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DESIGN AND CREATIVITY IN SYNTHESIS OF MULTIVALENT NEOGLYCOCONJUGATES

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ABBREVIATIONS

3TC, 2′,3′-dideoxy-3′-thiacytidine (lamivudine); AFM, atomic force microscopy; AGP, human α1-acid glycoprotein; AIBN, 2,2-azobisisobutyronitrile; ASF, asialofetuin glycoprotein; ATRP, atom transfer radical polymerization; BBA, bladder-binding assay; 9-BBN, 9-borabicyclo[3.3.1]nonane; BBV, *N*,*N*′-4,4′-bis(benzyl-3-boronic acid)bipyridinium dibromide; BIEMA, 2-(2-bromoisobutyryloxy)ethyl methacrylate; bis-MPPA, 2,2-bis(hydroxymethyl)propanoic acid; Boc, *tert*-butoxy carbonyl; BOP, benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; Cbz, benzyloxycarbonyl; CCI, carbohydrate–carbohydrate interactions; β-CD, β-cyclodextrin; CD69, cluster of differentiation 69; CFU, colony-forming units; CHO, chinese hamster ovary; CNT, carbon nanotube,; Con A, Concanavalin A; CPS, capsular polysaccharide; CRD, carbohydrate recognition domain; CSLM, confocal laser-scanning microscopic; CT, cholera toxin; CuAAc, Cu(I)-catalyzed azide–alkyne [1,3]-dipolar cycloaddition; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; DEG, diethylene glycol; DIBAL-H, diisobutylaluminium hydride; DIC, diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DLS, dynamic light scattering; DMAP, dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DMI, 1,3-dimethyl-2-imidazolidinone; DMPC, dimyristoyphosphatidylcholine; DMSO, dimethyl sulfoxide; DOSY, diffusion ordered spectroscopy; DPPC, dipalmitoyl phosphatidylcholine; EcorL, *Erythrina corallodendron* lectin; EDC or EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; EFV, efavirenz; EHEC, enterohemorrhagic *E. coli*; ELISA, enzyme-linked immunosorbent assay; ELLA, enzyme-linked lectin assay; EPR, enhanced permeation retention; ESI-MS, electrospray ionization-mass spectrometry; FITC, fluorescein isothiocyanate; fmoc, 9H-fluoren-9-ylmethoxycarbonyl; FRET, fluorescence resonance energy transfer; Gb_3, glycosphingolipid globotriaosylceramide; GM1, monosialotetrahexosylganglioside; GNA, *Galanthus nivilis* agglutinin; HAI, inhibition of hemagglutination; HATU, (2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HEK293, human embryonic kidney 293; HepG2, hepatocellular carcinoma cells; HIV, human immunodeficiency virus; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, hydroxybenzotriazole; HP, haematoporphyrin; HPA, *Helix pomatia* agglutinin; HPA-FITC, *Helix pomatia* agglutinin-fluorescein isothiocyanate; HRPO, horseradish peroxidase; HUS, hemolytic uremic syndrome; IC, inhibitory concentration; ICAM, intercellular adhesion molecule; IgG 2G12, immunoglobulin G 2G12 antibody; K_D, dissociation constant;

IT, inhibition titer; ITC, isothermal titration microcalorimetry; KLH, keyhole limpet hemocyanin; LCA, *Lens culinaris* lectin; LFA, *Limax flavus* lectin; LTBh, *E. coli* heat-labile toxin B; NHS, *N*-hydroxysuccinimide; NK, natural killer cell; NMR, nuclear magnetic resonance; MAG, multiple antigenic glycopeptides; MAIG, 3-*O*-methacryloyl-1,2:5,6-di-*O*-isopropylidene-D-glucofuranose; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MALS, multiangle light scattering; MBP, mannose-binding protein; Me-αMan, Me α-D-mannopyranoside; MDCK, Madin–Darby canine kidney cells; MHC, major histocompatability complex; MIC, minimum inhibitory concentration; MLCT, metal-to-ligand charge transfer; MM, mucin mimic; MMP, matrix metalloproteinase; MNPG, *m*-nitrophenyl α-D-galactoside; MPPI, mannosylated-polypropyleneimine dendrimers; MPR, mannose-6-phosphate receptor; MRI, magnetic resonance imaging; MSC, mesenchymal stromal cells; MT1-MMP, membrane type-1-matrix metalloproteinase; MUNeuAc, 2′-(4-methylumbelliferyl)-α-*N*-acetylneuraminic acid; MVK, methyl vinyl ketone; MWNT, multiwalled nanotube; OMPC, outer membrane protein complex; P_3CS, tripalmitoyl-*S*-glycerylcysteinyl-serine; PAII-L, *Pseudomonas aeruginosa* lectin II; PAMAM, poly(amidoamine) dendrimers; PAP, poly(*p*-*N*-acryloylamidophenyl); PAP-α-Glc, poly(*p*-*N*-acryloylamidophenyl) α-glucopyranoside; PBS, phosphate buffered saline; PePO, pentaerythrityl phosphodiester oligomer; PET, photoinduced electron transfer; PITC, phenylisothiocyanate; PDT, photodynamic therapy; POSS, polyhedral oligosilsesquioxane; PMBC, peripheral blood mononuclear cells; PPI, polypropyleneimine; PS, photosensitizer; PVK, poly(methyl vinyl ketone); PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; QSAR, quantitative structure–activity relationship; RCA_{120}, *Ricinus communis* agglutinin; RGP, radial growth polymerization; SCVCP, self-condensing vinyl copolymerization; SEM, scanning electron microscopy; SHC, Sonogashira—Heck—Cassar cross coupling reactions; SLT, Shiga-like toxin; SLT-Iie, Shiga-like toxin II edema variant; SPG-Lac, schizophyllan bearing lactosides; SPR, surface plasmon resonance; SPS, solid-phase synthesis; STEC, Shiga toxigenic group of *Escherichia coli*; Stx, Shiga toxin; SWNT, single-walled nanotube; TBAF, tetra-*n*-butylammonium fluoride; TBAH, tetra-*n*-butylammonium hydroxide; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEM, transmission electron microscopy; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TF, Thomsen–Friedenreich antigen; TFA, trifluoroacetic acid; TGA, thermogravimetric analysis; THF, tetrahydrofuran; TRIS, tris(hydroxymethyl)aminomethane; VAA, *Viscum album* agglutinin; VT, verotoxin; VV-HRP, horseradish peroxidase-labeled plant lectin *V. villosa*; VVA, *V. villosa* plant lectin; WGA, wheat germ agglutinin; YDS, yolk decasaccharide

I. Introduction

1. Multivalency: Definition and Role

Generally, the valency of a particle (namely a small molecule, oligosaccharide, protein, nucleic acid, lipid or aggregate of these molecules, a virus, bacterium, or cell) can be defined as the number of separate structural units of the same kind that can interact with other particles through ligand–receptor interactions.[1] Thus, one can consider that a molecule having two tethered and identical copies of binding components can be classified as a divalent entity. Similarly, multivalent or polyvalent interactions can be defined as specific simultaneous associations of multiple ligands (or epitopes) present on a molecular construct or biological surface that binds in a cooperative way to multiple receptors expressed on a complementary entity.

The ubiquity of these multivalent interactions at different levels in several biological mechanisms testifies to their essential role. In fact, multivalency in Nature is very often expressed by fractal or ''dendritic'' architectures that represent perhaps the most pervasive topologies observed in vegetal and animal kingdoms.[2] Typical examples of these patterns may be found at different scales of dimensional length (meters to microns), and typical examples can be observed in abiotic systems (such as snow crystals, fractal erosions, manganese dendrites in rock) or in the biological world. The reasons for such extensive mimicry of these dendritic topologies at virtually all scales of dimensional length are not entirely clear. However, one might speculate that these evolutionary architectures have been optimized over the past several billion years to provide structures manifesting maximum interfaces for optimum energy extraction/distribution, nutrient extraction/distribution, information storage/retrieval, and adhesive processes. For example, trees use fractal dendritic patterns above and beneath the ground in order to enhance the exposure of their leaves to sunlight to harvest light and maximize the photosynthesis process, and of their roots to collect water from the soil.

Most notable natural examples of this architecture at the molecular level are probably the glycogen and amylopectin hyperbranched structures that Nature uses for energy storage. Presumably, the many chain ends that decorate these macromolecules facilitate enzymatic access to glucose for high-demand bioenergy events. Another nanoscale example of dendritic architecture in biological systems is found in proteoglycans. These macromolecules appear to provide energy-absorbing, cushioning properties and determine the viscoelastic properties of connective tissues.

In addition, the tremendously complex dendritic respiratory network is composed of bronchioles and alveoli to give the maximum surface for efficient transfer of oxygen into the bloodstream. The arterial and central nervous system networks, together with the kidneys and lung structures also consist of a great number of cells growing into dendritic structures in order to gain the largest exchange of material or information with the surrounding tissues.[3] Recently, the implication of dendritic patterns observed under the feet of the gecko has also been clarified to explain the exceptional ability of these animals to climb rapidly up smooth vertical surfaces.[4] Microscopic examinations have shown that a gecko's foot has nearly five hundred thousand keratinous hairs or setae, each composed of an impressive dendritic network of tiny foot hairs ''spatulae.'' Measurements have revealed that one seta is 10 times more effective at adhesion than predicted from maximal estimates on whole animals. Values of the adhesive force support the hypothesis that individual setae operate by weak attractive quantum chemical forces from molecules in each foot-hair interacting through Van der Waals forces with molecules of the surface.

Finally, a very striking example is also afforded by Nature, particularly in exposing a wide array of complex dendritic glycoconjugates on mammalian and HIV-1 cell surfaces. These carbohydrate structures play critical roles in multiple key cellular events, such as cellular adhesion and recognition, regulation of physiological functions, and pathogenic infections. For HIV-1, several hypothesis have been formulated concerning their role in infection process, and it has been speculated that the N-linked hyperbranched high mannose oligosaccharide ($Man_9GlcNAc_2$), exposed at the exterior envelope glycoprotein gp120, helps the ''hidden'' virus to escape neutralizing antibodies.[5] Indeed, these glycans are produced by the host cell, and are largely unrecognized by the immune system machinery.

2. Multivalency in Protein–Carbohydrate Interactions

Although carbohydrates and their corresponding conjugated glycoforms have long been regarded as only space-filling matrices or post-transcriptional accessory elements in glycoproteins serving to protect them from premature degradation, it has become apparent that such glycoconjugates as glycolipids or glycosaminoglycans exhibit a broad variety of additional biological functions. Indeed, carbohydrates are expressed on the majority of mammalian cell surfaces, and are bound to proteins, glycoproteins, and glycolipids, or conjugated to such cellular constituents as proteoglycans that are entangled in the cell membrane and clustered in multiantennary configurations. In other terms, these oligosaccharides constitute signal transductors

between extra- and intracellular media.[6–8] Hence, these glycosylated structures are responsible for the presentation of target structures for microorganisms, toxins and antibodies, control the half-life of proteins, modulation of protein function, or provision of ligands for specific binding events. In addition, they have recently emerged as antigenic determinants in cell–cell recognition, signaling events, and as ligands for bacterial and viral infections.[9,10] As such, they constitute the first line of contact for the adhesion and tissue colonization by several pathogens expressing carbohydrate-binding proteins (lectins). Conversely, several infectious microorganisms use or escape the immune defense mechanisms by masking important receptors or antigenic determinants by exposing self-carbohydrate structures. As mentioned earlier, the consequences of these ''masking'' events are that the bacterial or viral pathogens are transported to target tissues by self cellular systems.

Despite their critical importance, carbohydrate–protein interactions are paradoxically characterized by rather weak association constants (millimicromolar), with limited specificity and selectivity on a per-saccharide basis.[11,12] Nature usually compensates for this situation by exposing numerous copies of the same carbohydrate ligands on the extracellular domains of the cells. Consequently, these interactions are transformed into very potent attractive forces, dramatically and naturally reinforced, when multiple ligand copies are presented to similarly clustered receptors. This phenomenon, resulting from a synergic and cooperative effect, is known as the ''glycocluster or dendritic effect,''[13] and has been initially observed with asialoglycoprotein receptors found on hepatocytes.[14] In its widespread version, it is usually assumed that this effect has its source in the enhanced affinity of a given multivalent glycoside toward a CRD (carbohydrate-recognition domain) by fully occupying one active site at a time.The phenomenon is now widely accepted of having its basis in stabilization by macroscopic ''crosslinking glycocluster effects.'' It has thus come well established that multivalency may offer numerous benefits in terms of affinity and receptor selectivity versus monovalent interaction, and can induce particular clustering organization on the cell surface, notably to provide a strategy for controlling signal transduction pathways within cells.[15]

Surprisingly, however, mammals utilize only nine different monosaccharides, which are organized in a massive amount of structural diversity. The variations of anomeric configurations and linkage positioning between saccharides are thus responsible for unraveling several distinctive ''glycocodes.''[16] As a consequence, the different architectures and topological expressions that result are at the origin of the required high affinity and selectivity for a particular tailored recognition event. Interestingly, even such complex multiantennary glycans as $Man_9GlcNAc_2$ are only partly involved in these binding processes and often, but not unexpectedly, only the

peripheral groups are bound in the receptors' active sites. Hence, multivalent interactions are now understood to be a ubiquitous strategy that has evolved in Nature for a wide range of functions, and which provide numerous benefits and unique roles not achievable with monovalent interactions. Consequently, from a simplistic point of view, most multiantennary glycans may be regarded as polyvalent neoglycoconjugates of well-defined architectures and multivalency, designed to mimic the complexity of the multiantennary oligosaccharide structures while emphasizing the recognition of the sole surface.

3. Synthesis and Applications of Multivalent Glycoconjugates

As mentioned before, multivalent carbohydrate–protein interactions mediate many important physiological and pathophysiological processes. However, their thorough understanding suffers from the natural complexity of the carbohydrates resulting from incomplete biosynthesis or subtle attachment of other functionalities at specific positions along the oligosaccharide sequences. Numerous key functions of carbohydrates depend on the observed microheterogeneity, added to well-defined cluster organization. In order to study, characterize, understand, and manipulate these critical interactions, striking advances have been made in isolation, purification, structural analyses, and partial or selective degradation processes. Alternatively, chemical or chemoenzymatic synthesis of multivalent carbohydrate ligands is, however, likely to remain the method of choice to afford tailored multivalent architectures developed as effectors or inhibitors of biological mechanisms. Accordingly, it is highly desirable to tackle the inherent problem of high specificity and increased affinity by simultaneously optimizing factors involved in both multivalency and intrinsic fine tuning of a ligand toward individually targeted carbohydrate-binding protein interactions.

To achieve these goals, glycochemists are actively pursuing an approach that can be summarized in Fig. 1. The steps to be undertaken may be expressed as follows. After assessing the bacterial/viral genomes, proteomic analysis, and search for carbohydrate-binding protein homology through bioinformatics, the presence of a carbohydrate-binding protein is then confirmed. For bacterial or viral lectins, the isolated proteins can be labeled with fluorogenic probes. The ligand-binding specificity of the fluorescent lectins is then determined by glycan microarrays such as the one freely accessible from the Consortium for Functional Glycomics (or the like) to identify the best oligosaccharide ''lead.''[17] The most recent microarrays are usually constituted of approximately 400 natural and synthetic glycans (version 3.10).[18–22] Once essential carbohydrate residues (epitopes) responsible for the biological activity of interest are

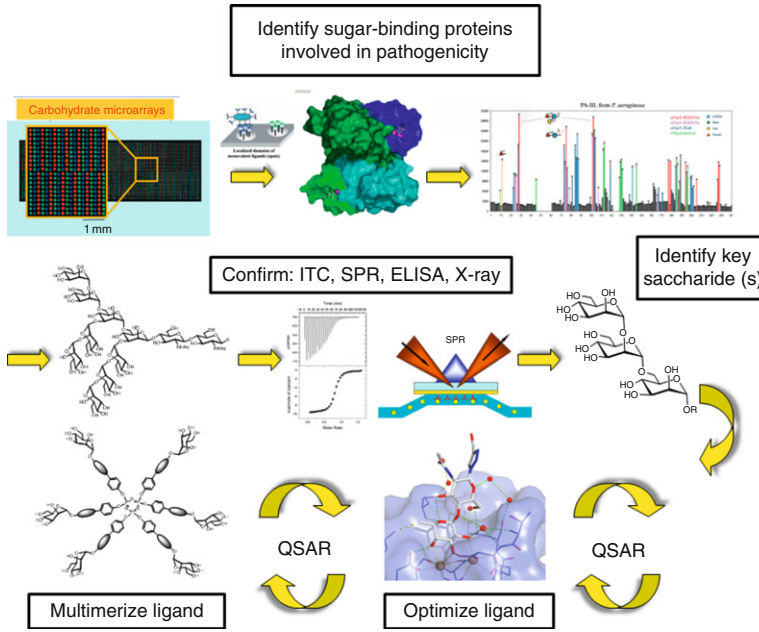


FIG. 1. Steps involved in the discovery of optimized multivalent glycodendrimers.

identified, the lead candidates are then validated by using a range of binding assays such as ELISA (enzyme-linked immunosorbent assay), ELLA (enzyme-linked lectin assay), ITC (isothermal titration calorimetry), SPR (surface plasmon resonance), FRET (fluorescence resonance energy transfer), X-ray crystallography, and analogous techniques. The relative binding affinities of confirmed oligosaccharide ligands are next evaluated with a panel of simpler oligosaccharides with, ideally, a monosaccharide as the simplest target to ease potential manufacturing purposes and lead optimization that can be obtained by classical QSAR (quantitative structure–activity relationship).

The resulting ''glycomimetics'' are further transformed into multivalent architectures such as glycodendrimers that should also undergo iterative scaffold optimization. In this context, ''artificial glycoforms'' have played crucial roles in our understanding of multivalent interactions, which encompass chelation,[23] receptor clustering and steric stabilization,[24] subsite binding,[25] and statistical rebinding phenomena.[26] Hence, research on synthetic multivalent macromolecules has intensified, giving rise to a myriad of original glycoconjugate structures which constitute

high-affinity multivalent ligands that target surface receptors (namely enzymes, lectins, toxins, and such pathogens as viruses or bacteria). These novel nanometer-size structures have thereupon shown great potential as potent effectors or inhibitors of surface–surface interactions, including cell–cell and cell–pathogen interactions that occur in biological systems.[27]

The following sections illustrate synthetic creativity recently devoted to generating a plethora of multivalent structures used in the biomedical field and with therapeutic goals in particular. Synthetic neoglycoconjugates in which carbohydrate residues are attached to carriers thus present numerous advantages in terms of characterization, structural uniformity, and availability. Hence, presentation of the sugar epitopes as multiple copies on an appropriate scaffold (molecular, dendritic, polymeric) creates a multivalent display that can efficiently mimic the natural mode of affinity enhancement that arises from multiple interactions between the binding proteins and the carbohydrate ligands. In general, the carbohydrate ligands are usually found at the periphery of these macromolecules. Efficient conjugation reactions are required for complete substitution and the structural integrities of the multivalent glycoconjugates are evaluated by such conventional techniques as NMR spectroscopy and mass spectrometry. Progress encountered in terms of their synthetic accessibility and efficiency has allowed optimization of their modulation and activity. These factors remain necessary for investigating how these multivalent structures can best influence the targeted binding activity. These investigations are critical for highlighting the potential of multivalent carbohydrate inhibitors as high-affinity ligands or as effectors capable of clustering cell-surface receptors, and may permit generation of structures having tailored biological activities.

In addition to useful but polydisperse multivalent glycomimetics of prominent components at the surfaces of mammalian cells [such as neoglycoproteins,[28,29] neoglycopeptides,[30–32] neoglycolipids (or glycoliposomes),[33,34] glycopolymers,[35–38] or glyconoparticles,[39–49] new synthetic families of well-defined and monodisperse glycosylated macromolecules have recently emerged, including glycoclusters, such glycosylated nanomaterials as glycofullerenes and glyconanotubes, glycosylated architectures from supramolecular assembly processes, and finally glycodendrimers. The uniformity of these multivalent neoglycoconjugates is ensured by the controlled arrangement of the constitutional building blocks. To this end, a large panel of linkage functions have been used, but the ones most frequently used are amides, thioureas, and recently 1,2,3-triazoles obtained via dipolar cycloadditions of alkyne and azide precursors through a process now termed ‘‘click chemistry.’’[50–53] The required functionalities are installed with equal success on either the sugars or on the scaffolds. This particular application has become widely used, since the sugar attachment can be

effected with both protected or free sugars. The protecting groups most repeatedly employed on the sugars have been esters because it is usually more difficult to remove large number of ether or acetal-protected sugars (for example, benzyl ethers). Moreover, the lectin-binding efficiency and specificity of multivalent glycoconjugates have been found to be dependent not only on the epitope density but also on the nature of the core and the geometrical characteristics of the multivalent assembly.

The main goal of this chapter consists in the description of the most recently conceived multivalent neoglycoconjugates, emphasizing the synthetic strategy required to afford well-defined glycoclusters, glycofullerenes, glyconanotubes, glycosylated self-assembled systems, and glycodendrimers, together with discussions concerning their respective relevant uses and perspectives in biomedical applications.

II. GLYCOCLUSTERS

''Glycoclusters'' (or ''cluster glycosides'') can be structurally considered as mimics of oligoantennary oligosaccharides of naturally occurring glycoconjugates. Arbitrarily, this chapter considers these clustered structures as multivalent glycoconjugates, regardless of the number of peripheral saccharides and they are built from ''home-made'' or commercially available scaffolds that do not contain repetitive units. These structural characteristics allow distinction between these generally rather low-valency architectures and the ''true'' multigeneration glycodendrimers that are presented in later sections.

Because of the straightforward synthetic access to such rather small structures, a large variety of glycoclusters, based on multivalent scaffolds and illustrated in Fig. 2, have been described in the past few years. Hence, branched aliphatic or aromatic scaffolds, calixarenes, porphyrins and derivatives, cyclic peptides, carbohydrates and cyclodextrins, or more exotic central cores have been efficiently used as core molecules, to afford small multivalent glycoconjugates having greatly enhanced avidity compared to corresponding monovalent carbohydrates. In particular, the design of such systems, including the valency, the introduction of suitable functions and flexible linkers with optimal lengths, has been tailored. Thus, their optimization has rapidly led to the dramatic enhancement of neoglycoconjugate–protein interactions, and has been useful for determining such critical parameters as optimal association geometries, including optimal distances between saccharide epitopes. It is noteworthy that the majority of these scaffolds have also been used for constructing more complex multigeneration glycodendrimers, in particular orthogonally derivatized AB_x systems, such as TRIS or gallic acid derivatives.

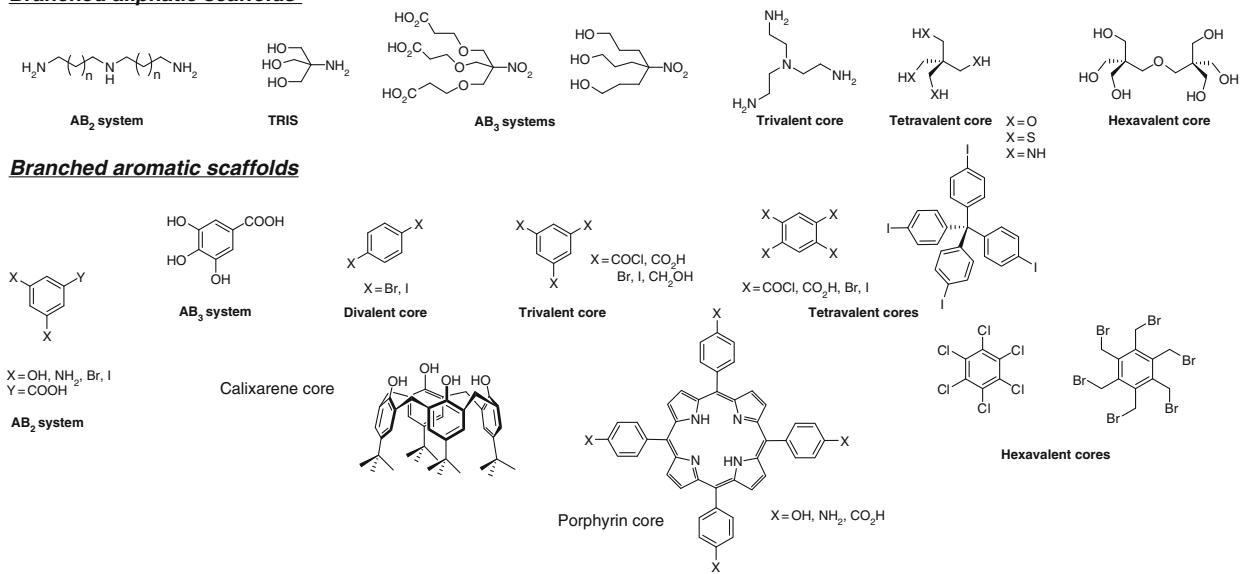


FIG. 2. Common scaffolds used in the design of glycoclusters and glycodendrimers.

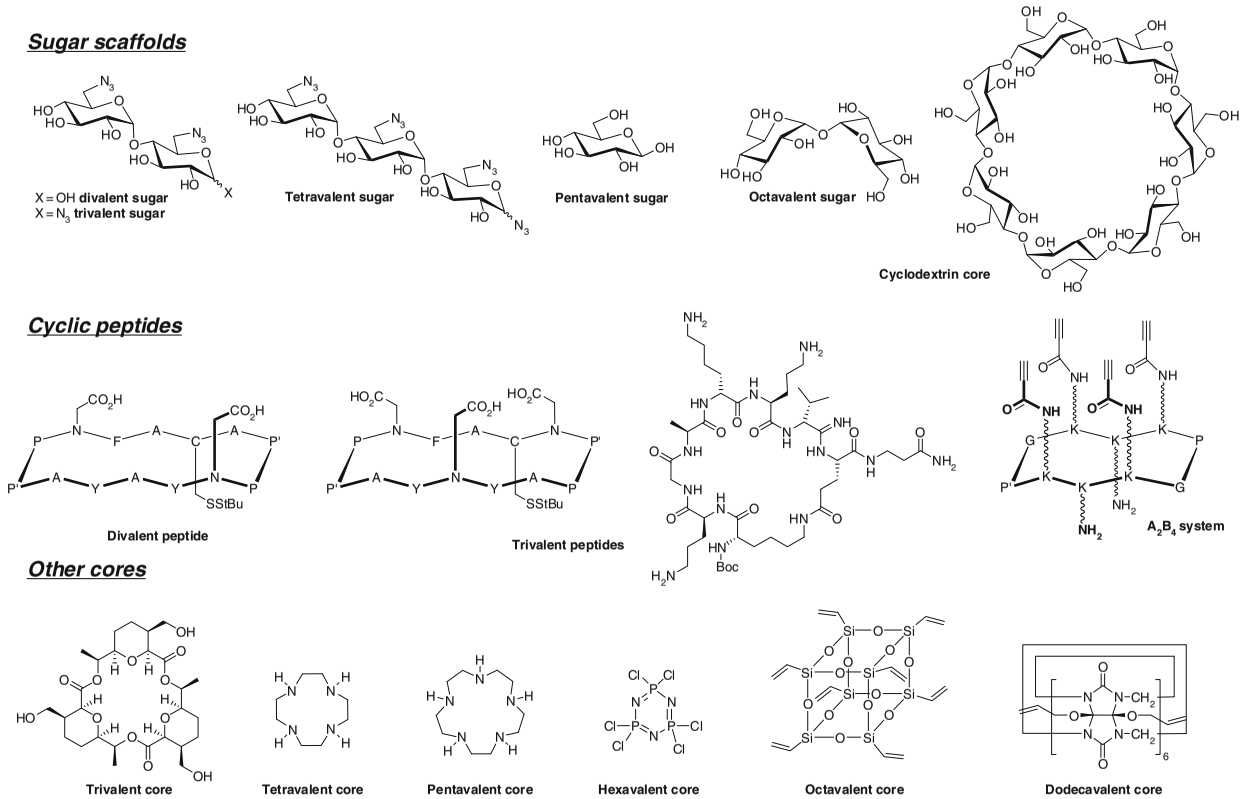


FIG. 2. (*Continued*)

Since a large panel of multivalent scaffolds has recently been reviewed by Roy *et al*.[54–56] and extensively detailed by the group of Santoyo-González for ''click chemistry,''[57,58] this section is dedicated to describing the synthesis of optimized glycoclusters, notably highlighting more recent synthetic advances that have led to multivalent candidates that are strikingly biologically relevant.

1. Glycoclusters from Branched Aliphatic Scaffolds

In order to synthesize the target complex glycoclusters, several commercially available or readily derivatized aliphatic scaffolds constituting small multifunctional building blocks have been used to permit rapid access to these multivalent structures (Fig. 2). Historically, one of the first branched glycoside clusters was based on a readily available AB_3 building block, tris(hydroxymethyl)aminomethane (**1**, TRIS), used by Lee in the late 1970s.[59] Owing to the synthetic advantages in terms of symmetry that ensure orthogonal and rapid functionalization, this building block constitutes an ideal candidate for clustering of saccharides. Hence, **1** and its derivatives have been extensively used by several groups for direct glycoside attachment on hydroxyl groups to generate trivalent clusters.[60] However, although the chemistry of glycocluster synthesis was established, the first such glycoconjugates were generally subject to unfavorable steric factors upon binding to proteins. In this context, Kötter *et al*. used extended TRIS derivatives, such as 4-(3-hydroxypropyl)-4-nitroheptane-1,7-diol (**2**) to prepare highly branched glycomimetics (Fig. 3).[61]

Based on these trivalent building block and peptide-coupling methodologies, the rapid synthesis of tri- (**3**, **4**) and nonavalent mannosylated clusters (**5**, **6**) varying in the chemical characteristics of their spacer moieties and lengths was accomplished.[62] The C-6-linked trimannosylated cluster **4** displayed the highest binding potency toward the type-1 fimbrial lectin from *Escherichia coli* (FimH), as tested by inhibition of agglutination and ELISA, for which **4** showed an IC_{50} of 11 μM. Unfortunately, the corresponding nonavalent dendrons **5** and **6** presented only very poor or no inhibitory potencies. To explain these *a priori* unexpected results, it has been postulated that the cluster did not present enough hydroxyl groups for efficient interactions with the lectin surface, or did not present hydroxyl or other functional groups in the appropriate orientation for lectin binding. Alternatively, it is well known that the FimH binds mannosides from the nonreducing end.

By virtue of promising preliminary biological results indicating that tri- and tetravalent glycoclusters constitute potent inhibitors of bacterial binding, which fitted particularly well into the CRD of bacterial lectins (especially for mannose-specific adhesion) straightforward syntheses of low-valency glycoclusters were initiated.

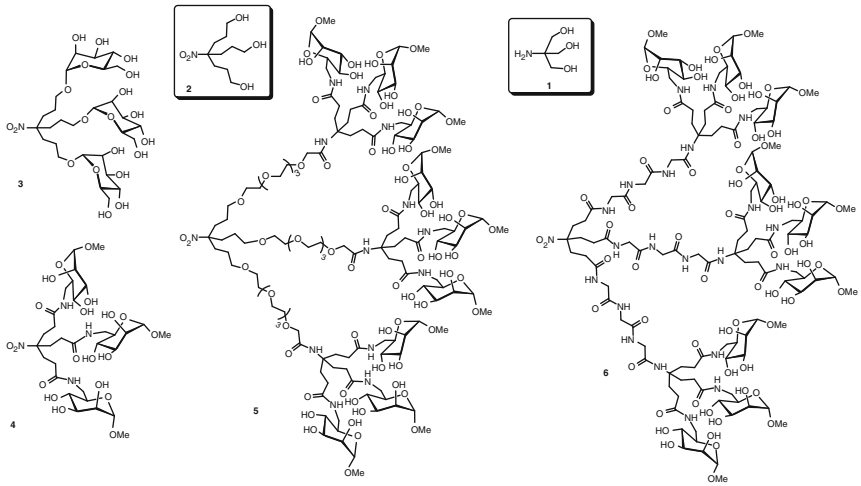
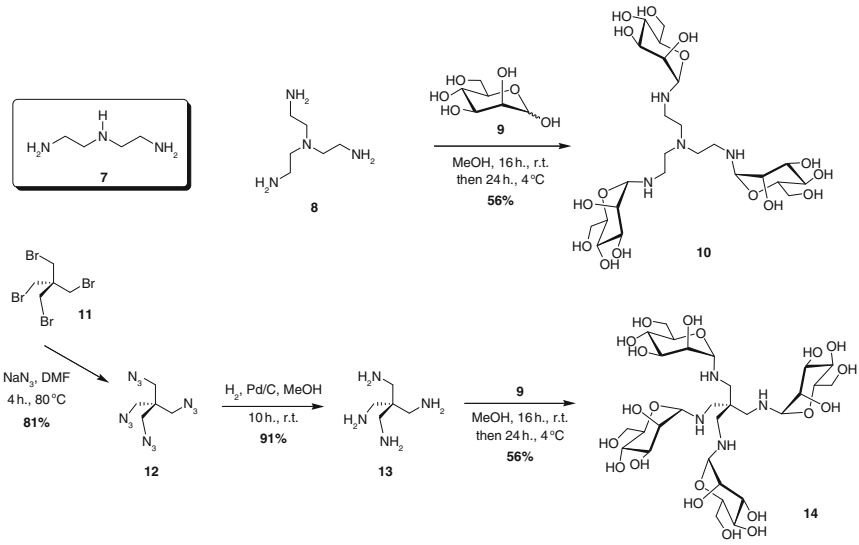


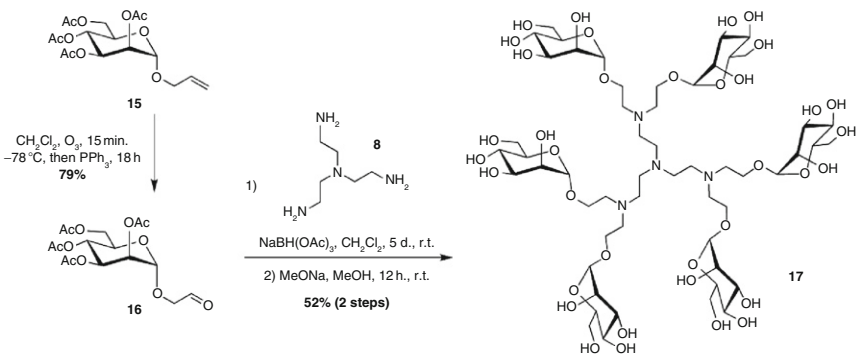
FIG. 3. Early mannoside clusters used as ligands for *E. coli* FimH.[61,62]

In this context, preparation of glycosylamines by direct condensation of amines with reducing sugars could be an appealing strategy. The direct conjugation of reducing sugars with diethylenetriamine (**7**) has been previously reported as allowing efficient and rapid access to divalent carbohydrate derivatives in excellent yields.[63] A few years later, Hayes *et al*. extended this strategy, describing an elegant one-pot methodology that allowed synthesis of higher valent derivatives through reaction of more-highly functionalized amine clusters with unprotected D-mannose (**9**) and dimannosides (Scheme 1).[64] Several linkers of different lengths, flexibility, and valency, including di-, tri-, and tetra-valent amines, were readily incorporated to generate multivalent targets in good yields. Notably, tris(2-aminoethyl)amine (**8**) and pentaerythrityl tetraamine (**13**), [prepared from tetrabromide (**11**) via azide substitution to **12** followed by reduction to **13** with hydrogen on 10% Pd/C], gave the corresponding tri- and tetra-mannoside clusters **10** and **14**, respectively, using the free reducing sugar D-mannose (**9**).

C_3-Symmetrical glycoclusters, based on a trivalent central core, and exhibiting three and six peripheral mannosides have similarly been prepared by Dubber and Lindhorst, who examined the potential of reductive amination for introducing an aldehyde group of a D-mannose derivative as the carbohydrate ligand on tris(2-aminoethyl)amine (**8**) as the branching core.[65] To this end, the glycoclusters were synthesized from (2-mannosyloxy)ethanal (**16**), which was obtained by ozonolysis of



SCHEME 1. Reductive amination leading to tri- and tetravalent mannosylated clusters.[63,64]



SCHEME 2. Double N-alkylation of a sugar aldehyde by reductive amination.[65]

acetylated allyl α-D-mannopyranoside (**15**), followed by treatment with sodium triacetoxyborohydride $NaBH(OAc)_3$ (Scheme 2). However, the instability of the trivalent conjugate arising from autocatalytic deacetylation led the authors to use an excess of aldehyde to ensure complete double N-alkylation. In more-complex carbohydrate-based multivalent architectures, this situation may afford undesired partial structures.

Another example has been described by Li *et al*., which proposed an efficient convergent one-pot synthesis of a trivalent mannoside cluster using a Ugi four-component reaction, involving the use of 2-carboxyethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside, benzaldehyde, methyl isocyanoacetate and the tris(2-aminoethyl) amine core **8** in methanol.[66] Biological investigations by ELLA assays indicated efficient inhibition of binding of yeast mannan to the phytohemagglutinin Concanavalin A (Con A) with all the synthesized ligands, with notably an IC_{50} of 30.6 μM for the trivalent derivative, corresponding to about a 10-fold enhancement after valency correction as compared to a methyl α-D-mannopyranoside standard.

As previously mentioned, pentaerythritol and its derivatives constitute another widely used family of aliphatic cores, allowing the construction of multivalent and branched structures via the attachment of four similar or different groups, two pairs of which are tilted at 90°. Accordingly, this tetravalent compound has received considerable interest as an orthogonally protected handle useful for the generation of combinatorial libraries, and as a building block that fits well into the general structure of oligonucleotides and peptides, providing additional functionalities. Hence, through the years, pentaerythritol-based multiantennary glycoclusters, exhibiting such relevant carbohydrates as β-D-galactopyranosides,[67] Galili antigen,[68,69] lactosides,[70] galabiosides, α-D-mannopyranosides or sialic acids, were efficiently prepared. These three last examples that furnished promising antiadhesins toward pathogenic infections will be described in more detail in the following section.

Historically, one of the first example of a glycocluster based on pentaerythritol was furnished in the late 1990s by Hanessian *et al*., who described the synthesis of di- and tri-haptenic clusters composed of the Tn (GalNAc) and the TF [β-D-Gal-(1→3)-GalNAc] antigens elongated with serine and glycolic acid spacers and attached through amide bonds to pentaerythritol amino derivatives (Fig. 4).[71]

A few years later, Hansen *et al*. proposed the first biological evaluation of galabioside clusters built around thiolated scaffolds with different valencies, including pentaerythrityl derivatives (Scheme 3). Efficient inhibition of hemagglutination (HAI) by the gram-positive bacterium *Streptococcus suis* at nanomolar concentration was achieved with these constructs.[72] Synthesis of the most potent tetravalent soluble inhibitors started with 2-bromoethyl galabioside (**18**), which was treated with sodium azide followed by O-deacetylation under Zemplén conditions and hydrogenolysis to afford 2-aminoethyl galabioside (**19**) in 80% overall yield. Concerning the central core, the commercially available pentaerythritol tetrabromide **11** was treated with methyl 3-mercaptopropanoate and cesium carbonate to give tetraester **20**, which upon hydrolysis led to the corresponding tetraacid **21** in excellent yield. Carboxylic acid activation via formation of a pentafluorophenyl ester, using pentafluorophenol and

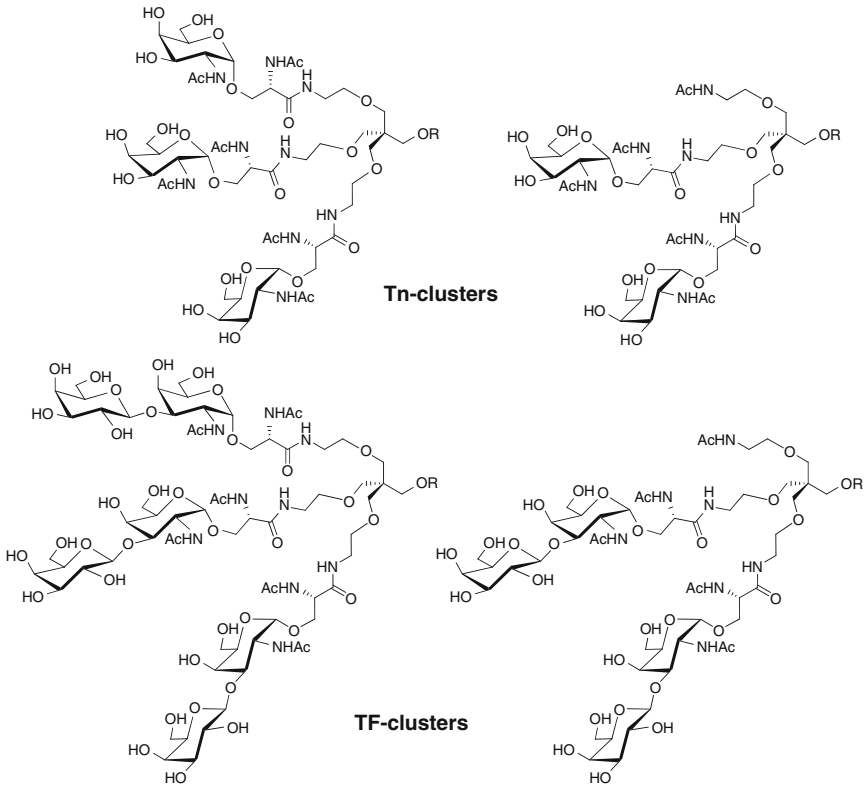
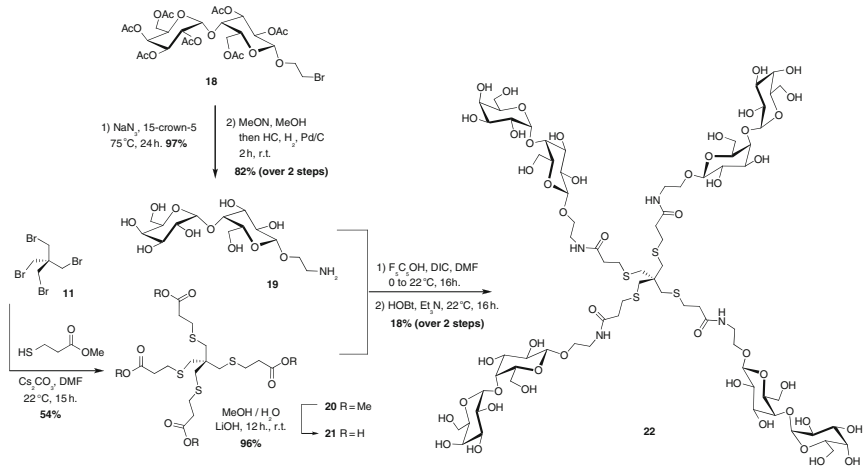


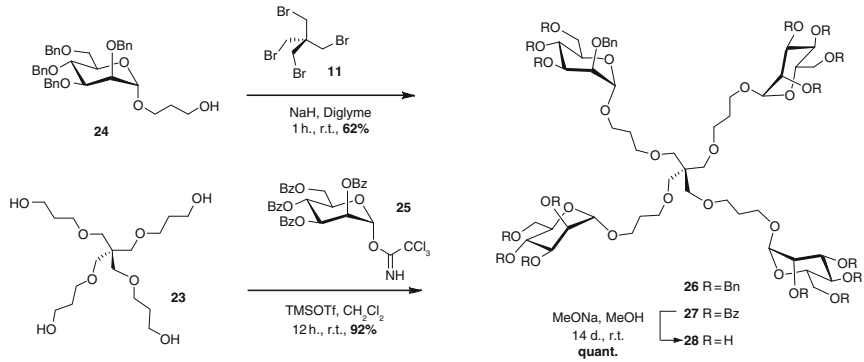
FIG. 4. Structures of trivalent tumor markers based on pentaerythritol derived with the Tn and the TF antigens.[71]

diisopropylcarbodiimide (DIC) and subsequent peptide coupling in the presence of amine **19**, allowed the synthesis in 18% yield of the desired deprotected glycocluster **22** containing four galabioside residues. Biological studies indicated, through the series of multivalent conjugates, a clear connection between inhibitory efficacy, the number of galabiose units present on the potential inhibitor, and the flexibility of the structures. Interestingly, the tetravalent galabioside was several hundred times more efficient than the monomeric galabioside in inhibiting the agglutination of human erythrocytes by the *S. suis* bacterium, resulting in complete inhibition at a concentration as low as 2 nM.

Inhibition of bacterial adhesion of fimbriated *E. coli* to pentaerythritol-based clusters bearing peripheral α-D-mannopyranoside residues has also been often addressed, albeit without systematic structure–activity relationships.[73,74]



SCHEME 3. Tetrameric galabioside having an IC_{50} of 2 nM in the inhibition of hemagglutination of human erythrocytes by *S. suis*.[72]



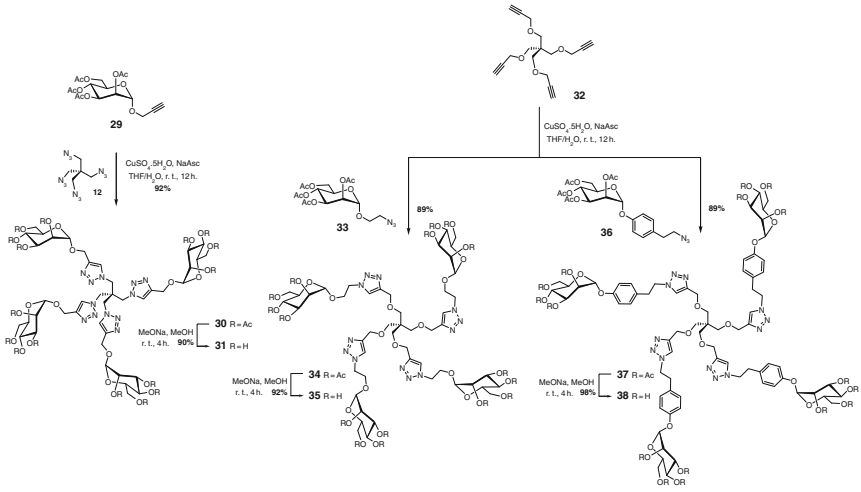
SCHEME 4. Synthesis of tetramannosylated pentaerythritol having an extended linker.[75]

In this context, Lindhorst *et al*. proposed biological investigations of pentaerythritol-based mannoside clusters to test their capacity to block the binding of *E. coli* to yeast mannan *in vitro*.[75] In all of the proposed structures, and according to a rational approach, pentaerythritol itself, as well as the included C_3 spacers, were used as structural components for substituting the inner regions of the monosaccharide moieties (Scheme 4). The tetravalent cluster **27** has been efficiently synthesized according

to two different synthetic pathways. In the first route, a C_3 hydroxy linker was introduced within the aglycone moiety of **24**, and a Williamson ether synthesis with pentaerythritol tetrabromide (**11**) led to a mixture of mono-, di-, tri-, and tetra-dentate (**26**) conjugates, even when an eightfold excess of the corresponding alcohol and forcing reaction conditions were used. In the best case, the protected tetravalent cluster **26** was isolated in 62% yield. To circumvent these difficulties, an alternative strategy was investigated, involving the use of a modified pentaerythritol derivative to serve as a longer spacer-equipped tetraol (**23**) for the subsequent glycosylation step, using imidate **25**. To this end, pentaerythritol was initially perallylated and the extended tetraol **23** was obtained by an hydroboration–oxidation sequence on the double bond in the presence of 9-BBN, NaOH, and H_2O_2. Then, the perbenzoylated mannosyl trichloroacetimidate **25** was used as the glycosyl donor for the Lewis acid-assisted mannosylation reaction. Deprotection of the resulting tetramer **27** under Zemplén conditions afforded the desired cluster **28** in excellent yield.

Evaluation of antiadhesive properties of the mannosylated clusters was assessed using ELISA inhibition assays, in comparison to the monovalent reference methyl α-D-mannopyranoside (MeαMan). Results indicated that tetravalent cluster **28** was more than 250 times more potent (thus 62.5 times on a valency-corrected basis) in inhibiting mannose-specific adhesion than MeαMan, with an IC_{50} of 12.6 μM. The observed inhibition of bacterial adhesion was most probably explained by the binding of cluster glycosides to single CRDs, which are distributed along type-1 fimbriae, rather than by multivalent binding, which would reflect interaction of the sugar clusters with more than one CRD.

Subsequently, Touaibia *et al*. described the efficient and systematic synthesis of a family of mannoside clusters built on pentaerythritol and dipentaerythritol scaffolds[76] using regioselective Cu(I)-catalyzed azide–alkyne [1,3]-dipolar cycloaddition (CuAAc) (''click chemistry'').[77–79] The synthetic strategy first involved the use of tetrazide derivative **12** with prop-2-ynyl α-D-mannopyranoside (**29**) under click chemistry conditions, thus providing tetramer **31** in good yield after O-deacetylation (Scheme 5).The conditions under which the Cu(I) catalyst was generated *in situ* from copper(II) sulfate and sodium ascorbate as the reducing agent generally provided slightly better yields than that using the Cu(I) species (CuI) directly. The second cluster was obtained by the treatment of tetrakis(2-propynyloxymethyl)methane (**32**), prepared via nucleophilic substitution of the corresponding pertosylated pentaerythritol and propargyl alkoxide, with 2-azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (**33**) under the same conditions already described. An excellent yield of the extended cluster **35** was similarly obtained after acetyl-group deprotection. The tetramannoside analogue **38**, bearing a more rigid aromatic spacer, was likewise



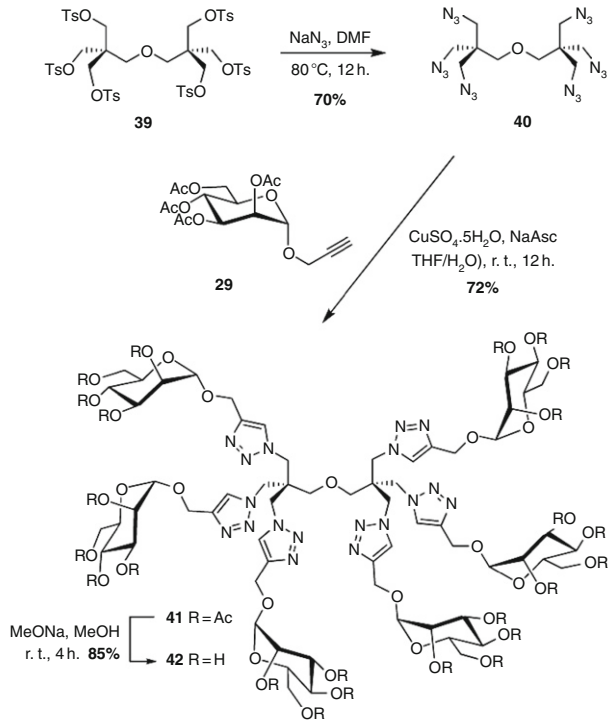
SCHEME 5. Extended tetramannosylated clusters prepared by Touaibia *et al.*[76]

obtained from the tyramine mannoside derivative **36** and the tetrapropargylated core **32**. Interestingly, the presence of the hydrophobic residue in the mannoside aglycone (triazole) was considered to play an important role for an adequate fit into the *E. coli* FimH CRD active site near tyrosine-48 and tyrosine-137.

Further, dendritic growths with new multiarmed clusters having more flexibility and various geometries have also been considered by the authors. Thus, the hexatosylated dipentaerythritol **39** was converted into hexaazide **40**, which upon treatment with propargylated mannoside **29** under standard conditions of click chemistry and subsequent O-deacetylation afforded the hexavalent cluster **42** in good (61%) yield over two steps (Scheme 6).

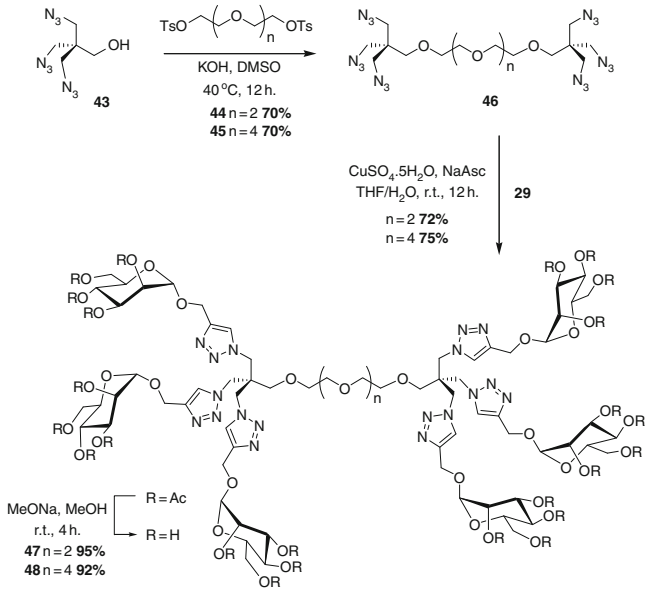
The elongated analogues **47** and **48** were then synthesized by the reaction of triazide **43** and ditosylates **44** and **45** under basic conditions (KOH, Me_2SO) (Scheme 7). The resulting hexakisazido pentaerythritol scaffolds **46** ($n = 2, 4$) were then independently ''clicked'' in the presence of prop-2-ynyl α-D-mannopyranoside (**29**), affording hexavalent clusters in 75% yields. Deprotection under Zemplén conditions furnished the corresponding conjugates **47** and **48** having respectively a distance of 11 and 18 Å between each the tripodal mannoside moieties.

Preliminary biological data on this series of mannosylated clusters indicated interesting potency in the inhibition of agglutination of *E. coli* x7122 by baker's yeast, with approximately a hundred times improved efficiency than those obtained with monomeric D-mannose.



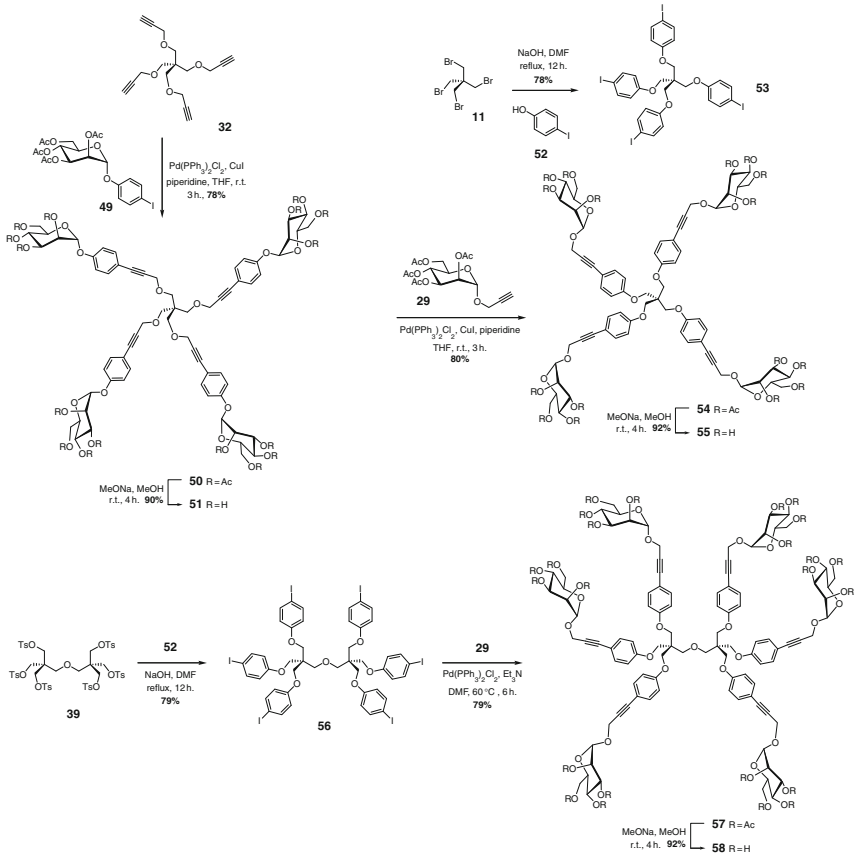
SCHEME 6. Hexakis tetramannosylated clusters for *E. coli* FimH binding.[76]

The pioneering observations of Sharon, who first demonstrated the binding preferences of type-1 fimbriated *E. coli* to mannopyrannosides bearing aromatic aglycones,[80] led to the hypothesis for the existence of ''subsite-assisted aglycone binding.''[81] Based on these premises, the group of Roy *et al*. pursued their investigations to enlarge the panel of mannopyranoside clusters via single-step multiple Sonogashira coupling.[82] The necessary carbohydrate precursors were built with either *p*-iodophenyl, propargyl, or 2-azidoethyl aglycones, whereas the central cores consisted of (di)pentaerythritol-based azide or propargyl derivatives. The first target tetramer (**51**) was synthesized from *p*-iodophenyl α-D-mannopyranoside **49**, previously prepared from peracetylated α,β-D-mannopyranose by glycosidation with triflic acid as a promoter. Then, Sonogashira coupling between tetrakis(2-propynyloxymethyl)methane (**32**) and **49**, followed by subsequent O-acetyl deprotection of **50** provided the key tetravalent cluster **51** in good yield (Scheme 8). Noteworthy is the fact that those transition metal-catalyzed cross-couplings have been optimized during



SCHEME 7. Oligoethyleneglycol interspaced, hexakis tetramannosylated clusters for *E. coli* FimH binding.[76]

this study, recommending the use of 5 mol% $Pd(PPh_3)_2Cl_2$ in the presence of 10 mol% Cu(I) catalyst, piperidine as a base in tetrahydrofuran (THF) or *N*,*N*-dimethylformamide (DMF), with slow addition of the tetrakis alkyne to prevent homocoupling and degradation. Alternatively, tetramer **55**, possessing the reversed linkage functionality, that is, the propargyl group installed on the mannoside residue and the aryl iodide on the pentaerythritol scaffold, was also similarly prepared to investigate the effect of the aryl pharmacophore positioning on binding. In this context, the central tetrakis[(4-iodophenyloxy)methyl]methane **53** was efficiently elaborated by nucleophilic substitution of pentaerythritol tetrabromide **11** using *p*-iodophenol **52** under basic conditions in 78% yield. Treatment of **53** with prop-2-ynyl α-D-mannopyranoside **29** under the optimized Sonogashira coupling conditions just described provided tetramer **54,** which upon further O-deacetylation gave unprotected tetramer **55** in 73% yield over two steps. According to the same strategy, but using Et_3N in DMF at 60 °C instead of the foregoing Sonogashira cross-coupling conditions, the corresponding hexavalent cluster **58** was efficiently obtained, using dipentaerythritol derivative **56** and mannoside **29** after conventional Zemplén deprotection of **57**.



SCHEME 8. Alternative strategy toward the synthesis of hexakis mannopyranosides.[82]

Three different types of biological assays were put in place to evaluate the relative binding properties of these tetra- and hexa-valent mannosylated clusters. Initially, the cross-linking abilities of these molecules were investigated by using a kinetic turbidimetric assay (nephelometry), with the tetrameric phytohemagglutinin Con A from *Canavalia ensiformis* as a model. Significant activity of the glycoclusters was observed when they were used as ligands in interactions with protein receptors, with the rapid formation of cross-linked lattices toward Con A, especially for tetramer **51**. In fact, this last example was the best candidate, and it induced an almost quantitative precipitation of the lectin within 2 min. Obviously, clusters having the

alternative triazole heterocycles or the extended series from the Sonogashira coupling were less efficient. These results were rationalized by the authors on the basis of the relative stability of the resulting insoluble complexes, with molecular modeling of tetramer **51** showing that each mannopyranoside residue was at the apex of a tetrahedron in which they were 18.6 and 16.0 Å apart. This distance could easily accommodate the clustering of four different tetrameric Con A lectins.

Each compound was then evaluated for its relative binding affinity by SPR measurements. The affinity of the lectin domain of isolated FimH of *E. coli* K12 toward clusters was obtained by competitive experiments between an immobilized anti-FimH antibody (1C10) and free mannosylated clusters. According to this study, tetramer **51** was designated as the best ligand known, with a K_D of 0.45 nM (1.8 nM/Man) in the subnanomolar range, corresponding to 1222-fold and 3-fold enhancement over the reference monomer methyl α-D-mannopyranoside and the strongest monosaccharide ligand known (HeptαMan), respectively. Once again, the position of the phenyl ring appeared to be rather important with regard to modulating the activity of tetravalent conjugate **55** (K_D = 273 nM), which differed from **51** only by the inverted alkyne–phenyl ring sequence. These results thus further demonstrated that clusters possessing an aryl moiety in the vicinity of the anomeric oxygen atom showed the best overall qualifications. Conjugates obtained by the click chemistry already described have also been studied. Spatial rearrangement of the hexamer having the triazole rings appeared to be a determinant for affinity, as **42** was nearly five times better than the analogous tetramer **31** (K_D of 3 and 14 nM, respectively), thus illustrating the influence of multivalency on this scaffold. The distance between the anomeric oxygen atom and the triazole ring was also a critical factor for affinity. On the other hand, the introduction of four or six mannopyranoside moieties using extended precursors and Sonogashira coupling had only a minor effect on the relative affinity.

Finally, the clusters were tested as inhibitors of hemagglutination of pig and rabbit erythrocytes by type-1 piliated UTI89 clinical isolate *E. coli*. The inhibition titer (IT), that is, the lowest concentration of the inhibitor at which no agglutination occurs, showed tetramer **51** to be the best inhibitor of hemagglutination, with an IT of about 3 μM, or a factor of 6000 as compared to its affinity, and corresponding to 1000-fold better inhibition than that induced by D-mannose. Overall, tetravalent cluster **51** was the best noncovalent cross-linker of Con A and the best ligand known to *E. coli* K12 FimH.

Extended biological investigations concerning structure–function studies were further initiated to evaluate the abilities of these clusters to inhibit Con A-induced membrane type-1-matrix metalloproteinase (MT1-MMP)-mediated pro-MMP-2 activation, cell death, and antiproliferative property in mesenchymal stromal cells (MSC).[83]

Mobilization of MSCs and recruitment by experimental vascularizing tumors involves MT1-MMP functions. Given that the mannose-specific lectin Con A induces MT1-MMP expression and mimics biological lectin–carbohydrate interactions, these clusters were tested to evaluate their potential to block Con A activities on MSC. The results indicated that specific tetra- and hexavalent mannoside clusters, especially **51**, **55**, and **58**, reversed Con A-mediated changes in MSC morphology, and antagonized Con A-induced caspase-3 activity and proMMP-2 activation. They also inhibited Con A, but not the cytoskeleton-disrupting agent Cytochalasin-D-induced MT1-MMP cell-surface proteolytic processing mechanisms, and effects on cell-cycle phase progression. The antiproliferative and proapoptotic impact of Con A on the MT1-MMP–glucose-6-phosphate transporter signaling axis was also reversed by these mannosides. In conclusion, this family of mannosylated clusters very effectively inhibited a spectrum of MT1-MMP-mediated cell responses that could be potentially transposed to target tumor-promoting processes. In addition, their noncytotoxicity allowed their use *in vivo* against experimentally implanted tumors.

Recently, Gouin *et al*. have designed a tetravalent cluster (**60**)[84] based on a potent *E. coli* FimH ligand heptyl α-D-mannoside (**59**)[85] that has been preliminarily recognized as a strong binder to FimH, with a K_D of 5 nM, as determined by SPR measurements (Fig. 5). Furthermore, this derivative inhibited both adhesion of

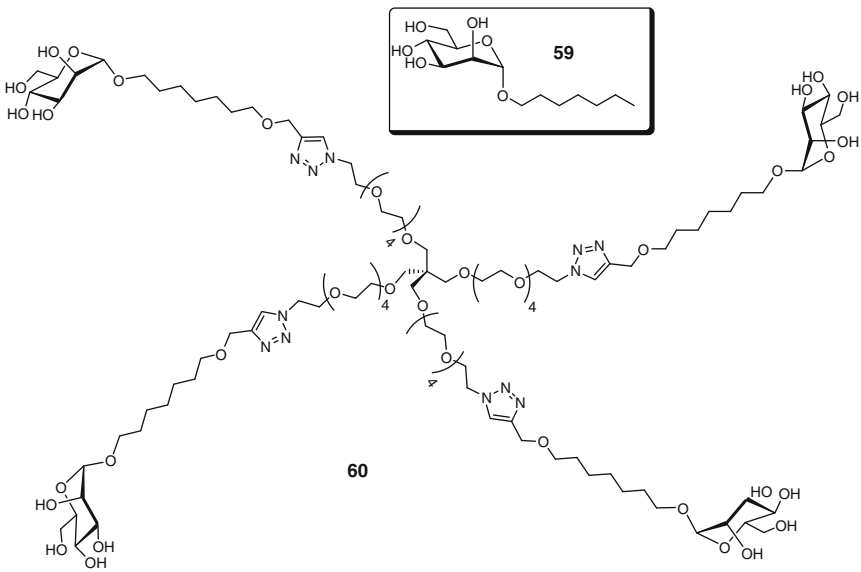


FIG. 5. Best ligands known for the inhibition of *E. coli* binding to murine bladder cells.[84]

type-1-piliated *E. coli* on a bladder cell line and biofilm formation *in vitro*, and also decreases bacterial levels in a murine cystitis model.[86] Suitably functionalized and flexible ethylene glycol linkers were used to tether **59** moieties in order to ensure water solubility and efficient conjugation on a pentaerythritol core via ''click chemistry'' to afford neoglycoconjugate **60**.

Binding affinities of the new flexible synthetic glycocluster toward type-1-piliated *E. coli* were evaluated by HAI and bladder-binding assay (BBA). The results indicated that, with an inhibition of bacterial bladder-cell binding at 12 nM ($\sim$6000- and 64-fold lower than mannose and **59**, respectively), the tetravalent compound **60** is currently one of the most promising antiadhesive drugs under development for the treatment of urinary tract infections.

Pentaerythritol-based glycoclusters have also been investigated as potential neuraminidase inhibitors. In this context, Linhardt's group has described a straightforward approach to generate clustered nonnatural *n*-glycosylamines of α-sialic acid, which are known to be resistant to neuraminidase-catalyzed hydrolysis as opposed to the natural *O*-glycosides.[87] Influenza viruses use their hemagglutinin to bind to sialic acid residues located on the surface of the host cell and gain entry into the cell. Once the cell is infected, the new virions use their neuraminidases (or sialidases) to escape from the infected cells. Thus, these neuraminidases have been targeted to stop viral infection by blocking the virus inside the infected cells. To this end, a small library of mono- and divalent 1,2,3-triazole-linked sialic acids has been constructed via click chemistry, together with corresponding tetravalent cluster, generated from tetrapropargylated core and α-sialic acid azide. Preliminary neuraminidase-inhibition assays, involving fluorescence measurements induced by the release of 4-methylumbelliferone produced by the hydrolysis of the substrate [2′-(4-methylumbelliferyl) *N*-acetyl-α-neuraminic acid] by the enzyme [neuraminidase from *Clostridium perfringens* (*Clostridium welchii*)], have been addressed. The results indicated micromolar IC_{50} values, notably for the tetramer with an IC_{50} of 20 μM, comparable to the known sialidase inhibitor Neu5Ac2en (67 μM).

2. Glycoclusters from Branched Aromatic Scaffolds

Inasmuch as only a few of the carbohydrate residues contained in large oligosaccharides can be involved in several binding-recognition process to trigger or inhibit various biological phenomena,[88] it has been assumed that the roles of the remaining sugars within the polymer were limited to a structural matrix, acting simply as spacers maintaining the epitopes at the proper distances to ensure optimal interaction with the

receptor-binding sites. Based on these preliminary assumptions, structural analogues of elicitors could result from molecules containing an aromatic core supporting pendant sugar epitopes. By substitution of structural sugar components with benzenoid groups, the rigidity of the polymeric matrix would be preserved with only minimal distortion to the three-dimensional framework. The first and closest analogy to such ''aromatic core'' harboring cluster glycosides was provided by Yariv *et al.* who first described phloroglucinol-based ''artificial antigens'' **61**, with the general formula 1,3,5-tris-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene. These early multivalent models had the ability to form brightly colored, cross-linked precipitates with antibodies directed against the appropriate and homologous carbohydrate determinants (Fig. 6).[89]

Through the years, the development of transition metal-catalyzed methodologies, notably involving cyclotrimerization and Sonogashira or Heck cross coupling, has paved the way for rapid and efficient access to aryl glycoclusters with desired and controlled valency.

a. Glycoclusters from Intermolecular Cyclotrimerization.—In 1982, Kaufman and Sidhu described the one-step synthesis of aromatic cluster glycosides via metal-catalyzed cyclotrimerization of appropriate acetylenic sugar precursors.[90] In this context, various 2-propynyl 2,3,4,6-tetra-*O*-acetyl-D-glycopyranosides and their corresponding thioglycopyranosides have been used to explore and validate the

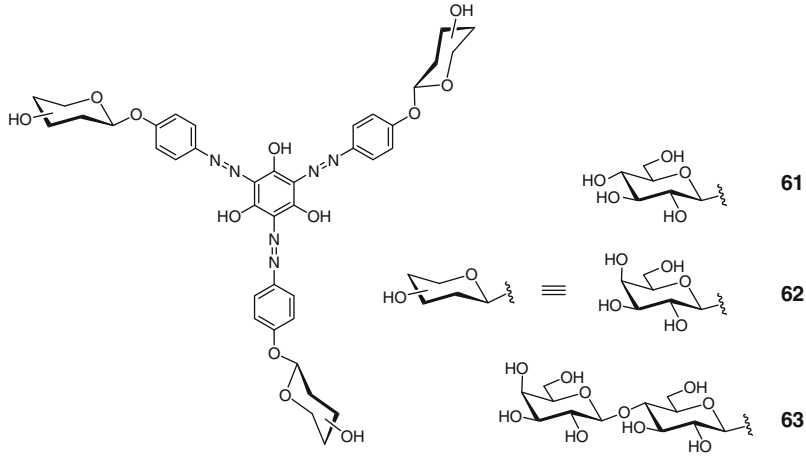
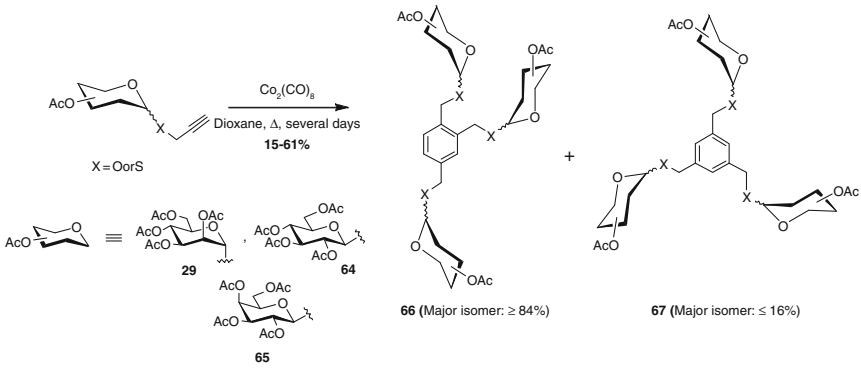


FIG. 6. Early (1962) glycoclusters used in quantititative immunoprecipitation of anticarbohydrate antibodies.[89]



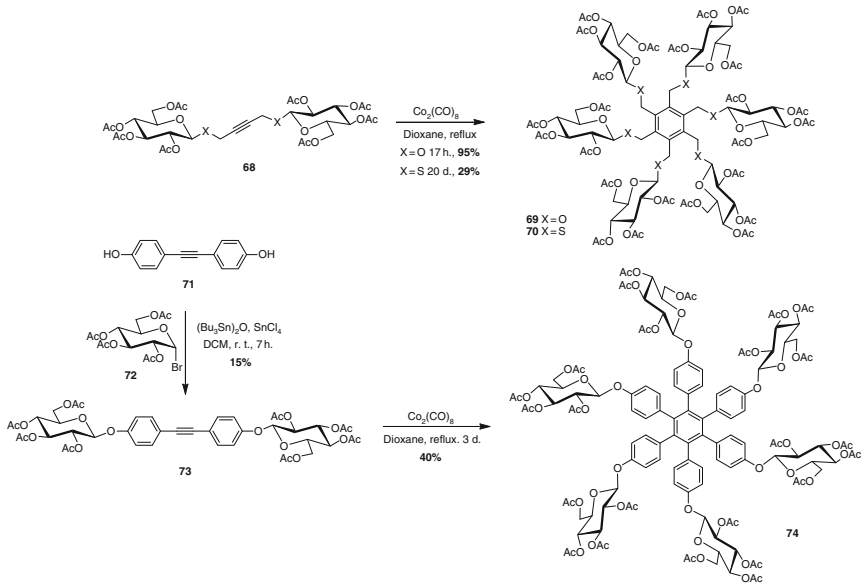
SCHEME 9. Cobalt-catalyzed cyclotrimerization of propargylated glycosides.[90]

feasibility of this type of benzannulation approach in glycochemistry. Treatment of acetylenic precursors (**29**, **64**, **65**) with the conventional cyclotrimerization catalyst, dicobalt octacarbonyl, $Co_2(CO)_8$, gave a mixture of the corresponding regioisomeric trimeric glycosides **66** and **67** (Scheme 9). Although the reaction appears to be quite general, the yields, ranging from 15% to 61%, tend to be markedly influenced by the nature of the starting material, and the thio analogues afforded poor yields, undoubtedly due to poisoning of the cobalt catalyst.

In the same study, the authors also proceeded to the construction of original ''octopus-like'' hexakis glycosides, readily accessible from the corresponding bis (glycoside) monomers (Scheme 10). Thus, cyclotrimerization of 2-butyn-1,4-diyl bis-(*β*-D-glucopyranoside) octaacetate (**68**) gave benzenehexaylhexamethylene hexakis-(*β*-D-glucopyranoside) tetraeicoacetate **69** in an excellent yield of 95%. The corresponding thio derivative (**70**) was also prepared according to similar conditions, but in lower yield (29%).

Hexavalent glycocluster **74**, organized around a hexaphenylbenzene core, has also been obtained via a synthetic strategy involving diphenol **71**, which was coupled with tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**72**) using stannate methodology to furnish divalent glycoconjugate **73** in moderate yield. Its cyclotrimerization under the standard cobalt-catalyzed conditions gave the desired glucosylated cluster **74** in 40% yield.

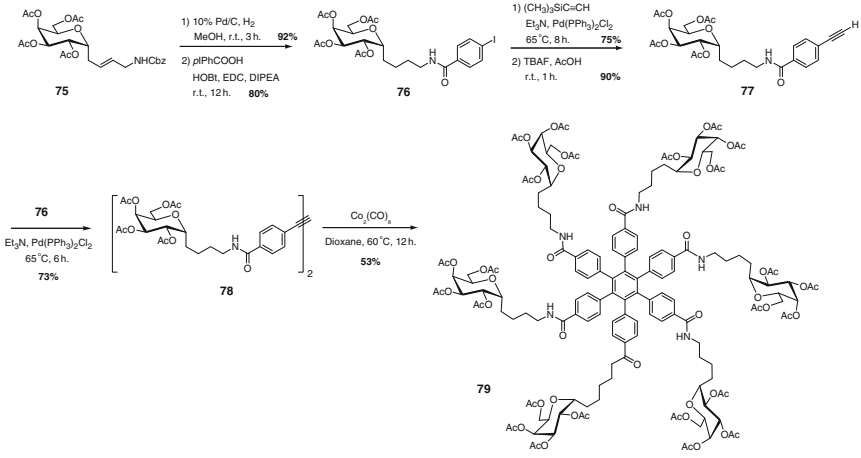
However, these elegant glycosylated nanostructures displayed only weak biological activity relative to their native glucan elicitor counterparts. Nevertheless, such glycoclusters provided unique environments for host–guest chemistry of amphipathic molecules owing to their lipophilic core surrounded by a hydrophilic periphery.



SCHEME 10. Synthesis of ''glycoasterisk'' using cobalt-catalyzed benzannulation.[90]

Subsequently, Roy *et al*. improved the methodology to provide hexavalent mannopyranosides via [2+2+2]-cycloadditions in the presence of the dicobalt octacarbonyl catalyst by refluxing in 1,4-dioxane for only 2 h (as compared to 21 days for the mannoside cluster obtained in the previous example).[91] Regioisomeric mixtures of 1,2,4- and 1,3,5-isomeric clusters were obtained in 63% yield and in 10:1 molar ratio. Interestingly, the reaction was general and could be equally applied to several other saccharides. Prompted by the success of the dicobalt octacarbonyl-catalyzed cyclotrimerization, the same group attempted similar reactions with symmetrical and mannosylated disubstituted alkynes. Under identical conditions as just described, the corresponding hexamer was obtained in 84% yield. The resulting deprotected cluster, obtained quantitatively under Zemplén O-deacetylation conditions, was water soluble and showed excellent cross-linking abilities with tetrameric plant lectins, indicating that the spatial orientation and rigidity provided by the extended inner aryl core offers great potential as neoglycoconjugates.[92]

A few years later, the same group extended this strategy in order to access metabolically stable *C*-glycosyl clusters containing long-arm spacers via a sequence of transition metal-catalyzed transformations (Scheme 11).[93] In this context, cross-metathesis reactions of various *C*-glycosyl compounds with alkenes having available



SCHEME 11. ''Molecular-asterisk'' bearing the hydrolytically stable *C*-galactosyl group as synthesized by Roy *et al.*[93]

amine groups were first studied. In particular, a peracetylated *C*-allyl α-D-galactopyranoside analogue, obtained in 81% yield (95:5, α/β) from the reaction of peracetylated galactopyranose in the presence of allyltrimethylsilane and $BF_3.Et_2O$ in acetonitrile,[94] was coupled to *N*-(benzyloxycarbonyl)allylamine using 20 mol% of Grubbs' catalyst (bis(tricyclohexylphosphine)benzylideneruthenium(IV) dichloride $[(Pcy_3)_2Cl_2Ru{=}CHPh)]$ to afford **75** in 45% yield as a single *trans* isomer. The synthesis of the desired hexavalent ''molecular asterisk'' was then initiated by transformation of **75** through hydrogenolysis into its amine derivative in 92% yield. The resulting amine was coupled with *p*-iodobenzoic acid under peptide-coupling conditions to afford aryl iodide **76** in 80% yield. The subsequent palladium-catalyzed Sonogashira reaction between **76** and (trimethylsilyl)acetylene efficiently gave an intermediate which, upon treatment with tetra-*n*-butylammonium fluoride (TBAF), gave the corresponding terminal alkyne **77** in 68% overall yield. With compound **77** in hand, subsequent Sonogashira cross-reaction was carried out with *p*-iodophenyl analogue **76** to afford the key acetylenic dimer intermediate **78** in 73% yield. Finally, the desired cyclotrimerized cluster **79** was isolated in 53% yield after treatment of **78** with dicobalt octacarbonyl in 1,4-dioxane at 60 °C for 12 h. Unfortunately, no biological data involving this deprotected extended cluster was available.

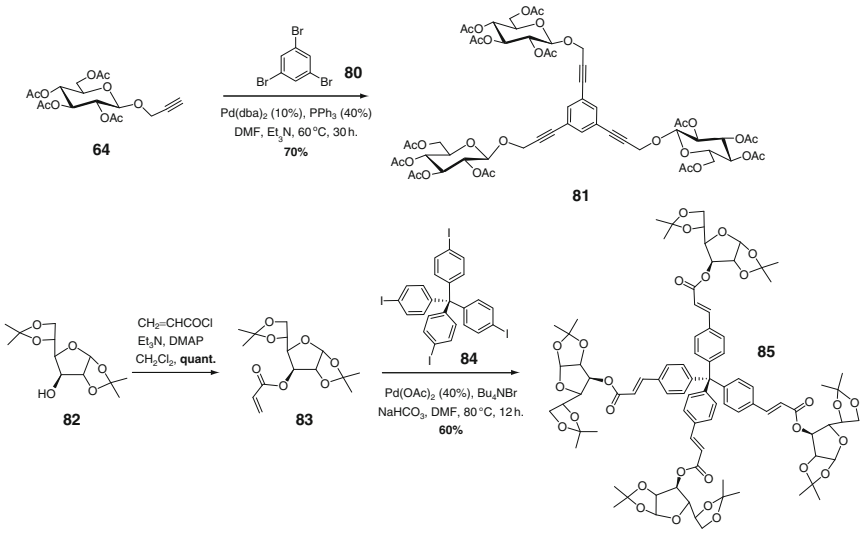
Following these investigations, Das and Roy first reported a Grubbs'-catalyzed version of these intermolecular cyclotrimerizations, using 2-propynyl derivatives and a ruthenium carbenoid catalyst, which thus afforded mixtures of regioisomeric aryl

mannopyranosides, in ratios similar to those observed with the dicobalt octacarbonyl-mediated benzannulation but with decreased reaction time and much improved yields.[95] Therefore, 2-propynyl glycosides, including the acetylated α-D-mannopyranoside, β-D-galactopyranoside, and lactoside were treated with Grubbs' catalyst (15 mol%) in dry dichloromethane at room temperature for 12 h. The desired trisubstituted benzene derivatives were isolated as typical mixtures of 1,2,4- and 1,3,5-regioisomers (90:10) in 66–75% yields. By contrast, the access to corresponding hexavalent analogue via cyclotrimerization of disubstituted symmetrical alkynes was not successful, perhaps because of steric hindrance.

b. Glycoclusters from Cross-Coupling Reactions.—Earlier work in the mid 1990s by several research groups highlighted the formation of cross-linked lattices induced when multivalent protein receptors were admixed with synthetic carbohydrate multimers, including dimers.[96,97] All of the reported evidence supported the notion that small-rigidified carbohydrate clusters bearing hydrophobic residues would form stable complexes. The result of the synthetic efforts as presented here was inevitably the formation of glycoside clusters wherein the sugar moieties were linked to the side arms emerging from the central platform through *O*-, *S*-, or *C*-glycosidic linkages.

Based on these observations, Roy *et al*. proposed efficient synthesis of oligomeric carbohydrate clusters, named ''sugar-rods,'' by using Sonogashira–Heck–Cassar (SHC) cross coupling reactions.[91,98] In this context, dimeric conjugates of constrained conformational flexibility, incorporating central hydrophobic aryl residues, were obtained under standard Sonogashira cross-coupling conditions. Hence, heating a mixture of protected propynyl glycosides with a *p*-diiodobenzene core in the presence of catalytic tetrakis(triphenylphosphine)palladium(0) in a 1:1 mixture of DMF–Et_3N quantitatively afforded the corresponding glycosylated bisethynylene derivative. It is worth noting that the reaction was effected in the absence of Cu(I) as cocatalyst, thus preventing the undesired oxidative homodimerization of the propynyl starting material (Glaser reaction). This general strategy, compatible with various glycosides and the usual acetate protecting groups, has also been applied toward the efficient synthesis of divalent ''rod-like'' thioglycosides, which represent potential enzyme inhibitors because of their resistance to enzymatic hydrolysis.[99]

Sengupta and Sadhukhan adapted this one-step Pd-catalyzed methodology to tri- and tetra-valent aromatic cores in order to generate multiantennary glycoclusters (Scheme 12).[100] A threefold Sonogashira cross-coupling reaction of propargyl β-D-glucoside **64** with 1,3,5-tribromobenzene (**80**) in the presence of $Pd(dba)_2$, PPh_3, in a 1:1 mixture of Et_3N and DMF at 60 °C smoothly gave rise to the centrally planar triantennary glycocluster **81** in 70% isolated yield. The authors also presented an



SCHEME 12. Pd(0)-catalyzed Sonogashira and Heck cross-coupling reactions leading to glycoclusters.[100]

example wherein multivalent glycoclusters could be rapidly assembled via a multiple Heck reaction strategy. For this purpose, 2-*O*-acryloyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**83**), derived from alcohol **82**, was used with the centrally tetrahedral core tetra(*p*-iodopenyl)methane (**84**) under phase-transfer-catalyzed conditions ($Pd(OAc)_2$, Bu_4NBr, $NaHCO_3$, DMF) at 80 °C. The resulting tetrahedral glycocluster **85** was obtained in 60% yield.

A few years later, Dondoni *et al*. extended the Pd-catalyzed cross-coupling strategy to *C*-glycosyl compounds, resistant to enzymatic degradation.[101] Dense *C*-glycosylated clusters built around benzene as a rigid platform were thereby synthesized via multiple SHC cross-coupling reactions, using various ethynyl *C*-glycosyl derivatives and polyiodinated benzenes. The limit of this type of conjugation was tested by the use of such crowded systems as those represented by vicinal polyiodobenzenes and short-arm sugar acetylenes. The study elegantly and efficiently afforded di- and tri-valent conjugates from *p*-diiodobenzene and *sym*-triiodobenzene, respectively, involving the use of diversely protected ethynyl glycosides (acetylated or benzylated). In addition, the deprotection via hydrogenolysis of the clusters generated from the conjugation of benzylated saccharides afforded the corresponding ethylene-bridged systems, since the triple bonds were also reduced through this operation. As the use of benzyl-protected ethynyl *C*-glycosyl derivatives turned out to be inefficient as reaction partner in the SHC with the tetraiodobenzene, the desired tetravalent neoglycoconjugate was

obtained in only moderate yield (49%). Further cross-coupling attempts under optimized conditions and involving hexaiodobenzene failed, affording only the tetraadduct in 35% yield. Interestingly, O-deacetylation and O-debenzylation, together with the reduction of triple bonds, yielded highly water-soluble structures.

Biological investigations of these rigid clusters were addressed by André *et al*., who described the preparation of lactoside-bearing glycotope bioisosteres having strong binding affinities to lectins (Fig. 7). According to the optimized Sonogashira conditions already described, and using the required di- and tri-iodinated benzene cores and per-*O*-acetylated 2-propynyl lactoside, they obtained divalent (**86**) and trivalent (**87**) glycosides in yields of 90% and 80%, respectively.[102] Complete O-deacetylation resulted in freely water-soluble clusters in almost quantitative yields.

The relative affinities of these lactoclusters were evaluated in a competitive solid-phase binding assay, using different labeled sugar receptor as probes, notably the *β*-trefoil mistltoe lectin (*Viscum album* agglutinin, VAA) and three mammalian galectins having different modes of presentation of their respective CRDs. Preliminary results indicated that glycoclusters could well surpass the inhibitory capacity of lactose. Of note is the fact that binding of the two homodimeric proto-type galectins-1 and -7 was not effectively influenced by the presence of multivalent compounds.

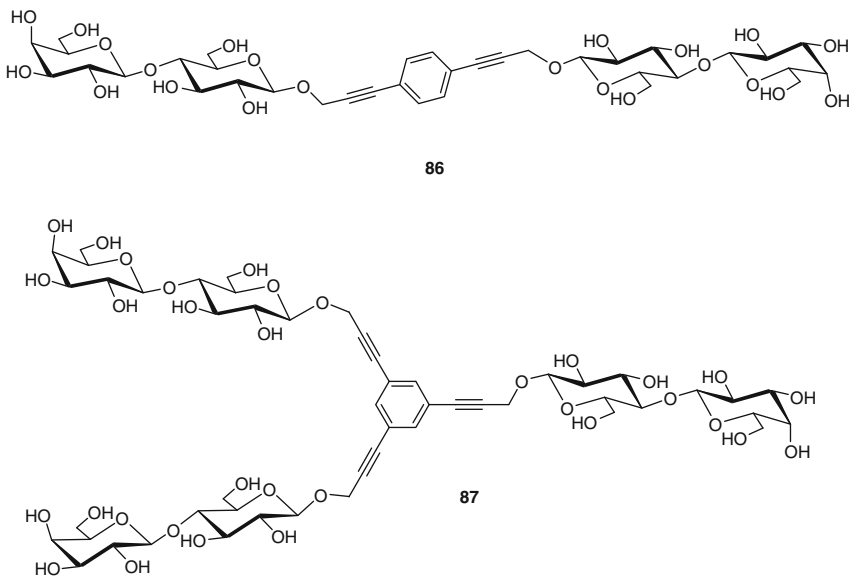
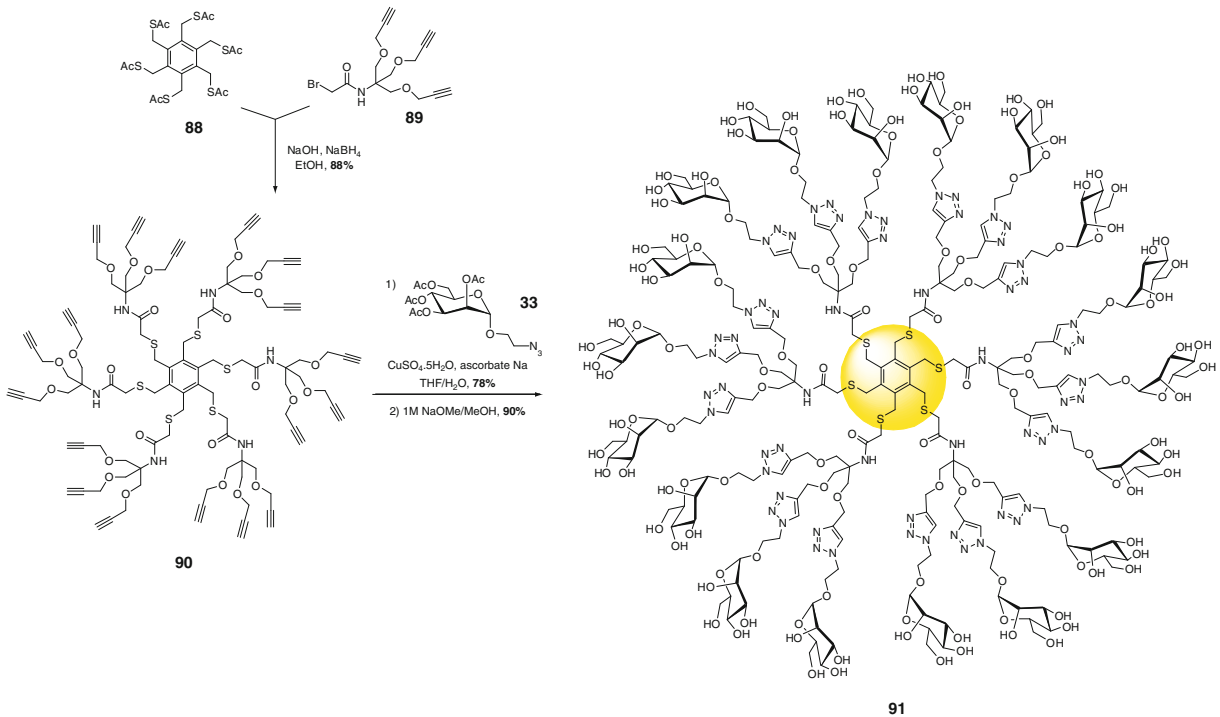


FIG. 7. Di- and tri-valent rigidified lactoside clusters having strong affinity to *V. album* lectin.[102]

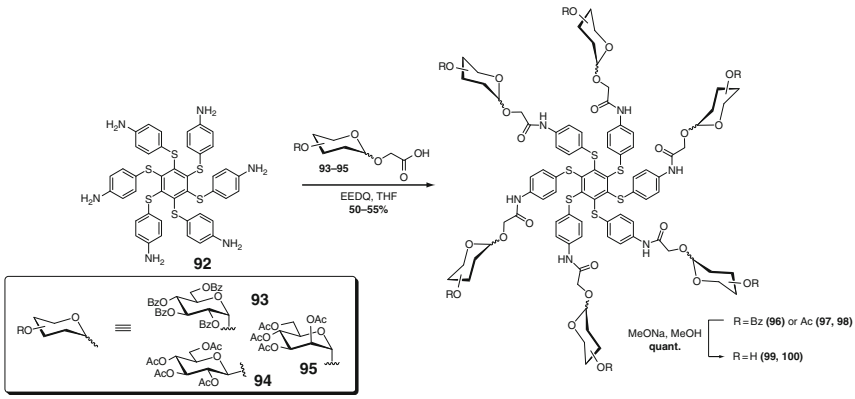
In fact, only a few of them manifested inhibitory capacity in the range of free lactose, demonstrating cases of negative correlation between carrier-dependent presentation of lactose and inhibitory efficiency. However, the trivalent cluster **87** induced the strongest cluster effect with the chimeric-type galectin-3, with an IC_{50} of 30.8 μM (700 μM for free lactose) in contrast to the corresponding divalent lactoside **86**. Its efficiency was independently confirmed by hemagglutination and by *in vitro* cytofluorometric tumor-cell binding analysis. The trivalent compound was a potent inhibitor of galectin-3, blocking its binding to native cell surfaces. Finally, these data underlined the feasibility of galectin-type target selectivity by compound design, despite the use of an identical headgroup for the synthesis.

c. Persulfurated Glycoclusters.—Recently, two research groups have independently described the synthesis of persulfurated glycoclusters, organized around an aromatic core, that showed interesting lectin-binding activities. in an ongoing research program to study mannose-binding proteins (MBPs) by the systematic syntheses and biological evaluation of multivalent glycomimetic inhibitors against bacterial adhesion, Chabre *et al*. have described the synthesis via ''click chemistry'' of dense sulfurated glycoclusters containing up to 18 peripheral α-D-mannopyranoside residues (Scheme 13).[103] The synthetic strategy employed hexavalent sulfurated scaffolds **88**, initially obtained by thioacetylation of a commercial hexabromomethylated precursor with potassium thioacetate, which was further functionalized with TRIS derivatives 2-bromoacetamido-tris[propargyloxy)methyl]aminomethane (**89**). This trivalent moiety was synthesized in an efficient three-step sequence using Boc N-protection of TRIS, followed by propargylation under basic conditions. Quantitative removal of the Boc protecting group under acidic conditions and reaction with bromoacetyl chloride led to precursor **89**, which was then treated with hexathioacetate **88** under basic and reductive conditions to afford the octadecapropargylated dendritic scaffold **90** in excellent yield. Its treatment with 2-azidoethyl tetra-*O*-acetyl-α-D-mannopyranoside (**33**) using ''click chemistry,'' and subsequent deprotection under standard Zemplén conditions provided the densely packed cluster **91** containing 18 peripheral mannoside residues. Initial evaluation against the BclA lectin from *Burkholderia cenocepacia* by ITC revealed promising candidate ligands having strong affinities (Chabre *et al*., unpublished results).

The second example has been furnished by Sleiman *et al*., who proposed a new class of persulfurated, semirigid, radial, and low-valent glycosylated ''molecular-asterisks'' possessing dual function as ligands and as probes by virtue of the conjugated electronic system.[104] Moreover, these glyco-asterisks could exhibit conformational preferences for alternating up-and-down patterns around the central core by exposing the phenylthio groups above and below the plane of the benzene ring, which could be



SCHEME 13. Persulfurated glycoclusters bearing 18 α-D-mannopyranoside residues.[103]



SCHEME 14. Persulfurated ''molecular asterisks.''[104]

potentially useful for optimized ligand orientation (Scheme 14). These glycoclusters were obtained from the hexaamino persulfurated benzene precursor **92**, readily accessible on a multigram scale by coupling sodium 4-acetamidothiophenolate with commercial hexachlorobenzene in 1,3-dimethyl-2-imidazolidinone (DMI) and subsequent deprotection in concentrated hydrochloric acid. Various protected carbohydrates containing terminal carboxylic acid function (**93**–**95**) were then introduced onto the multivalent amino-scaffold, using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as the coupling agent, in yields ranging from 50% to 55%. O-Deacetylation under Zemplén conditions occurred uneventfully to provide the hexavalent clusters **99** and **100**.

In order to assess their biological activity, the authors investigated their ability to inhibit the hemagglutination of rabbit erythrocytes by Con A. Results indicated that the α-glucose asterisk **99** efficiently inhibited hemagglutination at 11 μM, corresponding to a 60-fold enhancement per sugar relative to methyl α-D-glucopyranoside. A more significant impact was recorded for the α-D-mannoside **100**, which showed one of the best inhibitory potencies reported at a minimum concentration of 89 nM, and hence a greater than 3750-fold increase in relative activity per sugar compared to methyl α-D-mannopyranoside. Control experiments proved that nonspecific interactions of the scaffold were not responsible for the observed inhibition effects, which were more probably induced by a powerful cross-linking phenomenon. In addition, the authors observed a 60-fold amplification of selectivity between the mannose and the glucose clusters relative to the monovalent compounds. Dynamic light scattering (DLS) experiments further confirmed the strong aggregating effect of

Con A by the mannoside cluster at concentrations slightly above the nanomolar range, according to a mannose-dependent and partially reversible process. Finally, these results suggested that, rather than an increased avidity for Con A, the unusual potency of these molecular glycoasterisks was due to an efficient and kinetically controlled macromolecular assembly that strongly amplified the effect of these low-valency ligands.

d. Glycoclusters with a Calixarene Core.—Cyclic polyaromatic calix[*n*]arenes, owing to their oligomeric nature and shapes that can be tailored by the size of the macrocycle ring and by the nature of the substituents on the lower rim, are attractive multivalent scaffolds with controllable valency. Hence, they constitute valuable candidates onto which glycosides can be exposed at the periphery. More particularly and analogously to CDs, calix[4]arenes blocked in the cone conformation can, to some extent, mimic a small portion of the multiantennary cell surface, presenting a series of glycosylated residues on the exterior of a lipophilic cavity. Moreover, the cavity and the spacers between the upper rim of the calix[*n*]arenes and the sugar units can be useful for cooperatively binding molecules in a host–guest chemistry process. In this way, the inclusion complexes could be directed toward selected biological targets which specifically recognize the carbohydrate units, with the glycocalix[*n*]arenes acting as novel types of site-directed molecular delivery systems. They thus provide a versatile platform of well-defined shape for the construction of more-sophisticated structures, including neoglycoconjugates that may be named ''calyx sugars.'' Furthermore, because of their low cytotoxicity, calixarene derivatives have found wide applications in the biomedical field as enzyme inhibitors, anticoagulant and antithrombotic agents, antiangiogenic and anticancer, antiviral, antimicrobial, and antifungal products.[105]

Since the first examples of lower and upper rim glycocalixarenes were obtained in 1994 by Marra *et al.*,[106] employing the Mitsunobu reaction or copper(II)-catalyzed glycosylation, the development of efficient synthetic methodologies has allowed the emergence of several examples of *O*-, *N*-, or *C*-glycosyl calix[*n*]arenes, and these have recently been reviewed (**101**–**106**, Fig. 8).[107,108]

Severe drawbacks resulting from the absence of suitable spacer arms and low water solubility recorded for the first calix[*n*]arene-glycoconjugates have been overcome during subsequent years with the use of adapted chemical ligations and suitably derivatized carbohydrates. As example, 2-thio-α-sialosides have been efficiently conjugated to a calix[4]arene scaffold via nucleophilic substitution, providing extended and water-soluble clusters[109] that may exhibit up to 16 dense clustered epitopes.[110] Wittig olefination,[111,112] Pd-catalyzed Sonogashira cross coupling,[113] click chemistry,[114,115] and azide–nitrile cycloaddition[116] have also given rise to

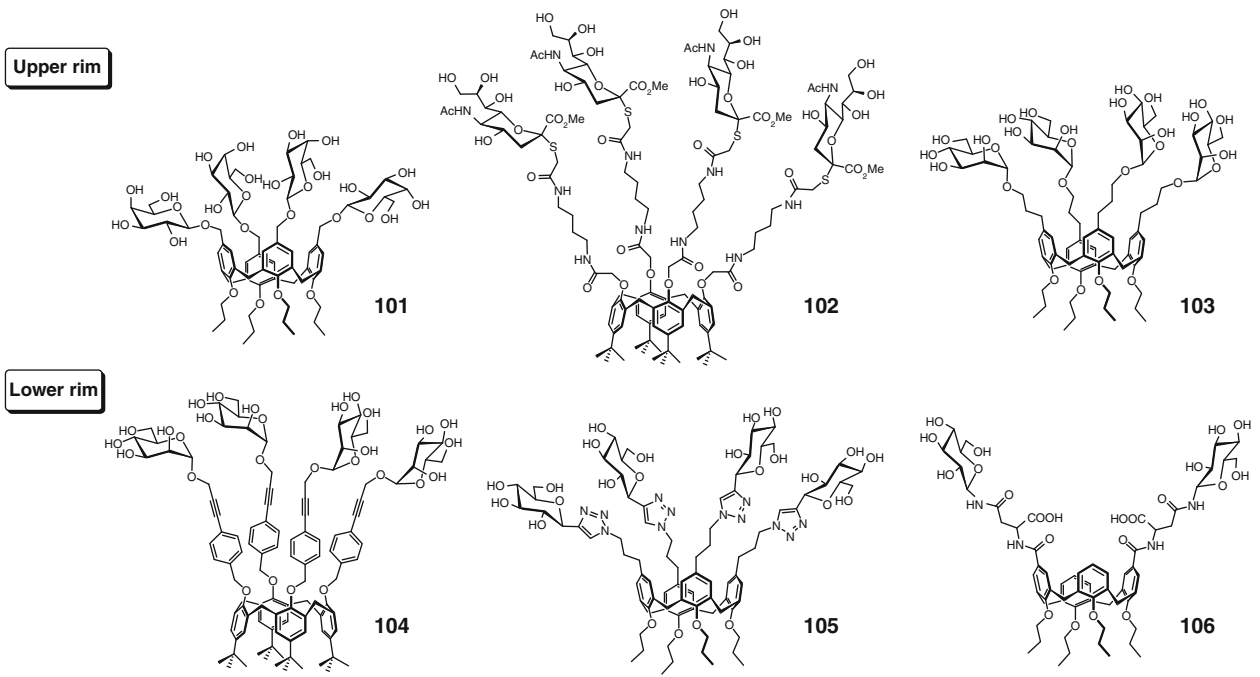


FIG. 8. Typical examples of calyx sugars prepared through various ligation strategies.

diversified calyx sugars. In addition, efficient introduction of biologically relevant carbohydrates on calix[*n*]arenes, through amide[117] or thiourea linkages[118–121] (which can act as hydrogen-bonding groups) have been described and have afforded elongated nanostructures with tailored spacer arms. These have shown promising applications as drug delivery systems via host–guest supramolecular chemistry.

Initial exploratory investigations, addressed by Dondoni *et al*. in the late 1990s,[122] have highlighted the ability of calyx sugar **101** to recognize such neutral and charged molecules as D-glucosamine hydrochloride and tetrabutylammonium dihydrogenphosphate, and showing interesting potential as receptors of phosphate or phosphonate-bearing molecules of biological relevance.

The same group then synthesized thiourea-linked upper rim calix[4]arene glycoconjugates with exposed two or four glucose, galactose, and lactose units, and they further investigated the recognition properties of these glycocalixarenes, together with their interactions with specific lectins.[123] Solution ^{1}H NMR and electrospray ionization-mass spectrometry (ESI-MS) experiments demonstrated their anion-recognition properties, enhanced with the presence of an aromatic ring in the guest, whereas turbidimetric analysis indicated the ability of the glycoclusters bearing four peripheral glucosides and galactosides to interact with Con A and with peanut lectin, respectively. These combined features made these new glycoclusters attractive as possible site-specific molecular delivery systems. Higher valency analogues, such as glucosylthioureidocalix[6]- and calix[8]arenes were further investigated.[124] Supramolecular studies suggested the formation of self-assembled small discoid-like particles in water (3–10 nm in size), in equilibrium with the monomeric macrocycle, which ideally could exhibit a working valency much higher than when compared with that of the monomeric species. Turbidimetric measurements and atomic force microscopy (AFM) assays indicated strong interactions with Con A, especially for the octameric glycoconjugate, causing agglutination with the formation of large supramolecular entities, which progressively evolved toward precipitation because of the extensive lectin cross-linking.

Besides these preliminary studies that have demonstrated the interesting potential of these calyx sugars, recent applications have afforded striking and concrete results. In 2005, elegant work disclosed from combined efforts of the Ungaro and Bernardi groups has described the synthesis of a divalent cholera toxin (CT) glycocalix[4]arene ligand (**109**) having higher affinity than the natural GM1 oligosaccharide **107** (Fig. 9).[125] The bacterial CT is produced by toxicogenic strains of the gram-negative bacillus *Vibrio cholerae*, the causative agent of cholera, the life-threatening acute diarrhea, that mainly affects third-world populations. This heterohexameric AB_5 complex is composed of structurally independent, catalytically active heterodimeric

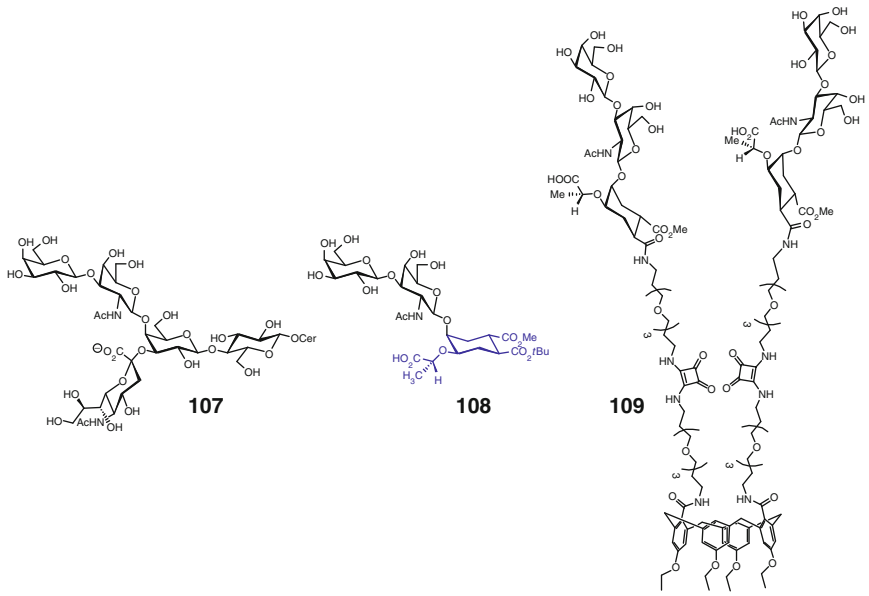


FIG. 9. Divalent GM1 mimic having high affinity against cholera toxin.[125]

A (CT-A, enzymatic) and homopentameric B (CT-B, targeting) subunits. This last moiety effects recognition and anchoring to the cell membrane. With 80% sequence homology in common with the heat-labile enterotoxin secreted by some strains of the *E. coli* bacterium (LT), their mode of action remain similar: both toxins exploit the intrinsic complicated trafficking mechanisms of the host cells to gain access to the cytosol, where they exert their detrimental activity. As a prelude to the infection, once in the lumen of the gastrointestinal tract of the human host, the toxins recognize the receptor ganglioside GM1 [βGal1→3βGalNAc1→4(αNeu5Ac2→3)βGal(1→4)βGlc1→1Cer] (**107** with an IC_{50} of 14 nM for CT)) on the surface of epithelial cells through the B subunits arranged in a pentameric pattern which trigger endocytosis.[126] Acute structural studies and biochemical data concerning the fundamental interaction of GM1 with CT demonstrate the critical role of the two sugars at the nonreducing end of GM1, namely galactose and sialic acid. Taking these specific structural properties into consideration, the rational design of artificial receptors for the toxin as monovalent or multivalent ligands has been envisaged.[127] While preparation of the ganglioside GM1 represents a tedious synthetic challenge, one of the strategies of impeding CT-B/GM1 interactions concerns the design and synthesis of functional and structural

mimics of the natural CT membrane receptor GM1. In this regard, Bernardi *et al*. described the synthesis of pseudosugar GM1 mimics, designed using molecular modeling techniques and presented as high-affinity binders of CT, including the second-generation conjugate **108**, having a K_D of 190 μM.[128]

In this context, divalent presentation of this promising candidate has been investigated by the authors using the fixed *cone* conformation of calix[4]arene to allow introduction of the molecules onto the *upper rim*, and their projection into the same portion of space, thus mimicking, to some extent, a small portion of the natural cell-surface ganglioside. The synthesis of the divalent neoglycoconjugate was based on initial functionalization of the corresponding diacid with an aminated spacer and further introduction of squaric acid moieties.

The interaction of the deprotected cluster **109** with CT was studied by fluorescence spectroscopy, which indicated that, together with ELISA assays, the ligand displayed a higher affinity for CT ($IC_{50} = 48$ nM) than the natural GM1 oligosaccharide under the same conditions ($IC_{50} = 219$ nM). An exceptionally high-affinity enhancement relative to that for the monovalent ligand **108** was thereby obtained, roughly 4000-fold (namely 2000 per sugar mimic). Although complementary detailed thermodynamic analysis is needed to determine the precise role of multivalency in this particular system, the result constituted another striking example highlighting the advantageous utilization of calixarene scaffolds together with the use of a glycomimetic.

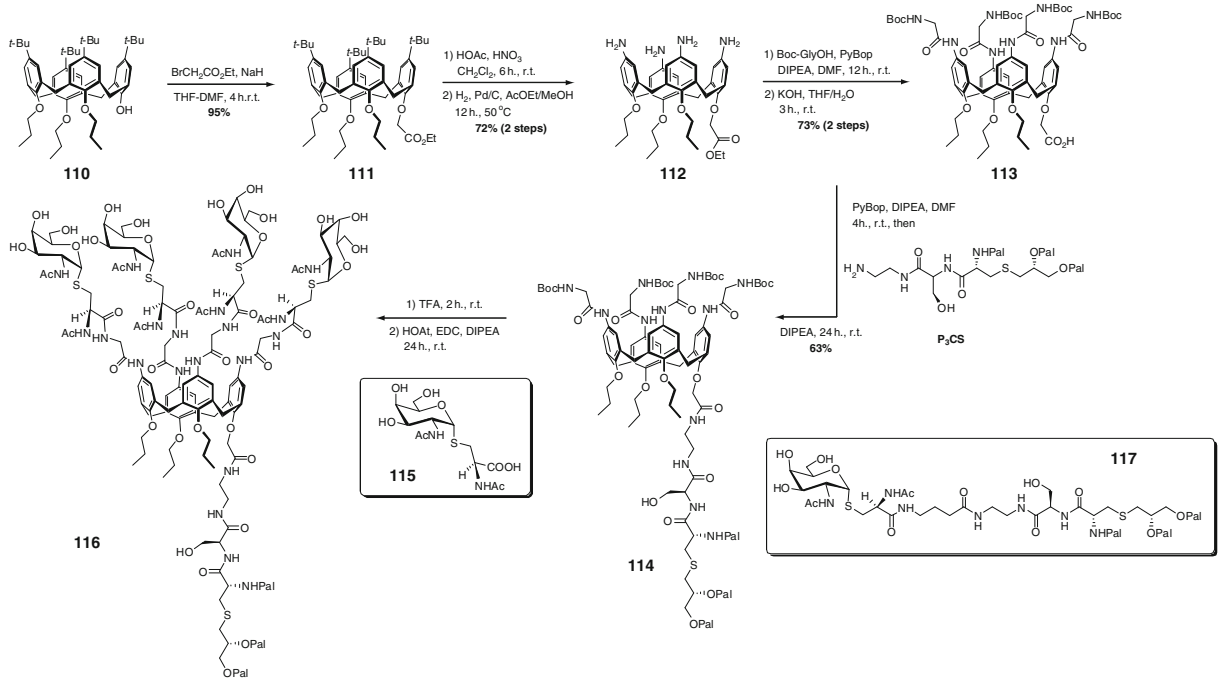
Subsequently, Křenek *et al*. proposed a series of calixarenes substituted with 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) directly linked by a thiourea spacer, and tested their binding activity to heterogeneously expressed activation of C-type lectin-like receptors of the rat natural killer (NK) cells NKR-P1, and the receptor CD69 (human NK cells, macrophage).[129] NK cells are important components of the innate immune response against tumors and early protection against viruses and other intracellular pathogens. Interestingly, high-affinity carbohydrate ligands for both major activating receptors, NKR-P1 and CD69, have been found. More particularly, GlcNAc was recognized as a high-affinity receptor in this study. Furthermore, multivalency through multiantennary, branched GlcNAc-containing structures was found to be crucial for a highly specific recognition and binding event to NKR-P1 and CD69 receptors *in vitro*. Hence, this study was initiated to determine differences in the binding capability of calix[4]arene-based glycoconjugates to recombinant receptors, as well as their effects on proliferation and cytotoxic cell-effector function. Results indicated that, in the case of NKR-P1, the binding affinity of β-D-GlcNAc-substituted calixarenes carrying two or four sugar units was comparable to that observed with known linear chitooligomers. The influence of

GlcNAc substitution of the calixarene skeleton on binding affinity for CD69 receptor was more profound, and the corresponding tetravalent cluster containing thiourea functionalities displayed very potent inhibitory activity, with $-\log IC_{50}$ as high as 9.3, making it one of the best ligands for this receptor. The use of glycoconjugates with lower valency led to a dramatic decrease of the binding activity. In addition, the immunostimulating activity of the tetravalent glycoconjugate indicated a proliferation and stimulation of natural cytotoxicity of human peripheral blood mononuclear cells (PBMC) at concentrations of 10^{-4} and 10^{-8} M, as observed for lipopolysaccharide. Moreover, at these concentrations, it induced an increase in the spontaneous death of tumor targets. Generally, calyx sugar derivatives were superior to the corresponding dendritic PAMAM-$GlcNAc_8$ analogues (see next).

The synthesis of a novel anticancer vaccine candidate built on a nonpeptidic scaffold, in which a cluster of four S-linked Tn antigen glycomimetics (S-Tn) was conjugated to an immunoadjuvant moiety, trihexadecanoyl-*S*-glycerylcysteinyl-serine (P_3CS), through a calix[4]arene scaffold has been described.[130] The glycomimetic *S*-Tn antigen was chosen as antigenic determinant because of its higher metabolic stability and capability to determine the maximal immunostimulating activity of a construct at lower doses with respect to the natural O-Tn analogue.

Preparation of the calix[4]arene platform started from disymmetrical 25,26,27-tripropoxy-28-hydroxy-*p*-*tert*-butylcalix[4]arene (**110**), which reacted with ethyl bromoacetate in the presence of NaH to furnish ester **111** quantitatively (Scheme 15). Direct replacement of the *tert*-butyl groups via aromatic nitration afforded the nitrocalixarene derivative which, upon subsequent reduction with H_2 at atmospheric pressure using Pd-C catalyst, afforded the amino analogue **112**. Afterwards, four Boc-glycine spacers were efficiently introduced into the upper rim of the macrocycle, via standard amide coupling in the presence of PyBOP and DIPEA. Saponification (aq. KOH, THF) of the ester group led to intermediate **113**, containing a single carboxylic acid functionality in the macrocycle lower rim, for amidation to the P_3CS immunoadjuvant unit leading to **114**. Removal of the Boc group with TFA and coupling of the S-Tn acid derivative **115** with HOAt/EDC in dry DMF allowed the formation of the targeted conjugate **116**.

Thus, in the designed construct, the four S-Tn antigen units were linked covalently through the glycine spacers of the calix[4]arene. Three propyloxy groups and one pendant P_3CS unit at the narrow rim block the synthetic core in a rigid cone-shaped conformation, ensuring the preorientation of the antigenic units on the same side with respect to the median plane of the macrocycle. The all-*syn* orientation of Tn antigens was crucial for better mimicking of the cancer cell surface, while the presence of a glycine spacer should avoid steric hindrance and ensure flexibility, both advantageous in the antigen–immune system interaction.



SCHEME 15. Glycosylated calix[4]arene.[130]

The authors evaluated the immunogenicity of the tetra-Tn construct **116** in mice by using a monovalent glycoconjugate **117** as reference compound. The aim was to investigate the role of the cluster effect in amplifying the antibody production with respect to a single presentation of the Tn antigen unit. In the serum-dilution range considered, mice immunized with tetramer **116** showed a substantial and significant production of antibodies. Even when the concentration of monovalent compound was increased fourfold with respect to the parent monovalent derivative **117**, the reactivity elicited by the clustered construct was significantly higher. These data clearly showed that a cluster effect provoked the higher immunostimulating activity of the tetravalent cluster as compared to the monovalent analogue. The authors thus presented still further convincing evidence for a vaccine candidate built on a nonpeptidic platform that induces a more-effective immune response due to the cluster effect and presentation in an optimized arrangement of Tn antigens. The rigid calix[4]arene scaffold was claimed to properly mimic the mucine surface encountered *in vivo*.

In a later investigation, a set of 14 calix[*n*]arenes ($n = 4$, 6, or 8) having thiourea-linked galactose or lactose moieties were prepared to analyze their reactivity toward the reference plant lectin from VAA relative to three human galectins (galectins-1, -3, and -4).[131] Despite their high degree of flexibility, the calix[6,8]arenes generally proved especially effective for the plant AB-toxin. In the solid-phase model system involving VAA lectin and using the absorbed glycoprotein asialofetuin (ASF), the inhibitory potency of the hexa- and octavalent galactoside clusters were 5 and 8 μM, respectively, corresponding roughly to a 100-fold enhancement as compared to free galactose. As demonstrated with solid-phase and cell-binding assays, clustering on macrocycles was also particularly effective toward galectin-4. In fact, among the human lectins, different response patterns were registered, the tandem-repeat-type galectin-4 reacted very sensitively to the presence of these test compounds. The IC_{50} value for the lactoside cluster was lowered by a factor of 300-fold relative to the free sugar, when calculating the sugar concentration in the assay. These bioassays underlined clear intergalectin differences and dependence of inhibition on the conformational properties of the calix[*n*]arenes scaffold, as well as on the shape and valency of the glycoclusters.

e. Glycoclusters with Porphyrin Cores.—The attachment of saccharide components to porphyrin macrocycles gives rise to various derivatives termed glycoporphyrins, which might become of great importance for medicinal and other applications, such as catalysis and molecular recognition.

Since the pioneering synthesis of this type of glycoconjugate first described by Maillard *et al*. in the late 1980s via condensation of 2-formylphenyl tetra-*O*-acetyl-β-D-glucopyranoside with pyrrole,[132] improved synthetic methodologies have generated

highly promising water-soluble glycoconjugates showing potential as photosensitizers (PSs) in photodynamic therapy (PDT),[133] or as antibiotics,[134] antiviral agents,[135] and drug sensors.[136]

Interestingly, despite the fact that carbohydrate and porphyrin derivatives constitute two groups of naturally occurring compounds of great significance to all existing organisms, only a few examples of natural glycoporphyrins are known. Tolyporphin A **118**, isolated from the blue-green alga *Tolypothrix nodosa*, whose total synthesis has been reported,[137,138] and the chlorophyll c_2-monogalactosyldiacylglyceride **119**, a derivative isolated from *Emiliana huxleyi*, are two examples of natural glycoporphyrin-like derivatives (Fig. 10).

The biological significance of glycoporphyrins, their limited natural occurrence, and their widespread applications have made the availability of such compounds a scientific challenge for several research groups. Synthetic approaches have consisted in the direct glycosylation of prefunctionalized porphyrins or chemical synthesis from suitable glycoconjugates precursors.

Two general approaches have thus been used to access glycoporphyrins.[139] The first is based on Lindsey's method involving the coupling of pyrroles or dipyrromethanes with glycoaldehydes species through acid-catalyzed condensations, followed by oxidation of the reaction mixture, for instance with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (Fig. 11).[132] By this strategy, O-,[140,141] C-, and S-linked glycosyl compounds[142] can be obtained, and have been examined in detail.[143] Interestingly, higher valency glycoporphyrins can be obtained by following the pyrrole–aldehyde condensation strategy, generating O-glycosylated porphyrin dimers possessing ether linkages.[144]

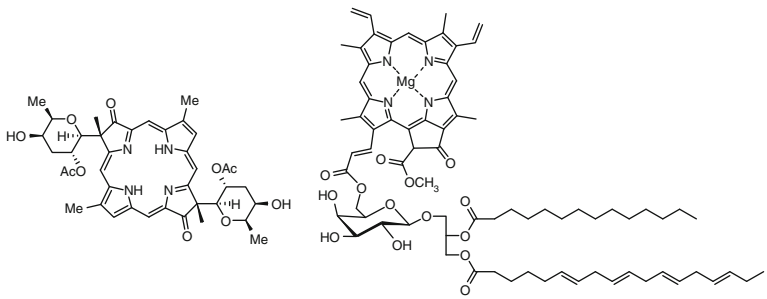


FIG. 10. Natural glycoclusters bearing a porphyrin core.

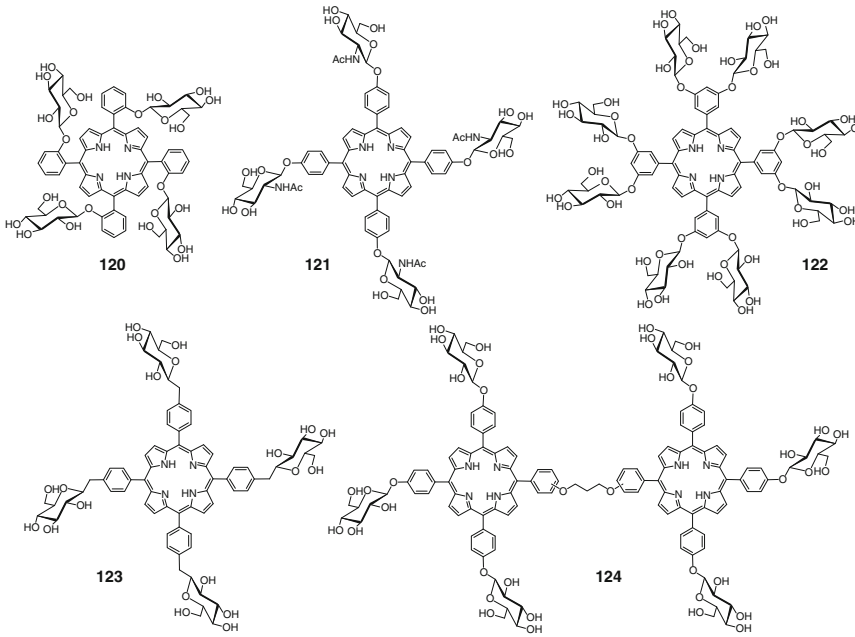


FIG. 11. Sugar aldehydes condensed directly on pyrrole derivatives by Lindsey's chemistry.

The second methodology involves direct introduction of glycosylated moieties onto a suitably functionalized *meso*-arylporphyrin scaffold, accessible from a natural source (protoporphyrin-IX) or by total synthesis. Several O-,[133,145,146] S-,[147] and N-glycoporphyrins[148] (**125–129**) have thus been prepared (Fig. 12). Moreover, in order to explore the influence of the clustered peripheral saccharides around the porphyrin scaffolds, and to evaluate their photophysical properties, the synthesis of dodecavalent porphyrins bearing four trivalent glycodendrons via amide ligation (**129**) has been achieved.[149]

PDT is certainly the most promising application for this kind of neoglycocluster. Its concept is based on the concentration of PS in target cells and upon subsequent irradiation with visible light in the presence of oxygen, leading to specific destruction of the target cells or tissues. There is general agreement that the principal mechanism whereby cell destruction occurs involves the disruption of the cellular, mitochondrial, or nuclear membranes by singlet oxygen ($^1O_2$) generated by the action of the PS.[150] Photofrin®, the first PDT formulation introduced, has been approved in several countries including USA, Canada, Netherlands, France, Germany, and Japan for

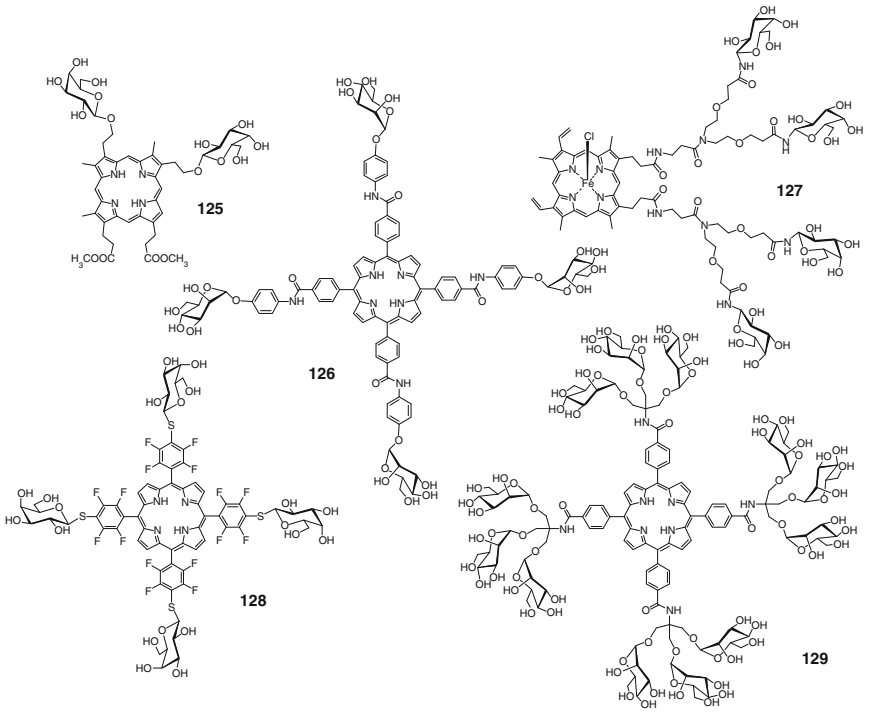


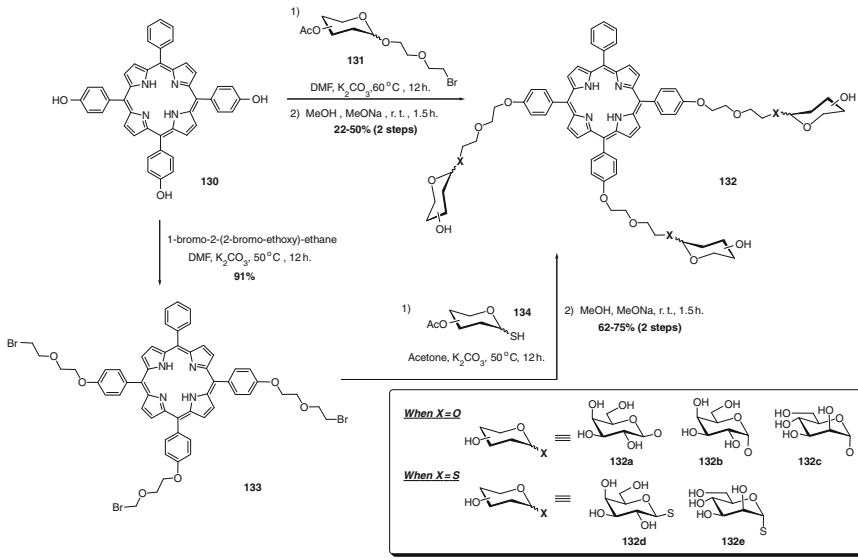
FIG. 12. Glycoconjugates incorporated on porphyrin scaffolds.

treatment of bladder, esophageal, gastric, cervical, and lung cancers, and several other diseases. Although Photofrin® is effective against a number of malignancies, it is not an ideal PS because of the lack of a well-defined structure, its weak absorbance in the red region of the visible spectrum and the long-lasting skin photosensitivity induced. Mainly because of these drawbacks, several other derivatives have been synthesized with the required peripheral substituents, such as carbohydrate residues to control water solubility, biodistribution, pharmacokinetics, and affinity–selectivity for cancer cells.[143,147] Hence, glycoconjugation can advantageously modify the amphiphilic–lipophilic balance of macrocycles and can favor specific interactions of the resulting conjugates with the lectin receptors usually overexpressed in certain malignant cells. Glycoconjugation may thus be a potentially effective strategy for targeting PS toward tumor cells and allow a more efficient and selective PDT treatment. To illustrate this concept, some recent studies leading to efficient glycosylated PS are presented later.

Five novel diethylene glycol (DEG)-linked O- and S-galacto or manno-conjugated *m*-tetraphenyl porphyrins have been prepared and their biological and photobiological properties investigated *in vitro* against a human retinoblastoma cell line (Y79).[133] Preliminary studies established that human retinoblastoma cells express sugar receptors that exhibit a preferential affinity for galactose and mannose residues.[151]

The precursor 5,10,15-*m*-tri(*p*-phenol)-20-phenylporphyrin (**130**) was used for synthesis of all of the glycoconjugates studied (Scheme 16). The trivalent O-glycosylated DEG porphyrins **132a** (β-Gal), **132b** (α-Gal), and **132c** (α-Man) were obtained by treating **130** with the requisite bromo-substituted glycosides (**131**), followed by deprotection under Zemplén conditions, with yields ranging from 22% to 50% over two steps. The corresponding thioglycosylated porphyrin analogues **132d** (β-Gal) and **132e** (α-Man) were obtained according to a slightly modified strategy involving the preliminary synthesis of the bromo-substituted porphyrin glycol **133** via standard Williamson etherification and final introduction of peracetylated 1-thioglycopyranoses **134** under basic conditions, with improved overall yields of 62–75%.

The amount of PS taken up by the Y79 retinoblastoma cells was first determined by fluorescence intensity measurements (reflecting the internalized drug concentration)



SCHEME 16. Galacto- and manno-substituted tetraphenyl porphyrins used for PDT against human retinoblastomas.[133]

using flow cytometry. The results indicated efficient internalization of the glycoconjugates, preferentially in the membranes of all cytoplasmic organelles, with the extent of uptake dependent upon the nature of the sugar component, its anomeric configuration, and the linker used. An increase in the spacer length linking the tetrapyrrolic ring and the sugar moiety resulted in higher cellular uptake in the case of **132a** (*O*-β-Gal), **132d** (*S*-β-Gal), and **132c** (*O*-α-Man), while a considerable decrease in drug internalization was observed for **132b** (*O*-α-Gal). Hence, these results highlighted the importance of the amphiphilic/lipophilic character of these clusters, indicating that high lipophilicity led to low cellular uptake. The binding affinity toward the sugar-specific receptors on Y79 cells has also been investigated by examining a possible competition effect between the glycoporphyrins and the corresponding glycosylated albumin, indicating inhibition of uptake values of 40–45%, possibly due to cell–sugar–receptor saturation. In addition, their phototoxicity in darkness, evaluated by measurements of the cell survival fraction, was found to be negligible in all cases. High photoactivity was observed for the two α-galacto–manno porphyrins **132b** and **132c** ($LD_{50} = 0.05$ and 0.35 μM, respectively) at 514 nm and low fluence (1 J/cm^2). In addition, glycoporphyrins can also exhibit possible cell–membrane interactions that can affect, for instance, the plasma lifetime of a particular drug.

Promising results have been obtained for both compounds **132b** and **132c** since each one showed high *in vitro* photobiological activity in human retinoblastoma cells. Very low doses of the drug associated with low light intensities were sufficient to observe a marked effect. Moreover, both compounds undergo only very limited cellular metabolic degradation.

The same group further investigated the *in vitro* phototoxicity in order to determine the influence of different structural parameters.[152] Despite lower photodynamic activity than that observed for hydroxylated PSs (in particular the common Foscan® or Temoporphyn®), glycoconjugates displayed phototoxicity against Y79 cells having significant intrinsic cytotoxicity. These results confirmed that the photoactivity could be strongly modulated by the presence of a DEG spacer between the chromophore and the glycoside, and by the anomeric configuration of the sugar. Trivalent α-O-galactosylated porphyrin **132b** was determined to be a better candidate than Foscan® in the clinical application of PDT for conservative therapy of retinoblastomas.

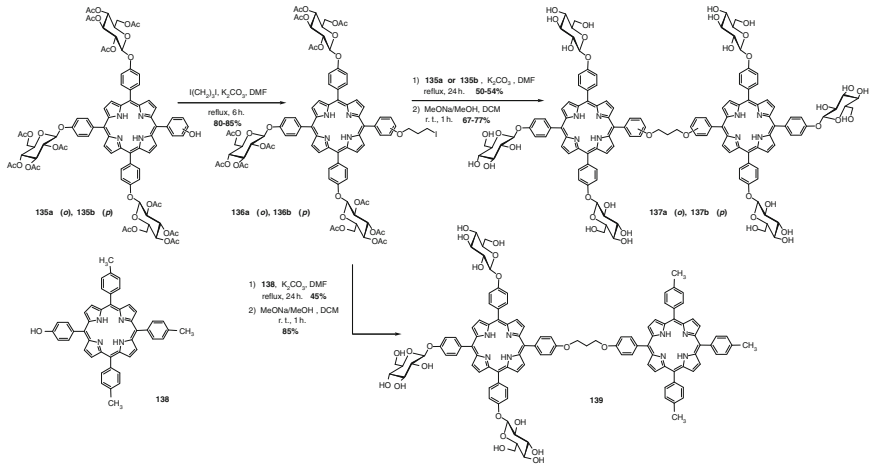
A second example has been addressed in which the synthesis and potential application of a series of neutral O-glycosylated porphyrin dimers and two original O-glycosyl cationic dimers were examined in PDT. In order to understand the influence of the number of glycosyl moieties, the spatial geometry and the ionic character on their photodynamic activity was evaluated.[144] The *in vitro* results

concerning their photocytoxicity against K562 human chronic leukemia cells compared favorably with those of the common PS Photoprin II®.

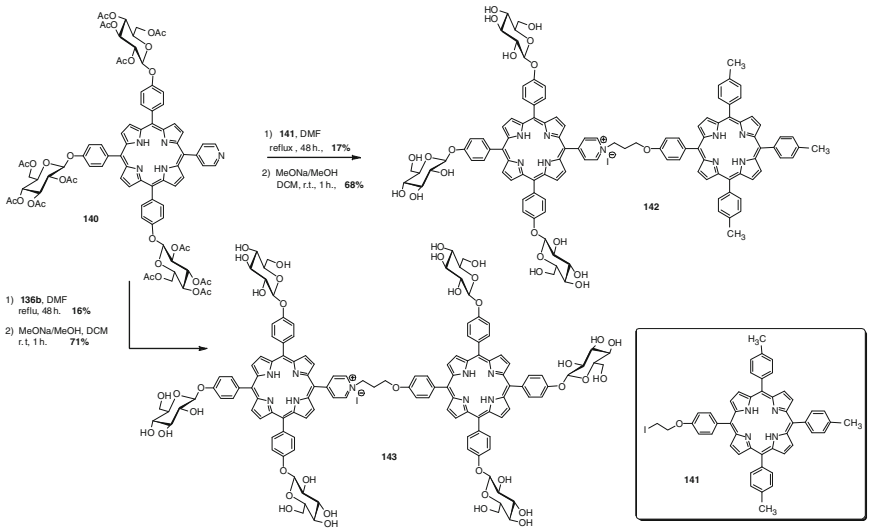
Two strategies for preparation of neutral and monocationic porphyrin models were used. The presence of a spacer arm directly attached to the *meso* phenyl position of the porphyrins gave access to a series of dimers with different substituents on the *meso* position and with different geometries.

Neutral bisporphyrins were synthesized according to Lindsey's methodology, namely the coupling of pyrrole with *p*-formylphenyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside and *o*- or *p*-hydroxybenzaldehyde with controlled stoichiometry and in the presence of $BF_3.Et_2O$ as catalyst (Scheme 17). Subsequent oxidation of the porphyrinogen intermediates with *p*-chloranil gave porphyrins **135a** and **135b** in 5% and 7% yields, respectively. Treatment of **135a** or **135b** with an excess of 1,3-diiodopropane (K_2CO_3, DMF, reflux) afforded derivatives **136a** or **136b** in 80–85% yields, respectively. Dimers **137a** and **137b** were formed by the reaction of **136a** or **136b** with the monohydroxylated precursors **135a** or **135b** via nucleophilic substitution, followed by O-deacetylation under modified Zemplén conditions. The analogous triglycosylated bisporphyrin **139** was similarly obtained from 5-(4-hydroxyphenyl)-10,15,20 tristolylporphyrin (**138**).

An elegant synthesis of a monomeric porphyrin intermediate bearing one pyridyl and three glycosyl units, precursor of cationic dimers, has also been described (Scheme 18).[153] Thus pyrrole, *p*-formylphenyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-



SCHEME 17. Tri- and hexavalent glucosylated porphyrins prepared by Sol *et al.*[144]



SCHEME 18. Synthesis of glucosylated porphyrins bearing cationic head-groups.[153]

glucopyranoside, and pyridine-4-carboxaldehyde were added to propanoic acid to provide the expected intermediate **140** in 7% yield. Condensation of **140** with the elongated analogue **141** of **136b** in DMF using potassium carbonate afforded adducts in 17% and 16% yields which, following sugar deprotection, provided monocationic dimers, **142** or **143**, respectively.

The authors first determined the partition coefficients of the deprotected bisporphyrin conjugates in order to evaluate their lipophilic character. Both triglycosylated conjugates **139** and **142** were found to be more lipophilic than **137a,b** and **143**. Next, studies concerning their photosensitizing properties, consisting in trapping reactions of $^1O_2$ with ergosterol acetate, indicated production of $^1O_2$ in very high yield, comparable to the reference hematoporphyrin (HP). Their *in vitro* photocytotoxicity was then evaluated against the K562 leukemia cell line. The amphiphilic character was found to be an essential factor for efficient PDT, since the amphiphilic triglycosylated dimers **142** and more particularly neutral **139**, were found to be more active, with a quasi-similar activity to Photofrin® for **142** after 120 min of irradiation, inducing 70–80% cellular death, probably through apoptosis. In addition, it has been shown that the presence of the glucosyl residues on the same side of the dimer conjugates was crucial for optimal activity.

A final illustration of the biological potential of glycoporphyrins has been provided by Ballut *et al.*, who described their incorporation into a dimyristoyphosphatidylcholine

(DMPC) liposome membrane and measured the interaction of the resulting conjugates with Con A lectin.[154] In this context, the authors designed a new family of glycoconjugated PSs bearing only one glycodendron moiety, with variable length for the spacer linking the carbohydrates to the porphyrin scaffold, on the *para* position of one *meso*-phenyl group (Fig. 13). Briefly, the Cbz-glycine spacer was introduced via peptide coupling onto the amino function of a trivalent precursor, followed by deprotection of the three terminal carboxylic acid functions of the scaffold and subsequent introduction of aminoethyl α-D-mannopyrannoside derivative through amide ligation. The suitably functionalized aminodendron (after Cbz-hydrogenolysis) was then coupled to 5-benzoic acid-10,15,20-triphenyl porphyrin through an amide linkage, which after standard O-deacetylation afforded the trivalent glycoporphyrins **144** and **145** in good overall yields.

In order to evaluate the conditions of incorporation of compounds **144** and **145** into a liposome membrane, the two derivatives were mixed with DMPC in a 1:1 ratio and the mixtures were spread at the air–water interface. Interestingly, incorporation of the elongated conjugate **145** in DMPC induced the formation of larger vesicles than the phospholipid ones (diameter of 218 vs. 185 nm) while compound **144** mixed poorly, leading to smaller and less stable vesicles probably because of repulsive interaction between sugar moieties in the vicinity of the phospholipid headgroups. Moreover, 1 h of contact between the mixed liposome containing **145** and Con A, led to a dramatic increase of the vesicle diameter (up to 2510 nm) and polydispersity was observed. These striking results were postulated to originate from the long spacer, which would increase the mobility of the mannoside moieties and thus facilitate their interactions with the lectin. In addition, the existence of Con A dimers and tetramers at the pH studied, allowed lectin interaction with more than one porphyrin molecules possibly borne by different liposomes. Such multiple interactions would lead to the formation of a network of vesicles bridged by Con A molecules, resulting in a dramatic increase

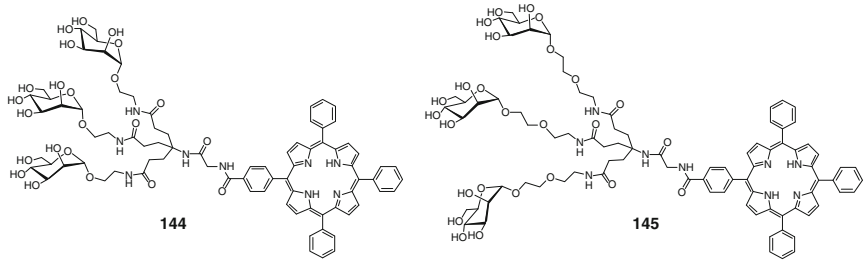


FIG. 13. Mannosylated prophyrin clusters prepared by Ballut et al. for liposome preparation.[154]

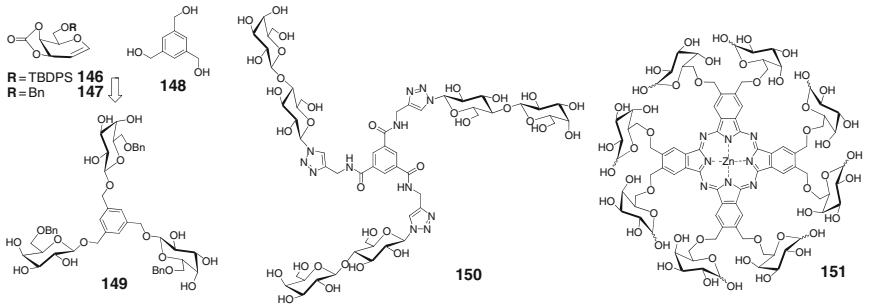


FIG. 14. Varied strategies for the coupling of carbohydrate derivatives to multivalent scaffolds.

in their apparent size. Such liposomes bearing glycodendronized phenylporphyrin could constitute an efficient carrier for drug targeting in PDT.

f. Other Aromatic Glycoclusters.—Clearly, access to other type of clusters has also been addressed, for instance, by using more conventional glycosylation strategy. In this context, the synthesis of trivalent β-D-galactoside **149** using stereoselective Lewis acid-catalyzed tris-glycosylation of 1,3,5-benzenetrimethanol (**148**) with galactal **147** derived from **146** has been described (Fig. 14).[155] A second example concerned the synthesis of trivalent clusters (**150**) containing such disaccharides as lactoside as ligands for galectin-1 and -3,[156] or the more-complex α-L-Fuc*p*-(1→4)-β-D-GlcNAc for the inhibition of binding of PAII-L lectin from *Pseudomonas aeruginosa,*[157] generated by click chemistry between an aromatic tripropargylated core (derived from trimesyl chloride), and the corresponding glycoside azide. Inhibitory properties of 20 μM against galectin-1 were observed for the conjugate **150** in HAI assays. This comprises a 40-fold enhancement as compared to free lactose, and 13.3-fold on a per-saccharide corrected basis. In addition, more-unusual macrocyclic scaffolds, such as phthalocyanine (**151**) have recently emerged to afford dense glycoclusters for which biological relevance remains to be determined.[158,159]

3. Glycoclusters from Carbohydrate Scaffolds

Structurally, carbohydrate derivatives are interesting polyfunctional platforms for the synthesis of oligosaccharide mimetics or glycoclusters. The possibility of selective functionalization of the various hydroxyl groups by well-established methodologies, and control of the relative orientation of the branches by configurational and conformational bias, makes these ''full-carbohydrate glycoclusters'' ideally suited for mapping the geometrical requirements for efficient lectin binding.

In this context, several synthetic strategies for efficient derivatization of various carbohydrate scaffolds have been described, notably including perallylation of non-reducing mono- or di-saccharides and subsequent ligation of *S*-glycosides by thioether linkages through photochemically promoted radical addition.[160] Controlled introduction of such desired functionalities as hydroxyl or amino from terminal allyl functions can also permit the construction of glycoclusters through ether,[161] or thiourea linkages (**154**–**156**, Fig. 15).[160,162]

Systematic studies aimed at evaluating their biological properties against uropathogenic *E. coli* have been initially addressed by measuring the relative affinities of Con A towards D-glucose-centered mannosylated clusters **152**α and **152**β and their corresponding analogues, **153**, **154**, and **155** by a competitive ELLA assay.[160] Hence, the known allyl 2,3,4,6-tetra-*O*-allyl-α- and β-D-glucopyranosides were chosen as the core building-blocks for the preparation of pseudosymmetric penta-branched derivatives. The versatile reactivity of the terminal alkene has been exploited in different ways to produce glycoclusters exhibiting a variety of linking functional groups, such as thioether, ether, and thiourea. With this strategy, the higher-valent D-glucose-centered glycocluster **155** containing 15 peripheral epitopes

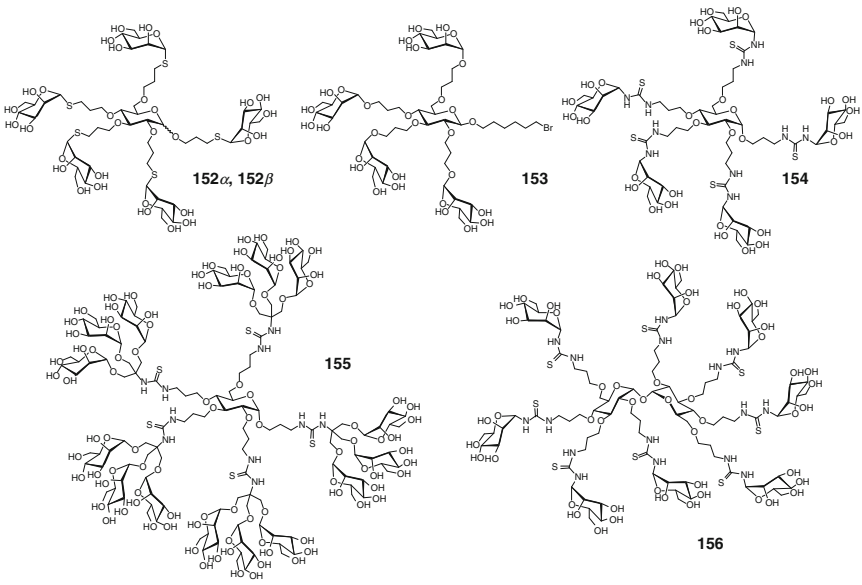


FIG. 15. Various mannosylated glycoclusters built around carbohydrate scaffolds.[160–162]

has also been obtained. The IC_{50} values of the pentavalent clusters **152**α and **152**β (27 and 31 μM, respectively) were indicative of strong Con A affinity relative to the reference methyl α-D-mannopyranoside, corresponding to a 6.4 and 5.5-fold enhancement on a per mannoside basis. A tetravalent analogue (**153**) containing an aglyconic oxygen atom presented identical avidity, indicating that multivalent presentation through four branches is sufficient to elicit a cluster effect. In sharp contrast, replacement of the 1-thiomannose wedges with α-D-mannopyranosylthioureido units virtually abolished any multivalent or statistic effects, along with a dramatic decrease of binding affinity. Alternatively, the 15-valent ligand **155**, possessing classical O-glycosidic linkages, was a very efficient inhibitor of the Con A–yeast mannan association (IC_{50} of 18.5 μM). However, the valency-corrected relative potency was about one-half that of the pentavalent derivatives. Those results illustrated the potential of carbohydrates as multivalent scaffolds for glycocluster synthesis and underlined the importance of careful design of the overall architecture in optimizing glycocluster recognition by specific lectins. As mentioned in the introduction, the TRIS scaffold is NOT optimized for simultaneous binding without the utilization of elongated spacers that allow several lectins to bind the maximum accessible sugar ligands.

In contemporary studies, several others multimannoside glycoconjugates based on carbohydrate scaffolds have been similarly constructed using ''click chemistry.'' The construction of these multivalent glycoclusters involves first the introduction of propargyl or azide functionalities on such mono- or oligo-saccharides as maltoside or maltotrioside as central cores and subsequent reaction with glycosylated ligands containing the complementary functionality (**157**–**159**, Fig. 16).[163,164]

A remarkable example of a biologically relevant D-glucose-centered glycocluster has been achieved by Kitov *et al*., who designed an oligovalent, water-soluble carbohydrate ligand named STARFISH having subnanomolar inhibitory activity toward Shiga-like toxin I (SLT-I).[23] Shiga toxin (Stx) and Shiga-like toxins (SLTs or VTs (Verotoxins) are ribosome-inactivating proteins that act as *N*-glycosylases, cleaving several nucleobases from the RNA, and thereby halting eukaryotic protein biosynthesis. Infection by bacteria that produce SLTs results in serious gastrointestinal and urinary tract disorders, and is known to cause a potentially lethal disease, the hemolytic uremic syndrome (HUS), that may result in kidney failure. Stx is produced by *Shigella dysenteria*, whereas SLTs are produced by the Shiga toxigenic group of *E. coli* (STEC), including serotype O157:H7 and enterohemorrhagic *E. coli* (EHEC). The *E. coli* toxins can be further divided into SLT-I and SLT-II having conserved structures in the binding sites, and the SLT-II edema variant (SLT-IIe). Like CT, SLTs belong to a family of bacterial enterotoxins harboring a hexameric AB_5 structure,

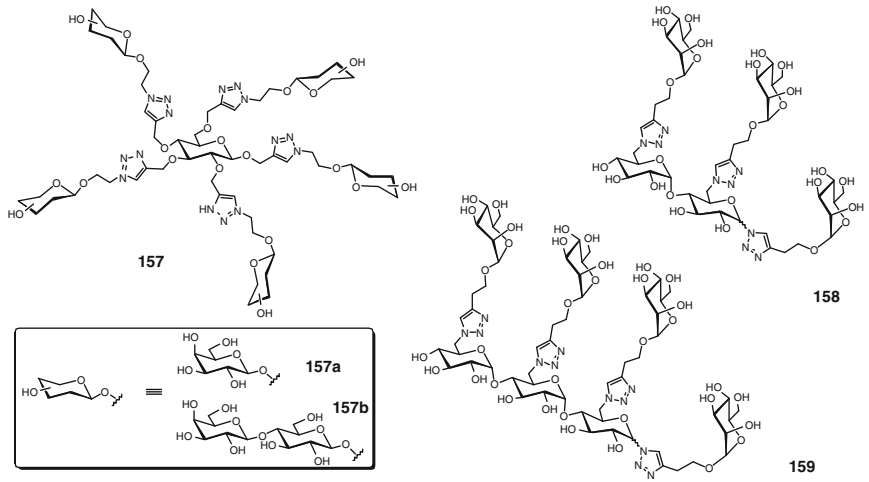


FIG. 16. Multivalent glycoclusters built around saccharide scaffolds using ''click chemistry.''[163,164]

where A denotes a cytotoxic enzyme located above the center of one face of the B_5-subunit, which represents a symmetrical homopentameric lectin-like carbohydrate-recognizing complex having a doughnut shape that facilitates delivery and entry of the A component into the cell of the host.[165] The SLT-I is virtually identical both in structure and mechanism of action to the toxin expressed by *S. dysenteria*, having similar B-subunits and differing only in one residue (Ser45 vs. Thr45) in their A-subunits. The *in vivo* receptor of Stx and SLTs is the trisaccharide portion ($\mathbf{P^k}$) of the neutral glycolipid, globotriosylceramide ($GbOse_3$ or Gb_3, αGal-(1-4)βGal(1-4) βGlc(1-1)ceramide), present in greater amounts on the surfaces of kidney glomerular endothelial cells, to which the renal toxicity of Stx may be attributed (Fig. 17).

When the crystallographic structures of the protein or carbohydrate-ligated proteins are known, it is possible to model multivalent oligosaccharide inhibitors. As mentioned, the SLTs possess five noncovalent B-subunits per molecule and each subunit presents three carbohydrate-binding domains. Therefore, oligovalency ''tailored'' to the structure of the B-subunit pentamer offers the best opportunity for designing higher affinity inhibitors, as 15 binding sites are symmetrically arranged across the toxin surface that engages the cell membrane. Thus, highly selective, potent binding of SLTs to $\mathbf{Gb_3}$ is mainly attributed to the multiple interaction of the B-subunit pentamer with the trisaccharide moiety on $\mathbf{Gb}_3$. On the basis of these facts, several SLTs antagonists in which this trisaccharide moiety was combined using various dendritic core structures have been reported. Among these, the foregoing study in

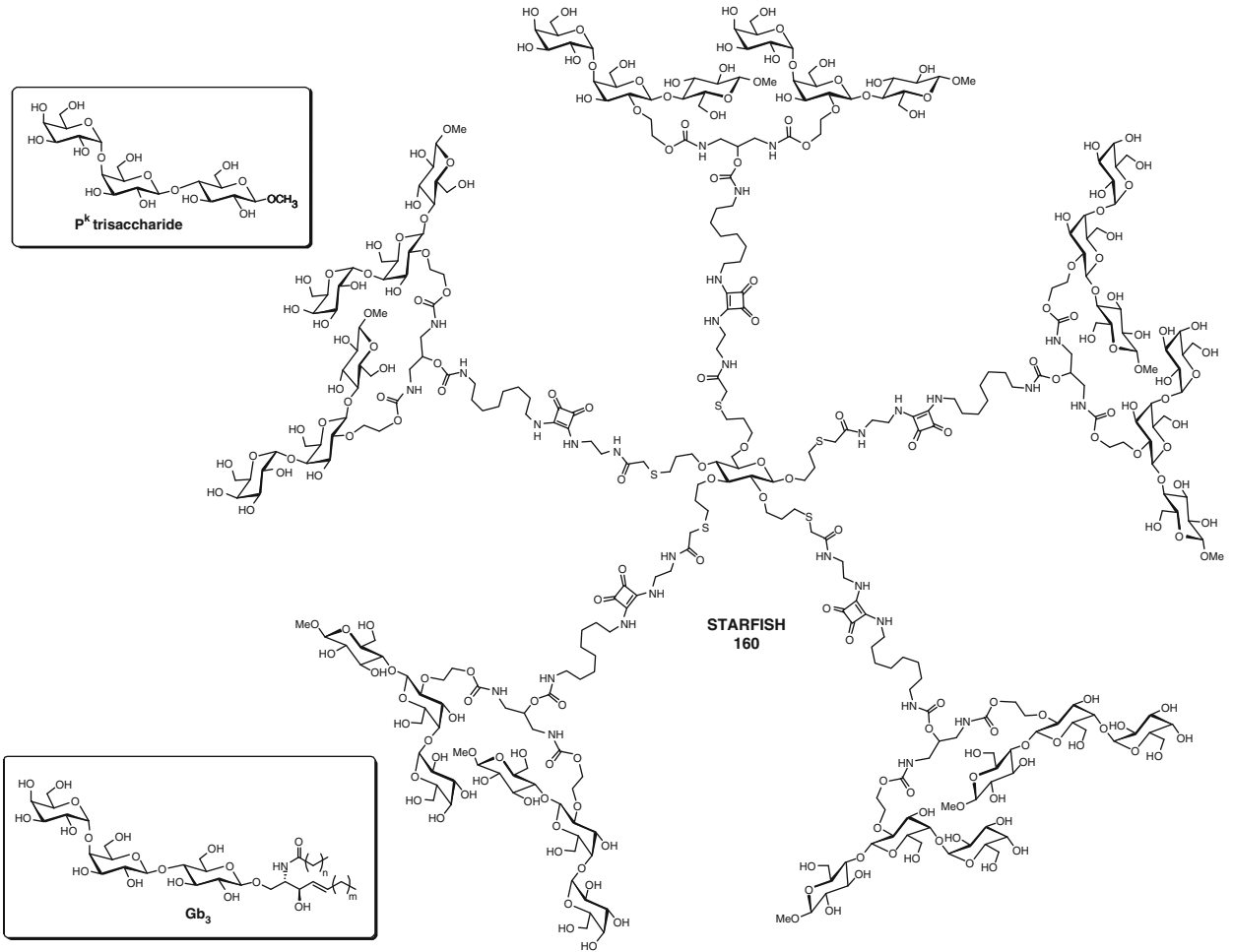


FIG. 17. The STARFISH dodecamer of Kitov *et al*. with the $\mathbf{P^k}$ *trisaccharide* linked laterally at 2′ and showing subnanomolar inhibitory activity against Shiga-like toxin I.[23]

particular afforded spectacular results and effectiveness in neutralizing SLTs infection. Hence, the use of the crystal structure of the B_5 subunit of *E. coli* 0157:H7 SLT-IB in conjunction with an analogue of its carbohydrate receptor allowed the design of tailored multivalent glycocluster **160** bearing 10 peripheral functionalized $\mathbf{P^k}$ residues. Structurally, this decavalent cluster, named STARFISH, comprised a radially symmetrical star-shaped carbohydrate backbone (D-glucose) with pairs of $\mathbf{Gb_3}$ trisaccharides at the tip of each arm, ideally spaced and oriented to be simultaneously engaged by each the B-subunits and thus to permit pentavalent interaction with SLTs. To achieve this display of tethered ligands, a glucose molecule was chosen as the central core for adequate pentavalent presentation, via its derivatization as pentaallyl ethers, free-radical reaction with thioglycolic acid, and subsequent chain elongation of the radial arms. Each of them was designed to span $\sim$30 Å from the central core of the toxin and the tether was adapted to bridge binding sites 1 and 2 on each B-subunit. ELISA and solid-phase inhibition assays indicated the highest molar activity of any inhibitor reported, with subnanomolar inhibitory activity against STL-I and II (IC_{50} of 0.4 and 6 nM, respectively), thus being 1–10-million-fold higher than that of monovalent ligand. Furthermore, the ability of the inhibitors to protect host cells against a lethal dose of SLT-I in culture could be determined by using Vero cells. Cytotoxicity assays indicated that STARFISH inhibitor **160** provided effective protection of Vero cells cultured in the presence of SLT-I (IC_{50} of 1.19 μM) and SLT-II (IC_{50} of 1.58 μM), even over a 2-day coincubation period. Interestingly, the crystal structure showed that one STARFISH molecule bound to not just one but to two B-subunit pentamers. Instead of binding sites 1 and 2, the tethered $\mathbf{P^k}$-trisaccharides of STARFISH bound to two B-subunit monomers from separate toxin molecules. As, in the experiment, the concentration of STARFISH was sufficient to form a 1:1 complex, it was suggested that the formation of a 2:1 sandwich must be thermodynamically favored, as for the CT. Extended investigations have been made by the same group in order to circumvent the differentiation of SLT-I and SLT-II during biological studies. In fact, STARFISH **160** protected mice when it was injected subcutaneously in admixture with a lethal dose of SLT-I but not SLT-II, and it also reduced the distribution of ^{125}I-STL-I but not ^{125}I-STL-II to the murine kidney and brain. Modification of the nature and length on the linkage between the oligosaccharide component and the backbone (thereby increasing the flexibility of the tether) was then necessary to protect against the more-toxic SLT-II.[166]

Despite a considerable decrease in its inhibitory activity in the solid-phase inhibition assays (with an IC_{50} of 300 nM, corresponding to a $\sim$50-fold increase compared with **160**), an equivalent decrease in performance of the so-called DAISY (**161**) in the more challenging SLT-I and SLT-II verocytotoxicity-neutralization assays was not

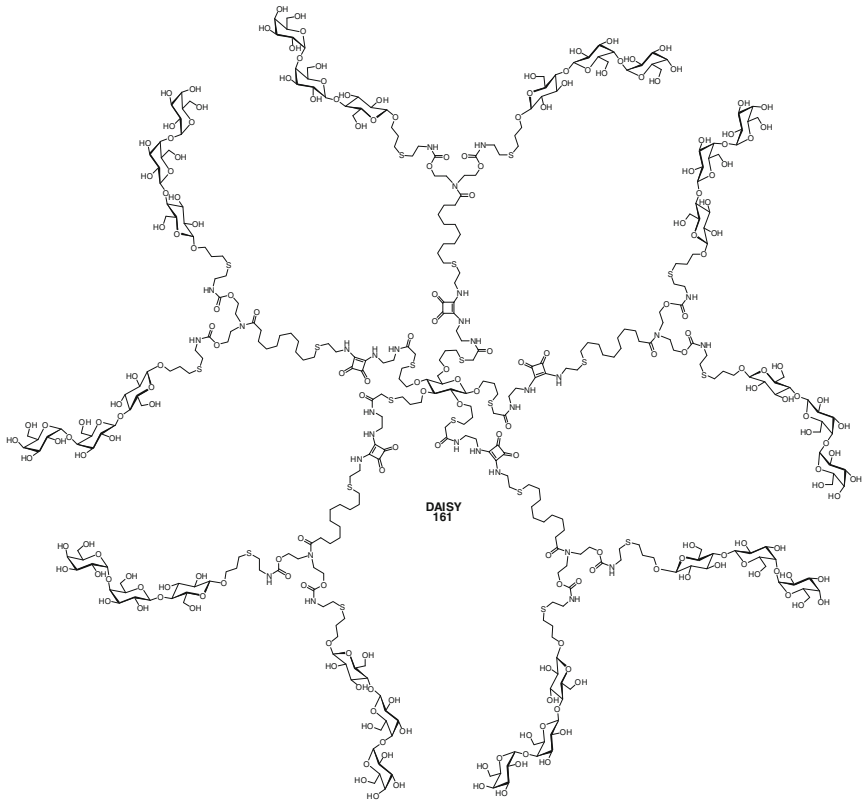


FIG. 18. A variant of the STARFISH 10-mer in which the $\mathbf{P^k}$ antigen is linked to the central glucose core at the anomeric position.[166]

apparent (Fig. 18). Subcutaneous injection of **161** protected mice against oral challenge with SLT-I and also STL-II-producing STEC. Interestingly, **161** did not interfere with the ability of the murine immune system to produce SLTs-specific protective antibodies.

The class of well-defined biocompatible CDs also provides an interesting alternative for construction of carbohydrate-centered clusters. This nonimmunogenic scaffold combines unique structural features, such as high symmetry and a truncated cone-shaped hydrophobic cavity, which can be exploited for efficient and radial epitope presentation, together with complexation of hydrophobic drugs in their cavities for targeted drug delivery.[167] More particularly, perglycosylated CDs showed much improved ability to form stable complexes with an antitumor

reagent as compared with the CD itself and they provide one of the most potent candidates among specific carriers toward drug-delivery systems.

In order to introduce peripheral carbohydrates on these macrocyclic scaffolds, a variety of selectively functionalized derivatives has been described, often generated without need for hydroxyl-group protection. Since the first synthesis of perthioglucosylated derivatives of β-CD in 1995,[168] several other groups have described alternative synthetic pathways for the multifunctionalization of CDs, either on their primary faces or on both faces simultaneously. Those methodologies, involving well-documented photoaddition of thioglycosides to polyallylated β-CD ethers in an anti-Markovnikov fashion,[169] followed by selective introduction of such reactive functionalities at the primary positions as iodine,[170,171] amine,[172] and chloroacetamide,[173] gave rise to a panel of neoglycoconjugates named ''glycoCDs.'' These can exhibit simple β-D-glucosides (**162** and **163**) or *N*-acetyl-D-glucosamines (**164**) through ether, thiourea, or amide linkages, or more-sophisticated carbohydrate appendages, such as elongated sialic acids (**165**)[173] or sialyl Lewis^X (**166**)[170] via thioether functions (Fig. 19).

Concerning the general biological relevance of glycoCDs, it was demonstrated that these systems often showed amplified inhibitory effects as compared to their monovalent analogues. For instance, the Roy group synthesized a small library comprising β-D-glucosylated, β-D-galactosylated, α-D-mannosylated, and *N*-acetyl-β-D-glucosaminated CDs. They evaluated their relative binding properties toward different natural carbohydrate-binding plant lectins, using both microtiter plate competitive-inhibition experiments, double-sandwich assays using horseradish peroxidase (HRPO)-labeled lectins, and turbidimetric assays.[174] In general, all persubstituted β-CDs showed good to excellent inhibitory properties, together with abilities to cross-link their analogous plant lectins. Their capacity to anchor both microtiter plate-coated lectins and their corresponding peroxidase-labeled derivatives further confirmed the usefulness of these multivalent neoglycoconjugates in bioanalytical assays.

Another example has been provided by Furuike *et al*. who proposed an efficient practical synthesis of CD-scaffolded glycoclusters via standard nucleophilic substitution of iodide from a heptakis(6-deoxy-6-iodo-β-cyclodextrin) precursor by various unprotected sodium thiolates derived from 3-(3-acetylthiopropanamido)propyl glycosides (Fig. 20).[175] Hence, novel glycoCDs having D-galactose (**167**), *N*-acetyl-D-glucosamine (**168**), lactose (**169**), and *N*-acetyllactosamine (**170**) residues were obtained in high yields ranging from 78% to 88%. In order to evaluate the effect of clustering on the biological potency of these four glycoclusters, the hemagglutination inhibitory activities were examined by means of wheat germ agglutinin (WGA) from *Triticum vulgaris* and *Erythrina corallodendron* lectin (EcorL), which are known as

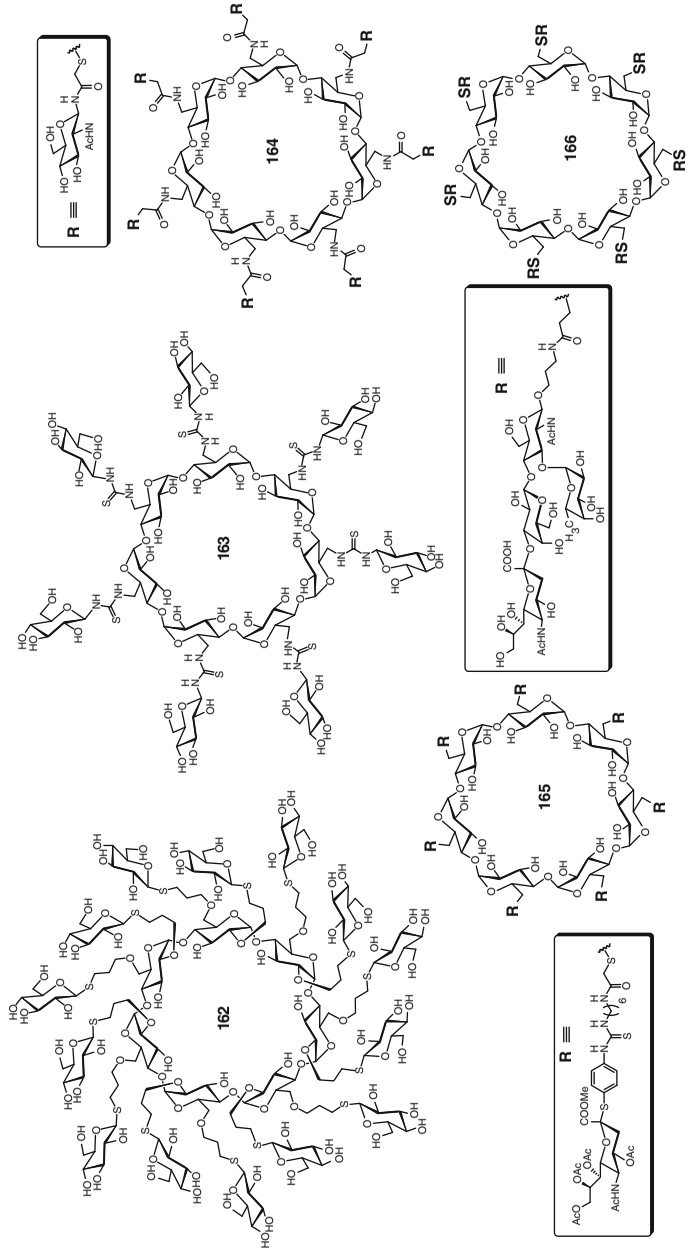


FIG. 19. Cyclodextrins decorated with sugars, including sialic acid and sialyl oligosaccharides.

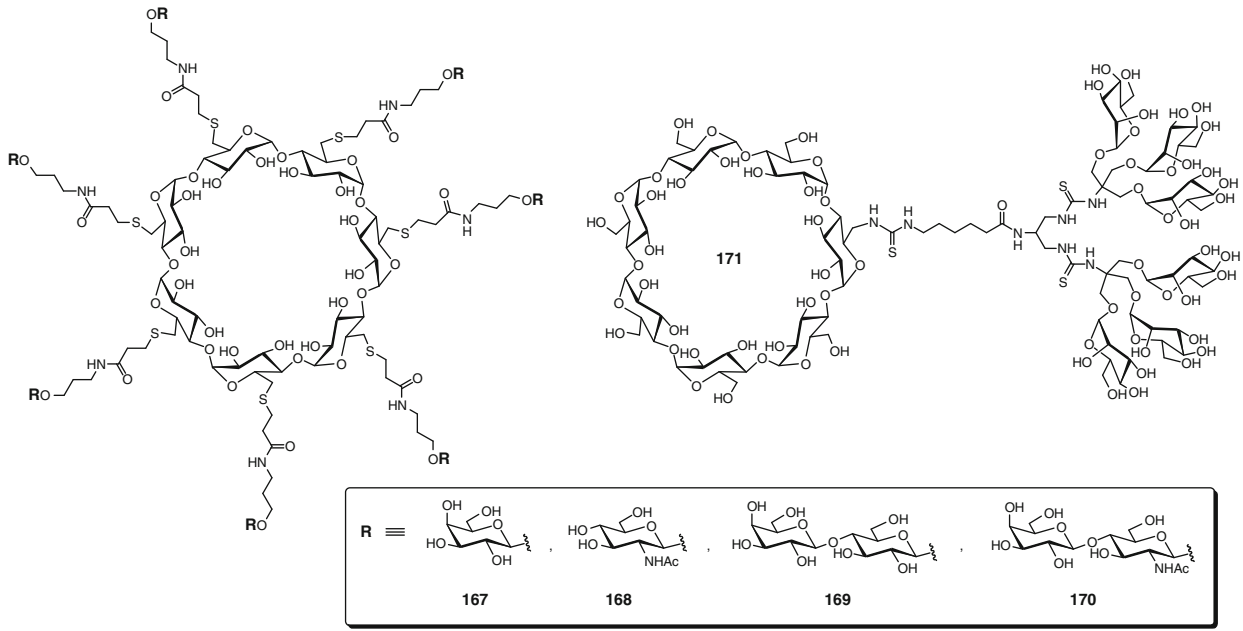


FIG. 20. Glycocyclodextrins and dendronized cyclodextrin-bearing mannose ligands.

N-acetyl-D-glucosamine- and *N*-acetyllactosamine-specific lectins. As anticipated, heptavalent GlcNAc-CD (**168**) showed drastically enhanced affinity against WGA in comparison with the monovalent analogue, corresponding to a 40-fold enhancement of the inhibitory effect. Similarly, the three other glycoconjugates efficiently inhibited hemagglutination induced by EcorL, notably compound **170** bearing *N*-acetyllactosamine residues, which showed the lowest inhibitory concentration (MIC of 89.2 μM).

Finally, another remarkable example has been designed in which a variety of glycoCDs bearing clustered mannosyl ligands were prepared and investigated for binding toward the tetrameric plant lectin Con A.[176,177] In this context, the key template was a 6^{I}-amino-6^{I}-deoxy-β-cyclodextrin,[178] the modified 1,2,3-triaminopropane branching component,[179] and an isothiocyanato-functionalized α-D-mannopyranosyl cluster prepared from TRIS. Coupling of the isothiocyanate derivative and the amine-functionalized trimannoside and the branching component gave the thiourea-bridged glycodendrimer–CD conjugates **171**. The monosubstituted hexavalent β-CD mannocluster showed a strong cluster effect in the inhibition of Con A binding to yeast mannan, with an IC_{50} of 10 μM that constituted up to a 22-fold increase on a molar basis as compared to monovalent derivative. In addition, the CD derivatives exhibited extremely high water solubility, more than 20-fold higher as compared to the parent CD (15 mM). Furthermore, the potential utility of such systems in active sugar-directed drug delivery to specific saccharide receptors on biological surfaces has been investigated. Thus, the cavity of β-CD was used to carry the anticancer drug Taxotere, for which the water solubility was greatly improved by the construct. Up to 4.5 and 4.7 g/L of Taxotere was solubilized in 25 mM aqueous solutions of a trivalent mannocyclodextrin derivative at 25 °C, corresponding to more than a 1000-fold solubility enhancement as compared to the water solubility of the isolated drug (0.004 g/L).[180]

4. Glycoclusters from Peptide Scaffolds

The class of peptide-based glycoclusters constitutes a valuable addition to the arsenal of glycoconjugates serving as important tools in glycobiology. Their structural properties allow them to act as mimetics of glycocalyx constituents and permit presentation of the glycans in particular and optimized orientations. Ideally, an unnatural peptide scaffold, cyclic or not, might provide much greater design flexibility than one afforded by the natural sequence. In general, manipulation of the length and nature of the amino acid sequence of the cyclic peptide provides opportunities for

designing multivalent ligands that are suitable for different geometric requirements. Moreover, variation in the number of glycan attachments, as well as the distances between glycans, together with subsequent introduction of a suitable chemical handle for conjugation to a carrier protein constitute parameters that could be readily tailored during the scaffold synthesis.

Although standard chemical processes have been used to access linear bioactive glycopeptides,[181–183] solid-phase synthesis (SPS) constitutes one of the most appealing synthetic methodologies and has led to a large variety of well-defined glycoclusters grafted onto synthetic peptide platforms. The numerous successes encountered in the SPS of various peptide-based conjugates, coupled to the ease of preparation and purification of complex derivatives, have motivated chemists to apply this methodology to synthesis of peptide glycoclusters. Hence, linear peptides containing a glycocluster head group (**172**, **173**),[184] multivalent cyclic neoglycopeptides including, for instance, three *N*-acetylglucosamine residues (**174**),[185] and multitopic biotinylated glycoclusters build on a topological cyclodecapeptide template (**175–179**)[186] represent remarkable illustrations of this powerful and straightforward methodology (Fig. 21).

Concerning biological investigations, Zhang *et al*. described the SPS of a pentavalent ligand having a cyclic decapeptide scaffold with built-in linkers in order to create efficient inhibitors for the CT B pentamer.[187] Varying the nature of the flexible amino acids lacking side chains (such as lysine, γ-aminobutanoic acid, and ϵ-aminohexanoic acid) allowed the authors to achieve the desired ring-size variations and to increase the likelihood that the peptides might adopt expanded conformations in solution (Fig. 22). The synthetic peptide-core (**180**) bearing five galactosylamine residues were assayed for their ability to block CT B pentamer binding to ganglioside-coated plates. Interestingly, ligands having longer or shorter linkers than optimal exhibited a loss in inhibitory power, demonstrating that when a ligand's effective dimension is not matching that of its target, there is a decrease in the ligand's affinity. Submicromolar IC_{50} values were obtained for the best derived ligand (**180**), depending on the core size, which ranged from 2.5 to 6 Å. The resulting glycoclusters presented a more than 10^5-fold increase over monovalent galactose, which had an IC_{50} of $\sim$100 mM in the same receptor-binding inhibitory assay.

Several glycopeptides, particularly those having mannosides or complex oligomannoside end-groups, have the potential to become entirely synthetic vaccines. More notable are the following examples directed at raising the protective immune response against HIV-1 infection. Noteworthy is the fact that several infectious microorganisms use or escape the immune defense mechanisms by masking important receptors or antigenic determinants through exposing self-carbohydrate structures.

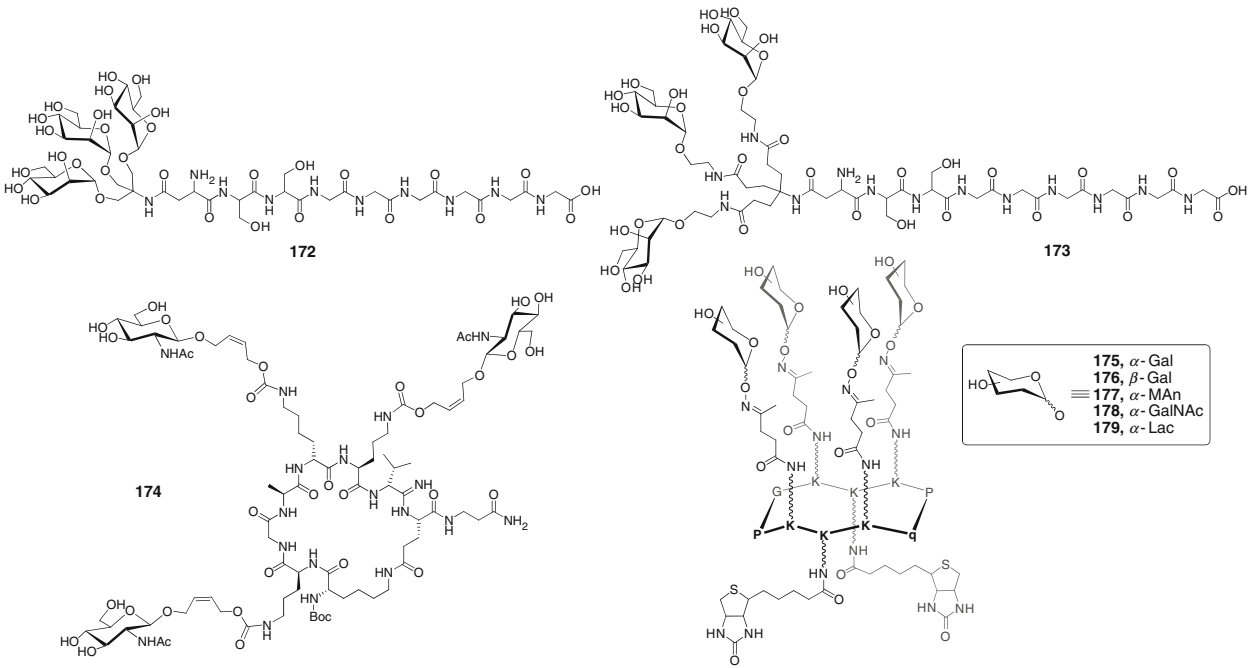


FIG. 21. Peptide-based glycoclusters at the wedge of linear peptide sequences or as cyclic scaffolds.

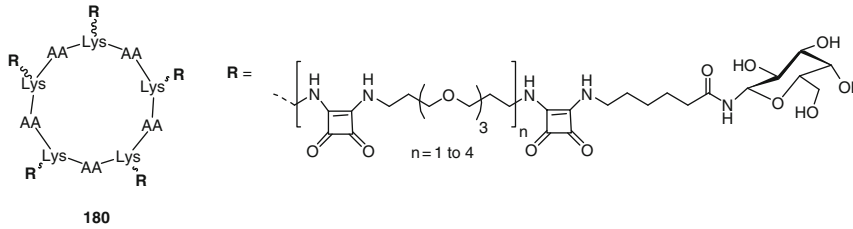


FIG. 22. Potent pentavalent D-galactosylamine inhibitors used by Fan's group for the cholera toxin B pentamer.[187]

The consequences of these ''masking'' events are that the bacterial or viral pathogens are transported to target tissues by self cellular systems. An emerging example of this irregular situation is the recognition, binding, and transport of the hyperbranched high-mannose oligosaccharide ($Man_9GlcNAc_2$) present on HIV gp120 by dendritic cells, with the ensuing transport of the ''hidden'' virus particles to lymph nodes.

Danishefsky's group using 14-residue cyclic peptides bearing β-turns provided by two D-Pro-L-Pro sequences appended at the extremity of the scaffolds (**181**), elegantly constructed the potent HIV-gp120 carbohydrate antigen **182**, which was strongly recognized by the human swapped and protective IgG 2G12 antibody (Fig. 23).[188] The cyclic peptide containing two or three handles for glycan attachment, together with a single cysteine residue for further carrier protein or biological marker conjugation, was built on a prolinated trityl resin using solid-phase peptide chemistry, while the synthetic oligosaccharide $Man_9GlcNAc_2$-NH_2, prepared by amination of the reducing sugar,[189] was attached to the aspartic acid residues properly positioned onto the cyclic peptides **181a** or **181b** using the Lansbury aspartylation procedure, thus providing dimer and trimer (**182**), respectively.[190]

Studies with SPR indicated that the control peptide lacking the saccharide portion, as well as the monovalent glycopeptides, showed no measurable response, thus confirming the importance of multivalent binding event in this critical recognition process. The corresponding divalent and trivalent glycopeptides proved to be strong ligands against the 2G12 bound surface, indicating topographical homology with the natural gp120 structure. With the confirmed antigenicity of both glycopeptides, the authors then used the sulfhydryl anchor of the divalent antigen for its attachment to the highly immunogenic, maleimido-derived outer membrane protein complex (OMPC) carrier from *Neisseria meningitidis*. The conjugate addition, performed at near-neutral pH, afforded a vaccine candidate bearing $\sim$2000 copies of the carbohydrate antigen which was also recognized by 2G12 antibody in an ELISA assay using

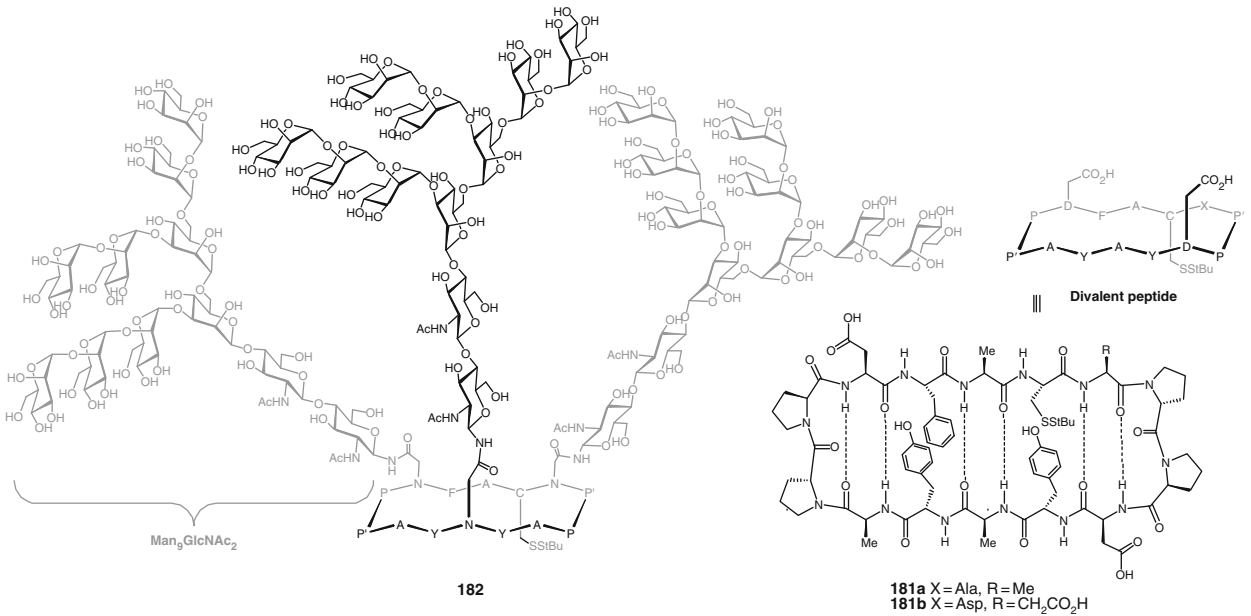


FIG. 23. Representative examples of a potential anti-HIV-1 gp120 conjugate vaccine.[188]

either gp160 or the glycoconjugate as coating antigens. Horse radish peroxidase-conjugated antihuman IgG (HRP-IgG) evidenced detection of the binding event. The results indicated that, while both antigens could bind to the 2G12 antibody, the natural gp160 bound more efficiently. The immunological properties of the semisynthetic vaccine are presently under investigation.

An analogous example has been provided for the construction of a tetrameric gp120 glycan epitope (**186**) by using the determined shortest version derived from the D1 arm of the $Man_9GlcNAc_2$ (**183**), and prepared as an extended azide-bearing aglycone **184** (Fig. 24).[191] The cyclic peptide scaffold **185** was built with 6 lysine, 2 glycine, and 2 proline residues. Four of the lysine ϵ-amino groups were acylated with propynoic acid for glycan attachment by ''click chemistry,'' and the remaining two ε-amines were coupled to the universal T cell epitope derived from tetanus toxoid 15-mer peptide $TT^{830-845}$ to afford a fully synthetic vaccine candidate **186**.

The binding of vaccine candidate **186** to the human antibody 2G12 was analyzed by SPR technology. While a synthetic monomeric oligosaccharide such as **184** as well as the natural high-mannose type N-glycan $Man_9GlcNAc_2Asn$ (**183**) did not show binding to antibody 2G12, the related synthetic oligosaccharide clusters carrying

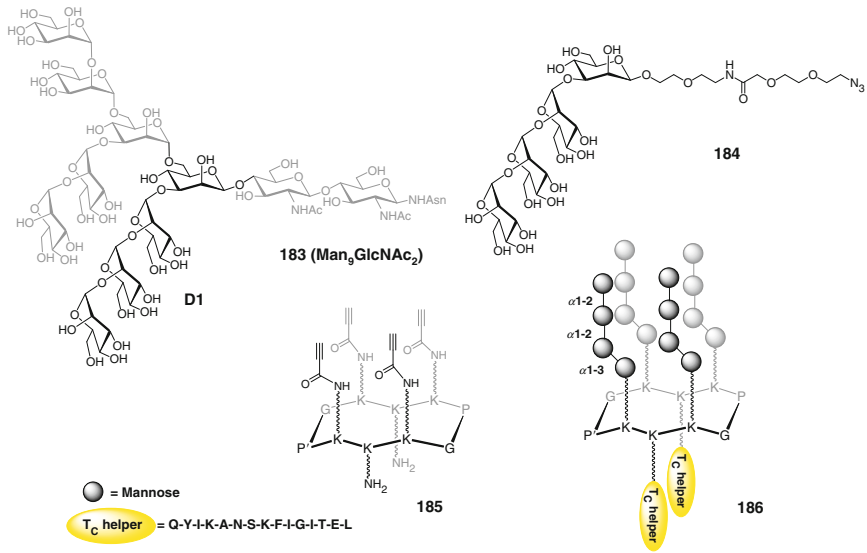


FIG. 24. Cyclic peptide vaccine candidate bearing the minimally epitopic D1 branch of the $Man_9GlcNAc_2$ antigen of HIV-1 gp120 recognized by the protective human antibody 2G12.[191]

four units of the D1 arm tetrasaccharide **186** have demonstrated affinity to this antibody. Furthermore, results suggested that an appropriate spatial orientation of the sugar chains in the cluster was crucial for high-affinity binding to antibody 2G12, and that the introduction of the T-helper epitopes onto the cyclic decapeptide template did not affect the structural integrity of the oligosaccharide cluster formed at the other face of the template. Therefore, compound **186** constitutes a valuable immunogen that might also be able to raise carbohydrate-specific neutralizing antibodies against HIV-1.

5. Other Glycoclusters

To better understand the critical role of oligosaccharide–receptor interactions and their molecular mechanisms through the cluster-effect, and thus access optimized synthetic ligands, several research groups have shown creativity in proposing original multivalent platforms that could allow for tailored valencies, dimensions, and epitope orientations.

For instance, Burke *et al*. investigated a templated ligand array based on a conformationally defined macrocyclic scaffold on which three mannoside residues, appended through a solubilizing linker, were displayed (Fig. 25).[192] The C_3-symmetric hydropyran cyclooligolide core was previously obtained according to an iterative multistep synthetic sequence notably involving key-step macrolactonization under Keck–Steglich high dilution conditions.[193] In particular, the template rigidity conferred a specific orientation of saccharide residues such that they emanate from a single face. In addition, molecular-modeling studies predicted a maximum separation between mannose residues of approximately 35 Å. Given that the binding sites within the Con A tetramer are separated by 65–70 Å, the trivalent ligand **187** could not simultaneously occupy two mannose-binding sites within the tetrameric lectin. Therefore, it could be used to explore mechanisms of multivalent ligand binding in the absence of the chelate effect. SPR competition-binding assay with Con A indicated the absence of nonspecific interactions induced by the template, and highlighted the fact that **187** promoted rather than inhibited binding of Con A to the mannose-substituted lipid surface. The data suggested that the glycocluster was able to bind two or three Con A tetramers simultaneously and to form soluble clusters with high avidity for immobilized ligands. Assay by FRET showed the reversibility of the phenomenon, and corroborated the fact that clustering of Con A by **187** did not depend on subsequent precipitation or cluster binding to a surface. In addition, this synergistic interaction favored the formation of a Con A cluster rather than the formation of a one-to-one complex of Con A and the trivalent ligand.

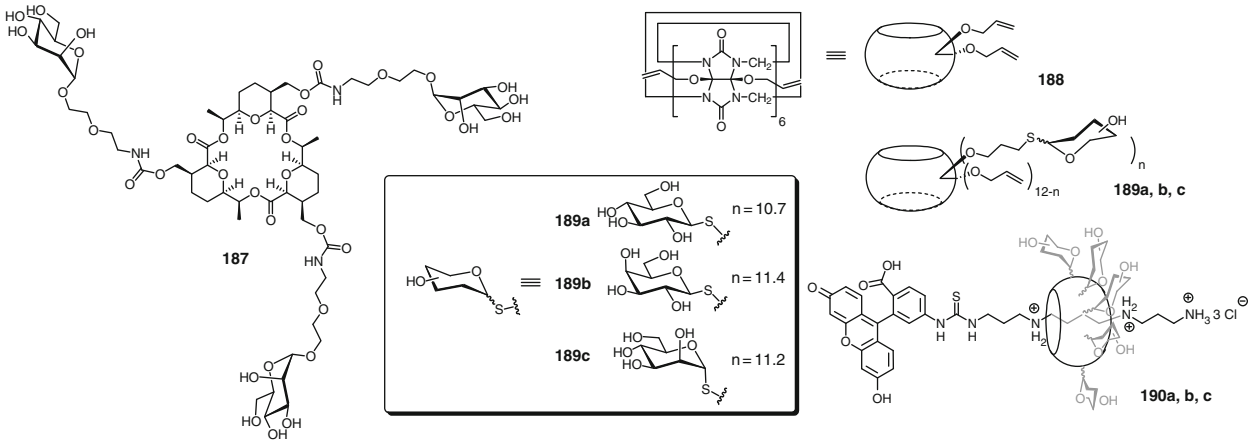


FIG. 25. Cucurbit[*n*]uril cavitand bearing sugar clusters and fluorescein probe.[194]

Another striking example has been provided which exploited cucurbit[n]urils (CB[n], $n = 5–10$) (**188**), a family of macrocyclic cavitands comprising n glycoluril units as multivalent scaffolds for carbohydrates.[194] They possess a hydrophobic cavity accessible through two identical carbonyl-fringed portals, and can form stable host–guest complexes with a wide range of guest molecules. Hence, CB[6]-based clusters **189a-c**, presenting an average of 11 carbohydrate moieties attached to the periphery (**189a**, β-glucose; **189b**, β-galactose; and **189c** α-mannose, respectively) were prepared via photoreaction of $(allyloxy)_{12}$CB[6] (**188**) with corresponding acetylthioglycosides, followed by standard O-deacetylation (Fig. 25). In particular, the mannoside cluster **189c** presented strong avidity for Con A and a 1100-fold excess of Me-αMan was necessary for the disruption of its cross-linking interaction with the lectin. ITC confirmed this affinity and indicated that **189c** behaved predominantly as a trivalent ligand to the lectin with a binding constant $K = (1.9 \pm 0.2) \times 10^{-5}\ M^{-1}$, which was 25 times higher than that for Me-αMan. The authors further exploited the cavity provided by such conjugates to incorporate, through host–guest interactions, the fluorescein isothiocyanate (FITC)–spermine conjugate **190** in order to estimate their potential as drug-delivery vehicles. Targeted delivery experiments *in vitro* were thus carried out with the (FITC)-spermine derivative as a fluorescent probe as well as a drug model, and the HepG2 hepatocellular carcinoma cells that overexpressed galactose receptors as target cells. Intracellular translocation has been estimated by confocal microscopy, and the results confirmed internalization of the CB[6]-galactose-(FITC)–spermine complex **190b** via galactose-mediated endocytosis. This study demonstrated the potential of glyco-cucurbituryl derivatives as targeted drug-delivery system and paved the way to other interesting therapeutic applications.

Touaibia and Roy have used biodegradable and biocompatible cyclophosphazene derivatives as multivalent platforms to afford densely functionalized α-D-mannoside clusters **191** (Fig. 26).[195] Use of a short and efficient strategy based on single-step Sonogashira and ''click chemistry'' has afforded a variety of glycoclusters around cyclotriphosphazene with different valencies, spacers, and epitope spatial arrangements. Hexavalent derivatives **191a–191d** were obtained from commercial hexachlorocyclotriphosphazene ($P_3N_3Cl_6$) onto which various phenol derivatives had been conjugated via nucleophilic substitution. Direct ligation of α-D-mannoside containing the adequate complementary function via optimized Pd-catalyzed cross-coupling or ''click chemistry'' and subsequent deprotection under Zemplén conditions afforded hexavalent conjugates in excellent yields.

Higher valency glycoclusters were also obtained by reaction of the hexabromomethylated P_3N_3 scaffold derivative with di- or triazide building blocks containing a hydroxy anchoring function (Fig. 27). Corresponding deprotected dodeca- and

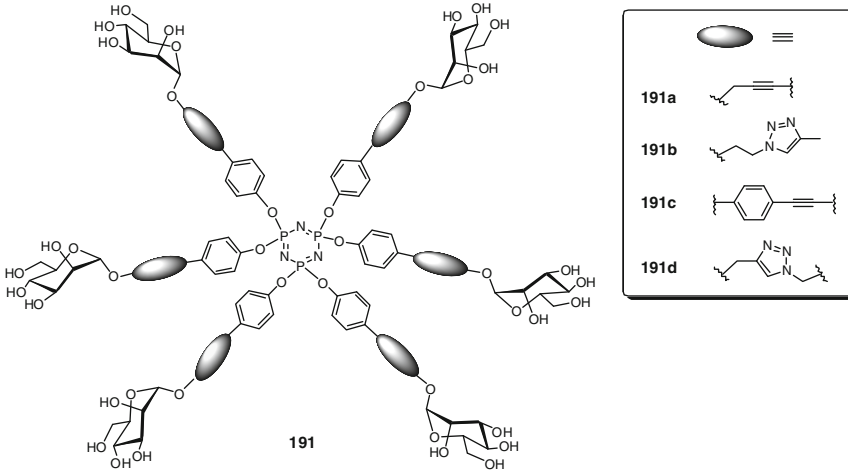
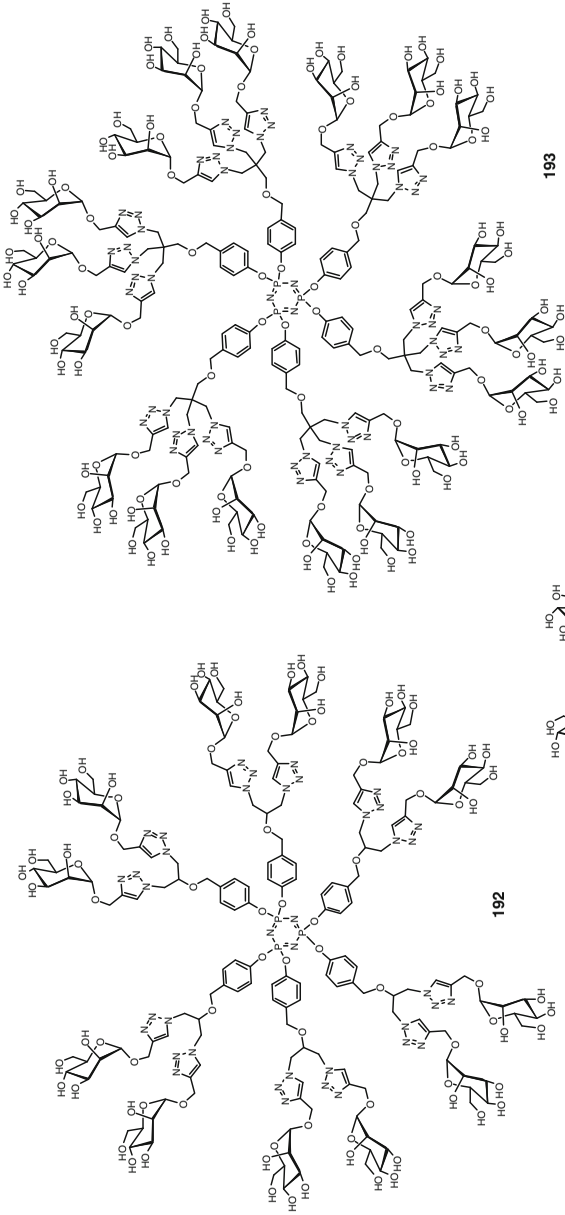


FIG. 26. ''Majoral-type'' glycodendrimers built around a cyclophosphazene core (P_3N_3).[195]

octadeca-mannoside clusters **192** and **193** were thereby isolated following click chemistry and O-deacetylation. Similarly, decamannoside **194** has been obtained from a dimer bearing an $N_3P_3Cl_5$ fragment prepared from the double monosubstitution of hexachlorocyclotriphosphazene with bisphenol A, using *n*-BuLi as a base and appropriate stoichiometry. Subsequent replacement of the remaining chlorides by iodoaryl groups allowed the preparation of the Sonogashira adduct **194**.

To evaluate the influence of structural parameters governed by the cyclophosphazene core concerning the valency and the spatial orientation of epitopes, as well as the nature of linkers directly related to the ligation technique used for the mannoside incorporation, the authors performed preliminary kinetic turbidimetric assays with Con A. Insoluble cross-linked complexes formed rapidly for all compounds, without marked difference for the hexavalent analogues. On the other hand, the incorporation of additional mannosyl units led merely to statistical binding-affinity enhancements, notably for the less-dense decamer **194**, which presents favorable extended intersugar distances.

The class of azamacrocycles also constitutes an interesting platform for controlled multivalent presentation of epitopes (Fig. 28). Although examples are quite scarce, their use has generated biologically relevant glycoclusters. For instance, the tetravalent α-mannosyl clusters **195** scaffolded on 1,4,8,11-tetraazacyclotetradecane (cyclam) have been described and showed interesting inhibitory properties in the adhesion of type-1 fimbriated *E. coli* to guinea pig erythrocytes[196] or binding properties toward Con A (**196**, **197**).[197]



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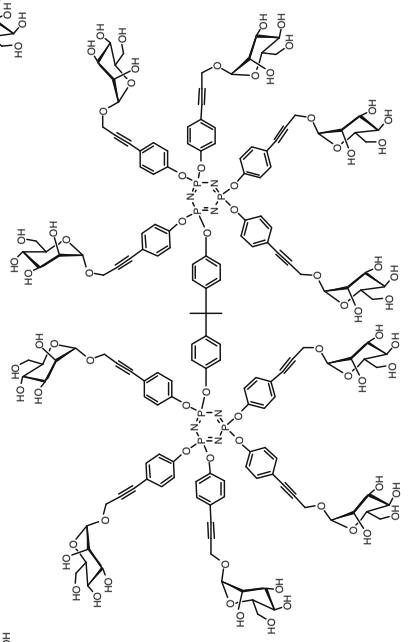

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FIG. 27. Mannosylated cyclophosphazenes prepared by Touaibia and Roy.[195]

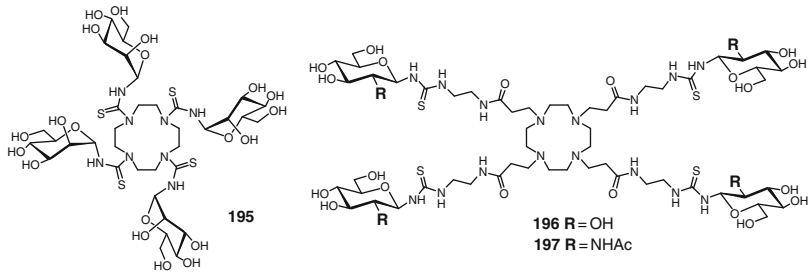


FIG. 28. Mannosylated clusters built around the azamacrocycle cyclam.[196,197]

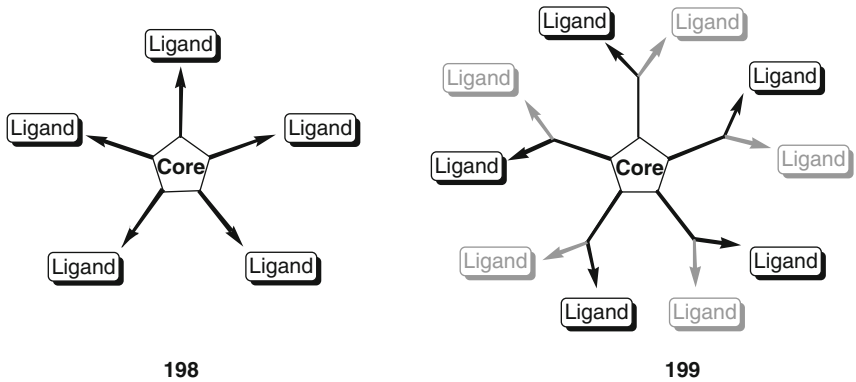


FIG. 29. Potent antiadhesins against the AB5 subunit of bacterial toxin.[198]

Significant enhancements have been obtained by Fan *et al*., who similarly included the pentavalent analogue (pentacyclen) into their arsenal of templates toward the high-affinity multivalent ligands **198** and **199** (Fig. 29).[198] The modular design of multivalent antagonists (antiadhesins) targeting members of the AB_5 bacterial-toxin family has been achieved, including CT and *E. coli* heat-labile enterotoxin (LT). The strategy exploited the fivefold symmetry of the binding sites on the toxins B pentamer.

This synthetic plan was based on difficulties in distinguishing ligand-mediated aggregation of the protein from an actual gain in effective affinity when highly multimeric ligands were studied. In this context, the authors expected substantial enhancement in affinity by using multivalent systems that allowed geometrically restrained presentation of the exposed ligands to match the specific arrangement of the target protein's binding sites. With this in mind, synthesis of various pentavalent derivatives **198** and **199** containing three distinct modules has been achieved.

A semirigid core, having ''fingers'' projecting outward in the direction of the receptor's binding sites and fitting perfectly into the active site have been assembled.

A study describing the syntheses of β-D-galactosylated pentavalent ligands built around a pentacyclen core and adopting a fivefold symmetry, and the systematic effects of flexible linkers has been published (Fig. 30).[198] Preliminary results detailed the affinity for LT, and the best pentavalent ligand **200** (R = R_1 with $n = 4$) showed an IC_{50} that was 10^5-fold higher than galactose. A few years later, the same group optimized the structure, incorporating five copies of the more potent *m*-nitrophenyl α-D-galactoside (MNPG) derivatives rather than five copies of β-D-galactose.[199] The $\sim$100-fold higher single-site affinity enhancement of MNPG for the binding site of the toxin relative to galactose was found to yield a proportionate increase in the affinity and IC_{50} measured for the respective pentavalent constructs. Indeed, ELISA experiments involving CTB pentamer revealed an IC_{50} of 0.9 μM for **201** (R = R^2), corresponding to about an 18-fold affinity enhancement over **200** (R = R^1). These results demonstrated that improved affinity for a single-site ligand confers a parallel improvement in the affinity of a pentavalent construct presenting five copies of the same ligand. Furthermore, from DLS studies and crystallographic data, it resulted that a 1:1 CTB/**201** complex was the major mode of association in solution between the ligand and the toxin. The lead structure **201** was then affined with the incorporation of guanidine-bridged poly(ethylene glycol)s of various lengths, in order to enhance its water solubility. Thus, compound **202** (R = R^3) having an optimized effective

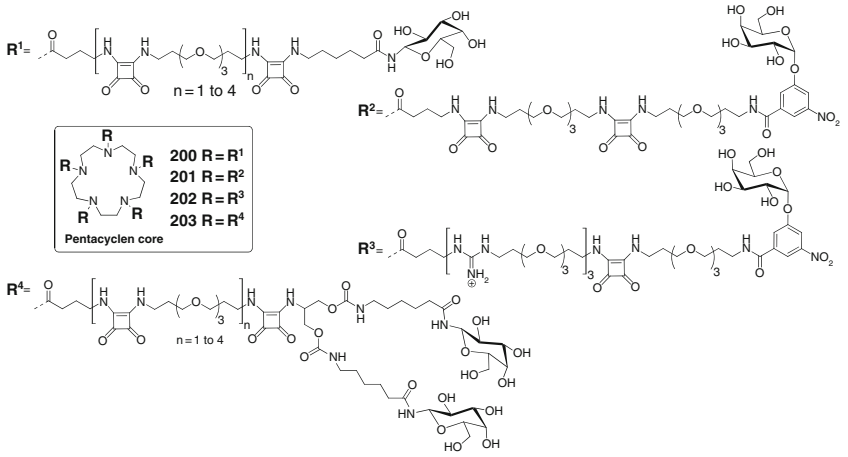


FIG. 30. The best ligands against *E. coli* heat-labile enterotoxin.[198–201]

dimension for strong affinity, displayed an IC_{50} of 6 nM, being lower than that of the natural receptor GM1, and which represented the first multivalent ligand based on a simple galactoside having nanomolar affinity against CTB.[200] During parallel investigations, spectacular improvement has been observed with the construction of a family of complex ligands having five flexible arms, each ending with a bivalent ligand **203** ($R = R^4$).[201] ELISA tests revealed that the improved decavalent derivatives were significantly more potent, showing affinities for CTB an order of magnitude better than the corresponding nonbranched ligands. More precisely, a more than 10^6-fold enhancement in inhibitory power over the monovalent ligand was achieved with the best decavalent candidate **203** (with $n = 4$) with an IC_{50} of 40 nM, a value that lies in the same range as the IC_{50} of the natural receptor (50 nM). Dynamic light-scattering studies demonstrated the formation of concentration-dependent unique 1:1 and 1:2 ligand–toxin discrete complexes in solution, with no sign of formation of large aggregates. Crystallographic studies confirmed that the decavalent inhibitor resulted in a ''sandwich arrangement'' of two B pentamers facing each other and bridged by the ligand. The improvement in IC_{50} displayed by the decavalent ligands might be attributed to a substantial difference in affinity between the galactose fragment within the pentavalent ligand and the rather short, nonspanning bivalent galactose moiety present in the decavalent ligand.

Finally, two other exotic scaffolds has been described that can be added to the large panel of glycoclusters described so far. The first consists in linear pentaerythrityl phosphodiester oligomers (PePOs) onto which galactosyl clusters were attached via combinatorial and automated synthesis on a solid support (Fig. 31).[202] The propargylated scaffold was synthesized by standard DNA solid-phase supported phosphoramidite chemistry, and azido-galactoside residues were conjugated by ''click chemistry.'' Microwave-assisted acceleration of the reaction time afforded the corresponding multivalent clusters **204a,b** with controlled length and valency. The elongated fucosylated analogues **205** have also been described by the same authors, and showed increased affinity in ELLA competition assay for *P. aeruginosa* lectin (PA-IIL), with IC_{50} values 10–20 times higher than the monovalent L-fucose but corresponding to only a modest twofold increase on a per saccharide basis.[203]

The second one concerns polyhedral oligosilsesquioxanes (POSS) that have been functionalized with carbohydrate moieties **206–208** via standard amide bonds[204] or more efficiently via photolytic thiol addition from a perallylated precursor.[205] Biological evaluation of the octavalent POSS-glycocluster **208** exhibiting elongated lactoside residues was investigated by measuring the inhibitory effect on the binding of asialo-oligosaccharides from human α1-acid glycoprotein (AGP) by RCA120 (a β-galactose specific lectin), using capillary-affinity electrophoresis. The first

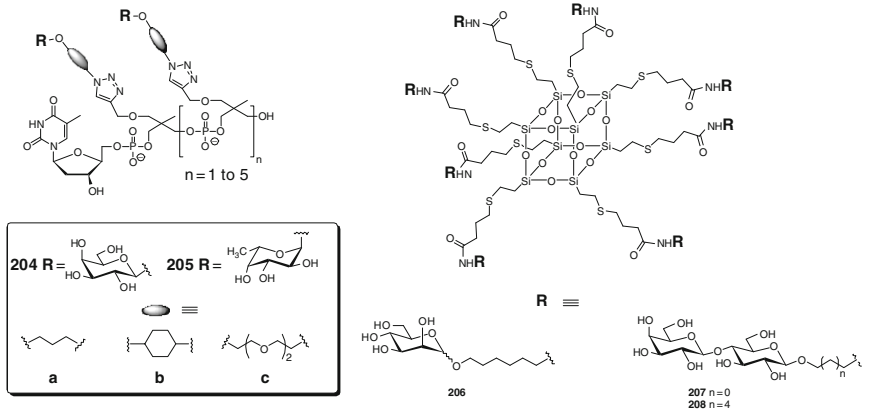


FIG. 31. Various multimeric glycoclusters.

results indicated strong inhibition, ~200 times higher than that induced by free lactose and being thus attributable to the cluster effect.

III. GLYCOSYLATED CARBON-BASED NANOSTRUCTURES

The past few years have witnessed the discovery, development and, in some cases, large-scale production and manufacturing of novel materials that lie within the nanometer size scale. More particularly, the incorporation of nanotechnologies in early-stage development of new drugs, diagnostics, or therapeutics constitutes a powerful addition to the arsenal of classical macromolecular structures. Consequently, this strategy opens new avenues for the original and efficient designs of adapted nanomedicine. In this context and owing to their three-dimensional architectures, the fullerenes and nanotubes (both carbon-based nanomaterials), constitute promising candidates for novel nanometric constructs having biological properties and offering challenging scaffolds toward multivalent presentation of saccharide units.

1. Glycofullerenes

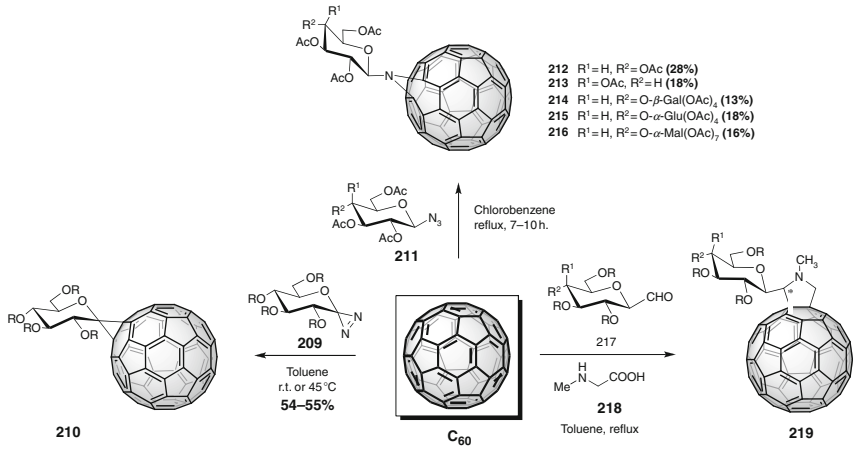
Fullerenes, the third allotropic form of carbon along with graphite and diamond, are a novel class of spheroidally shaped molecules made exclusively of carbon atoms. They have generated much enthusiasm and numerous research efforts during the past few years.[206] Hence, the chemical and physical features of C_{60}, also named

Buckminsterfullerene, the most representative example among the fullerenes, have been extensively explored. Their intrinsic properties such as their size, hydrophobicity, three-dimensionality, and electronic properties have made them extremely promising nanostructures, offering interesting features at the interface of various scientific disciplines, ranging from material sciences[207] to biological and medicinal chemistry.[208–210]

In order to demonstrate and illustrate their attractive potential in the foregoing applications, an array of studies has been recorded, including cytotoxicity investigations on both unmodified and functionalized fullerenes. Initial results have indicated that these novel and fascinating architectures were not carcinogenic when applied to the skin, nor did they affect the proliferation and the viability of cells when they are internalized. Hence, despite an observed dose-dependent toxicity phenomenon reported for certain related derivatives, the early observations indicate great promise for applications in DNA cleavage, PDT, enzymatic inhibition, antiviral, antibacterial, and antiapoptotic activity.[209–211] However, the total lack of solubility in aqueous or physiological media is a severe drawback for their quick and efficient development as suitable carriers. In order to circumvent the natural repulsion of fullerenes for water, several methodologies have been adopted, including their entrapment into tailored microcapsules, their suspension with the help of cosolvents, and their chemical derivatization, notably their introduction onto peripheral solubilizing appendages. Furthermore, it has been shown that the multivalent presentation of polar groups around the fullerene spheres can prevent clustering phenomena in reasonably dilute solutions and consequently increase the hydrosolubility of the resulting conjugates. In this context, a variety of chemical functionalities have been utilized to increase both the hydrophilicity (with groups such as OH, CO_2H, NH_2, quaternary ammonium, and CD) and to prepare novel compounds possessing biological and pharmacological activity.

Among the panel of known fullerene derivatives, the fulleroglycoconjugates (also termed glycofullerenes) exhibit a combination of interesting properties related to water solubility and biological relevance. Spherical topology of the fullerenes has furnished suitable scaffolds for multivalent presentation of peripheral carbohydrate residues, and chemistry has been adapted for their efficient and controlled conjugation. The first examples of glycofullerenes were monovalent conjugates which were subsequently tailored and optimized to generate multivalent glyconanostructures based on C_{60} where biological properties were enhanced by taking advantage of the ''glycoside cluster effect.''

a. Monovalent Structures.—In the early 1990s, Vasella *et al.* reported the first synthesis of a monoglycosylated fullerene, introducing one carbohydrate residue on C_{60} via the nucleophilic glycosylidene carbene precursors **209** and generating the

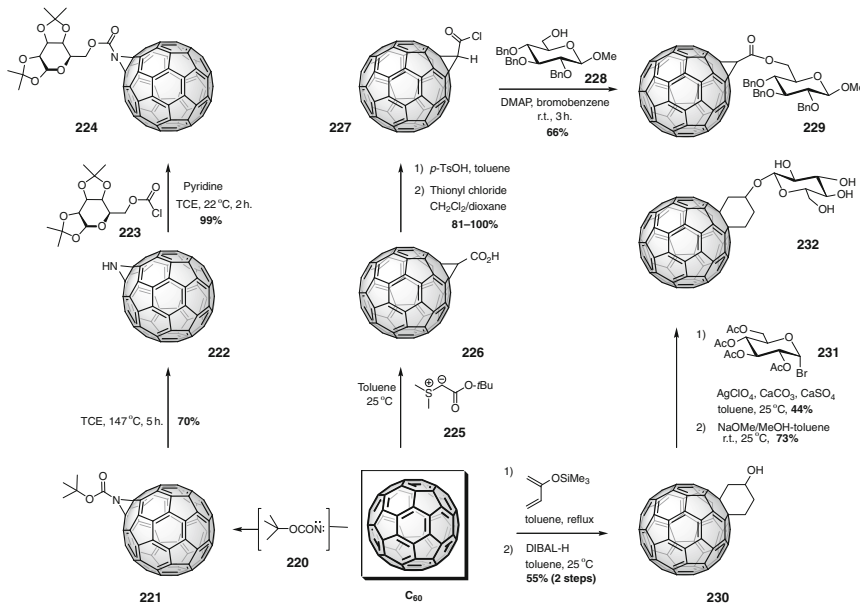


SCHEME 19. Synthesis of monovalent fulleroglycoconjugates directly introduced onto C_{60}.

enantiomerically pure spiro C-linked glycosyl-C_{60} derivatives **210** (Scheme 19).[212] Another approach permitted synthesis of fullerene glycoconjugates via the thermal cycloaddition of the per-*O*-acetyl glycosyl azides **211** in boiling chlorobenzene.[213] Although this methodology generated a mixture of two inseparable stereoisomers of *N*-*β*-glycopyranosyl [5,6]azafulleroids in relatively poor yields (13–28%), the generality of the reaction has been demonstrated with a series of mono- (**212**, D-glucopyranose and **213**, D-galactopyranose), di- (**214**, lactose and **215**, maltose), and trisaccharide (**216**) conjugates to C_{60}.

Soon afterwards, Dondoni *et al.* developed a three-component approach involving C_{60}, a carbohydrate aldehyde (**217**), and *N*-methylglycine **218** (sarcosine) leading to the formation of glycofulleropyrrolidine monocycloadducts (**219**) in poor yields (10–14%), via 1,3-dipolar cycloaddition reaction of the intermediate azomethine ylide to C_{60}.[214] The feasibility has nevertheless been demonstrated with the use of a series of 1-deoxy-1-*C*-formyl derivatives of galactopyranose, glucopyranose, and mannofuranose. Moreover, this methodology has been advantageously adapted for synthesis of C_{60} derivatives containing a 6-(*β*-D-glycopyranosylamino)pyrimidin-4-one unit.[215]

Alternative strategies utilizing the initial introduction of suitable chemical functionality onto the fullerene have been adopted by several research groups, allowing conjugation of the saccharides with complementary functions in the final synthetic steps. This approach has led to improved flexibility with the chemical functions on the carbohydrate moieties, especially the anchoring ones, and a better control on the number of attached saccharide residues.



SCHEME 20. Monovalent fulleroglycoconjugates introduced by preactivation of C_{60}.

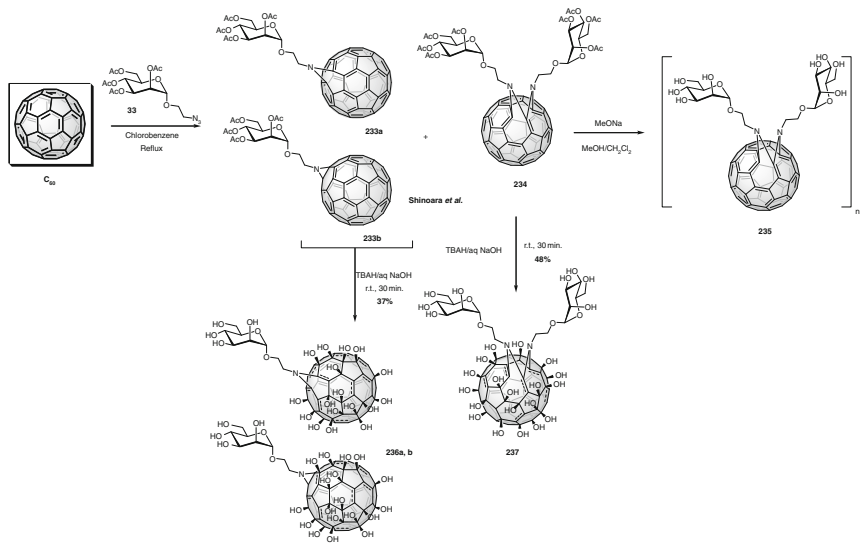
In this context, Banks *et al*. proposed the synthesis of glycofullerenes based on the use of aziridino[2′,3′:1,2][60]fullerene (C_{60}NH) (**222**) (Scheme 20).[216] Its preparation in good yield (55–60%) involved the initial formation of *N*-*tert*-butoxycarbonylaziridinol[2′,3′:1,2][60]fullerene **221**, generated by *in situ* trapping of the intermediate nitrene (*t*BuO_2CN:) **220** by C_{60}. Use of the D-galactose chloroformate derivative **223** through direct acylation under mild conditions led to the desired galactofullerene **224** in quantitative yield.

Another example has been provided by Ito *et al*., who described the use of methanofullerene derivatives as powerful and stable precursors for glycofullerenes.[217] Their study was based on the use of [60]fullerenoacetyl chloride (**227**), obtained from the *tert*-butyl [60]fullerenoacetate derivative **226**, which had been prepared in 56% yield by treatment of corresponding stabilized sulfonium ylides **225** with C_{60}.[218] Subsequent transformation with *p*-TsOH in toluene gave [60]fullerenoacetic acid, which was directly converted into the corresponding acyl chloride **227** by using thionyl chloride. Standard ester formation with methyl 2,3,4-tetra-*O*-benzyl-*β*-D-glucopyranoside (**228**) and 4-(dimethylamino)pyridine (DMAP) afforded the desired hybrid derivative **229** in 66% yield.

An additional study investigated the DNA- and protein-degradation properties of the novel glycofullerene derivative **232**.[219] Its synthesis was based on a Diels–Alder reaction of 2-trimethylsilyloxy-1,3-butadiene with $\mathbf{C_{60}}$, followed by the reduction of the cyclohexanone derivative with DIBAL-H in toluene to afford the corresponding racemic cyclohexanol **230** in excellent yield.[220] Glycosylation with glycosyl bromide **231** in the presence of $AgClO_4$, $CaCO_3$, and $CaSO_4$ in toluene and subsequent deprotection under Zemplén conditions gave the desired glycofullerene **232**, which was shown to effect selective degradation of the HIV-protease by photoirradiation.

Synthetic aspects for access to monovalent fullerene–carbohydrate hybrids were highlighted, but only a few biological applications were mentioned. In contrast, multivalent presentation of saccharides by multiple anchorages to the same structure, or their presentation as antennary glycodendrons, has generated promising results.

b. Multivalent Structures.—Divalent hybrid glyconanostructures have been prepared via the [3+2]-cycloaddition reaction between 2-azidoethyl glycopyranoside derivatives and C_{60}. The first example described a strategy for synthesis of [60] fullerenols carrying mono- and bis-α-D-mannopyranosides (Scheme 21).[221] The methodology was based on initial thermal coupling of 2-azidoethyl mannopyranoside (**33**) and an equimolar amount of $\mathbf{C_{60}}$ in chlorobenzene, giving a mixture of two isomeric

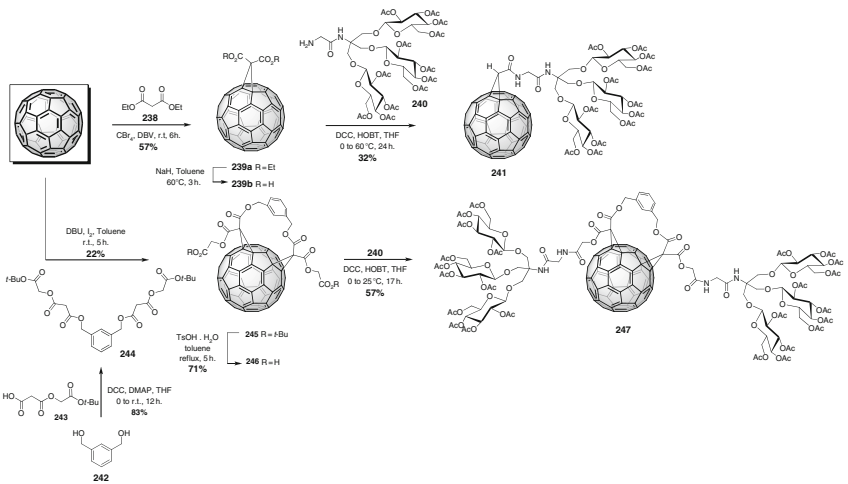


SCHEME 21. Synthesis of mannosylated fulleroglyco hybrids by [3+2]-cycloaddition of glycosyl azide onto C_{60}.[221–224]

monoadducts (α-D-mannosyl [5,6]-azafulleroid, **233a**, and α-D-mannosyl [6,6]-aziridinofullerene, **233b**)[222] and a bismannosylated adduct **234**. Subsequent simultaneous deacetylation and polyhydroxylation were then conducted in the presence of tetra-*n*-butylammonium hydroxide (TBAH) in aqueous NaOH, to afford deprotected mono- (**236**) and bisfullerenols (**237**) with an average of heterogeneously distributed 29 OH groups per C_{60}. Biological evaluations, notably involving lectin-induced HAI assay with an α-D-mannoside-specific lectin (Con A), revealed that mono- and bis-mannosyl fullerenols exhibited diminished activity for both binding to Con A and aggregating erythrocytes as compared to the activity of mannosylated neoglycopolymers. A few years later, a similar grafting methodology was used to generate a series of mono- and bis-sugar-pendant [60]fullerene derivatives prepared from a variety of carbohydrate-linked azides.[223] The phototoxicity of the corresponding deprotected glycoconjugates was evaluated for potential applications in PDT. Substantial production of singlet oxygen ($^1O_2$) under laser irradiation (355 nm) was observed for both adducts, notably for the monosugar derivatives, which shown a better photosensitizing ability. In addition, *in vitro* studies involving HeLa cells confirmed their photocytotoxicity and indicated a carbohydrate-dependent efficiency. In line with previous work, another application has been proposed which described the formation of stable self-assembling structures in aqueous solvents from the deprotected bis(α-D-mannopyranosyl)-[60]fullerene conjugate **235**.[224] The diameters of the resulting large aggregates (100–300 nm), resembling bilayer vesicles or unadulterated liposomes, were determined by DLS and AFM. These supramolecular structures were able to encapsulate Ba^{2+} ions and such organic molecules as Acridine Red, constituting for instance, promising candidates for slow drug delivery. The ability of these mannofullerenes to bind to a mannoside-recognizing lectin (Con A) was also investigated. Colloidal suspensions of the stable self-assembling structure **235** presented higher blocking activity than the two monoadducts, with an interesting submicromolar MIC (minimum inhibitory concentration) value in a lectin-induced hemagglutination assay. This strong protein-binding activity has been rationalized by the authors by the fact that the fullerene scaffold allowed a spatial arrangement of the bis(mannopyranoside) moiety that could mimic 3,6-branched α-D-trimannoside, a natural ligand of Con A. Unfortunately, this methodology often generates mixture of diversely substituted adducts, difficult to separate, in rather low yields and presenting only very limited solubility in water.

An alternative synthetic approach, first developed by Bingel[225] allowed the efficient nucleophilic cyclopropanation of fullerenes via their reaction with bromomalonate derivatives in the presence of base. This approach, the most reliable method for the synthesis of functionalized methanofullerenes, combined the advantages of mild

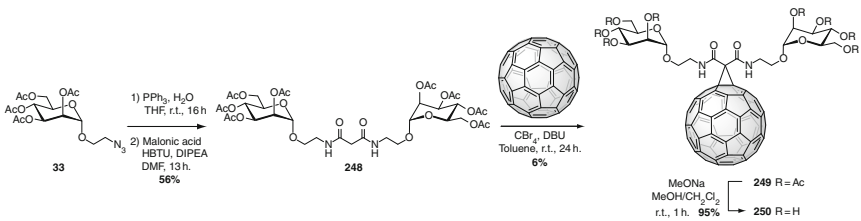
reaction conditions, good yields, and exclusive formation of [6,6]-bridged adducts. Furthermore, the degree of functionalization could be controlled by the stoichiometry of the malonate derivatives for access to higher adducts in one step (bis up to hexakis). This methodology suffers, however, from the complexity of preparing the bromomalonates, often generating mixtures of mono- and di-bromo derivatives with similar chromatographic properties. An optimized methodology has then been proposed by Camp and Hirsch that avoids the use of bromomalonates by direct treatment of the fullerene with malonates in the presence of CBr_4 (or I_2) and DBU (1,8-diazabicyclo-[5.4.0]undec-7-ene).[226] It has been exploited in collaborative work describing the synthesis of amphiphilic glycofullerodendrimers and their incorporation into Langmuir and Langmuir–Blodgett films (Scheme 22).[227] The synthesis of the first C_{60}-dendrimer conjugate containing one glycodendron headgroup started from the treatment of C_{60} with diethyl malonate (**238**) in the presence of CBr_4 and DBU to furnish the monoadduct **239a** in 57% yield (Scheme 22). A clean formation of **239b** from its diester **239a** was accomplished in toluene with a 20-fold molar excess of NaH at 60 °C.[228] DCC (*N*,*N*'-dicyclohexylcarbodiimide)-mediated double amide bond formation between **239b** and the O-acetylated trisglucoside wedge **240**,[229] accompanied by monodecarboxylation of the starting material allowed the formation of glycofullerodendron **241** in 32% yield. The bisadduct **247** was synthesized via the same synthetic pathway through preliminary coupling with DCC–DMAP-mediated esterification of *m*-benzenedimethanol (**242**) with malonic acid monoester **243** in 83% yield.



SCHEME 22. Synthesis of C_{60} malonates by Bingel's procedure, followed by glycodendronization.[227]

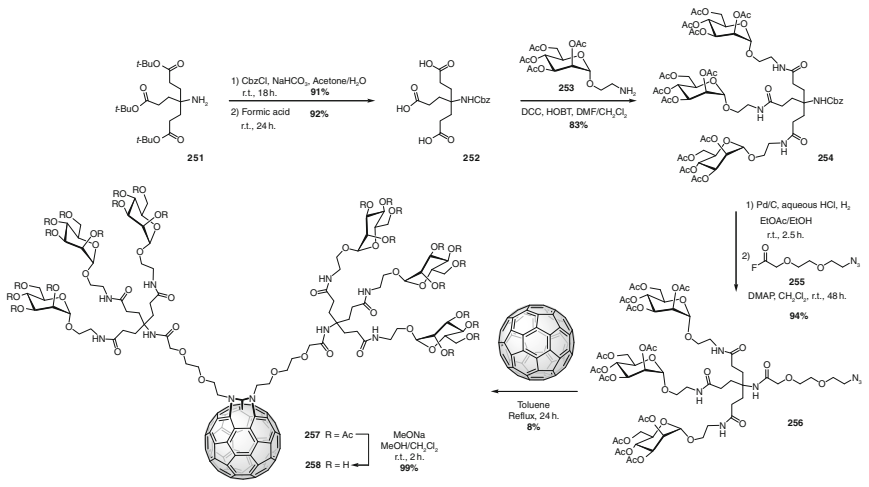
The resulting bismalonate derivative **244** was then engaged in double-Bingel macrocyclization with C_{60} to afford the C_s-symmetrical *cis-2* bisadduct **245** in 22% yield. Subsequent selective cleavage of the *tert*-butyl ester under acidic conditions provided the desired diacid core **246**,[230] which was finally treated with the glycodendron **240** under the foregoing conditions to yield glycofullerodendrimer **247** exhibiting six peripherally protected glucopyranoside residues. With this work, the authors highlighted the preponderant role of the bulky glycodendron headgroups on C_{60} that formed a compact insulating layer around the carbon sphere, avoiding the general propensity of amphipilic fullerene derivatives to aggregate irreversibly. Hence, both amphiphilic fullerenes **241** and **247** were able to form stable, ordered monomolecular Langmuir layers at the air–water interface and showed reversible behavior in successive compression–expansion cycles. In addition, the monolayers were transferred successfully as X-type Langmuir–Blodgett films onto quartz slides for anticipated applications as biosensors.

The group of Hirsch has reported the synthesis of ''sugar balls'' according to the two distinct methodologies already mentioned, but involving the direct grafting of glycosylated dendrons onto C_{60} (Scheme 23). They also described their supramolecular assembly in aqueous solution.[231] In this context, bis(α-D-mannopyranosyl)fullerene (**250**) has been synthesized by a sequence involving the bis-(α-D-mannopyranosylated malonate) **248**. This resulted from a peptide-coupling reaction between the corresponding 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside, quantitatively obtained from corrersponding azide derivative **33** after Staudinger reduction, and malonic acid, in 56% yield. The subsequent nucleophilic cyclopropanation of C_{60} with **248** under typical Bingel–Hirsch conditions, followed by O-deacetylation of **249** under Zemplén conditions afforded fully deprotected [6,6]-monoadduct **250** in an overall isolated yield of ~6%. It is noteworthy that the protected architecture, presenting robust amide linkages around the malonate anchor points, tolerated basic cleavage of the acetyl protecting groups without side reactions or decomposition of the fullereno sugar.



SCHEME 23. Synthesis of a bismannoside-C_{60} adduct using the Bingel–Hirsch procedure.[231]

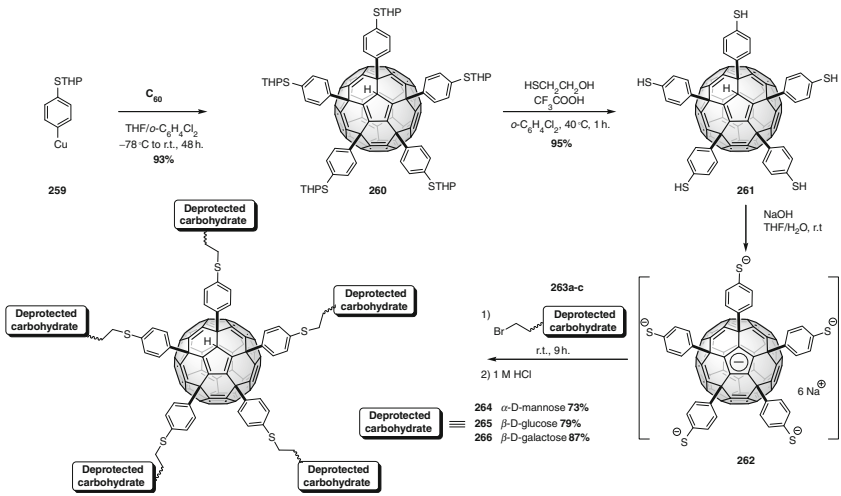
Unfortunately, its relatively low solubility in aqueous media compelled the authors to enhance the number of peripheral saccharidic units. Toward this goal, they developed fullerene glycoconjugate **258**, where two dendritic branches terminated by six deprotected α-D-mannopyranosyl building blocks were connected through two adjacent imino bridges to the all-carbon framework (Scheme 24). Moreover, in this type of C_{60} adduct, which constitutes a 1,9-dihydro-1a-aza-1(2)a-homo(C60-I_h)[5,6]fullerene derivative, the entire 60-π-electron system of the fullerene core was retained. For its preparation, AB_3 glycosylated dendritic building blocks were first synthesized by preliminary protection of Newkome's dendron **251**[232] with benzyl chloroformate (CbzCl), then treatment with formic acid for orthogonal and quantitative cleavage of the *tert*-butyl ester to give triacid **252** upon which, peptide coupling with 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **253** provided trimannoside **254** in 83% yield. Selective removal of the Cbz protecting group by hydrogenolysis gave the corresponding amine in quantitative yield, which was engaged without further purification with a suitable linker bearing a terminal azide group, namely 2-[2-(2-azidoethoxy) ethoxy]acetyl fluoride (**255**), in the presence of DMAP in CH_2Cl_2 to provide the azido oligo(ethylene glycol)-terminated glycodendron **256** in 94% yield. Its subsequent thermal coupling with C_{60} in toluene afforded a mixture of mono- and bisglycodendron adducts. Separation and purification by column chromatography gave regioselectively the twofold cluster-opened diazabishomofullerene adduct **257**,



SCHEME 24. Direct anchoring of azide-bearing mannosylated dendrons onto C_{60}.[231]

but in only 8% yield. Finally, quantitative deprotection under standard Zemplén conditions provided **258**. This C_{60} glycoconjugates having six peripheral mannoside residues is the most water-soluble fullereno sugar (> 40 mg/mL) known. Its amphiphilic nature, with a cone-shaped structure, triggered the formation of small supramolecular aggregates in aqueous solutions (observed in DOSY NMR and corroborated by TEM investigations), as uniform spherical micelles with an extremely narrow size distribution. These micellar sugar balls, with a diameter below 5 nm, furnish an original arrangement of saccharides that opens the gate to new biomedical applications.

Another fruitful methodology, initially developed by Nakamura's group[233] allowed the introduction of five carbohydrate moieties onto a C_{60} scaffold using the efficient synthesis of C_5 symmetric fullerene derivatives (Scheme 25).[234] A thiolate–alkyl halide coupling reaction in aqueous basic media provided their one-step synthesis in good yields, taking advantage of the high nucleophilicity of the thiolate anions generated. Synthesis of the key fulleropentathiol intermediate **261** involved the fivefold addition reaction of the copper derivative **259**, prepared *in situ* from the corresponding Grignard reagent and $CuBr{\cdot}SMe_2$. The desired fullerene cyclopentadiene bearing tetrahydropyranyl-protected thiophenol moieties (**260**) was obtained in 93% isolated yield, which was then deprotected in *o*-dichlorobenzene in the presence 2-mercaptoethanol and trifluoroacetic acid to afford the pentathiol derivative **261** in

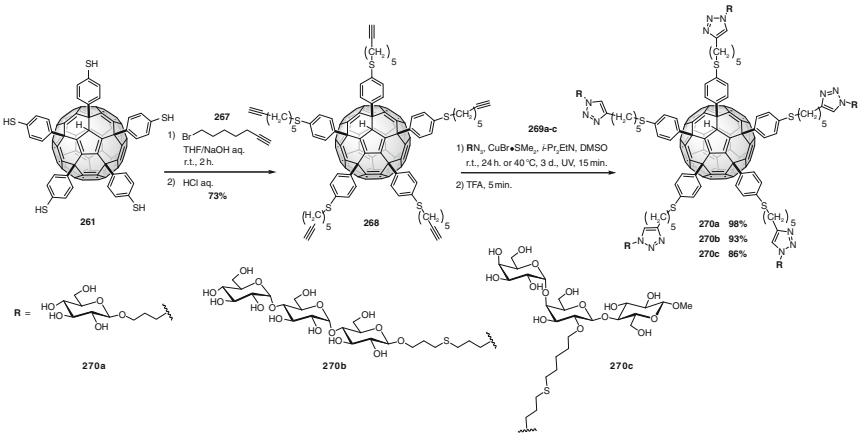


SCHEME 25. Pentavalent fulleroglycoconjugates.[234]

95% yield. Subsequent installation of five saccharide moieties via a nucleophilic substitution reaction in aqueous THF with 6 equivalents of sodium hydroxide and 2-bromoethyl glycopyranosides **263a–c** afforded the fullerene glycoconjugates α-D-mannoside **264**, β-D-glucoside **265**, and β-D-galactoside **266**, in 73%, 79%, and 87% yields, respectively.

Despite the generality of this methodology for simple carbohydrate derivatives, its application for introduction of larger oligosaccharides via direct nucleophilic substitution produced side products and led to lower yields. To circumvent this situation, the same authors described the optimization and adaptation of this methodology for the quantitative synthesis of fullerenes bearing five saccharides, such as the glucose **270a**, maltotriose **270b**, and globotriaosylceramide Gb3-**P^k** trisaccharide **270c** derivative (Scheme 26).[235] Hence, more-sophisticated nanometer-scale pentavalent molecular architectures could be readily prepared by use of the key pentaalkynylfullerene intermediate **268** obtained directly by the derivatization of fulleropentathiol **261** with 7-bromohept-1-yne **267** in 73% yield under the foregoing conditions. Click chemistry allowed efficient conjugation of the functionalized saccharide moieties with a terminal complementary azide function (**269a–c**) on the pentaalkynylated core **268** under mild conditions. Hence, in the typical presence of CuBr.SMe_2 and DIPEA in Me_2SO, deprotected fullero-derivatives **270a–c** were obtained in nearly quantitative yields through optimized conditions involving controlled heating or microwave irradiation.

Similarly to the STARFISH design already described (Fig. 17), the peripheral and radial presentation of five **P^k**-trisaccharide residues presenting tailored spacers could



SCHEME 26. Alkylation of fulleropentathiol by an alkyne spacer followed by ''click chemistry'' with various azido sugars, including the **P^k** trisaccharide.[235]

furnish adapted structures for efficient and specific interactions with SLTs. In line with promising results obtained with the functionalization of fullerenes, several research groups have recently described the derivatization of carbon nanotubes (CNTs) with biologically relevant carbohydrates.

2. Glyconanotubes

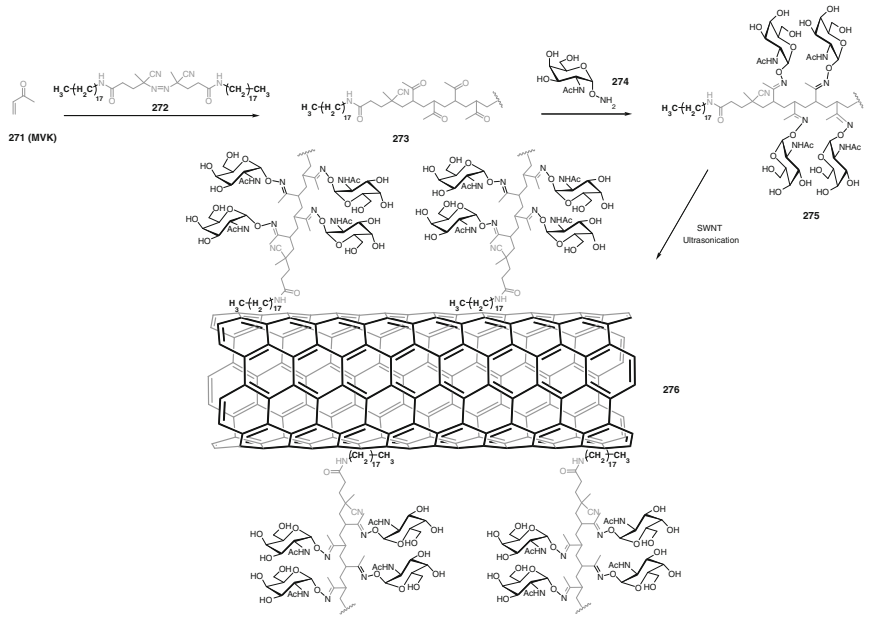
CNTs are members of the fullerene structural family and consist exclusively of carbon atoms arranged in a series of condensed benzene rings, organized in graphitic sheets that are rolled up into a tubular structure.[236] CNTs made up of a single graphene layer wrapped into a cylindrical structure constitute single-walled CNTs (''SWNTs''), whereas multiwalled CNTs (''MWNTs'') are generated from a central tubule of nanometric diameter and surrounded by several graphitic layers spaced by a distance of about 0.34 nm. Most commonly, the diameter range of CNTs varies between 0.4 and 2 nm for SWNTs and between 1.4 and 100 nm for MWNTs, while their length is in the range of micrometers (up to 100 nm). Moreover, CNTs typically form bundles that are entangled together in the solid state, giving rise to a highly complex ''spaghetti'' network.[237] Intrinsically, CNTs possess very interesting and unique physicochemical properties such as: high surface area, ordered structure with a high aspect ratio, ultralight weight, excellent chemical stability, high electrical conductivity, high thermal conductivity, and metallic or semiconducting behavior according to the arrangement of the hexagon rings along the tubular surface. For these reasons, these nano-objects have raised great enthusiasm and expectations in many different applications, including material, biological, and medical sciences.[238] More particularly, their intriguing structures and properties have led to the emergence of functionalized CNTs as new biologically relevant alternatives for therapeutic and diagnostic applications,[239] including their use as vectors for delivery of therapeutic molecules[240] (plasmid gene delivery,[241] peptides/proteins,[242] and antibiotics[243]). Indeed, prior to become viable and effective nanomedicines, the toxicological and pharmacological profiles of CNTs had been determined by several research groups and reviewed by Lacerda *et al.*[244] As with biological studies on fullerenes, functionalized CNTs offer potential as nanomedicines, whereas nonfunctionalized CNTs usually contain amorphous carbon, carbon nanoparticles, and residues from metal catalysts, regardless of the procedures for their production. Indeed, their inherent hydrophobic properties, associated with their innate propensity to form bundles via aggregation through van der Waals forces, constitute major technical barriers for their utilization in medicinal applications. To overcome these drawbacks, modification of

their surface is typically achieved by adsorption, electrostatic interaction, or covalent bonding of different molecules and chemical procedures that render them more soluble in biological media.[245] Such modifications generally improve the water solubility of CNTs, decrease the aggregation phenomenon, and transform their biocompatibility and biodistribution profiles.

It has been shown experimentally that such bioactive proteins, nucleic acids, genes, and more particularly carbohydrates can be successfully conjugated to CNTs. In particular, because of versatile modes of chemical modification and solubilization, the one-dimensional nanostructure of SWNTs, with high surface area-to-weight ratio and some structural flexibility, can be exploited as platforms for multivalent carbohydrate arrays under physiological conditions. The resulting densely attached sugars, as pendant groups on the CNT hybrids, serve as multivalent carbohydrate ligands and show potential biological activities as well as behaving as glycoconjugate polymers. Multivalent presentation of saccharidic residues has been realized via noncovalent stabilization processes through favorable hydrophobic, π–π stacking or electrostatic interactions, or via sidewall- or defect-targeted covalent functionalization. Several groups have reported multivalent carbohydrate–CNTs conjugates, such schizophyllan polysaccharides, lipid-terminated glycopolymers, and carbohydrate ligands toward bacterial toxins and viral proteins.

a. Glyconanotubes from Noncovalent Interactions.—The initial challenge of obtaining SWNTs soluble in organic or aqueous media by a supramolecular approach was motivated by the necessity for retaining their intrinsic physical properties without affecting the all-carbon scaffold. The first successful example was realized through noncovalent functionalization by wrapping synthetic and biocompatible water-soluble polymers around the SWNTs. The formation of supramolecular complexes in solution between SWNTs and the preformed helical structure of starch (a polymer composed of the linear amylose and branched amylopectin) has been investigated.[246] The enhanced water solubility of the supramolecular complexes was significant, and further investigations suggested that the presence of the preformed helical structures of amylose may not be required.[247] Other studies examplifying complexation of the SWNTs with CDs have also yielded some interesting results.[248]

As a natural extension of these preliminary studies, promising applications in the area of glyconanotechnology have been directed toward artificial glycoconjugates carrying bioactive carbohydrates, densely distributed onto CNT platforms, to mimic cell-surface oligosaccharides. A striking example describes a biomimetic surface modification of CNTs, using glycosylated polymers designed to mimic cell-surface mucins (Scheme 27).[249] These high molecular weight glycoproteins are involved in



SCHEME 27. Noncovalent functionalization of SWNTs by using an amphiphilic glycopolymer, ending with a lipid tail for mucin mimicry.[249]

specific molecular cell–cell recognition processes. Additionally, the dense clusters of O-linked glycans confer rigidity, provide strong hydration and passivation against biofouling (cell adhesions), thus ensuring protection of structural functions against nonspecific biomolecular interactions. It has been demonstrated that, in native mucins, the clustered *N*-acetyl-α-D-galactosamine (α-GalNAc) residues proximal to the peptide were the major contributors to the rigidification of peptide backbones.

Based on these observations, the authors prepared synthetically tractable mucin mimic (MM) in which α-GalNAc residues were linked through an oxime bond to a poly(methyl vinyl ketone) (PVK) lipid-teminated backbone. Its preparation started with the initial introduction of a C_{18} lipid chain at the extremities of 4,4′-azobis(4-cyanopentanoic acid) via peptide coupling to give **272**. Polymerization of the functionalized initiator via radical polymerization of methyl vinyl ketone (1-buten-3-one, **271**, MVK) afforded C_{18}-poly(MVK) **273** onto which aminooxy-functionalized α-GalNAc **274** residues were grafted through oxime bonds, affording C_{18}-α-MM polymer **275**. An aqueous solution of the resulting amphiphilic glycopolymer was then subjected to ultrasonication in the presence of SWNTs which were thereupon

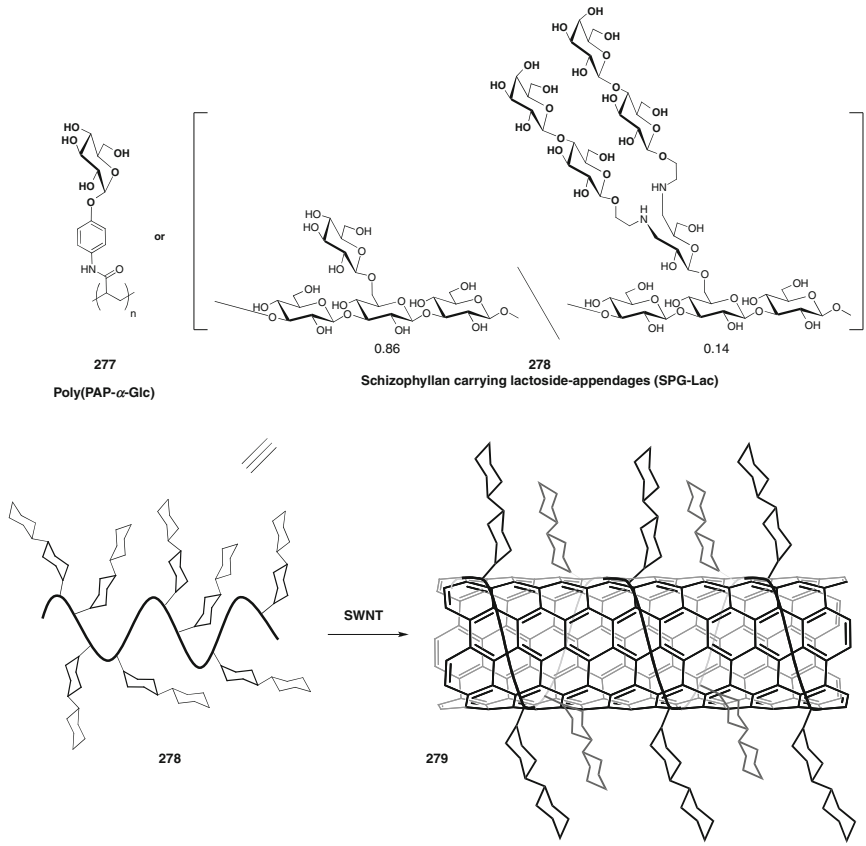
fully solubilized, suggesting the formation of hydrophilic surface coating through the self-assembly of the terminal lipid chains on the nanotubes via hydrophobic interactions, affording glycoconjugates C_{18}-α-MM-SWNTs (**276**). Evidence for mucin mimetic coating was provided by three different imaging techniques, namely AFM, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

As anticipated, substantial enhancement of water solubility was observed and the previously entangled SWNT bundles dissociated to form much finer bundles, stable in aqueous solution for several months. Despite the inherent difficulties from the imaging technique conditions, the formation of individual nanotubes with fairly uniform diameters of 65–70 nm was observed. The recognition and resistance properties of these nanotube–mucin mimetics were investigated with the use of *Helix pomatia* agglutinin (HPA). Incubation of **276** with a solution of HPA conjugated with fluorescein isothiocyanate (HPA-FITC), and appropriate subsequent treatment led to significant fluorescence that was attributed to a dependent receptor–ligand interaction. Moreover, studies under similar conditions but involving glycoconjugates bearing the β anomer of GalNAc manifested no significant fluorescence labeling. Hence, the lectin did not interact with the coated nanotubes in the absence of its favored ligand. These results clearly demonstrated that SWNTs coated with a MM could engage in specific molecular recognition with protein receptors and resist nonspecific protein binding.

A few years later, the same group extended studies with functionalized CNTs–cell interactions.[250] In order to exploit their ability to interact with cells, the advantage of the hexavalency of HPA and its capability to cross-link cells and glycoproteins was explored. Two parallel experiments were conducted, on one hand involving the preformed complex of HPA-FITC with C_{18}-α-MM-coated CNTs incubated with chinese hamster ovary (CHO) cells, and on the other the preincubation of CHO cells with unmodified HPA and subsequent treatment with modified C_{18}-α-MM-coated CNTs containing the fluorescent dye Texas Red. In both instances, analysis by fluorescence microscopy and flow cytometry suggested the formation of the tricomponent α-GalNAc-HPA–CNT–cell surfaces complex, and with a dose- and precomplexation-dependent labeling for the second study. Control CNTs modified with C_{18}-β-MM-CNTs indicated no significant fluorescent labeling at low concentration. In conclusion, demonstration that lipid-terminated poly(MVK)-based glycopolymers could coat CNT surfaces and promote their binding to cells through receptor–ligand interactions has been demonstrated. Modified CNTs were nontoxic to cultured cells (CHO and Jurkat cells), but irregular surface and nonuniform thickness of the CNTs coating might be explained by the high polydispersities (>1.7) of the polymers employed. The surface heterogeneity generated could

constitute a drawback to the systematic use in a reproducible way of glycopolymer-coated CNTs as sensors of protein binding.

In 2004, Hasegawa *et al*. demonstrated that SWNTs could be literally wrapped within a helical polymeric superstructure composed of schizophyllan, a polysaccharide bearing lactoside appendages (SPG-Lac, **278**), in aqueous solution (Scheme 28).[251] Water-soluble SPG-Lac–SWNTs nanocomposites (**279**) were obtained by mixing the glycopolymer in Me_2SO with SWNTs dispersed in water. Their formation was unequivocally observed and confirmed by a battery of such analytical techniques as UV–vis spectroscopy, colorimetry, thermal gravimetry, and AFM. Molecular recognition of the resulting composite was assessed by SPR, using

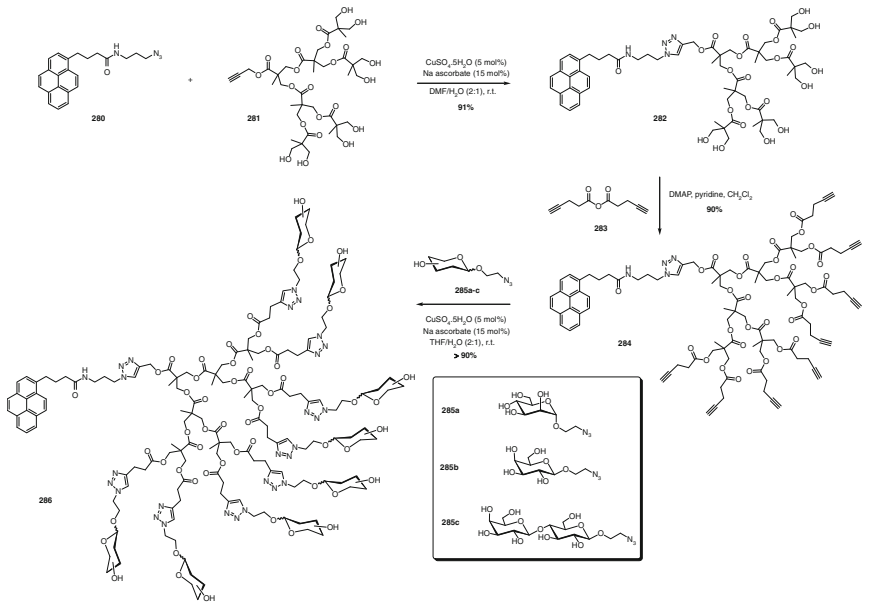


SCHEME 28. Solubilization of SWNTs (**279**) with artificial (**277**) or natural (**278**) schizophyllans.[251,252]

immobilized RCA_{120} lectin (*Ricinus communis* agglutinin, β-Lac-specific) on gold surfaces, and the results indicated highly specific affinity toward the lectin. This specificity was also confirmed by confocal laser-scanning microscopic (CSLM) observations using FITC-labeled RCA_{120}. Promising applications as SWNTs-based sensory systems have been demonstrated by the authors, who succeeded in constructing a layer-by-layer structure composed of the SPG-Lac–SWNTs composite and RCA_{120}.

By the same methodology, an individual dispersion of water-soluble SWNTs randomly wrapped by a helical glycoconjugate polymer (poly(*p*-*N*-acryloylamidophenyl)-α-D-glucopyranoside, PAP-α-Glc, **277**) has been obtained.[252] The characteristic sharp photoluminescence signal observed by near-infrared fluorescence spectroscopy, associated with the multivalent presentation of peripheral carbohydrates, made this system promising as new sensing approach for detecting carbohydrate-recognition proteins.

The preparation of biocompatible SWNTs, noncovalently functionalized with bioactive glycodendrimers, has been reported (Scheme 29).[253] A bifunctional dendritic scaffold **284** was built using the 2,2-bis(hydroxymethyl)propanoic acid as a building block **281**, which was linked to an azidopyrene tail (**280**) capable of binding the surface



SCHEME 29. Syntheses of glycodendrons with hydrophobic and fluorescent pyrene head-groups for π–π stacking to SWNTs.[253]

of SWNTs through π–π interactions. The orthogonal synthetic approach was based on sequential condensation of the dendrititic polyol **282** with 2,2-bis(hydroxymethyl)-propanoic anhydride (**283**).[254] At the desired generation, azide-functionalized pyrene **280** was chemoselectively ligated to the propargylated and hydroxylated dendritic scaffold **281** by CuAAC methodology. A panel of deprotected 2-azidoethyl mono- or disaccharides (**285a**, α-mannoside; **285b**, β-galactoside; and **285c**, β-lactoside) were then efficiently grafted onto the multivalent dendrimer **284** by click chemistry**.** The uniformity and purity of the resulting amphiphilic glycodendrimers **286a–c** were confirmed by NMR spectroscopy and MALDI-TOF mass spectrometry. The glycodendrimers were then adsorbed onto SWNTs by ultrasonication in aqueous solution.

Analysis by SEM and TEM revealed small bundles and individual SWNTs coated entirely with a thin uniform layer of glycodendrimers, contrasting markedly with the thick and heterogeneous coatings obtained by use of the glycopolymers mentioned earlier. Specific binding of SWNT-bound glycodendrimers to receptors was observed with the FITC-lectins: *C. ensiformis* agglutinin (Con A), *Arachis hypogaea* agglutinin (PNA), and *Psophocarpus tetragonolobus* agglutinin (PTA), which recognized [G(3)] Man-SWNTs **287a**, [G(3)] Gal-SWNTs **287b**, and Lac **287c**, respectively (Fig. 32). Similar results were obtained with the corresponding [G(2)] glycodendrimers. In addition, experiments in which SWNTs were initially functionalized with a mixture of [G(2)] Man **287a** and [G(2)] Lac **287c** using various molar ratios, and then incubated with a 1:1 mixture of Texas Red-conjugated PNA and FITC-Con A, demonstrated that multiple epitopes displayed on SWNTs could bind simultaneously to discrete proteins. The [G(2)] Man-SWNTs **287a** were then successfully engaged with FITC-Con A and CHO cells in the experimental protocol earlier employed to promote specific binding of modified SWNTs to the cell membranes. Similar results were obtained with the other [G(2)] glycodendrimer-functionalized SWNTs with corresponding labeled lectins. Finally, incubation of HEK293 cells with [G(3)] glycodendrimer–SWNTs conjugates did not affect their proliferation, in contrast to the unmodified SWNTs, indicating that glycodendrimers mitigated the cytotoxicity.

Besides the preparation of glyconanotube conjugates by supramolecular interactions, another synthetic pathway commonly used involved covalent attachment of the saccharidic units on the CNT scaffolds. The progress recently recorded concerning the derivatization of unfunctionalized CNTs, allowing introduction of suitable anchoring functions at their surface, are the basis of this synthetic alternative.[255]

b. Glyconanotubes from Covalent Interactions.—Multivalent presentation of peripheral saccharides around functionalized CNTs has been mostly assessed through covalent processes. In most applications, the use of CNTs containing carboxylic acid

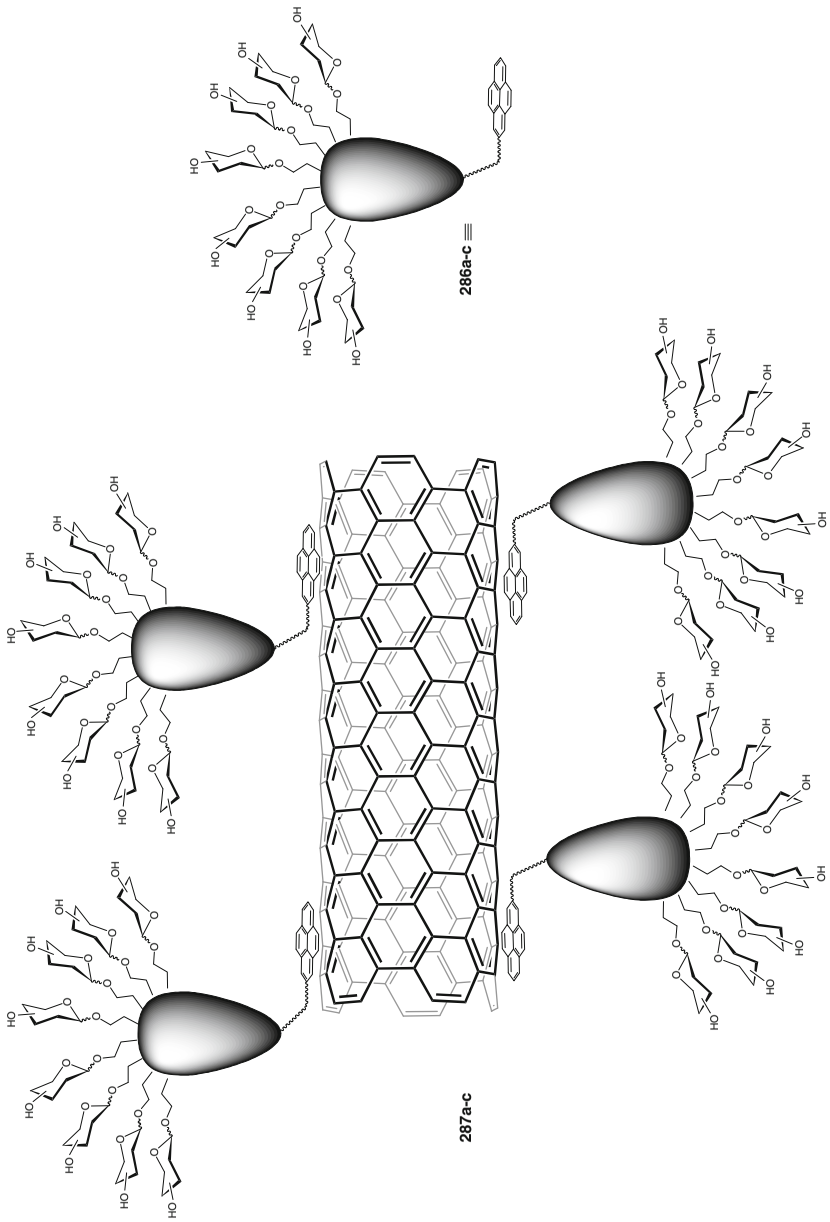
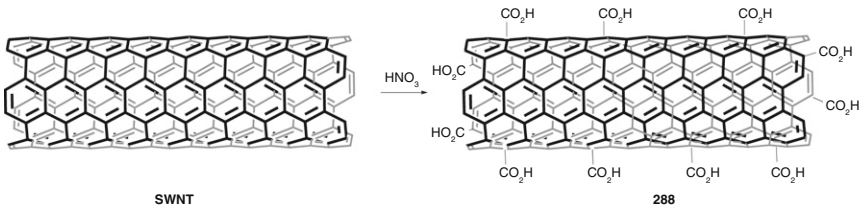


FIG. 32. Pyrene-based glycodendrons adsorbed onto SWNTs.[253]

groups as suitable anchoring sites constitutes the favored covalent methodology for installing suitable functional group that render the CNT soluble in appropriate solvents.[256] Typically, carboxylic acid functions are generated on CNTs through the oxidation of surface defects, by suspending the CNTs in 3 M HNO_3, and then treatment with aqueous HCl (Scheme 30).[257] As already presented in this chapter, in addition to their biological relevance, the conjugation of unprotected carbohydrates onto all-carbon scaffolds can solve the problem of their intrinsic low solubility.

One of the first examples of a water-soluble SWNTs glycoconjugate was obtained by covalent grafting of glucosamine via amide bonds.[258] The carboxylic acid functions of **288** were first converted into the corresponding acyl chlorides by suspending the functionalized SWNTs in a solution of thionyl chloride ($SOCl_2$) in DMF. The resulting solid was then mixed with an anhydrous solution of glucosamine in THF at reflux to afford glucosamine grafted-SWNTs. The new glycoconjugates were characterized by UV–vis and AFM analysis, and their solubility in water ranged from 0.1 to 0.3 mg/mL, depending on the temperature.

The synthesis of the first β-D-galactoside-modified SWNT (Gal-SWNT, **296**) with high water solubility has been described, along with its interactions with galactose-specific lectins that formed supramolecular junctions at the surface of the glycocoated SWNTs (Fig. 33).[259] Using a similar strategy, analogous Man–SWNTs conjugates (**297**) effective in the capturing of pathogenic *E. coli* under physiological solution was also synthesized.[260] The synthesis was based on the use of 2-aminoethyl β-D-galactopyranoside (**290a**) grafted onto SWNT-bound carboxylic acids (**288**) under sonication conditions, through carbodiimide-activated amidation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in aqueous KH_2PO_4 buffer. Complete instrumental characterization included solution-phase NMR, SEM and TEM, Raman, and near-IR spectroscopy, optical absorption, thermogravimetry, and spectrophotometry. The resulting galactosylated-SWNTs (**296**) were exfoliated and well dispersed, and presented a total sugar content of $\sim$65%. The high sugar ratio and large surface area of the covalently galactosylated SWNTs permitted the display of abundant sugar arrays



SCHEME 30. Oxidation of defect sites of SWNTs to provide carboxylated SWNTs.[257]

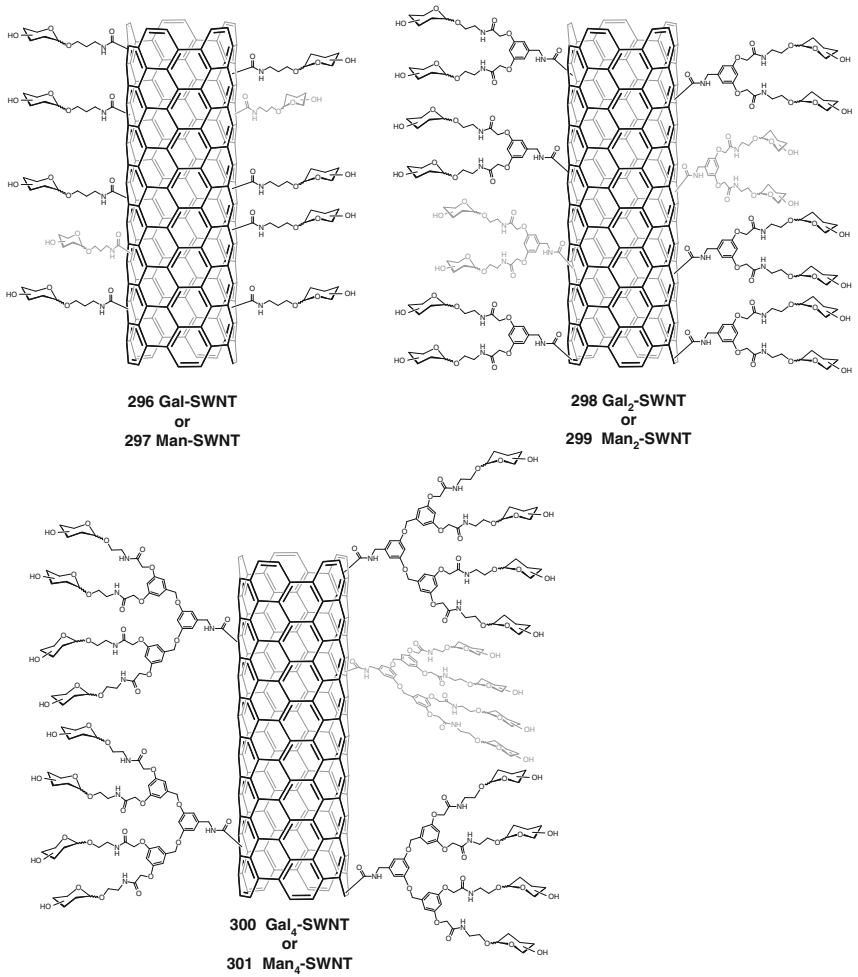


FIG. 33. Carboxylic acid-functionalized SWNTs bearing series of galactosylated or mannosylated mono-, di-, or trivalent dendrons.[262]

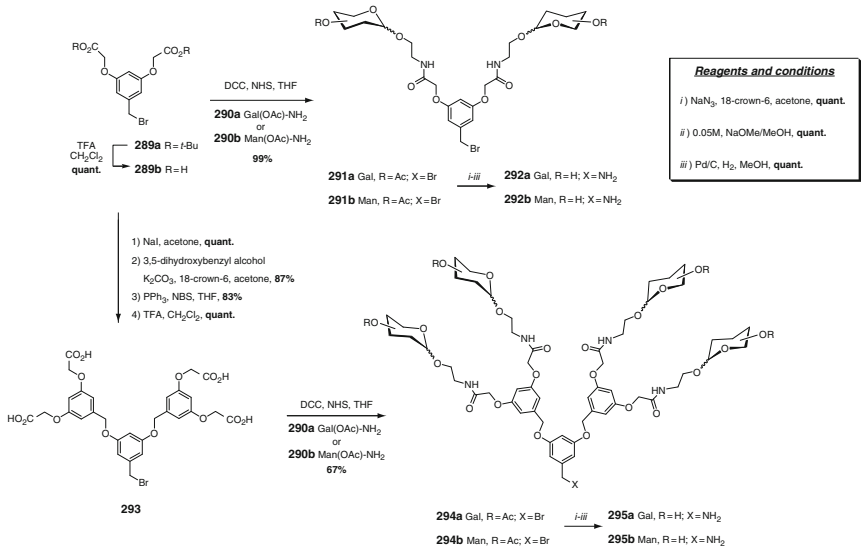
implicated in their strong binding interaction with receptors on pathogenic *E. coli* 0157:H7 cells. Significant cell agglutination was observed by SEM, with multiple nanotubes binding to one cell and some nanotubes ''bridging'' adjacent cells.

The same research group went further into detail concerning the antimicrobial properties of monosaccharide-coated SWNTs, advantageously exploiting their application for efficient and specific recognition of a nonvirulent strain of *Bacillus*

anthracis.[261] In this context, similar nanostructures containing a large number of monosaccharides (mannose or galactose) have been synthesized by conjugation of the corresponding 2-aminoethyl glycopyranosides (**290a,b**) onto **288** through amidation. The resulting water-soluble conjugates both strongly bound to *B. anthracis* spores, leading to significant aggregation with the mediation of the Ca^{2+} divalent cation. This binding phenomenon was unique to the nanotube-displayed monosaccharide molecules, and was not available with other displaying platforms such as polymeric nanoparticles (polystyrene nanobeads for instance), indicating specific arrangements of the carbohydrate ligands on the nanotube scaffolds. Furthermore, the associated substantial reduction in colony-forming units (CFU) could potentially find valuable applications in efforts for detection and decontamination.

Another series of hydrophilic glycodendron-functionalized SWNTs was also synthesized and the lectin-binding interactions studied.[262] It was assumed that the limited defect sites on the original nanotube surface could generate only a limited number of carboxylic acid functions. The desired dense population of multivalent ligands could therefore be reached by covalently grafting sugar dendrons containing a suitable anchoring functionality. Toward this goal, β-D-galactopyranoside dendrons Gal_2-NH_2 (**292a**), Gal_4-NH_2 (**295a**), and their analogous α-D-mannopyranoside dendrons Man_2-NH_2 (**292b**), and Man_4-NH_2 (**295b**) were efficiently prepared by a systematic synthetic sequence based on the use of trisubstituted benzene cores (Scheme 31). Divalent **292a,b** and tetravalent glycodendrons **295a,b** were obtained from brominated derivatives **291a,b** and **294a,b**, respectively**.** Synthesis of the required dendritic building blocks was initiated with the trisubstituted benzene core **289a**, from which *t*-butyl ester removal with TFA afforded brominated precursors **289b**. The tetravalent bromine-ending analogue (**293**) was synthesized by selective etherification of 3,5-dihydroxybenzyl alcohol with **289a** in order to double the surface functionalities, followed by the introduction of bromine function with the PPh_3/NBS tandem. The acetylated amine-tethered monosaccharides **290a,b** were then conjugated to **289b** or **293** by carbodiimide-activated amidation yielding protected glycodendrons **291a,b** and **294a,b** in 99% and 67% yields, respectively. Finally the amine-ending glycodendrons were prepared by a common synthetic protocol involving treatment with NaN_3, O-deacetylation under Zemplén conditions, and subsequent hydrogenolysis with H_2 on Pd/C.

The amine-derivatized glyco wedges were finally used for the functionalization of SWNT acids **288** by peptide coupling as before, to afford **298–301** (Fig. 33). The resulting larger number of displayed ligands per SWNT was responsible for the significant increase of water solubility, increasing with the functionalization, and the enhancement of biocompatibility. In addition, they enabled more quantitative



SCHEME 31. Amine-ending glycodendrons bearing either galactoside or mannoside residues.[262]

characterization, permitting a better understanding of the structural details of SWNTs exhibiting multivalent carbohydrate ligands. Solution and gel-phase NMR studies, SEM, Raman, optical absorption studies, spectrophotometry, and thermogravimetric analysis (TGA) have all been employed in order to determine the morphology, the arrangement of glycodendrons around the SWNT scaffolds, and the sugar content. No meaningful differences in sample composition between the Gal- (**296**) and Man- (**297**), Gal_2- (**298**) and Man_2- (**299**), Gal_4- (**300**), and Man_4- (**301**) glyconanotubes as functional moieties were observed. More sugar residues were displayed on the same amount of SWNTs for the divalent conjugates than for the monovalent ones. Furthermore, and interestingly, the electronic properties of the CNTs were largely preserved during the functionalization.

These carbohydrate-functionalized SWNTs were evaluated in binding assays with pathogenic *E. coli* and with *Bacillus subtilis* (a nonvirulent form of *B. anthracis* or anthrax). As compared with the binding properties of Gal-SWNT (**296**) to *E. coli* O157:H7 and the induced decrease of CFU evoked earlier, considerable improvements were recorded with divalent Gal_2-SWNT (**298**). In the presence of the same *E. coli* strain, the amount of recovered cellular aggregates was clearly larger, and with a more significant reduction of CFU. This more favorable binding process by

Gal_2-SWNTs (**298**) seemed to suggest that the paired β-D-galactosides are more effective in the specific interactions. Moreover, Man_2-SWNTs (**299**) and Man_4-SWNTs (**301**) were both capable of binding and aggregating *B. subtilis* spores in the presence of calcium cations. Despite preliminary encouraging observations, quantitative evaluations of the foregoing binding assays were complex: a lack of major tendencies and inconsistent results over a same series rendered the interpretation difficult and inaccurate.

Finally, a subsequent study furnished a clear demonstration that CNTs could provide suitable support for rapid and efficient polymeric growth for the synthesis of novel and biocompatible linear and hyperbranched glycopolymers by the ''grafting from'' strategy, with good and high reproducibility (Fig. 34).[263] The successful and controlled linear glucopyranoside-polymer grafting onto MWNTs was achieved by atom-transfer radical polymerization (ATRP), while hyperbranched glycopolymers were introduced by self-condensing vinyl copolymerization (SCVCP) of the corresponding functionalized 1,2:5,6-di-*O*-isopropylidene-3-*O*-methacryloyl-D-glucofuranose (**305**) and inimer 2-(2-bromoisobutyryloxy)ethyl methacrylate (**306**) via ATRP. The MWNT-CO_2H precursor was generated by oxidation of the crude MWNT with 60% HNO_3, and then modified with ethylene glycol, generating MWNT-OH. The CNT-based macroinitiator MWNT-Br (**302**) for ATRP was formed by treating MWNT-OH with 2-bromo-2-methylpropanoyl bromide. The morphology and nanostructures of the resulting multihydroxylated conjugates **303** and **304** were observed by SEM, TEM, and SFM, indicating the formation of nanotube–supported polymer brushes for linear polymers and a polymer layer in a core-shell structure for hyperbranched structures, implying their uniform growth on the surfaces of the MWNTs. These original water-soluble glycopolymer-grafted CNTs offer promise in the fields of tissue engineering and bionanomaterials.

In conclusion, such three-dimensional carbon-based nanostructures as fullerenes and nanotubes constitute scaffolds whose efficient functionalization and derivatization

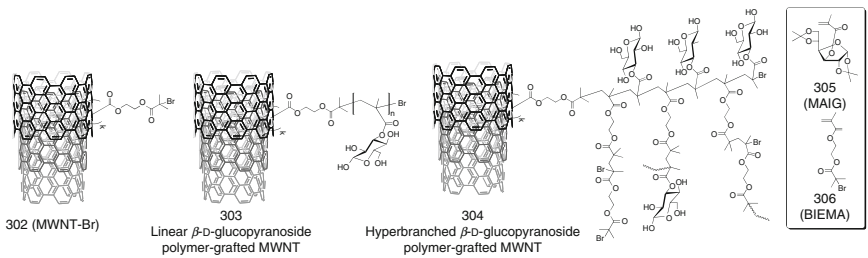


FIG. 34. Glycopolymer grafted onto SWNTs.[263]

has been developed in recent years. Accurate control of the introduction of biologically relevant moieties has been achieved, giving rise to original nanostructures with intriguing and promising properties. In particular, monovalent carbohydrates, more complex oligosaccharides, or glycodendrons have been successfully anchored at the surfaces, through covalent or noncovalent methodologies. Although practical applications remain as yet limited, preliminary results suggest that these nanomaterials offer unique and favorable arrangements of multivalent carbohydrate arrays that are not readily available with other displaying platforms.

IV. Multivalent Glycoconjugates by Self-Assembly

To enhance the number of saccharidic units exhibited, construction of multivalent scaffolds through supramolecular chemistry provides an interesting alternative for the rapid synthesis of glycodendrimers. Dynamic and reversible self-assembly process consisting in coordinating metal-assisted association of ligands is one of the most striking examples of supramolecular organization.[264] Over the years, optical and electrochemical properties of various complexes have been widely studied and exploited in different domains for material and biological applications.[265,266]

To target specific biological events, cells or tissues, the introduction of relevant recognition molecules such as carbohydrates have been investigated during recent years. In this context, metalloglycoclusters-initiated self-assembly of ligands containing simple saccharides or more dense glycodendrons around a coordinating metal has contributed valuable additions of original and promising structures to the range of available multivalent glycoconjugates. It is rather surprising that only a few examples have been described, because the metal-assisted association of carbohydrate components offers a straightforward access to carbohydrate clusters in which the number and the relative orientation of the carbohydrate residues can be modulated almost at will by changing the structure of the ligand and the nature of the metal. In addition, the intrinsic photophysical properties of the metal complexes can be advantageously exploited, for instance to create efficient biosensors. Furthermore, a second example of multivalent presentation of carbohydrates through self-organization process resulting from lipophilic cores has been furnished through the aggregation of glycodendrimers in aqueous solution, allowing the homogenous and controlled formation of hypervalent structures with distinct recognition properties as compared to the individual precursors.

1. Self-Assembly Using Coordinating Metals

Historically, the first multivalent glycoconjugate obtained by metal-assisted self-assembly was described in 1994 by Sakai and Sasaki (Fig. 35).[267] The methodology was based on the trimerization of unsymmetrical Bipy-GalNAc (**307**) induced by the presence of Fe(II) salts, to give the tridentate Fe^{II}(bipy-GalNAc)$_3$ cluster **308** as a mixture of four diastereomeric isomers (Λ-*fac*, Δ-*fac*, Λ-*mer*, and Δ-*mer*).

A comparative study involving bipy-GalNAc (**307**), Fe^{II}(bipy-GalNAc)$_3$ dendrimer (**308**), and asialoglycophorin A using the GalNAc-specific plant lectin, *Vicia villosa* B_4, has been assessed by the authors to determine and compare their relative binding potential. Better binding affinities were observed for the trivalent complex **308** over the monovalent precursor **307**, indicating the role played by the cluster formation of GalNAc residues around the metal template. More interestingly, the similar strong binding affinities observed for asialoglycophorin A, a natural glycoprotein containing several repeating units of GalNAc linked to Ser or Thr residues, and Fe^{II}(bipy-GalNAc)$_3$ were attributed to the adapted organization of GalNAc residues in the complex with adequate intercarbohydrate distances.

A few years later, the same group proposed further investigations to explain the strong binding affinities earlier observed with the self-assembled complex, describing the dynamic molecular recognition of their synthetic ligands by lectins.[268] Studies involving *V. villosa* B_4 lectin and the complex highlighted the fact that the ratio of the four diastereoisomers gradually changed during the binding experiment. After 32 h at room temperature, the authors recorded enrichment in the Λ-*mer* isomer, which in the end comprised 85% of the total isomers (vs. 15% in the native mixture). These experimental observations suggested that the dynamic equilibrium seen at room

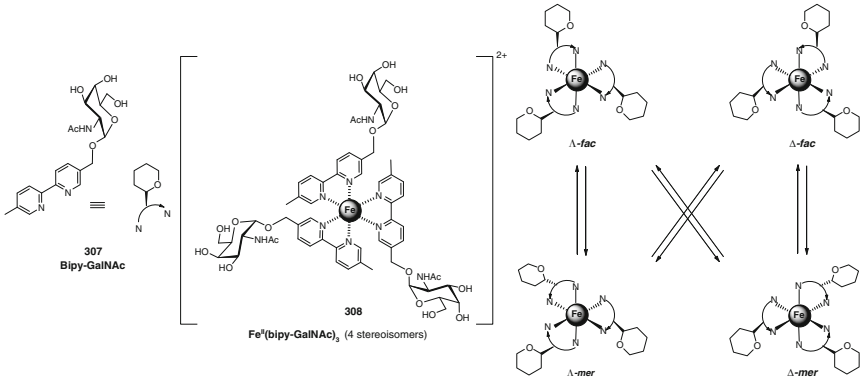


FIG. 35. Self-assembled glycodendrimers around a Fe(II)-bipyridyl complex.[267]

temperature allowed the spatial arrangement of the three GalNAc residues to self-adjust in order to provide a better complementarity to the multivalent carbohydrate-binding sites of the lectin.[269] This tendency was confirmed with studies involving other GalNAc-specific lectins, such as *Glycine max*, specific for a terminal α-D-GalNAc residue attached to the 3′-OH group of galactose. Selective enrichment of other diastereoisomers has been observed, indicating the adaptability of the process for lectins presenting different shape and functionnalities in their carbohydrate-binding pockets. The intramolecular lectin's binding sites, being too distant from one another to be reached by the close GalNAc residues, strongly suggest a dynamic cross-linking process.

Kobayashi *et al*. employed hexavalent metal complexes based on the self-assembly of three symmetric 2,2′-bipyridine ligands tethering two functionalized glycopyranosides around ruthenium(III)[270] or iron(II)[271] as the core component (Fig. 36). They undertook complete comparative studies involving glycosylated Tris-bypyridine iron and ruthenium complexes in order to develop robust biosensors for monitoring saccharide-binding phenomena. Synthesis of the ligands was based on the use of a 2,2′-bipyridine-4,4′-dicarboxylic acid core onto which amino glycopyranoside derivatives were conjugated via amide functions. Hence, self-assembled glycoclusters

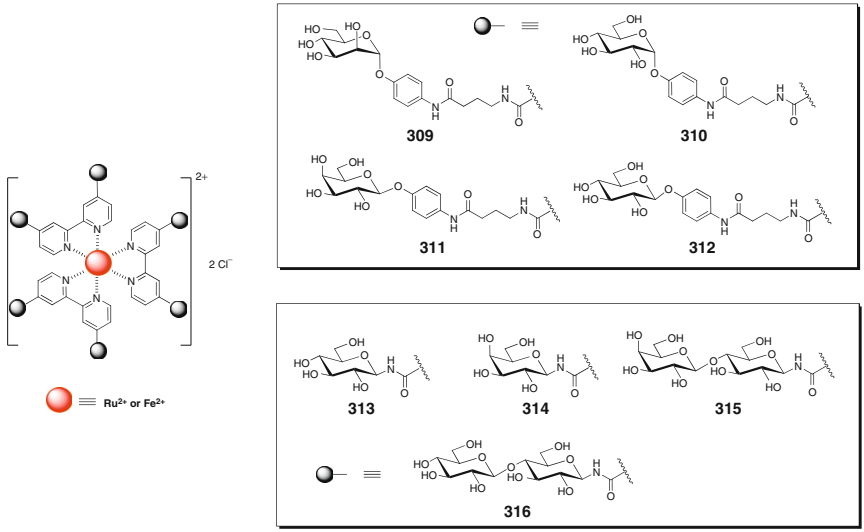


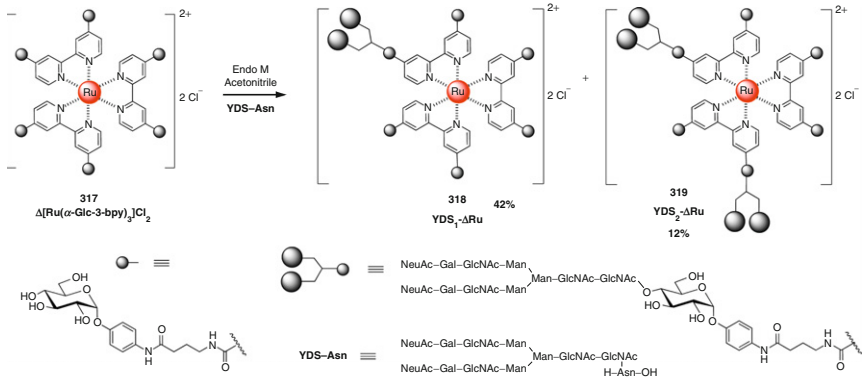
FIG. 36. Self-assembled glycodendrimers using chelating metal cations and divalent bipyridine scaffolds.[270,271]

consisted of direct *N*-linked glycosylamine appendages to the complex, or through a O-linked *p*-butanamidophenyl spacer, were produced. Three equivalents of the resulting conjugates were then treated with $RuCl_3$ or $FeCl_2$ in a minimum amount of boiling water–alcohol mixture to afford the corresponding tris-bipyridineruthenium and iron complexes as mixtures of separable diastereoisomers.

To determine the importance of the structural parameters, the first study involved ferrous O- (**309–312**) and N-(**313–316**) glycoclusters. In this context, the influence of the spacers on the lectin affinity was investigated. Their relative binding properties to specific lectins were evaluated by a HAI assay and by SPR, using α-glucoside- and α-mannoside-specific ConA and β-galactoside-specific RCA_{120} lectins. Lectin affinities observed for the N-linked-glycoclusters **313–316** were low, but high affinities (IC_{50} in the micromolar range) were recorded for the elongated conjugates, thus highlighting the importance of the flexibility and the density of the carbohydrate scaffolds. Flexibility seemed essential for reaching high and specific lectin-recognition, and this was probably induced through optimized conformational organization of the epitopes to fit the binding sites of the lectins. On the other hand, strong binding avidities to lectins were not observed, because of the lability of the iron complexes, which readily dissociated in dilute aqueous solution ($< 10^{-6}$ M).

For this reason, the authors further investigated more stable elongated ruthenium O-linked glycoclusters, and showed their avidity to the corresponding lectin to be enhanced, reaching IC_{50} in the nanomolar range, comparable to known potent glycopolymers. The results indicated that dense saccharide clusters played essantial roles in their specific and strong binding, as compared to monovalent precursors. Furthermore, the complexes exhibited intense emission spectra after excitation of the metal-to-ligand charge transfer (MLCT) band in water, as compared to nonglycosylated ruthenium complex. The strong luminescence was attributed to the peripheral carbohydrate shell caused by closely packed saccharide clusters, which could possibly isolate the luminescent core from that lost to the outer aqueous environment. The structures were considered by the authors to resemble natural proteins having redox and luminescent cores embedded in the polypeptide shells. Interestingly, binding of lectin to these glycoconjugates decreases their luminescence by disrupting their outer carbohydrate shell structure.

In 2003, combined efforts of the two research groups just mentioned developed a one-pot transglycosylation strategy for the construction of complex-type disialooligosaccharides starting from an α-D-glucopyranoside residue self-assembled onto a tris-bipyridineruthenium complex $\Delta[Ru(\alpha\text{-Glc-3-bpy})_3]Cl_2$ (**317**) (Scheme 32).[272] They succeeded in this commendable chemoenzymatic one-pot transformation by



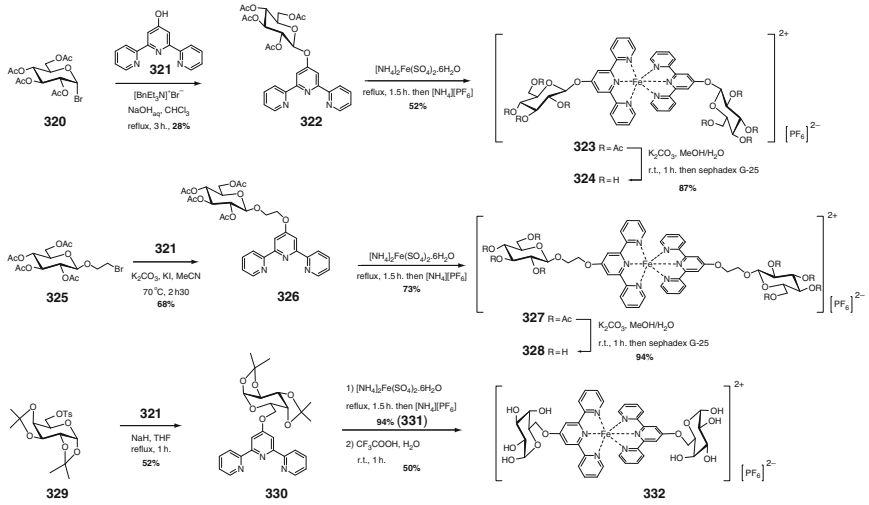
SCHEME 32. Enzymatic transglycosylation of a preformed bisglucobipyridine core self-assembled as a hexavalent cluster around a ruthenium cation.[272]

using endo-β-D-GlcNAc-ase from *Mucor hiemalis* (Endo M), which effectively catalyzed transfer of the egg-yolk decasaccharide (YDS) from the asparagine-bonded complex-type disialooligosaccharide (YDS-Asn) onto the 4-OH position of a α-D-glucoside unit of **317**. Therefore, a mixture of two separable products, consisting of mono- (YDS_1-ΔRu) (**318**) and bisadducts (**319**), was obtained in 42% and 12% yields, respectively.

The binding properties of **318** and **319** in the presence of influenza type-A viruses (A/Memphis/1/71) were evaluated, using an inhibition test with virus-infected MDCK cells. The results revealed that, despite their small molecular weight, the YDS-adducts and more especially **319**, exhibited strong inhibitory potency, with an IC_{50} of 8.4 μM, being values of two orders of magnitude higher than that of the parent YDS-Asn. The origin of this excellent virus affinity was unknown, but speculations concerning favorable electrostatic and hydrophobic interactions between the cationic and aromatic-rich complex center and certain amino acid residues of the hemagglutinin have been formulated. In addition, **318** and **319** exhibited strong luminescence at 605 nm, the intensity of which increased with the number of YDS residues. As evoked in the earlier work, this enhancement was attributable to the bulky oligosaccharides spanning around the luminescent core. Interestingly, the addition of influenza type-A viruses to the **318** and **319** in PBS resulted in luminescence quenching, probably caused by disruption of the saccharide shell and the resulting exposure of the luminescent core to the aqueous environment. Although the exact mechanism was not clear, this strategy could allow for the construction of robust and efficient sensitive biosensors for various toxins, viruses, and bacteria.

This strategy constitutes an extension of the work of Constable *et al*.[273] who initially described the self-assembly of peripheral saccharides covalently linked to terpyridine moieties. Divalent iron(II) and ruthenium(II) complexes bearing functionalized β-D-glucopyranosides or β-D-galactopyranosides have been efficiently prepared by this approach (Scheme 33). The peracetylated glucosylated terpyridine ligand **322** was obtained under phase-transfer conditions involving the reaction between the nucleophilic 4′-hydroxy-2′2′:6′,2′′-terpyridine **321** and tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**320**). The elongated analogue **326** was synthesized by simple nucleophilic substitution using K_2CO_3, KI, **321** and 2-bromoethyl tetra-*O*-acetyl-β-D-glucopyranoside (**325**). 1,2:3,4-Di-*O*-isopropylidene-6-*O*-tosyl-α-D-galactopyranose (**329**) was similarly treated with **321,** using sodium hydride in THF to afford **330**. Divalent complexes (**323**, **327**, and **331**) were typically formed by treating their terpyridine intermediates with $[NH_4]Fe(SO_4)_2.6H_2O$ in acetonitrile or ethanol and precipitation with ammonium hexafluorophosphate. Subsequent deprotection of the saccharide portions with aqueous methanolic K_2CO_3 or trifluoroacetic acid in water, respectively, afforded the glycoadducts **324**, **328**, and **332**.

The authors conducted preliminary biological investigation on these complexes, studying their stability toward enzymatic hydrolysis. Interestingly, the glucoside residue was not released from the complexes under the action almond β-glucosidase,

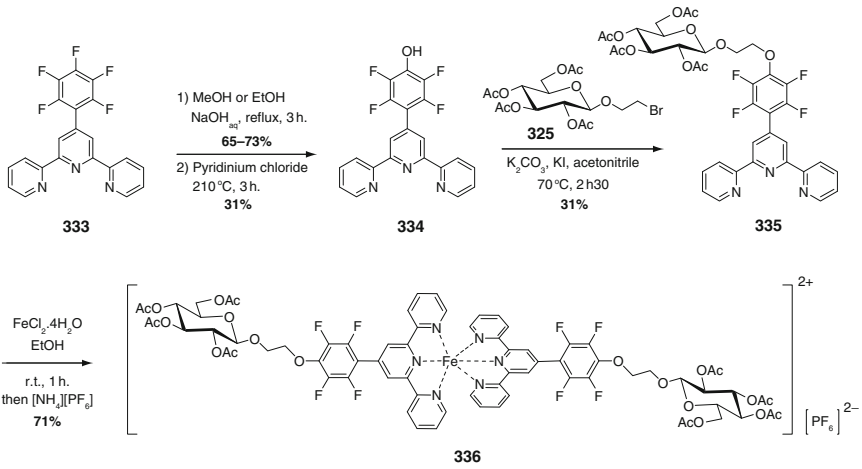


SCHEME 33. Self-assembled glycoclusters using a monosubstituted terpyridine ligand.[273]

while free ligands were hydrolyzed under similar conditions. The precise molecular origin of these effects was, however, not determined.

Later, the same group attempted to improve the efficiency of coupling of the carbohydrates onto the terpyridine scaffold by introducing a reactive pentaflurophenyl group onto the 4′-position (**333**) with the use of simple alcohols or saccharides (Scheme 34).[274] Somewhat surprisingly, it appeared that the nucleophilic $S_N$aryl substitution could not deliver the expected conjugates. They then relied on an alternative strategy using a 4′-tetrafluorophenoxy spacer (**334**), which allowed synthesis of the intermediate **335**, via attack of the electrophilic sugar **325**. Complex formation between **335** with ferrous chloride furnished the divalent iron complex **336** in good yield. The lengthy synthetic sequence, together with the modest yields obtained for the starting materials, rendered the strategy unsuitable for further development.

In order to prove the generality of the concept and enhance the stability of the resulting complexes, several research groups investigated the use of various ligands or coordinating metals. With this concept in mind, a versatile synthetic methodology leading to the self-assembly of galactose–oligopyridine conjugates around various metal cores has been disclosed.[275] The simple and successful strategy allowed structural variation in the components and linkers, generating multivalent adducts of different sizes, shapes, valencies, and conformational mobilities. The synthesis of the necessary galactosylated precursors started from tetra-*O*-acetyl-*β*-D-

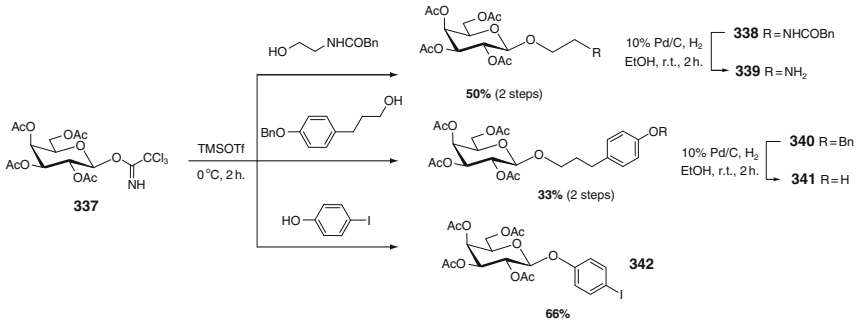


SCHEME 34. Alternative strategy used by Constable et al. for the construction of a dimeric iron(II) complex built around a terpyridine ligand.[274]

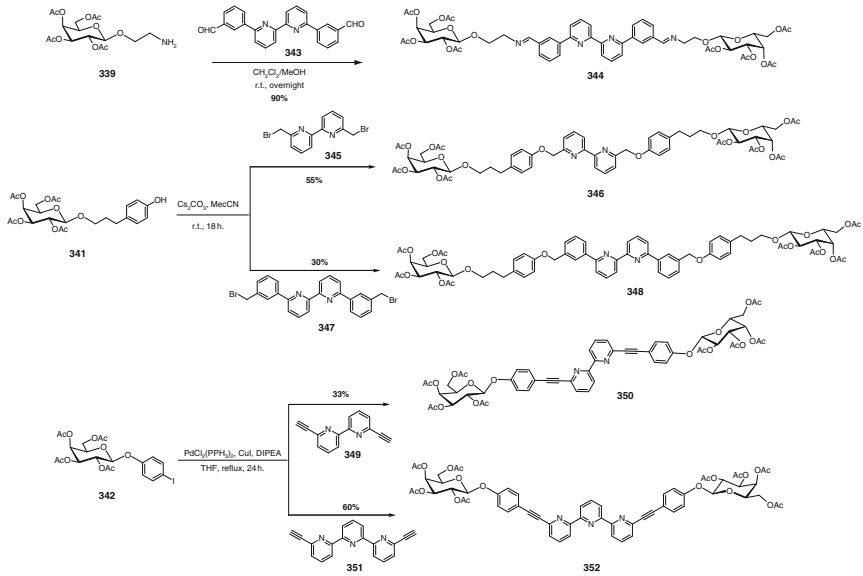
galactopyranosyl trichloroacetimidate (**337**), further functionalized under standard trimethylsilyl triflate-promoted glycosidation reaction conditions and subsequent transformation with various alcohols (Scheme 35). Three peracetylated galactoside conjugates (**338**, **340** and **342**), containing terminal amino, phenol, and aryl iodide functions, respectively, were thereby generated.

Functionalized oligopyridines consisting in bi- (**343**, **345**, **347**, **349**) and terpyridine (**351**) scaffolds were synthesized according to classical chemical strategies to allow efficient anchorage of the complementary functions of the β-D-galactosylated moieties **339**, **341**, and **342** (Scheme 36). The bisimine conjugate **344** was first prepared by condensation of 2-aminoethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**339**) with the bisaldehydo bipyridine **343** in excellent (90%) yield. In order to obtain the elongated analogues **346** and **348**, 3-(4-hydroxyphenyl)-1-propyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**341**) was anchored to the dibromomethyl bipyridines **345** and **347** via nucleophilic substitution in 55% and 30% yields, respectively. Finally, Pd-(0)-catalyzed Sonogashira coupling was used efficiently to introduce 4-iodophenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**342**) on the ethynylated bi-**349** and ter-pyridine **351** to afford divalent glycoconjugates **350** and **352**, in 33% and 60% yields, respectively.

Complexations of the bipyridine ligands with CuOTf and terpyridine ligand with $Zn(OTf)_2$, afforded the corresponding 2:1 (**353**) and 1:1 (**354**) complexes, respectively, as single species (Fig. 37). Depending on the metal ligands, spectroscopic evidence indicated the formation of tetrahedral structures in the Cu complexes, regardless of the length or the nature of the spacers, while a trigonal-bipyramidal geometry was observed for the self-assembled glycoconjugates resulting from the



SCHEME 35. Synthesis of β-D-galactopyranoside precursors, bearing amine, alcohol, and iodide as anchoring functionalities, for metal-based self-assembly.[275]



SCHEME 36. Divalent β-D-galactopyranosides built on oligopyridine metal ligand scaffolds.[275]

Zn metal. In addition, unprotected bipyridine–Cu(I) complexes were obtained via O-deacetylation under Zemplén conditions, in contrast to the terpyridine–Zn(II) adduct, where the complex decomposed.

A later investigation describes the attachment of different 1-thiosaccharides to 2,2′-substituted bipyridines to obtain biologically stable ligands and radiolabelled metal complexes of Re and Tc (Scheme 37).[276] The novel ligands were obtained by coupling 4,4′-dibromomethyl-2,2′-bipyridine **355** with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose (**356**), 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose (**357**), and 2,3,4,6-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-α-D-mannopyranose (**358**), under basic conditions**.** The ensuing thioglycosidically linked bipyridine derivatives were O-deacetylated under standard Zemplén conditions to afford the water-soluble ligands **359–361**. Luminescent rhenium complexes (**362–364**) were formed by refluxing a methanolic suspension of the corresponding ligand with rhenium pentacarbonyl chloride $[Re(CO)_5Cl]$. New glycoconjugated complexes of the general formula $[Re(L)(CO)_3Cl]$, considered as luminescent probes, were characterized by mass spectrometry, elemental analysis, ^1H and ^{13}C NMR, IR, UV–vis, and fluorescence spectroscopy. One of the structures was unequivocally characterized by X-ray crystallography, indicating a surprising nonsymmetric metallocomplex architecture. In addition, the rather stable radiolabelled ^{99m}Tc

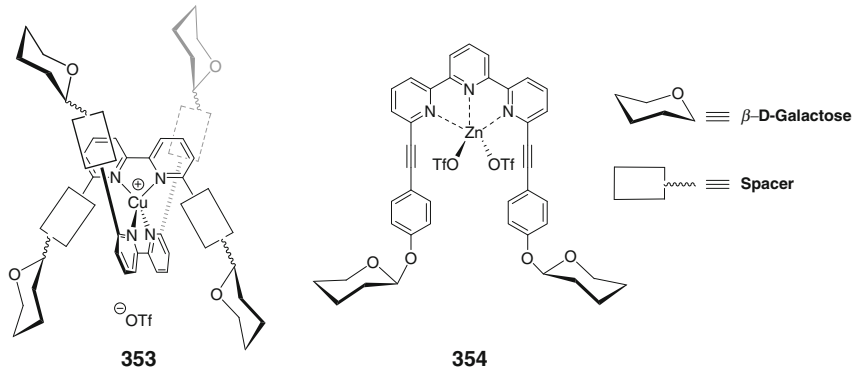
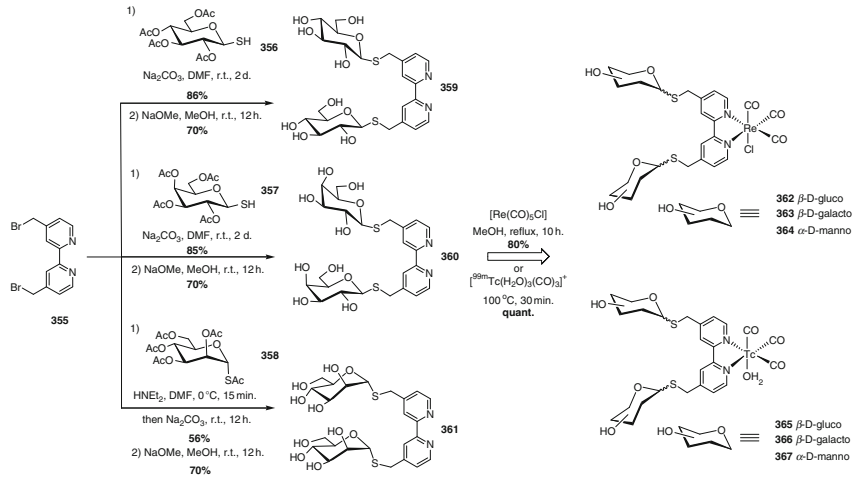


FIG. 37. Metal complexes resulting from the glycosylated bi- or ter-pyridine ligands.[275]



SCHEME 37. Preparation of luminescent Re and ^{99m}Tc glycoclusters obtained by self-assembly.[276]

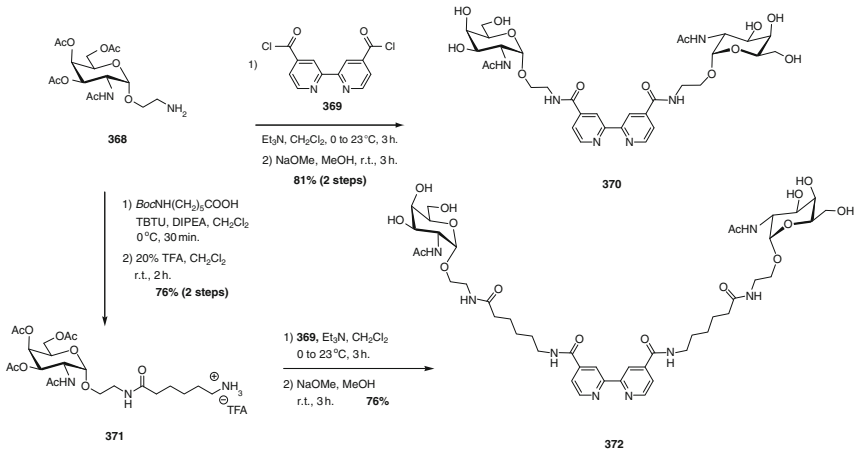
complexes **365–367** were synthesized similarly in quantitative yields, using $[^{99m}Tc(CO)_3(H_2O)_3]^+$ as the metal ion source.

In order to increase the complexity of the peripheral epitopes resulting from the self-assembly process, glycodendrons have been successfully grafted onto various ligands, allowing the formation of dense glycocomplexes. Indeed, in most of the examples, branching of the dendrimers produces a microenvironment that

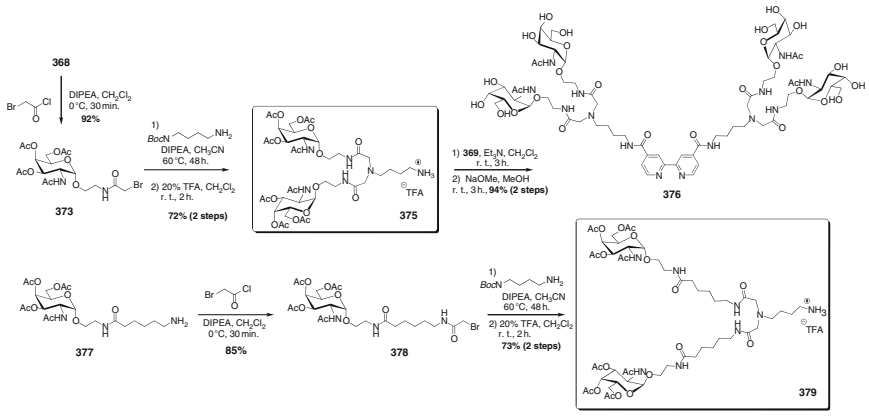
encapsulates the coordinating metal core and can drastically modify its photochemical and electrochemical properties, and these can be modulated at will for suitable applications.

The first such example was proposed by Roy and Kim[277] who described the synthesis and the relative lectin-binding properties of square planar complexes organized around a Cu(II) coordinating core, and glycodendrons containing four or eight peripheral Tn-antigen units (α-GalNAc known as a cancer marker), covalently linked to a bipyridine core (Scheme 38). 2-Aminoethyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranoside (**368**) and its analogue **371** containing an elongated linker (obtained by peptide coupling with *N*-Boc-protected 6-aminohexanoic acid followed by acid deprotection with TFA, CH_2Cl_2, 76%) were used for anchoring. Compounds **368** and **371** were then coupled with 2,2′-bipyridine-4,4′-dicarboxylic acid chloride (**369**) to provide divalent Tn antigens **370** and **372**, respectively, after Zemplén deprotection.

In order to provide access to self-assembling structures of increasing carbohydrate valencies, the authors described a convergent strategy for dimeric GalNAc building blocks having both short- and long-space arms. Thus, aminated derivatives **368** and **371** were treated with bromoacetyl chloride in the presence of DIPEA to afford bromoacetylated GalNAc derivatives **373** and **378** in excellent yields (Scheme 39). N,N-Dialkylation involving mono-*N*-Boc-1,4-diaminobutane (**374**) and bromoacetyl derivatives **373** and **378**, followed by trifluoroacetolysis afforded dimers **375** and **379** in 72–73% yields. By a synthetic pathway similar to that described earlier, their



SCHEME 38. Tn-Dimers built on 2,2′-bipyridine core for self-assembly.[277]



SCHEME 39. Other elongated Tn-antigen (α-D-GalNAc) clusters for self-assembly.[277]

amidation with 2,2′-bipyridine-4,4′-dicarboxylic acid chloride (**369**) in the presence of Et_3N, followed by O-deacetylation yielded the elongated bipyridyl tetramer **376**.

The preformed dendrons **370**, **372**, and **376** were clustered around copper(II) ($CuSO_4$ $5H_2O$) in aqueous media to provide efficiently the symmetric and hydroxylated glycoclusters and glycodendrimers **380**–**382** (Fig. 38), respectively, exhibiting four and eight GalNAc residues. These metallated α-D-GalNAc-bearing glycodendrimers were fully characterized by standard spectrometric analyses, ^1H and ^{13}C NMR, MALDI-TOF mass spectrometry, and UV–vis spectroscopy.

The potential of these self-assembled complexes to cross-link and to precipitate horseradish peroxidase-labeled plant lectin *V. villosa* (VVA-HRP) was confirmed by a solid-phase competitive inhibition assay (ELLA) with the use of asialoglycophorin as coating material. All of the neoglycoconjugates, including simple divalent and tetravalent ligands, inhibited the binding of asiaglycophorin to VVA-HRP with greater efficacy than the monomeric standard inhibitor allyl α-D-GalNAc (IC_{50} of 158.3 μM). As anticipated, the elongated dimeric analogue **381** was the most potent (IC_{50} of 1.82 μM) and the tetravalent ligand **380** showed less potency (IC_{50} of 4.09 μM), probably because of the shorter intrasugar distances between each of the branches. As compared to their nonmetal complexes, the metallic clusters clearly exhibited enhanced inhibitory potencies following the expected ''cluster effect.'' Indeed, the Cu(II)-(bipy-GalNAc) tetramers, **380** and **381** were 61- and 251-fold, respectively, more potent than the monovalent control, while octamer **382** exhibited a 259-fold enhanced avidity. Interestingly, the best binding affinity was recorded for the tetramer Cu(II) nucleated derivative **381** (63-fold on a per saccharide basis) that has

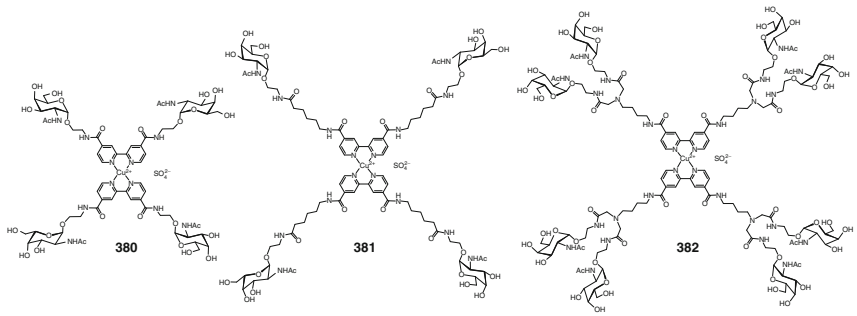
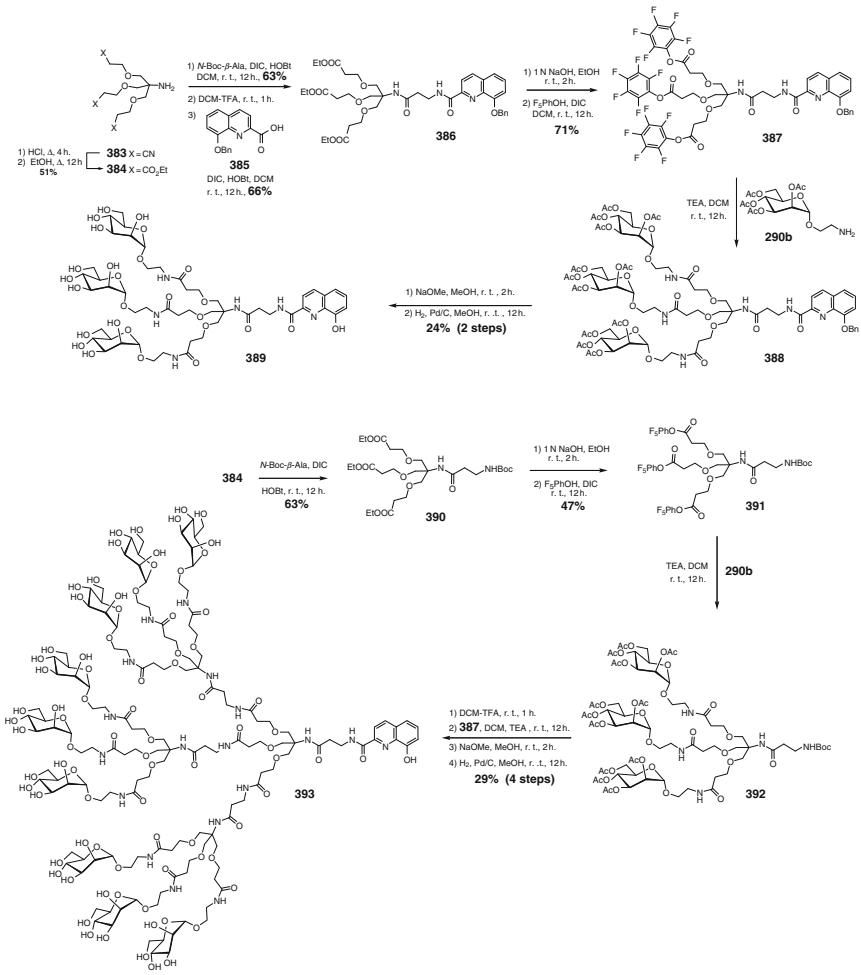


FIG. 38. α-D-GalNAc 2,2′-bipyridine oligomers self-assembled around a copper(II) salt.[277]

the longest intersugar distances. These results clearly demonstrate that the structural arrangement and flexibility of the molecules play crucial roles in their relative binding affinities. This work confirmed that the aglycone spacer and valency enhancement of sugar residues in neoglycoconjugates were responsible for an increase in binding affinity in carbohydrate–protein interactions.

This supramolecular assembly process has also been directed toward the synthesis of metallic and fluorescent glycodendritic architectures.[278] Thus, homogenous fluorescent and sugar-functionalized metallic dendrimers, containing various numbers and types of monosaccharides have been prepared. The chelation of transition or lanthanide metals was ensured by the presence of 8-hydroxyquinoline (Scheme 40). Metallic glycodendrimers containing up to 18 peripheral mannoside residues have been prepared starting from *N*-(tris[(2-cyanoethoxy)methyl])methylamine (**383**), which was treated with concentrated HCl in ethanol to yield triester **384**. Successive peptide couplings with Boc-*β*-alanine and then with 8-*O*-benzylquinoline-2-carboxylic acid (**385**) yielded the functionalized tripod **386**, which after hydrolysis and further coupling with pentafluorophenol afforded the active triester **387** in 71% yield. Tetraacetylated mannose containing an anomeric 2-aminoethoxy linker (**290b**) was then coupled to **387** to generate the trivalent neoglycoconjugate **388**. O-Deacetylation and further hydrogenolysis afforded the corresponding complex precursor **389** in 24% yield over two steps.

Second-generation mannosylated dendrons were synthesized by the process employed for **389**, starting with the introduction of Boc-*β*-alanine on triester **384** to provide **390** in 63% yield. Saponification and coupling with pentafluorophenol under standard conditions afforded triester **391**. The 2-aminoethyl mannoside **290b** was then coupled to **391** to give **392**. *In situ* removal of the *N*-Boc-protecting group (TFA) and treatment with **387** afforded, after deprotection, the nonavalent glycodendron **393** in



SCHEME 40. Mannosylated dendrons having an 8-hydroxyquinoline ligand at the focal point for self-assembly.[278]

29% yield over four steps. Finally, treatment of the foregoing precursors under appropriate stoichiometry with $Zn(OAc)_2$, [or $Al(OAc)_3$ or $GdCl_3$ for lower generation] in hot methanol afforded the self-assembled complexes **394** and **395** in 75–82% yields, containing either 6 or 18 peripheral mannopyranosylated residues (Fig. 39).

After assessing the interesting optical properties of the complexes, specific protein interactions with ConA as a model lectin were investigated in preliminary

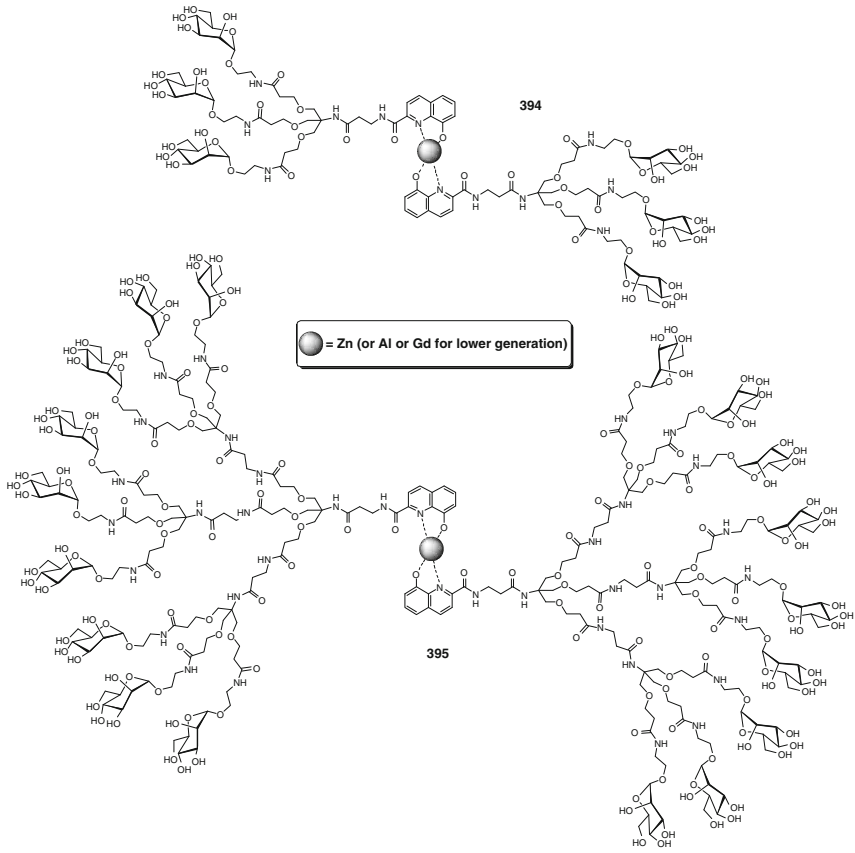
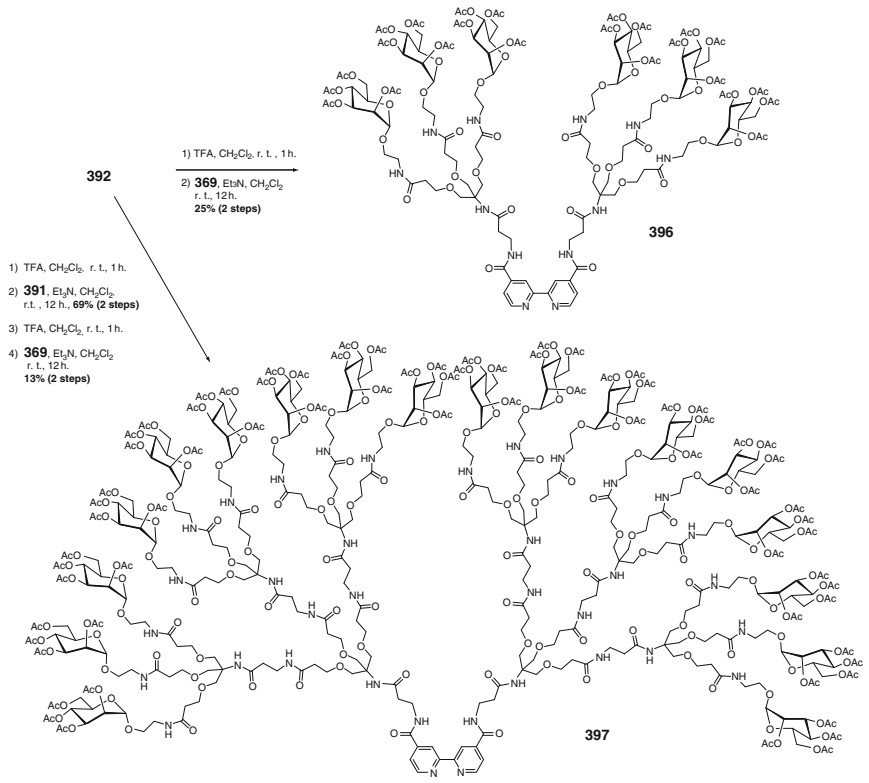


FIG. 39. Self-assembled mannodendrons.[278]

turbidimetry assays. Initial results indicated that metallic second-generation glycodendrimer **395** induced strong binding because of the large and dense glycocluster at the surface, and was a highly efficient lectin sensor. This approach to lanthanide-glycodendrimers [for instance with Gd(III)] might thus provide tunable fluorescent or MRI reagents for imaging.

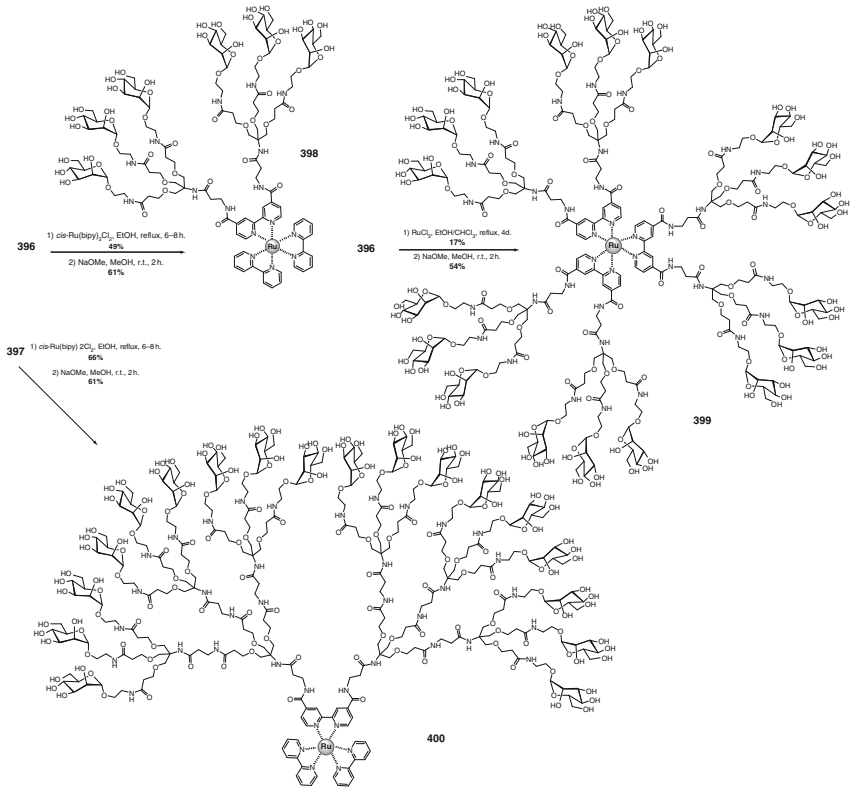
The same research group extended their studies, proposing a systematic investigation of the core activities for different carbohydrate densities during biosensing processes.[279] Three new mannosylated dendrimers with a $Ru(bipy)_3$ core unit were synthesized, and the influence of the number and size of dendritic branches on the rate of electron and energy transfer, as well as the lectin-biosensing abilities (Scheme 41),



SCHEME 41. Mannosylated glycodendrimers on a bipyridine core for ruthenium complexation.[279]

were investigated. Mannose-capped dendrimers **398**, **399**, and **400** were prepared using glycosylated bipyridine precursors by treatment with $RuCl_3$ or *cis*-Ru-$(bipy)_2Cl_2$. A first-generation dendron (**396**) was prepared in modest yield (25%) via the previous strategy, by removal of the *N*-Boc protecting group of **392** and subsequent peptide coupling with 2,2′-bipyridine-4,4′-dicarboxylic acyl chloride (**369**). A second-generation dendron, containing nine saccharidic units (not shown), was synthesized in 69% yield by the introduction of an amine derived from **392** and coupling to **391**. Following *N*-Boc deprotection (TFA) and subsequent peptide coupling with bipyridine derivative **369** gave **397** in 13% yield over two steps.

Boiling the first- and second-generation glycodendrons **396** and **397** in ethanol in the presence of *cis*-Ru$(bipy)_2Cl_2$ or $RuCl_3$ followed by O-deacetylation under Zemplén conditions yielded dendritic complexes **398–400** (Scheme 42) containing 6 or 18 peripheral mannopyranosides, respectively.



SCHEME 42. Preparation of photoinducible electron-transfer Ru(II)-complexes.[279]

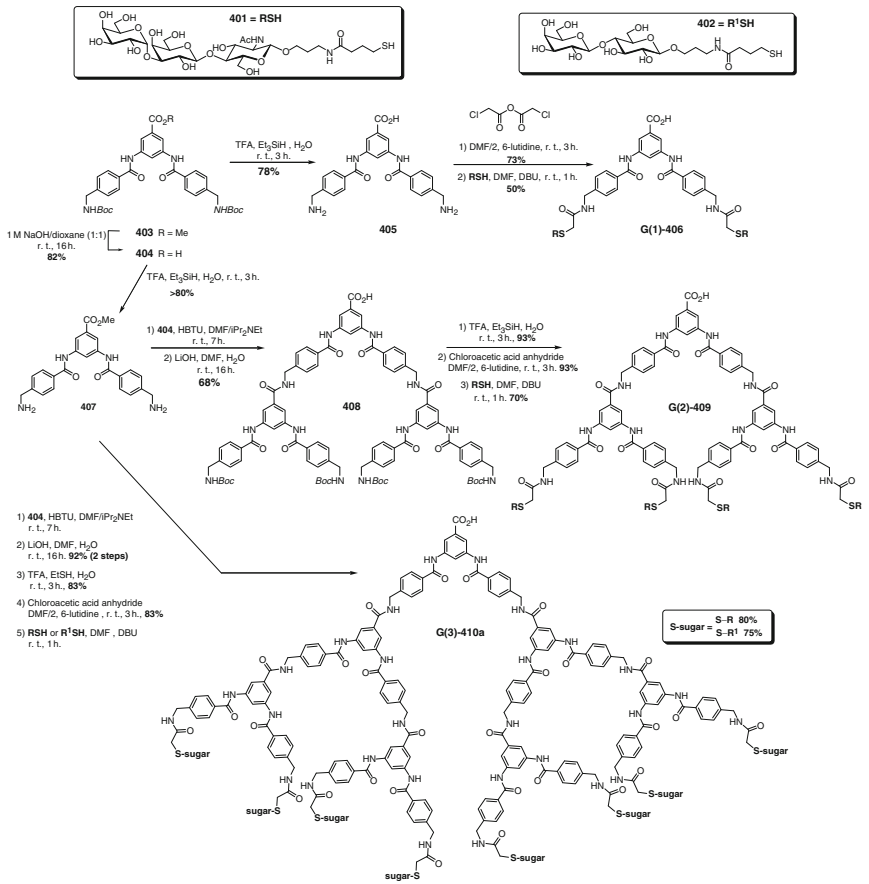
The rate of electron transfer within the complexes was investigated by photoinduced electron transfer (PET) between photoexcited Ru(II)-templates and a suitable quencher (*N*, *N*′-4,4′-bis(benzyl-3-boronic acid)bipyridinium dibromide, BBV) having high affinity for sugar. As expected, all compounds exhibited different extents of quenching, depending on the degree of peripheral carbohydrate density that efficiently insulated the core properties. The rate of energy transfer induced by photoexcitation of the complexes was also evaluated by quantification of molecular oxygen in the singlet state trapped by a quencher (TEMPO). The rate of appearance of the generated stable species (TEMPO) decreased from **398** to **400**, thus supporting the notion that dense carbohydrate insulation of the Ru(II) template presents a shield that stopped effective energy transfer to dissolved oxygen. In addition, the selectivity and sensitivity of these processes have been studied by use of complexes as lectin

biosensors. ConA and *Galanthus nivilis agglutinin* (GNA), both tetramers that contain one and three mannose-binding sites per subunit, respectively, were selected for these investigations. The concept was based on the reconstitution of the fluorescent signal when preliminary complexes between the BBV quencher and mannosylated-complexes were disrupted in favor of the tight interactions that take place between glycodendrimers and lectins. A spontaneous gain in fluoresecence was observed with the addition of 75 nM of ConA to the initial mixture of hexavalent complex and BBV. In contrast, more modest gains in fluorescence were obtained for dendrimers containing 18 mannosides under the same conditions, but a steady and linear increment continued at higher concentrations of ConA. Similar experiments with the higher valency lectin GNA induced the same tendency. Based on these results, detection limits for each Ru-complex were evaluated and compared, indicating that hexavalent glycoconjugate **398** was the most sensitive; whereas a higher detection limit was determined for **399** and **400** which exhibited a broader limited range of linear response. In conclusion, although complex **398** represented the best compromise between encapsulation and efficient quenching properties for sensitive lectin sensing, glycodendrimers **399** and **400** were also suitable sensitive biomarkers to study lectin–carbohydrate interactions, owing to their high quantum yield and excellent lectin-affinity through their high carbohydrate density.

2. Self-Assembly of Glycodendrons in Solution

In the early 2000s, Thoma *et al*. presented an alternative concept for the polyvalent presentation of ligands, based on the supramolecular chemistry of rather small molecules that fulfill single-molecule entity criteria.[280] They described the noncovalent and dynamic self-assembly of original and functionalized dendrons capped with carbohydrate ligands in aqueous solution, generating polyvalent glyconanoparticles. Dendritic scaffolds were prepared by a convergent ''outside-in'' approach.[281] This iterative methodology was based on a single building block (**403**) obtained by the coupling of methyl 3,5-diaminobenzoate and 4-(*tert*-Boc-aminomethyl)benzoic acid. Selective cleavage of the methyl ester group to give **404**, and subsequent removal of the *N*-Boc group liberated **405**, comprising the G(1) dendrimer generation with two amine end groups. Its coupling with **404**, followed by orthogonal methyl ester cleavage furnished **408**, a G(2) dendrimer generation containing four terminal anchorage functions. The third-generation dendrimer, with eight end groups, was obtained by treatment of **408** with **404**. The same procedure was repetitively applied to yield dendrimers with up to 64 end groups, corresponding to the sixth generation.

The peripheral amines of the glycodendrons of each generation were isolated as TFA salts and further transformed by introduction of chloroacetamide groups, using a large excess of chloroacetic anhydride in DMF, leading to complete conversion. Subsequent introduction of thiolated oligosaccharides such as **401** (Galili antigen) and **402** (Lac-SH) onto the dendritic cores was realized in the final step of the synthetic sequence in the presence of DBU, to afford water-soluble glycodendrimers **406**, **409**, **410a**, and **410b** (not shown; sixth generation) presenting a controlled number of peripheral oligosaccharidic residues based on the generation (Scheme 43).



SCHEME 43. Glycodendrimers scaffolded on hydrophobic repeating-units, forming stacked aggregates in aqueous solutions.[280,281]

Aggregation of all glycodendrimers in water was observed by ^{1}H NMR and quantified by using multiangle light scattering (MALS). Measurements indicated the formation of small aggregates of 50 kDa for the first generation (**406**) whereas the tetravalent analogue **409** formed large particles of 7000 kDa, corresponding to the aggregation of more than 1500 individual molecules per particle. Surprisingly, the particle weight obtained with higher generation dendrimers decreased with increasing mass of the individual molecule. Furthermore, the third-generation glycodendrimers **410**, containing eight (RS- or R^{1}S-) residues, exhibited similar size and weight, indicating that particle structure depended primarily on the dendritic core architecture, and less on the size and the nature of the carbohydrate group. The synthesis of several dendritic cores containing more branched aliphatic components in their structures, such as cyclohexyl rings, linear alkyl chains, or methylated amides demonstrated that only highly aromatic architectures induced aggregation as a result of π-stacking, hydrophobic interactions, and rigidity.[281] The authors assumed that intramolecular π-stacking led to preorganization, allowing efficient core–core contacts. An optimal core–core interaction was recorded for the second generation, whereas the lowest generation dendrimer was too small and the largest ones were too shielded by the dense carbohydrates. In addition, both the shape and the size of these homogenous nanoparticles (within a factor of 2) was investigated by AFM, indicating a disk-like morphology, having average diameters decreasing with increasing mass of the individual molecule, thus resembling their tendency in solution.

The biological activity of these noncovalent multivalent ligands was investigated via the inhibition of decavalent IgMs directed against the αGal (Galili) xenoantigen. To assess the compounds as polyvalent IgM ligands, the authors employed two *in vitro* assays in which the inhibition of both the anti-αGal IgM binding to the xenoantigen and the αGal-mediated lysis of pig erythrocytes were measured. In agreement with previous results, monomeric αGal was inactive at 100 μM in both assays. Whereas no activity was observed for the divalent conjugate, the second- and third-generation dendrimers **409** and **410** were highly potent in both assays (0.025, 0.035 μM and 0.010, 0.010 μM, respectively). The fourth-generation analogue, containing 16 peripheral αGal residues, also showed high potency in the binding assay (0.019 μM) but was significantly less potent in the hemolysis assay (0.18 μM). Generally, potency decreased drastically for the larger dendrimers which did not induce aggregation. The avidity thus correlated with the size of the aggregates, but not with the size of the individual particles. Preliminary *in vitro* results highlighted the involvment of polyvalent aggregates in antibody binding, without nonspecific interactions of the dendrimer backbone. The most potent compound (**410**) bearing eight trisaccharidic epitopes, was selected for *in vivo* profiling in cynomolgus monkeys.

Within 5 min after injection, the anti-αGal IgMs detected by ELISA were diminished to 20% of the initial value determined prior to the administration, and remained at low levels for more than 4 h. Most importantly, anti-αGal antibody-mediated hemolytic activity was completely eliminated.

Even though examples remain rather scarce, the preparation of multivalent structures via supramolecular chemistry involving the self-organization of ligands around a coordinating core has afforded glycoconjugates in which the valency, the size, and the structures can be efficiently tailored with the nature of the coordinating metal and the chemical composition of the ligand. In most instances, the stable complexes generated constitute promising organizations with enhanced biological properties as compared to the corresponding monomeric ligand. Moreover, this noncovalent, dynamic, and reversible self-assembly process can, ideally, allow the utilization of the polyvalent receptor (lectin) as a template to optimize its own polyvalent inhibitor with the organization of carbohydrate moieties to fit perfectly into the recognition sites. This adaptability has also been emphasized by the significant improvement of biological activities as compared to those of individual species that has been observed for homogenous noncovalent glycoparticles that self-assembled in aqueous media via a similar dynamic equilibrium process.

In the light of promising applications offered by these supramolecular glycoconjugate structures, notably for the intrinsic optical and electrochemical properties of metal complexes, this underexploited research area is undoubtedly in its infancy and holds promise for such systems as biomarkers or sensitive biosensors.

V. Glycodendrons and Glycodendrimers

1. Introduction

a. Definition and History.—A dendrimer is a synthetic highly branched monodisperse and polyfunctional macromolecule, constituted by repetitive units (so-called ''generations'') that are chemically bound to each other by an arborescent process around a multifunctional central core.[282,283] Thus, as opposed to traditional polymers, which often have poorly defined molecular structures that are clearly an important drawback for medical application in terms of product characteristics, dendrimers are structurally well defined and can be synthesized from a fully controlled iterative approach. Although differences exist in terms of rigidity and compaction, dendrimers are often compared to ''artificial proteins'' with their semiglobular or globular structures, mostly with a high density of peripheral functionalities and a small

molecular ''volume.''[284,285] It is now accepted that dendritic polymers are the fourth major class of polymeric architecture, consisting of three subsets that are based on the degree of structural control, namely: (a) random hyperbranched polymers, (b) dendrigraft polymers, and (c) dendrimers. The concept of repetitive and controlled synthetic growth with branching was first reported by Vögtle, who achieved the construction of a low molecular weight ''cascade'' polyamine.[286] However, it was not until 1985 that the groups of Newkome[287] and Tomalia[288] independently described a divergent macromolecular synthesis, giving birth to the first well-characterized true dendrimers, named ''arborols'' and ''PAMAM'' [poly(amidoamine)] dendrimers, respectively. Their strategy thus efficiently avoided problems of low yields, purity, or purification encountered by Vögtle in his cascade synthesis. In their chapter, Tomalia *et al.* paved the way to dendritic structures: their definition and construction, and introducing for the first time the term ''dendrimer,'' which arises from the Greek *dendron* meaning ''tree'' or ''branch,'' and *meros* meaning ''part.'' Their original and efficient methodology still constitutes the preferred commercial route to the trademarked Starburst® dendrimer family, with molecular weights ranging from several hundred to over 1 million Daltons (namely, generations 1–13). Until the mid-1990s, the synthetic challenge of such aesthetic structures stimulated numerous research groups to investigate intensively a range of synthetic strategies. These efforts gave rise to original dendritic structures emerging from two main synthetic strategies used to construct perfectly branched dendrimers: the divergent and the convergent approach. Over time, an accelerated version of the convergent strategy has been developed in order to increase its throughput and efficiency by using clever adaptations of cores or dendrons.

Initial efforts gave rise to well-characterized dendritic macromolecules, but applications remained limited because of the lack of specific functionalities. An exponential increase of publication volume observed for about 15 years testified the growing interest for dendrimers and has led to versatile and powerful iterative methodologies for systematically and expeditiously accessing complex dendritic structures. The perfect control of tridimensional parameters (size, shape, geometry) and the covalent introduction of functionalities in the core, the branches, or the high number extremities, or by physical encapsulation in the microenvironment created by cavities confer such desired properties as solubility, and hydrophilic/hydrophobic balance. Thus, creativity has allowed these structures to become integrated with nearly all contemporary scientific disciplines.

Undoubtedly, biology and nanomedecine, more particularly biomedical and therapeutic applications, are the domains that have generated the highest infatuation for these architectures. However, the complexity of mechanisms involved in biological

processes presents an important challenge for efficient structure design. In fact, historically, problems concerning toxicity, hydrosolubility, degradability, targeting specificity, pharmacokinetic, and biodistribution profiles for this kind of applications have been recurrent when using monomeric systems. Application of polymeric systems has been exploited advantageously to enhance hydrosolubility, biocompatibility, lack of toxicity, immunogenicity in order to improve drug stability, and selectivity toward malignant tissues. This enhancement of therapeutic properties observed with the use of ''prodrugs'' has been explained by the physical properties of these polymeric structures, presenting high hydrodynamic volumes that facilitate blood persistence and accumulation in tumoral tissues in particular.This phenomenon, called the ''EPR effect'' (i.e., enhanced permeation retention), may be roughly explained by physiological and biochemical differences observed between tumoral and healthy tissues. In fact, tumor tissues present specific vascularization with a defective lymphatic drainage system that allows macromolecules (with molecular weight $\geq$ 20 kDa) permeability, retention, and accumulation.[289] However, a high polydispersity index and low drugs loading are often responsible for critical lack of reproducibility and efficiency.

Dendrimers, combining several of the advantages described for polymers, along with monodispersity and a high density of functionalities with a small molecular volume, have been exploited for about 15 years to surpass the problems usually encountered. Thus, their particularly unique structures and properties have motivated their use in numerous applications as drug or gene delivery devices in anticancer therapy, and as antibacterial, antiviral, or antitumoral agents. The use of dendrimers in biological systems, and also systematic studies of the most common dendritic scaffolds to determinate their biocompatibility, such as *in vitro* and *in vivo* cytotoxicity, their biostability or immunogenicity have been extensively reviewed.[290,291] One typical example concerns the use of dendrimers as ''glycocarriers'' for the control of multimeric presentation of biologically relevant carbohydrate moieties that are useful for targeting modified tissue in malignant diseases for diagnostic and therapeutic purposes. In such molecules, termed ''glycodendrimers,'' the saccharide portions are conjugated according to the principles of dendritic growth or are ligated to preexisting highly functionalized and repetitive dendritic scaffolds having varied molecular weights and structures. Since they first appeared in the literature in 1993,[292] glycodendrimers and related glycodendrons, with their spheroidal or dendritic wedge structures, have been initially designed as bioisosteres of cell-surface multiantennary glycans that stimulated wide interests within the scientific community.[293–298] As with conventional dendritic structures, glycodendrimers can be obtained as dendrons, spherical or as globular architectures, or ''hybrid dendronized

polymers'' according to divergent, convergent, or accelerated approaches. All of these original synthetic clusters were constructed in such a way that their valencies, shapes, and carbohydrate contents could be varied at will with a controlled integration of dendritic building blocks. Hence, recent progresses in synthesis of dendritic structure can allow easier optimization, to afford tailored glycoconjugates with biologically adapted and optimized properties.

b. Glycodendrimer Syntheses.—Historically, the divergent strategy was used to prepare the first dendritic structures. Dendrimers are built iteratively out from a central core, layer by layer, requiring activation/addition steps to afford the desired dendritic structures: the focal and multifunctional molecules are systematically expanded outward using various chemical linkages. The first-generation dendrimer is simply formed by attaching branching units to the core molecules. To form the second-generation dendrimer, the peripheral functional groups then react with a complementary chemical function presented on the branched building blocks. To avoid hyperbranched polymerization, this step has to be carefully controlled by using protected (or inert) groups on the building blocks. Activation (deprotection or chemical transformation) of the newly attached surface groups leads to the second-generation dendrimer. The generation growth quickly allows exponential multiplication of active terminal functions, and the process is repeated until the required degree of branching is obtained. For glycodendrimers, the sugars are then appended at the periphery of the molecules. Although this approach is conceptually straightforward, synthetic problems are sometimes encountered, involving the use of very large excess of reagent (or monomer) in each synthetic step to ensure complete functionalization and the necessity of efficient coupling in regard to the exponentially increasing number of functions. To accentuate the difficulty, and although monomers are generally easy to remove, separation of the required dendrimer from the structurally flawed by-products usually remains challenging, because of their mass, size, and general properties that are very close to the perfect dendrimer. For the sake of simplicity and diminished cost, the inner scaffold portion of the glycodendrimer can be either synthesized by a one-pot procedure using hyperbranched polymer methodologies, or purchased directly. Indeed, several dendrimers having various surface functionalities and building blocks are commercially available: polyamidoamine dendrimers (PAMAM, Starburst®, Dendritic Nanotechnologies), polypropyleneimine (PPI, Astramol®, DSM Fine Chemicals), polyglycerol dendrimers, and hyperbranched dendritic polymers (Boltorn®), are most commonly used as multibranched dendritic core or glycodendron precursors, and most of them are known to be nontoxic and nonimmunogenic.

An alternative and more efficacious convergent strategy was reported in 1990 by Hawker and Fréchet,[299] using the symmetrical nature of these structures, in order to overcome some of the synthetic and purification problems associated with the divergent methodology. It involves the preliminary synthesis of peripheral branched dendritic arms, named ''dendrons'' or ''glycodendrons,'' from the ''outside-in.'' This concept can be described by envisaging the attachment of X terminal units containing one reactive group to one polyfunctional monomer possessing orthogonally protected functionalities, resulting in the first-generation dendron. Transformation of the unique focal site, followed by treatment with $^{1}/_{X}$ equivalent of the protected monomer or a polyfunctional central core affords the next higher generation dendron or a dendrimer. The advantages of the convergent strategy lie with the diminished number of reactions carried out in each step. Moreover, purification of the desired dendrimer becomes less problematic than in the divergent case, fewer by-products are generated and they are structurally very different from the perfect target dendrimer. However, the fact that the focal functionalities of the wedge may be sterically inaccessible from within the infrastructure (depending on the generation) causes difficulties toward subsequent linkage to the core, thus resulting in slower and less efficient reactions as the generation grows. Nevertheless, it is now well established that a large number of surface glycan moieties impede accessibility of the carbohydrate by carbohydrate-binding receptors. Considering these synthetic advantages, this methodology has been used successfully for access to dissymmetric dendritic structures.[300]

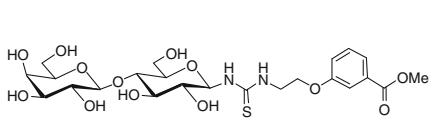
Cumbersome purifications and synthetic disadvantages observed with both iterative approaches have motivated investigations toward accelerated synthetic methodologies. To improve synthesis efficacy and limit synthetic steps, while preserving monodispersity and functionalization versatility, new strategies have been successfully addressed. These include the development of larger building blocks involved in ''multigeneration'' coupling, as largely described by Fréchet *et al.* In this way, high molecular weight dendrimers and dendrons have been synthesized using highly polyfunctional dendritic cores (''hypercores''),[301] and high-generation dendrons for subsequent coupling reaction (''hypermonomers''),[302] which can also be obtained by an orthogonal protected functions approach (''double exponential growth strategies'').[303] An elegant application has been described[304] based on this design to allow for more rapid dendritic construction, starting with small glycoclusters, which are attached to branching units to form glycosylated hypermonomers and then finally to a suitable central part. More recently, another clever strategy based on ''orthogonal monomer systems'' has been designed.[305,306] The judicious use of functionalized building blocks that are coupled together without need of protection/deprotection steps has allowed rapid and easy access to homogeneous and heterogeneous dendrimers.

2. Glycodendrons

The convergent alternative for the synthesis of glycodendrimers is attractive, since early synthesized glyco-coated molecular wedges present readily available and interesting multivalent candidates for biological investigations. Obviously, by analogy to classical synthesis of globular structures, their growth can emanate from the controlled succession of suitable functionalized building blocks provided by standard AB_2 or AB_3 systems.

a. AB_2 Systems.—Aromatic AB_2 dendritic building blocks have been widely used to construct glycodendrons possessing interesting biological activities. One of their first applications was to prevent tissue infection by the pentameric bacterial toxin from *V. cholerae*. The approach for preventing binding of CT-GM_1 consisted in considering the terminal galactose residue (Fig. 9) as the anchoring fragment, to which various pharmacophores could be attached to provide optimized small-molecule antagonists against CT. This strategy was based on the fact that this galactose residue bound very specifically at a buried pocket of the receptor-binding site. However, the rest of the binding site is very shallow and lacks well-defined hydrophobic pockets that can be exploited using traditional structure-based drug design to arrive at potent inhibitors. Initial studies were made by screening commercially available galactose derivatives. Using ELISA, it resulted that *m*-nitrophenyl α-D-galactopyranoside (MNPG) was the best inhibitor identified, with an IC_{50} of 720 μM, corresponding to a 100-fold better affinity for CT than that of the ''lead'' galactoside.[307] In addition, a small library of antagonists showing up to 14-fold improvement as compared to the best MNPG candidate was designed. The library consisted of 3,5-disubstituted phenyl galactosides (as α/β anomeric mixtures), in which a hydrophobic ring-system was linked via a short, flexible aliphatic linker through an amide linkage at the remaining *meta* position of the MNPG core.[308]

Further improvements were obtained by using a simplified strategy based on the use of relatively simple lactose derivatives (Fig. 40).[309] The potent monovalent



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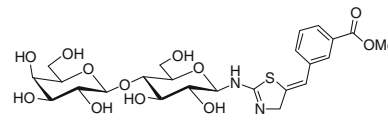


412

FIG. 40. Simple lactoside analogues as surrogates for the more complex GM_1 oligosaccharide ligand against the pentameric bacterial toxin from *Vibrio cholerae*.[309]

inhibitor (**411**) for CT was developed and had a K_D of 248 μM from a direct fluorescence-binding assay. This rather low value was attributed to the presence of the thiourea moiety, and the aryl group that seemed to contribute to the 70-fold binding enhancement as compared to unsubstituted lactose (K_D = 18 mM). An improvement in binding affinity was further observed by the same group with a new lactose 2-aminothiazoline conjugate (**412**), formed by a cyclization of the thiourea sulfur atom onto a triple bond and containing a more rigid spacer between the sugar and the aryl group.[310] Fluorescence studies revealed one order of magnitude enhancement in its affinity (K_D = 23 μM) for the CTB subunit, as compared with that of thiourea derivative.

In order to improve their respective inhibitory potencies against CT, the optimized monovalent inhibitors just described were linked to various AB_2 building blocks, thus adding the concept of multivalency. Therefore, an improvement in receptor-binding activity was expected with the use of the glycodendron approach, taking advantage of the symmetrical arrangement of five identical binding sites on the toxin B pentamer, as mentioned in earlier sections. This context allowed the synthesis of generation 1, 2, and 3 of lactosylated dendrons based on a 3,5-bis(2-aminoethoxy)benzoic acid repeating unit and containing 2 (**413**), 4 (**415**), and 8 peripheral lactoside residues (**417**), respectively, bound to the dendritic scaffold via thiourea linkages (Fig. 41).[309] Binding affinities with the CT subunit were determined by fluorescence assay (FRET) and ranged from 18 mM for lactose to 33 μM for the octavalent glycodendron **417**.

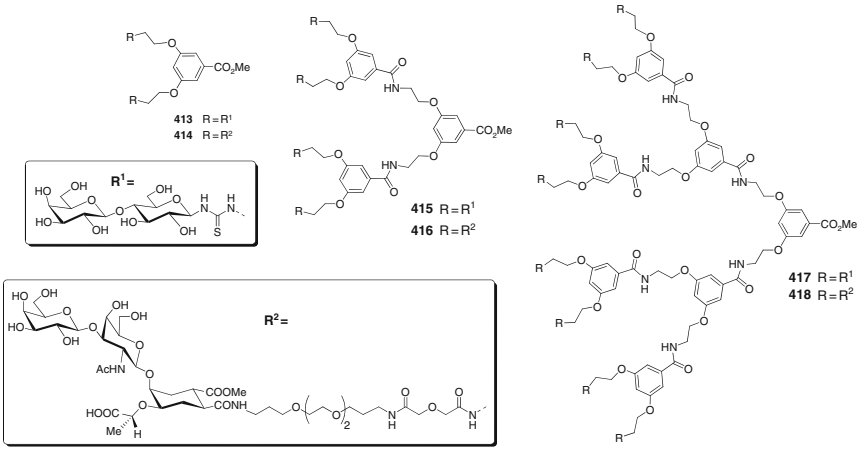


FIG. 41. Glycodendrimers bearing lactoside and Bernardi's GM_1os surrogates.[311]

This value corresponded to an eightfold enhancement as compared to corresponding monovalent lactoside derivative under the same conditions. However, an increase in the branching of the dendron (namely valency) provided only a modest increase in the potency of the ligand, corresponding to a rather constant relative potency per lactose, regardless of the number of peripheral epitopes.

To further advance the effectiveness of such glycodendrimers, the same group incorporated two modifications to the system.[311] The first alteration implicated glycodendrons having a slightly modified scaffold, outfitted with elongated arms, and peripheral attachment of the GM_1 mimics proposed earlier by Bernardi *et al.*[125] using the modified GM_1 analogue **108** (Fig. 9). Unlike GM_1, the modified **108** derivative was obtained on a gram scale and the synthetic sequence was adapted to achieve differentiation of the carboxyl group in the cyclohexanediol moiety to allow further functionalization. Using the SPR inhibition assay, the products showed good inhibition, with IC_{50} of 13 μM for the divalent **414** and 0.5 μM for the tetravalent **416**, corresponding to a 440-fold improvement over its monovalent counterpart. The octavalent analog **418** was the most potent compound, as determined using an ELISA assay.

In ongoing efforts, highly effective CT inhibitors were obtained by using similar architectures with a combination of several critical factors in their design: the use of authentic or modified GM_1 oligosaccharide sequences as the optimal monovalent ligand (**419–421**), bound to multivalent dendritic scaffolds presenting elongated spacer arms of optimal length (Fig. 42).[312]

Compound **419** and its galacto-modified analogue **421**, each bearing an azido group, can be conjugated to polyalkynic dendritic scaffold by ''click chemistry.'' To evaluate the potency of the inhibitors **419** and **421** and their corresponding di-, tetra-, and octavalent derivatives, an ELISA assay was used which indicated unprecedented affinities and potencies, notably for octavalent **422**, which exhibited a very

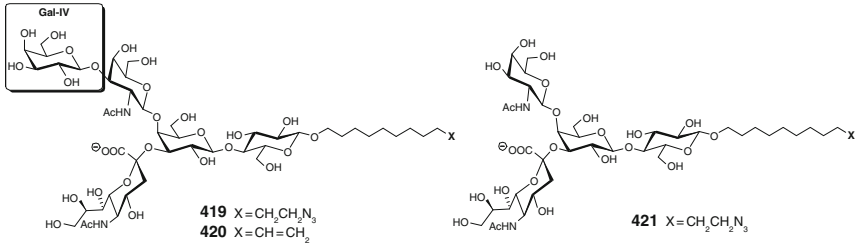


FIG. 42. GM_1 and agalacto-GM_1 ligands.[312]

low IC_{50} (around 50 pM), meaning that each GM_1os moiety bound 47,500-fold more strongly than the corresponding monovalent **419**. Di- and tetravalent GM_1os systems had IC_{50} values roughly 4–5 orders of magnitude lower than the octamer. In the agalacto dendrimer series related to **421**, the octavalent **423** was a weaker inhibitor than its tetravalent counterpart, having an IC_{50} of 0.1 μM.

Further studies concerning the activity of glycodendrons containing two or four peripheral GM_1os against the *E. coli* heat-labile toxin (LTBh) B-pentamer have been similarly described (Fig. 43).[313]

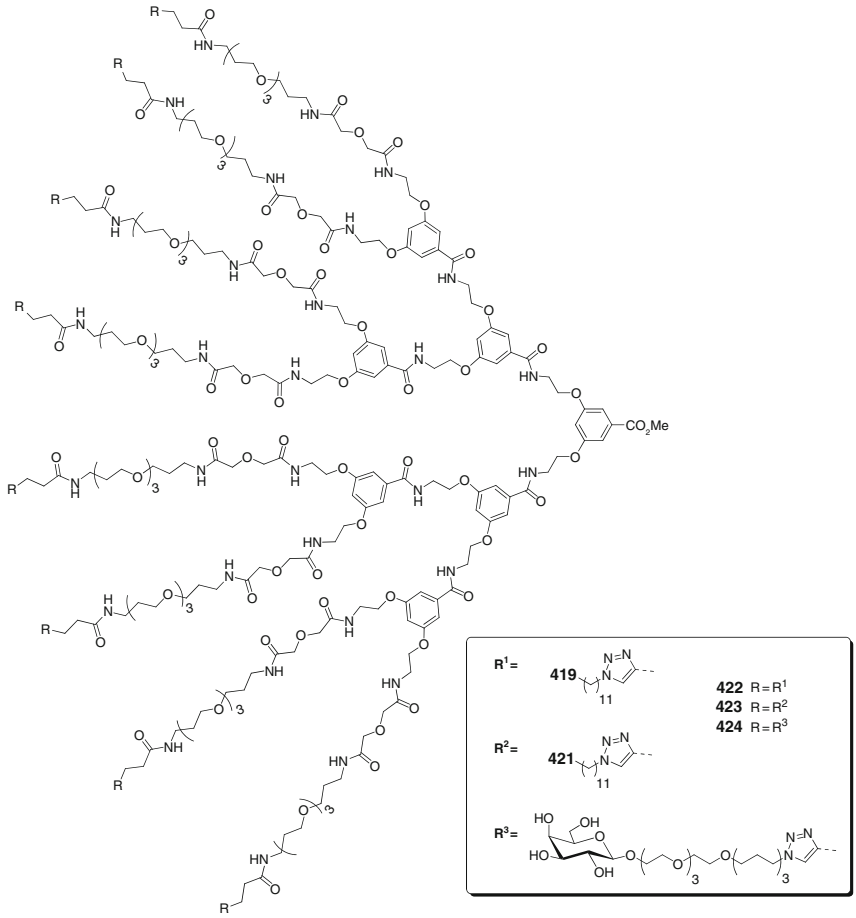


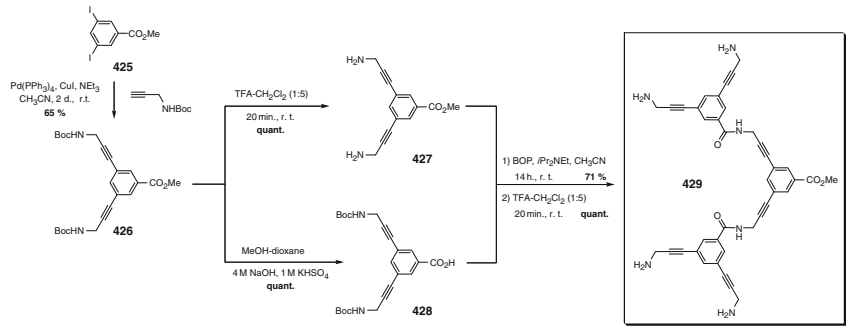
FIG. 43. Octameric glycodendrons bearing GM_1 analogues.[312–314]

Analytical ultracentrifugation and DLS have been used to demonstrate that the multivalent inhibitors induced protein aggregation and the formation of space-filling networks. This aggregation process appeared to take place when using ligands that did not match the valency of the protein receptor. Interestingly, the valency of the inhibitor had a dramatic effect on the mechanism of aggregation, influencing both the kinetics of aggregation and the stability of intermediate protein complexes. In addition, structural studies employing AFM have revealed that a divalent inhibitor induced head-to-head dimerization of the protein toxin, which either prevented the LTBh pentamer from sitting flat on the surface, or gave rise to a protein bilayer. Considering that tetravalent structures were shown to be more potent inhibitors than pentavalent analogues of similar size, the development of a strategy based on the use of mismatched valencies may provide more relevant multivalent therapeutics against pentameric bacterial toxins, thus adding to the arsenal of multivalent strategies.

To avoid the tedious preparation of GM_1 derivatives, the direct and efficient preparation of dendritic inhibitors based on a simple galactoside was also proposed by the same group.[314] Hence, a simple β-D-galactopyranoside bearing a poly(ethylene glycol) unit, crudely mimicking the other sugar rings of GM_1os and ending with a lipophilic part having a terminal azido function, was attached to keep this factor the same as in the systems just mentioned. The compounds of highest valency (**424**) and the corresponding tetravalent system showed IC_{50}s in the same range as the GM_1os derivative **422**. The multivalency effect, as expressed by the relative potency per sugar, still increased from di- to tetra-valent (923 vs. 2400), while remaining basically the same for the octavalent analogue (2500). Although results obtained with dendrimers coated with a sole galactoside residue were less spectacular than those observed with the agalacto-GM_1os derivatives, they again constituted an important step toward potent ligands against CT of low cost and ready availability.

Parallel investigations by the same group described the construction of new and rigidified multivalent structures bearing up to four lactose-2-aminothiazoline units at the periphery.[315] The synthesis of the dendritic scaffold started with aromatic diiodide **425**, which was treated with Boc-protected propargylamine under modified Sonogashira coupling conditions, to yield the branching unit **426**, fully orthogonally protected as the key building block (Scheme 44). Part of this material was exposed to a CH_2Cl_2–TFA mixture to afford quantitatively the N-deprotected compound **427**. The other part was smoothly hydrolyzed with Tesser's base to lead to carboxylic acid derivative **428**. The two fragments were then coupled under standard peptide conditions and subsequently treated with TFA to yield the tetraamino compound **428**.

Coupling di- (**427**) and tetraamines (**429**) to the lactosyl β-isothiocyanate (**430**) in the presence of $i\text{Pr}_2\text{NEt}$ and subsequent acid treatments to facilitate formation of the



SCHEME 44. 3,5-Diaminobenzoic acid scaffolds prepared using Sonogashira chemistry.[315]

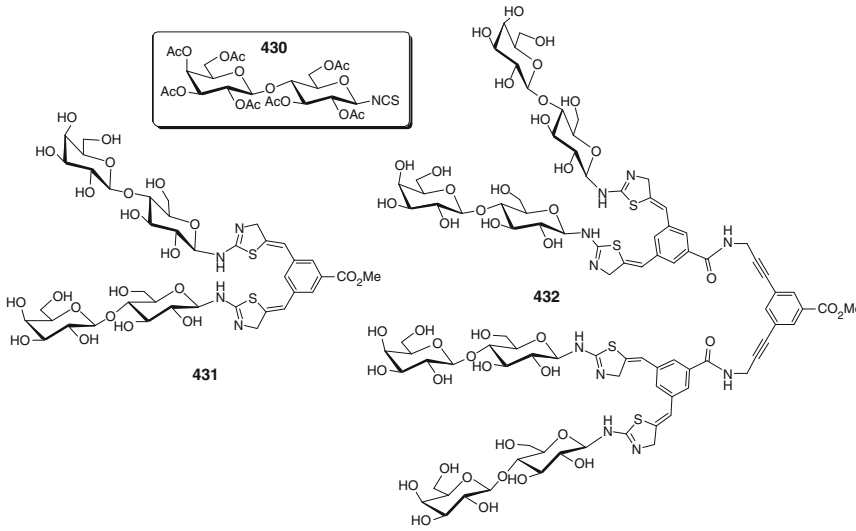


FIG. 44. Dendrons with 2- and 4-aminothiazole lactosides.[315]

aminothiazole afforded the glycoclusters in moderate yields of 65% and 33%, respectively (Fig. 44). Standard deacetylation under Zemplén conditions yielded di- (**431**) and tetra-valent dendrons (**432**).

The deprotected lactosides were evaluated as inhibitors against lectin binding in a solid-phase inhibition assay with immobilized ASF on the surface of microtiter plate wells, mimicking cell-surface presentation, while mammalian galectins-1, -3, and -5 were in solution. Strong multivalency effects and selectivity were observed for the

tetravalent lactoside in the inhibition of galectin-3 binding, even better than for ASF, with an IC_{50} of 70 nM, corresponding to a 4300-fold enhancement compared to lactose (thus a factor of 1071 in the relative potency of each lactose unit). On the other hand, although rigidified glycodendrons generally generated more interesting results than the corresponding analogues **415** and **417**, no marked multivalency effects in the inherent binding affinities to galectin-3 were observed by fluorescence spectroscopy with all components in solution.

A biotinylated glycopeptide dendron, based on a dendritic L-lysine scaffold and bearing four T-antigen tumor markers [β-Gal-(1→3)-αGalNAc, T-Ag **433**] (Fig. 45), was first proposed by Baek and Roy in 2001.[316] The doubly associative binding interactions between the heterobifunctional biosensor **433** and the coating streptavidin, together with mouse T-Ag monoclonal antibody, were demonstrating using conventional solid-phase double sandwich ELISA. Hence, the virtue of the T-Ag glycodendrimer used as coating agent was a very effective anchoring motif of high avidity (nanomolar coating), and constituted an efficient cell-surface model.

Using analogous aromatic AB_2 building blocks, a modular approach leading to glycoconjugates with multiple copies of **Gb3** analogues that can induce differentiation between structurally homologous Shiga 1 (Stx1) and Shiga 2 (Stx2) toxins from complex samples has been developed.[317] To this end, divalent systems bearing **Gb3** (Fig. 17) analogues, or those of a neutralizing polysaccharide corresponding to serogroup O117 (O117 LPS, **434**), have been constructed (Fig. 46). Interestingly, O117 LPS resembles **Gb3**, but there are significant structural differences since **Gb3**

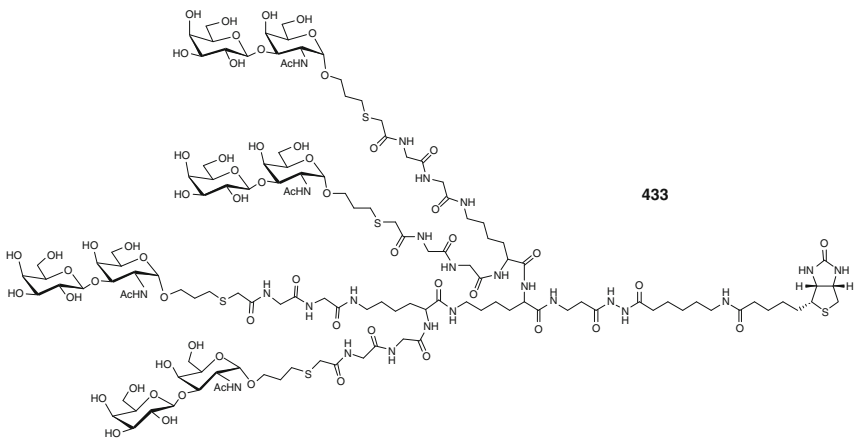


FIG. 45. Biotinylated dendritic T-Antigen synthesized by Baek and Roy.[316]

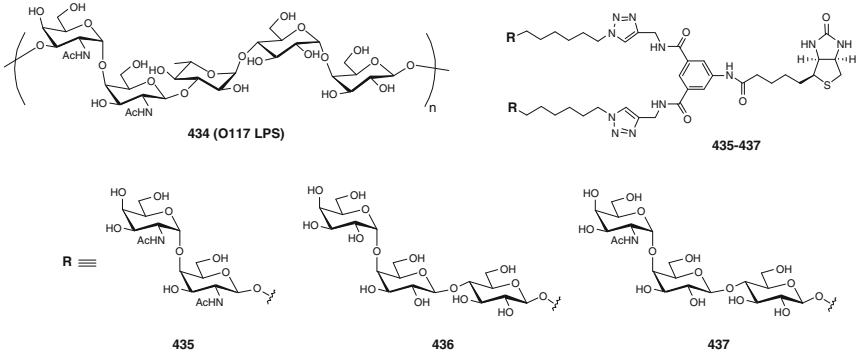


FIG. 46. Dimers of Shiga toxin analogues.[317]

has a terminal α-(1→4)-digalactoside moiety, whereas the neutralizing polysaccharide has a modified terminal digalactose moiety possessing a bulky *N*-acetyl group at each 2-position. In contrast to **Gb3**, which binds to both Stx1 and Stx2, O117 LPS was not able to bind Stx1 or to neutralize its effect on Vero cells, suggesting that the *N*-acetyl group sufficiently modifies the binding specificity toward Stx1. Tailored biantennary glycoconjugates (**435**–**437**) consisting of three structural components including peripheral carbohydrate-recognition components, flexible aliphatic spacers, and a biotinylated dimer have been synthesized with varied N-acetylation patterns. Biotin was used because it afforded ready access to multivalency as one streptavidin tetramer binds to four biotin molecules, and it can be conjugated to commercial streptavidin matrices for toxin capture. Molecules were designed such that the biotin and, hence, streptavidin were attached to the opposite end of the rigid scaffold to minimize interference by biotin in the binding event.

Binding of Stx1, Stx2, and Stx2c, a variant found in human clinical samples, was assessed by ELISA analysis. Results indicated that Stx2 bound to the di- and mono-*N*-acetyl-substituted galactosamine **435** and **437**, respectively, whereas Stx1 failed to bind to either compound. More precisely, **437** appeared to be a better substrate for Stx2 than **435**. In contrast and surprisingly, **436**, constructed with the **Gb3** analogue, bound exclusively to Stx1, probably due to the biantennary architecture with a short spacer that constrained binding to Stx2. Finally, the authors proved the ability of **436** to capture Stx1 in clinical applications, without any interference from a complex sample, indicating the feasibility of highly selective and sensitive synthetic glycoconjugate-based detection reagents for Stx by introducing simple manipulations in the structure of known saccharide receptors.

Using the same versatile modular synthetic strategy, the same group developed biotinylated bi- (**438**) and tetra-antennary (**439**) mannosylated glycoconjugates to capture and detect *E. coli* cells, and compared the relative capturing ability of these molecules to commercial polyclonal antibodies (Fig. 47).[318] Instead of aliphatic spacers, tetraethylene glycol linkers were used to diminish nonspecific binding and to impart flexibility for a better fit in the active sites.

Biotinylated glycoconjugates or antibodies were grafted on commercial streptavidin-coated magnetic beads and the resulting material was used to capture, isolate, and quantify bacterial recovery by using a luminescence assay. In this context, ''glyco-magnetic'' beads completely covered with glycans were incubated with two isogenic strains of *E. coli*, ORN178 and ORN208. The ORN178 *E. coli* bears numerous fimbrial adhesins (FimH) possessing binding preferences to α-mannosides. The second strain is a mutant lacking pilus expression. In initial experiments, strain ORN178 mediated the aggregation of beads coated with mannose-bearing divalent compound **438** within minutes of addition to the beads, whereas strain ORN208 did not. Bacterial aggregation has been shown to be dependent on multivalency, and these results suggested that a single bacterium could bind to multiple beads. Moreover, at higher concentrations of *E. coli*, the tetravalent conjugate **439** was responsible for an increase in capture effectiveness. The authors further compared their competence relative to standard antibody-coated beads for the capture of bacteria. The results indicated that the glycoconjugate-coated magnetic beads outperformed traditional antibody-coated magnetic beads in sensivity and selectivity when compared under identical experimental conditions. In addition, these systems could capture *E. coli* from environmental samples of stagnant water, with the possibility for targeting specific pathogenic

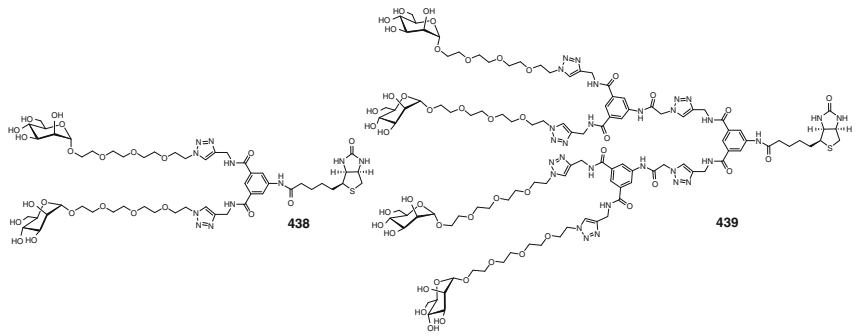
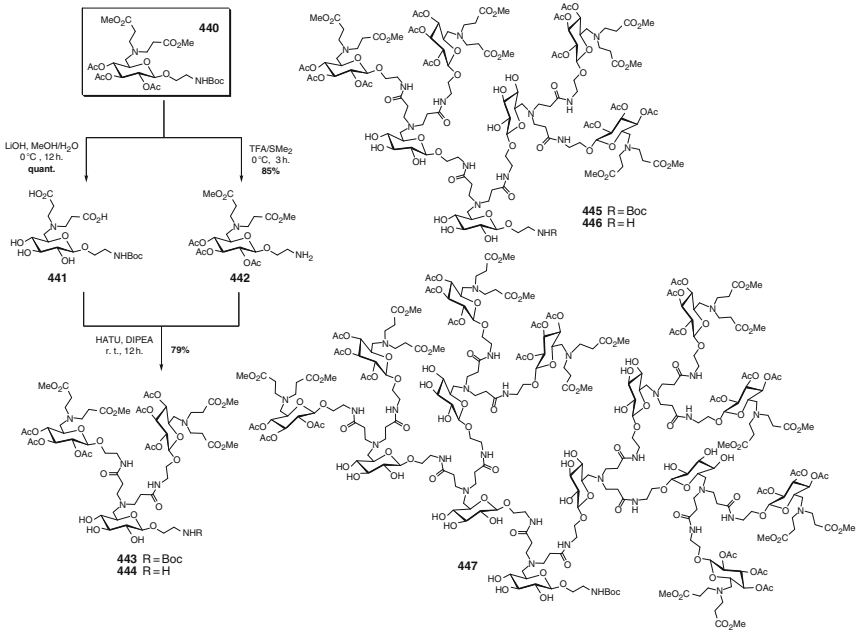


FIG. 47. Mannosylated dendrons with biotin end group for *E. coli* capture.[318]

bacteria modulating the nature of the carbohydrate recognition component. These experiments thus clearly revealed the power of glycans in biosensing, and demonstrated that these stable and inexpensive glycomagnetic beads could be used for capture and isolation of pathogens from other complex matrices.

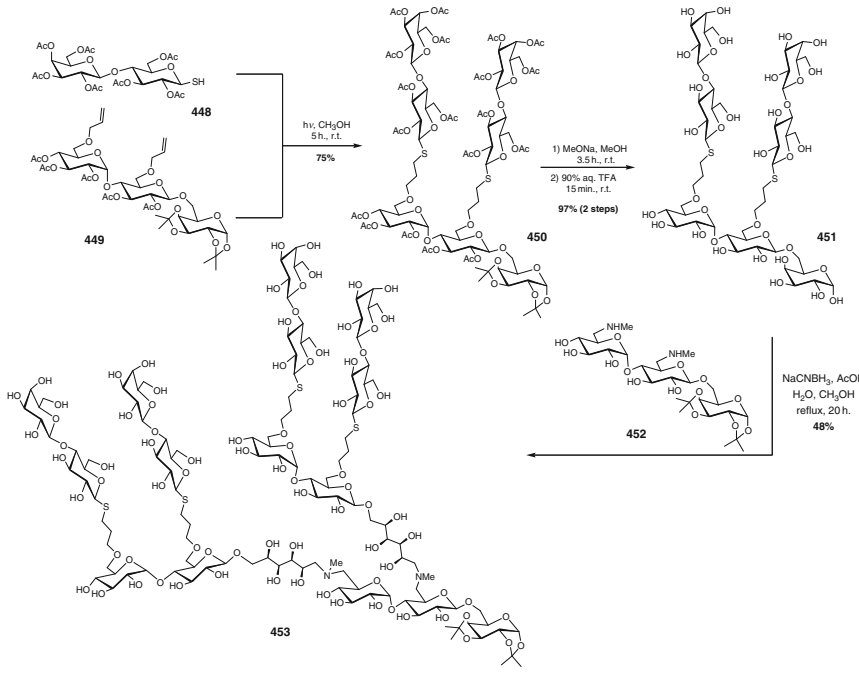
In 2000, Saladapure and Lindhorst reported the synthesis of glycopeptide dendrons in which the peptide coupling of orthogonally protected AB_2-type carbohydrate units formed the basis for an iterative sequence leading to the various generations of glycodendrons in either a divergent or convergent manner.[319] The synthetic strategy was based on the use of efficient peptide chemistry in the linking step, avoiding sophisticated glycosylation techniques for each coupling step, and allowing possible adaptation to solid-phase chemistry (Scheme 45). The key AB_2-type glucoside building block **440** was used for the preparation of multigeneration glycopeptide dendrons from its orthogonal deprotection, to yield complementary building blocks **441** and **442**. Hence, mildly alkaline hydrolysis of the methyl ester groups led to dicarboxylic acid **441**, whereas removal of the *N*-Boc protecting group with a TFA–dimethyl sulfide mixture afforded amino-functionalized glucoside **442**. HATU-mediated



SCHEME 45. AB_2-sugar scaffold used in glycodendrimer synthesis.[319]

peptide coupling, together with DIPEA gave protected first-generation glycodendron **443** in 79% yield. Using the *N*-Boc-deprotection and peptide-coupling reaction sequence, the synthesis of higher generations of glycopeptide dendrons was carried out convergently. Hence, **443** was converted into its amine derivative **444**, whose coupling with **441** led to protected G(2) glycopeptide dendron **445** in 60% yield. The same convergent strategy was applied for the construction of the third-generation glycodendron **447** containing eight peripheral glucoside units, via peptide coupling between **446** dendron and **441**. Although no biological investigation was proposed by the authors, the stability of O-deacetylated G(1) glycodendron against β-glycosidases from almonds was evaluated and no degradation was observed over several hours.

Four years later, Nelson and Stoddart designed di- and tetra-valent lactosylated dendrons according to a convergent pathway and under mild and chemoselective conditions (Scheme 46).[320] The strategy was based on the photochemical addition of hepta-*O*-acetyl-1-thio-β-lactose (**448**) onto bisallyl trisaccharide **449** to form divalent adduct **450**. Two nearly quantitative deprotection steps consisting in standard



SCHEME 46. Photochemical construction of lactosylated dendrons.[320]

Zemplén O-deacetylation followed by cleavage of the acetal protecting groups with aqueous TFA afforded G(0) dendron **451**, whose reducing end was reductively aminated with bismethylamino trisaccharide **452** using cyanoborohydride in 1:1 MeOH–H_2O to furnish the G(1) dendron **435** in 48% yield.

In 2007, Heidecke and Lindhorst detailed an original approach toward glycodendron synthesis using a 3,6-diallylated precursor serving as an AB_2 system, and in which a hydroboration–oxidation sequence or radical addition of mercaptoethanol as an activating step, and subsequent glycosylation with branched or unbranched sugar trichloroacetimidates constituted key steps toward dendritic growth (Fig. 48).[321] A collection of six new hyperbranched mannosylated glycodendrons (**454**–**459**) was thereby prepared, and tested using ELISA for their potential as inhibitors of type-1 fimbriae-mediated bacterial adhesion of *E. coli* to yeast mannan polysaccharide from *Saccharomyces cerevisiae*.

The branched oligomannosides differed with regard to both their carbohydrate content and to their spacer characteristics. Binding data to *E. coli* indicated that all these glycodendrons performed better than the monovalent MeαMan, exceeding its inhibitory potency by one or two orders of magnitude. The small conjugate **454**, consisting of three α-D-mannosyl moieties, presented the weakest inhibition (IC_{50} of

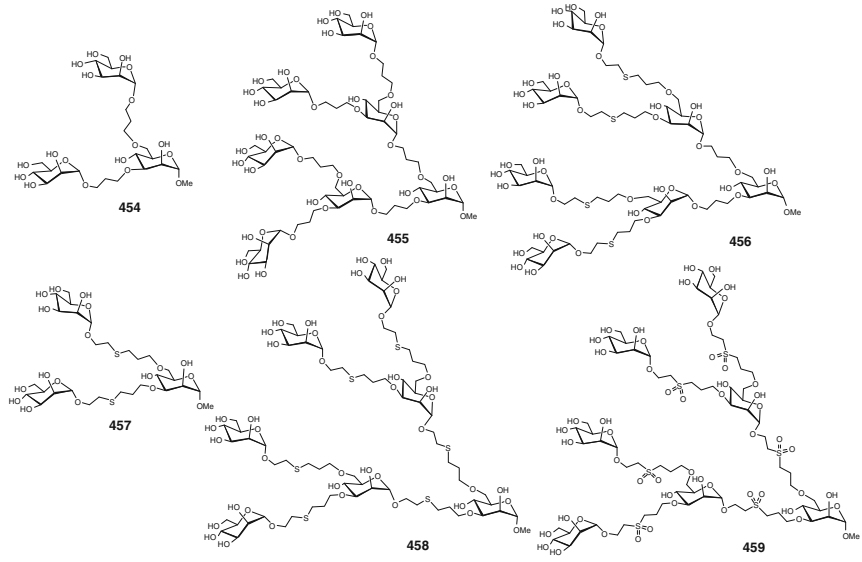
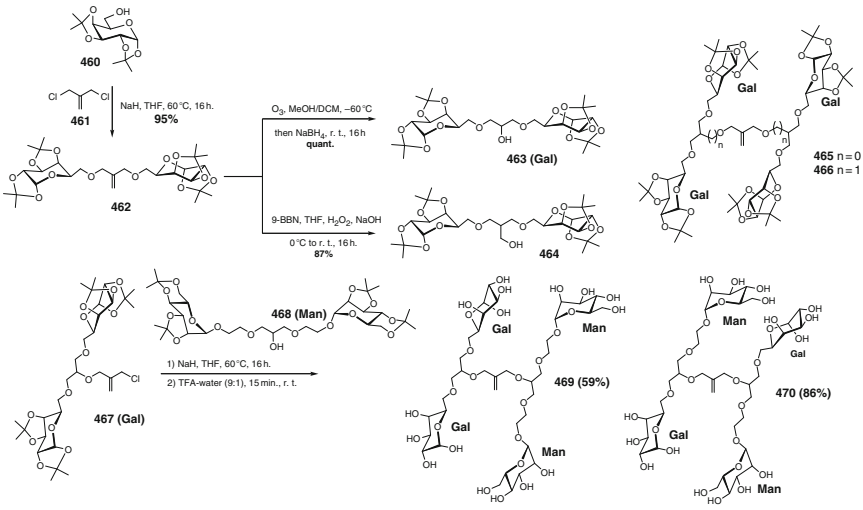


FIG. 48. Mannosylated dendrons built on an AB_2-mannoside scaffold.[321]

5.9 mmol). Its extended analogue **457**, in which two α-D-mannoside units were tethered on thiahexyl spacers, performed unexpectedly well. For instance, it showed average inhibitory potency (89 μM) exceeding that of the larger glycodendron **455** (IC_{50} of 0.14 mmol) in which the carbohydrate moieties were spacered by propyl units. Thus, with regard to the influence of the spacer characteristics, it was concluded that conformationally flexible glycodendrons containing four peripheral mannosyl units and the longer thiahexyl spacers (such as **456** and **458**, IC_{50} of 31 and 55 μmol, respectively) showed increased inhibitory potencies relative to their counterparts bearing propyl spacers (such as **455**, the shorter analogue of **456**). Furthermore, oxidation of the sulfide groups of **458**, providing sulfone **459**, had a pronounced negative effect on its inhibitory potency, indicating that the lipophilic properties of the spacers might also promote the affinity of a given glycoconjugate, as previously concluded.

Finally, a polyether AB_2 system, initially developed by Jayaraman *et al.*[322] have been used for the synthesis of di- and tetravalent clusters decorated with β-D-galactoside moieties (Scheme 47).[323] In addition, the convergent construction allowed the preparation of more complex systems consisting of ''mixed'' glycodendrons carrying both galactoside and mannoside moieties as biologically important ligands. This strategy involved a sequence of repetitive simple or double Williamson etherifications with methallyl dichloride (**461**), followed by generation of alcohols **463** and **464** via



SCHEME 47. Polyether glycodendrons built from methallyl dichloride (**461**).[323]

subsequent ozonolysis–reduction or by hydroboration–oxidation of the double bond with 9-BBN transformations. To this end, double etherification of **461** with 1,2:3,4-di-*O*-isopropylidene-D-galactopyranose (**460**) under basic conditions afforded the symmetrical alkene **462** bearing two galactos-6-yl residues in 95% yield. Ozonolysis followed by reductive work-up with sodium borohydride proceeded quantitatively to yield the corresponding alcohol **463**. Next-generation glycodendron **465**, containing four peripheral galactose units was then obtained in excellent yield by Williamson etherification with **461**. Application of the hydroboration–oxidation sequence with 9-BBN, leading to **464** together with the same subsequent etherification afforded the more flexible analogue **466**. Deprotection of both glycodendrons in TFA–water proceeded in good yields within 15 min.

Toward better mimetics of highly complex natural oligosaccharides, the authors also embarked on the synthesis of ''mixed'' glycodendrons bearing carbohydrate moieties of different kinds, employing two different routes. The first one involved the bisgalactose-substituted alcohol **463** with an equimolar amount of **461** to allow the synthesis of chloride **467** in 67% yield. Subsequent etherification with bismannose-modified alcohol **468** furnished **469** which after acidic deprotection provided an example of a mixed-type polyether glycodendron. The second way was based on the preliminary desymmetrization of **461** with one of the saccharides, followed by attachment of the other one on the remaining reactive function. Generation of the alcohol group from the double bond and subsequent Williamson etherification on **461** afforded, after TFA–water deprotection, the second ''mixed'' glycodendron **470**.

c. AB_3 Systems.—Among the most widely used AB_3 dendritic building blocks, derivatives of the aliphatic TRIS and of the aromatic 3,4,5-trihydroxybenzoic acid (gallic acid) provide systems of choice for constructing dense glycodendrons according to an iterative and orthogonal synthetic strategy. Since the pioneering work of Newkome and coworkers in the early 1980s describing the synthesis of ''arborols,'' the widespread use of TRIS and its derivatives has afforded highly functionalized structures.[287,324] TRIS thus offers synthetic advantages in terms of symmetry for ensuring an accelerated dendritic growth: the amine can serve as an anchoring function and the hydroxyl groups can allow efficient Tris-functionalization.

For instance, TRIS-based glycodendrons have recently been designed by the group of Wong, based on an efficient synthesis of structures displaying multivalent oligomannosides in high density, notably to mimic the glycans on HIV-1 gp120 (Fig. 49).[325] Their interaction with the antibody 2G12 and DC-SIGN lectin has been characterized by a glycan microarray binding assay. An AB_3 type dendritic skeleton **473** (functionalized TRIS) was chosen as a precursor for constructing

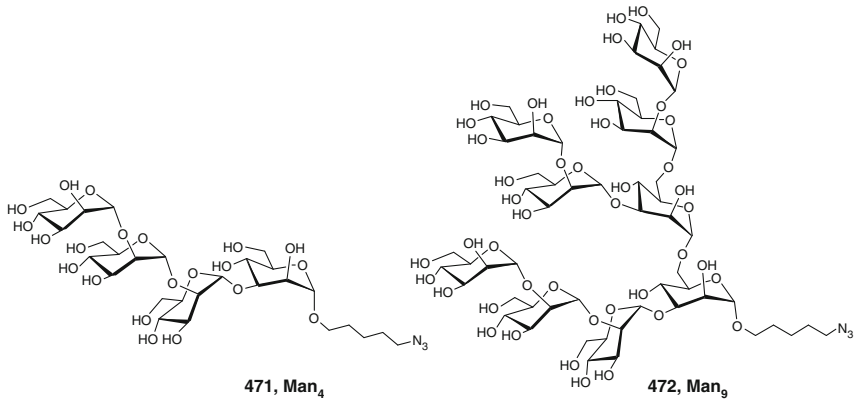
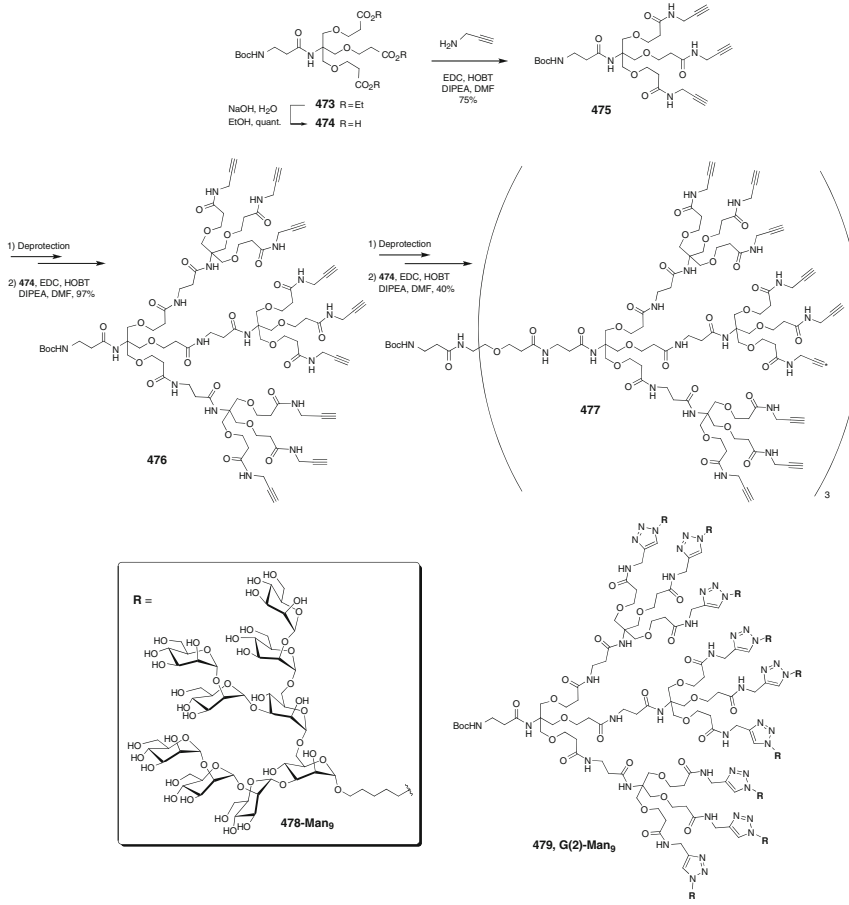


FIG. 49. Oligomannoside-ending azides used in ''click-chemistry'' toward HIV-1 gp120 mimetics recognized by human antibody 2G12 and DC-SIGN.[325]

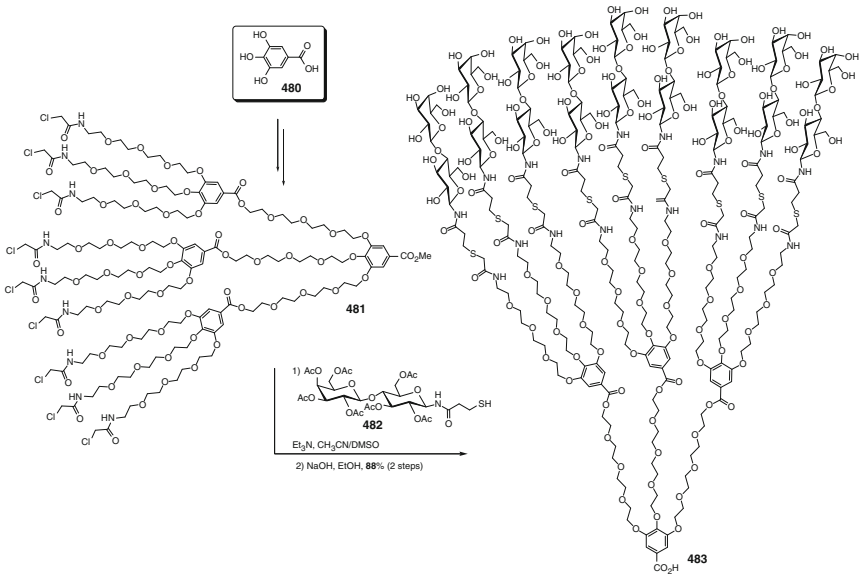
densely packed glycodendrons that were achieved in a few generations. The versatile ligation was ensured by the use of catalyzed alkyne–azide 1,3-dipolar cycloaddition reaction (CuAAC) to conjugate the sterically demanding azido oligomannosides **471** and **472**, designated as Man_4 and Man_9, respectively, to the polypropargylated dendrons (Scheme 48). A convergent approach was also designed to facilitate the homogeneity of the dendritic scaffolds, using an iterative sequence based on *N*-Boc removal and subsequent EDC–HOBt-mediated peptide coupling on tricarboxylic acid derivative **473**, to afford polypropargylated compounds **475**–**477** for the first, second, and third generations, respectively.

Interesting results from biological studies indicated that G(2)-Man_9 **479** appeared to be an effective mimic of the HIV-1 gp120 surface glycan, suitable for conjugation to a carrier protein as a vaccine candidate. Furthermore, evaluation of inhibition of DC-SIGN with **479** was studied via gp120/Fc-DC-SIGN ELISA tests. Excellent inhibition activity in the nanomolar range was demonstrated, in contrast to the millimolar range from the reference mannoside. In these experiments, no inhibition was observed for the corresponding nonglycosylated alkynyl dendron **477** (up to 0.1 mM), indicating that the multivalent oligomannose residues were responsible for DC-SIGN binding. The inhibition of glycodendrons interacting with antibody 2G12 and DC-SIGN indicated that these dendritic architectures, especially for G(2)-Man_9 glycodendron **479**, had the potential for use in the development of both carbohydrate vaccine candidates and as antiviral agents.



SCHEME 48. Propargylated dendrons using a modified TRIS scaffold.[325]

As with TRIS, the commercially available gallic acid **480** constitutes an ideal candidate as branching unit for rapid dendritic growth. It allows dendron and dendrimer scaffolding to reach 3^n surface groups at the *n*th generation. Thus, gallic acid and its derivatives can afford highly functionalized glycosylated structures, taking advantage of its geometry for orthogonal transformation. It was initially used by Roy *et al*. for the construction of hyperbranched dendritic lactosides presenting up to nine peripheral saccharide residues (Scheme 49).[326] The convergent strategy described relied on the synthesis of a thiolated lactoside derivative **482**, to be added to a



SCHEME 49. First gallic acid-based glycodendrons synthesized by Roy *et al.*[326]

preformed gallic acid derivative containing G(0) or G(1) dendrons (**481**) capped with functionalized tetra(ethylene)glycol, to afford such glycodendrons as **483**. The hydrophilic spacer was chosen to ensure advantageous water solubility of the resulting dendrimer and to counteract the hydrophobic effect of the aromatic gallic acid.

Two years later, the same group described the synthesis of hyperbranched glycodendrimers containing sialic acid residues, according to a similar strategy involving gallic acid derivatives and oligo(ethylene)glycol as dendritic backbones.[327] The foregoing conditions were used to conjugate α-thiosialosides onto an N-chloroacetylated dendritic precursor (**481** for instance) by nucleophilic substitution, affording the anticipated sialodendrimers in high yields. Interestingly, turbidimetric analysis confirmed the strong potential of G(1) sialodendrimers having nine readily accessible sialic acid residues to efficiently bind, cross-link, and precipitate two different lectins: the wheat-germ agglutinin WGA and the lectin from the slug *Limax flavus* (LFA).

Similar structures were later employed to create original dendronized polymers **485** and **486**, based on a chitosan backbone and using such sialodendrons as **484** (Fig. 50).[328] Chitosan itself is nontoxic, biodegradable, and has widespread biological activities, but major intrinsic drawbacks such as low solubility in both organic solvents and water have hampered its development as a bioactive polymer. Thus, the synthesis of water-soluble

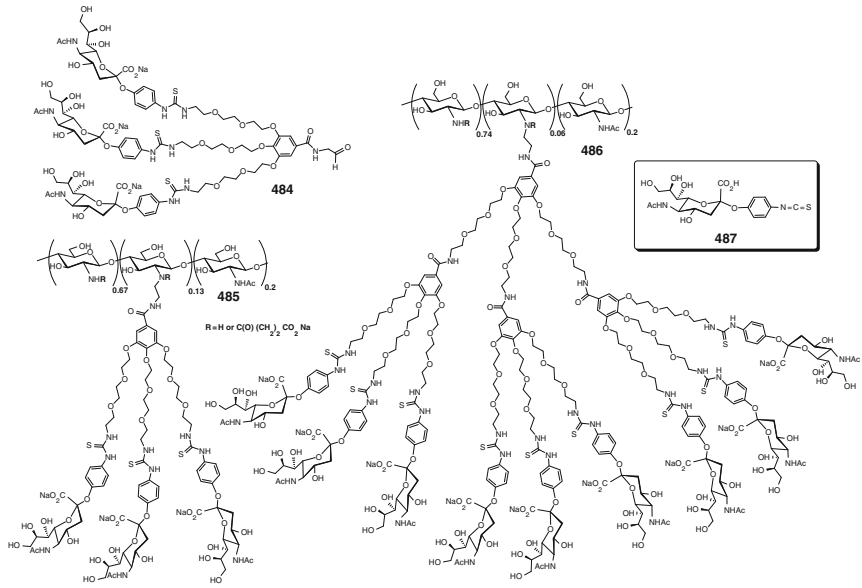


FIG. 50. Polysialic acid dendronized chitosan.[328]

dendronized chitosan–sialic acid hybrids was successfully achieved, using gallic acid as focal point and tri(ethylene)glycol as hydrophilic spacer arm, in order to investigate their potential to inhibit viral pathogens, including the flu virus.

Sialodendrons bearing a focal aldehyde end-group (**484**, for a trivalent dendron) were synthesized by a reiterative amide-bond strategy, based on the use of polyamine-ending trivalent or nonavalent dendritic scaffolds having gallic acid as the branching unit and capped with an acetal as a precursor for the aldehyde function. Sialic acid *p*-phenylisothiocyanate (PITC) derivative **487** was conjugated via thiourea linkages, followed by hydrolysis of the resulting aldehyde acetal with TFA to provide aldehyde **484**. The same procedures were followed efficiently to access the next generation of dendrimers. Finally, the focal aldehyde sialodendrons were grafted convergently onto the chitosan polysaccharide backbone by reductive amination in good yields, with the degrees of substitution indicated in Fig. 50. The water solubility of these original hybrids was further enhanced when unmodified amino groups of the chitosan backbone were succinylated with an excess of succinic anhydride.

An improved strategy using microwave-assisted synthesis, involving a gallic acid core and copper-catalyzed [3+2] cycloaddition (CuAAc), afforded a series of glycodendrons.[329] The straightforward synthesis of this series of glycodendrons was

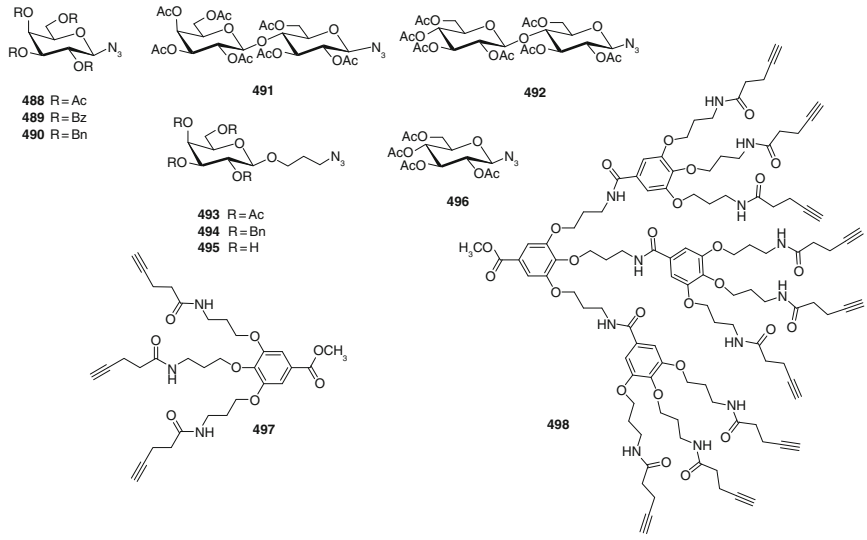


FIG. 51. Azides and propargylated dendrons for the rapid assembly of glycodendrimers.[329]

achieved in high yields, starting from azido sugar derivatives (**488–496**) and their subsequent Cu(I)-catalyzed cycloaddition with acetylene-bearing dendrimers **497** and **498** (Fig. 51). This strategy allowed the rapid preparation of triazole glycodendrimers up to the nonavalent level and the successfull use of unprotected carbohydrates. The direct introduction of unprotected carbohydrates provides an interesting approach, avoiding tedious final deprotection steps and allowing, for steric reasons, more efficient couplings.

An additional example provided by Fernandez-Megia *et al*. describes a quick, efficient, and reliable multivalent conjugation of unprotected alkyne-derived carbohydrates to three generations of azido-terminated gallic acid-triethylene glycol dendrons (Fig. 52).[330] In this work, azide-terminated dendrons were favored over those incorporating terminal alkynes because of the potential bias of the latter to Cu(II)-catalyzed intradendritic oxidative coupling. Under aqueous conditions and employing typical ''click chemistry,'' glycodendrimers containing up to 27 [G(3)] unprotected fucose (**499a**), mannose (**499b**), and lactose (**499c**) residues were efficiently isolated in high yields, after practical purification of the reaction mixture by ultrafiltration.

Further investigations by the same group led to the synthesis of three generations of a new family of block copolymers PEG-(**R**)-saccharide **500a**, **500b**, and **500c** with very good to excellent yields.[331] Interestingly, an NMR relaxation study of the

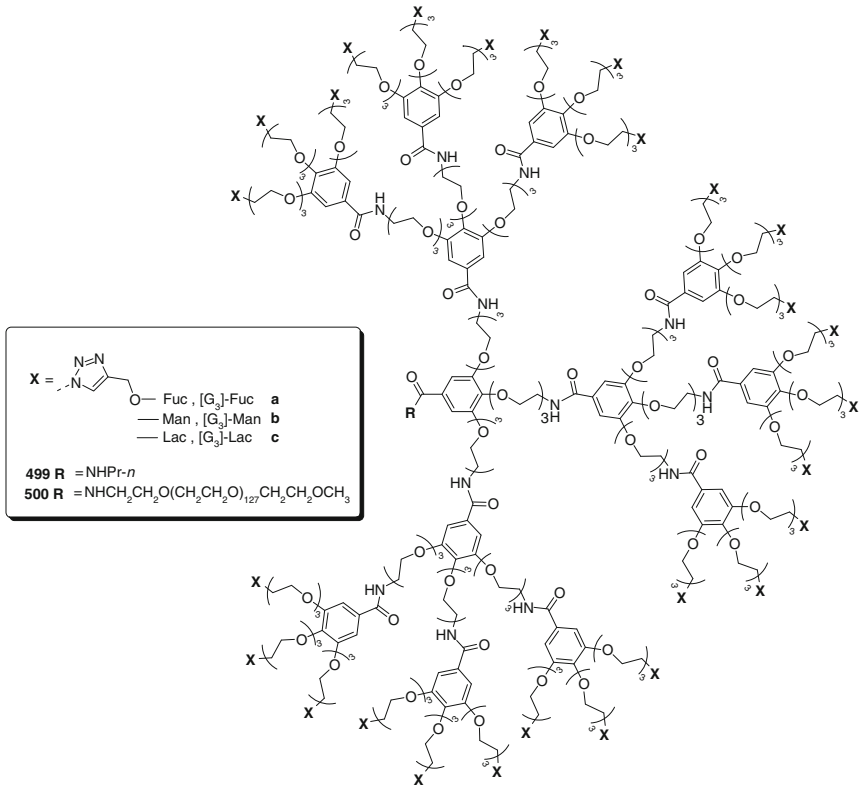


FIG. 52. Gallic acid-based glycodendrimers prepared using ''click chemistry.''[330,331]

azido-terminated PEG-dendritic block copolymer precursors revealed a radial decrease of density from the core to the periphery, becoming more intense on increasing the generation of the dendritic block. Furthermore, the resulting PEGylated and mannosylated glycodendrimers demonstrated an increased capacity to aggregate lectins with increasing generation.

3. Glycodendrimers

a. Glycopeptide Dendrimers.—As stated earlier, the first glycopeptide dendrimers were described in the literature in 1993.[292] They were built using divergent solid-phase peptide Fmoc-chemistry and L-lysine as a repeating amino acid on a

Wang resin. The initial sugar attached was sialic acid, which was introduced to confer strong inhibitory properties against flu virus hemagglutinin, a lectin-like protein recognizing α-sialosides on respiratory mucins. This early hyperbranched L-lysine scaffold was elongated with *N*-chloroacetylglycylglycine and efficiently coupled to a peracetylated α-thiosialoside. Using dendrons bearing eight surface α-thiosialosides, Roy *et al*.[332] demonstrated that each saccharide moiety was a 1000-fold better, on a per saccharide basis, than the corresponding monomer, while being stable to viral neuraminidase. These interesting architectures were, however, not as potent as sialopolymers since they could not properly cover the entire surfaces of the spherical virions, a property well exploited by random-coiled polymers.[333,334] It was later argued that dendronized polymers had improved potencies in this respect (see following PAMAM section).[335] When the same poly-L-lysine scaffold was utilized with mannoside residues bearing an optimized arylated aglycon, the resulting 8- and 16-mer glycodendrons showed 100,000-fold increased inhibitory potency against fimbriated *E. coli* K12 on a per mannoside basis.[336] The structure of the 8-mer mannosylated dendrimer (**501**) is illustrated in Fig. 53. The octameric aminophenyl α-D-mannopyranoside had an IC_{50} of 2.8 nM (22.4 nM/Man). It was found that a competitive binding assay measuring the binding of ^{125}I-labeled, highly mannosylated neoglycoprotein (BSA) to type-1 fimbriated *E. coli* (K12) strain in suspension gave much lower IC_{50} values than the equivalent values obtained by hemagglutination or in assays that involved microplate immobilization. Two key features strongly influencing the affinity to *E. coli* adhesin were: (1) the presence of an α-oriented aglycone bearing a hydrophobic group, and (2) the presence of multiple mannoside residues that can span a distance of 20 nm or longer. The two best inhibitors were a highly mannosylated neoglycoprotein with the longest linking arm between a mannose and protein amino group, and the 16-mer mannosylated dendrimer (fourth generation), with an IC_{50} of 0.9 nM (14.4 nM/Man).

First described and patented by Denkewalter *et al*. in the late 1970s,[337] dendronized lysine is one of the most widely used core structures in dendrimer[338] and glycodendrimer synthesis. An excellent series of reviews by the group of Ježek on the synthesis and biological applications of lysine-based glycodendrimers has been published.[339–342] Such glycosylated lysine dendrimers have been prepared both on solid supports[343–345] and in solution.[346–348] Reaction of the peripheral amino groups with a variety of electrophiles carrying pendant sugar residues (such as isothiocyanates, carboxylic acid, and reducing sugars) have lead to glycodendrimer thioureas, amides, and amines, respectively. Sugars bearing activated ester groups have also been coupled to dendritic lysines to provide galactoside- and *N*-acetylglucosamine-capped glycoclusters,[316,349] including the important Thomsen Friedenreich (TF) disaccharide

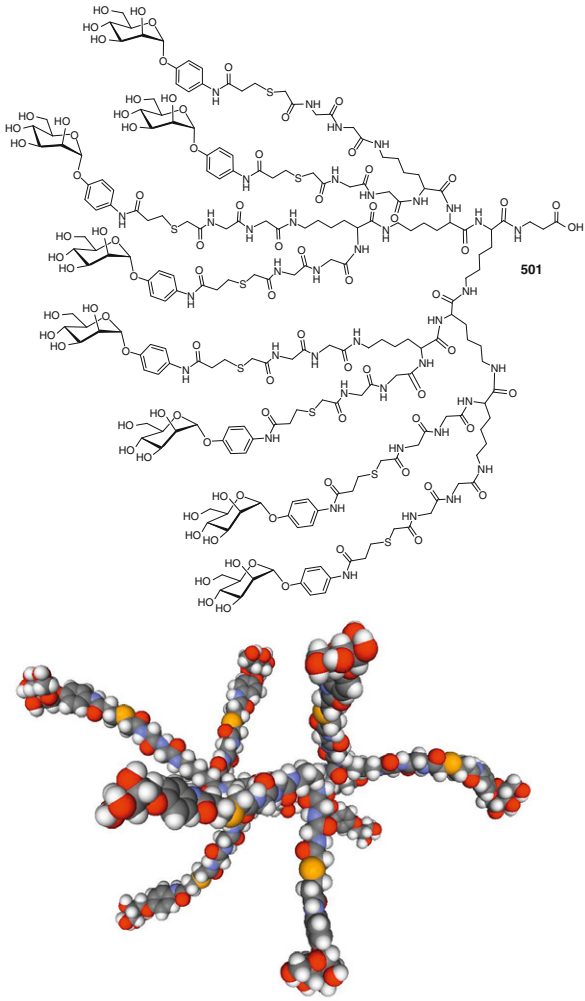


FIG. 53. Mannosylated dendron based on a poly-L-lysine scaffold. This construct leads to subnanomolar inhibitory potency against uropathogenic *E. coli*.[336]

antigen [β-D-Gal(1→3)-α-D-GalNAc] known as a breast cancer marker and against which monoclonal antibodies were raised (see, for instance, Fig. 45).[350,351] The corresponding TF-bearing glycodendrimers bound to the antibodies, and were shown to be adsorbed strongly onto the surface of microtiter plates. The analogous

β-D-GlcNAc glycodendrimer was also further elaborated as the Lewis^X tetrasaccharide antigens **502**, using multiple chemoenzymatic processes (Fig. 54).[352]

Analogous mannosylated architectures have also been proposed. MBP acting as receptors can mediate uptake and internalization of both soluble and particulate glycoconjugates, and as such they take part in innate immunity.[353,354] The broad pattern-recognition displayed by mannose receptors, together with their implication in adaptive immunity, has stimulated considerable efforts toward the selective delivery of enzymes,[355,356] drugs,[357–359] oligonucleotides or genes,[360–363] and antigens[364–366] to cells expressing them, for therapeutic and vaccine strategies. Another important mannose receptor is a membrane-associated protein restricted on sinusoidal liver cells, peripheral and bone marrow macrophages, and dendritic cells. It also recognizes and internalizes mannosylated glycoconjugates from pathological microorganisms, tumor and yeast cells, such glycoproteins as type-I procollagen, tissue-type plasminogen activator, or various lysosomal enzymes. As such, this mannose receptor contributes to the nonimmune host-defense system. In addition, the macrophage receptor is implicated in major histocompatability complex-mediated antigen (MHC) presentation by dendritic cells (DC-SIGN) (Dendritic Cell-Specific ICAM-3 grabbing nonintegrin) (ICAM-3 = intercellular adhesion molecule). DC-SIGN also belongs to the family of C-type lectins able to bind high-mannose glycoproteins of HIV-gp120,

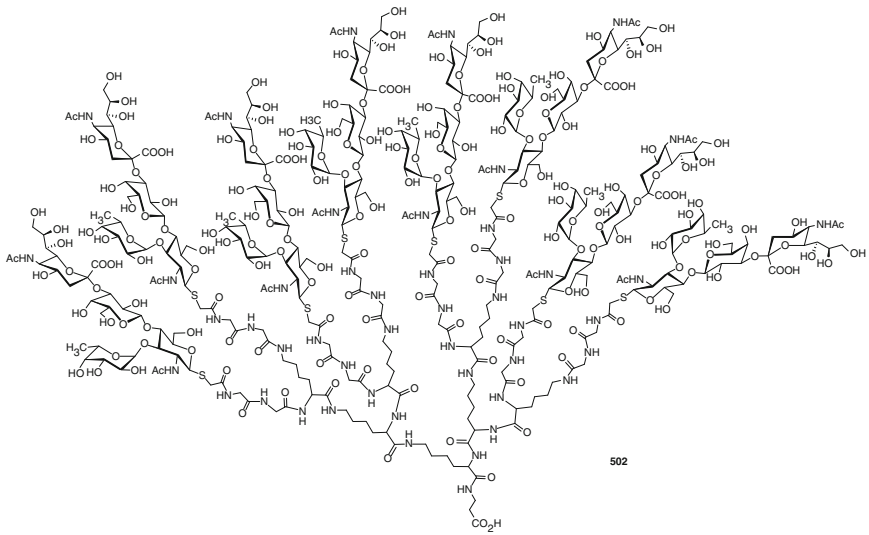


FIG. 54. Chemoenzymatically prepared sialyl Lewis^X glycodendrimer.[352]

Ebola-gp 1, or Dengue-gp E. DC-SIGN is also known to oligomerize, and it is therefore particularly important to understand the intrinsic binding and multivalent binding requirements of this lectin.

In this context, Biessen *et al*. conceived oligomeric linear L-lysine mannosides bearing the same arylated aglycone just described for **501**.[367] Its associated *p*-isothiocyanatophenyl α-D-mannopyranoside was coupled to the poly-L-lysine backbone through an isothiourea linkage. The structure of the 6-mer **503** is illustrated in Fig. 55. The affinity of these mannoclusters toward the mannose receptor increased steadily from 18-23 mM (dimer) to 0.5-2.6 nM (6-mer). As a consequence of its high affinity, **503** is a promising targeting device for cell-specific genes and delivery of drugs to liver endothelial cells or macrophages in bone marrow, lungs, spleen, and atherosclerotic plaques.

Another investigation targeting the mannose receptor expressed by the human dendritic cell (DC-SIGN) has been presented by Grandjean *et al*. but adding carbohydrate mimicry to the multivalent concept.[344,368] As with the strategy just described, quinic and shikimic acid derivatives used as mannose bioisosteres have been linked to dendritic L-lysine scaffolds to afford novel hyperbranched glycomimetics (Fig. 55). Fluorescein-labeled pseudoglycodendrimers with valencies of two to eight were tested by competitive-inhibition assays with mannan, which was evaluated by confocal microscopy using mannose receptors expressed in transfected COS-1-cells. Cells expressing mannose receptor-mediated uptake were assayed on monocyte-derived human dendritic cells by cytofluorometric analysis. The synthetic clusters were shown to be effective ligands against the dendritic cells, with an optimum affinity

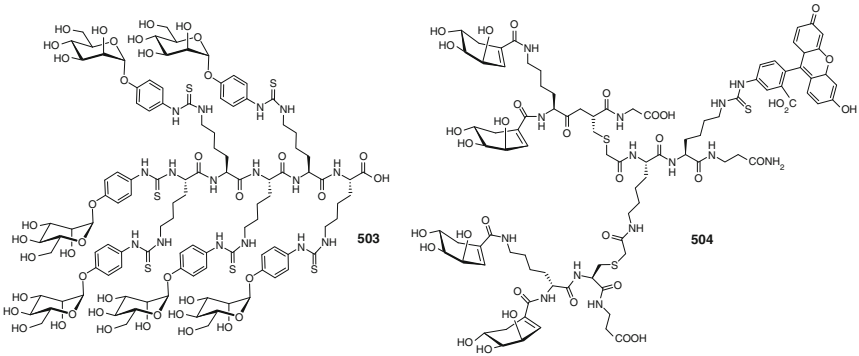


FIG. 55. Linear oligo-L-lysine bearing aryl mannosides (**503**) and the quinic acid bioisostere of mannose (**504**).[367,368]

toward clusters having a valency of four (**504**). However, the glycomimetics as well as the natural mannosides did not perform , although the results indicated that the mannose receptor could accommodate structures that diverged significantly from previously identified natural ligands and which could be further optimized using QSAR.[369,370] Additionally, monodisperse lysine dendrimers capped with 2–64 mono-, di-, and tri-α-D-mannopyranosyl residues did not induce dendritic cell maturation.[348]

As stated, DC-SIGN is a key mannoside receptor for exogenous pathogens that is used by viruses for entry into the lymph nodes. The inhibition of this process has thus been sought as an interesting strategy for blocking viral adhesion. Consequently, a series of mannosylated Boltorn® dendrimers was prepared (see later section) that were rather efficient in this respect.

An interesting extension of this strategy was proposed using various fluorescent probes: fluorescein, rhodamine, pyrene, and dansyl groups (**505**, Fig. 56). They were incorporated onto mannosylated dendrons prepared entirely by SPPS (Fmoc-chemistry) and used for the imaging studies of mannose receptor-mediated entry into dendritic cells by confocal fluorescence microscopy.[371] After pathogen capture, internalization, and digestion, peptide fragments were expressed on MHC molecules.

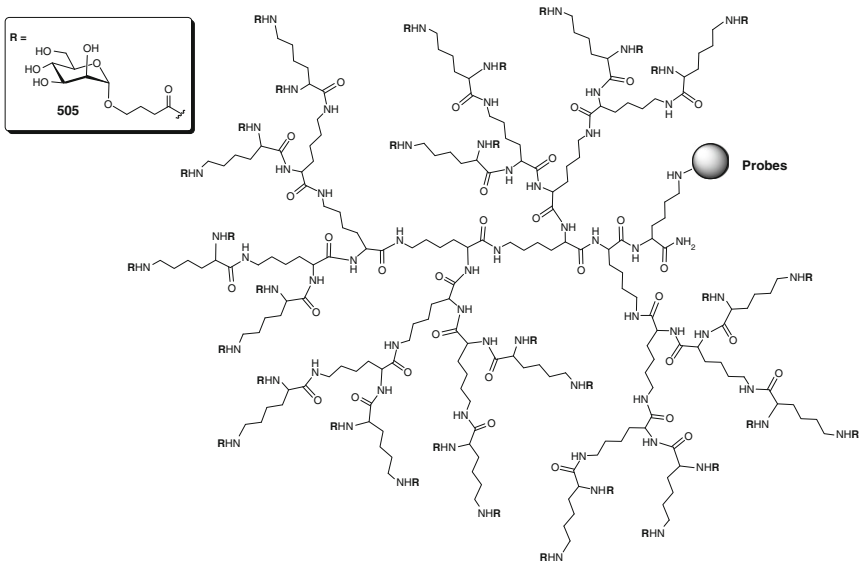


FIG. 56. Fluorescently labeled mannodendrimers for MHC capture and imaging.[371]

A T cell-specific immune response was thereby initiated. This sequence of biological events was exploited for vaccine design (see next). The heterobifunctional, high-affinity multivalent ligand was assembled on nontoxic, nonimmunogenic poly-L-lysine dendrimers to which the required number of mannosyl residues can be attached simultaneously via a 4-hydroxybutanoic acid linker, a substance occurring naturally in mammals. The synthesis of the model poly-L-lysine dendrons was initially performed on a commercial Tentagel resin preloaded with Fmoc-Sieber amide linker. The low loading level of the commercial resin (0.16 mmol/g) ensured that sufficient space was allowed for the large fourth and fifth-generation dendrons to be assembled without problems of steric hindrance. At the same time, the mild cleavage conditions required for this type of resin did not cause significant acid-promoted glycoside hydrolysis. The 4-(mannopyranosyloxy)butanoic acid was then introduced by classical peptide-coupling reagents.

The established protocol was next applied toward the fluorescently labeled analogues by introducing at the focal point a lysine residue possessing the acid-labile 4-methyltrityl (Mtt) protecting-group. The side chain of the focal ε-amine, protected by the Mtt group, was used for labeling, in conjunction with the considerably less acid-labile commercial Rink amide linker for maximum versatility. Thus, the Rink amide Tentagel resin was loaded with Fmoc-Lys(Mtt)-OH and the *N*-terminal Fmoc group was removed by classical treatment with 20% piperidine in DMF. After the reiterative insertion of bis-Fmoc lysine was terminated, the Mtt group was removed at low TFA concentrations with minimal losses of the dendron, and the newly freed amine was directly labeled onto the resin. Accordingly, treatment of the polymer-supported G(4) dendron with 3% TFA in CH_2Cl_2 led to complete removal of the Mtt group within a short time. Lower concentrations of TFA were inefficient. The free amine group of the glycodendrons was then treated with the appropriate fluorescent probes to provide desired mannosylated glycodendrons such as **505** (Fig. 56).

Spherical and hemispherical glycodendrimers containing a polylysine scaffold have been elaborated (Fig. 57).[372,373] Third-generation polylysine dendrimer **506** was prepared as a TFA salt from tris(2-ethylamino)amine (**8**) as a trivalent core and the stepwise condensation of diBoc-lysine, according to a published strategy. The *N*-Boc-protecting group was removed using standard conditions (2 M TFA) to provide the free polyamine. The polylysine dendrimer **506**, having 24 terminal amino groups on the surface, was then treated with the peracetylated cellobioside **507,** using BOP-mediated peptide coupling. Deprotection of the crude peracetylated glycodendrimer was achieved (Zemplén conditions: NaOMe, MeOH, 3 h), and the free dendritic glycopeptide **508** was obtained in 58% yield and fully characterized by ^{1}H NMR and MALDI-TOF MS. It was anticipated that, after random sulfation, the synthetic

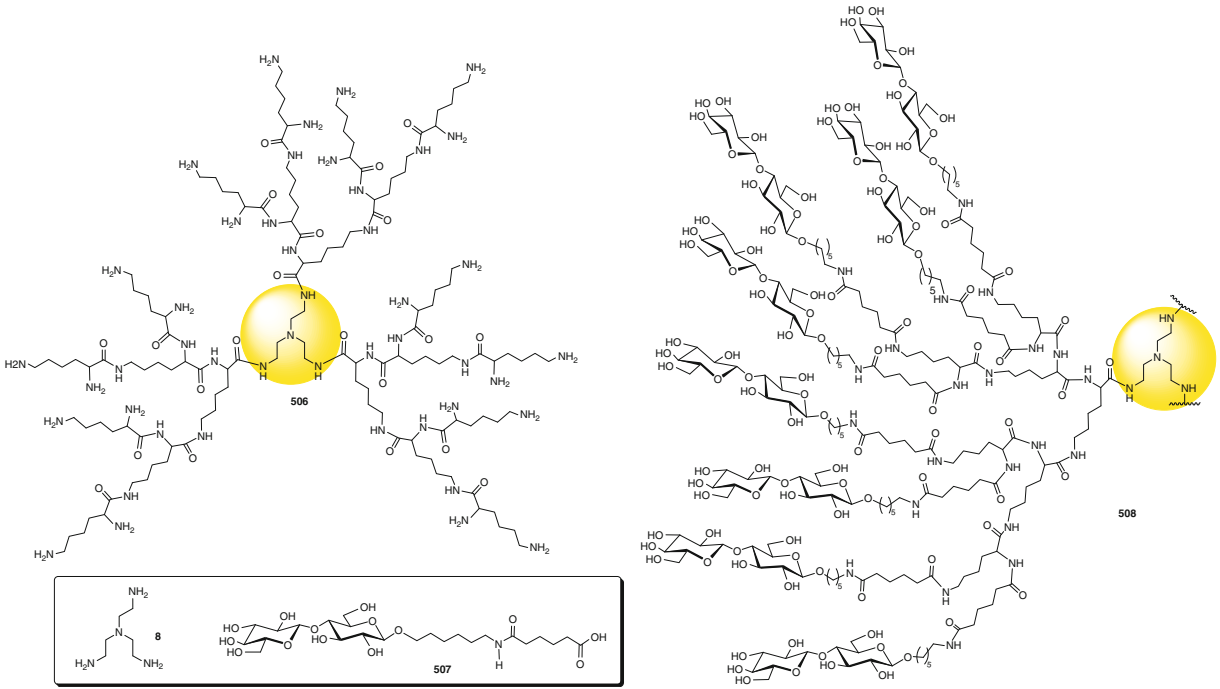


FIG. 57. Tris(2-ethylamino)amine (**8**) used as central core for the build up of poly-L-lysine cellobioside dendrimers.[372,373]

cellobiosyl glycopeptide dendrimer would show key biological activities such as anti-HIV and blood anticoagulant activities.

Solid-phase combinatorial synthesis furnished a large (390,625-members) neoglycopeptide dendrimer library ending with *C*-fucoside derivatives.[374] A tetravalent dendrimer **509** (Fig. 58) showed the strongest binding affinity (IC_{50} of 0.6 μM) against the *P. aeruginosa* PA-IIL lectin, a virulence factor in cystic fibrosis (CF) patients. The optimized ligand combined multivalency with the presence of positive guanidine charges in proximity to the carbohydrate residues, and which happen to be better in comparison to a divalent analogue lacking the N-terminal lysine residues (IC_{50} of 5.0 μM). An improved 15,625-membered peptide dendrimer library was prepared analogously.[375] Dendrimer **510** (α-Fuc-CH_2CO-Lys-Pro-Leu)$_4$(Lys-Phe-Lys-Ile)$_2$Lys-His-Ile-NH_2 was the most potent ligand against the model plant lectin *Ulex europaeus* (UEA-I), with an IC_{50} of 11 μM and the bacterial lectin PA-IIL from *P. aeruginosa* (IC_{50} of 0.14 μM). Glycopeptide libraries have also been synthesized with divalent carbohydrate structures to optimize multivalent carbohydrate-binding protein interactions at subsite at the vicinity of the carbohydrate-recognition domain (CRD).[81]

As stated earlier, dendritic cells and macrophages are valuable antigen-presenting cells (APCs). These cells express MBPs and, as such, they constitute important entry mechanisms for vaccine targeting. Thus, instead of linking short peptide antigens to

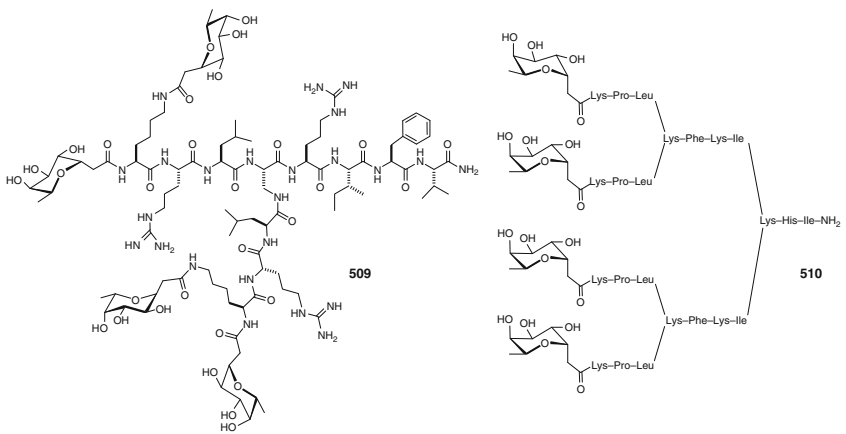


FIG. 58. Optimized *C*-fucosylated glycopeptides identified in a chemical library of 390,625 members produced by SPPS.[374,375]

classical immunogenic protein carriers, such as keyhole limpet hemocyanin (KLH) or tetanus toxoid and the like, a study was evaluated toward the possibility of using mannoside-capped polylysine glycodendrimers (**511**) constructed at the N-terminal of several immunogenic peptides (Fig. 59).[376] Peptide sequences from HIV-1 gp41 protein (541–555 bearing the LLSGIV motif capable of inhibiting viral fusion, 553-567),[377] SARS-CoV S2 (1081–1105, 1144–1187), and influenza hemagglutinin HA2 (1–25) were built on a Rink amide resin. The lysine moieties were then introduced as a G(3) lysine dendron followed by mannosylation, using 4-(mannopyranosyloxy)butanoic acid (**512**) at the terminal (ϵ) amino groups of the lysyl-peptide dendrimer (**513**). Preliminary data from the resulting vaccine candidate **511**, containing the LLSGIV motif, demonstrated that it could elicit a polyclonal antibody response in rabbit much stronger than the KLH constructs. It was concluded that the mannosylated dendron was stabilizing the peptide from proteolysis. N-Terminally mannosylated peptides carrying one to six mannose residues were also shown by Koning and coworkers to elicit an immune response, with efficiency up to 104-fold greater than peptide antigens alone.[378]

The capture of antigenic determinants by APCs that will ultimately appear in MHCs for T helper cell stimulation depends on several factors. If APCs are represented by B cells, multivalency becomes an issue, because both the recognition and the binding events are triggered by cell-surface immunoglobulins. A classical example of successful applications in this regard is presented by the high immunogenicity of bacterial capsular polysaccharides (CPS) which, on their own, can elicit protective antibodies.[379,380] However, the resulting antibodies are usually limited to low-affinity IgMs, and the immune responses lack memory effects because of the absence of T

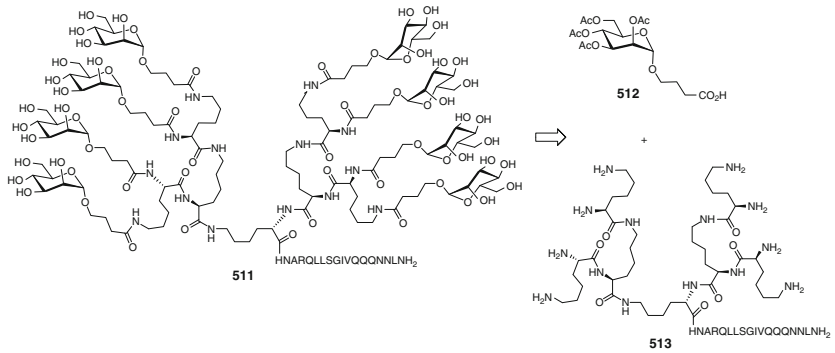


FIG. 59. Entirely synthetic anti-HIV vaccine candidate capable of APCs uptake.[376,377]

cell-dependent antigens normally associated to peptides. CPS are thus said to be T independent antigens. Alternatively, if the B cell antigens are not multivalent, such as those found in small peptides or oligosaccharides, they will greatly benefit from being mounted on dendritic scaffolds. Moreover, the key T cell epitopes, needed in the context of MHC complexes, can be typically short 15-mer peptides and yet, be too small for efficient uptake by APCs. Consequently, the design of dendritic glycoconjugates bearing multiples copies of both oligosaccharides (B cell epitopes) and short peptides (T cell epitopes) has been proposed as entirely synthetic vaccine candidates (**514**) (Fig. 60).

A few elegant applications of this principle have been recently proposed for triggering neutralizing antibodies against the V-3 loop glycoprotein of gp120 from HIV-1 isolates that recognize and bind to the high mannose oligosaccharide epitope $Man_9GlcNAc_2$ **183** (Fig. 24) that is present in numerous copies on gp120. The proposal was based on the observation that a swapped human antibody, named 2G12, was identified in a patient that successfully mounted a protective anti-HIV immune response (Fig. 61).[381]

Besides glycoclusters based on a central peptide, earlier presented in Section II.4, the group of Kunz was the first to fully demonstrate the feasibility and efficacy of eliciting complete immune responses with memory effects, using carbohydrate cancer antigens built on multiple antigen glycopeptide scaffolds.[382,383] Thus, the immunogenicity of synthetic multiple antigenic glycopeptides (MAGs) displaying four clustered T_N-epitopes anchored to an oligomeric branched lysine core was examined (Fig. 62). Conventional SPS of the peptidic structure was performed by Fmoc-methodology on Wang resin. The dendritic immunogen **516**, containing clusters of three consecutive T_N-epitopes linked to the poliovirus T cell epitope KLFAVWKITYKDT, was tested in mice as both a prophylactic and as a therapeutic cancer vaccine. As the analogous MAG **515**, harboring only four monomeric T_N-antigens that have been shown to increase the survival of tumor-bearing mice, added results indicated that **516** showed high immunogenicity and good protection against the development of T_N-expressing tumor cells.[382]

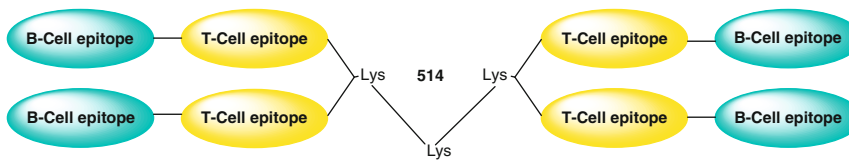


FIG. 60. Schematic representation of a fully synthetic carbohydrate-based vaccine.[379,380]

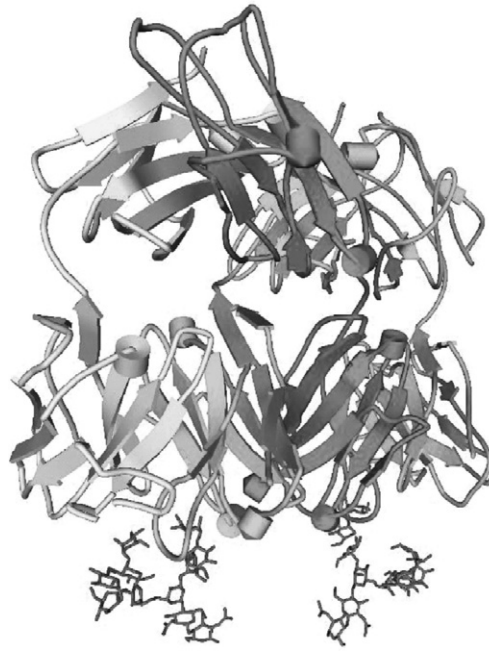


FIG. 61. Crystal structure of the HIV-1 neutralizing human antibody 2G12 bound to the oligomannoside $Man_9GlcNAc_2$ present on the ''silent'' face of the gp120 envelope glycoprotein (PDB 1OP5).

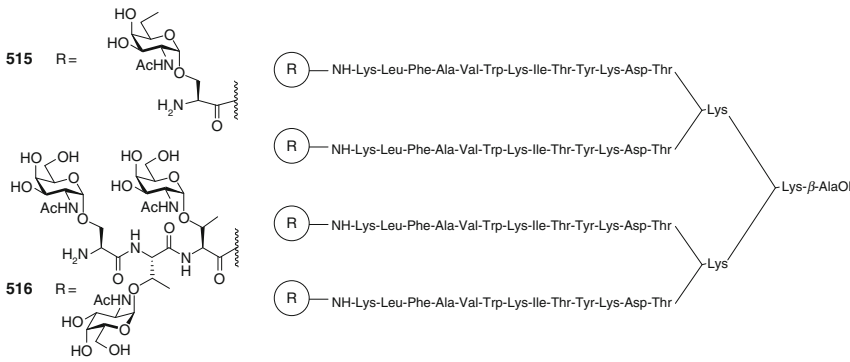


FIG. 62. Poliovirus T cell epitopes capped with monomeric (**515**) and trimeric T_N-antigens (**516**) that confered high murine immunogenicity.[382,383]

The N-linked pentasaccharide core $Man_3(GlcNAc)_2$ glycopeptide bearing the extracellular MMP inducer sequence **517** (emmprin 34–58) has been successfully linked to G(1) PAMAM aminodendrimer by thioester activation (Fig. 63). The resulting octameric 30 kDa construct was obtained in low yield, but was purified by preparative electrophoresis and fully characterized by MALDI-TOF mass spectrometry. The multivalent architecture was built to evaluate the requirement of emmprin multimerization for inducing MMP expression.[384]

The numerous successes achieved in the solid-phase syntheses (SPS) of glycopeptides dendrimers, coupled to their ease of preparation and purification, has also triggered chemists to apply the SPS nucleotide chemistry toward the synthesis of phosphodiester-linked glycodendrimers. As mentioned earlier in the glycoclusters section, the chemistry and geometry of phosphodiesters and triesters are obviously appealing factors for their use as glycodendrimer scaffolds. The following strategy was based on the synthesis of key phosphoramidite building blocks bearing either *N*-chloroacetyl or alcohol end groups for further branching.[385] DEG spacers were initially transformed into the mono *tert*-butyldiphenylsilyl derivative **518** which upon treatment with *N*,*N*-diisopropylphosphoramidous dichloride (Cl_2P-N-$(iPr)_2$, DIPEA, CH_2Cl_2), provided the essential building block *N*,*N*-diisopropyldiphosphoramidite (**519**) in 84% yield (Fig. 64). Alternatively, the mono *n*-chloroacetylated DEG derivative **520**, prepared from commercially available 2-(2-aminoethoxy)ethanol ($ClCH_2CO_2H$, EEDQ, 45 °C, 4 h, 82%) was similarly treated to provide phosphoramidite **521** (68%). Further couplings of **519** or **521** with alcohols **518** or **520** in the presence of 1*H*-tetrazole gave the corresponding phosphotriesters **522** or **523** after oxidation with *t*-butyl peroxide (70–71%). Coupling tris(*N*-chloroacetyl) derivative **524** (MeOH, Et_3N) with the thiolated *N*-acetylgalactosaminide **525** afforded a first-generation phosphoglycodendrimer (63%) having three α-D-GalNAc moieties (not

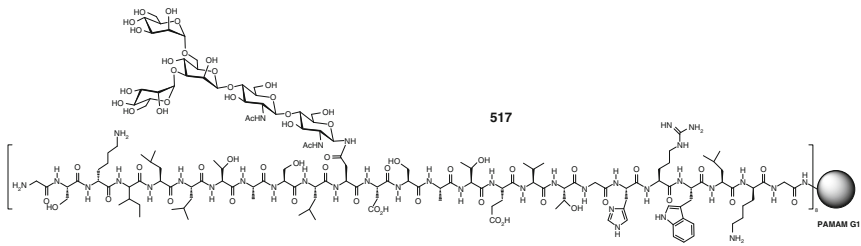


FIG. 63. $Man_3GlcNAc_2$ anchored to PAMAM-based matrix metalloproteinase (MMP) inducer sequence (emmprin 34-58).[384]

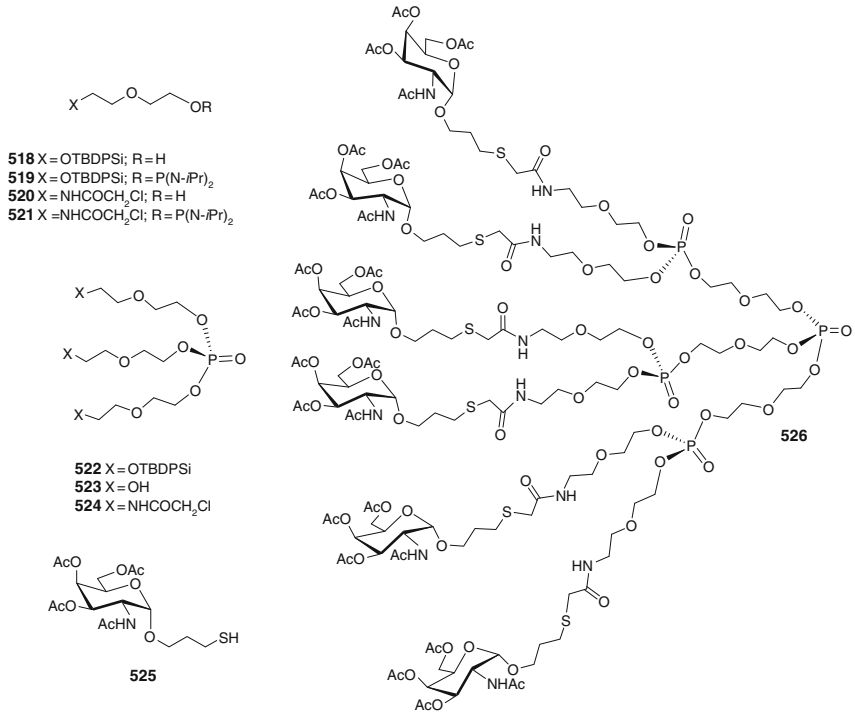


FIG. 64. An α-D-GalNAc phosphotriester dendron built using phosphoramidite chemistry (Kratzer and Roy, unpublished data).[385]

shown). Thiol **525** was prepared from allyl *N*-acetyl-α-D-galactosaminide by reaction with thioacetic acid (HSAc, MeOH, AIBN, reflux, 10 h, 77%) followed by Zemplén S-deacetylation.

tert-Butyldiphenylsilyl-protected phosphotriester **522** was deprotected by fluoride anion (Bu_4NF, THF, 78%) to give a triol **523**, which was further branched with building block **521** as before to provide a second-generation phosphotriester (57%) having six N-chloroacetyl residues after oxidation of the phosphite. Nucleophilic displacement of the hexavalent N-chloroacetylated precursor by **525** as before, provided hexavalent dendrimer **526** (52%). Further processing of the synthetic sequence, coupled with tethering strategies with various spacers, gave access to a family of dendrimers having many different valencies. All of the GalNAc-bearing phosphodendrimers thus produced were tested as inhibitors of the plant lectin *V. villosa* binding to asialoglycophorin, a natural ligand for hepatocytes receptors. The results indicated a 3–10-fold (hexamer) enhanced

affinity over the monovalent ligand, thus supporting once again the glycoside cluster effect. Interestingly, sugar **525** is part of a key determinant known as the TF-antigen overlay expressed on breast cancer tissues and other melanoma cancers. Its structure and higher oligosaccharide homologues have formed the basis for the dendritic glycopeptides vaccines already described.

Additional examples of dendritic phosphodiesters have been highlighted by Dubber and Fréchet with mannoside and galactoside oligonucleotide conjugates, using a DNA synthesizer (Fig. 65).[386] The SPS of multivalent glycoconjugates enables the custom tailoring within hours of their valency and structural requirements toward the biological targets, as opposed to more cumbersome traditional approaches. A tetrameric mannoside (**530**) was thus prepared from simple precursors **527**–**529**, using phosphoramidite chemistry. A glycodendron was built with a fluorescein probe and another one with a thiolated oligonucleotide coded for the antisense inhibition of inducible nitric oxide synthase. Once again, the constructs, prepared on a 1 μmol scale, were satisfactorily characterized by standard ^{1}H NMR and MALDI-TOF MS.

b. Commercial Dendritic Scaffolds.— *(i) PAMAM Dendrimers*. Several dendrimer scaffolds having various surface functionalities and valencies, constructed from diverse building blocks and according to both general synthetic strategies (Fig. 66, see also Section V.1.b) are commercially available. Some of the most commonly used

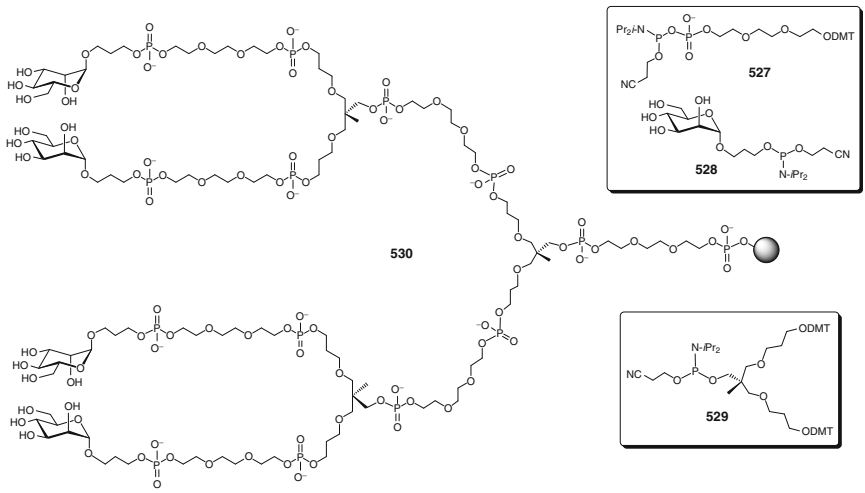


FIG. 65. Solid-phase synthesis of mannosylated dendrons for antisense gene delivery.[386]

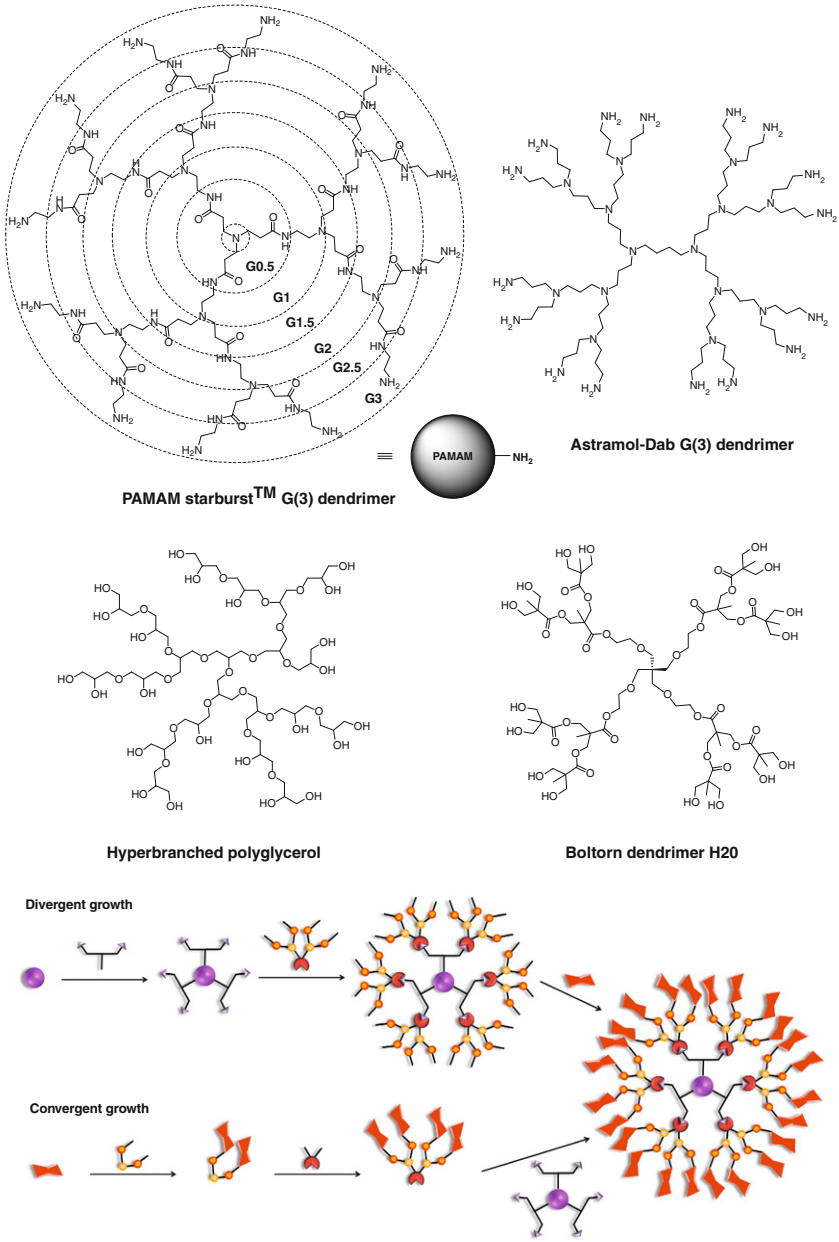


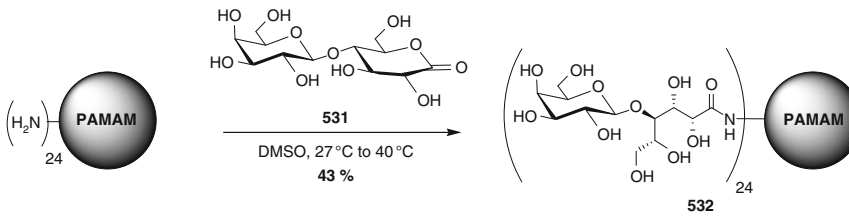
FIG. 66. Commercially available dendrimer scaffolds commonly used for glycodendrimer syntheses, and general synthetic strategies for synthesis of glycodendrimers.

ones are illustrated in Fig. 66. Among these readily available and widely used dendritic scaffolds, poly(amidoamine) dendrimers (PAMAM, Dendritic Nanotechnologies) constitute candidates of choice that have been extensively exploited by many groups of glycochemists, notably for the rapid synthesis of highly branched glycodendrimers. Since the pioneering synthesis of poly(amidoamine) dendrimers proposed by Tomalia *et al.* using a divergent growth procedure, these attractive molecules have constituted an exciting new class of macromolecular and highly branched architectures with well-defined size, shape, and geometry. They have drawn much interest in several research areas.[284,288]

Accordingly, PAMAM-based dendrimers, having built-in surface amine functionalities, have been the first and most frequently used scaffolds for attachment of sugars. The very first example of saccharide-substituted PAMAM dendrimers was proposed by Okada and coworkers, who described the synthesis of ''sugar balls'' **532** via amide bond formation, starting from sugar lactones (**531**).[387] Although this process is a straightforward manipulation, it suffered from the disadvantage of sacrificing the reducing sugars, which alternatively served as extended linkers (Scheme 50).

Several other strategies have been used to adequately functionalize PAMAM dendrimers with carbohydrates, involving: (a) introduction of thiourea linkages formed by treating amino dendrimers with isothiocyanated saccharide derivatives, (b) direct amide linkages with sugar-bearing carboxylated or activated ester derivatives, (c) reductive amination, or (d) incorporation of chloro- or bromo-acetamido groups onto PAMAM dendrimers or saccaharides, to afford highly electrophilic species that can for instance react with thio or amino derivatives. All of these synthetic approaches are illustrated in the following section.

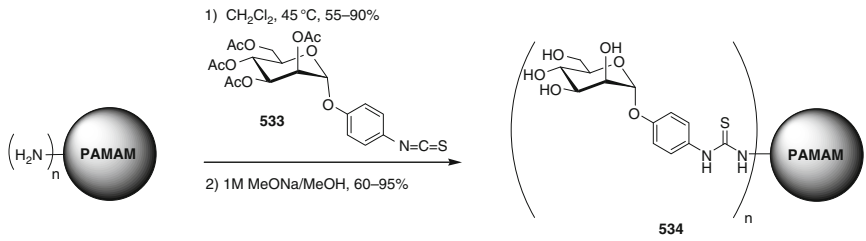
Thiourea Linkages. Attachment of saccharide units to the surface of PAMAM through thiourea linkages offers one of the most efficient ways to develop multivalent ligands quickly and efficiently for the study of protein–carbohydrate interactions.



SCHEME 50. ''Sugar-balls'' obtained by direct ring-opening of sugar lactones by PAMAM-ending polyamines.[387]

Historically, Lindhorst and Kieburg first published the simple and efficient coupling of different low-generation polyamine scaffolds, including PAMAM, to β-D-gluco-, α- and β-D-manno-, β-D-galacto-, β-cellobio-, and β-lacto-configured glycosyl isothiocyanates.[388] An improved version, using a hydrophobic aryl aglycone, later permitted the syntheses of the first four generations of monodispersed neoglycoconjugates having up to 32 mannoside units (**534**). *p*-Isothiocyanatophenyl α-D-mannopyranoside **533**[389] was used as key precursor (Scheme 51). The resulting glycodendrimers were evaluated as ligands for the phytohemagglutinins from Con A and *Pisum sativum* (pea lectin), using ELLA and turbidimetric analyses. The relative binding data indicated that incorporation of terminal α-substituted mannoside residues furnished glycodendrimers showing an up to 400-fold increase in binding capacities. Moreover, their ability to bind and form insoluble carbohydrate–lectin complexes was also demonstrated by radial double immunodiffusion and turbidimetric assays, and their capacity to precipitate selectively their homologous protein receptors from crude lectin mixtures made them convenient tools for the rapid and simple isolation of proteins.[390] The same procedure was successfully adapted by the same group a few years later, allowing the introduction of *p*-isothiocyanatophenyl β-D-lactoside onto PAMAM scaffolds to study their relative binding behavior toward the family of galectins, the influence of the generation, and the binding-site orientation of receptors.[391]

In 1997, a similar but simplified procedure, involving aqueous solutions that and avoiding protecting groups, for the synthesis, from methylthiourylene α-D-mannopyranoside or the aromatic derivative, of a G(2) PAMAM dendrimer containing up to six peripheral mannosyl residues was proposed.[392,393] Parallel investigations by Thompson and Schengrund involved the synthesis of glycodendrimers from poly(propylene)-imine and Starburst® (PAMAM) dendritic scaffolds, and provided potent inhibitors of CT.[394] They contained four to eight peripheral oligo-GM1 [βGal-(1→3)-βGalNAc-(1→4)-αNeu5Ac-(2→3)-βGal-(1→4)-βGlc-(1→1)Cer] group with IC_{50} of 14 nM against CT, that were covalently attached to the central core via thiourea linkages,



SCHEME 51. PAMAM-based mannosylated dendrimers incorporating the key aryl aglycone.[389]

using the PITC derivative of oligo-GM1. The IC_{50}s values were determined for the oligo-GM_1-PITC dendrimers, native GM_1, and the oligosaccharide moiety of GM_1 (oligo-GM_1), and studies revealed that the glycodendrimers inhibited binding of the CT to GM_1-coated wells at molar concentrations 5- to 15-fold lower than native GM_1 and more than 1000-fold lower than that of the free oligosaccharide.

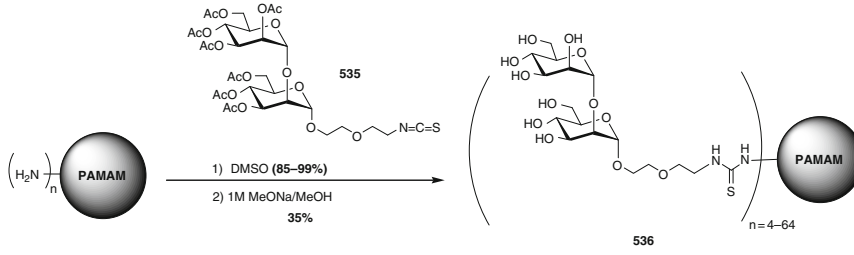
G(2) and G(3) sialic acid-containing PAMAM glycoconjugates with *p*-isothiocyanatophenyl α-sialoside (**487**) (Fig. 50) as precursor have also been described by Zanini and Roy, and their lectin-binding properties evaluated.[395] Preliminary turbidimetric studies with G(2) and G(3) α-thiosialodendrimers, presenting 16 and 32 peripheral sugar units, respectively, demonstrated their ability to bind the slug lectin from LFA, showing well-organized precipitation patterns. Furthermore, their relative efficiency to inhibit the binding of HRPO-labeled LFA to human AGP (orosomucoid) was determined by a competitive ELLA. Interestingly, an increase in multivalency resulted in a steady increase of inhibitory potential, with IC_{50} values in the nanomolar range (2.89 and 1.13 for G(2) and G(3), respectively) corresponding to an ∼210-fold jump in inhibitory potential over the monomeric analogue. Subsequently, Baker and coworkers evaluated similar sialic acid-conjugated G(4) PAMAM structures as a mean of preventing adhesion of three influenza A virus subtypes, and furnished one of the first documentations of the function of dendrimer conjugates as antiinfective agents *in vivo*.[396] HAI *in vitro* showed the glycodendrimers to inhibit some specific influenza subtype strains at concentrations 32–170 times lower than those of sialic acid monomers. The *in vivo* studies also demonstrated the ability of the glycodendrimers to protect against experimental infection by influenza A X-31 H3N2 virus in mice.

In 2001, Woller and Cloninger described the largest glycodendrimer then built, prepared on generation G(6)-PAMAM dendrimer and containing 256 mannoside residues.[397] When compared to methyl α-D-mannoside taken as the monomeric control, dendrimers G(1) and G(2) did not show any increase in activity toward the phytohemagglutinin Con A, and the G(3) dendrimer bound roughly one order of magnitude better than G(1), G(2), or methyl mannoside. This was suggestive of a glycoside cluster-effect (enhanced local concentration). As with G(1) and G(2) glycodendrimers, G(3) was too small for multivalent binding to occur (chelate effect). Dendrimers G(4) to G(6) showed a two orders of magnitude increase in activity against the tetrameric Con A, indicating that multivalent binding was occurring. It is also possible that the change in shape from circular [G(1)- to G(3)-PAMAM] to spherical [G(4) to G(6)] caused the observed binding enhancement.[398] Further studies led the same group to the efficient incorporation of more complex saccharide units such as αMan-(1→2)Man disaccharide **535**, manifesting the fact that dimannose

functionalized G(3) and G(4)-PAMAM dendrimers **536**, having an average of 26 and 48 peripheral groups were very competent for the recruitment of cyanovirin-N (CV-N), an HIV-inactivating protein that blocks virus-to-cell fusion through high mannose-mediated interactions (Scheme 52).[399]

Finally, a subsequent investigation provided the first example of the use of glycodendrimers as model systems for studying carbohydrate–carbohydrate interactions (CCI).[400] More particularly, lactosylated PAMAM dendrimers with various peripheral carbohydrate numbers, depending on the generation, were synthesized to examine the CCI of lactose with mixed Langmuir monolayers containing GM_3 and dipalmitoyl phosphatidylcholine (DPPC). Thiourea ligation between glycosyl isothiocyanate derivatives and the aminated dendritic core, following standard protocols afforded glycodendrimers containing appropriate amounts of lactose, which were further capped with poly(ethylene)glycol chains or other carbohydrates. The results corroborated the concept that lactosyl dendrimers were engaged in a CCI with GM_3 in a Langmuir monolayer. This CCI was dependent on both the carbohydrate density and the density of glycolipid within the monolayer. Moreover, a specific CCI was observed only in the presence of calcium ions and when at least one-fifth of the monolayer was composed of GM_3.

Amide linkages. Strategies based on the direct formation of amide bonds via carboxylic acid or activated ester-containing saccharides and PAMAM dendrimers have afforded sophisticated dendritic architectures having promising biological properties. For instance, using a similar approach to the one just described, Aoi *et al.* described the synthesis of dendrimer-based star polymers (‘‘oligoglycopeptide sugar balls’’) by the original macromolecular design of living polymerization.[401] The actual dendritic oligoglycopeptides were thus synthesized by living oligomerization of glyco-*N*-carboxy anhydrides (glyco-NCAs) with poly(amidoamine) (PAMAM) dendrimer as a multifunctional macro initiator. This polymerization system was termed as ‘‘radial-growth polymerization (RGP),’’ since dendrimer-based living polymerizations



SCHEME 52. Manα1-2Man disaccharide attached to PAMAM via thiourea linkages.[399]

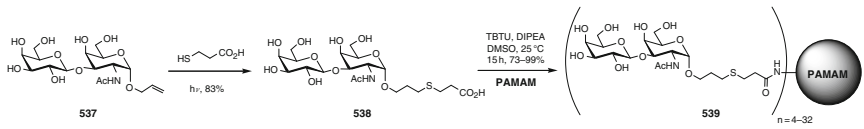
offered highly ordered star-shaped macromolecules with a number of arms, which should be different from conventional star polymers. The resulting sugar balls in this study are dendritic nanocapsules surrounded radially by oligoglycopeptide chains.

Roy *et al*. described the systematic preparation and biological evaluation of glycoPAMAMs containing up to 32 TF-antigen units [βGal-(1→3)-αGalNAc], known to be a cancer-related epitope and as an important antigen for the detection and immunotherapy of carcinomas, particularly relevant in breast cancer patients.[351,402,403] The synthetic sequence was based on the efficient acid functionalization of the allyled TF-antigen derivative **537**, accomplished using 3-mercaptopropanoic acid, to provide acid **538** in 83% yield. GlycoPAMAMs **539**, presenting up to 32 peripheral TF-antigens, were prepared by direct amide bond formation between unprotected **538** and PAMAM, using the efficient TBTU coupling reagent (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) and DIPEA as a base (Scheme 53).

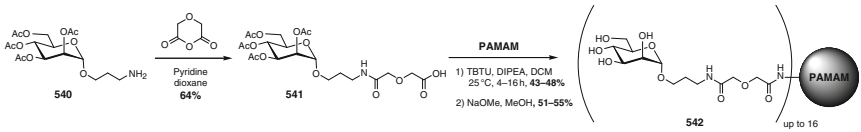
The protein-binding properties of these glycodendrimers were evaluated using peanut lectin from *Arachis hypogaea* and a mouse monoclonal IgG antibody. Based on bulk conjugates, the glycoPAMAMs with the highest carbohydrate density exhibited the strongest inhibitions, clearly indicating a cluster effect. All of these conjugates were antigenetically active and their IC_{50} values of 5.0, 2.4, 1.4, and 0.6 nmol, respectively, for G(0) to G(3) correspond to inhibitor abilities 460, 960, 1700, and 3800 times higher than that of the monomer **537** toward antibody-coating antigen interactions.

Based on an identical approach, mannosylated PAMAM dendrimers were constructed in order to evaluate their relative inhibitory properties against the type-1 fimbriated uropathogenic *E. coli*.[404] As an alternative to the synthetic strategy just described, the authors chose 3-aminopropyl α-D-mannopyranoside (**540**) pretreated with diglycolic anhydride. The resulting extended acid derivative **541** was then coupled to peripheral PAMAM amino groups with typical peptide coupling reagents such as BOP (benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate) or TBTU (Scheme 54).

The deprotected glycodendrimers **542** were tested using a newly developed ELISA-based inhibition assay for their ability to inhibit the binding of recombinant type-1 fimbriated *E. coli* (FimH) to a monolayer of T24 cell lines derived from human



SCHEME 53. Antigenic PAMAM-based TF-antigens linked by amide coupling.[402]



SCHEME 54. Amide-linked mannosylated PAMAM dendrimers.[404]

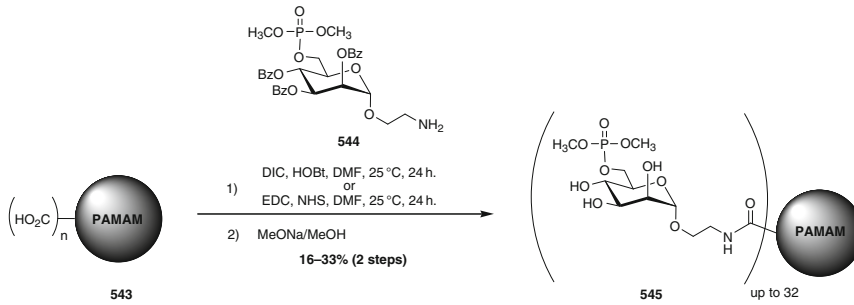
urinary bladder epithelium. The PAMAM mannodendrimers displayed potent affinity toward the target FimH, with IC_{50} of 37 and 19 μM for G(1) and G(2), respectively, although their relative potency per mannose was rather low.

The synthesis of mannose 6-phosphate-functionalized PAMAM dendrimers was also investigated and studied for their ability to bind goat-liver mannose-6-phopshate receptor (MPR 300).[405] The preparation of these glycodendrimers was achieved by the initial preparation of the amine-tethered Man-6-P derivative **544**. Since the amino function was present on the saccharide residue, the authors used preformed half-generation G(0.5) to G(3.5) PAMAM dendrimers **543** exhibiting 4, 8, 16, and 32 carboxylic acid groups, respectively. The resulting dendritic conjugates **545** were obtained by direct amide bond formation, using DIC–HOBt (1-hydroxybenzotriazole) or EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)–NHS (*N*-hydroxysuccinimide) as coupling reagents (Scheme 55).

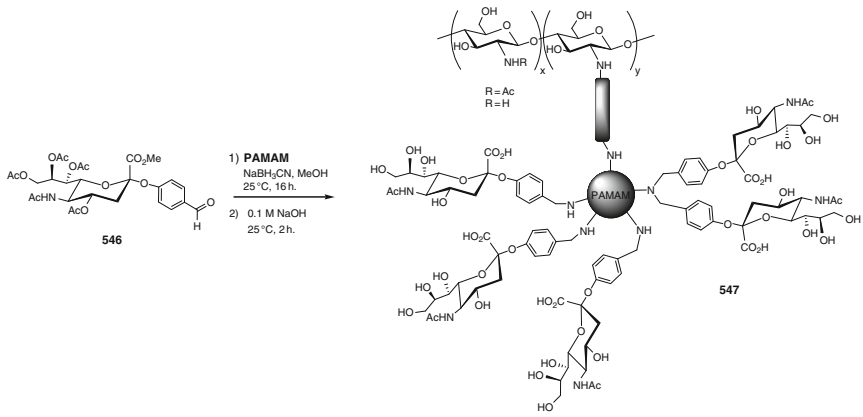
Preliminary biological studies suggested that the newly synthesized Man-6-phosphate-containing dendrimers could act as adsorbents in related affinity chromatography, with an interesting potential to bind the purified goat-liver mannose-6-phopshate receptor (MPR 300) protein. Additional conjugation of various nonprotected glycosides has been similarly described using *p*-nitrophenyl activated saccharide esters to conjugate disaccharides or sialic acid components onto PAMAM scaffolds.[406]

Reductive Amination. Sashiwa *et al.* have used reductive amination to provide dendronized chitosan–sialic acid hybrids containing different spacers (Scheme 56).[407–409] The sialic acid residues were successfully attached to each PAMAM dendrimer by reductive N-alkylation with *p*-formylphenyl α-sialoside **546**, using $NaBH_3CN$ in MeOH, to furnish hybrid chitosans **547**. However, since an excess of aldehyde must be used for high incorporation of sialic acid, the procedure also gave rise to double N-alkylation. The reaction thus gave complex sialic acid constituents, affording undesired mixed structures. To circumvent these drawbacks, N-methylamino derivatives (see Scheme 46) have been subsequently used for the reductive amination, and sialodendrimers have been prepared to target the immunoglobulin exposed Siglecs.[410]

Nucleophilic Substitution via Incorporation of Haloacetamido Groups. In 1996, Zanini and Roy described the design and synthesis of a novel family of symmetrical



SCHEME 55. Synthesis of mannose-6-phopshate PAMAM conjugates.[405]



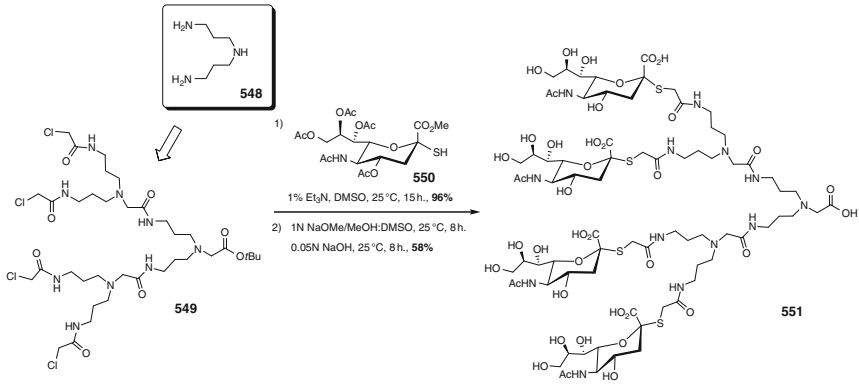
SCHEME 56. Synthesis of dendronized chitosan–sialic acid hybrids.[407–409]

dendrimers presenting even valencies between 2 and 16 residues, based on a 3,3′-iminobis(propylamine).[411] The synthetic approach was based on convergent assembly of suitable multibranched dendrimers containing N-chloroacetylated end groups to provide electrophilic species that could readily react with thiolated carbohydrate derivatives. Many synthetic benefits emanated from this strategy. First, the method was general, high-yielding, and readily amenable to such existing commercially available amine-terminated dendrimers as PAMAM that could be similarly treated with chloroacetic anhydride. Second, the synthesis of this dendritic family presented a viable alternative to PAMAM dendrimers, avoiding the need for large excesses of the reagents commonly used to ensure complete conversion. More importantly, these

structures are not susceptible to base-catalyzed retro-Michael degradations, in contrast to classical PAMAMs, since the substituents are not β-carbonyl positioned. The authors thus accomplished the efficient preparation of dendritic α-thiosialosides **551**–**553**, starting from the AB_2-type core 3,3′-iminobis(propylamine) (**548**), which was subsequently transformed to afford various symmetrical N-chloroacetylated dendrons, such as tetramer **549** (Scheme 57). A slight excess of thiosialoside **550** allowed the synthesis of glycodendrimers under mild conditions. They were deprotected to afford the dendritic architectures containing between four (**551**, Scheme 56) and up to 16 sialoside residues (**552**, Fig. 67), respectively.

The same group conducted a systematic study of similar but tethered sialodendrimers with even valencies between 4 and 12 (**553**), based on the same AB_2 core **548**, and according to the same synthetic strategy.[412] The potential of these sialodendrimers to cross-link and precipitate LFA was confirmed by preliminary turbidimetric analysis. When tested in enzyme-linked lectin inhibition assays using human AGP (orosomucoid) as coating antigen, and HRPO-labeled LFA for detection, tetravalent dendron **551** showed IC_{50} values of 11.8 nM, while **552** presented a higher value of 425 nM. On the other hand, these assays indicated that for the corresponding tethered dendrimers, the inhibitory potency increased with a corresponding increase in valency. The highest IC_{50} value was obtained for **553** (8.22 nM) and constituted a 182-fold increases in inhibitory potential over the monovalent 5-acetamido-5-deoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosyl azide used as standard (IC_{50} of 1500 nM). The same group then extended this approach using solid-phase chemistry.[413]

Despite the demonstration that PAMAM dendrimers can exhibit up to 256 peripheral saccharide units, a general consensus highlights the fact that the most highly



SCHEME 57. α-Thiosialodendrons built on base stable AB_2 amine core.[411]

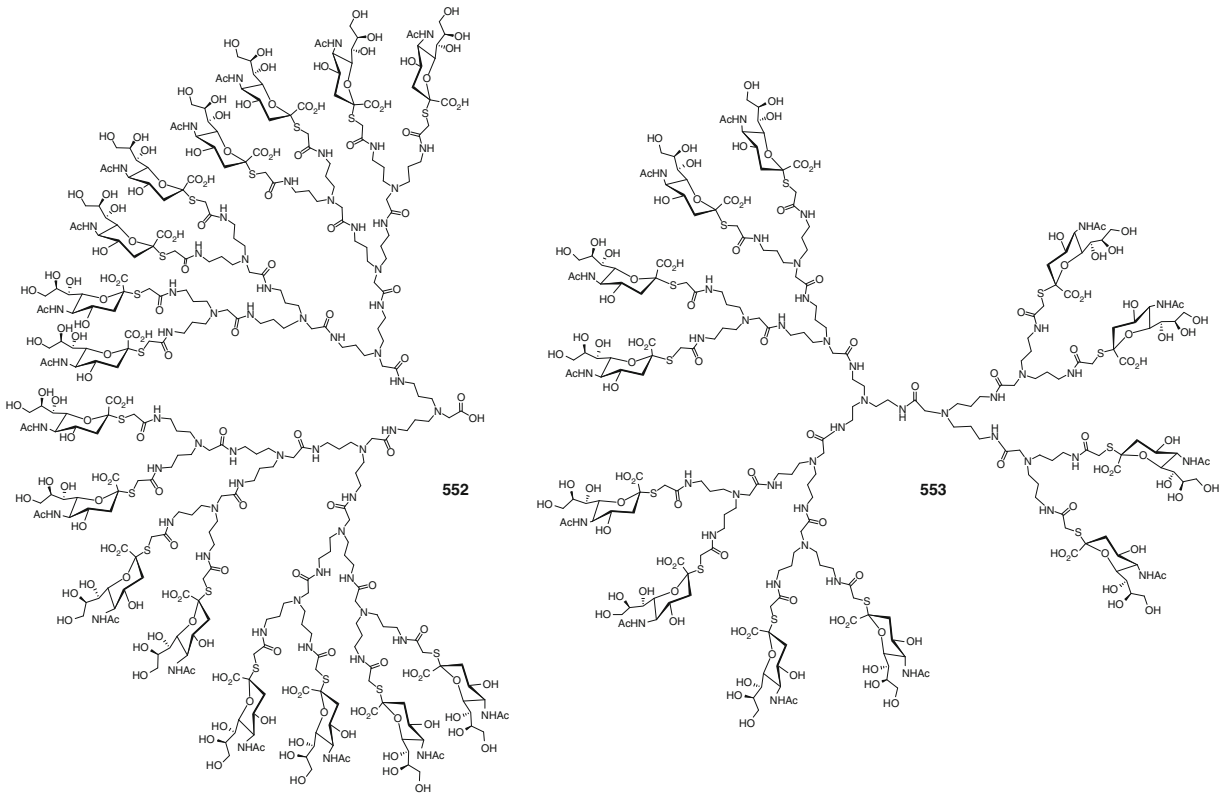


FIG. 67. α-Thiosialodendrons (**552**) and dendrimers **553** built on AB_2 amine scaffolds.[412]

functionalized scaffolds are not always the ones with the highest protein-binding activity. In fact, less-functionalized ligands may often present optimal potencies. A plausible explanation for this phenomenon has been suggested and highlights the fact that at high loading, the peripheral saccharide portions might become less accessible to proteins because of an increase in steric hindrance. Another hypothesis is based on the drawbacks of using large dendrimers bearing aromatic aglycones, which can intramolecularly undergo π-stacking, thus further tightening the saccharide units at the dendrimer surface. Furthermore, solubility problems are often encountered with PAMAM dendrimers bearing more than 64 arylated mannoside moieties.

Straightforward solutions to this problem have been addressed in the following publications describing the synthesis of sialoside dendrimers **554**, where the glycans are interspaced by TRIS residues used as ''dummy'' functionalities (Fig. 68).[335]

Aiming to validate these concepts, Wolfenden and Cloninger conducted the controlled preparation of a series of heterobifunctionalized mannoside and hydroxyl-G(3) to G(6)-PAMAM dendrimers **555** (Fig. 69).[414,415] A systematic study was addressed of the effect of carbohydrate loading on the activity of the dendrimer for a lectin and the influence of the conjugates' size. The degree of functionalization of mannose–hydroxyl groups via thiourea linkages was controlled by the stoichiometric amount of isothiocyanate derivatives of comparable reactivity. MS data allowed determination of the average number of mannose surface residues for each dendrimer generation. Hemagglutination assays were performed by adding rabbit erythrocytes to preincubated solutions of Con A and varying the concentrations of deprotected dendrimers. Comparison of the fourth through sixth PAMAM generations with different loading of surface mannose residues suggested that the binding efficiency was the highest for all generations at 30% to 50% loading. The most interesting observation was that the highest activity did not correlate with the maximum sugar loading, but rather occurred at slightly closer packing of the sugars as the generation increased, suggesting that unfavorable steric interactions precluded optimal binding at high carbohydrate density.

A follow-up investigation described the simultaneous anchoring of various sugars, such as mannose, galactose, and glucose residues via thiourea linkages, in order to quantify the effect that functionalization of dendrimers with monomers of varying affinities would have on its multivalent activity with lectins (**556**).As mentioned in earlier work, MALDI-TOF MS was used to determine the exact number of carbohydrate residues of each type on the resulting glycodendrimers. Both the change in MW after each sequential addition and the change in molecular weight after deacetylation were used for accurate measurement of the extent of functionalization. The association of the corresponding deprotected dendrimers with Con A was studied by

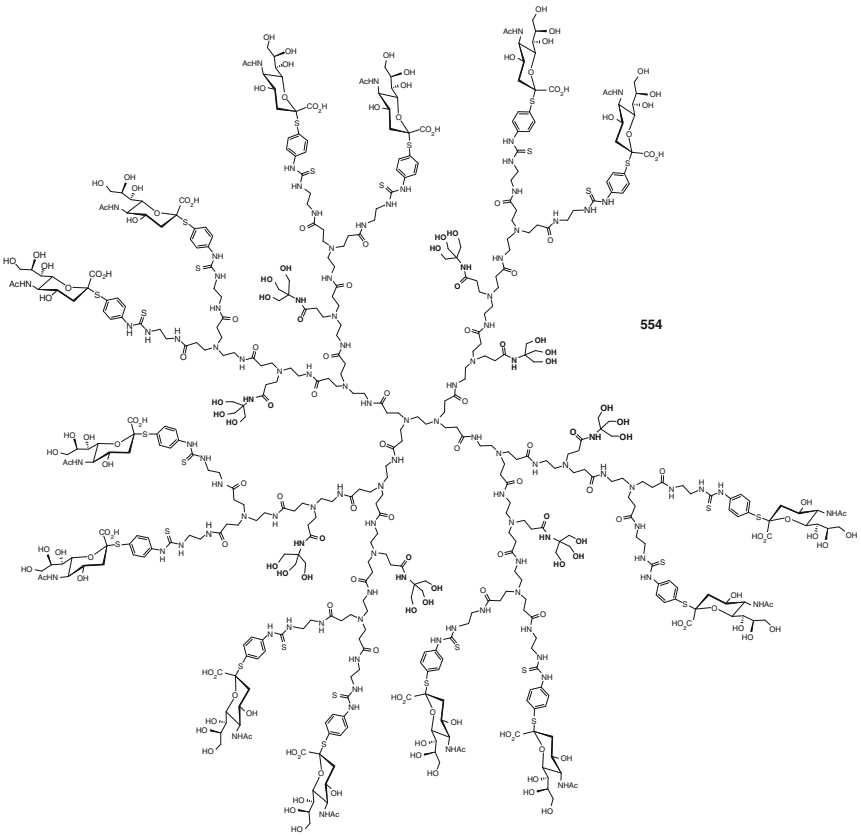


FIG. 68. Modified PAMAM scaffold bearing inter-spacing TRIS residues to promote better accessibility to surface saccharides.[335]

precipitation and hemagglutination assays. As expected, increasing the number of mannose residues, while decreasing the number of glucose residues, caused an increase in the relative affinity toward Con A. As with the dendrimers bearing 50% mannose: glucose loading, a linear relationship between Man:Glc loading and assay activity was observed for compounds of generations 4–6. However, the relative binding differences between mannosylated dendrimers and glucosylated dendrimers varied from one generation to another.

Alternatively, the G(3)-mannosylated PAMAM had a fivefold higher relative affinity toward Con A compared to the glucose-functionalized analogues. In summary, the reported results suggested that multivalency can be influenced in

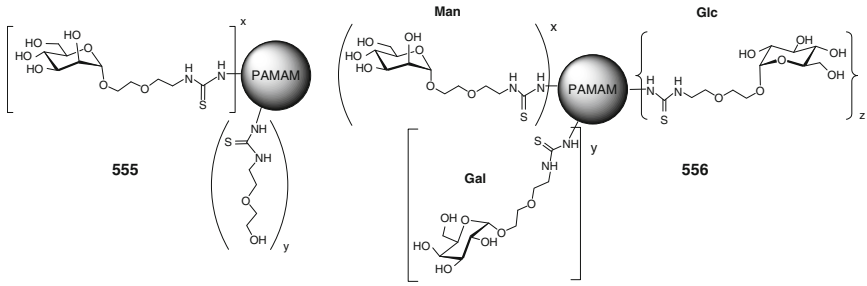


FIG. 69. Heterofunctional GlycoPAMAM dendrimers.[414,415]

predictable and therefore tunable ways. Monovalent differences are amplified by multivalent associations, and mixtures of low- and high-affinity ligands can be used to attenuate multivalent binding activities.

Given their commercial accessibility, PAMAMs have been the scaffold most widely used in modern investigations. They have been modified with a large variety of sugar derivatives and with varied sugar densities (Fig. 70). They have been used for several important binding phenomena and represent the most deeply studied scaffold for toxicity evaluations. In addition, scaffolded glycodendrimers were shown to be nonimmunogenic, a key property if they are to be used as bacterial or viral antiadhesins.

b. PPI Dendrimers.—A convenient reaction sequence for large-scale synthesis of PPI dendrimers [poly(propyleneimine) or DAB-*dendr*-$(NH_2)_x$] (Fig. 66) has been described by Meijer's group, who nicely adapted the original strategy proposed earlier by Vögtle's group (which suffered from cumbersome purifications).[286,416] By a repetition of double Michael addition of acrylonitrile into primary amines, followed by metal-catalyzed hydrogenation, the preparation of well-defined DAB-*dendr*-$(NH_2)_x$ containing up to 64 primary amine surface groups was efficiently achieved. As with PAMAM dendrimers, this family of commercial dendritic scaffolds has been widely functionalized. Obviously, saccharide-based PPI dendrimers have also been synthesized.

The first example of glycodendrimers using PPI scaffolds was proposed by Ashton *et al*. who described their use for the rapid and facile construction of high molecular weight G(1) to G(5) carbohydrate-coated dendrimers. A divergent approach was followed and glycodendrimers containing, respectively, 4, 8, 16, 32, and 64 D-galactose and lactose peripheral groups, were efficiently synthesized.[417] The saccharide residues were attached to the aminated dendritic scaffold was by amide

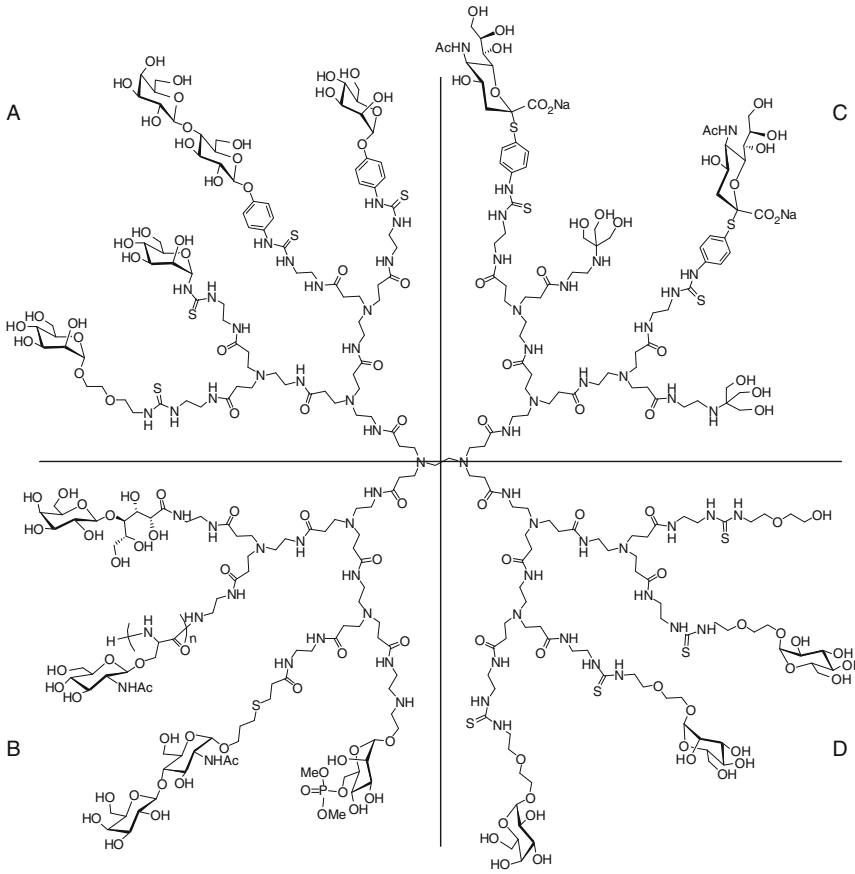
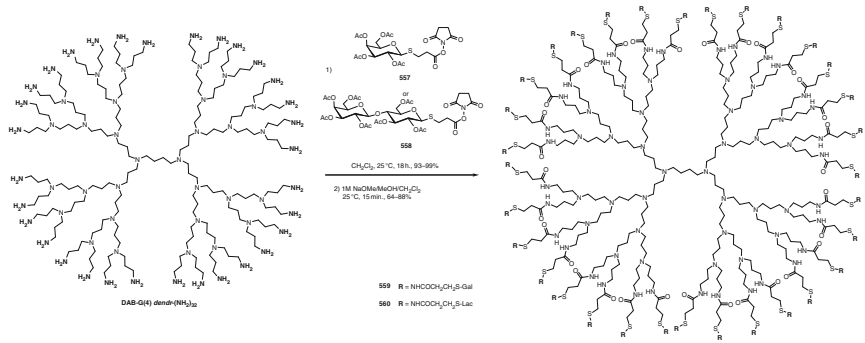


FIG. 70. PAMAM can be modified with varied linkage functionalities: thioureas (A); amides (B); heterobifunctional interspacing groups (C); and mixed sugars (D).

bonding through glycosides ending with the *N*-succinimidyl esters **557** and **558**. Highly globular glycodendrimers were obtained essentially quantitatively and were deacetylated under Zemplén conditions [to give G(4)-PPI glycodendrimers **559** and **560**] (Scheme 58).

In continuing efforts, the same authors constructed tris(galactoside)-modified DAB dendrimers by the accelerated convergent strategy. In this particular approach, the saccharides were connected first to a small TRIS branching derivative to form a cluster which served as a building block, once suitably functionalized.[417] N-Succinimidyl-activated ester **561** was then coupled onto G(1) and G(2)-PPI dendrimers with



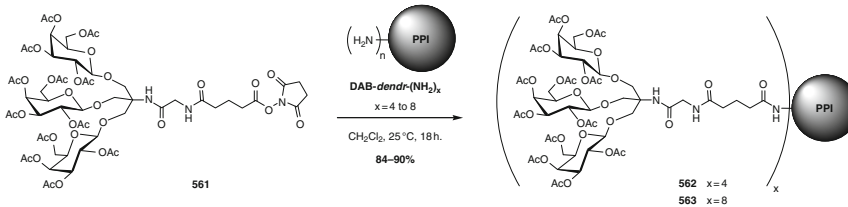
SCHEME 58. First poly(propyleneimine) glycodendrimers (PPI) coated with thioglycosides through amide bond formation.[417]

4 and 8 primary end groups, respectively, to afford glycoconjugates **562** and **563** containing respectively, 12 and 24 peripheral galactosides (Scheme 59).

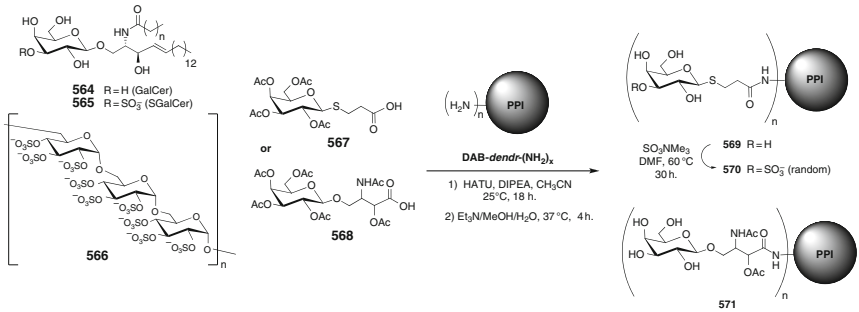
The same group modified the linker by using different numbers of carbon atoms (1, 5, 10) to afford variations of the local saccharide concentrations at the dendrimer surface.[418] This study was aimed to determine the influence of this linker parameter on the glycodendrimer–protein interactions, the relationship between structure and water solubility, and to investigate amphiphilic properties.

In 2004, additional poly(sulfogalactosylated) dendrimers based on DAB scaffolds were investigated to study their antagonist properties against HIV-1 infection.[419] First, to assess the interaction of HIV-1 gp120 with its reported alternative glycolipid receptors, a series of glycodendrimers built from mimics of galactosyl ceramide (GalCer **564**) and its sulfated derivatives **565** were synthesized, analyzed by SPR as ligands for rgp120 IIIB, and evaluated for their ability to inhibit HIV-1 infection on CXCR4- and CCR5-expressing indicator cells. The synthesis was based on direct amine bond formation with carboxylated galactoside residues **567** or **568** and G(1) to G(5) DAB-dendrimer (Scheme 60), and afforded compounds **569** and **571**. Polysulfated dendrimers **570** were obtained by random sulfation of **568**, and the average number of sulfates was determinated by MALDI-TOF MS analysis. The *in vitro* studies of their effectiveness at inhibiting infection of U373-MAGI-CCR5 cells by HIV-1 Ba-L indicated that the sulfated glycodendrimers were better inhibitors than those of the nonsulfated compounds, but not as effective as the sulfated dextran control **566**.

Further investigations led the same group to increase the degree of sulfation on galactosylated dendrimers, based on the hypothesis that glycosphingolipid SGalCer



SCHEME 59. Convergent synthesis of PPI glycodendrimers.[417]



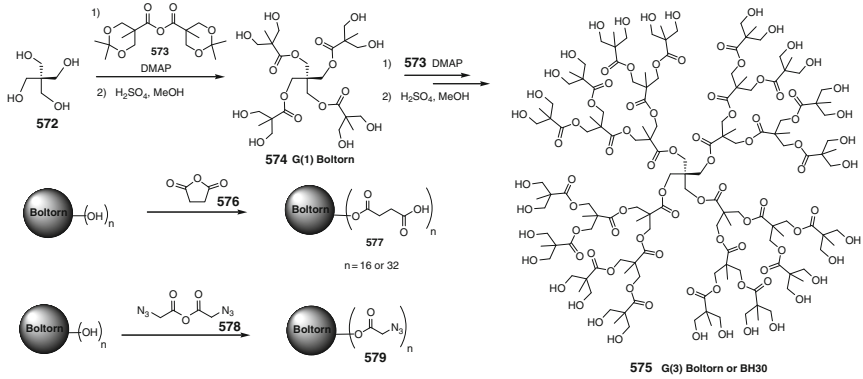
SCHEME 60. Galactosylated PPI dendrimers used as inhibitors of cell infectivity by HIV-1.[419]

565 was the best ligand for both recombinant and virus-associated gp120.[420] Therefore, the synthesis of G(5) DAB-dendrimer **570**, containing up to 64 peripheral galactoside groups and with an average of two sulfate groups per galactose residues (versus about 0.5 for the first example) was performed. The ability of **570** to inhibit infection of cultured indicator cells by HIV-1 was compared to that of dextran **566**. The results showed that **570** inhibited HIV-1 IIIB as well as dextran sulfate (a known potent inhibitor of HIV-1 infectivity) with EC_{50} values in the nanomolar range, and both were comparable in their ability to inhibit HIV infection of both X4 and R5 indicator cells. Furthermore, cytotoxicity studies revealed that neither the glycodendrimer nor **566** were toxic to the cells at the highest concentration (3 mg/mL) tested. Critical parameters such as the number and the position of sulfate groups (especially at C-3), which may constitute a key structural component of an efficient inhibitor, were clarified by this study.

An interesting application against HIV was developed, using G(5)-mannosylated PPI dendrimers (MPPI) as carriers for the controlled and targeted delivery of

antiretroviral nonnucleoside reverse-transcriptase inhibitors, such as lamivudine (3TC) and efavirenz (EFV).[421,422] Numerous benefits over the standard treatment based on the free drug or its PPI-encapsulated version, were demonstrated *in vitro*. These nanocontainers ensured efficient entrapment of the bioactive drugs (~45%), which allowed their prolonged release profile for up to 144 h (vs. 24 h with PPI). The toxicity of these systems was generally found negligble. In cellular-uptake experiments, MPPI interacted with the lectin receptors present on the surface of MT2 cells or monocytes/macrophages, leading to cellular uptake of drugs 12 times higher than that of the free drugs. Furthermore, this phenomenon may be responsible for the significant, but preliminary anti-HIV activity displayed by MPPI. Despite these promising results, further studies are needed to consider those encapsulated systems as potent carriers for efficient control and targeting the delivery of anti-HIV drugs.

c. Hyperbranched Boltorn® Dendrimers.—Hyperbranched dendritic Boltorn® polymers (Perstorp Speciality Chemicals) are also useful scaffolds, which have been used for the multivalent presentation of various functional groups. Their selection is based on the fact that second, third, and fourth generation polymers (H20, H30, and H40, respectively) are commercially available at a low cost since they are prepared in bulk quantities by polymerization. They are, however, polydisperse.[423,424] Nevertheless, they can also be synthesized by a simple and iterative approach, using pentaerythritol (**572**) as the central core and 2,2-bis(hydroxymethyl)propanoic anhydride (bis-MPA anhydride, **573**) (Scheme 61). Deprotection in acidic media furnished G (1)-Boltorn® polymer (**574**) containing 8 peripheral hydroxyl functions, and the sequence can be repeated for subsequent generations, leading, for instance to G(3) BH30 (**575**).



SCHEME 61. Idealized Boltorn® hyperbranched polyols.

To mimic the natural organization of high-mannose structures notably present in pathogens, mannosylated glycodendrimers obtained from these multivalent scaffolds have been synthesized. The commercial hyperbranched polymers of the second, third, and fourth generations were first functionalized with succinic anhydride (**576**) to furnish poly(acid) **577**, which was then capped with 2-aminoethyl α-D-mannopyranoside to give mannosylated dendrimers of various generations [**580** for G(3) (Fig. 71)]. Preliminary studies of these glycodendrimers, perfectly soluble under physiological conditions and nontoxic against several cell lines, evaluated their interactions with *Lens culinaris* lectin (LCA).[425] Data obtained by STD NMR

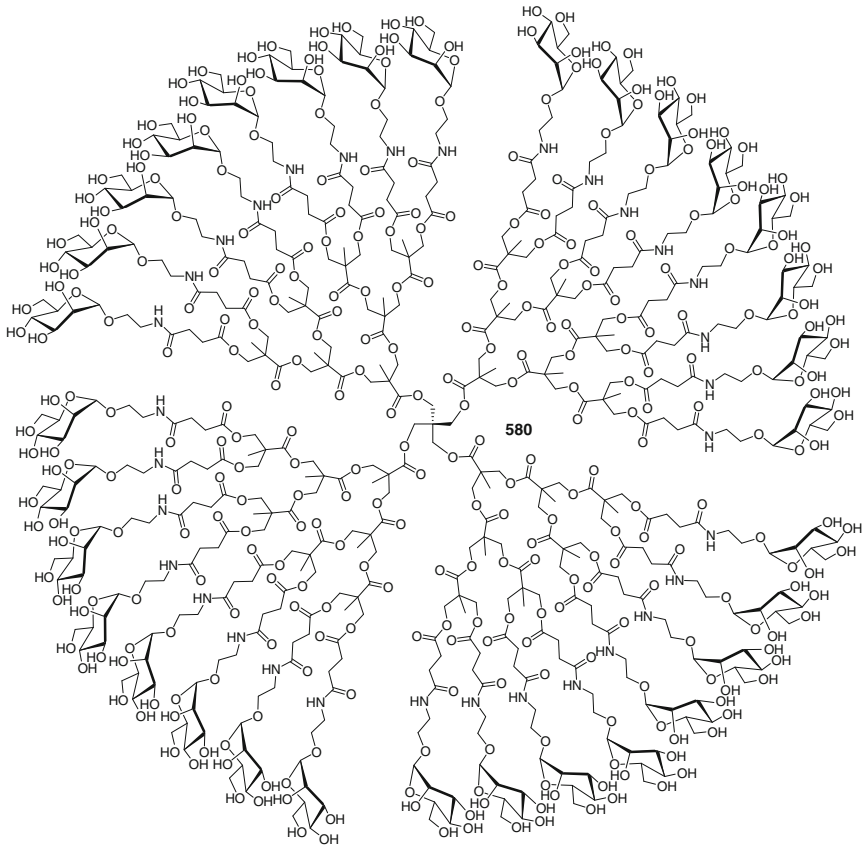


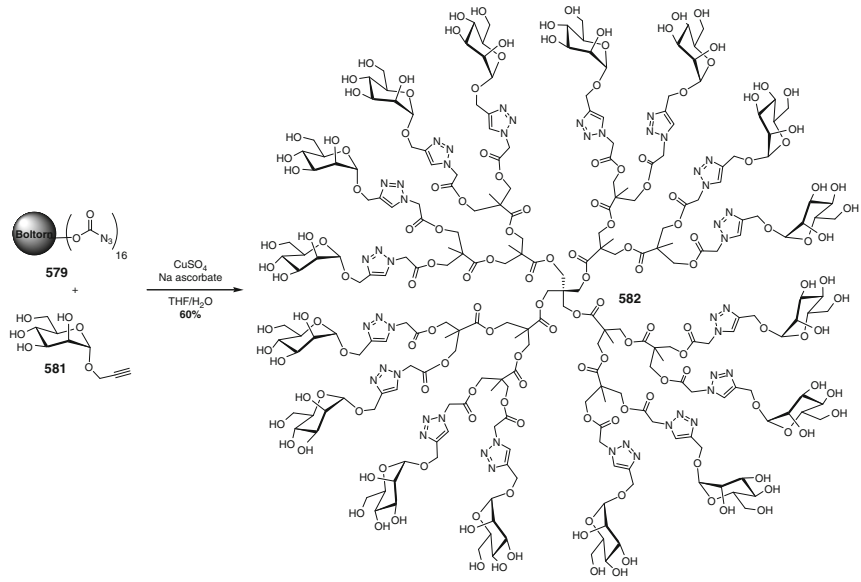
FIG. 71. Idealized mannosylated hyperbranched Boltorn® dendrimer.[425]

experiments and quantitative-precipitation assays demonstrated that glycodendrimers on the Boltorn® platform effectively interact with such biological receptors as plant and mammalian lectins.

The same group demonstrated that these mannodendrimers block the interaction between DC-SIGN and Ebola virus envelope glycoprotein.[426] Interestingly, dendrimer **580**, containing an average of 32 peripheral mannosides, was able to inhibit selectively the DC-SIGN-mediated Ebola infection in an efficient dose-dependent manner (IC_{50} of 337 nM), and showed no inhibitory effect in infection experiments using DC-SIGN-negative cell lines. These results showed that **580** was a potent inhibitor of Ebola infection mediated by DC-SIGN both in *cis* and *trans* (intra- and inter-cellular) presumably via the same inhibition mechanisms involving the interaction between the lectin and the viral envelope. In addition, a carbohydrate-dependent inhibitory effect was confirmed and a multivalent effect of two orders of magnitude demonstrated, since the monovalent manoside was able to inhibit this interaction at only millimolar concentrations.

A related application used ''click chemistry'' for synthesis of azido-functionalized Boltorn® dendrimer **579** (BH20) (Touaibia and Roy, unpublished data). Dendrimer **579** was obtained by treatment of hexadecahydroxylated BH20 (on average) with azidoacetic anhydride (**578**). IR analysis demonstrated complete hydroxyl-group transformation and introduction of azide function. The ''clicked'' hyperbranched dendrimer **582** was obtained under typical reaction conditions ($CuSO_4$ and sodium ascorbate) using nonprotected propargyl α-D-mannoside **581** (Scheme 62). The relative inhibitory potency of the mannosylated dendrimer for the inhibition of agglutination of *E. coli* by yeast mannan was approximately 400 times higher than that of the respective methyl mannoside (Benhamioud and Roy, unpublished data).

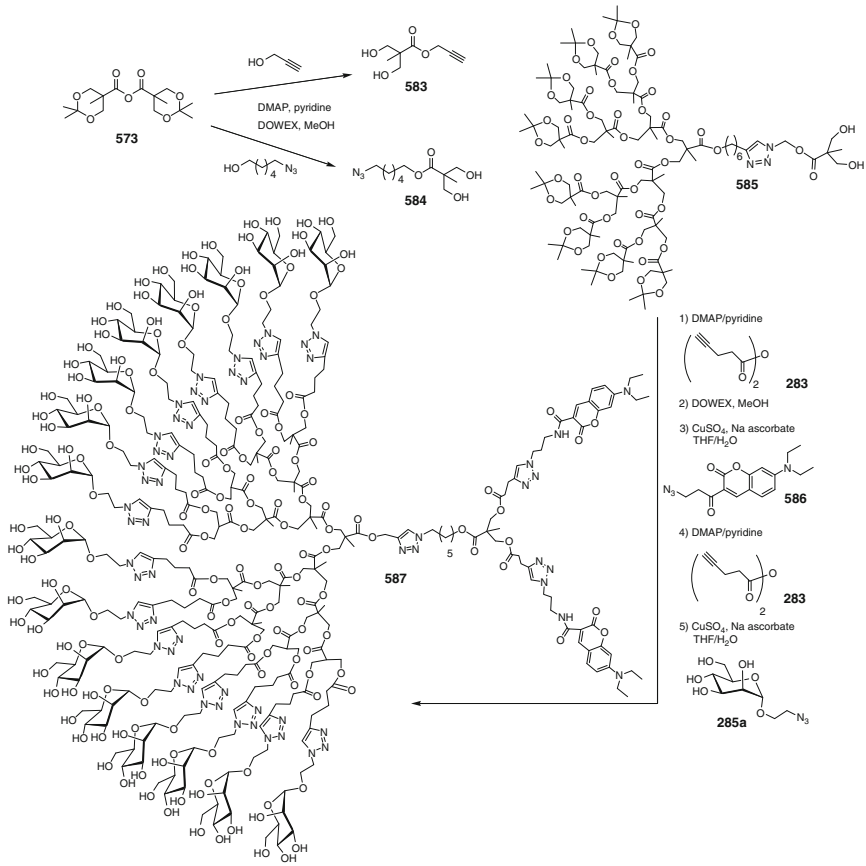
For most practical and biological applications of glycodendrimers, three functional units are usually required: a targeting moiety, a biologically active agent, and a probe. A general and facile strategy for functional-group introduction at defined positions on dendrimers is best achieved when dendrimers are synthesized stepwise. From this perspective, Sharpless, Hawker, and their group have provided an example of sophisticated, multifunctional materials that can be constructed in a stepwise, yet facile manner, using the efficient ''click'' methodology, and with a fashion-controlled strategy toward unsymmetrical glycodendrimers in which two distinct moieties (targeting and detection probe) were placed at the chain ends.[254] Dendritic block copolymers up to the fourth generation were coupled via the key step of Cu(I)-catalyzed azide–alkyne cycloaddition to prepare rapidly these dual-purpose and multifunctional materials without use of protecting groups. The orthogonal synthetic approach was based on 2,2-bis(hydroxymethyl)propanoic acid (bis-MPA), which constitute



SCHEME 62. ''Clicked'' mannosylated Boltorn® H20 (Touaibia and Roy, unpublished data).

the main building block in synthesis of the commercial Boltorn® dendrimers. The corresponding bis-MPA anhydride (**573**) provided access to alkyne ester **583** and azide ester **584** by condensation with the appropriate alcohol (Scheme 63). Removal of the protecting groups and subsequent condensation by ''click chemistry'' allowed the generation growth of the core and afforded the asymmetrical structure **585** having a diol as the focal point. Two molecules of the functionalized coumarin-type fluorescent dye **586** were then introduced by esterification of the two free hydroxyl groups of **585** with pent-4-ynoic anhydride **283**, followed by the [3+2] cycloaddition. Acetal hydrolysis and subsequent introduction of the 16 alkynes via esterification, followed by [3+2] cycloaddition with an unprotected 2-azidoethyl α-D-mannopyranoside (**285a**) in THF–H_2O furnished the asymmetrical heterobifunctional dendrimer **587**.

The performance of the resulting mannosylated dendrimer **587** was investigated in a standard hemagglutination assay using the MBP Con A and rabbit red-blood cells. When compared to the activity of a single mannose, **587** exhibited 240-fold greater potency, corresponding to a relative activity of 15 per sugar moiety, thus demonstrating the synergistic benefit provided by the multivalent dendritic array of sugar groups.



SCHEME 63. Synthesis of bifunctional glycodendrons with two distinct moieties containing targeting and detection probes.[254]

d. AB_2 and AB_3 Subunit-containing Glycodendrimers.— *(i) Aromatic AB_2 Systems*. As mentioned earlier, aromatic AB_2 building blocks constitute privileged components for rapid design of highly branched glycodendrimers possessing biological potential. As well as examples presented earlier, the approach has involved the design and synthesis of G(1) and G(2) glycodendrimers containing building blocks of 3,5-dihydroxybenzoic acid.[427] Homo- and heterobifunctional glycodendrimers ending with up to 16 fucoside and/or galactoside residues, installed via the ''click'' 1,3-dipolar cycloaddition, were synthesized via an ''outside in strategy,''

from a diazide derivative **588** of methyl 3,5-dihydroxybenzoate (Fig. 72) and alkyne-functionalized carbohydrates. The saccharides were selected based on the fact that the bacterium *P. aeruginosa* (responsible for chronic lung colonization and the major cause of morbidity and mortality in CF patients), expresses two lectins, PA-IL (LecA) and PA-IIL (LecB) specific for D-galactose and L-fucose residues, respectively. Polyazido dendritic scaffolds **589–593**, containing up to 16 peripheral azido functions were synthesized by successive formation of amide linkages between dendritic subunits. The polyazides were then totally functionalized with L-fucoside units to give **594–597** (Fig. 73) while the heterobifunctionalized dendrimer **598** bearing both D-galactoside and L-fucoside residues was also prepared, providing binding access to either PA-IL and PA-IIL, simultaneously. The sugar heterogeneity was incorporated in order to increase the biological activities of the dendritic architecture and afford potent new antiadhesin agents against *P. aeruginosa*.

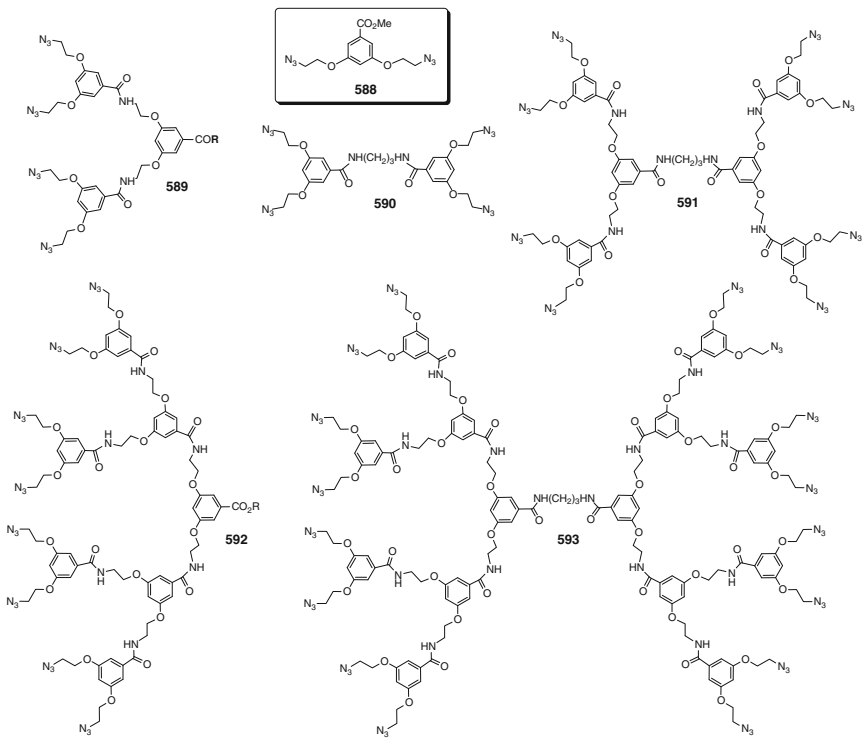


FIG. 72. AB_2-based polyazides used in ''click chemistry'' with propargylated β-D-galactosides and α-L-fucosides.[427]

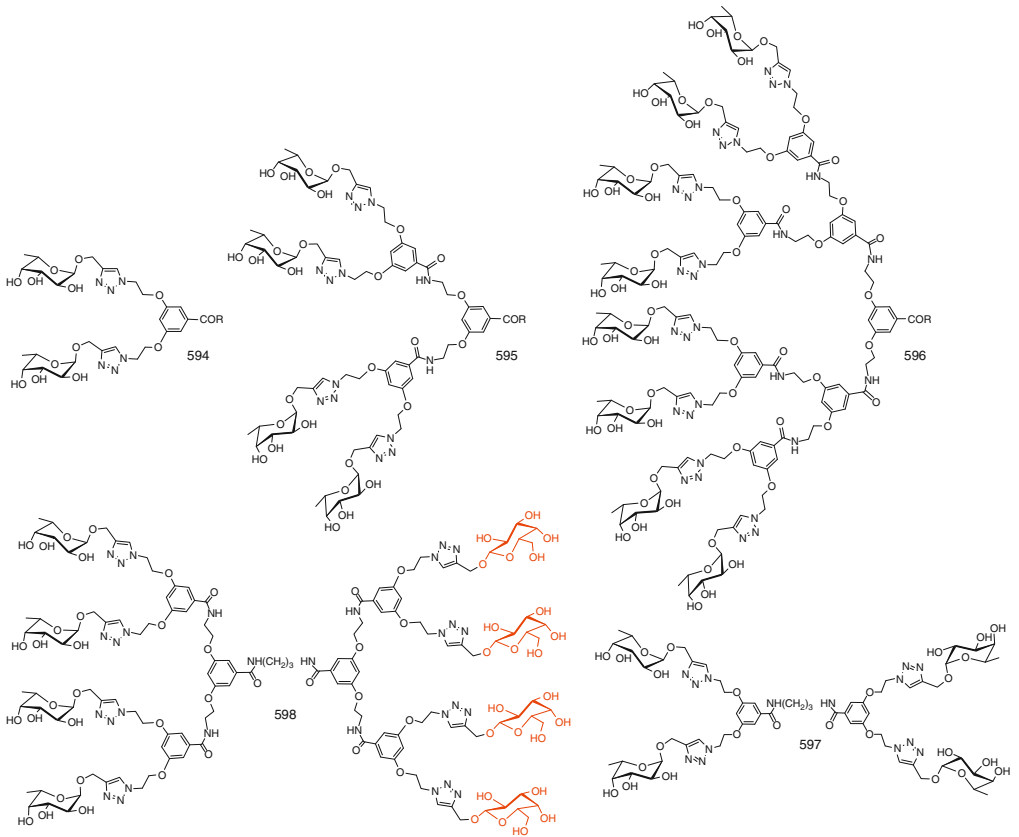
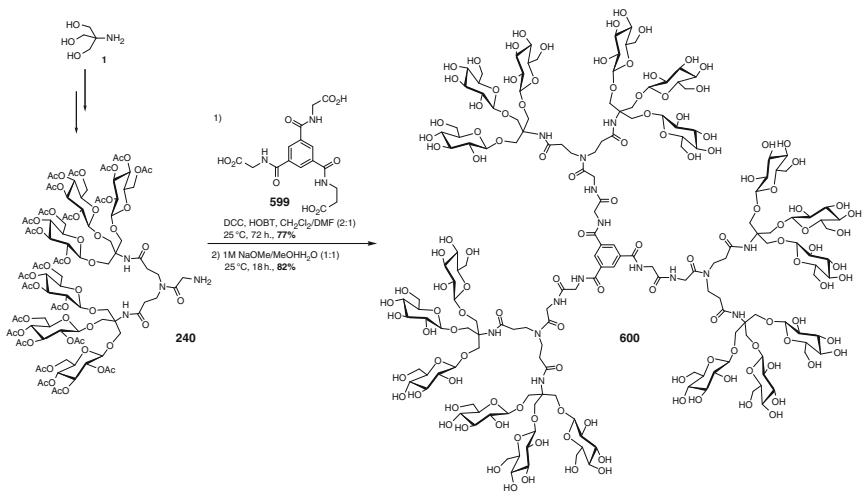


FIG. 73. Galactosylated and/or fucosylated *P. aeruginosa* lectin ligands built on an AB_2 scaffold.[427]

Turbidimetric assays indicated that glycodendrimers possessing a minimum of four fucoside residues on the same side showed rapid cross-linking abilities with tetrameric *P. aeruginosa* PA-IIL lectin, by forming insoluble complexes. As expected, heterodendrimer **598** containing 4 fucosides and 4 galactosides had the ability to recognize both binding-site domains of PA-IL and PA-IIL.

(ii) AB_3 Systems. One of the first examples of glycodendrimers based on AB_3 building blocks was proposed by Stoddart *et al.* who designed dense multivalent neoglycoconjugates in which the carbohydrates were located at the periphery of short peptidic chains emanating from an aromatic central core and TRIS-containing branching system.[229] The convergent approach was adopted, with preliminary construction of dendrons based on initial glycosylation of the three hydroxylmethyl groups of TRIS (**1**) with glucose (Scheme 64). The availability of the single free amino group in **1** following deprotection enabled further elaboration through formation of amide bond. This could be accomplished directly with triacid **599** or, preferably, to avoid steric problems through the intermediacy of the 3,3′-iminodipropanoic acid-derived dendron **240**. The glycine-derived glucosylated dendron **240** was thus connected with trifunctional scaffold **599** to afford glycodendrimer **600** decorated with up to 18 peripheral glucosides in a single step.

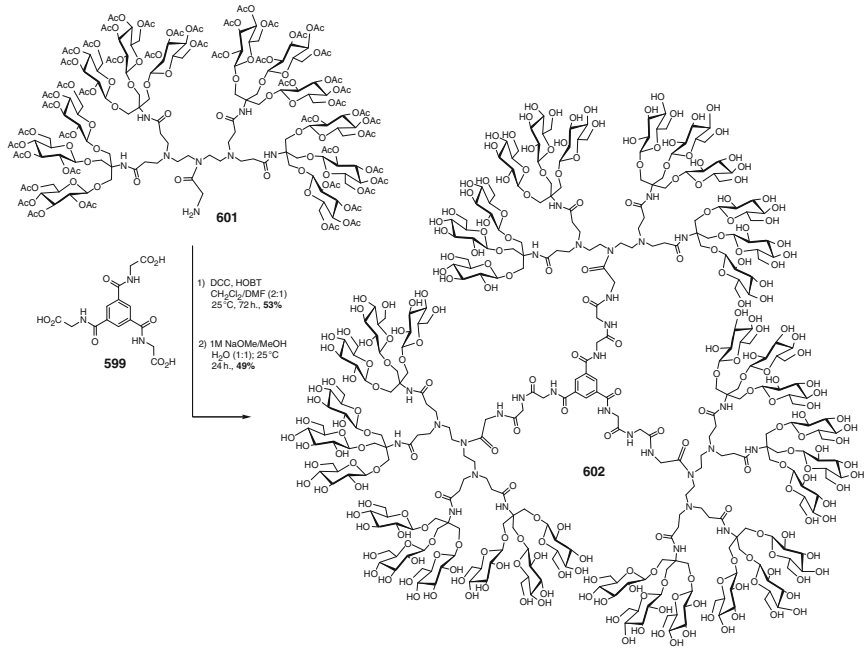


SCHEME 64. Convergent strategy for the construction of glucosylated dendrimers using TRIS as the branching component.[229]

The authors then prepared even larger dendritic derivatives employing two different, yet closely related, convergent accelerated strategies.[304] The resulting D-glycopyranoside-containing glycodendrimers (**602**), bearing up to 36 saccharide residues, were obtained in good yields by a so-called 12×3 reaction sequence, involving three equivalents of dendritic wedge **601** (12-mer) and one equivalent of the trifunctional core **599** (Scheme 65). This advanced strategy circumvented the problem of incomplete dendrimer formation arising from limited reactivity of the core and accessibility impeded by the decreased interstitial space around it. High-generation glycodendrimers were thus obtained with very precise molecular sizes and shapes, and were completly monodisperse, despite the densely packed surface groups.

Furthermore, to avoid the anticipated limitation upon the growth of this type of dendrimer, common when saccharides contain protecting groups, Jayaraman and Stoddart focused on synthesis of glycodendrimers wherein the saccharides were totally unprotected during construction.[428]

e. Carbosilane Glycodendrimers.—In 1999, Matsuoka *et al.* synthesized the trisaccharide moieties present in globotriaosylceramide by using carbosilane-based



SCHEME 65. Advanced convergent strategy used by Ashton *et al.*[304]

dendrimers.[429] As well as an efficient methodology for constructing multivalent carbosilane scaffolds, the authors simultaneously developed a general synthetic strategy for the attachment of the carbohydrate moieties, employing an efficient SN2 substitution of an alkyl halide by the highly reactive thiolate anion. In addition to typical advantages encountered for dendrimers, multivalent carbosilane architectures offered (*i*) synthetic simplicity to extend the generation at will and thus provide access to derivatives of definite molecular weight and number of terminal functions, (*ii*) the neutral nature of dendritic scaffolds having chemical and biochemical stabilty, and (*iii*) biological inertness. These dendrimers (**603**–**606**), displaying unique shapes (fan-shape, ball-shape, and dumbbell-shape, respectively) were called ''SUPER TWIGs'' and are presented in Fig. 74.

The same group studied these ''SUPER TWIGs'' as therapeutic agents for treatment of infections by Stx-producing *E. coli* O157:H7, and demonstrated their inhibition potential.[430] SPR studies showed that dendrimers **604** and **606** presented very low K_D values of 4.2 and 1.4 μM, respectively, for STL-I, while the value for **603** was 30 times higher. This indicated that ''SUPER TWIGs'' **604** and **606** bound directly with high affinity to the STL-I B-subunit. In addition, six trisaccharides situated at an appropriate distance (span of $\sim$30 Å from the central silicon core) in one molecule allowed full embrace of the predicted Gb_3-binding sites (CBPs 1 and 2 notably) and were sufficient for high-affinity binding. Furthermore, *in vivo* inhibitory effects of the ''SUPER TWIGs'' on the lethality of intravenously administred SLT-II in mice, indicated that **604** was a better candidate than **606** for this specific application. SLT-II was used in this study because it is more toxic than SLT-I both *in vitro* and *in vivo*, and clinically more significant. The assays indicated that **606** completely supressed the lethal effect of STL-II when administrated along with the toxin: **604**-treated mice survived more than 2 months without any pathological symptoms,

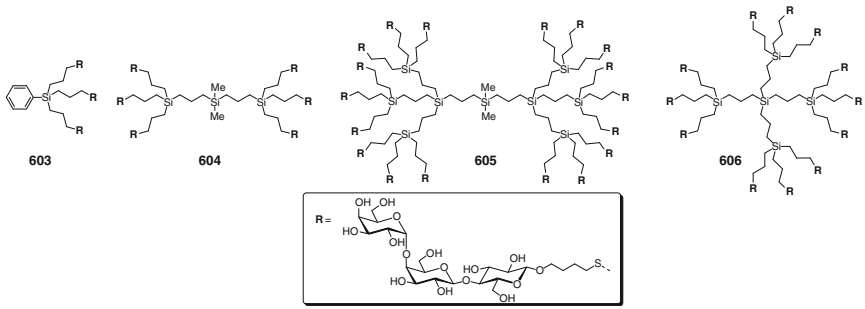


FIG. 74. Carbosilane glycodendrimers prepared by Matsuoka *et al.*[429]

whereas 100% of nontreated mice died within 5 days. The biological results suggested that **604** suppressed the lethality of SLT-II by diminishing the deposition of SLT-II in the brain and consequent fatal damage. Also, **604** protected mice from a challenge with a fatal dose of *E. coli* O157:H7, even when administrated after establishment of the infection. In the light of these results, a unique dual mechanism of action was proposed to explain this spectacular efficiency: (i) **604** bound to SLTs with high affinity and inhibited its Gb_3-dependent incorporation into target cells; (ii) it induced active uptake and subsequent degradation of SLTs by macrophages present in the reticuloendothelium.

A few years later, the same group identified the optimal dendritic structure, namely optimal valency and shape, required for SLTs neutralization.[431] They identified the 18-mer **605** as another potent SLTs neutralizer, functioning *in vivo*. Examination of additional structural features allowed the identification of crucial structural parameters. A dumbbell-shaped structure in which two clusters of trisaccharides, symmetrically located through their hydrophobic core and having an optimal length of at least 11 Å was first required. This indicated that grouping of the trisaccharides was more important than the number of trisaccharides. In the dumbbell shape, at least six trisaccharides needed to be involved for *in vivo* activity, and terminal trisaccharides with spacers had to be branched from the same terminal silicon atom to be clustered in high density. These structural requirements were essential for the appropriate induction of macrophage-dependent incorporation and degradation of SLT-II, further supporting the pivotal role for this mechanism in the *in vivo* SLTs-neutralizing activity of ''SUPER TWIGs'' having optimal structure. Interestingly, the data demonstrated the crucial implication of the CBP 3 present on the SLT-II B-subunit, and showed that it was an essential and sufficient site for high-affinity binding of **605**. In addition, the core length was a major structural parameter for perfectly embracing the three sites in a multiple way and, consequently, to provide an adequate hydrophobic volume for recognition by macrophages.

Considering the fact that galabiose is also known to bind SLTs with high affinity, Matsuoka *et al*. considered that enhancement of the binding ability by the clustering of galabiose would be advantageous as compared to clustering of globotriaose, both in terms of synthetic accessibility, and from the viewpoint of practical use for SLTs neutralization.

According to the synthetic strategy just described, a series of water-soluble carbosilane dendrimers were made, bearing peripheral galabiose trimers interspaced by 29 Å, adjusted to the binding-site distances (Fig. 75).[432] The binding affinity to SLT-I and SLT-II B subunits were determined by Scatchard plot analysis. Hexavalent compound **610** showed K_D values of 1.3 and 1.6 μM for SLT-I and SLT-II,

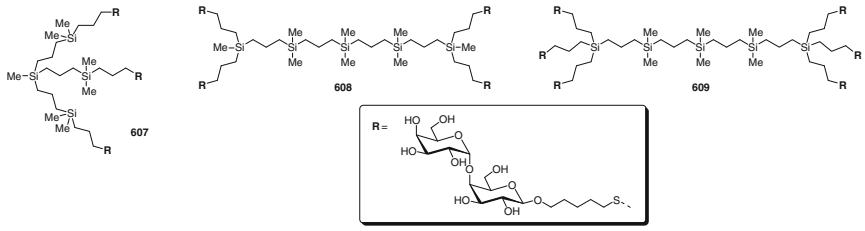


FIG. 75. Carbosilane glycodendrimers with pendant galabiose disaccharides.[432]

respectively, constituting one-tenth of the potency of the homologous **604**. Tri- and tetravalent analogues **607** and **608**, respectively, also presented weaker affinities (~60 and 10 μM, respectively, for both SLTs). On the other hand, further evaluations with ^{125}I-labeled SLTs binding and cytotoxic assays showed that multivalent **607–609** inhibited the binding of ^{125}I-labeled SLTs to vero cells, and presented very weak inhibitory effects in the cytotoxic assay as compared to the best dumbbell **604.**

In summary, results of *in vivo* experiments showed the effectiveness of carbosilane dendrimers having clustered $\mathbf{P^k}$ carbohydrate moieties, and the complete neutralization potency against STL-II was discovered when dumbbell-shaped dendrimers were identified as potent candidate inibitors. Although the precise mechanism of action remains to be elucidated, this type of inhibitor provided a new strategy for the detoxification of SLTs present in circulation.

The same research group further reported synthesis of αsialyl- (2→3)-lactosyl moieties [αNeu5Ac-(2→3)-βGal-(1→4)-βGlc-(1→] using a series of carbosilane dendrimer scaffolds and the products had interesting biological activities against various such human influenza virus strains as A/PR/8/34 (H1N1) and A/Aich/2/68 (H3N2). These dendrimers were thus uniformly functionalized with αsialyl-(2→3)-lactose residues, with different degrees of freedom of the sugar moities depending on the spacer length and core shape, and thus being responsible for a variety of 3D structures (Fig. 76). The inhibitory activity against the hemagglutination of influenza viruses to erythrocytes suggested that dumbbell amide **610**, having the longest spacer-arms and most carbohydrate epitopes, possessed the highest activity of the glycolibrary (IC_{50}s < 10 μM for both H1N1 and H3N2 viruses).[433]

Following this initiative, other complex glycosylylated architectures have been designed, finishing a series of large multigeneration glycodendrimers containing 27 [G(1), **615**], 81 [G(2), **616**], and 243 [G(3), **617**] terminal-modified D-xylose branches obtained by ''click chemistry'' from the corresponding polyalkynylated dendritic

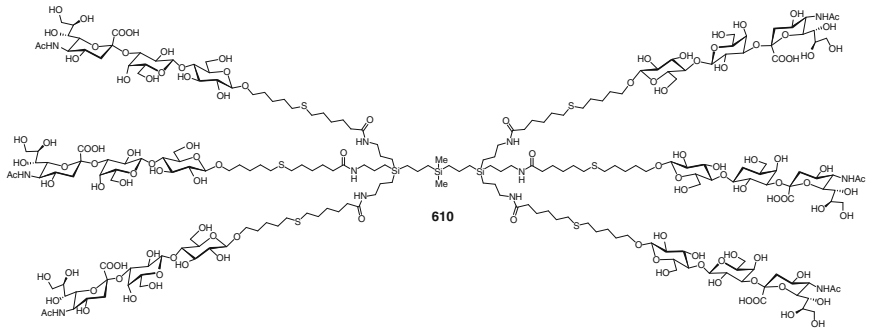


FIG. 76. Sialyloligosaccharide-capped carbosilane dendrimers successfully used as ligands against various strains of influenza virus hemagglutinins.[433]

cores (**612–614**) and 2-azidoethyl 2,3,4-tri-*O*-acetyl-*β*-D-xylopyranoside (**611**) (Fig. 77).[434] Characterization by ^{1}H- and ^{13}C NMR, elemental analysis, and IR spectroscopy confirmed their low polydispersity (1.04–1.05). The growth in size of the dendrimer from G(1)-27 to G(3)-243 and also upon functionalization within each generation, was shown by diffusion light scattering (DSL), DOSY NMR, and size exclusion chromatography (SEC), all of which satisfactorilly agreed with the expected size of the nanostructures.

f. Other Glycodendrimers.—Several other types of glycodendrimers have been synthesized from a wide variety of multivalent scaffolds.[435] Three of the most relevant exampes as described in the following sections.

Another interesting example illustrates the use of *scyllo*-inositol, the all-equatorial stereoisomer of *myo*-inositol. It has also been added to the growing selection of suitable scaffolds for synthesis of multivalent neoglycoconjugates.[436] The *N*-Boc-protected amino *scyllo*-inositol scaffold **618** constituted the key intermediate for subsequent glycosylation, as well as for chain elongation and multiplication directed toward the next dendrimeric generation (Scheme 66). Hence, conjugate 1,4-addition between triamine **618** and excess methyl acrylate, followed by amidation of the resulting esters with ethylenediamine afforded an amidoamine dendrimer under conditions previously seen for the synthesis of PAMAM dendrimers. Reiteration of this two-step sequence successfully doubled peripheral amino functionalities toward a G(2)-dendrimer **620**. Glycoconjugation of the dendrimers was then achieved by the thiourea linkages discussed previously based on mannosyl isothiocyanate **619**.

Another, architecture as yet exotic, has been proposed by Sakamoto and Müllen, who designed original glycodendrimers based on shape-persistent polyphenylenes building

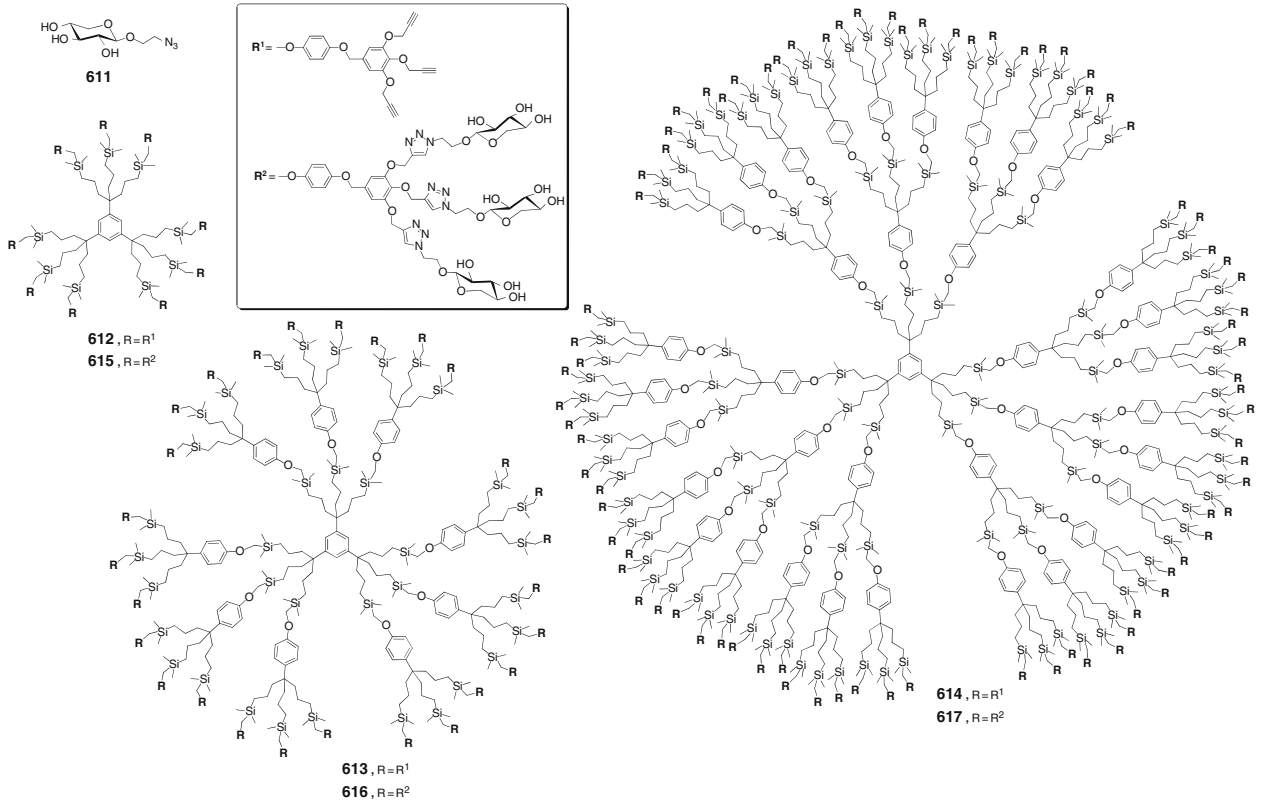
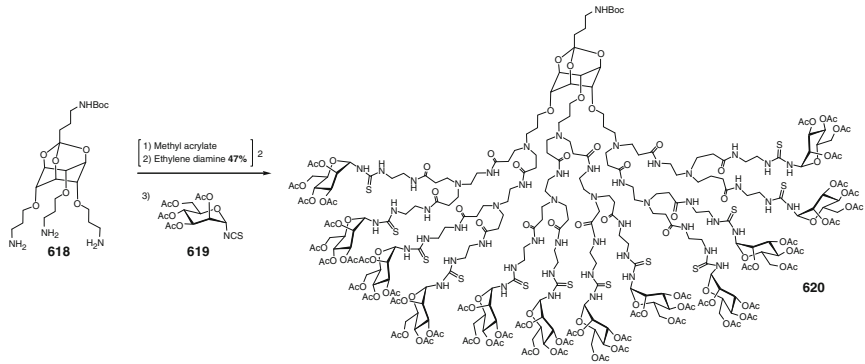


FIG. 77. D-Xylose-carbosilane dendrimers.[434]



SCHEME 66. Synthesis of *scyllo*-inositol repeating unit toward the construction of novel mannosylated dendrimers.[436]

blocks (**621** and **622**, Fig. 78).[437] Both convergent and divergent routes were used to allow for sugar installation on not only the dendrimer surface but also within the hydrophobic internal scaffold, employing successive Schmidt glycosylation and the Diels–Alder reaction. All of the described glycodendrimers exhibited good solubility in weakly acidic aqueous solutions, and those possessing interior sugar moieties (reminescent of an active center situated inside a hydrophobic pocket of natural enzymes) were identified as interesting candidates for host–guest molecular recognition.

Finally, the group of Majoral prepared additional phosphorus-based glycodendrimers involving hydrazone groups, derived from D-xylose and containing up to 48 peripheral epitopes, in excellent yields and through very simple precipitation-based purification processes (Fig. 79).[438] Such multivalent derivatives were obtained via substitution of the chloride atoms of terminal $-P(S)Cl_2$ groups with a triacetylated phenolic derivative **623** of D-xylose under basic conditions [Cs_2CO_3 (4 eq./Cl atom)]. Consequently, G(1) to G(3) glycodendrimers, containing 12, 24, and 48 xyloside residues, respectively, were readily purified through filtration and extraction steps, and their uniformity was confirmed, notably by ^{31}P NMR spectroscopy. Controlled Zemplén O-deacetylation allowed full deprotection to furnish such valuable amphiphilic glycodendrimers as **624** without decomposition.

VI. CONCLUSION

This chapter has highlighted different strategies leading to a vast range of neoglycoconjugate architectures.[439,440] Since their discovery by Roy *et al*. in 1993, the field

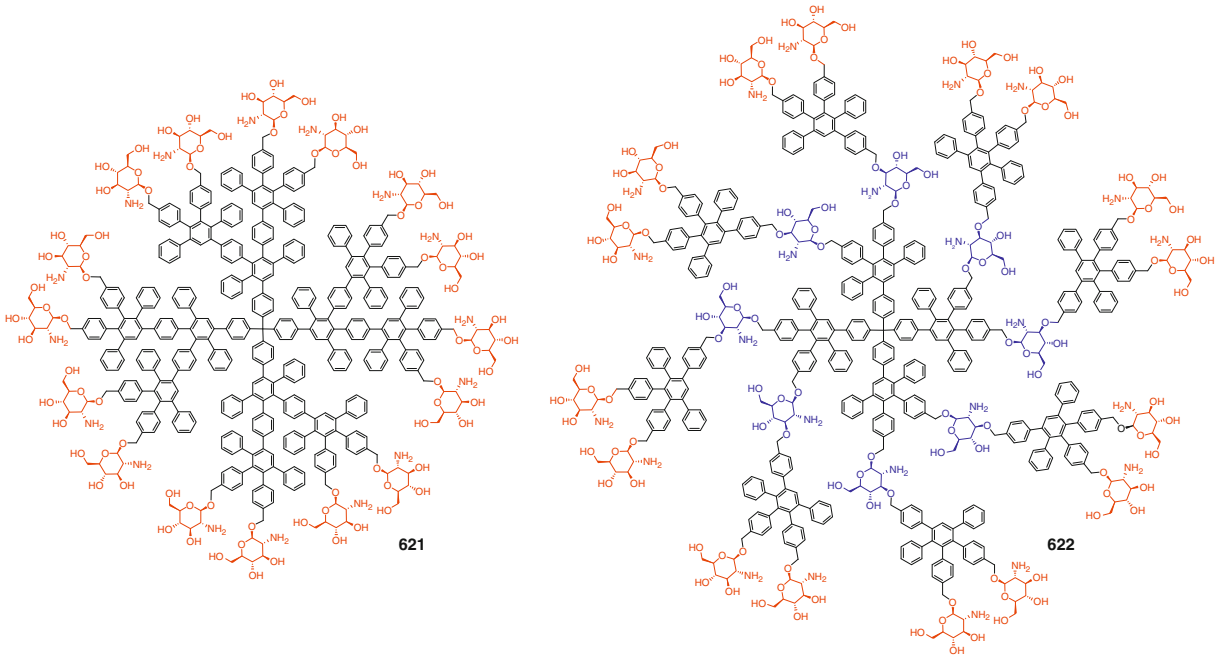


FIG. 78. Polyphenylenes building blocks used by Sakamoto and Müllen for glucosamine dendrimers.[437]

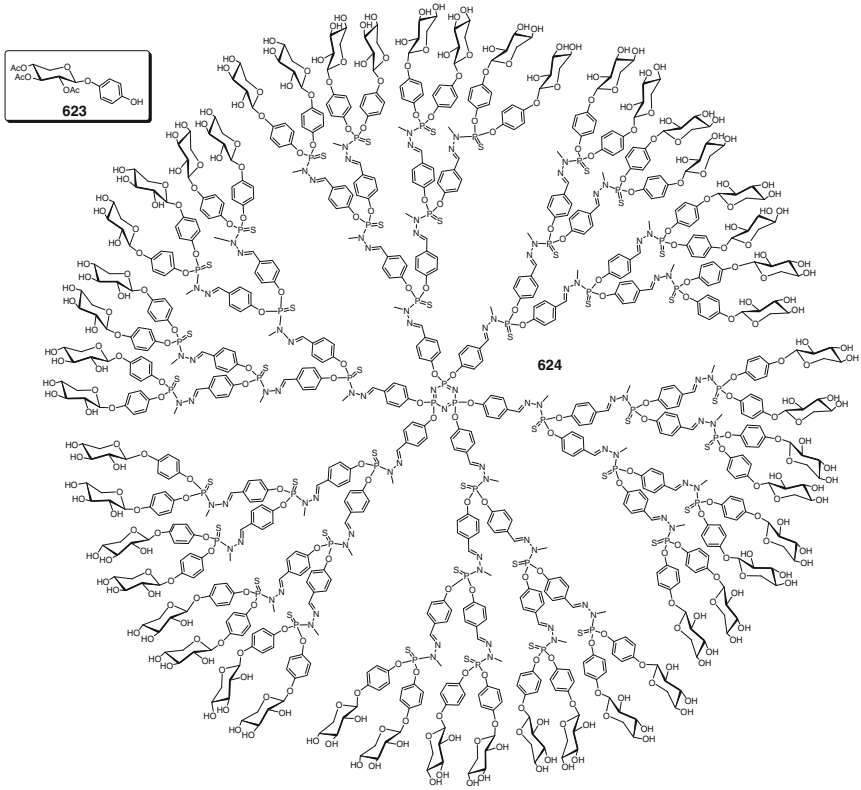


FIG. 79. ''Majoral-type'' glycodendrimers bearing peripheral D-xylose residues.[438]

of glycodendrimers has matured and expanded to unpredicted levels. The synthetic strategies leading to these nanostructures have also witnessed considerable progress, that including organometallic chemistry, chemoenzymatic build-up, silicon chemistry, and self-assembly. Most of the initial challenges in complete substitution by the carbohydrate, and structure determination, have been resolved. Undoubtedly, a fundamental contribution toward this goal has been the utilization of classical [1,3]-dipolar cycloaddition, using the soft and efficient copper-catalyzed, coupling reaction of azide to alkyne. This method, extensively used in this field, has proven extremely versatile. An additional enhancement has been ''accelerated convergent strategies,'' offering clear synthetic advantages and ease of purification. An important addition to the arsenal of sophisticated glycodendrimer methodologies is the recognition that most complex carbohydrate epitopes can be advantageously replaced by simpler

saccharides, and most importantly by glycomimetics. In this way, by combining the multivalent scaffolding of carbohydrate ligands, together with their replacement by simpler but higher affinity mimetics, the research community has delivered very potent antiadhesin candidates against viral and bacterial infections, including HIV and Ebola viruses. Key to these discoveries and applications, glycodendrimers have permitted a better understanding and appreciation of the very complex nature of multivalent carbohydrate–protein interactions. The upcoming challenges must address the issues of fine-tuned specificity faced by numerous families of carbohydrate-binding proteins having common and conserved CRDs, such as those identified for instance in galectins and MBPs. Another challenge will be to determine the toxicities of glycodendrimers to allow development of safe therapeutic agents.

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