = 17 mM, with relative capacity to separate Glc from Gal of about 20. Although Glc binding is still very weak in absolute terms, such affinities could be practically relevant for biological fluids where this level tends to be quite high [204], and the presented details on chemical substitutions give substance to the potential of this toolbox for optimal receptor design. Having hereby illustrated receptor design for a monosaccharide, the route is open to proceed to a 'two-letter' sugar word. Will the temple architecture also be suited for disaccharide recognition?

Respective structural extensions have been started and seen to accommodate all-equatorial disaccharides. As a first example, the tetracyclic **48** (Fig. 10d) was designed to possess a rigidly enforced cavity complementary to ligands such as cellobiose (Glcβ1,4Glc, **50**, the building block of cellulose) and its *O*-methyl derivative **51**. Again, the system has been proven efficient and remarkably selective, binding **51** with K_d = 1.1 mM and ~80-fold selectivity vs lactose (**52**) with its axial 4′-hydroxyl group in Gal [205]. The simpler tricyclic design **49** was originally thought to be too flexible and less likely to prove effective. However, when eventually studied, it succeeded well beyond expectations [206] (please see comment on [206] in Table 1, entry 22). For example, β-cellobioside **51** was bound with K_d = 0.2 mM, a six-fold

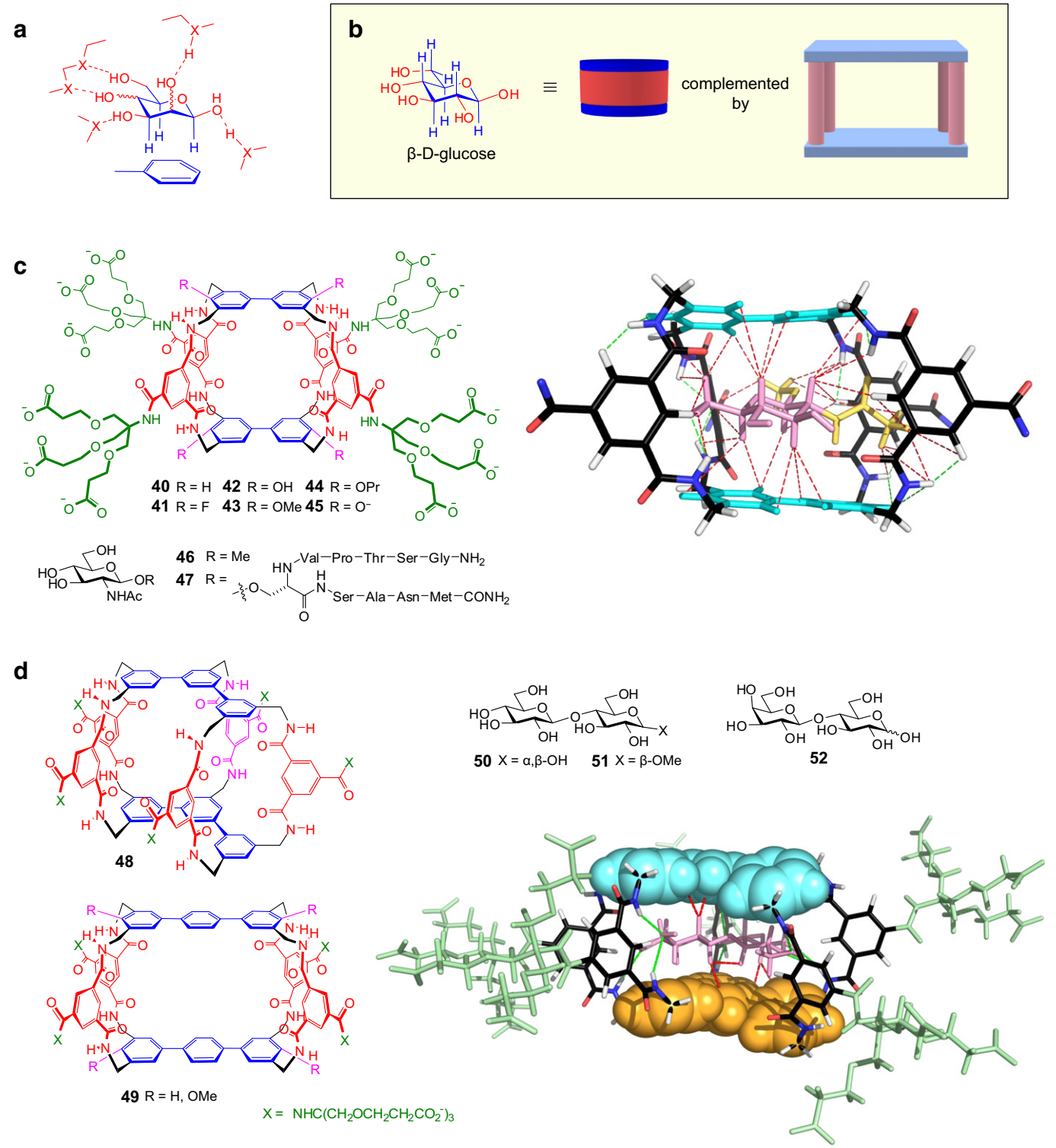


Fig. 10. The "temple" architecture for synthetic carbohydrate receptors. Basic design requirements are that hydrophobic patches on the carbohydrate ligand should be matched by aromatic surfaces, polar hydroxyl groups by H-bond donors and acceptors (a), leading to the "temple" concept, complementary to all-equatorial carbohydrates (b). As example, biphenyl-based temple receptors **40**–**45**, binding the β-*N*-acetylglucosaminyl-based ligands **46** and **47**, are shown (c). The structure of the complex between **40** and **46** (derived from NMR-based information), illustrating through-space connections established by 2D-NOESY (please see also Fig. 5), is given on the right. As further example, terphenyl-based temple receptors **48** and **49**, their disaccharide ligands **50**–**52** and the NMR-data-based structure of the complex between **48** and **50** are shown (d).

enhancement over **48** (Fig. 10d). This affinity is currently the highest known for well-characterized biomimetic recognition of an uncharged carbohydrate ligand, and calorimetry pinpointed the binding to be driven both by enthalpy and entropy changes. The contribution of entropy tended to decrease as the binding became stronger [205]. For a complex of rather high affinity (**49** + **50**, with K_d ~ 0.3 mM), the entropic term became almost insignificant. The high affinities measured for **48** and **49** reflect the principle, perhaps obvious, that larger ligands present easier targets. Limited flexibility as delineated for various carbohydrates by NMR spectroscopy combined with computational techniques (please

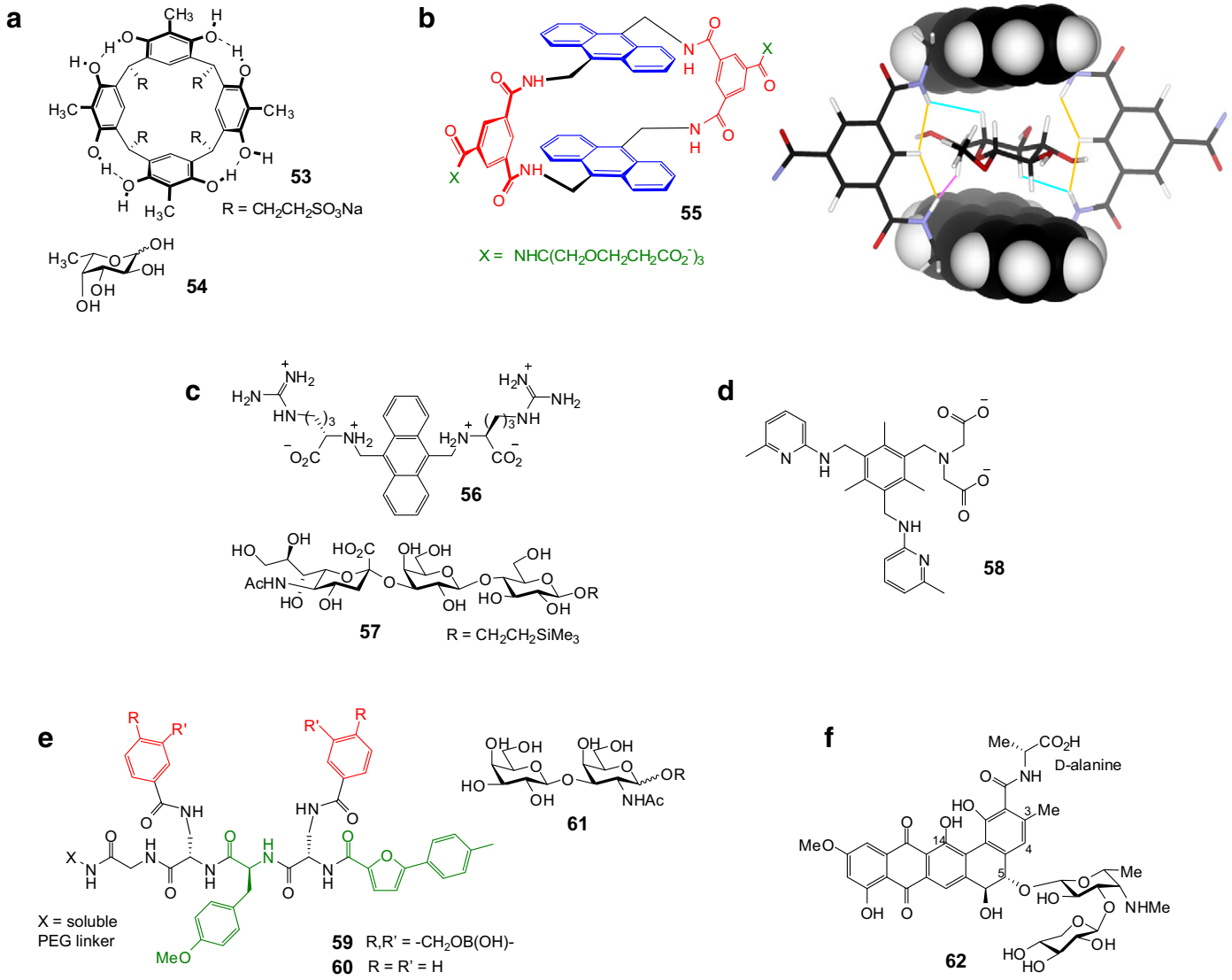


Fig. 11. A graphic survey of synthetic carbohydrate receptors with monocyclic and acyclic architectures. Aoyama's calixresorcarene **53**, with the preferred ligand fucose **54** (a), an anthracene-based macrocyclic receptor **55** (left) and the NMR-data-based structure of **55** binding methyl β-D-glucoside (right) (b), an acyclic anthracene-based receptor (**56**), with its ligand (**57**), the trisaccharide of ganglioside GD3 (c), a cellobiose-binding tripodal receptor (**58**) (d), Hall's boronopeptide **59** and boron-free analog **60** with their preferred substrate, the core 1 *O*-glycan disaccharide **61** (e), also shown in Fig. 2, and pradimicin A **62**, a carbohydrate-binding secondary metabolite (f).

see Section 3 above and also Table 1, entries 1 and 11) may also help them slip into a preformed cavity. Disaccharides possess more functional groups and larger hydrophobic surfaces than monosaccharides, allowing more possibilities for binding interactions to a receptor. This reasoning is rather likely reflected in the way proteins bind sugars: the actual contact area is expected to be like a cavity in proteins responsible for transport of monosaccharides to optimize affinity and selectivity, whereas receptors recognizing code words can perform well even with a flat surface [191]. Before we turn to these aspects, a further look at synthetic biomimetic models other than temple receptors is warranted. After all, although the temple receptors have nicely proven to bind mono- and disaccharides, they are topologically complex and not so easy to synthesize, reasons to encourage efforts to gain access to alternatives. Indeed, aromatic and polar groups can also be combined in simpler architectures, and testing some of these indicated potential for applications.

Early examples were provided by resorcinol cyclic tetramer [207] and by water-soluble calix[4]resorcarene macrocycles, exemplified by **53** (Fig. 11a) [208]. This system showed no detectable affinity to common hexoses such as Gal, but did exhibit very weak binding to the 6-deoxy sugar fucose (**54**; K_d = 170 mM), a constituent of the ABH/Le histo-blood group determinants. In related molecules, it was found that deprotonation of phenolic hydroxyl groups could raise affinities significantly, pointing to a role for C–H/π-interactions [208]. A glycophane (interestingly, glycophanes are also suited scaffolds for glycocluster design offering potential as targeting cargo transporters [209,210]) built from α,α-trehalose and naphthalene molecules (a kind of cyclodextrin-cyclophane hybrid) could host nitrophenyl glycosides, with some changes in values between sugars (up to 0.5 kcal/mol) [211]. Very recently, the macrocyclic bis-anthracene **55** (Fig. 11b) has been proven remarkably effective for β-glucosyl units [212]. Like the temple receptors, this system features paired aromatic surfaces

separated by isophthalamide spacers. It is, however, a great deal simpler to prepare. Remarkably, it is competitive with **44** (shown in Fig. 10c) as a Glc receptor (K_d = 18 mM) and is even more selective, showing almost no reactivity with Gal or Man. Practically useful for experimental monitoring, it exhibits strong fluorescence that is substantially enhanced on binding, a property also exploitable for measuring affinity of sugars to natural receptors involving Trp in binding (for details and comments on [212], please see Table 1, entries 6 and 7). NMR-spectroscopical studies confirm that the Glc moiety is inserted between the two aromatic surfaces (Fig. 11b). This sandwiching is biomimetic to how certain proteins accommodate their ligand, i.e. type B carbohydrate-binding modules (CBMs: please see Section 7 for further information), which let chains of Glc-type sugars become fixed between two aromatic rings [191,213]. In a broader context, the principle of molecular sandwiching has been found beyond glycosciences, for instance for the 5′-m^{7}G-cap structure of eukaryotic mRNA, with two Tyr residues of the cap-binding protein 20 (CBP20) involved in nuclear export or two Trp rings in the case of the eukaryotic translation-initiating factor 4E (eIF4E) [214,215]. Modeling for ligand complexes of the bis-anthracene suggests that the fit is tight, an assumption proven by experimental data (Fig. 11b), and, accordingly, the entropy of binding is quite strongly negative [212].

The receptors described thus far have all been macrocycles, but sugars have also been found to associate with non-macrocyclic structures. For example, the simple mono-anthracene derivative **56** (Fig. 11c) has been reported to bind both α2,3-sialyllactose (**57**) and the fucose-containing tetrasaccharide sialyl Le^x with K_d ~ 10 mM [216]. Although these glycans qualify as putatively easy targets, being both large and negatively charged, the actual detection of binding to such a simple chemical compound is incentive to come up with refinements. Several carbohydrate receptors have been constructed from 1,3,5-tris-aminomethylbenzene units [193,217]. While most have been designed to operate in organic solvents, the bis-carboxylate **58** (Fig. 11d) has been proven effective in water. Analysis of NMR titration data for interaction of **58** + **50** (cellobiose) suggested a 1:2 stoichiometry, with successive K_d-values of 3 and 15 mM [193]. A particular advantage of acyclic designs is ease of variation, and thus suitability for testing combinatorial approaches. Their application identified the bis-boronate **59** (Fig. 11e) from a library of 400 similar compounds to bind a disaccharide, i.e. the Thomsen–Friedenreich (TF) antigen **61** (K_d estimated at 0.5 mM) [218], a core 1 *O*-glycan (Galβ1,3Gal*N*Ac) (structure given in Fig. 2). **59** itself is not biomimetic as binding entails formation of a covalent B – O bond. However, interestingly, the boron-free "control" compound **60** was also effective, albeit with the K_d of about 2.5 mM. This result may point to a future strategy, in which libraries of peptide-like compounds, perhaps containing specially designed components, are screened against relevant targets in attempts to generate suites of complementary binders for further analysis and iterative affinity improvement.

Finally, coming closer to natural compounds, pradimicins and benanomicins have attracted interest due to their ability to bind D-mannosides in the presence of Ca^{2+} [219]. The best-characterized is pradimicin A (**62**, Fig. 11f), which binds methyl α-D-mannoside with K_d ~0.1 mM (measured by calorimetry) [220] (for further details, please see respective comment in Table 1, entry 29). Unfortunately, this system is difficult to study due to complex stoichiometries, but progress has been made by using solid-state NMR spectroscopy, pointing to coordination bonds between the sugar and Ca^{2+}. In addition to its active role in natural sugar receptors, this cation can also be involved in carbohydrate–carbohydrate interactions (for further information, please see [221,222]).

Having herewith documented information on how synthetic receptors and sugar interact prompts the question on how natural systems accomplish carbohydrate recognition, in terms of specificity and selectivity. Closest to the described synthetic receptors are peptides. Respective sequences can be obtained by screening of phage-displayed libraries for sugar binding. Also, natural receptors are a source either by performing proteolytic degradation of the protein while in contact to a protective ligand or by a knowledge-based sequence selection. Binding studies for di- and oligosaccharides, for example the TF antigen mentioned in the two paragraphs above, and 15mer peptides yet highlight the enormous drawback of high-level peptide flexibility and reveal rather minor contributions of stacking [223,224]. Intramolecular disulfide bonding reduces this dynamics so that hereby constrained peptides can acquire affinity for sugars. Reactivity for the glycan part of neoglycoproteins and activity in hemagglutination assays (for details on the method, please see Table 1, entry 21) indicated potential of sugar binding for the α- and Θ-defensins, cyclic peptides of 18 (Θ) and 28–35 (α) amino acids with a trisulfide ladder, and propensity for multimer formation [225,226]. Although selectivity for sugars could be ascertained in these studies, as was also the case with peptides from two natural receptors, which expectedly required maintained presence of distinct amino acid residues such as the Trp unit for binding [227] (for further details, please see respective comment in Table 1, entry 16), to learn from Nature necessitates to turn to the analysis of protein–carbohydrate recognition. Its affinity levels surpass the μM limit, and intricate selectivity is reached even between structurally related oligosaccharides. As noted above, the protein, as sequence with proper folding together with disulfide bonds and Ca^{2+} for local positioning of amino acid side chains, will present the contact region(s) in suited shape for complementarity, directing interest to see how proteins work as sugar receptors. Before turning to structural aspects, it is instructive to take a look at the historical development of the field and the roots of the current terminology.

7. Natural carbohydrate receptors: reading the sugar code

The origin of our knowledge on presence of such receptors can be traced to assays with erythrocytes, already described in the 19th century when using venom from the rattlesnake (*Crotalus durissus*) or extracts of *Ricinus communis* beans (for an overview on history, please see [187]). This receptor-mediated cross-linking of the blood cells, mentioned above for defensins, led to formation of aggregates, an agglutination process (for further information, please see Table 1, entry 21) as antibodies against blood-group antigens were known to build [185,228]. These proteins, consequently termed hemagglutinins, resembled antibodies also in being epitope selective. Simple sugars turned out to be active as inhibitors (haptens) of agglutination. First, α-methyl *L*-fucopyranoside (four-fold more active than *L*-fucose and eight-fold more than the β-methyl anomer) was successfully assayed using O-type erythrocytes and eel (*Anguilla anguilla*) serum, later for example *N*-acetylchondrosamine (now known as *N*-acetyl-D-galactosamine, Gal*N*Ac) when working with certain plant extracts and A-type erythrocytes [229,230] (for further details, please see comment on [229] in Table 1, entry 21). In her own words, W. M. Watkins (a co-author of the seminal study) described the way to this discovery based on K. Landsteiner's hapten assay by writing that "by 1952 we had accumulated a large selection of anti-H reagents of human and animal origin and we decided, with no great expectation of the outcome, to screen them for inhibition of the agglutination of O cells with the component sugars in the blood-group active substances. Somewhat to our surprise one of the many reagents, that from the eel, *A. anguilla*, was quite strongly inhibited by *L*-fucose and to a greater extent by α-methyl *L*-fucoside and not by the other monosaccharides. Our conclusions were somewhat tentative at first because this was an isolated result with an exotic reagent, but the inference that *L*-fucose in α-linkage is more important than the other sugars for H specificity was reinforced when we were given some plant agglutinins …. The failure of many human anti-H and anti-A antibodies to be inhibited by the simple sugars told us that the complete determinant sugars recognized by these antibodies must be larger than the monosaccharides to which the lectins bound. Nevertheless, these simple experiments pointed to the carbohydrate nature of the blood-group determinants and gave the first indications that for each specificity one of the component

sugars was playing a more dominant role than the others. Until that time, the complete substances had been seen as the blood-group antigens but now we were able to perceive that probably only a small part of the macromolecule was involved in the blood-group specific properties" [186].

In contrast to antibodies, the production of these agglutinins in animals did not depend on an antigenic challenge and, as just mentioned, agglutinins are also present in plants. In his own words, seeing the need for a new distinctive term, W. C. Boyd noted that "it would appear to be a matter of semantics as to whether a substance not produced in response to an antigen should be called an antibody even though it is a protein and combines specifically with a certain antigen only. It might be better to have a different word for the substances and the present writer would like to propose the word *lectin* from Latin *lectus*, the past principle of *legere* meaning to pick, choose or select" [231]. Hereby, the author intended "to call attention to their specificity without begging the question as to their nature" [232], and, as given above, specificity is directed to carbohydrates. In laboratory assays, it is defined in terms of mono- and disaccharides, as done by inhibition of hemagglutination. Physiologically, specificity means selecting glycan determinants of glycoconjugates or polysaccharides, presented in a suitable spatial mode. This direct association to a receptor specificity helped the term to find general acceptance, beyond application in hematology [187,233].

Experimentally, sugar reactivity of monovalent lectins, which can bind to but cannot cross-link glycans and thus not agglutinate cells, was a strong argument to focus the definition on the property of sugar binding, dropping a connection to "positive and easily testable properties" [234] such as hemagglutination, which requires at least bivalency. Currently, the term 'lectin' implies that the protein to be classified binds carbohydrates, and here two terminological issues need to be addressed to preclude confusion, dealing first with the realization that a name for a protein will often not cover all structural aspects.

In Nature, many proteins are composed of several domains (modules). The modular design of these proteins, which underlies functionality of a protein in more than one respect, entails that a lectin domain can become linked to other functional elements. That this association is functionally relevant, in a kind of inter-domain cooperation, is illustrated most easily for the endocytic receptors with their transmembrane domain. Looking exemplarily at extracellular proteins, the slime mold lectin discoidin I with its lectin and cell attachment (RGD) sites, the first operative in lectin-site-dependent externalization and the second then in mediation of ordered cell migration, or galectin-3/serum collectin MBL, with collagen-like tails for protein aggregation to enable multimer formation for high-affinity glycan binding, provide instructive cases of proteins with a carbohydrate recognition domain (CRD) associated to other types of bioactive regions [235–238]. The aggregation brings CRDs into suited spatial vicinity, for the defense protein MBL yielding multi-point contact to the surface of bacteria or yeast (please see respective entry on recognition of foreign glycosignatures in Table 2). Lectin activity can thus reside in modular proteins, as a part of their functional profile. Looking at binding partners, it is therefore possible to assign a certain protein to different receptor classes: the cation-independent P-type lectin (please see Gallery of Lectins for folding) is also an insulin-like growth factor II receptor [239,240]. The same holds true for galectins, which react with glycans extracellularly and with other types of molecules such as apoptosis-regulating proteins as bcl-2 or oncogenic H-ras intracellularly [241]. Thus, the presence of an active lectin domain, regardless of its context and the protein's name, is the common denominator.

As the historical origin of the term 'lectin' attests, it deliberately excludes antibodies. By definition, altering the biochemical nature of the ligand is also excluded. In other words, the bound glycan is not a substrate. The refinement that only carbohydrate-binding proteins "other than an enzyme or an antibody" [242] and other than sensor/transport proteins for free sugars are meant when using the term 'lectin' gives the term the required precision [187]. As a special group, the non-catalytic CBMs, which are closely associated to the catalytic centers in glycoside hydrolases degrading cell wall or storage polysaccharides (mostly in bacteria and fungi), are set apart, too (the ability of type B CBMs to sandwich sugar ligands between aromatic amino acids had been mentioned above) [191,213]. They appear to assist the enzymatic sites by guiding their positioning. Having herewith delineated the meaning of the term, the question arising next is whether lectins are rarely or often found in Nature. In the concept of the sugar code, the frequency with which domains or protein folds are adapted to accommodate glycan epitopes as ligands should be fairly high, to match glycome complexity.

The answer to this question is that presently up to 14 different folds have been identified in human/animal lectins ([191,243]; for a graphical survey, also giving an idea on the range of ligands, please

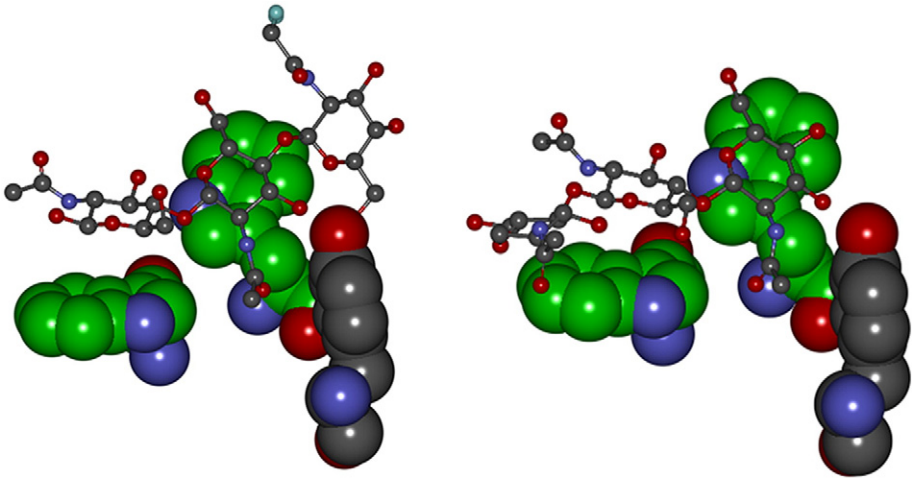
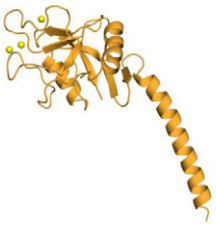
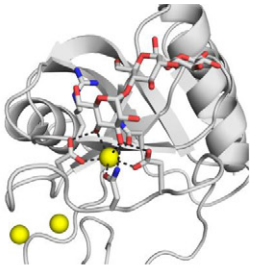
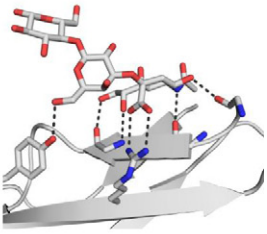
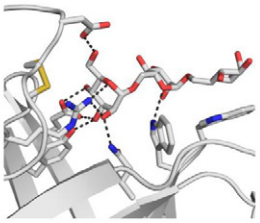
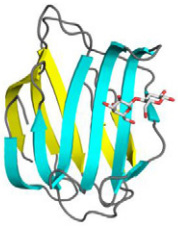
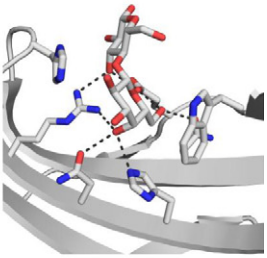
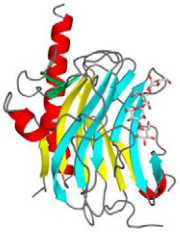
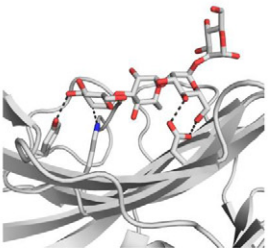
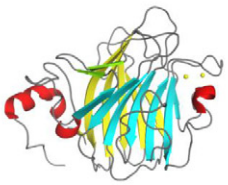
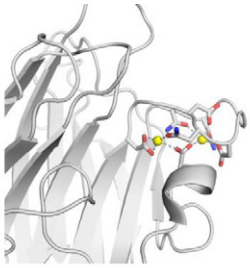
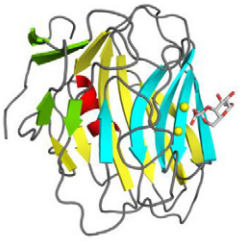
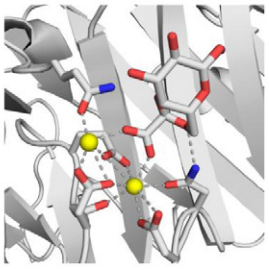
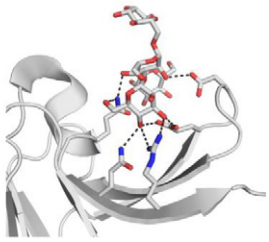
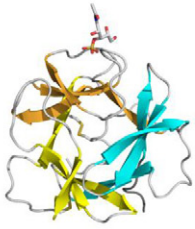
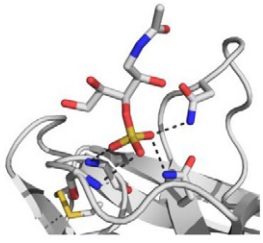
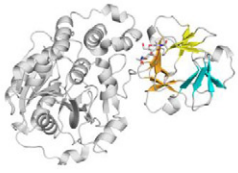
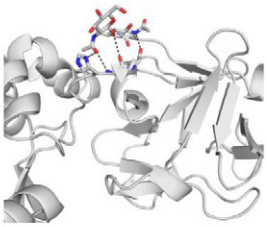


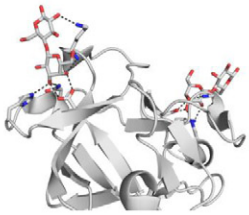
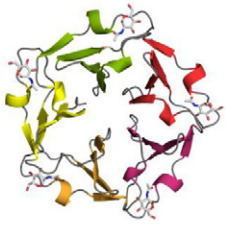
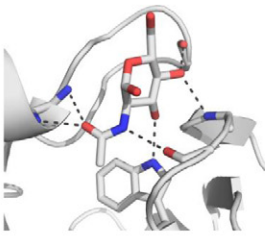
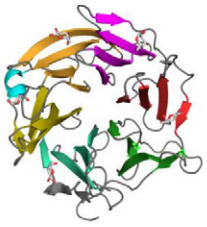
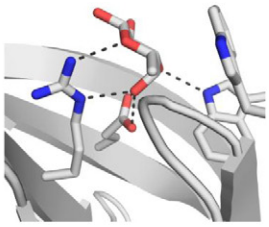
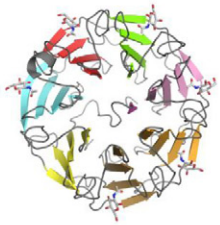
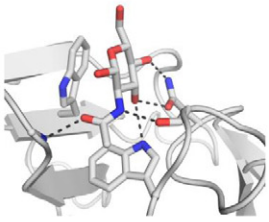
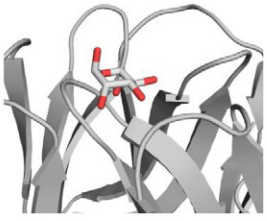
Fig. 12. Dissecting protein–carbohydrate contacts: the hevein/chitooligosaccharide case. Two possible binding modes of the chitin trisaccharide to hevein are shown. In both cases, CH–π stacking interactions between two sugar moieties and two Trp rings (in green) stabilize the complex. Additionally, the Tyr ring (in grey) provides further contacts with the methyl group of the acetamide of a Glc*N*Ac residue. The two binding modes differ in the sugars that provide the stacking interactions. In the left part, the non-reducing terminal residue interacts with one Trp ring and the Tyr moiety; on the right side, the position of the trisaccharide is shifted in the binding site, with the central sugar making the simultaneous contacts to the two aromatic rings. Both binding modes are detected experimentally by NMR spectroscopy. Moreover, two hydrogen bonds additionally stabilize the complexes.

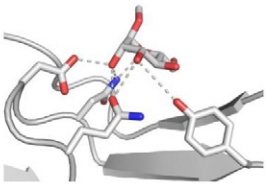
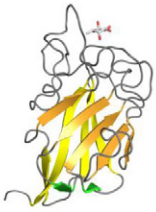
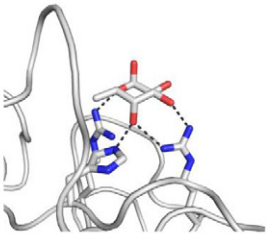
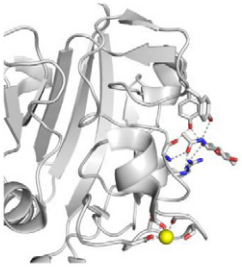
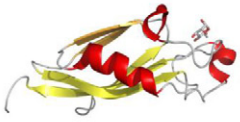
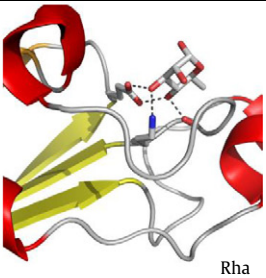
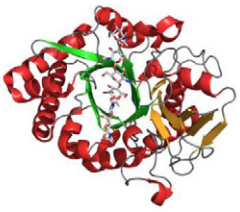
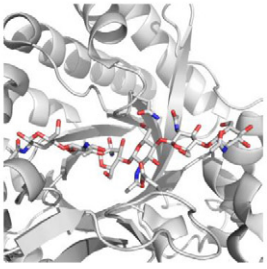
Gallery of Lectins

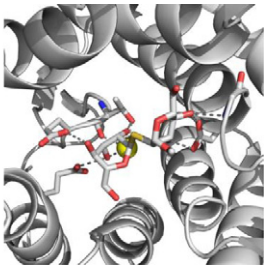
This and the following four pages present a compilation of the folds of animal/human lectins (starting on the left column with a view on each type highlighting the secondary structure and a close inspection of the carbohydrate-binding site accommodating a ligand, with details on the network of hydrogen bonding); examples of group members (for information on functions of mentioned lectins, please see Table 2) and finally of PDB codes (on the right column) are then given, completing the overview.

Type of fold	Overall fold	Carbohydrate-binding site #	Example for lectin	Example for PDB entries
C-type		 Glcα1,4Glcα1,4Glc	asialoglycoprotein receptor, collectins, selectins	asialoglycoprotein receptor: **1DV8**, **1BCH**; lung surfactant protein: **2GGU***, **1PW9**, **1PWB**, **3IKN**; C-type mannose-binding protein: **1AFB**, **1HUP**, **2KMB**, **2MSB**; selectins: **1G1Q**, **1G1R**, **1G1T**, **1KJA**, **3CFW**
I-type		 Neu5Acα2,3Galβ1,4Glc	N-CAM, TIM-3, siglecs	neural cell adhesion molecule (N-CAM): **1EPF**; TIM-3: **3KAA**; siglecs: **1QFO***, **1O7V**, **2ZG1**, **2ZG3**, **2DF3**, **2HRL**, **2BVE**
P-type		 Manα1,6Manα1,6Man	mannose-6-phosphate receptors (MR) and proteins with MR homology domain (erlectin, also called XTP3-B [XTP3-transactivated protein], OS-9)	cation-dependent mannose-6-phosphate receptor: **1M6P**, **2RL7**, **2RL8**, **3K41**; cation-independent mannose-6-phosphate receptor: **1Q25**, **1SY0**, **1SZ0**; OS-9 MRH domain: **3AIH***
β-sandwich (galectins)		 Galβ1,4Glc	animal, fungal and human galectins	galectin-1: **1GZW***, **1SLA**, **1SLB**, **1SLC**, **1SLT**; galectin-2: **1HLC**, **2YMZ;** galectin-3: **1A3K**, **3ZSJ**; galectin-4: **3I8T**; galectin-7: **1BKZ**, **2GAL**, **3GAL**, **4GAL**, **5GAL**; galectin-8: **3AP4**, **3AP5**, **3AP6**, **3AP7**, **4BMB**, **4BME**, **4FQZ**, **4GXL**, **4HAN**; galectin-9: **2D6M**, **2D6N**, **3LSD**, **3LSE**, **3NV2**, **3NV4**; galectin-10: **1G86**
β-sandwich (chaperone)		 Glcα1,3Manα1,2Manα1,2Man	calnexin, calreticulin	calnexin: **1JHN**; calreticulin: **3O0X***, **1HHN**, **3O0V**, **3O0W**, **3POW**, **3RG0**

β-sandwich (transport-mediating protein; ER and Golgi)			ERGIC-53, ERGL, VIPL, VIP36	ERGIC-53: **1R1Z***, **1GV9**; VIP36: **2DUO**, **2DUP**, **2DUQ**, **2DUR**, **2E6V**
β-sandwich (pentraxin)		 methyl-4,6-O-(1,R-carboxyethylidine)-βGal	C-reactive protein, serum amyloid P component	C-reactive protein: **1GNH**, **3PVN, 3PVO;** serum amyloid P component: **1GYK*, 1LGN**, **3FLP**
β-sandwich (*N*-glycanase)		 Manα1,3(Manα1,6)Manα1,6Manα1,3 Man	CRD of Fbs1 in SCF E3 ubiquitin ligase and peptide-*N*-glycanase (PNGase)	PNGase: **2I74***, **2G9G**, **2G9F**
β-trefoil (mannose receptor family)		 Gal*N*Ac-4-SO_4	cysteine-rich domain of C-type macrophage mannose receptor; phospholipase A2 receptor; ENDO180; DEC-205	cysteine-rich domain of macrophage mannose receptor: **1DQO***, **1DQG**, **1FWU**, **1FWV**
β-trefoil (ppGal*N*Ac-transferases)		 Glc*N*Acβ1,4Glc*N*Acβ1,4Man	lectin domain in ppGal*N*Ac-transferases	ppGal*N*AcTase-1: **1XHB***; ppGal*N*AcTase-2: **2FFU**, **2FFV**

β-trefoil (invertebrate lectins)		 Galβ1,4Glc	hemolytic lectin CEL-III of sea cucumber and lectin EW29 of earthworm	hemolytic lectin CEL-III: **1VCL**, **2Z48**, **2Z49**; galactose-binding lectin EW29: **2ZQN***, **2ZQO**, **2DRY**, **2DRZ**, **2DS0**
β-propeller (5-bladed)		 GlcNAc	tachylectin-2	tachylectin-2: **1TL2***
β-propeller (6-bladed)		 Fuc	tachylectin-1	fucose-specific lectin (*Aleuria aurantia* lectin): **1OFZ***, **1IUB**, **1IUC**
β-propeller (7-bladed)		 GlcNAc	lectin from the mushroom *Psathyrella velutina*	lectin from the mushroom *Psathyrella velutina*: **2C4D***, **2BWM**, **2BWR**, **2C25**
β-prism I		 putative binding region for Man/heparan sulfate	ZG16p and ZG16b	Human zymogen granule (membrane) proteins 16p/b (paralogues): **3APA***, **3AQG**; jacalin: **1KU8**, **1KUJ**, **1UGW**, **1UGY**; banana lectin: **2BMY**, **3MIT**, **3MIU**

β-prism II		 Man	pufferfish (*Fugu rubripes*) lectin (no 3D structure currently available); the so-called pufflectin shares homology with monocotyledonous lectins such as GNA.	*Galanthus nivalis* agglutinin (GNA):**1MSA***,**1NIV**, **1JPC**; *Allium sativum* agglutinin: **1BWU**, **1KJ1**
jelly-roll barrel		 Fuc	horseshoe crab tachylectin-4, eel (*Anguilla anguilla*) agglutinin, *Xenopus* X-epilectin.	*Anguilla anguilla* agglutinin: **1K12***
fibrinogen-like		 GlcNAc	ficolectins (H, L and M); intelectins (mammalian, *Xenopus*); tachylectin-5; slug (*Limax flavus*) lectin	H-ficolin: **2J5Z**; L-ficolin: **2J0G**, **2J3O**; M-ficolin: **2D39**, **2JHH**, **2JHI**, **2JHK**, **2JHM**, **2WNP**; tachylectin-5: **1JC9***
α + β		 Rha	lectin domain of mouse latrophilin-1, a G-protein-coupled receptor (GPCR)	mouse latrophilin-1 GPCR lectin domain: **2JXA***, **2JX9**
TIM barrel		 $(GlcNAc)_8$	YKL-40 (human cartilage glycoprotein-39, chitinase-like lectin)	human cartilage glycoprotein (HCGP-39): **1HJW***, **1HJV**, **1HJX**, **1NWR**, **1NWS**, **1NWT**, **1NWU**; YM1 lectin: **1E9L**

$(\alpha\alpha)_7$ barrel		 methyl-2-S-(αMan)-2-thio-αMan	EDEM1, 2, 3 (ER-associated degradation-enhancing -α-mannosidase-like proteins); Mnl1	endoplasmic reticulum mannosyl-oligosaccharide α1,2-mannosidase: **1X9D***, **1FMI**, **1FO2**, **1FO3**

* selected PDB files used for graphical representation
# Calcium ions are represented by yellow spheres

see our Gallery of Lectins). This compilation of illustrations (for animal/human lectins) presents, in each case, the general fold along with structural details of a member of this family (for information on plant lectins, please see [244]; for information on bacterial/viral lectins, please see [109]; by the way, the references attest the ubiquitous presence of lectins matching the range of glycosylation). How broad the range of physiological consequences of lectin presence is documents the overview on functions of animal/human lectins given in Table 2. Here, individual proteins and groups are listed. Tracing the course of phylogenesis, a general fold adapted to host sugars becomes the scaffold for development of descendants, each the starting point for ensuing evolutionary diversification. On the level of protein structures, C-, I- or P-type (the two P-type receptors and lectins in the secretory pathway containing a homologous domain) lectins and galectins are the product of gene duplications [240,245–250]. On the level of genomic representation, copy-number variation with species-dependent changes can occur, as tracked down recently for a galectin by systematic data base mining [251]. A general master fold such as the β-sandwich can even prove so versatile to let several lectin groups arise (please see respective information on β-sandwich proteins in the Gallery of Lectins). In aggregate, this fairly large number of folds and the described high level of intrafamily diversification clearly argue in favor of a high potential of the sugar receptors to be physiologically significant. The common property of all these proteins is their affinity to carbohydrates, prompting the question on similarities seen in the binding site. In view of the principles gleaned from work with synthetic receptors, they are assumed to be manifested by shared presence of certain amino acids.

On the sequence level, looking at the distribution of relative occurrence of amino acids in the contact area of 19 non-homologous carbohydrate-binding proteins, "certain amino acids show a strong propensity to be in the binding site. This applies in particular to the aromatic rings, which can pack against the hydrophobic face of the sugar, and arginine, aspartate and glutamate residues, which can all form bidentate interactions, with adjacent hydroxyls on the sugar" [252]. As the entries to our Gallery of Lectins document, a wide panel of ligands (code words), charged and neutral, is reactive with animal/human lectins, in full agreement with the concept of the sugar code. From the protein side, the complete sandwiching between aromatic side chains of amino acids noted above and an extensive H-bond network in a deep cavity are the two poles of the mode how molecular interaction is accomplished [191]. Chemical or spectroscopic techniques are outlined in Table 1 for how to detect and determine contributions/involvement of these modes to/in binding (please see entries 2, 12, 19, 22 and 26 as prominent examples). In addition to the directional H-bonds, the presentation of a Ca^{2+} adds to sensing distinct hydroxyl-group presentation making flawless epimer distinction possible [188,191]. Here, C-type lectins, for which either Man or Gal satisfies the topologically imposed requirements to allow a network of coordination and H-bonds, take a reliable way to separate cognate from inactive epimers without recruiting a large apolar surface [191,247], an instructive lesson for designing synthetic receptors. Overall, no new chemical forces are recruited by lectins, even when targeting oligosaccharides. Here, complementarity of shapes for making contacts possible is a key feature, and this is shown in each entry presented in the Gallery of Lectins.

A paradigmatic case of how already a small lectin, i.e. the 43-amino-acid hevein briefly mentioned at the beginning of Section 6, can discern the correct binding partner is illustrated in Fig. 12. Measuring the pattern of chemical shift perturbations for amino acids, along with NOE signals from the protein (total of 475 constraints) and protein (Ser19, Trp21, Trp23, Tyr30)–sugar (total of 15 intermolecular contacts) complexes, resulted in this structural view on carbohydrate recognition by the lectin (for further information, please see Table 1, entries 14 and 27). By combining non-polar contacts to two Trp moieties and a Tyr residue with H-bonding, using Tyr30 and Ser19 hydroxyl functionalities, this small-sized plant lectin (and an engineered variant truncated to 32 amino acids) can target different sections of the chitin chain via its preformed contact site [253–257] (for further details on [253], please see Table 1, entry 14). Of note, the ΔG value increases considerably from −3.8 kcal/mol for the Glc*N*Ac dimer to −7.8 kcal/mol for its pentamer [256]. Complex dissociation is getting less and less likely with length extension of the chain, to which hevein can bind at different positions (Fig. 12).

As a principle, the lectin presents a kind of molecular 'lock', into which a certain 'key' can fit, drawing on E. Fischer's famous allegory based on his work on glucoside hydrolysis with two different enzyme preparations [258]. In his own words, enzyme and glucoside should fit like lock and key to exert a chemical effect on each other. Indeed, sugar binding takes place at a preformed site and does then not require major conformational changes, an observation made throughout the survey presented in our Gallery of Lectins. Having learned in Section 4 that glycans in solution can adopt more than one conformation (inviting the comparison to "a bunch of keys" [259]), it appears energetically favorable (in entropic terms) that a lectin will select a certain (preformed) conformer ('key') (or, if flexibility is high, will target a single sugar unit without impeding the mobility around its glycosidic linkage). And this actually happens, as just seen for hevein and in other instances such as binding of the suited conformer of β-galactosides by a galectin or a plant lectin [130,137,149,260] (for further details on [130,137], please see Table 1, entries 1 and 27) or of the sialyl Le^x tetrasaccharide by the three C-type lectins of the group of selectins [261,262] (please see also the PDB information for selectins in the entry on C-type lectins in the Gallery of Lectins). How will a flexible ligand become associated to a lectin? Two modes of binding have developed for accommodating the α2,6-sialylgalactoside. It can be bound either by a network of H-bonding/stacking to both sugar units, selecting the most populated rotamer of the ω-angle in the *gt* orientation as seen in complexes with viral or fungal receptors [110,263], or exclusively via the sialic acid without impeding free rotation around the α2,6-linkage, seen in human siglec-5 and the knob domain of adenovirus serotype 37 [264,265]. Compared to the current generation of synthetic receptors

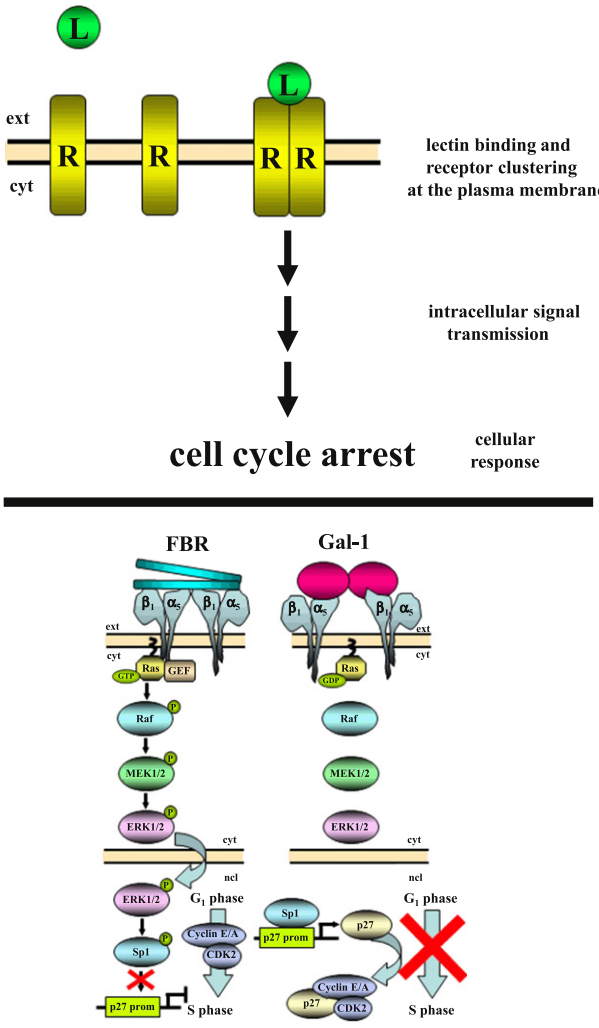


Fig. 13. Translating sugar-encoded information into effects: illustration of signaling for cell cycle arrest by a human lectin. Top panel: binding of a lectin (L), for example galectin-1, to a cell surface receptor (R) induces receptor clustering and activation. The ensuing signaling route results in a cellular response, for example cell cycle arrest. Bottom panel: illustration of signal transmission and cellular response to binding of the extracellular matrix glycoprotein fibronectin (FBR) or a lectin (galectin-1, Gal-1) to the fibronectin receptor ($\alpha_5\beta_1$-integrin). The specific interaction involves different sites of the integrin: peptide motifs for fibronectin and glycan motifs for the lectin. Bottom panel, left column: the binding process induces integrin clustering and activation. This leads to recruitment of plasma-membrane-anchored Ras protein and its activation upon exchange of GDP by GTP, a process catalyzed by a guanine nucleotide exchange factor (GEF). The GTP-loaded Ras then initiates the sequential activation of a chain of protein kinases including Raf, mitogen-activated protein kinase (MAPK)/ERK kinases 1 and 2 (MEK1/2) and extracellular-signal-regulated protein kinases 1 and 2 (ERK1/2) by sequential transphosphorylation. The active (phosphorylated) form of interconvertible proteins is marked by a small-encircled P. The phosphorylated ERK1/2 translocates from the cytosol (*cyt*) to the nucleus (*ncl*) and here phosphorylates the transcription factor signaling protein 1 (Sp1). As a consequence, the binding of this protein to the promoter of the $p27^{KIP1}$ gene (p27 prom) is abrogated. The protein $p27^{KIP1}$ is a cyclin-dependent kinase inhibitor. It acts on cyclin E/A-cyclin-dependent kinase 2 (CDK2) complexes blocking cell cycle progression. As a result of the downregulation of its expression, cells can now enter the S-phase. Bottom panel, right column: in contrast, the interaction of the Gal-1 homodimer with the integrin does not trigger engagement of Ras, which remains in its inactive (GDP-loaded) form. This prevents the activation of the MAPK pathway, its components remain inactive (unphosphorylated). Without being phosphorylated, Sp1 induces transcription of the $p27^{KIP1}$ gene after binding to its promoter (p27 prom). The produced $p27^{KIP1}$ protein (p27) then associates with cyclin E/A-CDK2 complexes, causing G_1 arrest. The acronyms *ext*, *cyt*, and *ncl*, respectively, denote the extracellular, cytosolic and nuclear compartments. The two crosses signify that the respective process is blocked (for experimental details, please see [291]; from [292], with permission).

with dissociation constants around the mM limit, lectin binding to cognate glycan counterreceptors can reach the nM level and even beyond (for a survey of methods to determine affinity of lectin–glycan interaction including in vitro/in vivo assays, please see Table 1 starting at entry 16). One factor explaining this affinity increase is that binding sites to glycans can often be extended, accommodating a larger area for contact than the current synthetic products (please see Fig. 12 for hevein and the Gallery of Lectins for further examples). Here, the rather rigid presentation of oligosaccharides (for case studies, please see Table 1, entry 1) is a boon to keep entropy losses upon binding small (when compared with highly flexible peptides). With attention paid to entropy, emerging evidence from NMR-spectroscopical analysis with ^{15}N-labeled proteins (e.g. galectin-3) indicates a sizeable contribution of increases of structural entropy in the protein to the thermodynamics of binding (for further information, please see comment on [343] in Table 1, entry 11). Obviously, loading the binding site with ligand engenders an increase in conformational entropy at other sections of the protein. This factor for affinity enhancement will likely not be operative for the small and rigid synthetic receptors. From the side of the ligand and with impact on entropy, too, binding is strengthened by local clustering, on both sides (e. g. by the mentioned non-covalent aggregation via a collagen-like tail). Clearly, the access to natural oligosaccharides and to glycoclusters by synthesis, combined with the application of modeling protocols to correctly interpret the NMR-spectroscopical information reported by the ligand and by isotopically labeled lectin to detect any structural changes in the binding site and beyond (for details and examples, please see Table 1 entries 1, 11, 12, 14, 26 and 27), will immensely add to our understanding of the molecular rendezvous involving more than a mono- or disaccharide.

Using this combination, i.e. interaction analysis and modeling, the branched pentasaccharide of ganglioside GM1, a cell surface counterreceptor for human galectin-1 in growth regulation of neuroblastoma cells and effector T cells relevant for autoimmune regulation [266–268] (please see comment on [267] in Table 1, entry 37), has been shown to offer more than a single Gal unit to the lectin: the Galβ1,3Gal*NA*c terminus and the α2,3-sialyl branch both contribute to the overall interaction energy [130], and in one of the glycan's three low-energy conformations. Adding another graphic example, the binding site of the lectin covers more than a monosaccharide. It is in contrast adapted for recognition not only of a chain like Lac*NA*c repeats noted in Section 3 but also of a suitably branched glycan [130,269]. Only one of the three conformers of the α2,3-sialylgalactoside reaches complementarity. Intriguingly, the lectin part of cholera toxin, a bacterial AB_5 toxin, hosts a different low-energy conformation [270]. This biomedically relevant case of *differential conformer selection* between two receptors sets the basis to preclude cross-reactivity between the human effector and the bacterial toxin, when designing reagents for lectin blocking. In addition, the differences in the spatial presentation of the lectin sites between the two proteins, as homodimer or homopentameric (disc-like) ring, can be exploited by modular structure-based glycocluster design to further increase selectivity and specificity, for bacterial AB_5 toxins [271] and also for other bacterial lectins underlying the first step in infection [272–274], aglyconic extensions enhancing the affinity [275]. This chemical approach can also work for reaching the aim to selectively hit a group of lectins (e.g. selectins as illustrated in Table 1, entry 39). Even more challenging, a certain member of a lectin family might be targeted by an inhibitor with discriminatory design of i) the headgroup, ii) the aglyconic extension, and iii) the cluster, an attractive long-term aim for the chemist, as noted at the end of the third section. The mentioned trivalent clusters for asialoglycoprotein receptors, using only Gal (not lactose) as ligand to avoid cross-reactivity with galectins [276,277], are a role model, as are enzymatically trimmed glycoproteins for enzyme replacement therapy (for further information, please see Table 1, entry 39). Systematic activity assays with carbohydrate derivatives and with scaffolds, in different experimental systems of increasing biomedical relevance (for further information, please see Table 1, e.g. entries 9, 19, 30 and 39), are the prerequisite to identify viable options, such a step

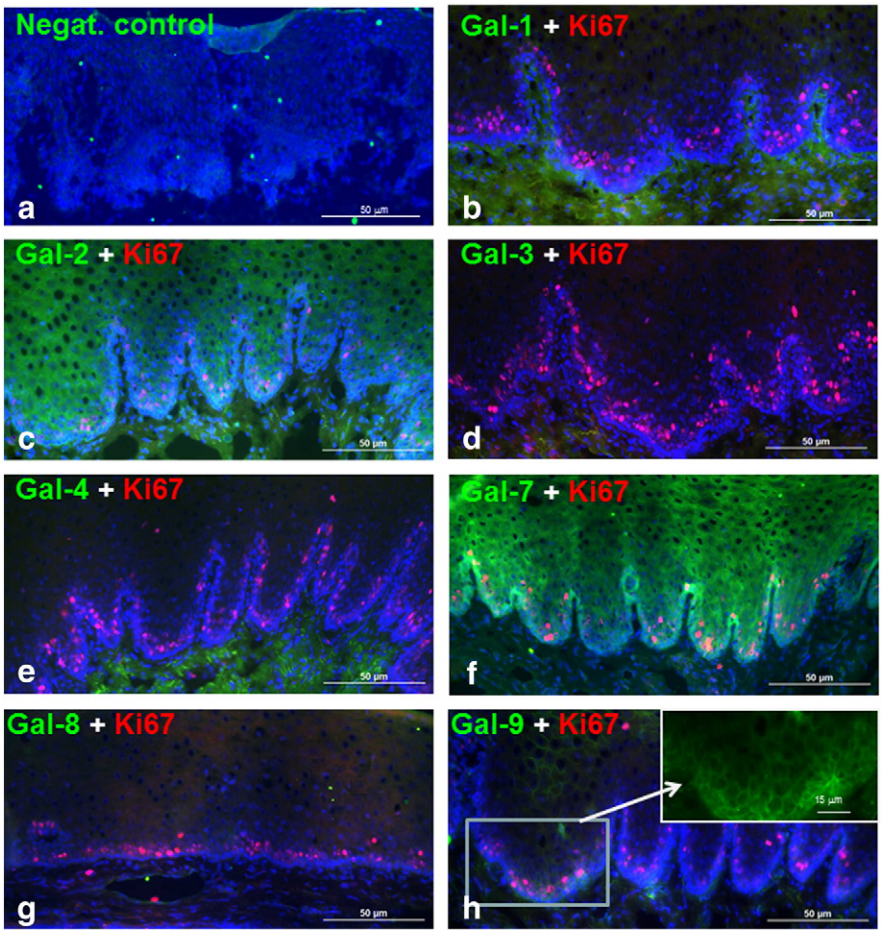


Fig. 14. The network of galectins. Presence of selected representatives of the galectin family (green signal) in the oral epithelium obtained from a donor with long smoking history: negative control by processing with a rabbit polyclonal antibody to an antigen not present in the tissue (a), galectin-1 (Gal-1, b), galectin-2 (Gal-2, c), galectin-3 (Gal-3, d), galectin-4 (Gal-4, e), galectin-7 (Gal-7, f), galectin-8 (Gal-8, g) and galectin-9 (Gal-9, h) as well as proliferation marker Ki67 (red signal, b–h) were detected by immunofluorescence. The epithelium has slightly hyperplastic character, and nuclei positive for the proliferation marker Ki67 are located suprabasally. The cytoplasm of epithelial cells was positive for galectin-2 (c) and for galectin-7 (f). Galectin-9 was located mainly in membranes of cells of basal and lower spinous layers (h). Subepithelial connective tissue was positive for galectin-1 (b), galectin-2 (c) and galectin-4 (e); here, galectins were present predominantly in the extracellular matrix. FITC-labeled swine anti-rabbit serum and TRITC-labeled mouse anti-human immunoglobulins were used as second-step reagents. Nuclei were labeled by DAPI. Bar is 50 and 15 µm, respectively.

taken within the galectin family for a pair of functionally antagonistic proteins, i.e. galectins-1 and -3 [174,278] and galectin-3 with/without its aggregation-promoting tail [279], the latter case with a glycophane, a scaffold for synthetic receptors (please see above [211]). Physiologically intriguing, growth regulation by the homodimeric galectin-1 can locally be switched off by galectin-3. This lectin thus acts as endogenous antagonist when galectin-1 reduces neuroblastoma proliferation or induces anoikis (programmed death of cells not attached to a matrix) of pancreatic carcinoma cells [280,281]. In consequence, galectin-3 becomes a target for inhibition to let galectin-1 set limits to cell growth.

The insights into structure–activity correlations emerging from such studies with glycoclusters and related lectins differing in topology of CRD presentation will then contribute to explain the exquisite target specificity of endogenous lectins. After all, despite an abundance of related carbohydrate epitopes on cell surfaces, tissue lectins home in on distinct counterreceptors (cognate glycans presented on particular lipids/proteins), and spatial factors appear to be a crucial part of the selection process. Its dissection identified six levels, from the lectin's monosaccharide specificity (please note that the structural context of a glycan has a bearing on epitope binding so that the information on monosaccharides is not necessarily predictive [282]) to its preference to facing the cognate glycoconjugates clustered in microdomains [191,243]. As a consequence, spatial factors of the lectin matter, too. Obviously, a lectin with two contact sites pointing into different directions (homodimer or tandem-repeat-type protein) can act as a cross-linker. Altering the length of a linker peptide between CRDs of a tandem-repeat-type lectin by engineering or impairing the aggregation status by a single nucleotide polymorphism, without any change of the set of residues in the binding site, can affect the profile of binding partners and the affinity [283,284]. By the way, natural mutants arising from polymorphisms in the coding region, especially when connected to a disease, as seen for a galectin recently [285], are promising case-study objects in this research area. The interplay between rational engineering of lectins and preparing glycoclusters/cell-like test models such as glycodendrimersomes guides the way to solidify the meaning of the statement that structural features of the glycan/lectin pair (sequence and shape) and context-dependent spatial characteristics of presentation on both sides determine specificity and selectivity, the process of 'reading' the sugar code. Its 'translation' into the documented cellular effects (for a survey of lectin functions, please see Table 2) will engage different types of post-binding mechanisms, from connecting glycans by a lectin bridge (as in hemagglutination) or routing

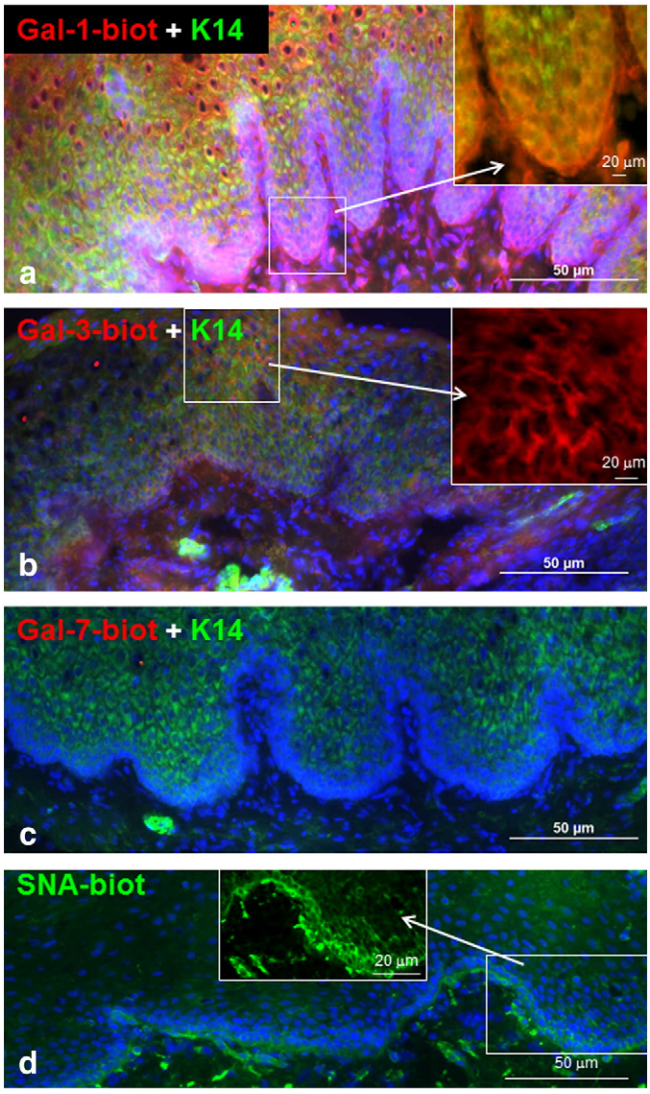


Fig. 15. Spotting tissue reactivity for (ga)lectins. Detection of binding sites for galectin-1 (Gal-1-biot, red signal, a), galectin-3 (Gal-3-biot, red signal, b), galectin-7 (Gal-7-biot, red signal, c), for the *Sambucus nigra* agglutinin reactive with α2,6-sialylated Gal/GalNAc (SNA-biot, green signal, d) and of intermediate filament keratin-14 (K14, green signal, a–c) in sections of the same specimen as in Fig. 14. Binding sites for galectin-1 were detected in the cytoplasm of cells located in all layers of epithelium, with maximum signal intensity in basal and lower spinous layers (a). Binding sites for galectin-3 were located suprabasally predominantly at the cell surface (b), whereas no binding sites for galectin-7 were detected (c), demonstrating reactivity differences. Binding sites for the plant lectin SNA were seen predominantly in the cytoplasm and membrane of cells of the basal layer of the epithelium (d). Cells reactive with SNA are usually not recognized by galectin-3, indicating the lack of reactivity of galectins with α2,6-sialylated LacNAc epitopes. Keratin-14, which is normally strictly located in proliferating basal cells, exhibited a highly aberrant suprabasal expression profile (a–c). FITC-labeled swine anti-rabbit serum and TRITC- or FITC-labeled ExtrAvidin were used as second-step reagents. Nuclei were labeled by DAPI. Controls for carbohydrate-dependent binding of lectins were performed with lactose as haptenic sugar (for galectins) or neuraminidase treatment (for SNA), for antigen-specific binding by a control monoclonal antibody. Bar is 50 and 20 μm, respectively.

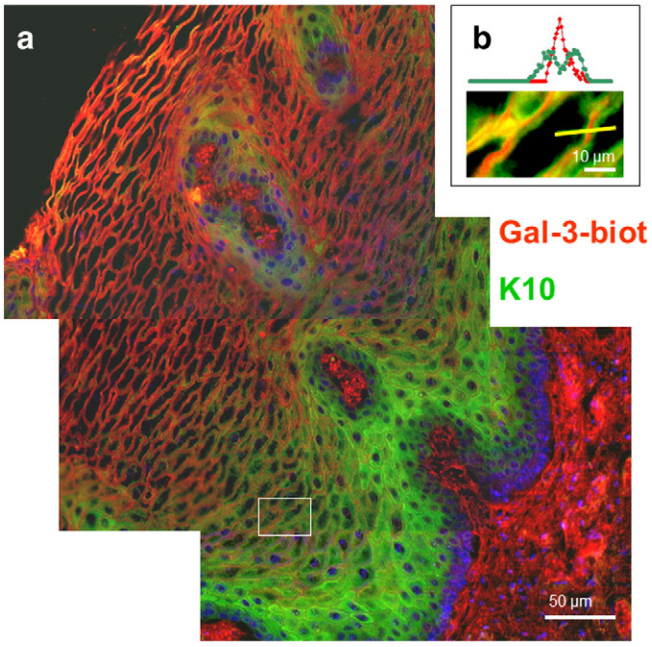


Fig. 16. Tissue lectin as histochemical tool. Section through a specimen of leukoplakia of human esophagus, showing the profile of binding sites for galectin-3 (Gal-3-biot, red signal) and keratin 10 (K10, green signal) (a, b). Keratin 10 was in the cytoplasm of cells of the lower spinous layer, binding of galectin-3 was detected strictly in suprabasal intercellular contacts, as also documented in the inset showing the boxed area at higher level of magnification (b); a fluorescence profile of both parameters is added, line of measurement of the fluorescence intensities is marked by a yellow bar. The subepithelial connective tissue was also positive for binding of the biotinylated galectin. FITC-labeled swine anti-mouse serum and TRITC-labeled ExtrAvidin were used as second-step reagents. Nuclei were counterstained by DAPI (for specificity controls, please see legend to Fig. 15). The figure is composed of three independently recorded microphotographs to present different regions of the specimen, with zonal distribution of the galectin reactivity. Bar is 50 and 10 μm, respectively.

glycoconjugates to certain destinations to triggering intricate signaling.

8. Natural carbohydrate receptors: translating the sugar code

Post-binding events lead to a broad range of activities, building bridges in adhesion being the simplest one. The entries into Table 2 summarize functions of animal/human lectins. They start, like our review, with the eminent role of lectins in quality control and the ER-to-Golgi partitioning, then turning to endocytosis by e.g. asialoglycoprotein receptors of glycoproteins, the starting point of biochemical work on mammalian lectins, and the other aspects of functionality (structures of the folds of listed proteins are given in the Gallery of Lectins, Table 2 and our Gallery covering structural and functional aspects). Besides serum glycoproteins, lectin activity can regulate, too, the survival of platelets in circulation, depending on the actual structure of the termini of their *N*-glycan chains [286,287]. Co-transport processes in routing depend on several glycan signals beyond the terminus of complex-type *N*-glycans. Man-6-phosphate on *N*-glycans of lysosomal enzymes, the recognition signal for P-type lectins, or Gal-3-sulfate as sulfatide headgroup on a ceramide backbone with long-chain (up to C26) fatty acids, in apical cargo delivery the contact site for galectin-4, similarly are molecular addresses [240,288].

What's more, the selection of distinct cell surface targets enables to trigger outside-in biosignaling, directly eliciting effects or acting in cross-talk with other effector cascades [289,290]. Fig. 13 illustrates the principle of a signal-transfer cascade and a case leading to cell cycle arrest of adherent carcinoma cells by a cross-linking galectin [291, 292]. At the heart of the entire process, the glycan part of the cell surface counterreceptor (glycoprotein) is responsible for the docking of the lectin, the protein part is then decisive for the post-binding signaling, the two working together (as noted at the beginning of Section 7 for lectins with more than one functional site such as discoidin I). This interplay heightens the respect for the prophetic (and concise) statement by P. J. Winterburn and C. F. Phelps that the significance of the glycans "is to impart a discrete recognitional role", here to effector proteins [67]. The elaborate glycosylation machinery in the Golgi, to connect to Section 1, equips the proteins with the means to become part of an

intricate regulatory system. Turning back to tumor growth regulation, it also works on cells in suspension. When not in contact to substratum, this type of receptor (galectin-1)–glycan (presented by the glycoprotein $\alpha_5\beta_1$-integrin) interplay starts the route to caspase-8-dependent anoikis (please see above), unless α2,6-sialylation precludes the interaction like a molecular switch set on 'off' [293]. A single carbohydrate at the right place (here a sialic acid residue) can thus let cells leave growth control. Mechanistically, caspases are also involved in driving activated T cells into apoptosis, each human proto-type galectin eliciting its own effector profile [289,294]. The clonal selection of T cells based on counterreceptor expression (here glycoprotein CD7) and susceptibility to galectin-induced cell death in patients with Sézary syndrome takes galectin activity to the clinical level [295]. Only T cells lacking CD7 will survive, explaining the clinically apparent clonal selection toward CD7-negative cells during disease progression. For onset of autoimmune disease, another aspect of functionality of lectin-integrin rendezvous is relevant. In the communication between regulatory and effector T cells, complexes of galectin-1 and this integrin together with ganglioside GM1, structural details in the 'reading' given above, transfer the initial signal to eventually let TRPC5 channels (belonging to the transient receptor potential (canonical) ion channel subfamily and upregulated upon effector T cell activation) be opened and intracellular Ca^{2+}-concentration in activated effector T cells be increased, to dampen effector T cell activity [267,268]. In other words, lectin made available by activated regulatory T cells "tells" effector T cells presenting cognate glycan to take it easy.

Signal initiation critically depends on the availability of the lectin and its counterreceptor at the right time and place, this ensured by their co-regulation during T cell activation [267,296,297]. A dysfunction at either side can apparently be a factor for unrestrained effector T cell auto-aggression in a mouse model (NOD) for type I diabetes [298]. Of course, more than a single factor appears to partake in the pathogenesis of this disease. To illustrate the network concept for galectins and then beyond galectins, galectin-9 has potential to attenuate Th1 immune responses in this model by inducing apoptosis of $CD4^+$ Tim-3^+ T_H1 cells [299,300]. Beyond the galectin aspect, the GPI-anchored glycoprotein CD52 on the lymphocyte surface is not only a marker for antigen-activated $CD4^+$ T cells but also a mediator of suppressive activity by lectin (siglec) binding (for information on the siglec fold, please see Gallery of Lectins). $CD52^{high}$ $CD4^+$ T cells release CD52, its binding to siglec-10 on T cells via α2,6-sialylated glycans reduces phosphorylation of kinases (Lck/Zap70) associated with the T cell antigen receptor, and hereby opens another route toward confining auto-aggression [301]. The cases of a tumor suppressor ($p16^{INK4a}$) orchestrating lectin and glycan expression toward a pro-anoikis (glyco)phenotype in pancreatic carcinoma cells [117,281,302] (for further information, please see Table 1, comments on [117,302] in entries 32 and 38) and of inflammatory stimuli to do the same to prepare leukocytes for selectin-dependent homing to the inflicted site [303] underscore the intricate integration of the individual parts of the glycobiological toolbox into cell physiology. Most likely, the mutual adaptation is a rather general phenomenon to let lectin–glycan display match. Fittingly, glycophenotyping by plant lectins is increasingly complemented by applying tissue lectins to detect functionally active constellations.

As further given in detail in the entry 33 of Table 1, monitoring the status of expression and the spatial presentation on both sides of a recognition system is experimentally amenable by applying neoglycoconjugates/lectin-specific antibodies and the lectin. Used as a tool, the reagents will bind to sites with complementarity, that is the glycan of neoglycoconjugates to sugar-binding proteins, the antibody to antigenic determinants and the lectin to cognate epitopes. Labeling of the reagents ensures spotting presence of an interaction by microscopy, as done with fluorescent dyes for recording the microphotographs presented in the final set of figures. Exemplary localization profiles from running such protocols are illustrated for galectins in Fig. 14 (immunohistochemistry) and for binding sites in Fig. 15 (galectin histochemistry). Detailed explanations for the staining pattern are given in the respective legends. Obviously, cellular positivity can cover extra- and intracellular regions, illustrating the aspect of multifunctionality mentioned in Section 6. To highlight an assumedly functional role in cross-linking cellular glycans, intense labeling in the region of intercellular contacts by a galectin is presented in Fig. 16. In sections of head and neck squamous cell carcinomas, a similar type of staining with galectin-3 was a prognostic indicator for relapse-free ($p = 0.011$) and overall survival ($p = 0.0259$): with 5% of the tumor cell population set as threshold for positive/negative classification, reactivity above this limit was correlated with prolonged periods, thus favorable [304]. In order to highlight that intrafamily divergence can engender disparities in glycan preferences, reactivity to galectin-9 required enzymatic desialylation [305] (please see also Table 1, comment on [325] in entry 4). These figures from histochemical work also reveal, by applying the fingerprinting approach (measuring profiles of family members [306–308]), that a network of related lectins, with both distinct and overlapping staining characteristics, can exist. Considering the potential for functional antagonism, as described for neuroblastoma growth regulation above, the design of localization studies should take functional diversity into account and prefer fingerprinting to single-protein detection. And the set of histochemical figures teaches another lesson: illustrated intracellular presence of lectin and binding sites attests reactivity of lectins to proteins residing in the cytoplasm and nucleus [309–311] (please see also Section 7). Studying clinical specimen, an indication for actual in vivo activities of a lectin can be gained by the correlation of its presence and localization to tissue parameters, e.g. growth or invasiveness of tumors. Before reaching conclusions on biomedical perspectives, caveats must be adequately considered: the more often a lectin is expressed in different cell types with evidence for context-dependent multifunctionality, the more difficult it will be, especially when also being part of a network and present at different sites in a cell, to define it as a promising target for medical application [241]. In other words, the hepatic asialoglycoprotein receptor or selectins will likely be more suitable as object for drug development than galectins.

Conversely, the molecular analysis of disease states or engineered models can delineate important clues on lectin/glycan functionality (for further information, please see Table 1, entries 35–38). To give an example, the occurrence of recurrent infections in patients with leukocyte adhesion deficiency type II (CDG-IIc) is attributed to reduced extent of cell surface fucosylation by defective transport of activated fucose (GDP-Fuc) into the Golgi to feed the enzymatic assembly line, the resulting lack of the sialyl Le^x signal precluding selectin binding and selectin-dependent leukocyte rolling [40,312]. Engineering of mouse models by introducing both equivalent defects in glycan synthesis and selectin deficiencies fully corroborates this evidence [43]. On other occasions, however, interspecies differences in glycosylation/lectin aspects can inevitably hamper extrapolations. On the level of emerging therapeutic options, connecting insights from basic science to applications, the knowledge on sugar-encoded 'postal codes' on the 'cargo' can help to let cells [313] or enzymes [314] reach desired destinations by glycoengineering (for information on treating Gaucher disease with engineered enzyme, please see Table 1, entry 39). Overall, at each section of Table 2, the natural glycan tailoring for lectin reactivity is operative, together with lectins 'reading' and 'translating' the sugar-encoded information.

9. Concluding remarks

Our view on complex carbohydrates has gone through a significant transition. Instead of feeling uneasy about the enormous structural variability, it is now appreciated as a sign of the unsurpassed coding capacity of glycans. Having mastered the challenges of their synthesis was crucial for structural analysis (for glycans, their receptors and complexes), illustrating the essential nature of interdisciplinary cooperation (for survey of experimental techniques, please see Table 1). This

intimate interplay will in the same manner be fruitful to delineate how structure and topology of presentation on both sides of the recognition system are teamed up in vivo to reach the exquisite specificity observed for endogenous lectins and their counterreceptors. Physiologically, orchestrated (re)programming of their expression, as exemplarily mentioned for anoikis induction by a tumor suppressor or preventing onset of auto-immune diseases by effector/regulatory T cell communication, underscores the far-reaching significance of the sugar code. In the words of N. Sharon after having delivered seminal lectures on complex carbohydrates, we (sharing his mission and enthusiasm) "trust that what we have just told you has conveyed to you the feeling of where we stand at present in the study of carbohydrates, both simple and complex. It is also our hope that we have convinced you why this field is of such great importance, and why it is so exciting" [315].

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