Calixarene and Calixresorcarene Glycosides: Their Synthesis and Biological Applications

Alessandro Dondoni* and Alberto Marra*

Dipartimento di Chimica, Laboratorio di Chimica Organica, Università di Ferrara, Via L. Borsari 46, 44100 Ferrara, Italy

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Contents

1. Introduction

1.1. Calixarenes and Calixresorcarenes as Scaffolds of Glycoclusters

It is firmly established that cell surface carbohydrate−protein interactions are at the forefront of a myriad of vital cellular events.[1] Such interactions, however, are usually weak, with K_D values of the order of mM for monosaccharides. However, when a set of multivalent saccharides is clustered together with the right structure and spatial disposition, the interaction becomes strong and specific. This augmented association is more than what would be expected on the basis of the increased carbohydrate local concentration, and it has been termed glycoside cluster effect.[2] It is quite obvious that in order to intervene efficiently on these interactions, multivalent external agents have to be employed. In this context, one can envisage the use of glycoclusters firmly anchored to a conformationally rigidified calixarene or calixresorcarene scaffold as suitable systems for protein recognition and consequently protein activity inhibition. This is especially important in those cases in which carbohydrate−protein interactions are detrimental such as viral and bacterial infections, inflammations, and cancer metastasis. The aim of this review is to present the various approaches that have been carried out for the synthesis of multivalent calixarene and calixresorcarene glycosides (calixsugars) displaying different anomeric linkages and carbohydrate-macrocycle tethers. The validation of the biological activity of suitably designed calixsugars as well as of complex molecular systems in which they were introduced will be also reported. The synthesis and biological applications of calixarene glycoconjugates represented a new way of considering

* To whom correspondence should be addressed. E-mail: adn@unife.it (A.D.); mra@unife.it (A.M.).

Alessandro Dondoni has been Professor of Organic Chemistry at the University of Ferrara from 1975 to 2008. At present he is Senior Scientist at the same University. He has been the recipient of several awards, including A. Mangini Gold Medal from the Italian Chemical Society (1996), Avogadro−Minakata Lectureship Award of the Chemical Society of Japan (1999), Ziegler−Natta Lectureship Award of the German Chemical Society (1999), Lincei National Academy Prize in Chemistry (1999), and G. Berti Gold Medal from the Italian Chemical Society (2010). His present research interests are in new synthetic methods, organocatalysis, and carbohydrate chemistry. He recently started a new program dealing with the use of free-radical thiol-ene coupling as a click ligation tool for peptide and protein modification. A large part, if not all, of his chemistry is being developed with the aim of providing new products for biochemical studies and pharmaceutical and biomedical applications.

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Alberto Marra graduated in Pharmaceutical Sciences from the University of Pisa (Italy) in 1985 and obtained his Ph.D. degree in Organic Chemistry from the Université Pierre et Marie Curie (Paris VI, France) in 1989 under the supervision of Professor P. Sinaÿ. He spent one more year in Paris at the Ecole Normale Supérieure as Associate Researcher of the French National Research Council (CNRS). He was postdoctoral fellow at the University of Zurich (Switzerland) with Professor A. Vasella (1991). In 1992, he was appointed to a lectureship in Organic Chemistry at the Faculty of Engineering of the University of Ferrara, where he joined the group of Professor A. Dondoni. In 1998, he was promoted to the position of Associate Professor at the same university. Since 2002, he has been teaching Organic Chemistry and Natural Products Chemistry at the Faculty of Sciences of the University of Ferrara. He was Visiting Professor at the Université de Picardie "Jules Verne", Amiens, France (May 2008), and the Université de Cergy-Pontoise, France (June 2010). His research interests are on the synthesis and biological properties of natural and unnatural carbohydrates, in particular calixarene-based glycoside clusters. Recent work also deals with the synthesis and properties of ionic liquids.

calixarenes in particular with respect to their traditional role as ionic and molecular receptors.[3] Thus, it can be pointed out that the invention of these compounds has substantially broadened the scope of calixarenes as auxiliaries in organic and bioorganic chemistry. The importance of calixsugars has been highlighted by Fulton and Stoddart in an excellent review partly dedicated to them and covering the literature up to 2001.[4]

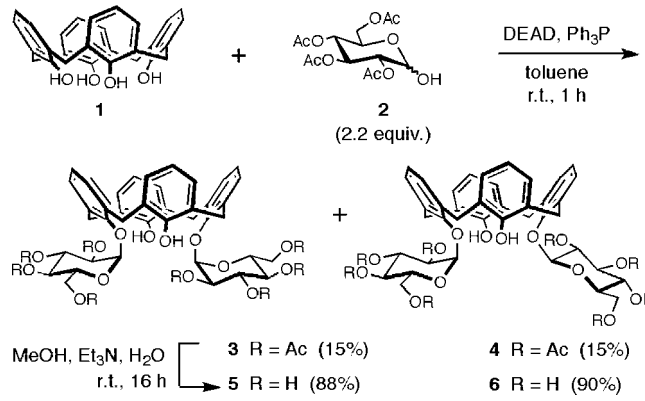
2. Calixsugar Assembly and Biological Testing

2.1. Calix[4]arene *O*-Glycosides

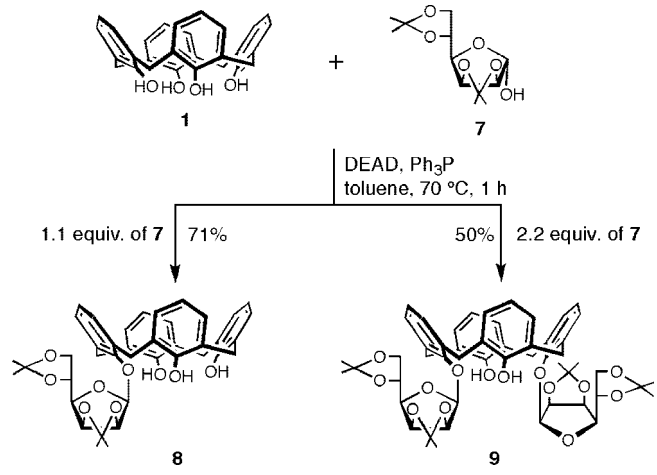
2.1.1. Assembly via Mitsunobu Reaction

While different families of calix[n]arenes are known depending on the number of phenol residues, calix[4]arenes (cyclic compounds constituted of four phenol residues connected through methylene bridges) have been employed as substrates for glycosidation because their formation can be favored by adopting suitable phenol-formaldehyde condensation conditions[5] and their conformational mobility can be controlled by placing appropriate substituents in the phenyl rings. Thus, fixed cone conformation of calix[4]arenes appeared to be the most suitable for the preparation of clusters with a well-defined arrangement around the macrocycle. The first calix[4]arene *O*-glycoside synthesis was pioneered in the mid 1990s in Dondoni laboratory at Ferrara University in collaboration with the Ungaro group at Parma University.[6] The method employed for the glycosylation of calix[4]arene **1** at the lower rim consisted of the Mitsunobu reaction with tetra-*O*-acetyl-α,β-D-glucopyranose **2** (2.2 equiv) (Scheme 1). The reaction afforded a mixture of α,α-bisglucoside **3** and α,β-bisglucoside **4** in ca. 1:1 ratio. Both

Scheme 1



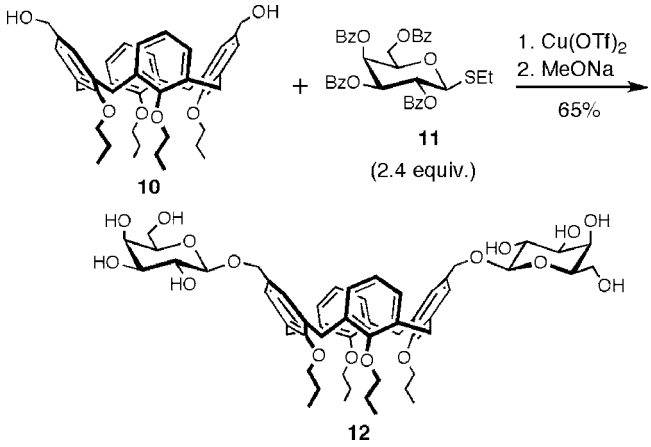
Scheme 2



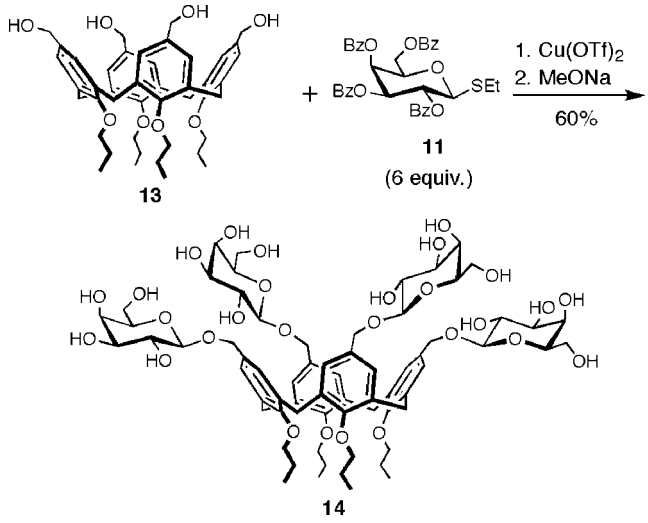
products were isolated in pure form by HPLC in modest yield (15% each). The ^{1}H and ^{13}C NMR spectra clearly indicated the assigned configuration of the anomeric carbons and the cone conformation of the macrocycle.[7] Moreover, the diametrical 1,3-functionalization in **3** was demonstrated by the ^{13}C NMR spectrum as well, whereas the same structural motif was assigned to **4** by analogy with **3**. It was also shown that upon deacetylation both compounds could be transformed into the corresponding unprotected calix[4]arene glucosides **5** and **6**. Unfortunately, these compounds showed scarce water solubility, thus preventing their use in molecular recognition processes.

In a subsequent and more extensive report,[8] Dondoni et al. described improved conditions targeting calix[4]arene *O*-glycosides whose synthesis was inefficient or failed in earlier work. Thus, mono- and bis-glycosylation at the lower rim of calix[4]arene **1** was successfully performed via Mitsunobu reaction with the configurationally stable α-D-mannofuranose diacetonide **7** to give the calix[4]arene mannosides **8** and **9** in good yields (Scheme 2). The mannosyl residues of these compounds showed a β-D-linkage in agreement with the well-known inversion of configuration of secondary alcohols via Mitsunobu reaction. Quite interestingly, both calixsugars **8** and **9** appeared to be fixed in the cone conformation as demonstrated by NMR analysis.[7] It was demonstrated that this structural arrangement was maintained in a wide range of temperature (from −80 to 160 °C). Attempted deacetonization of these glycosides was unsuccessful, and therefore unprotected compounds could not be prepared.

Scheme 3



Scheme 4



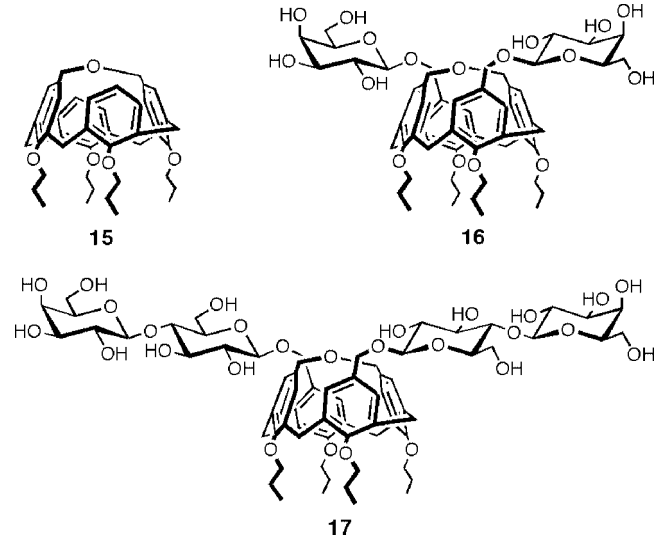
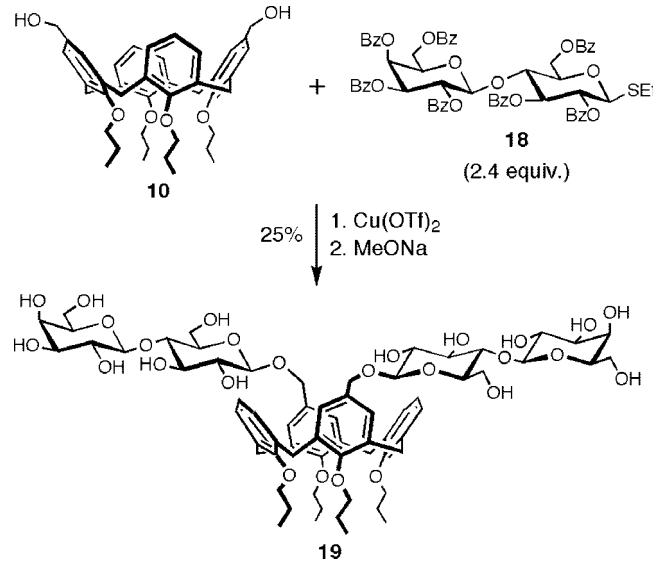


Figure 1. Ether-bridged calix[4]arene byproducts derived from the *O*-glycosylation of diol **10** or tetrol **13**.

Scheme 5



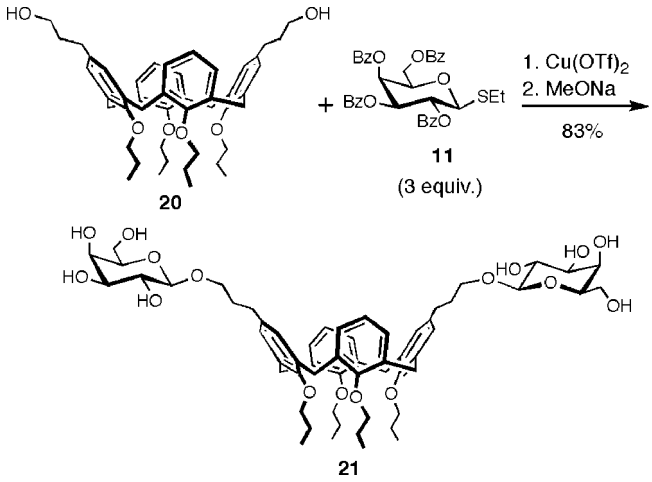
2.1.2. Assembly via O-Glycosylation

In the same joint paper,[6] Dondoni, Ungaro and co-workers reported on the efficient synthesis of the upper rim calix[4]arene bisgalactoside **12** (Scheme 3) via glycosylation of the bishydroxymethylene-substituted calix[4]arene **10** that was rigidified in the cone conformation by the four *O*-propyl groups at the lower rim.[9] Ethyl tetra-*O*-benzoyl-1-thio-β-D-galactoside **11** was used as a suitable glycosyl donor because the presence of participating benzoyl group at C2 ensured a totally β-D-stereoselective glycosylation reaction. The copper(II) triflate ($Cu(OTf)_2$) promoted coupling between **11** (2.4 equiv) and **10** occurred readily in acetonitrile at room temperature. Then, removal of the benzoyl protective groups by treatment with sodium methoxide afforded the expected β-linked bisgalactosyl calix[4]arene **12** in 65% yield. In a similar way, the β-linked tetra-*O*-galactosyl calix[4]arene **14** (60% yield) was prepared by glycosylation of calix[4]arene **13** with 6 equiv of thiosugar **11** (Scheme 4). Compound **14** represented the first tetravalent glycocluster anchored to a structurally well-defined calixarene platform. Quite interestingly, this calixarene turned out to be water soluble up to 5 mM and therefore was employed for binding studies with various organic compounds including carbohydrates and amino acids.[10] Unfortunately, no tests were carried out for galectins recognition by this compound.[11]

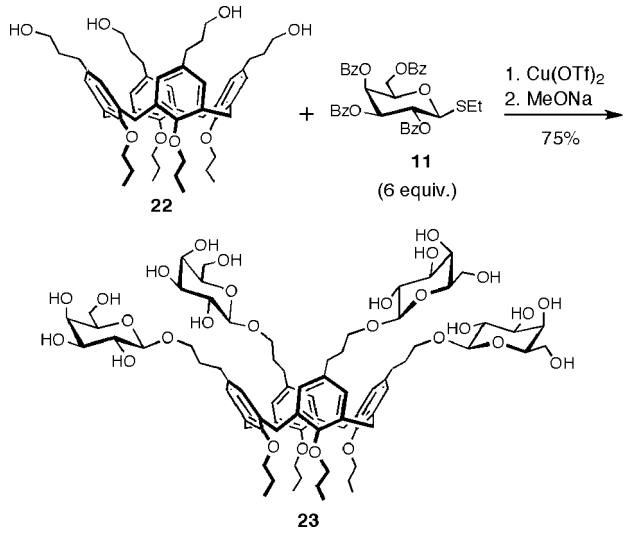
The same authors investigated[8] the reactions of calix[4]arene **13** with other thioglycosides. Side products were isolated and characterized as ether-bridged calix[4]arenes deriving from acid-catalyzed intramolecular coupling of two distal hydroxymethyl groups. The structures of these side products are shown in Figure 1. The substrate scope of the upper rim glycosidation was also broadened by performing the copper triflate promoted reaction of the bishydroxymethylene-substituted calix[4]arene **10** with thioethyl heptabenzoyl-β-D-lactoside **18** (Scheme 5). After methanolysis of the crude reaction mixture, the bis-*O*-lactosyl calix[4]arene **19** was isolated in modest yield. Unfortunately, attempts to introduce four lactosyl moieties by reacting the calix[4]arene tetrol **13** with lactoside **18** were met with failure as the only isolated product (25% yield) was the capped calixsugar **17** (see Figure 1). Evidently, the formation of the intramolecular ether bridge was favored over the introduction of two additional sugar fragments.

The effect of the alkyl chain length on the coupling of calix[4]arene polyols with Lewis acid activated glycosyl donors was investigated some years later by the same authors.[12] The aim of this study was to overcome the problem of ether-bridged side product formation (see Figure 1) and provide new structures to probe the glycoside cluster effect.

Scheme 6

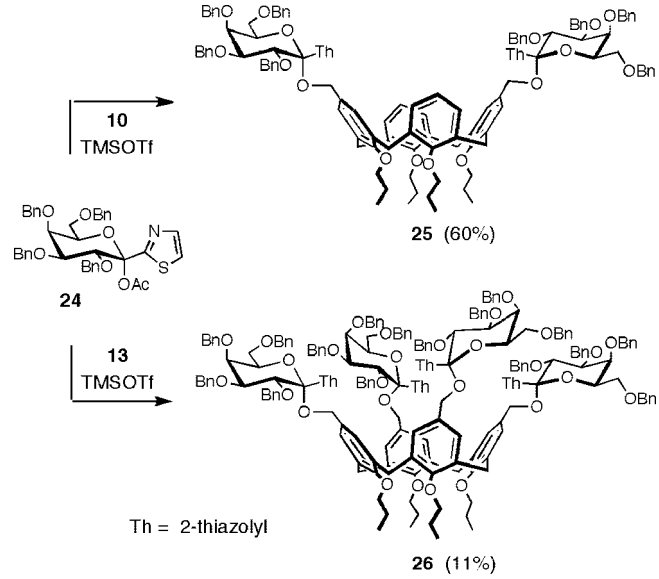


Scheme 7



It was becoming ever clearer that this still not completely understood phenomenon was very sensitive to the structural features of the carbohydrate multivalent partner.[13] Thus, it was first confirmed the difficulty to achieve an extensive glycosylation of calix[4]arene tetrol **13** bearing short hydroxymethyl arms (see Scheme 4) by using *O*-benzoyl-protected thioethyl gluco- and mannopyranoside. On the other hand, calix[4]arene diol **20** and tetrol **22** bearing long spacer arms (hydroxypropyl groups) turned out to be effectively glycosylated by *galacto*, *gluco*, and *manno* thioglycosides to give the corresponding *O*-glycosides in very good isolated yields (70−80%) and without the formation of ether-bridged side products. Examples of these reactions are shown in Schemes 6 and 7. A detailed study of the NMR spectra of the bis-glycosylated products as well as NOE experiments (in CD_3OD) indicated that the *galacto* derivative **21** adopted preferentially the open (also called far) flattened cone conformation. The same conformation was adopted by the *manno* derivative, while the *gluco* term did not show any preferred conformation. The flattened cone conformation of the *galacto* and *manno* derivatives in the gas phase was supported by calculations of molecular mechanics. It was suggested that the close conformation was indicative of the presence of intramolecular hydrogen bonds between the two sugar units and that this interaction was not disrupted by the deuterated methanol used as a solvent.

Scheme 8



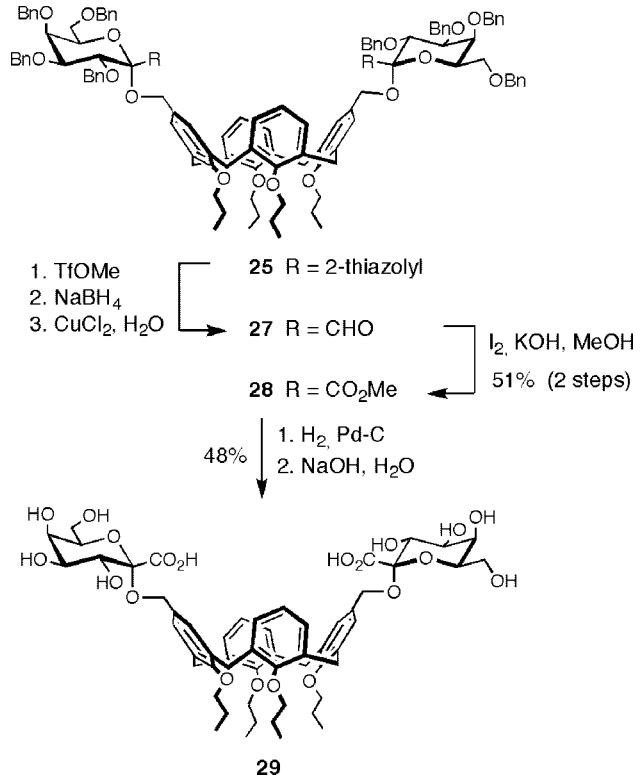
A special kind of calix[4]arene *O*-glycosides featuring ketosyl residues linked to the macrocycle platform were prepared by Dondoni and co-workers.[14] This work relied on the ready access to thiazolylketoses via thiazole-based chemistry[15] and the high reactivity of their anomeric acetates as glycosyl donors.[16] Thus, it was demonstrated that excess of thiazolyl galactoketose acetate **24** under Lewis acid catalysis (trimethylsilyl triflate, TMSOTF) gave rise to a highly stereoselective glycosylation of the model hydroxymethyl-substituted calix[4]arenes **10** and **13** (see Schemes 3 and 4) to give exclusively the α-D-linked di- and tetra-*O*-galactosides **25** and **26**, respectively (Scheme 8). The low yield (11%) of isolated tetravalent calixsugar **26** was very likely due to the increasing congestion of the system by the sequential insertion of the bulky ketosyl fragments. Accordingly, di- and triadducts were also formed. Nevertheless, the multiple glycosylation of diol and tetrol calix[4]arenes **10** and **13** by **24** confirmed the high and unexpected reactivity of thiazolylketose acetates as glycosyl donors.[16] Moreover, the exclusive formation of the α-glycosidic linkage was noteworthy. This stereochemical outcome was interpreted as due to a chairlike transition state derived from an axial attack of the hydroxyl group of the acceptor to the less hindered face of the sugar oxycarbenium ion intermediate.

The facile transformation of the thiazole ring into formyl group[17] was conveniently exploited for the conversion of **25** into the dialdehyde **27** that in turn was oxidized to the diester **28** (Scheme 9). Debenzylation of the sugar fragments and saponification of the ester group afforded the totally unprotected glycoconjugate **29**, a calix[4]arene bis-ulosonide.

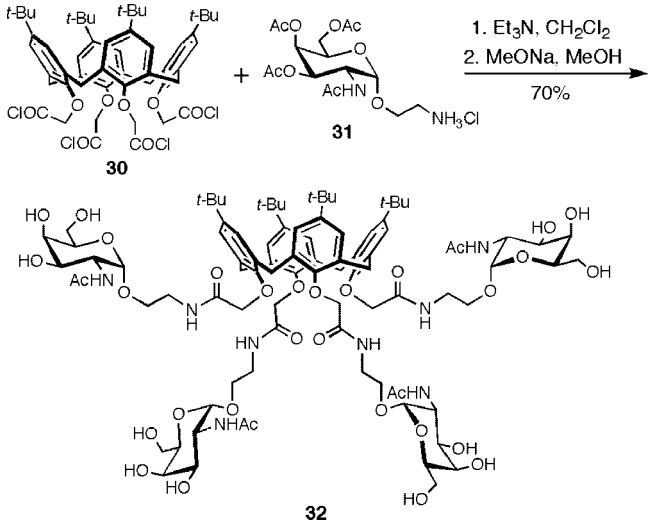
2.1.3. Assembly via Amide Bond Formation

Very soon after the Italians' work, other groups became interested in calix[4]arene *O*-glycosides. Roy and Kim identified *p-tert*-butylcalix[4]arene glycoconjugates as amphiphilic systems that assembled on a hydrophobic surface through the lipophilic *tert*-butyl groups could mimic the cell saccharide surface through the hydrophilic carbohydrate ligands.[18] Having chosen the α-linked *N*-acetyl-D-galactosamine (α-GalNAc) residue as ligand, these authors prepared the lower rim tetravalent calix[4]arene-based glycocluster **32** (Scheme 10). The synthesis involved the

Scheme 9



Scheme 10



amidation of the calix[4]arene-based tetra-acyl chloride **30** by the free amine derived from the peracetylated *O*-glycoside **31** followed by de-*O*-acetylation.

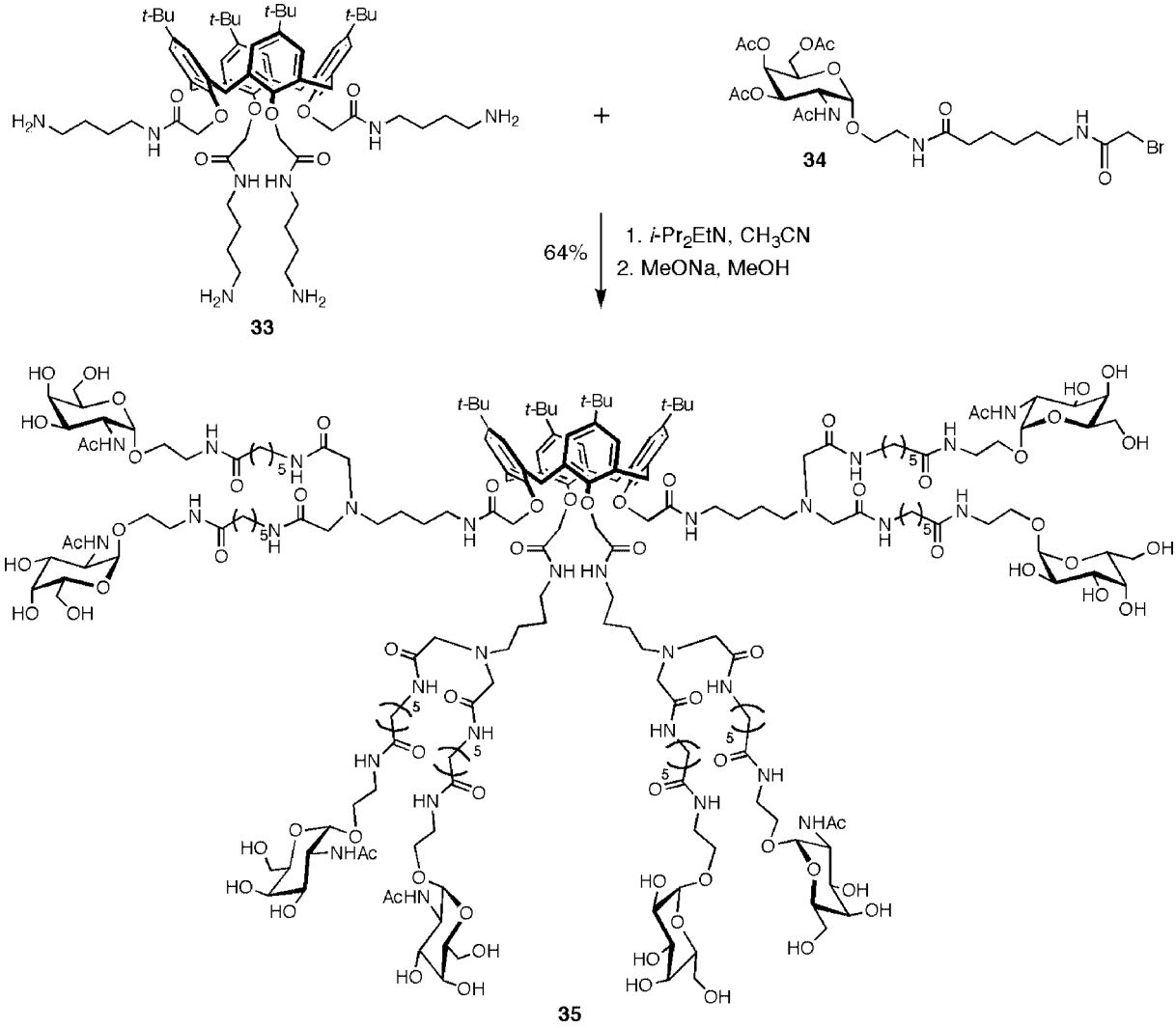
Roy and Kim prepared also the octavalent calix[4]arene-based glycocluster **35** featuring long spacer arms between the macrocyle and the GalNAc residues. In this case, the calixarene-based tetraamine **33** was allowed to react with 10 equiv of the GalNAc bearing bromide **34** in the presence of *N*,*N*-diisopropylethyl amine (DIPEA) and the reaction product was deacetylated to give the target *O*-glycoside **35** in very good yield (Scheme 11). This synthetic approach was expanded in a remarkable manner by reacting 20 equiv of **34** with a calix[4]arene-based octameric amine to give the hexadecameric glycocalix[4]arene **36**, a true dendrimer (Figure 2).

Quite interestingly, compounds **32**, **35**, and **36** were water soluble and therefore their binding properties against the plant lectin *Vicia villosa* agglutinin (VVA) were evaluated.[18] Turbidimetric analysis (rapid formation of insoluble precipitates and determination of the optical density at 490 nm at various times) revealed that all three glycoclusters exhibited direct binding abilities to VVA while the use of allyl 2-acetamido-2-deoxy-α-D-galactopyranoside (allyl α-D-GalNAc) as an inhibitor for the cross-linking of octavalent **35** with VVA demonstrated the sugar specificity of the binding. The interaction between **35** and VVA was so strong that a 250-fold molar excess of the above inhibitor was necessary to disrupt the cross-linking interaction. The efficiency of the glycoclusters to inhibit the binding of asialoglycophorin to VVA was also measured by enzyme-linked lectin assay. The most efficient inhibitor was the hexadecavalent conjugate **36** (IC_{50} = 13.4 μM) that in fact was 12-fold more potent than allyl α-D-GalNAc (IC_{50} = 158.3 μM). It was also demonstrated, as probably the most important finding, that the GalNAc containing glycoclusters **35** and **36** have the ability to form monolayer assemblies on polystyrene microtiter plates. This hydrophobic interaction was inhibited by an excess of allyl α-D-GalNAc. It was suggested that this property can be exploited for analytical purposes.

Calix[4]arene *O*-glycosides assembled through amide bonds have been the target of synthetic efforts by the Ungaro group in the mid 2000s.[19] The contribution of the amide tether to molecular recognition processes by binding to either acid or basic substrates was emphasized. Initial attempts to introduce carbohydrate fragments by coupling the tetrapropoxy calix[4]arene dicarboxylic acid **37** with 2,3,4,6-tetra-*O*-acetyl-galactosamine or glucosamine in the presence of a benzotriazole-based activating agent (*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HBTU) and a base (Et_3N) were unsuccesful. Also, the use of dichloride of the diacid **37** and a base did not produce any desired glycoconjugate. As the low reactivity of the glycosylamines appeared to be responsible for the unsuccessful coupling reactions,[20] the use of *O*-ethylamino glycosides was considered. In the event, the successful coupling took place between the known galactose-derived amine **38** with the diacid **37** in the presence HBTU and Et_3N to give, after deacetylation of the carbohydrate residues, the target upper rim calix[4]arene *O*-glycoside **39** in fair overall yield (34%) (Scheme 12). Comparable results were obtained using a glucose-derived analogue of **38**. Interesting comments were reported on the structure of these amide-linked calixsugars. In contrast to that expected on the basis of intramolecular hydrogen-bonding between the two amide groups, NMR spectra in $CDCl_3$ were consistent with an open flattened cone conformation as shown for compound **39**. The same spectra revealed extensive intermolecular aggregation that was attributed to the free hydroxy groups. The chiral carbohydrate fragments did not affect the NMR signals of the macrocycle, a result that was attributed to the distance of the two sectors.

The cholera toxin, a protein featuring five sugar-binding sites on a single face, is known to selectively bind to the cell-surface ganglioside GM1 **40** (Figure 3) through specific interactions with the terminal galactose and sialic acid residues.[21] In earlier reports, Bernardi and co-workers described the synthesis of the pseudotrisaccharide **41** (Figure 3), both in monovalent[22] and multivalent form,[23] as mimic of the oligosaccharidic portion of **40**.

Scheme 11



Aiming to mimick the external surface of the cell where the GM1 pentasaccharide units protrude from the lipophilic cell membrane, Bernardi and Casnati and their co-workers prepared the bis-glycosylated calix[4]arene **48** starting from the diacid **37** (Scheme 13).[24] First, two long chains were installed at the upper rim of **37** by amide coupling with the monoprotected diamine **42**. Then, the removal of the Boc

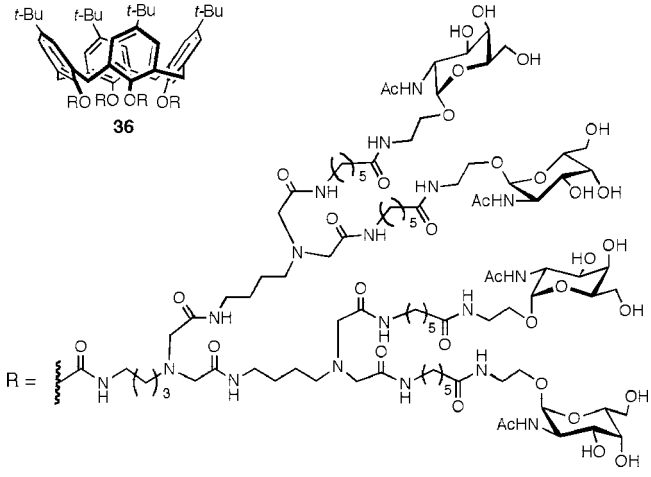
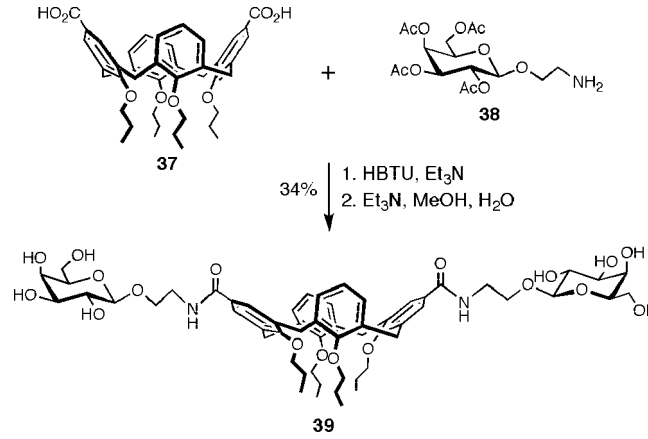


Figure 2. Hexadecaglycosyl calix[4]arene prepared by Roy and Kim.[18]

Scheme 12



group, followed by the reaction with squaric acid dimethyl ester **43**, afforded **44** in 57% overall yield. Further chain elongation using **42** and hydrolysis of the *t*-butyl carbamate function gave the calixarene diamine **45**, which was allowed to react with the monoacid **46** in the presence of HBTU and triethylamine. The amide coupling took place at a moderate extent (35%), affording, after debenzylation and transesterification of the acetyl groups, the divalent calixsugar **48** (54%). The interaction of the latter compound with the cholera toxin was studied using fluorescence titration and

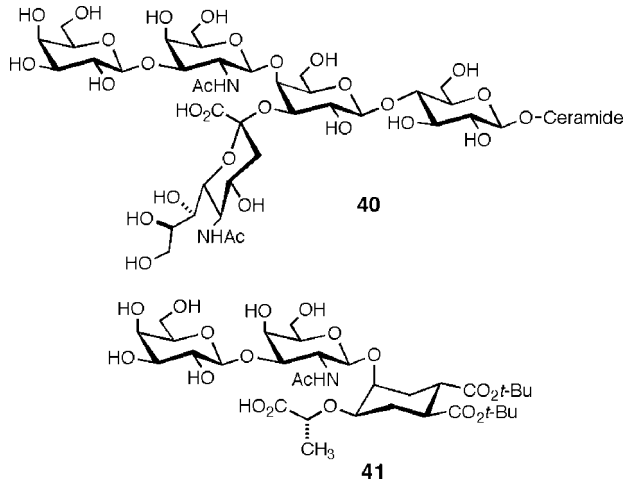


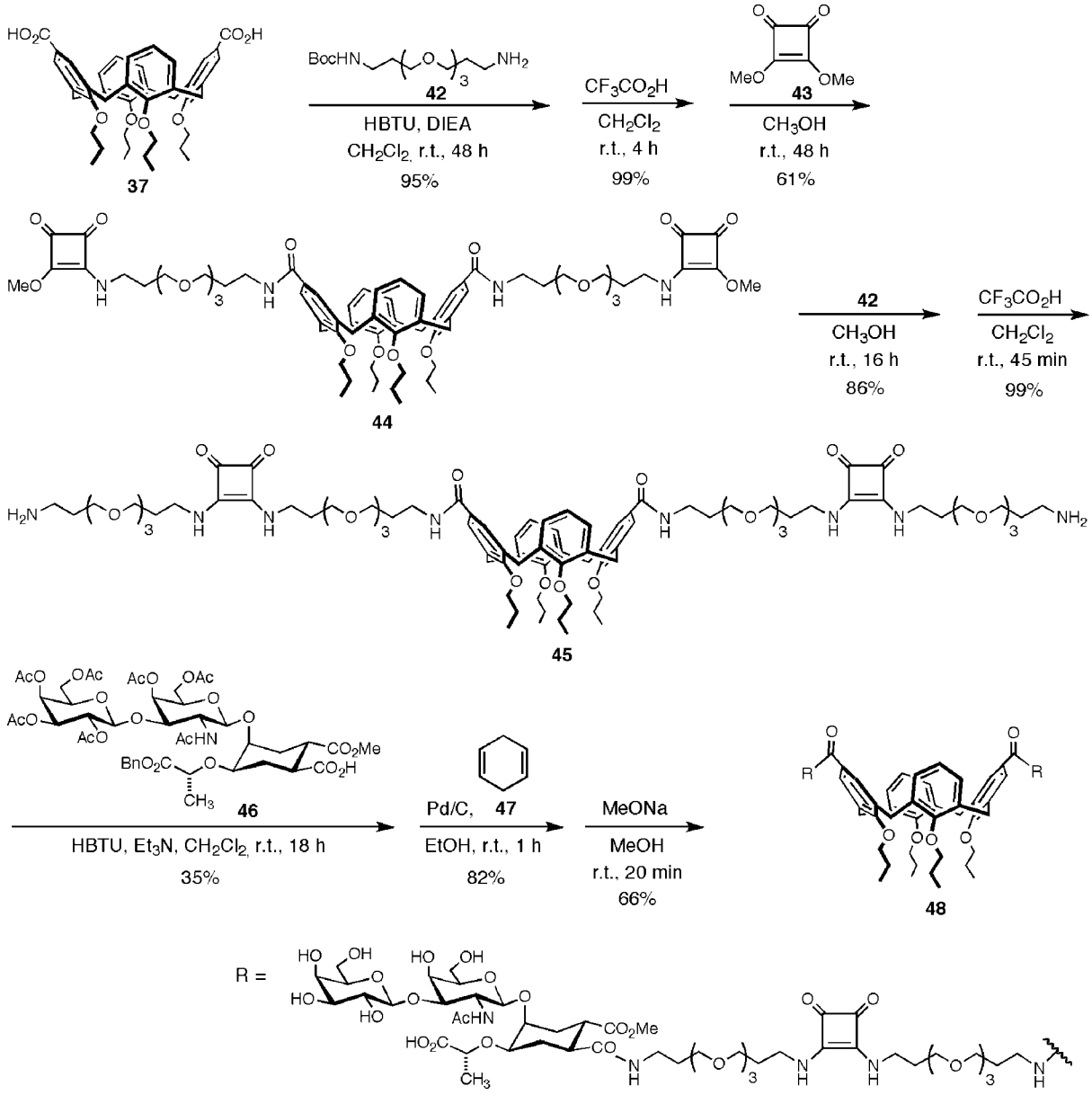
Figure 3. Ganglioside GM1 (**40**) and one of its mimics, the pseudotrisaccharide **41**.

ELISA experiments. Unfortunately, the adsorption of **48** at the GM1-coated ELISA plates occurring at high concentrations (>0.2 mM) of ligand prevented an extensive study of its inhibition properties. On the other hand, the fluorescence titration data indicated that the affinity of **48** for the cholera toxin was 2000-fold (per pseudotrisaccharide unit) higher than the affinity observed for the monovalent mimic **41**. This result was surprising because a dendrimer-based divalent analogue of **41** showed only a 9-fold (per ligand unit) affinity enhancement.[23] Therefore, it was concluded that a more detailed thermodynamic analysis based on other multivalent ligands was required to explain those controversial results.

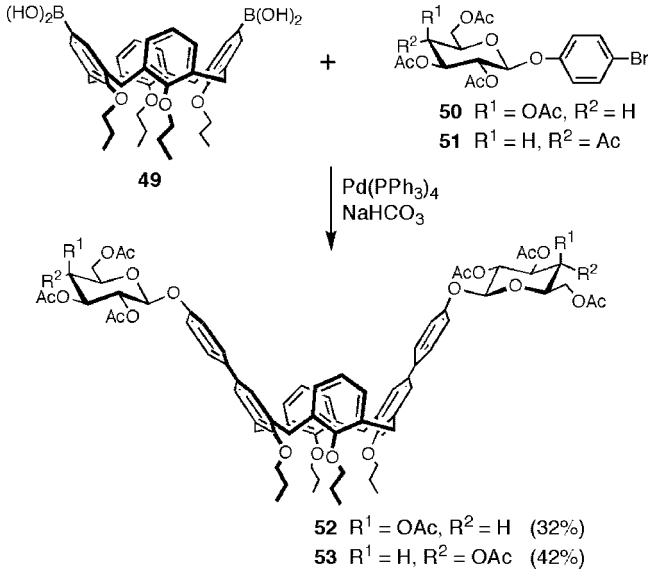
2.1.4. Assembly via Suzuki Coupling

Another interesting route to calix[4]arene *O*-glycosides was reported by Parrot-Lopez and co-workers.[25] It consisted of Suzuki type coupling in the presence of Pd(0) between a *p*-bromophenyl glycoside and the boronic acid derivative of a calix[4]arene. The work aimed to both increase the size of the macrocycle cavity for a better binding of potential guest molecules and incorporate saccharide antennae for specificity of transport and recognition properties. Thus, the $Pd(PPh_3)_4$

Scheme 13



Scheme 14

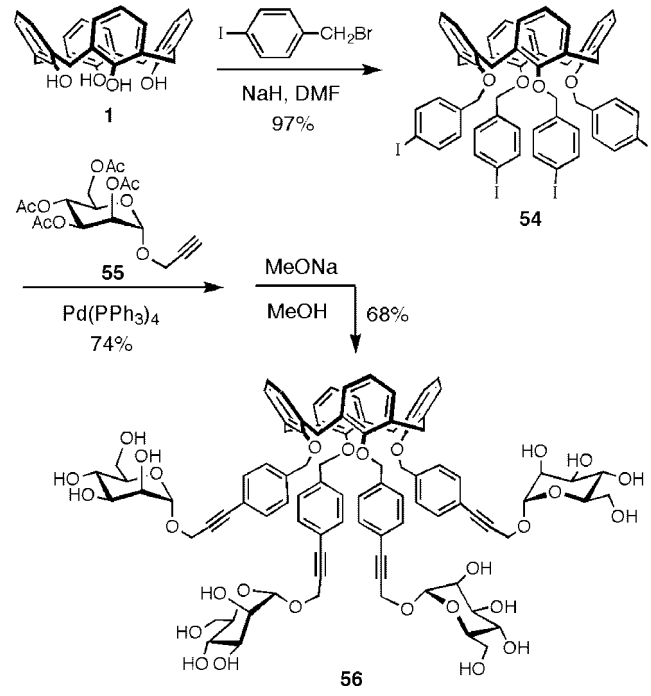


catalyzed cross-coupling of diboronic acid calix[4]arene **49** with 2 equiv of peracetylated *p*-bromophenyl galactoside **50** or glucoside **51**, afforded the corresponding upper rim bis-glycosylated calix[4]arenes **52** and **53**, respectively, in fair yields (Scheme 14). Then the acetyl protecting groups were removed using sodium methoxide. It was reported, however, that the unprotected products were difficult to be purified due to their amphiphilic character so that they were isolated in very low yields (ca. 10%). The same research group developed also a one-pot procedure, again via Suzuki coupling, leading to a calix[4]arene bearing a single phenyl *O*-maltoside fragment at the upper rim.

2.1.5. Assembly via Sonogashira Coupling

A paper was next reported by Pérez-Balderas and Santoyo-González on the use of the Pd(0)-catalyzed Sonogashira reaction for the glycosylation of a calix[4]arene at the lower rim.[26] A deeper lipophilic cavity was constructed by this approach because the first step introduced four iodophenyl rings at the lower rim by ether formation between calix[4]arene **1** and *p*-iodobenzyl bromide (Scheme 15). The functionalized calix[4]arene **54** obtained in excellent yield was then reacted with the peracetylated propargyl α-D-mannopyranoside **55** using catalytic $Pd(PPh_3)_4$. The four carbohydrate residues of this Sonogashira product were deacetylated by transesterification to give a tetravalent *O*-glycocluster grafted onto the calix[4]arene platform through phenylethynyl tethers (compound **56**). The upper rim tetra-*t*-butyl derivative analogous of **56** was efficiently prepared by the same procedure. Unfortunately, the low water solubility of the *tert*-butyl derivative prevented its use for biological testing. On the other hand, the recognition properties of water-soluble **56** toward the plant lectin Concanavalin A were determined by enzyme-linked lectin assays (ELLA)[27] using methyl α-D-mannopyranoside as a reference standard. Unfortunately, no glycoside cluster effect turned out to be operative because **56** showed lower inhibitor properties than that of the reference monosaccharide.

Scheme 15



2.1.6. Assembly via Cu(I)-Catalyzed Azide−Alkyne Cycloaddition

In a paper dealing with the construction of multivalent structures anchored to various scaffolds, Santoyo-González and co-workers prepared a calix[4]arene *O*-glycoside via thermal azide−alkyne cycloaddition.[28] Given the lack of regioselectivity of this coupling reaction, a mixture of isomers was obtained as the carbohydrate residues were linked to the macrocycle platform through 1,4- and 1,5-disubstituted triazole rings. Neither isolation nor characterization of individual regioisomers was reported.

The Cu(I)-catalyzed azide−alkyne cycloaddition-(CuAAC),[29] the quintessential click reaction,[30] leading to the regiospecific formation of 1,4-disubstituted triazoles, did not escape researcher attention as a formidable tool for calixarene decoration with biologically active molecules. Dondoni and Marra reported in 2006 on the use of this ligation tool for multiple carbohydrate anchoring to calix[4]arene scaffolds (see section 2.2.2.1). One year later (2007), a research group headed by Bew described the synthesis of two triazole-linked calix[4]arene *O*-glycosides via CuAAC[31] (Figure 4). In both cases, peracetylated carbohydrate residues were introduced at the upper rim of the macrocycle, namely two glucosyl and two lactosyl moieties, as shown in compounds **57** and **58**, respectively.[32] While no comments were made on the structure of these compounds, it is likely that they adopted an open flattened conformation as demonstrated for the bis-glycosylated calix[4]arene derivative **39** shown in Scheme 12. A tetrakis-*O*-lactosyl calix[4]arene[32] was also prepared, and this was mono- and bis-sialylated using a *trans*-sialidase. No bioassays, however, were carried out with these compounds.

A series of partially and fully *O*-galactosylated calix[4]arene clusters in cone, partial cone, and 1,3-alternate conformation was prepared by Imberty, Matthews, Vidal, and co-workers via CuAAC.[33] The di- (**59** and **60**), tri- (**61**), and tetrapropargyl (**62**) *t*-butyl-calix[4]arenes, rigidified in the cone conformation, were allowed to react with the 8-azidodiox-

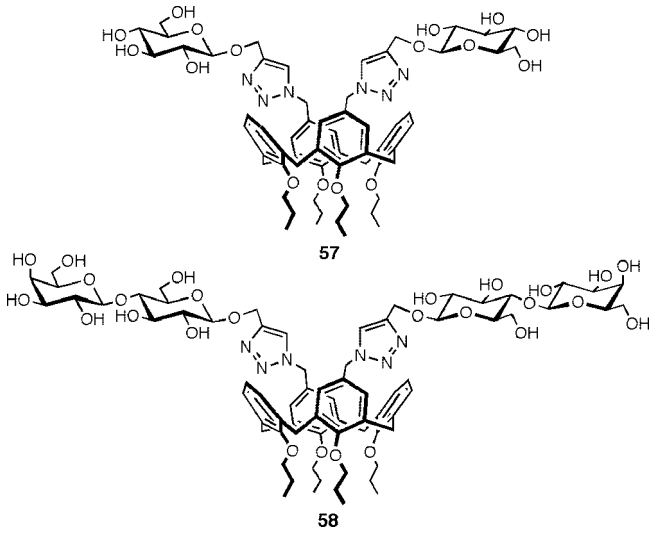
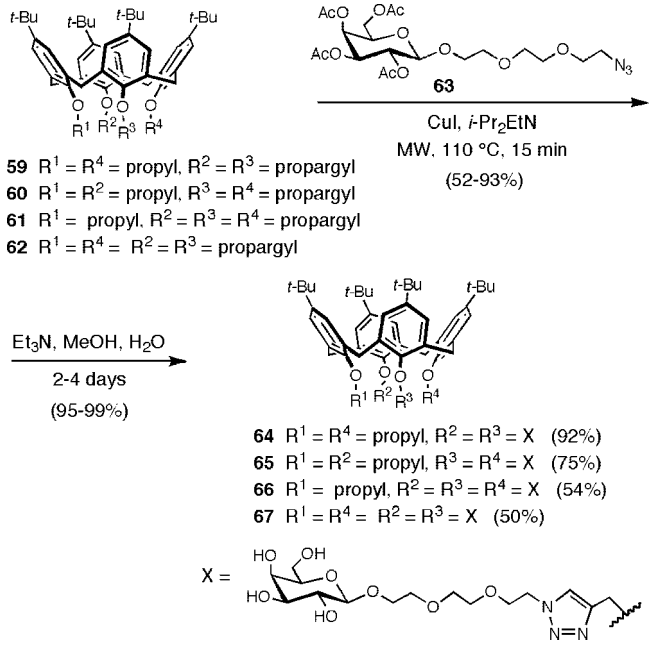


Figure 4. Bis-*O*-glycosylated calix[4]arenes prepared via Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) by Bew and co-workers.[31]

Scheme 16



aoctyl galactopyranoside[34] **63** in the presence of catalytic CuI and *N*,*N*-diisopropylethylamine (Hünig's base) under microwave irradiation to give, after deacetylation, the corresponding triazole-linked lower rim glycoclusters **64**−**67** in 50−92% overall yield (Scheme 16). The diastereomeric tetragalactosyl calixarenes displaying 1,3-alternate (**68**) or partial cone conformation (**69**) were also prepared in good yields by the same click azide−alkyne cycloaddition (Figure 5).

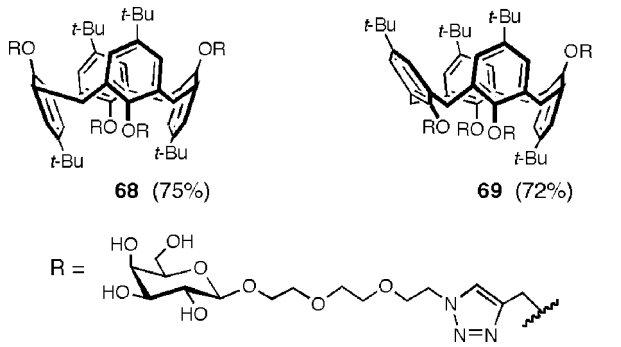


Figure 5. Tetra-*O*-glycosylated calix[4]arenes in 1,3-alternate (**68**) or partial cone conformation (**69**) prepared via CuAAC.[33]

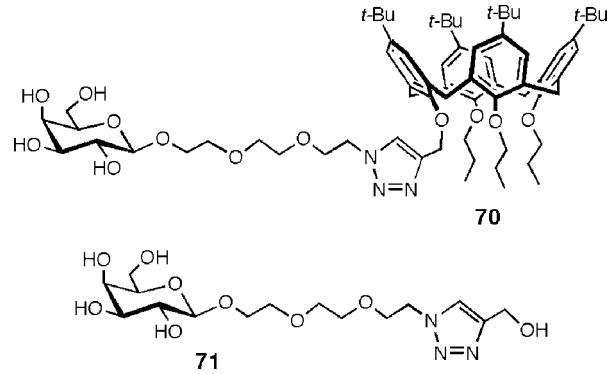


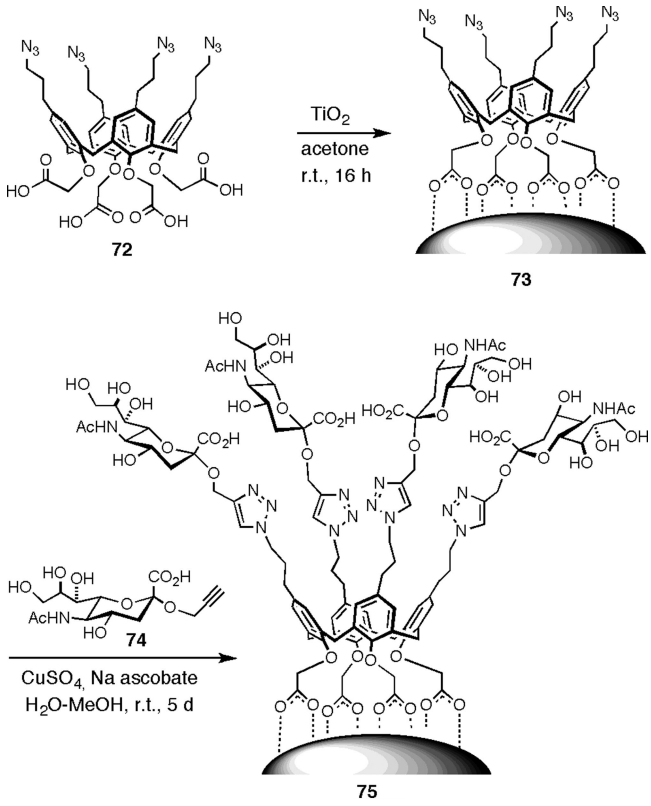
Figure 6. Monovalent ligands of *Pseudomonas aeruginosa* lectin PA-IL.

The affinity of these glycoclusters toward *Pseudomonas aeruginosa* lectin PA-IL, which selectively recognizes D-galactosides, was determined by isothermal titration calorimetry (ITC) and compared to the affinity of a monovalent ligand. Unfortunately, the monogalactosylated calixarene **70** (Figure 6) could not be used in this study because of its poor solubility in H_2O and H_2O−DMSO mixtures. Therefore, the *O*-glycoside **71** (Figure 6), although devoid of the hydrophobic macrocyclic scaffold, was used as monovalent ligand. Titration experiments showed that the bis-galactose clusters **64** and **65** did not bind to PA-IL, whereas the trivalent glycocluster **66** as well as the tetravalent ones (**67**−**69**) displayed an enhancement in binding up to 200-fold larger (per D-Gal unit) than that observed for **71**. Docking calculations allowed the authors to establish that the best ligand of the series, the tetragalactosyl cluster **68**, adopting a fixed 1,3-alternate conformation, was well suited for the topology of the rectangular shaped PA-IL tetramer. The modeling study indicated that two galactose units located on the opposite sides of **68** were linked to the adjacent binding sites on the small face of one PA-IL tetramer, whereas the other two galactose residues were available for binding to another PA-IL tetramer. It was found that the two lectin tetramers face each other without steric conflict.

Glycoclusters **68** and **69** were also efficient inhibitors of the adhesion of PA-IL to a biotinylated polygalactosylated surface onto a streptavidin-coated chip as proved by surface plasmon resonance analysis. Therefore, it was concluded that these antiadhesive molecules could find potential applications as antimicrobials.

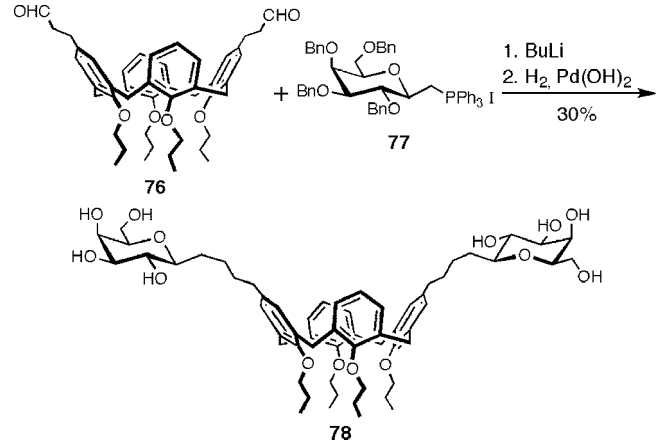
Immobilization of triazole-tethered calix[4]arene *O*-glycosides on TiO_2 nanoparticles was reported by Dondoni and Marra and their co-workers at the beginning of 2010.[35]This represented the first example of TiO_2 nanoparticle-calixsugar hybrid materials. As reported earlier by de la Fuentes and Penadés,[36] metal and metal oxide nanoparticles coated by biologically relevant oligosaccharides, named as glyconanoparticles, constitute a good biomimetic model of carbohydrate presentation at the cell surface. As glyconanoparticles present carbohydrates in a globular and polyvalent configuration on their surface, they can give rise to multivalent interactions, such as those occurring in natural systems, to compensate for the low affinity of carbohydrates for their receptors.[2b,11,37] While the direct immobilization of glycoside units on TiO_2

Scheme 17



nanoparticle surface represented a difficult task, the use of a suitable functionalized calix[4]arene as a platform was considered. Thus, given the well-known ability of carboxylate groups to form strong bonds with TiO_2 surfaces,[38] the orthogonally fuctionalized calix[4]arene **72** displaying azido and carboxyl groups at the upper and lower rim, respectively, was grafted on TiO_2 nanoparticles (Scheme 17). In this way, the calix[4]arene **72** acted as a tetrapodal ligand using the four carboxylate groups while the azido groups were preserved for the execution of click reaction with alkyne functionalized carbohydrates. In the event, treatment of the calixarene-coated TiO_2 nanoparticles **73** thus obtained with free hydroxy propargyl *O*-sialoside **74** in the presence of $CuSO_4$ and sodium ascorbate gave rise to multiple triazole forming reactions on each calix[4]arene platform anchored to TiO_2 surface. This resulted in the formation of the target glyconanoparticles **75**. The occurrence of the azide−alkyne cycloaddition was monitored by infrared analysis, while thermogravimetric analysis (TGA) allowed for evaluating the molar concentration of the glycocluster per gram of TiO_2 as well as the number of organic molecules (0.3 per nm^2) on the surface of the nanoparticles. The biological relevance of these hybrid materials is apparent when considering that the 5-*N*-acetyl-neuraminic acid (Neu5Ac) fragments that constitute the sugar cluster sector are the most common members of the large family of sialic acids that are incorporated at the terminal positions of natural glycoproteins, glycolipids, and oligosaccharides. As such, they play an essential role in biological molecular recognition processes such as cell adhesion and differentiation phenomena.[39] Hence, biological assays with the sialonanoparticles thus prepared became of great interest. Preliminary experiments from Dondoni and Marra laboratory have shown that compound **75** acted as inhibitor of the BK virus-induced hemagglutination.[40]

Scheme 18



2.2. Calix[4]arene *C*-Glycosides

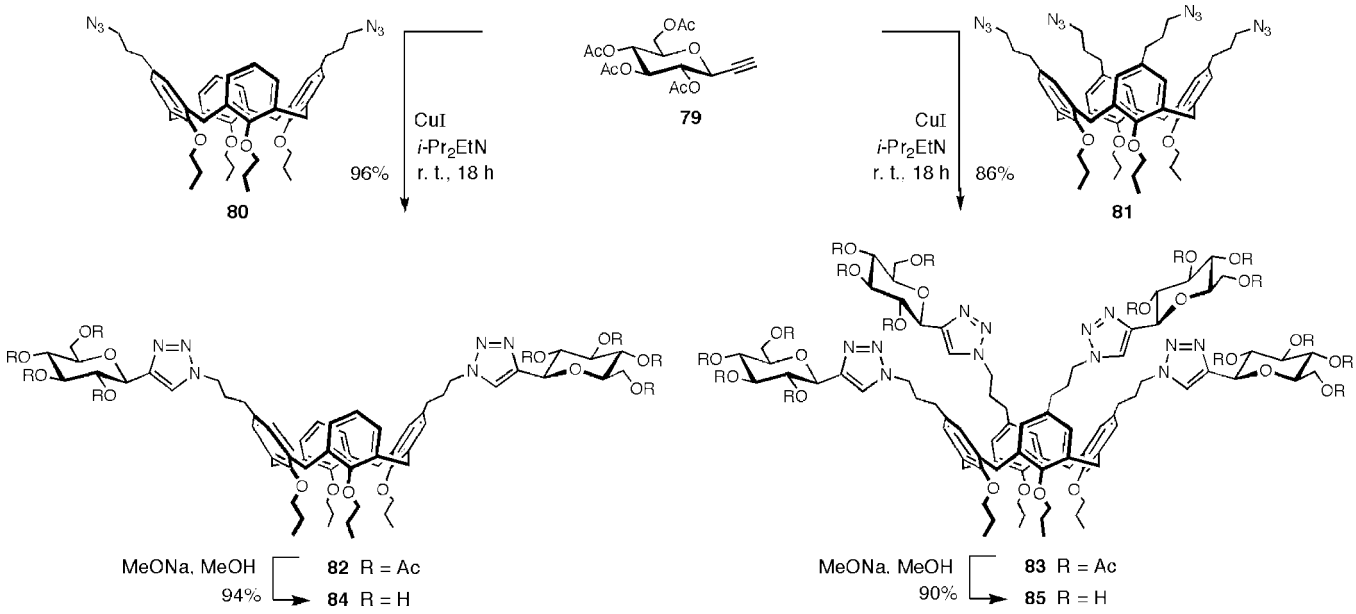
2.2.1. Assembly via Wittig Olefination

It is well-known that *O*-glycosides are intrinsically delicate compounds that are easily degraded via chemical or enzymatic hydrolysis. This is due to the labile exocyclic carbon−oxygen bond (anomeric acetal). The substitution of this bond with a more robust carbon−carbon bond to give *C*-glycosides provides higher stability.[41] This issue was addressed within the context of calix[4]arene glycosidation by Dondoni, Marra, and their co-workers since the early stage of calixsugar invention. The first approach to an all-carbon tethered calixsugar dated back to 1997 and consisted of multiple Wittig olefination of a tetraformyl-calix[4]arene with sugar phosphoranes followed by the reduction of the double bonds of the alkene-linked calix[4]arene glycoconjugate intermediates.[42] The products thus formed, however, could not be considered as *C*-glycosides because the carbohydrate fragments were linked to the alkyl chain by their nonreducing end. The first genuine calix[4]arene *C*-glycoside **78** was prepared some years later[12] via the same Wittig-based approach by using a sugar phosphorane generated from tetra-*O*-benzyl galactosylmethylphosphonium iodide **77** and the long arm calix[4]arene dialdehyde **76** (Scheme 18). Compound **78** appeared to be as the methylene isostere of the *O*-glycoside **21** shown in Scheme 6. Unfortunately the entry to a tetravalent *C*-glycocluster was precluded by the failure to prepare the required long arm calix[4]arene tetraaldehyde.

2.2.2. Assembly via Cu(I)-Catalyzed Azide−Alkyne Cycloaddition

2.2.2.1. Triazole-Linked Calix[4]arene *C*-Glycosides. A successful approach to calix[4]arene *C*-glycosides was reported in 2006 by Dondoni and Marra via click azide−alkyne cycloaddition (CuAAC).[43] In these products, the glycosyl residues featured an anomeric carbon−carbon bond and were anchored to the calixarene platform through 1,4-disubstituted triazole rings. The presence of this heterocyle as a linker was considered to be quite beneficial by virtue of its stability under a wide range of reaction conditions and its potential to contribute to molecular recognition processes through hydrogen bonding and dipolar interactions. Another advantage of this approach relied on the high efficiency of the CuAAC and the operational simplicity for product isolation due to the absence of side products. Thus, di- and tetravalent carbon linked glycoclusters were anchored to the conformationally rigidified tetrapropoxy-calixarene scaffold

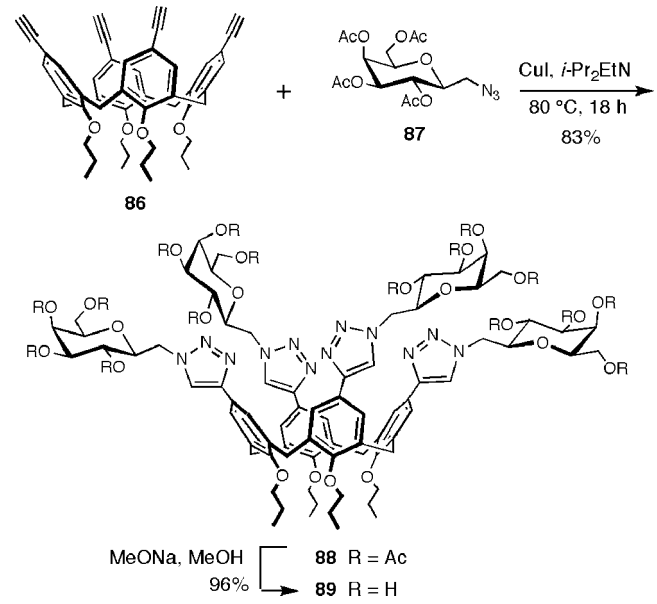
Scheme 19



using two complementary reagent combinations. The first was based on the coupling of an ethynyl *C*-glycoside, for example, the peracetylated *gluco* derivative **79**, with the bis-azidopropylcalix[4]arene **80** and the tetra-azidopropyl derivative **81** in the presence of catalytic CuI (Scheme 19). Both reactions afforded the corresponding calix[4]arene-based di- and tetravalent *C*-glycoclusters **82** (96% yield) and **83** (86% yield) featuring the triazole ring as a tether. The high yields of isolated products indicated that each cycloaddition has occurred in nearly quantitative manner. The *O*-acetyl group removal by transesterification was key to the transformation of calixsugars **82** and **83** into compounds **84** and **85** suitable for bioassays.

The second approach consisted of the coupling of an ethynyl functionalized calix[4]arene with a sugar azide. To this aim, a glycosylmethyl azide was used in order to produce a glycoconjugate with a stable methylene bridge between the sugar and triazole residue. This approach is exemplified in Scheme 20. This presents the CuI catalyzed cycloaddition between the galactosylmethyl azide **87** and the tetra-ethynyl-calix[4]arene **86** to give the calix[4]arene-based tetravalent glycocluster **88** in excellent yield.[43] Evidently, this compound differs from the tetravalent calixsugar **83** for the position of the carbohydrate and calixarene fragments attached to each triazole ring. This structural diversity broadens the scope of these robustly assembled calixsugars as substrates for biological assays. The structure of calixsugars thus prepared was established from their NMR spectra. Hence, it was easily demonstrated from typical ^{1}H signals and coupling constants that the original anomeric configuration of the carbohydrate residue and the cone conformation of the macrocycle had been preseved. Moreover, the 1,4-disubstitution pattern in the triazole rings was confirmed on the basis of the large and positive $\Delta(\delta_{C4}-\delta_{C5})$ values (ca. 20 ppm) in agreement with earlier findings on simple molecules.[44] Worth mentioning is also a special study that was carried out on the synthesis of the *galacto* analogue of the tetra-glucosyl-calixarene **83** via CuAAC in nonconventional solvents such as ionic liquids.[45] The use of three different ionic liquids combined with microwave dielectric heating confirmed the high efficiency (yields up to 90%) of the reaction described in Scheme 19 and demonstrated the viability to calixarene

Scheme 20



glycoconjugate synthesis via a green approach. The excellent yields of isolated tetravalent glycocluster and the lack of products arising from partial coupling of the sugar acetylene with tetra-azido-calix[4]arene **81** led to a suggestion that once the first triazole ring was formed, subsequent coupling reactions occurred much readily due to the formation of Cu(I)-triazolide-ethynyl complexes.

2.2.2.2. Triazole-Linked *C*-Glycocluster-Oligonucleotide Hybrids. The CuAAC reaction was employed for the preparation of calix[4]arene-based *C*-glycosides **90** and **91** featuring a single azido group attached to the lower rim through suitable tethers[46] (Figure 7). The synthesis of these compounds was carried out in the fulfillment of a program by an Italian−French consortium directed toward the construction of multivalent glycocluster-oligonucleotide hybrids **92** and **93** and evaluation of their affinity to galactose recognizing lectins (Figure 8). While the synthesis of the tetravalent *C*-glycoclusters **90** and **91** was developed taking advantage of the expertise of the Italian team on calix[4]arene

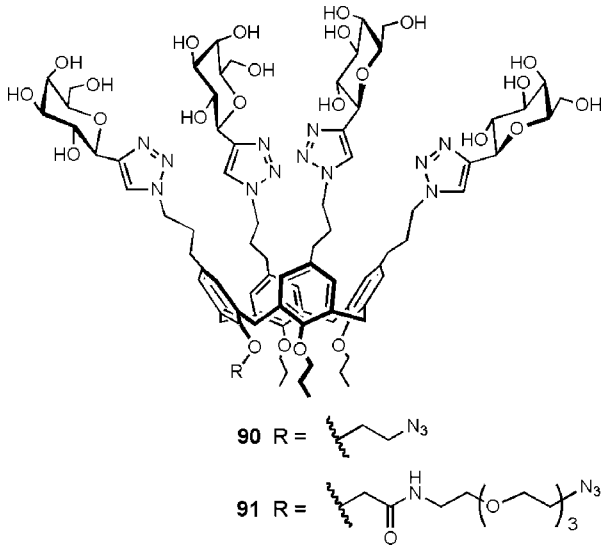


Figure 7. *C*-Glycosyl calix[4]arenes bearing an azido group at the lower rim.

functionalization,[47] the assembly of the multivalent complex sugar-nucleotide hybrids **92** and **93** relied on the DNA-based microarray methodology developed by the French group.[48] Succinctly, **90** and **91** were coupled via CuAAC to oligonucleotides bearing the fluorescent dye Cy3 and one or two propargyl groups to give **92** and **93**, respectively.

2.2.3. Bioassays with Calix[4]arene C-Glycosides

The glycocluster-oligonucleotide hybrids **92** and **93** were immobilized on DNA chips constituted of complementary oligonucleotide sequences covalently linked to 52-well glass slides. Next, the *Pseudomonas aeruginosa* lectin (PA-IL) and *Ricinus communis* agglutinin (RCA 120), labeled with specific fluorescent dyes, were deposited in the wells and the fluorescent signals of the conjugates were measured. The results indicated that the hybrids **92** and **93** had no affinity to the PA-IL lectin, while they displayed good affinity to the RCA 120 lectin. The level of affinity of the latter was similar to that observed for both a linear conjugate bearing three galactose moieties and a cluster bearing 10 galactose residues in an antenna spatial arrangement. Moreover, IC_{50} values, i.e., the concentration of lactose required for the removal of 50% of RCA 120 bound to the immobilized glycoconjugates, were measured and compared with those determined for linear monovalent and trivalent galactoside conjugates. From these assays, it appeared that the glycoconjugates **92** and **93** displayed a modest glycoside cluster effect as the affinity per galactose moiety was only five and seven times higher than that displayed by the monovalent

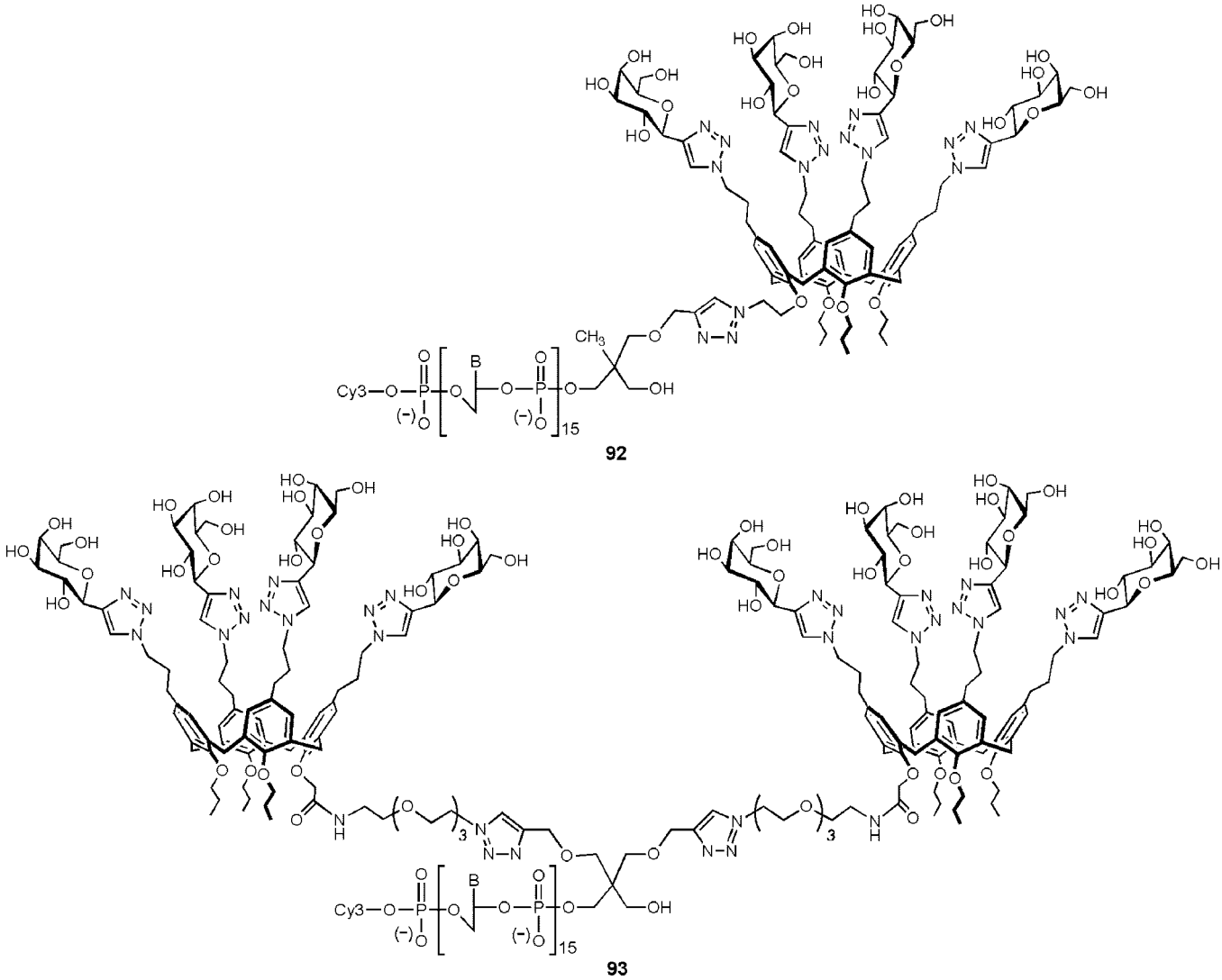


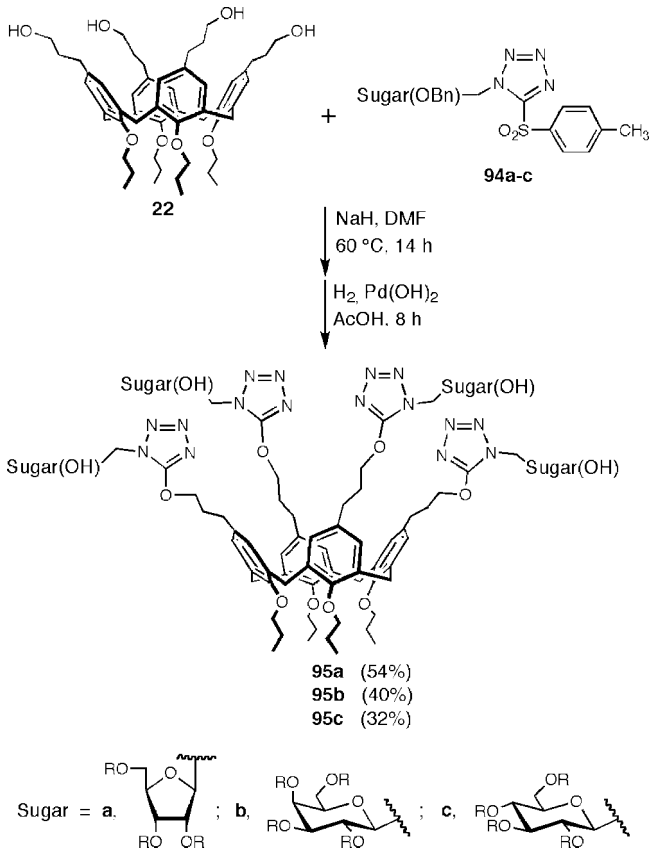
Figure 8. Multivalent glycocluster-oligonucleotide hybrids.

conjugate. Thus, it was concluded that for the attainment of multivalency, the spatial arrangement was more important than the number of sugar residues because the linear trivalent cluster was capable of binding to lectins more efficiently than calixarene and antenna systems bearing four, eight, and 10 galactose moieties.

2.2.4. Assembly via Nucleophilic Substitution in Tosyl Tetrazoles

Because of stability to both acids and bases as well as oxidizing and reducing reagents, also tetrazole ring was considered by Dondoni and Marra a useful system for establishing a robust connection between carbon-linked carbohydrate fragments and a calix[4]arene platform.[49] They envisaged that tetrazole, similarly to triazole, being a multinitrogenated heterocyclic, could actively participate in hydrogen bonding processes and therefore provide additional recognition properties to the system in which it was embedded. It was also pointed out that the presence of the heteroaromatic tetrazole group next to the carbohydrate residues may be highly beneficial, as it would enhance the interaction with lectins by increasing the hydrophobicity. A simple approach to tetrazole formation was the thermal azide-nitrile 1,3-dipolar cycloaddition that in fact was known to afford 1,5-disubstituted tetrazole derivatives.[50] This reaction, however, is of preparative value only with nitriles activated by electron-withdrawing groups. Thus, to give access to a wide range of disubstituted tetrazoles, Sharpless and Demko had developed a simple and expedient two-step sequence.[51] This involved the coupling of an organic azide with *p*-toluensulfonyl cyanide and then the displacement of the tosyl group in the 5-sulfonyl-tetrazole thus formed by a suitable nucleophile. This approach was successfully used for introducing four ribofuranose, galactopyranose, and glucopyranose units in the calix[4]arene tetrol **22** (Scheme 21). First, 1-glycosylmethyl-5-tosyl-tetrazoles **94a−c** were prepared in good to excellent yields (62−96%) by coupling *O*-benzylated glycosylmethyl azides with *p*-toluensulfonyl cyanide. Then, each of these compounds (6 equiv) was allowed to react under basic conditions with the polydentate alcohol **22** to give, after removal of the benzyl protective groups from the sugar fragments, the corresponding calix[4]arene *C*-glycosides **95a−c**. These compounds all featured the 1,5-disubstituted tetrazole ring (the substituents are adjacent) in the carbohydrate-to-platform tether. Hence these tetravalent carbon-linked glycoclusters showed a connectivity pattern around the heterocycle ring that was different to that established in triazole-tethered glycoclusters in which, in fact the substituents were distant. The access to this structural diversity may be of great value in the construction of suitable multivalent substrates.

Scheme 21



2.3. Calix[4]arene *N*-Glycosides

2.3.1. Assembly via Isothiocyanate-Amine Addition

Ungaro and co-workers realized the potential of the classical isothiocyanate-amine coupling for introducing amino acids, or small peptides, and carbohydrate fragments into calix[4]arenes through a thiourea spacer.[52] These authors envisaged the strong hydrogen bonding donor thiourea group as a point of interaction in molecular recognition processes. Accordingly, this interaction was invoked[53] in the mode of binding of the benzylphosphonate anion (**97**) to the thioureido-tethered calixsugar **96** (Figure 9). Moreover, the convenient choice of this ligation tool resided on the easy access of carbohydrates and calix[4]arenes functionalized with isothiocyanate and amino group. The only serious drawback, never mentioned however, that was associated with *N*-glycosides that are formed by this approach was their limited stability due to the intrinsically labile aminal bond holding the carbohydrate fragments.

The first report on calix[4]arene *N*-glycoside synthesis via reaction of glycosyl isothiocyanates with lower rim dialkylamino calix[4]arene **98** was reported in 1999 by Santoyo-González and co-workers.[54] Scheme 22 exemplifies this approach by showing the reaction of **98** with peracetylated lactosyl isothiocyanate **99** to give the calix[4]arene *N*-glycoside **100**. The same approach was successfully applied to *gluco*, *galacto*, and *manno* configured isothiocyanates. Quite interestingly, NMR spectra as well as NOE experiments in $CDCl_3$ revealed that these calix[4]arene glycocon-

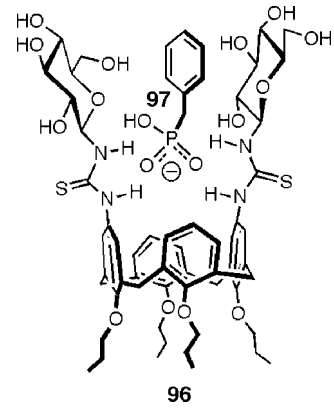
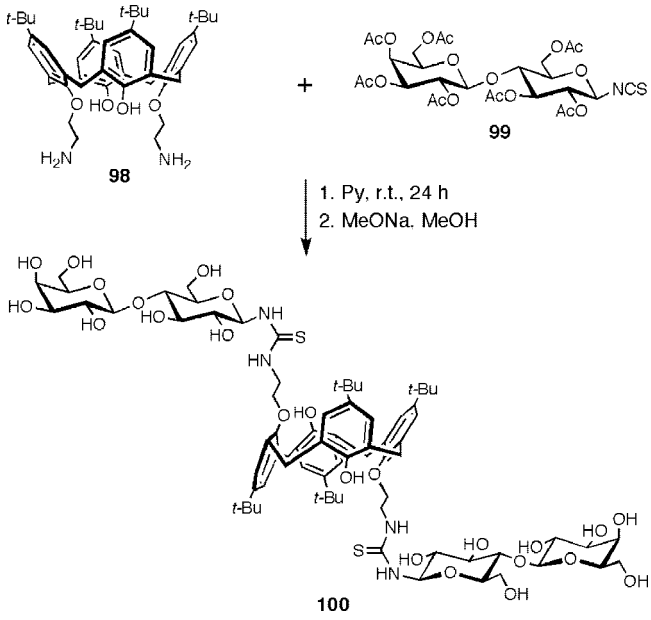
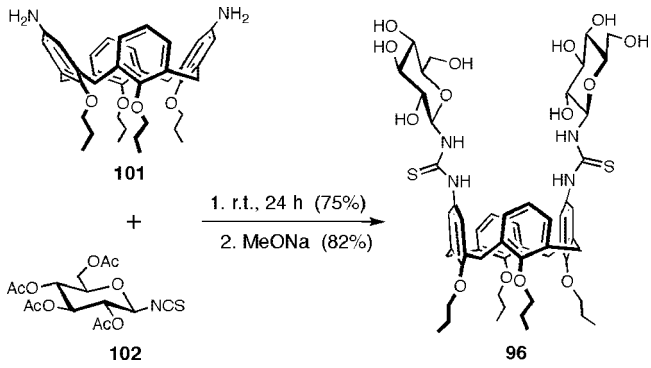


Figure 9. Complex between the thioureido-tethered diglucosyl calix[4]arene **96** and the benzylphosphonate anion.

Scheme 22



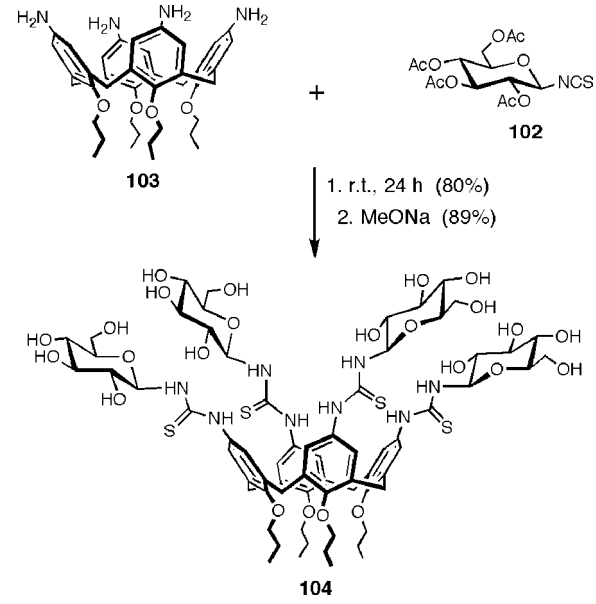
Scheme 23



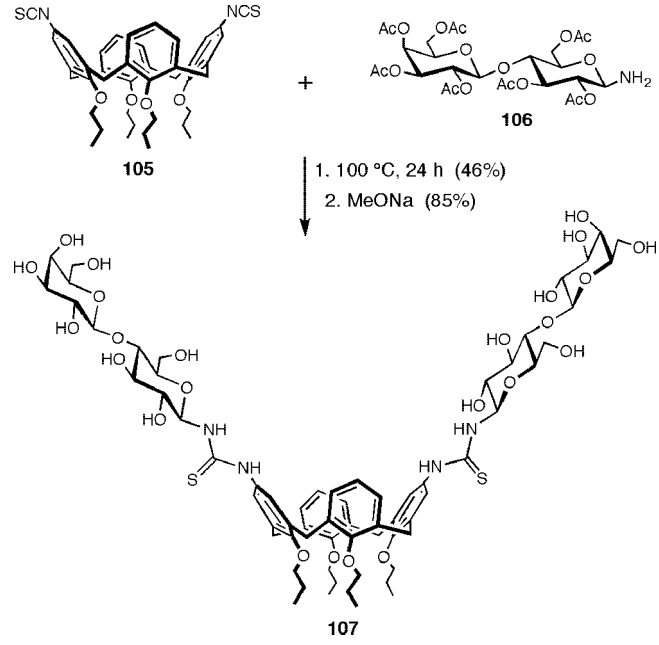
jugates adopted a 1,2-alternate conformation as shown for **100** and form dimers through intermolecular hydrogen bondings. This structural arrangement did not appear to be suitable for probing the multivalent effect with these compounds.

The thioureido-tethered calix[4]arene *N*-glycoside **96** assembled in a well-defined cone conformation was prepared in the Ungaro laboratory in the early 2000s[53] (Scheme 23). To this aim, the conformationally rigidified tetrapropoxycalix[4]arene diamine **101** was allowed to react with the peracetylated glucosyl isothiocyanate **102** and the product was deacetylated to give **96** in good overall yield. The close flattened cone conformation in $CDCl_3$ and CD_3OD of this compound was supported by a circumstantial NMR study. This preferential structure was attributed to hydrogen bonding interactions involving the thiourea group of one tether and the glycoside endocyclic oxygen in the other chain. The hydrogen bonding interactions disappeared in DMSO-d_6 so that the less hindered open flattened cone conformation (not shown) was adopted. Ungaro and co-workers prepared also the tetrafunctionalized calix[4]arene *N*-glycoside **104** by addition of glucosyl isothiocyanate **102** to the calix[4]arene tetraamine **103** (Scheme 24). In this case, the NMR spectra showed the expected C_4 symmetry and no particular conformational feature connected with the calix[4]arene ring mobility. It was, however, pointed out that the NMR spectra of calixsugars **96** and **104** at room temperature showed some

Scheme 24



Scheme 25

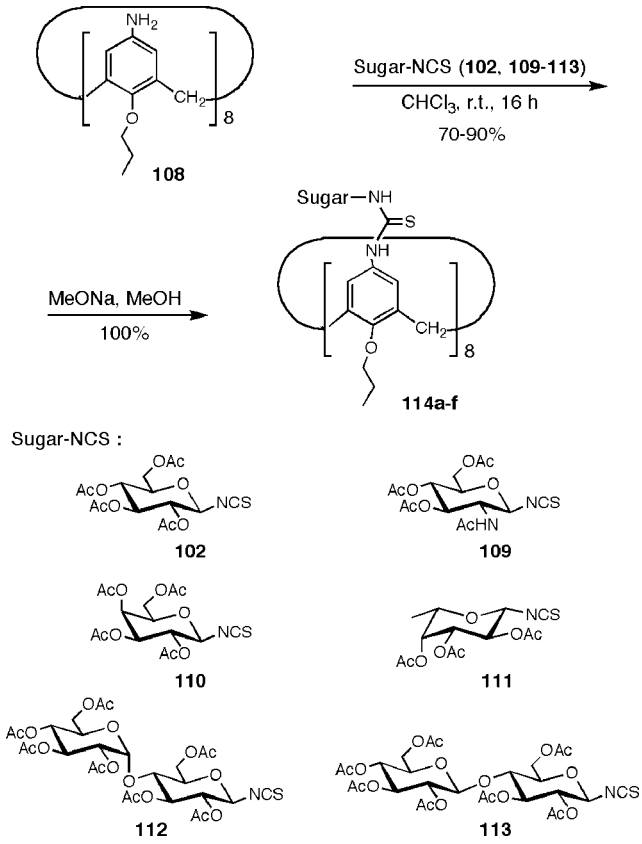


broad signals, in particular those corresponding to the NHs, the aromatic, and the anomeric protons. This was attributed to rotamers involving the thiourea groups. Accordingly, sharpening of the signals was observed at higher temperatures.

In the same paper,[53] Ungaro and co-workers demonstrated the viability to calix[4]arene *N*-glycosides by a complementary approach to that shown in Scheme 23. Specifically, the synthesis of the *N*-lactoside **107** involved the coupling of the peracetylated lactosyl amine **106** with the diisothiocyanate calix[4]arene **105** followed by deacetylation (Scheme 25). The yield of **107**, however, was much lower than those registered for **96** and **104** and therefore this approach was abandoned.

Thioureido tethering for calix[4]arene glycoclustering was used by Consoli and co-workers as well.[55] These researchers focused on the use of a calix[8]arene platform instead of the traditional calix[4]arene. They speculated that the major number of carbohydrate residues, the larger size of the

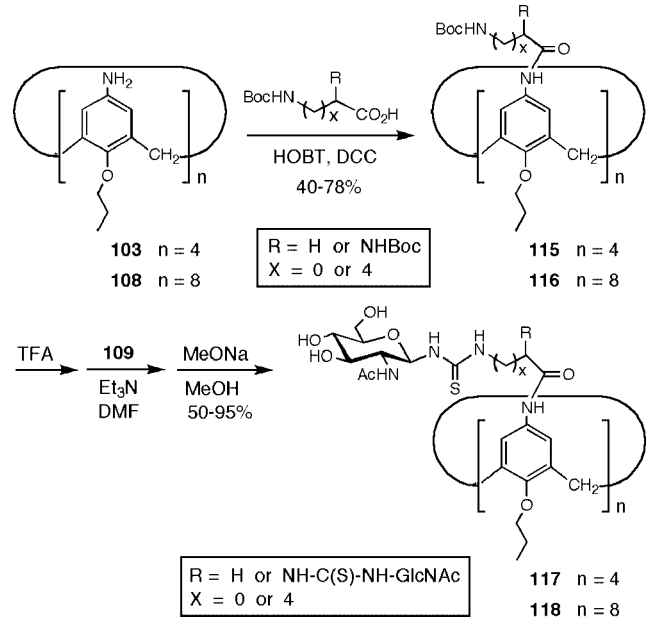
Scheme 26



hydrophobic cavity, as well as the flexibility of the eight-membered ring would be beneficial for intense molecular recognition processes. Thus, the octaamino-calix[8]arene **108** that was prepared for the first time was allowed to react with peracetylated glycosyl isothiocyanates **102** and **109**−**113** (D-glucosyl, 2-acetamido-2-deoxy-D-glucosyl, D-galactosyl, L-fucosyl, D-maltosyl, and D-cellobiosyl isothiocyanate, respectively) to give the corresponding thioureido-tethered calix[8]arene *N*-glycosides in very good yields (Scheme 26). The removal of the *O*-acetyl protecting groups by transesterification afforded the target octavalent calix[8]arene-based glycoclusters **114a**−**f**, all showing a good solubility in pure water. Complexation experiments followed by NMR titration revealed the formation of complexes between D-glucosamine hydrochloride and octagalactosyl (**114c**) and octacellobiosyl derivatives (**114f**) with association constants of 934 ± 90 M^{-1} and 764 ± 65 M^{-1}, respectively. Hence, it appeared a higher affinity for the guest by the monosaccharide bearing receptor with respect to the disaccharide-based one. This demonstrated that, in contrast to the above premises, increasing the number of sugar hydroxy groups in the host did not necessarily imply an increase in affinity.

Consoli and co-workers carried out also the synthesis of *N*-glycosylated calix[4]arenes and calix[8]arenes, exposing *N*-acetyl-D-glucosamine (GlcNAc) clusters at the upper rim.[56] The carbohydrate fragments were grafted on the macrocycle platform through a rather complex and chiral tether constituted of amino acid and thioureido groups. The selected *N*-Boc protected amino acid residues (for instance L-glycine) were introduced on the calixarene platform by multiple amidation of the calix[4]arene tetraamine **103** or calix[4]arene octa-amine **108** (Scheme 27). Then the *N*-Boc groups in **115** and **116** were removed by acid treatment and the polyamines thus formed were reacted with 2-acetamido-2-deoxy-D-

Scheme 27



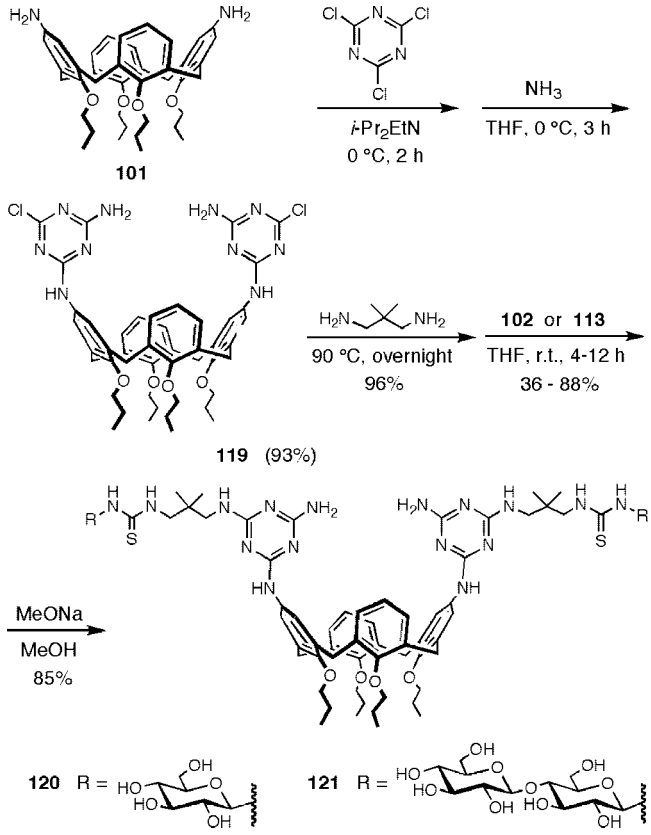
glucosyl isothiocyanate **109** to give, after de-*O*-acetylation, the target thioureido-amino acid bridged calix[n]arenes *N*-glycosides **117** ($n = 4$) and **118** ($n = 8$) in very good isolated yields. The NMR spectra confirmed the usual cone conformation for **117** and a flexible arrangement for **118**.

Targeting natural receptor mimicks, Crego-Calama, Reinhoudt, and co-workers[57] reported on the synthesis of calix[4]arenes diametrically substituted at the upper rim with two melamine units bearing simple achiral groups (alkyl, pyridyl, etc.) as well as biologically relevant moieties (amino acids, carbohydrates). The diamino-calixarene **101** was first coupled[58] with 2,4,6-trichloro-1,3,5-triazine (cyanuric acid) in the presence of *N*,*N*-diisopropylethylamine (*i*-Pr_2EtN) (Scheme 28). Then, the crude adduct was treated[59] with gaseous ammonia to give the bis(chlorotriazine)calixarene **119**, which was allowed to react with 1,3-diamino-2,2-dimethyl-propane to afford the corresponding bis-melamine derivative in 96% yield. The reaction of the latter compound with peracetylated glucosyl (**102**) or cellobiosyl isothiocyanate (**113**) (see Scheme 26) gave, after removal of the acetyl groups, the *N*-glycosylated calixarenes **120** (31%) and **121** (75%), respectively. The self-assembly of these calixsugars with barbituric or cyanuric acid derivatives into hydrogen-bonded, double-rosette nanostructures was studied by NMR and circular dicroism spectroscopies.

The preparation of the *N*-acetyl-D-glucosamine (GlcNAc) containing calix[4]arenes **122**, **123**, and **124** (Figure 10) featuring only the thioureido group as a tether was reported by Krenek and co-workers via the usual isothiocyanate-to-amine coupling reaction.[60] The geometrical arrangements of these compounds as shown in Figure 10 were supported by the method of synthesis[61] as well as NMR data. Thus, the tetravalent glycoclusters **122**, previously described by Consoli and co-workers,[56] was disposed in the fixed cone conformation whereas the isomer **123** featured the 1,3-alternate structure. The divalent compound **124** was assigned the usual open flattened-cone conformation.

Recently, Casnati and co-workers described[62] the synthesis of the glucosylthioureido-calix[6]arene (**127**) and calix[8]arene derivatives (**128**) starting from the corresponding hexa- and octaamino calixarenes **125** and **126** (Scheme 29), respec-

Scheme 28



tively, the latter being the methylated analogue of **108** (see Scheme 27). The isothiocyanate-to-amine coupling, followed by transesterification under basic conditions, gave the hexa- and octavalent *N*-glucoside clusters **127** and **128** in good yields. Although these compounds were fairly soluble in water (0.1−1 mM), they showed a tendency to form self-assembled aggregates as proved by atomic force microscopy (AFM) and dynamic light scattering (DLS) analyses. Moreover, AFM was also employed to study the interaction of **127** with plasmid DNA. In the presence of the glucocluster **127**, the DNA filaments of the supercoiled plasmids aggregated significantly but did not lead to filament condensation or changes in the DNA folding. The lack of the latter behaviors prevented the use of **127** as a synthetic gene delivery system (see also section 2.5.3.3).

In the same year, the Ungaro group, in collaboration with Gabius and co-workers, reported on the preparation and biological properties of another series of thioureido-linked calixsugars (Figure 11).[63,64] Exploiting the synthetic approach outlined in Scheme 29, they prepared *N*-galactosyl- and *N*-lactosyl-calix[4]arenes in cone (**129**−**132**) or 1,3-alternate conformation (**133**, **134**) as well as *N*-galactosyl- and *N*-lactosyl-calix[6]arenes (**135**, **137**) and calix[8]arenes (**136**, **138**) and evaluated their binding activity toward medically relevant lectins (see section 2.3.2.2).

2.3.2. Biological Tests with Calixarene N-Glycosides

The final goal that spurred the preparation of the compounds reported in section 2.3.1 was aimed at probing the biological activity of calixarene-based glycoclusters in various directions. It was clear that although precise rules had not been established, the occurrence of an effective glycoside cluster effect in carbohydrate−protein interactions depended on several factors, including the shape and flexibility of the

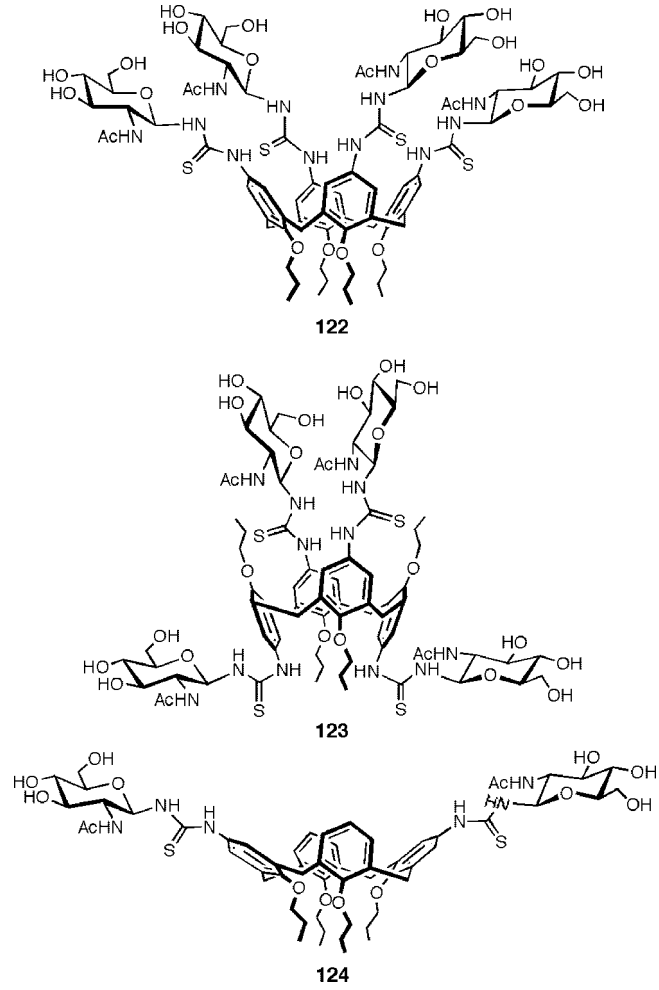
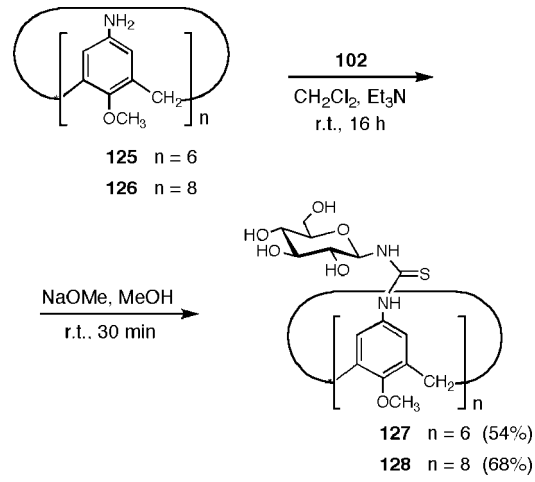


Figure 10. Thioureido-tethered glycosyl calix[4]arene prepared by Krenek and co-workers.[60]

Scheme 29



scaffold, the length, structure, and type of the tether between scaffold and carbohydrate residues, and the number, kind and spatial disposition of the latter key components.[13]

2.3.2.1. Binding Assays with Lectins. Ungaro and co-workers focused their attention[53] on the interaction of Concanavalin A (ConA) and peanut lectin (PNA, *Arachis hypogaea*) with the tetravalent glucocluster **104** (see Scheme 24) and the galactocluster **130** (see Figure 11) by turbidimetric analysis. These lectins have different glycoside receptors as ConA binds selectively α-D-glucoside and α-D-

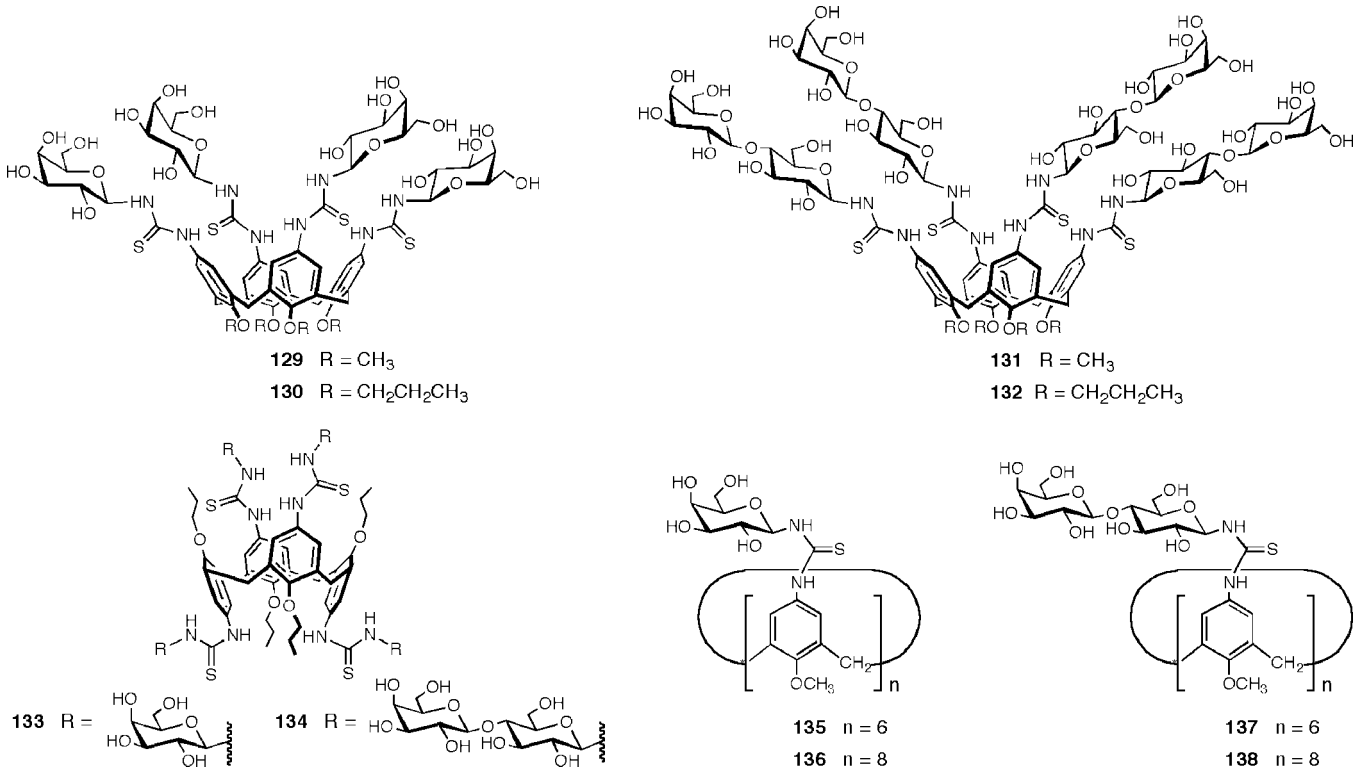


Figure 11. Thioureido-tethered glycosyl calixarene prepared by Ungaro, Gabius and co-workers.[63]

mannoside residues, whereas PNA specifically binds β-D-galactosides.[11] The absorbance increase for a mixture of ConA and calix[4]arene *N*-glucoside **104** in water at various time intervals up to 4.5 h, followed by an absorbance decrease upon addition of a large excess of D-glucose, revealed the specific involvement of the glucose residues of **104** in the interaction with the lectin. On the other hand, no turbidity was observed by mixing PNA with **104** while the phenomenon became apparent by adding the calix[4]arene *N*-galactoside **130**. The latter process was inhibited by adding an excess of D-galactose. From this evidence, it was concluded that calixsugars **104** and **130** specifically bind to ConA and PNA, respectively, through multivalent interactions.

Similar turbidimetric experiments were carried out using the hexa- (**127**) and octavalent (**128**) glucoclusters (see Scheme 29) and Concanavalin A.[62] Although both clusters agglutinated the lectin, the binding of the octamer **128** was considerably stronger than that showed by **127**. On the other hand, the two *N*-glucosylated calixarenes did not bind to the peanut lectin, which is selective for the D-galactoside moieties.

Consoli and co-workers carried out a circumstantiated study on the lectin-binding ability of the calixarene *N*-glycosides they had prepared.[56] Interaction of tetra- (**117**, **122**) and octavalent (**114b**, **118**) glycoclusters with wheat germ agglutinin (WGA), a lectin known to be GlcNAc specific, was examined by turbidimetric analysis and hemagglutination inhibition tests. Thus, the WGA-glycocluster cross-linking was evidenced by turbidity formation while the disruption of such GlcNAc−protein interaction was demonstrated by addition of large excess of D-GlcNAc. In support to the recognition specificity of WGA by GlcNAc residues were the observations that D-galactose did not induce any disruption of the observed aggregation and that PNA lectin, specific for β-D-galactosides, did not bind to any of the glycoclusters **114b**, **117**, **118**, and **122**.

The occurrence of the glycoside cluster effect was demonstrated by the ability of **114b**, **117**, **118**, and **122** to inhibit the WGA-dependent hemagglutination at 0.1−1 mM concentrations. All the prepared glycocluters turned out to be more potent than D-GlcNAc (up to 312-fold in the case of **117** having R = H, X = 4). Two other important observations were made about the three-dimensional arrangement of the scaffold and the linker holding the cluster domain. Thus, the tetravalent compound **117** blocked in the cone conformation and bearing both the amino acid and thioureido groups as spacers was shown to be more potent than the tetravalent and octavalent derivatives **122** and **114b**, respectively, featuring only the thioureido group as a spacer. Therefore, the fine combination of special structural features appeared to be crucial for an effective glycoside cluster effect could operate. Moreover, it was also demonstrated that a higher valency glycocluster such as a hexadeca derivative displayed lower inhibitory activity than the tetra- and octavalent derivatives. It was suggested that the cross-linking with crowded glycoclusters was prevented because of an improper spacing or geometry of the carbohydrate fragments.

2.3.2.2. Calix[4]arene *N*-Glycosides in Glycomedicine. A study by Krenek and co-workers[60] considered the binding activity of their GlcNAc-based calixsugars to C-type lectin-like activation receptors NKR-P1 (CD161) expressed by rat natural killer (NK) cells, and CD69, expressed by human NK cells. Natural killer cells are part of the human innate immune system and act as a protection against tumors as well as viruses and other pathogens by producing chemokines and cytokines.[65] Although ManNAc and GalNAc have the highest affinity for NKR-P1, the convenient choice of calix[4]arene-based GlcNAc clusters relied on their easy preparation and low cost of the monosaccharide. Moreover, the CD69 receptor displayed higher affinity for GlcNAc than for GalNAc. On the basis of the results obtained from a set of experiments, it was concluded that di- and tetravalent

glycoclusters **124** and **122** (see Figure 10) were better inhibitors for NKR-P1 than **123** featuring a blocked 1,3-alternate conformation. Parallel experiments demonstrated that the tetraglycosylated calixarene **122** was the best ligand for the CD69 receptor. Moreover, the latter glycocluster was also tested for its immunomodulatory effect in a functional cytotoxicity assay in vitro. It was found that **122** was more active, at picomolar concentrations, than a known[66] octavalent polyamidoamine (PAMAM) GlcNAc dendrimer used as a reference compound.[67] Moreover, unlike the above-mentioned dendrimer, the glycocluster **122** was able to induce the apoptosis of tumor cells.

Sortino and co-workers[68] found that the glycocluster **114b** (see Scheme 26), exposing eight GlcNAc residues,[55] was able to bind strongly to the β1,4-galactosyltransferase V, a cell surface enzyme responsible for the invasion, growth, and survival of glioma tumor cells. Because of this efficient recognition process, **114b** inhibited rat C6 glioma cell migration and proliferation, although the latter effect did not depend on the presence of sugar units but was related to the multiple ureido functions.

Gabius, Ungaro, and co-workers[63] submitted the galactoside and lactoside clusters shown in Figure 11 to lectin inhibition solid-phase assays using the *Viscum album* agglutinin (VAA). To this aim, the glycoprotein asialofetuin was adsorbed onto a microtiter plate while the biotinylated VAA and the glycoclusters, at various concentrations, were in solution. This study revealed that, among the galactoclusters under investigation, only the hexamer **135** and the octamer **136** inhibited the binding of VAA to the immobilized asialofetuin more efficiently than the corresponding monomeric ligand, i.e., free D-galactose. When similar experiments were performed with the lactoside clusters, it was found that also in this case the hexavalent and octavalent derivatives **137** and **138**, respectively, were stronger inhibitors than free D-lactose. Moreover, the tetravalent lactoside clusters **131**, **132**, and **134** were slightly stronger VAA inhibitors than D-lactose.

The solid-phase assays were employed also to evaluate the inhibition activity of the lactoside clusters toward human galectins-1, -3, and -4, important factors in tumor progression. Galectins-1 and -3 were inhibited to a variable extent by the lactosylated calixarenes, whereas galectin-4 was strongly inhibited by these compounds, being the hexamer **137** (see Figure 11) and the bis-lactoside calix[4]arene derivative **107** (see Scheme 25), the most active glycoclusters (IC_{50} values 300-fold lower than that of free D-lactose).[63] In aiming to develop potential medical applications, the activity of the above-mentioned glycoclusters was studied by means of cell-binding assays. Under these conditions, the lactosylated calixarenes were found active for plant lectin VAA and human galactins as well, although the IC_{50} values determined in the solid-phase assays did not automatically translate into a direct ranking in the cell assays.

2.4. Calix[4]arene *S*-Glycosides

This family of glyconjugates is essentially constituted of *S*-sialosides because *N*-acetyl-neuraminic acid (Neu5Ac), the most common sialic acid, was the carbohydrate component in most of the compounds prepared. The anomeric carbon−sulfur bond is the main yet important structural feature of these compounds thanks to which they are sufficiently stable to chemical and enzymatic degradation. Given the involvement of Neu5Ac in a variety of biological functions,[39] some of which are detrimental such as the adhesion of viruses and bacteria to human cells, clusters of sialic acids with a well-defined structure and composition are of great biological relevance. This was the essence of a reasoning that spurred two research groups to prepare calix[4]arene *S*-glycosides and evaluate their biological properties.

2.4.1. Assembly via S-Alkylation

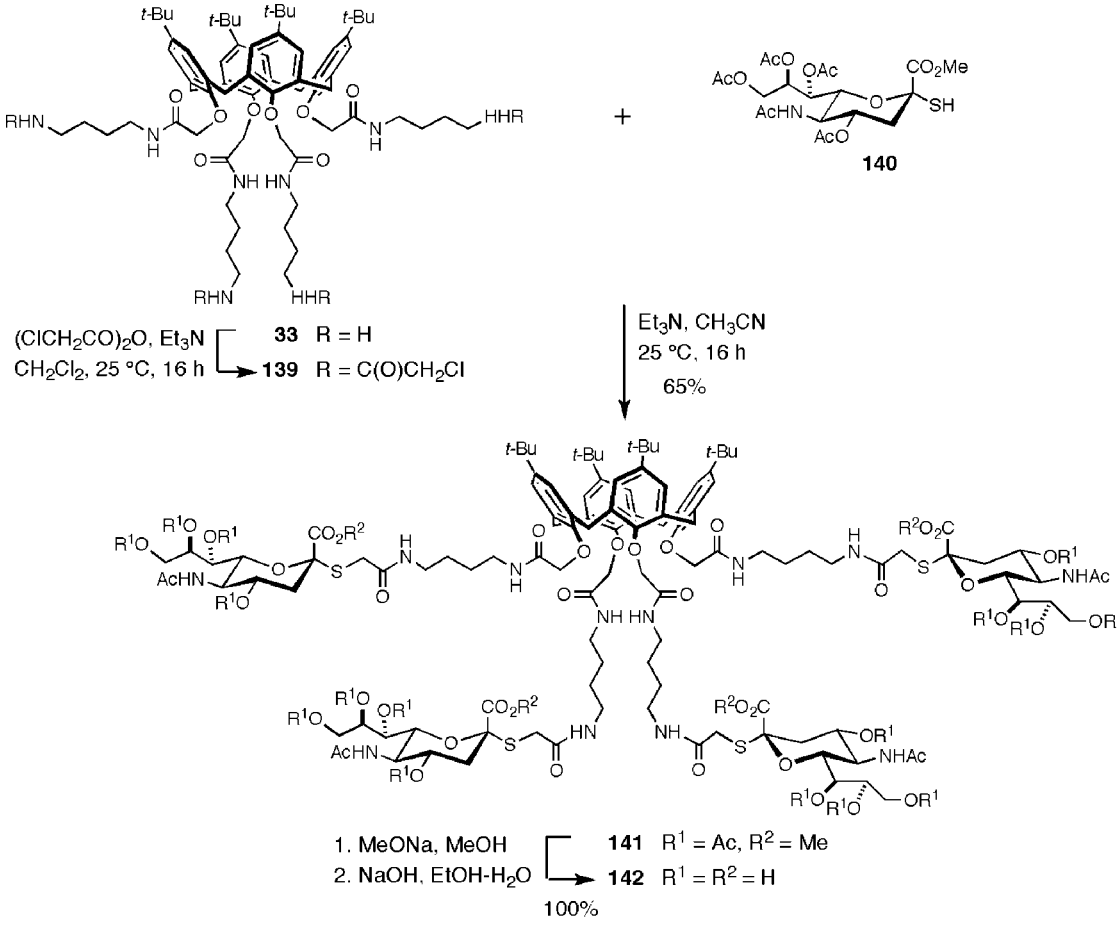
The first example of a calix[4]arene *S*-sialoside synthesis and lectin binding study was reported as early as 1996 by Meunier and Roy in a brief communication.[69] The *p-tert*-butylcalix[4]arene tetraamine **33** was transformed into the tetra-*N*-chloroacetylated calix[4]arene **139**, and this was treated with an excess of sialyl thiol **140** in the presence of triethylamine to give the tetrasialylated calix[4]arene **141** in 65% yield (Scheme 30). This compound upon basic hydrolysis gave the water-soluble (ca. 1 mM) deprotected calix[4]arene *S*-glycoside **142** suitable for lectin binding studies. Standard turbidimetric analysis revealed the formation of cross-linking aggregates between **142** and wheat germ agglutinin (WGA) that was known to bind sialosides. The lattice thus formed was disrupted by addition of a monovalent ligand, i.e., phenyl α-D-*S*-sialoside. As the methyl ester of **142**, water-soluble as well, formed stable aggregates with WGA, it was concluded that simple electrostatic binding interactions were absent.

2.4.2. Assembly via Cu(I)-Catalyzed Azide−Alkyne Cycloaddition

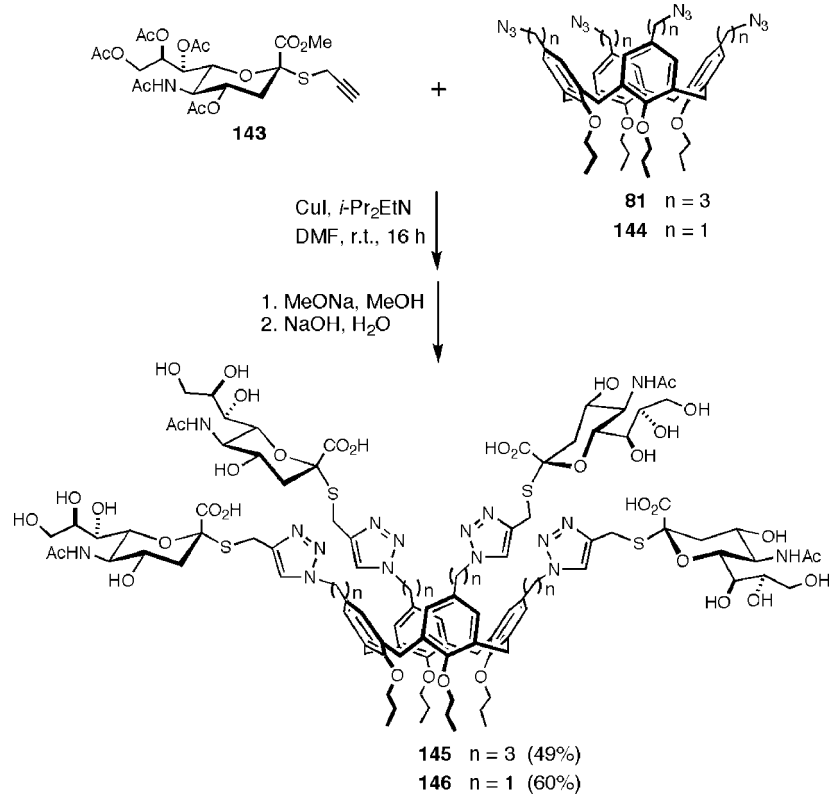
2.4.2.1. Triazole-Linked Calix[4]arene *S*-Sialosides. Twelve years after the Meunier and Roy report,[69] Dondoni, Marra, and their co-workers considered sialyl clustering on calix[4]arene scaffold an important issue to be addressed because of the potential applications in glycobiology (see section 2.4.2.2). At first, synthetic efforts were made toward the preparation of polyvalent calix[4]arene *S*-sialosides with different structure and composition.[70] The final goal was to achieve a substantial response in recognition processes involving sialic acid binding viruses. To this end, a synthetic route was paved by the use of the Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC),[29] as this powerful ligation mean was previously exploited by the same research group for calix[4]arene *C*-glycoside assembly.[43] The key reagent in this approach was the known[71] propargyl thiosialoside **143** that served as cycloaddition partner with azidocalix[4]arenes.[72] An excess of this sugar alkyne was allowed to react with the calix[4]arene tetraazide **81** in the presence of CuI and *i*-Pr_2EtN to give, after removal of the ester protecting groups, the upper rim tetravalent calix[4]arene *S*-sialoside **145** in good isolated yield (Scheme 31). Thus, the sialyl residues in **145** formed a tetravalent cluster with all sugar moieties anchored to the macrocycle platform through an *N*-propyltriazole tether that ensured enough flexibility to the whole system. The calix[4]arene *S*-sialoside **146** displaying shorter connecting arms was prepared in a similar way by reacting **143** with the calix[4]arene tetraazide **144**. NMR data of **145** and **146** confirmed both the cone conformation of the macrocyclic platform and the 1,4-disubstitution pattern of the triazole spacers. The latter structural motif was firmly established by ^{13}C NMR spectroscopy determination of the $\Delta(\delta_{C4}-\delta_{C5})$ values (see also section 2.2.2.1).

In a second instance, sialyl groups were introduced at the lower rim of the calix[4]arene platform. This new structure

Scheme 30

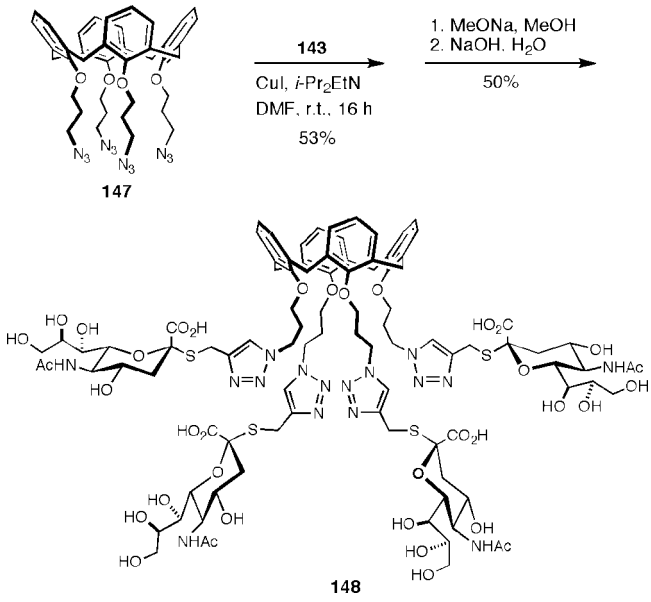


Scheme 31



was aimed at positioning the sialyl residues closer one to the other than in the upper rim functionalized compounds **145** and **146**. Also, the synthesis of the new target calix[4]arene *S*-glycoside **148** was performed by CuAAC that

Scheme 32



in this case involved the tetraazide **147** and the propargyl thiosialoside **143** (Scheme 32).

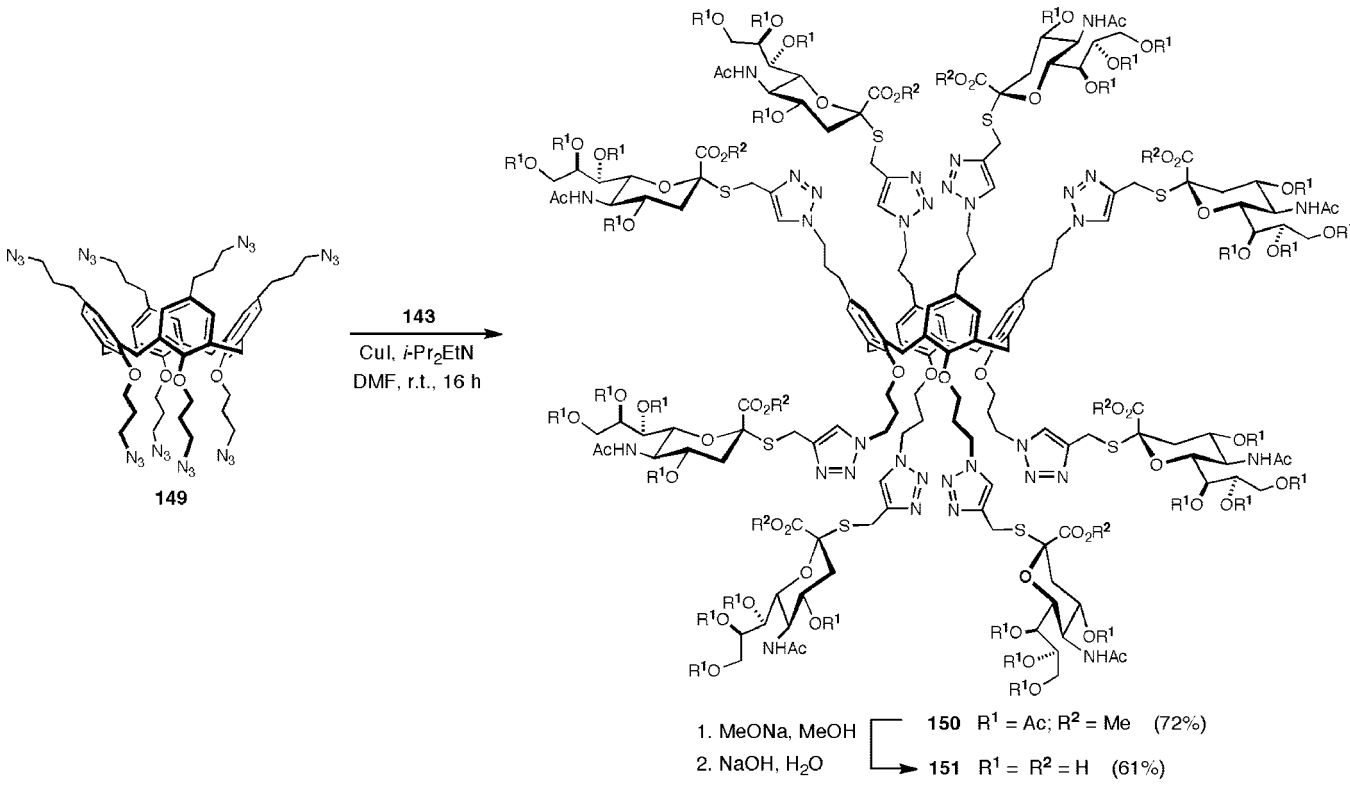
As a final endeavor, the introduction of sialyl residues at both the upper and lower rims of a calix[4]arene was considered. It was assumed that such a densely sialylated molecule could bind simultaneously to a couple of hemagglutinin trimers located onto a single virion or to two distinct viral particles. The coupling of the sugar alkyne **143** with the calix[4]arene octaazide **149** in the presence of CuI and *i*-Pr_2EtN proceeded smoothly to give the octavalent sialocluster **150** featuring all sialyl residues firmly anchored to the rigid calix[4]arene platform (Scheme 33). The high yield of isolated **150** (72%), although slightly contaminated by copper salts, indicated that each CuAAC had occurred in nearly quantitative yield. The NMR spectrum of a pure sample of **150** registered in DMSO-d_6 at 120 °C showed sharp signals that allowed confirmation of the structure of the platform and the triazole substitution pattern. Compound **150** was liberated of all protective groups to give the octavalent sialocluster **151** in pure form.

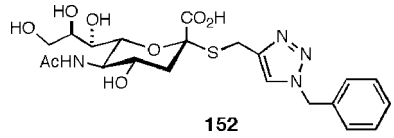
2.4.2.2. Inhibition Studies of Calix[4]arene *S*-Sialosides against BK and Influenza Viruses. The consortium constituted of synthetic organic chemists (Dondoni, Marra, Moni) and virologists (Corallini, Pazzi, Bridi) set out to investigate the interaction of calix[4]arene-based sialoclusters they had prepared (see section 2.4.2.1) with the pathological agents BK and influenza A viruses.[70]

BK virus (BKV) is a human polyomavirus that is known to be the etiological agent of a severe form of nephropathy, a complication that often leads to organ loss.[73] The extensive immunosuppressive treatment used to prevent kidney rejection enables a dramatic enhancement of the BKV replication resulting in the polyomavirus associated nephropathy. Since no reliable drugs against BKV are available to date, it is of great interest to develop new antiviral molecules based on the key role exerted by 5-*N*-acetyl-neuraminic acid (Neu5Ac), linked to human *N*-glycoproteins or gangliosides, as a ligand of the viral capsid proteins.[74] It is expected that artificial Neu5Ac-decorated structures able to interfere with this sugar−protein recognition process may act as anti-BKV agents.

The influenza A viruses, which include the human, avian, swine, and equine influenza viruses, are responsible for annual flu epidemics in humans. Moreover, these viruses can cause sporadic pandemics, these being characterized by excess mortality and morbidity. These single-stranded RNA viruses carry two surface glycoproteins, a trimeric lectin (hemagglutinin, HA)[75] and a tetrameric glycosidase (neuramini-

Scheme 33





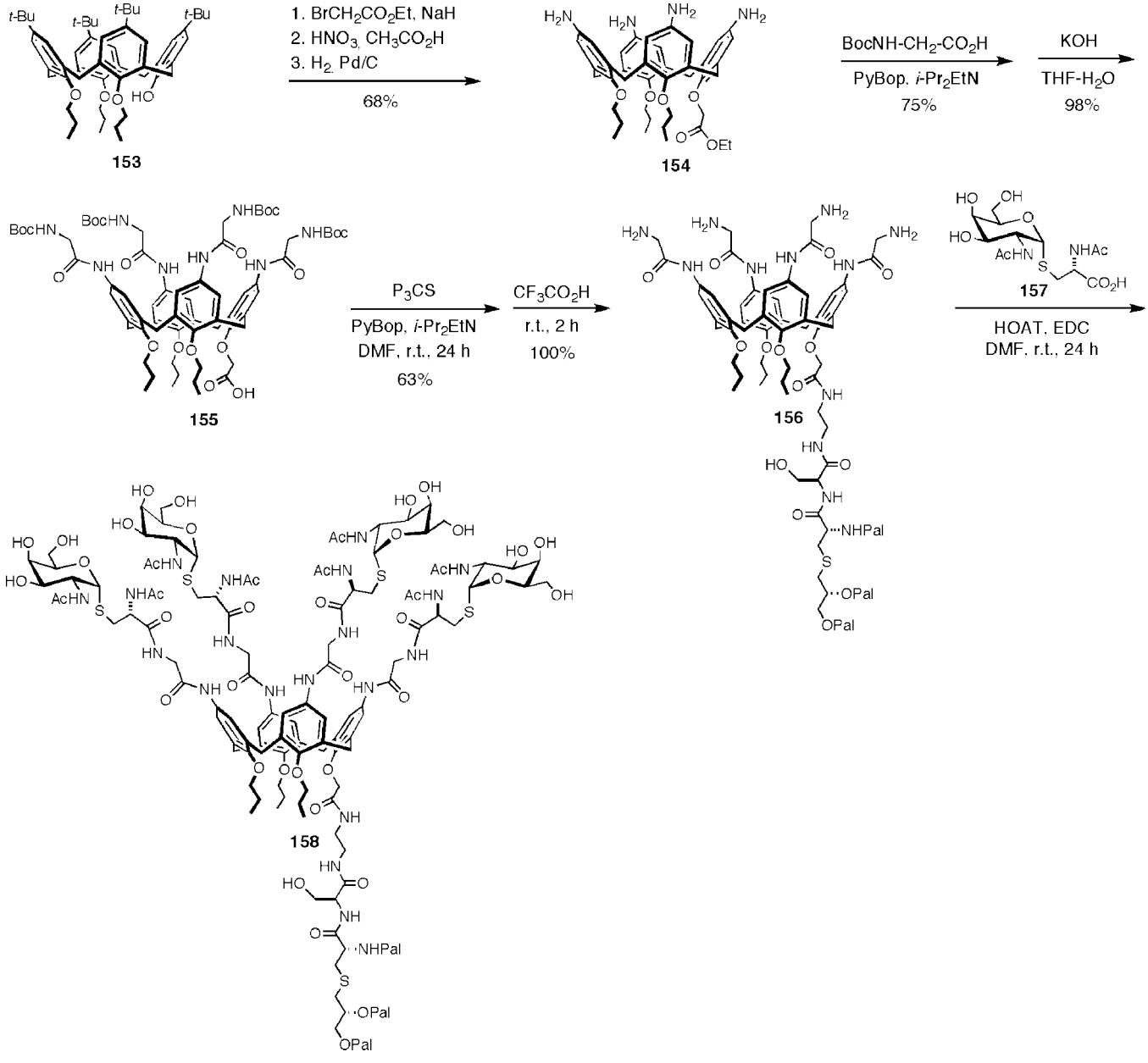
152

Figure 12. Monovalent sialoside ligand of viral hemagglutinin.

dase, NA),[76] each one having various subtypes. Both proteins are present in multiple copies on the virus surface and recognize the same carbohydrate in the host cell, i.e., Neu5Ac. Each protein plays a different role in the viral infection and diffusion. HA mediates the adhesion of the virus to the cell, whereas NA cleaves the terminal sialic acid residues that are linked to the glycoconjugate receptors to allow progeny virus release and consequently the spread of the infection. Therefore, designed molecules that bind stronger than the natural ligand to either HA or NA would be, in principle, good antiflu drugs.

The capability of calix[4]arenes *S*-sialosides **145**, **146**, **148**, and **151** to bind BKV particles was tested using the hemagglutination inhibition (HI) assay and compared to that of the monovalent analogue **152** (Figure 12), which featured a similar aglycon structure. All compounds were able to inactivate BKV at submillimolar concentrations, although each multivalent sialoside was less active than **152** when the actual concentration of sialic acid moieties was taken into consideration. The HI assay demonstrated also that the sialoclusters **145**, **148**, and **151** inhibited the influenza A virus-induced hemagglutination, whereas **146** failed to show HI activity at concentrations up to 50 mM and the monovalent sialoside **152** was a very weak inhibitor. The data allowed one to assess that, in this case, a moderate glycoside cluster effect was operative because, considering the inhibition activity per sialic acid unit, the multivalent sialosides were 50−83 times more active than the monovalent derivative **152**. Finally, the HI activity of the octasialoside **151** was close to that of the tetrasialosides **145** and **148**. This indicated that only one of the two sets of sialic acid units linked at both calixarene rims was involved in the interaction

Scheme 34

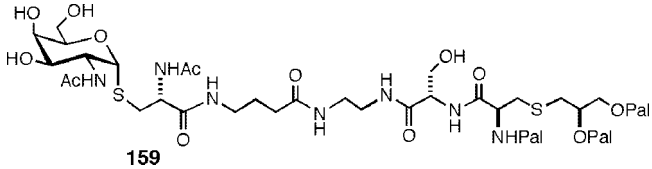


with the influenza A hemagglutinin. Moreover, experiments of viral infectivity proved that the above sialoclusters inhibited the adsorption of both BK and influenza A (H3N2 strain) virions to the host cells (Vero and MDCK cells, respectively). Therefore, the use of the non-natural calixarene scaffold and triazole linker did not prevent the molecular recognition properties of the Neu5Ac ligand.

2.4.3. Assembly via Amide Bond Formation

The natural Tn antigen (2-acetamido-α-D-galactosyl L-serine or L-threonine) is a well-known carbohydrate antigen overexposed on the surface of the tumor cells. However, this and similar tumor-associated sugar antigens are self-antigens and therefore weak immunogens. To enhance their immunogenicity, the antigen epitopes have been conjugated to carrier proteins or macrolipids.[77] Moreover, to increase the antibody response and mimick the mucine surface, where Tn antigens are present as di- to pentavalent clusters, multivalent vaccine candidates have been prepared by several research groups.[78] In 2008, Geraci, Spadaro, and co-workers[79] described the synthesis and immunological properties of calixsugar **158** (Scheme 34). This compound featured four 2-acetamido-α-D-galactosyl L-cysteine units linked to the upper rim of the macrocycle through four glycine residues and a lipopeptide as immunoadjuvant moiety at the lower rim. The latter derived from the *N*-terminal sequence of the principal lipoprotein of *Escherichia coli*. The *S*-glycosyl amino acid moiety was used as a hydrolytically stable mimic of the natural Tn antigen, which is an *O*-glycosyl amino acid. The synthesis of **158** was carried out through the following steps. In a first instance, the functionalized platform **154** featuring three *O*-propyl groups and one carboxylate group at the lower rim was prepared. Compound **154** was obtained in 68% overall yield starting from the known[9] tripropoxycalix[4]arene **153**, which was first alkylated with ethyl bromoacetate then submitted to an ipso nitration followed by catalytic hydrogenation. The amino groups at the upper rim of **154** served for the introduction of four Boc-protected glycine units via standard amide coupling (75%), while saponification of the ester group at the lower rim with a strong base gave the free acid **155** in nearly quantitative yield. The glycine spacers were introduced in order to avoid steric hindrance among the sugar units and ensure flexibility to the clustered antigen epitopes. Compound **155** was then coupled with the immunoadjuvant moiety P_3CS (tripalmitoyl-*S*-glycerylcysteinyl-serine) and treated with trifluoroacetic acid to afford the tetraamino-calixarene derivative **156** in 63% overall yield. This compound was allowed to react with the GalNAc-Cys derivative **157** (2 equiv per amino group), obtained from the corresponding protected precursor[80] in 60% yield, under HOAT/EDC activation to give the target product **158**. This was purified by size-exclusion and reverse-phase chromatography (yield not given).

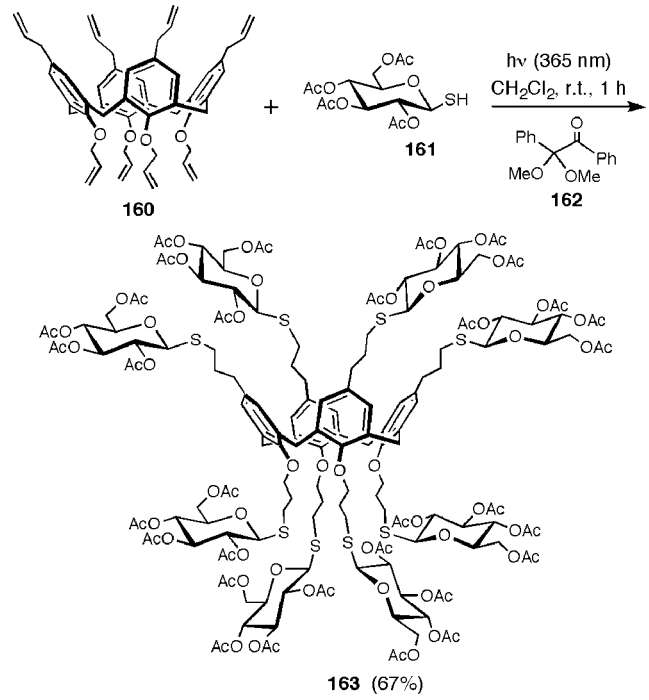
The immunogenicities of the glycocluster **158** and the previously reported[80] monovalent analogue **159** (Figure 13) were evaluated at different concentrations by ELISA experiments. It was found that the tetravalent cluster **158** showed a 4-fold increase of the end point titer when compared to that of **159** at the same molar concentration. Therefore, no cluster effect appeared to be operative.



159

Figure 13. Monovalent mimic of the natural Tn antigen.

Scheme 35



2.4.4. Assembly via Thiol-Ene Coupling

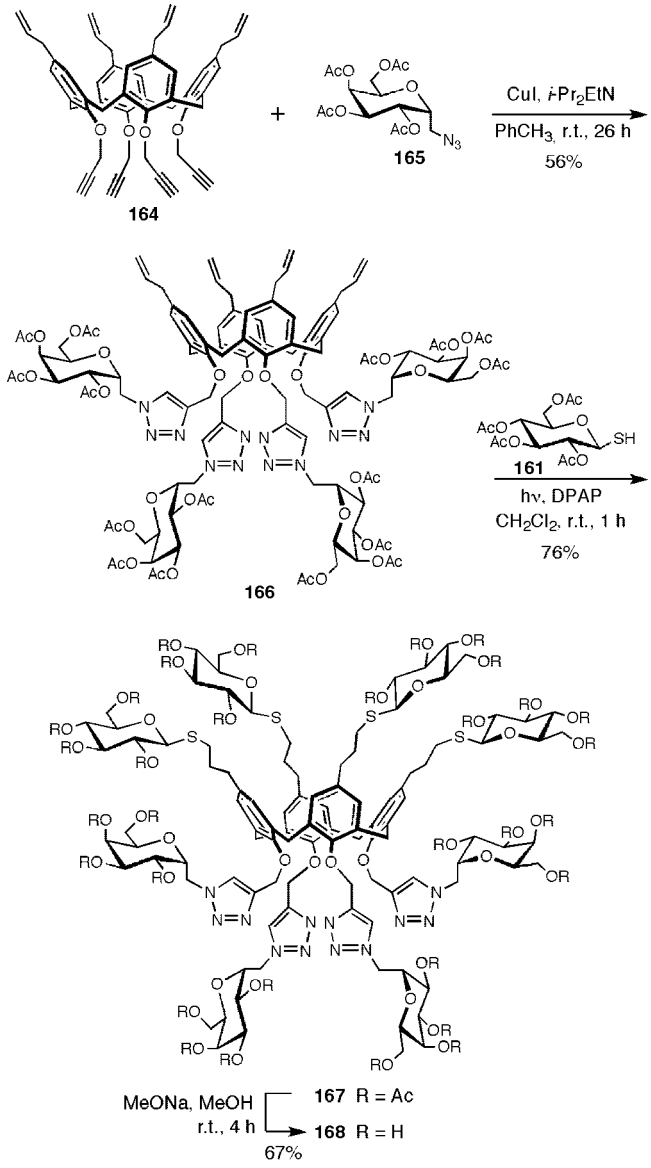
The free-radical addition of thiols to terminal alkenes, known as thiol−ene coupling, is a century-old reaction that proceeds with anti-Markovnikov regioselectivity and leads to linear thioethers.[81] This ligation tool was exploited by Dondoni and Marra for the preparation of calix[4]arene *S*-glycosides.[82] Typically, the photoinduced (λ_{max} = 365 nm) addition of the glucosyl thiol **161** to the octaallyl calixarene **160** in the presence of 2,2-dimethoxy-2-phenylacetophenone (DPAP) **162** as the sensitizer gave the octavalent glycocluster **163** in 67% yield, corresponding to a 95% average yield for each thiol-ene coupling (Scheme 35).

An efficient dual labeling of a calix[4]arene platform with two different carbohydrate fragments at the lower and upper rim was carried as shown in Scheme 36. The method involved a sequence of thiol-ene coupling and Cu(I)-catalyzed azide−alkyne cycloaddition. Thus, tetraene and tetrayne functionalized calix[4]arene **164** was first reacted with galactosylmethyl azide **165** and then with glucosyl thiol **161**. Compound **168** thus prepared was the first example of differentially glycosylated calixarene. Given the specificity of carbohydrate−lectin recognition, it was anticipated that **168** can interact at the same time with two different lectins.

2.5. Calix[4]resorcarene *O*-Glycosides

Calix[4]resorcarenes are cyclo-oligomers formed from the condensation of aldehydes with resorcinol in the presence of acids.[3a,b,83] These macrocycles exist in various diastereomeric forms due to the different relative configurations of the four substituents linked to the bridging methylene groups (derived from the aldehyde used in the condensation) and the cone−cone interconversion, similar to that of calix[4]arenes.[3a,84] For

Scheme 36



example, the bowl-shaped conformation adopted by **169** (Figure 14) displays four R groups at the lower (narrower) rim in an all-*cis* arrangement and axial orientation while the eight resorcinol hydroxy groups are located on the upper (wider) rim. Upon inversion of the conformation, **169** is converted into **170** that shows the four R groups in the equatorial positions (Figure 14). Although the conformations shown in Figure 14 are commonly adopted by calix[4]resorcarenes, for the sake of clarity these macrocycles are drawn in the present review using a planar projection as reported in the original articles.

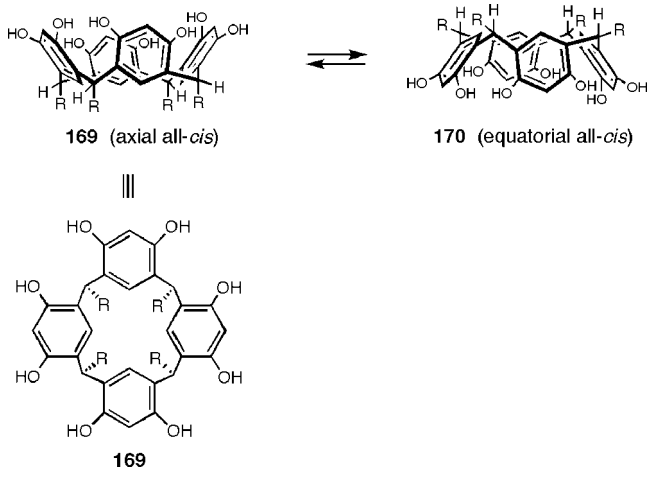


Figure 14. Cone conformations and planar projection of calix[4]resorcarenes.

2.5.1. Assembly via Amide Bond Formation

Research on the synthesis and biological applications of calix[4]resorcarene glycoconjugates was almost all carried out in the laboratory of Aoyama in Japan starting from mid 1990s. Thus, the octagalactopyranosyl calix[4]resorcarene-based cluster **173** was prepared[85] by multiple amide bond formation between the octa-*O*-ethylamino calix[4]resorcarene **171** and the D-lactonolactone **172** (Scheme 37).

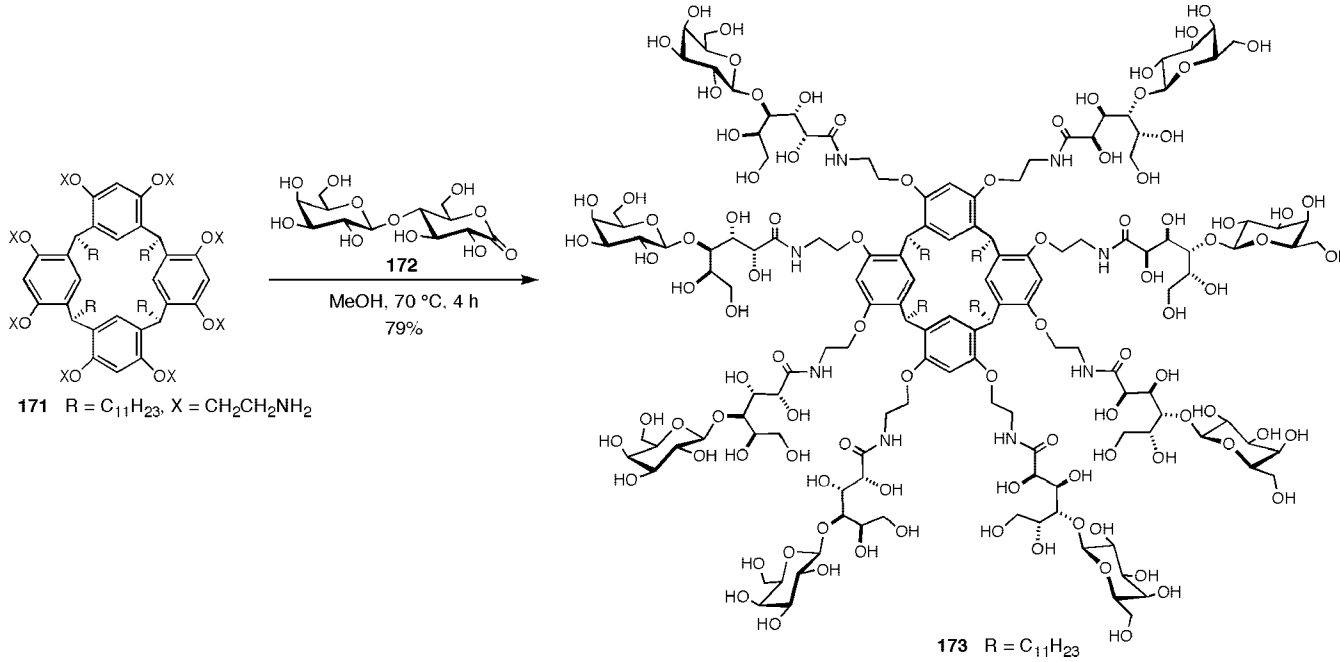
Quite interestingly, despite its amphiphilic nature, the densely glycosylated compound **173** was highly soluble in water. Nevertheless, it was readily adsorbed on the surface of quartz plate (silica glass) dipped in an aqueous solution of the former. The adsorption was practically irreversible, as **173** could not be rinsed off the plate by repeated washing with water. Aqueous solutions of base such as ethanolamine and pyridine were required in order to desorb the galactosyl cluster from the quartz plate. These results suggested that glycocluster **173** formed a closely packed monolayer on the quartz surface via multiple hydrogen bonding involving the free hydroxy groups of the sugar fragments and the silanol and silyl ether functionalities of the silica glass. The **173**-silica hydrogen bonding set could be disrupted only by a nitrogenated base. Moreover, galactose and other carbohydrates neither inhibited the adsorption of **173** on the quartz surface nor promoted desorption of **173** adsorbed thereon. This result indicated that **173** was anchored to the quartz surface in a multivalent fashion involving the above-mentioned hydrogen-bonding systems. As sugar binding via hydrogen bonding was known to be effective only in apolar organic media, it was concluded that the work provided a new strategy for sugar binding in water.[85a]

Another interesting property of compound **173** was its ability to form a stable 1:1 complex with 8-anilinonaphtalene-1-sulfonate (ANS) in aqueous solution.[85] ANS was located within the resorcarene cavity of **173**. Although ANS was not adsorbed onto a quartz surface, it was adsorbed onto this surface in the presence of the glycocluster **173**. The dual role of **173** as host as well as an adsorbate suggested a potential utility of this compound for the delivery of particular guest molecules as drugs or probes to the specific saccharide-receptor biological surfaces.

Finally, the adsorption of **173** to various functionalized monolayers was studied by Okahata and co-workers using a quartz crystal microbalance (QCM).[86] To this end, monolayers made by cationic, anionic, zwitterionic, and nonionic amphiphilic guests were immobilized onto the QCM plate and then exposed to the macrocyclic host **173**. The experiments showed that **173** preferentially adsorbed to anionic surfaces.

In the same way as **173** was the octaglucopyranosyl diastereoisomer **174** (Figure 15) prepared using the D-maltonolactone (yield not given).[87] A highly glycosylated calix[4]resorcarene-based cluster **175** displaying 32 α-D-glucopyranose residues (Figure 15) was obtained[88] in 61% yield from the reaction of the octaamino derivative **171** with maltopentaonolactone in methanol at 60 °C for 6 h. The

Scheme 37



amide bonds anchoring the sugar residues to the macrocycle platform ensured remarkable stability. The densely glycosylated compounds **174** and **175** were shown to bind various phosphate anions in water.[88] The interaction of phosphate anions with the glycoclusters induced the agglutination of the latter compounds. This aggregation phenomenon was almost certainly due to the ability of the phosphate anions to interact via hydrogen bonding with more than one cluster molecule at the time, sticking the clusters together. In this way, simple salts soluble only in water can be readily extracted from water into the clusters that act as macrosolvents for anions.

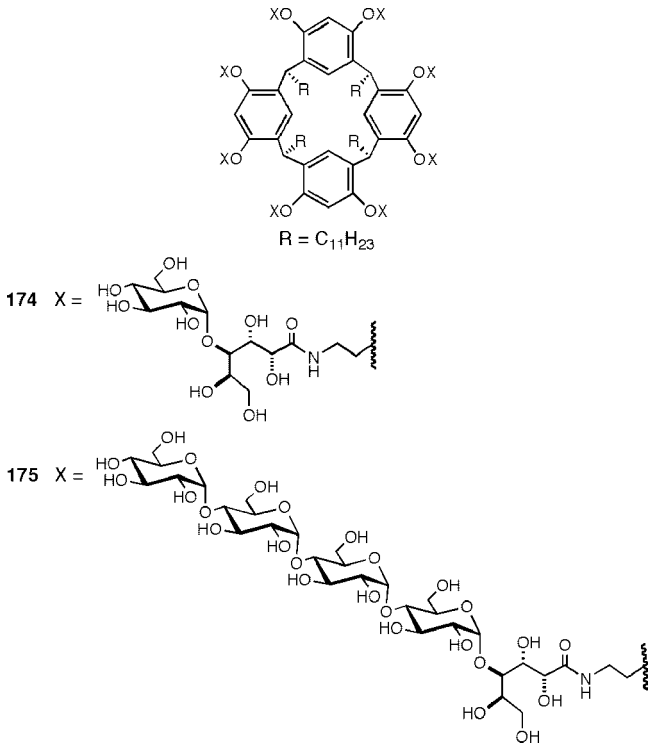
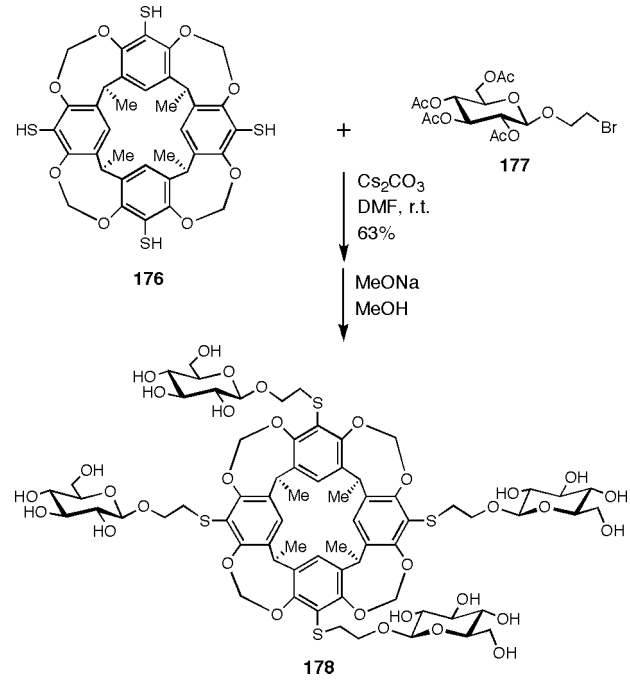


Figure 15. Highly glycosylated calix[4]resorcarenes prepared by Aoyama and co-workers.[87,88]

2.5.2. Assembly via Sulfide Bond Formation

A set of three calix[4]resorcarene-based carbohydrate clusters was prepared[89] by Aoyama and co-workers by introduction of glucose, maltose, and maltotriose fragments at the upper rim of the bowl-shaped macrocyclic tetrathiol[90] **176** (Scheme 38) via alkylation with suitable sugar bromides. Succintly, the cavitand tetrathiol **176** was treated with peracetylated 2-bromoethyl β-D-glucoside **177** in the presence of Cs_2CO_3 to give, after deacetylation, the corresponding product **178** (Scheme 38). In a similar way were obtained the tetravalent clusters **179** and **180** (Figure 16), displaying four maltose and maltotriose units, respectively, linked to the macrocyclic skeleton through sulfide bridges (yields not given).

Scheme 38



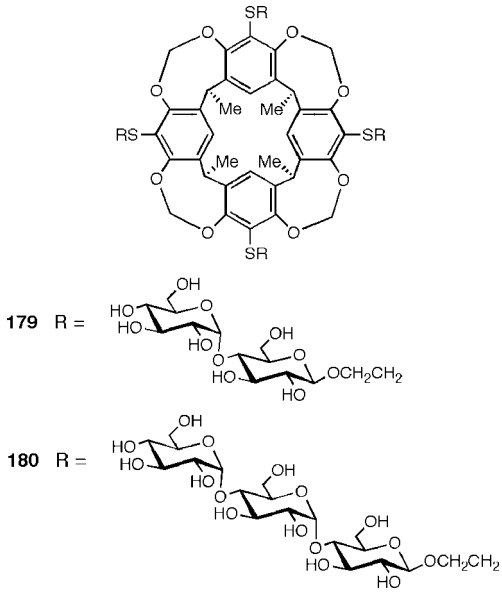


Figure 16. *O*-Glycosyl calix[4]resorcarenes prepared by alkylation of the macrocyclic tetrathiol **176**.

The guest binding behavior of hosts **178**−**180** in water toward 8-anilinonaphtalene-1-sulfonate (ANS), a fluorescent probe whose emission is sensitive to change in microenvironmental polarity, was examined. The formation of host−guest complexes in 1:1 molar ratio was revealed by the change in fluorescence intensity in the following sequential order with respect to the hosts: **180** > **179** > **178**. Calculated binding constants K and microenvironmental polarity parameters E_T^N appeared to be subjected to change by the size, shape, and hydrophobic character of the three-dimensional internal cavity of the hosts. Finally, it was demonstrated by turbidimetric analysis that glycoclusters **178**−**180** all recognize Concanavalin A (ConA), the major binding affinity being shown by **179** having four terminal α-D-glucosyl residues. On the other hand, peanut lectin, a galactose binding lectin, did not interact with the above clusters.

2.5.3. Biological Tests with Calix[4]resorcarene O-Glycosides

2.5.3.1. Binding Assays with Lectins. Calix[4]resorcarene *O*-glycosides **173** and **174** proved to recognize lectins in an efficient and selective manner.[91] Binding tests were carried out by passing a solution of the selected lectin over the cluster **173** or **174** immobilized on a hydrophobic surface plasmon resonance (SPR) sensor chip. The binding of the lectin to the immobilized cluster was monitored by measuring the refractive index change of the SPR chip. Thus, a strong interaction was detected between the octagalactopyranosyl cluster **173** and peanut agglutinin (PNA), a lectin that selectively binds galactosyl residues. On the other hand, no substantial interaction appeared to occur between **173** and Concanavalin A (ConA), a lectin that binds both mannosyl and glucosyl residues. This lectin was instead recognized by the octaglucopyranosyl cluster **174**, which in turn did not bind to PNA. Unfortunately, the glycoside cluster effect was not addressed in this study.

The specificity of lectin recognition by glycoclusters **173** and **174** was confirmed by agglutination−deagglutination experiments followed by turbidimetric analysis.[87] A solution of ConA became turbid when treated with compound **174**, while a solution of PNA remained unaltered. Deagglutination of the former solution occurred upon addition of a large excess of glucose as a competitive inhibitor. In a similar way, a solution of PNA, but never ConA, was agglutinated upon addition of compound **173** and deagglutination occurred only with galactose in large excess. Unfortunately, in both cases, the precipitation of the lectin-cluster adducts did not allow the determination of the binding constants.

The specific binding of octaglucosyl calix[4]resorcarene-based cluster **174** with ConA was also proved by using an immobilized ConA-Sepharose gel and by monitoring the absorbance change at 283 nm for **174** in the aqueous phase.[87] It was shown that upon addition of the gel to an aqueous solution of **174**, 73% of the latter was adsorbed on the gel. The adsorption was reduced to 15% in the presence of a large excess of glucose. Finally, a very low affinity (6−8% of adsorption) was detected in the case of the octagalactosyl cluster **173** and this was not affected by glucose. It was suggested that the very small adsorption of **173** was due to nonspecific interactions with the Sepharose matrix in the ConA-Sepharose gel.

2.5.3.2. Guest Delivering to Immobilized ConA-Sepharose and to Cells. Aoyama and co-workers reported a fine study demonstrating the ability of octaglycosyl calix[4]resorcarene-based clusters to deliver guest molecules to immobilized ConA-Sepharose gel.[87] The simple machinery of the system relied on the specificity of the carbohydrate−lectin interaction. Although both galactose- and glucose-based clusters **173** and **174** proved to form stable 1:1 complexes with the dye eosyn Y **181** (Figure 17), only the cluster **174** served to transport the dye **181** onto the surface of the ConA-Sepharose gel due to the specific recognition of glucose, not galactose, by ConA. The dye **181** was in fact encapsulated by both the clusters **173** and **174**, but only the **174**·**181** complex containing glucosyl residues formed a gel/host/guest ternary complex with Concanavalin A immobilized on Sepharose.

Calix[4]resorcarene-based carbohydrate clusters proved to be valuable molecular carriers to transport guest molecules even to cells.[92] Aoyama and co-workers examined the delivery of the fluorescent dye phloxine B **182** (Figure 17) to hepatocytes (liver cells) of mice, which contained a cell surface lectin (the asialoglycoprotein receptor) that was known to specifically bind terminal galactose residues. The multivalency of glycoside clusters was thought to overcome the problem of nonspecific affinities occurring via hydrophobic interactions. The octagalactosyl calix[4]resorcarene-

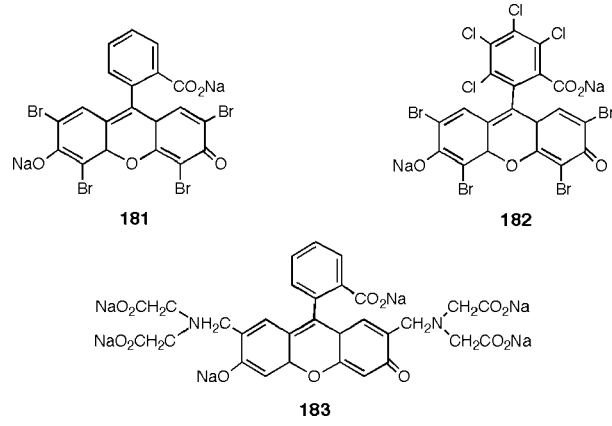


Figure 17. Dyes used in complexation experiments with octaglycosyl calix[4]resorcarene-based clusters.

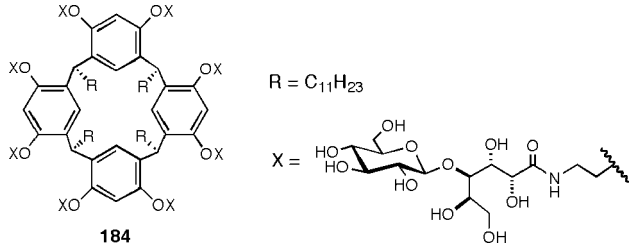


Figure 18. Octaglucosyl calix[4]resorcarene prepared by Aoyama and co-workers.[94a]

based cluster **173** and the octaglucosyl diastereomer **174** were once again selected as suitable host molecules in this study. First, it was shown that in the absence of any host, guest **182** bound to rat hepatoma (liver cancer) cells as a consequence of hydrophobic interactions. Then, it was shown that when the complex **173·182** was added to the same cancer cells, it caused the cells to fluoresce, thus demonstrating that the complex was bound by the cells as a consequence of the specific interaction between the terminal galactosyl residues present in the cluster **173** and the asialoglycoprotein receptor on the cell surfaces. On the other hand, no fluorescence was detected from the cells when the cluster **174** was used as a carrier of the dye **182**, thus indicating that the complex **174·182** was inert to the cells. The importance of the guest delivery by specific host−cell interaction was further demonstrated by using calcein **183** (Figure 17), a less hydrophobic dye, which in fact, when alone, showed little affinity to the hepatoma cells. On the other hand, **183** could be delivered to the cells by the galactose cluster **173** and not by the glucose analogue **174**. Moreover, **173**-mediated cell delivery was also cell-specific. In fact, when mouse spleen LT4Tr cells lacking the galactoside receptors were used instead of hepatoma cells RLC-16, the galactose cluster **173** inhibited the adsorption of the guest **182** on the cells. In the conclusion of these studies, the authors stressed the importance of hydrophobicity masking for the carbohydrate-directed cell recognition and the crucial role played by the identity of the sugar moieties of the clusters. In particular, the octagalactosyl calix[4]resorcarene-based cluster underwent specific saccharide receptor interactions with the complementary hepatic cells, while the octaglucosyl analogue was not recognized at all by the cells. The included guest molecules were either delivered to the target cells or protected in solution away from the cells.

2.5.3.3. Formation of Nanoparticles and Artificial Glycoviruses. While the issue of multivalency effects was addressed by a study of self-aggregation, phosphate-induced agglutination, and micellar nanoparticles formation by various calix[4]resocarene glycoconjugates,[93] the saccharide-dependent self-aggregation of a set of glycocluster amphiphiles and the formation of artificial glycoviruses was investigated by the Aoyama group.[94,95] The glycoclusters were prepared by the usual amide bond formation by coupling the calix[4]resorcarene-based octaamine **171** with three disaccharidic lactones (D-*lacto*, D-*malto*, D-*cello*). The use of large excess of lactone (32 equiv) provided the octavalent clusters **173** (Scheme 37), **174** (Figure 15), and **184**[94a] (Figure 18), respectively, while the use of limited amounts of lactone (5−7 equiv) gave rise to mixtures of regioisomers bearing five pyranosidic residues (β-D-Gal, α-D-Glc, or β-D-Glc, respectively) and three unreacted amino groups in in 33−43% yield.[94b]

All glycocluster amphiphiles irreversibly formed micellar aggregates, called glycocluster nanoparticles (GNPs), in water as shown by combined evidence from gel permeation chromatography (GPC), dynamic light scattering (DLS), and transmission electron microscopy (TEM). Thus, molecular weight (20000−22000), aggregation number (5−7), as well as the size (4−6 nm) of GNPs were determined. It was pointed out that GNPs arise from micellization of the cone-shaped glycoclusters where the sugar moieties play a key role as evidenced by the fact that octaamine **171** failed to give GNP-like particles. In analogy to natural viruses in which a single nucleic acid (DNA or RNA) is coated with a well-defined number of capsid proteins, **184**-derived GNPs were found to bind to a supercoiled pCMVluc plasmid DNA having a reporter gene for firefly protein luciferase and a cytomegalovirus promoter.[94a,96] The result of this complexation was the formation of an artificial glycovirus with a size of 54 nm and having a stoichiometry of 2 GNPs per helical pitch. This maximal accommodation was understandable on the basis of steric ground. It was suggested that the GNP−DNA complexation was driven by multiple hydrogen bonding between the OH groups of the carbohydrate residues of GNP and the phosphate groups of DNA. The octavalent clusters **173** and **174** as well as all the pentavalent clusters were also able to bind to the above-mentioned plasmid, but the resulting GNPs underwent self-aggregation.[94b] Finally, it was demonstrated that the glycoviral particles pCMVluc·(∼1600GNP) served as artificial viral vectors.[94] They transfected Hela cells in a serum-free medium and more effectively in the presence of fetal bovine serum with little cytotoxicity. Also hepatic cells HepG2 proved to be transfected by these artificial glycoviruses via a nonspecific but highly size-regulated endocytic pathway. Thus these viruses were endowed with the intrinsic function of natural viruses, that is cell invasion followed by gene expression. Later on, the same research group found that calix[4]resorcarene-based glycoclusters bearing only three β-D-galactopyranoside units (mixtures of regioisomers) were more suitable for the gene delivery to the hepatocytes than the octavalent (**173**) or pentavalent analogues.[97]

2.5.3.4. Inhibition of Cell Adhesion. Chondroitin sulfate proteoglycans, polypeptides bearing several glycosaminoglycan polysaccharidic chains, are components of the extracellular matrix that inhibit the cell adhesion by interaction with specific cell-surface receptors. Because the cell adhesion is involved in many cellular processes such as differentiation, growth, translocation, and angiogenesis, its inhibition may find therapeutic applications. The glycocluster **185** (Figure 19), displaying four chondroitin sulfate chains, was obtained[98] from the corresponding lactone[99] and the octaamine **171** in 16% yield. This cluster constituted a new proteoglycan mimic endowed with strong antiadhesive properties. Aoyama and co-workers found that the adhesion-triggered growth of BHK cells, incubated in a fibronectin-coated polystyrene plate, was totally suppressed in the presence of **185** at picomolar concentrations.[98] The glycocluster **186**, obtained from **185** by further functionalization with fluorescein, was used in control experiments to demonstrate that these bulky (MW ∼60000) polysaccharide clusters were irreversibly adsorbed onto the hydrophobic surface of the plate through their undecyl chains located at the lower rim. It was also found that a monovalent analogue of **185** as well as the glycocluster **173** (Scheme 37) bearing eight galactopyranosyl moieties, were totally inactive in the inhibition of the BHK cell adhesion.

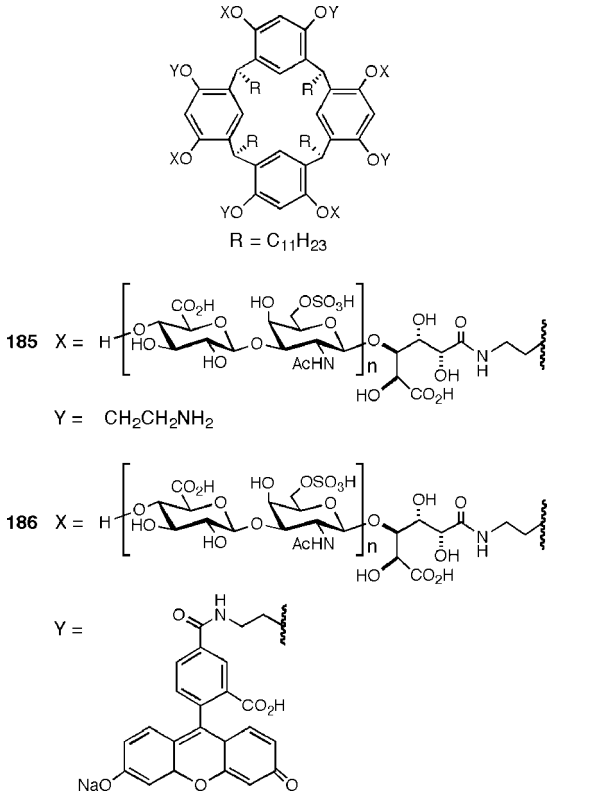


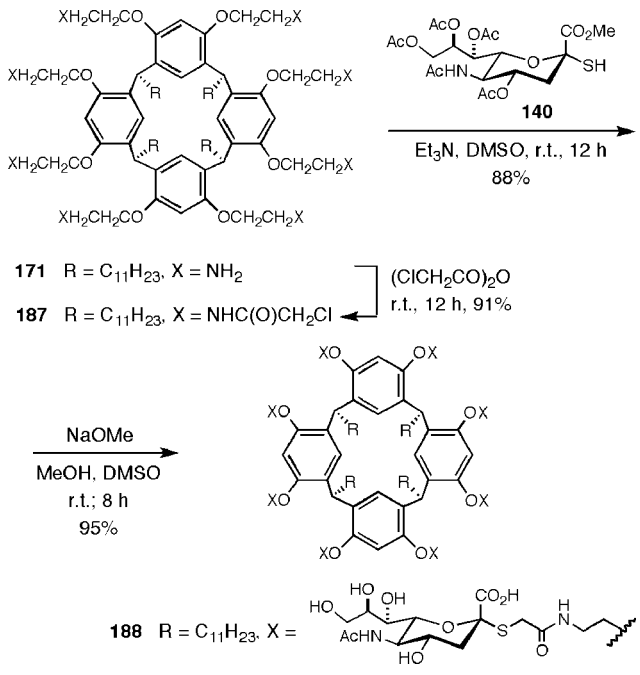
Figure 19. Calix[4]resorcarene-based clusters bearing chondroitin sulfate chains.

2.6. Calix[4]resorcarene *S*-Glycoside

2.6.1. Assembly via Sulfide Bond Formation

A single compound **188** was prepared in which eight thiosialic acid residues were installed at the upper rim of the calix[4]resorcarene scaffold bearing four undecyl groups as alkyl tails at the lower rim (Scheme 39).[100] The starting compound was the usual calix[4]resorcarene-based octaamine **171**. This was transformed into the octa(chloroacetamide) **187** which in turn was allowed to react with the protected sialyl thiol **140** from which hydrolysis of the methyl ester and *O*-acetyl groups of the sugar moiety afforded the target calix[4]resorcarene-based sialocluster **188**.

Scheme 39



The sialocluster **188**, owing to the four undecyl chains, was immobilized from an aqueous solution on a SPR sensor chip (gold) hydrophobized by long-chain alkanethiol molecules. From adsorption determination by surface plasmon resonance (SPR), it was inferred that the adsorbate **188** formed a closely packed monolayer with its long alkyl chains embedded in the hydrophobic surface of the chip. A sialo-targeting Japanese horseshoe crab lectin was readily adsorbed on the saccharide residues exposed to bulk water. The increase of resonance units indicated a **188**-lectin interaction much larger than that for the mannose/glucose-specific lectin Concanavalin A.

Given the well-known ability of sialylated oligosaccharides to act as ligands for influenza viruses (see also section 2.4.2.2), it was also demonstrated that cluster **188** interacted with influenza A viruses. Positive tests were obtained from the determination of the inhibition by **188** of the hemagglutination of human erythrocytes and cytopathic effect on MDCK cells, mediated by three different strains of influenza A viruses, at concentrations of 5−10 μM.

3. Conclusion

As concluded by Fulton and Stoddart in their 2001 review,[4] also the present review points out that the development of synthetic methods to calix[4]arenes glycosides by a variety of ligation strategies has been more deeply considered than the application in glycobiology, particularly with respect to molecular recognition of lectins and cells. The progress in preparative approaches, however, is well documented by impressive results in the synthesis of new glycoconjugates, especially by the fashionable Cu(I)-catalyzed azide−alkyne cycloaddition. Nevertheless, in these nine years, the gap between chemistry and biology of calix[4]arenes has been certainly reduced as even synthetic chemists in various laboratories have combined their efforts toward the preparation of calixsugars by new methods with studies in glycobiology and glycomedicine. Also, the issue regarding the glycoside cluster effect has been addressed in various papers. Another important novelty is the study of the interactions of calix[4]arene-based carbohydrate clusters, i.e. sialoclusters, with viruses.

An opposite scenario is presented in the field of calix[4]resorcarenes where biology is overwhelming synthetic chemistry. Using a few molecular structures constituted of the macrocyclic scaffold decorated with carbohydrate fragments at one rim and four lipophilic alkyl chains at the other rim, a huge number of impressive biological studies have been reported by a Japanese group. These spanned from specific recognition of lectins to interaction with influenza viruses.

In conclusion, numerous synthetic and biological studies have emerged from the simple idea conceived in an Italian laboratory (1994) and consisting of installing carbohydrate moieties at each rim of a calix[4]arene scaffold. The studies that followed in many laboratories have considerably broadened the scope of calix[4]arenes from merely ion receptors to efficient auxiliaries in supramolecular chemistry. It is likely that the latter is the new direction toward which the use of calixsugars will be oriented in the next years.

4. References

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