Recent Advances on Cyclopeptide-Based Glycoclusters

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Abstract: Many important biological processes including fertilization, tissue formation, cell adhesion, antigen/antibody interactions, cancer metastasis or pathogen infection involve polyvalent interactions between oligomeric proteins and carbohydrate-based ligands. The synthesis of multitopic, molecularly-defined structures that display both clustered sugars and immobilization or bioactive agents represents an attractive approach for the discovery of new diagnostics and therapeutics. This mini-review updates the current developments in this field by focusing on the recent interests on cyclopeptide-based glycoclusters.

Keywords: Cyclopeptide, glycocluster, multivalency, carbohydrate, solid-phase synthesis, oxime ligation.

1. INTRODUCTION

Due to their involvement in a wide range of physiological and pathological processes, the interactions between carbohydrates and proteins have been extensively explored over the last few years [1, 2]. The recent emergence of glycomics has strengthened this interest and notably contributed to spectacular progress in understanding how carbohydrates mediate and regulate these complex biological events [3-5]. By contrast with monovalent interactions which are, in principle, weak and poorly specific, it is now well established that a multivalent display of carbohydrates warrants simultaneous and cooperative contacts with multiple binding sites of proteins. This so called 'glycoside cluster effect' results thereby in a strong and specific recognition between a multivalent ligand and its receptor [6, 7]. For this reason, the design of a glycocluster that is capable of mimicking the multivalent display of the cell surface glycocalix should be considered, not only to extend the deciphering of the thermodynamic and kinetic parameters of biological processes, but also to discover new diagnostic and therapeutic agents [8, 9, 10].

Glycocluster chemistry and related biomedical applications represent a fascinating research area as proved by the increasing number of studies in this field [11]. The typical approach for the preparation of bio-recognizable glycoclusters consists, on the basis of the three-dimensional structure of the target protein, in grafting multiple copies of identical carbohydrates on the surface of a well-defined molecular scaffold (or template) through a suitable spacer. Because it was shown that the biological potency is closely related to the structural feature of the ligand architecture rather than the number of sugar copies [12], the nature of the scaffold should be carefully selected so that the multivalent presentation of oligosaccharides matches with the target binding sites. To this end, a wide panel of structures offering variable topology, valency and density was designed so far. For example, some linear polymers [12], cyclic cores such as calixarenes [13] and cyclodextrins [14] or branched structures such as dendrimers [15] expanding cluster of oligosaccharides in various spatial orientation (Fig. **1**) were shown to exert impressive biological properties. Indeed, further association with a suitable bioactive agent may provide not only antibacterial and anticancer vaccines but also delivery and detection systems. These topics were largely covered by excellent reviews published earlier [11-15]. Among other impressive approaches, cyclopeptides are subjected to recent interests because they were recognized as attractive templates for the assembly of biomolecule-based ligands [16, 17]. This mini-review is not intended to give an entire over view of the glycocluster story but rather focuses on the chemistry of cyclopeptide-based molecules that exhibit a multivalent display of oligosaccharides as well as their utilization for various biological applications.

2. HISTORICAL

In 1988, Mutter and co-workers proposed a new molecular scaffold, namely Template-Assembled Synthetic Protein (TASP), as a versatile tool for the design of artificial proteins [18]. Further extensions have lead to the exploration of various fields of application related to protein mimicry and molecular recognition [16, 19]. In this innovative concept, a conformationally restricted cyclopeptide core is used to stabilize the secondary structure of peptide building blocks in a well-defined spatial orientation and native-like folding topology. This scaffold, also known as Regioselectively Addressable Functionalized Templates (RAFT) [20], is a cyclic decapeptide composed of two proline-glycine (Pro-Gly) β turn that stabilize its conformation into an antiparallel β sheet (Fig. **2**).

Up to six amino acid residues containing easily functionalizable side-chain (e.g. lysine represented in the molecular model shown in Fig. **2**) which are equatorially oriented on both sides of the cycle plane can be incorporated into the peptide backbone, hence providing two independent domains with regioselectively addressable anchoring sites (Fig. **2**). Together with this structural feature, the large diversity of commercial orthogonally protected amino acids makes this modular scaffold easy to handle and useful for the stepwise attachment of various structural or functional units in controlled manner.

By analogy, Kunz *et al.* have more recently exploited the conformational properties of a cyclopeptide scaffold for the multivalent assembly of carbohydrate-based ligands [21]. This study, which represents the first synthesis of biologically active glycocluster graft to a cyclopeptide backbone, was then followed by diverse approaches differing by the design, the synthetic method and the expected biological applications.

3. SYNTHESIS OF CYCLOPEPTIDE-BASED GLYCOCLUSTERS

The chemistry of glycoconjugate is far from being trivial, mainly because of the polyfunctionality of both peptide and oligosaccharide and the relative instability of glycosidic linkages under certain conditions. The incorporation of a carbohydrate moiety to the peptide backbone in a predefined position and with an expected configuration is essential to warrant the well-defined structure of the target molecule. It requires the selection of proper orthogonal protecting groups, activations steps and involves in most cases time-consuming purifications. While major progresses were made to secure the molecular assembly of structurally complex molecules, it still remains difficult to propose a general synthetic route because each molecule might be considered as a particular case. Thus, various synthetic approaches were explored, going from solution and solid-phase protocols to chemoselective ligation.

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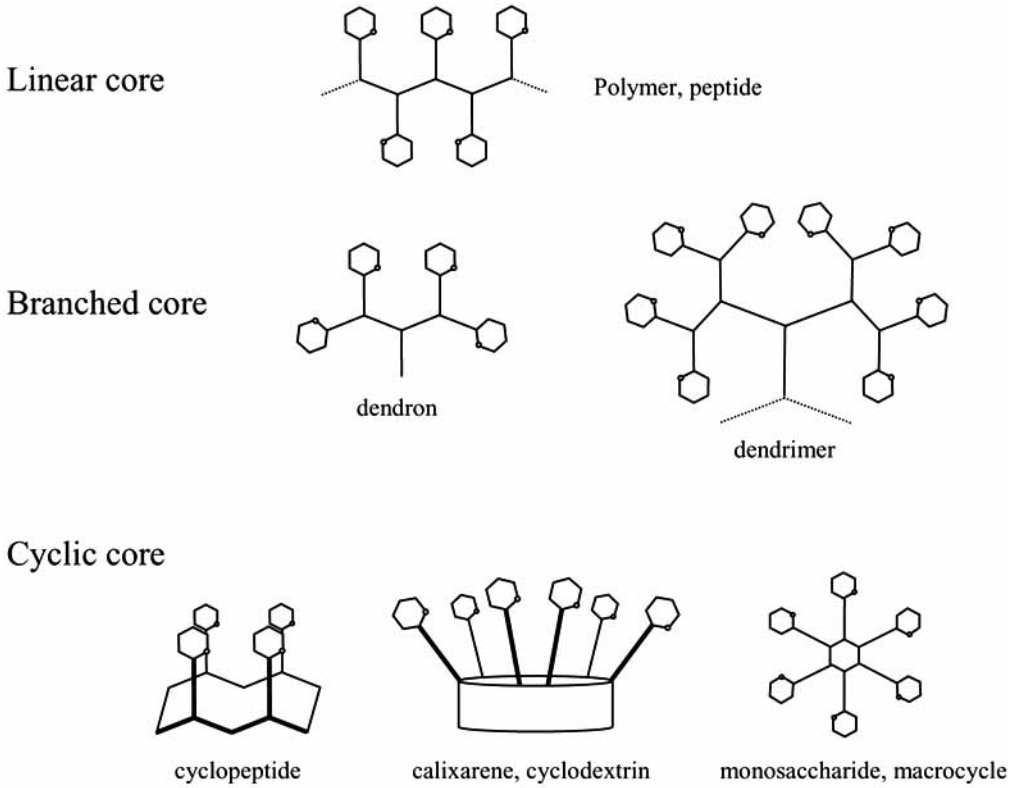


Fig. (1). Different type of glycocluster architectures.

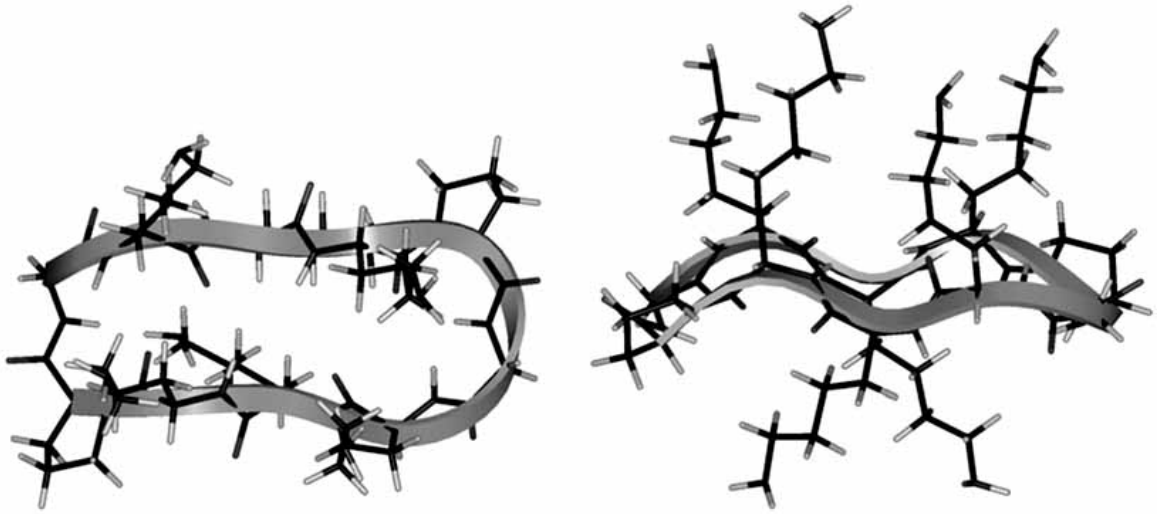
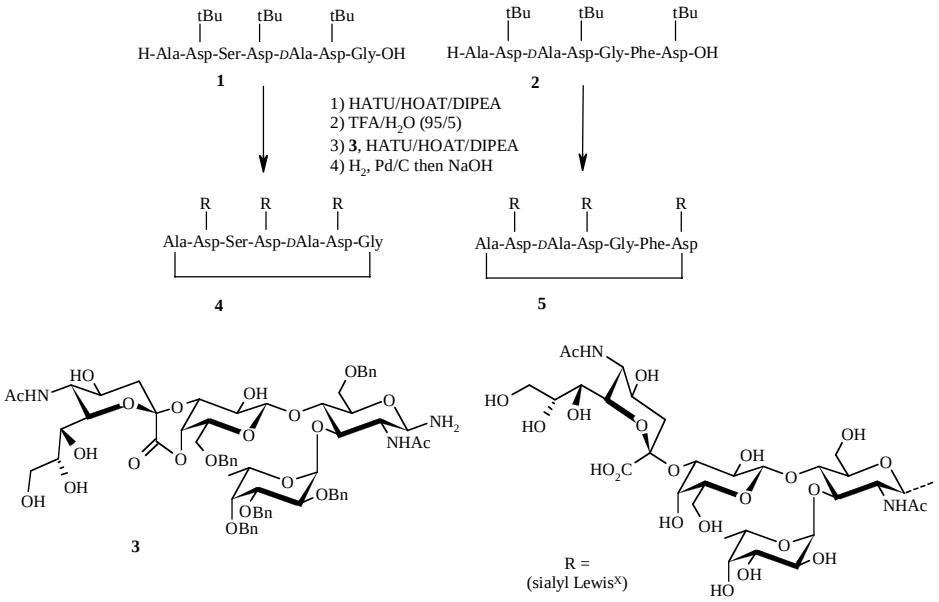


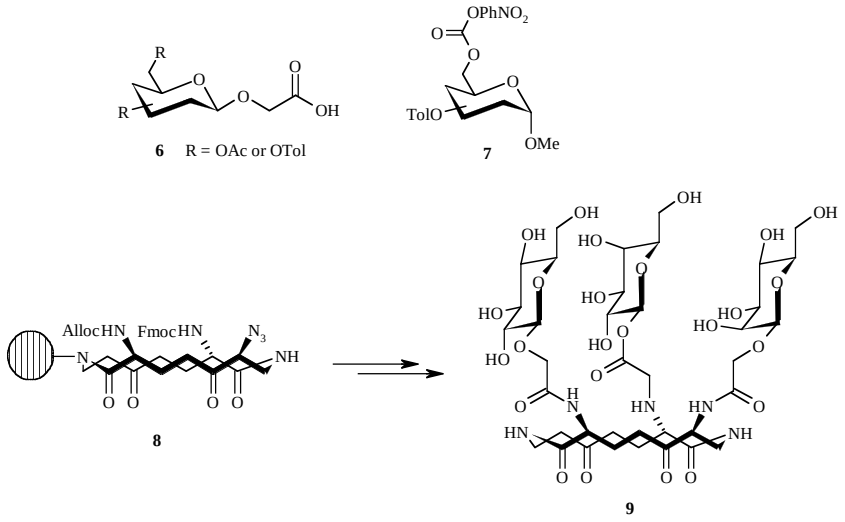
Fig. (2). Molecular model of the RAFT platform composed of two Gly-Pro *β*-type II turn inducers and six addressable lysine pointing on both sides of the template. (left: top view; right: side view). This model was kindly prepared by Prof. Julian Garcia.

3.1. Convergent Synthesis by Fragment Condensation

Kunz *et al.* have reported the synthesis of a sialyl-Le^x containing cycloheptapeptides by a combined supported/ solution protocol [21] in which both peptide and oligosaccharide counterparts are prepared independently (Scheme **1**). Two linear, orthogonally protected heptapeptides **1**-**2** containing D-alanine and three aspartic acids (Asp) were first designed and synthesized on solid-phase peptide synthesis (SPPS) following the standard Fmoc/*t*Bu (9-Fluorenyl-methyloxycarbonyl/*tert*-butyl) strategy on acido-labile SASRIN (Super Acid Sensitive Resin) resin. After release from the support under mild acidic conditions, the head-to-tail cyclization step of **1**-**2** was subsequently realized in solution under high dilution to secure the intramolecular reaction. This reaction has happened between both free *N*- and *C*-terminal ends using HATU/HOAT (*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyl-uronium hexafluorophosphate/1-hydroxy-7-aza benzotriazole) coupling reagents and DIPEA (Diisopropyl ethylamine) as a base. The fully deprotected cyclic peptides were obtained after removal of the *t*Bu protecting groups by acidolysis with TFA (trifluoroacetic acid) in the presence of 5% of water as carbocation scavenger. NMR experiments have confirmed the sequential integrity and the restricted conformation of the scaffold. The further incorporation of the sugar moiety was realized in solution to the Asp side chain by amide bond formation using the Lansbury aspartylation [22]. This coupling reaction occurred with a sialyl Lewis^x **3** bearing a β-amino group at the anomeric position and partially protected with benzyls and 4'-lactone to afford an asparagine linked glycan. Des-



Scheme 1.



Scheme 2.

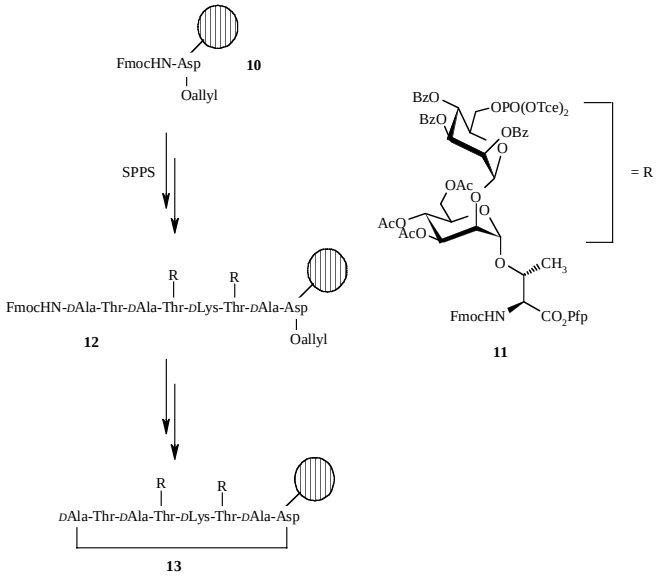
pite of the presence of adjacent bulky amino acid near to the Asp anchoring site, the *N*-glycoside formation step was found to be complete using the standard amide coupling conditions. The trivalent cyclopeptides **4**-**5** were finally obtained after deprotection of the tetrasaccharide moieties.

A similar strategy was recently adapted on solid support [23]. Orthogonally protected scaffolds were synthesized and subsequently functionalized on solid phase with various sugar units by carbamate or amide bond formation (Scheme **2**). These cyclo-β-tetrapeptides are composed of β-alanine and 2,3-diaminopropanoyl (Dpr) residues to confer a disklike conformation to the scaffold. The α-amino group of the Dpr derivatives was protected by Fmoc, Alloc (Allyoxycarbonyl) or masked with azide to provide orthogonally addressable sites and offer the possibility to introduce different sugar units in the same molecule. Different acetyl- (Ac) or toluoyl- (Tol) protected sugar derivatives **6**-**7** from glucose, galactose and mannose series bearing either carboxymethyl group at the anomeric position or functionalized at C-6 with 4-nitrophenyloxycarbonate active ester were prepared. The linear β-tetrapeptide was assembled on an aminomethyl polystyrene resin after incorporation of the 1-methyl-phenylethyl protected β-alanine derivative by reductive amination. The peptide backbone was then elongated using Fmoc strategy and either HATU or PyAOP (7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate) as coupling reagent. After removal of both *N*- and *C*-terminal ends by treatment with a solution of 1% TFA in dichloromethane, the protected peptide was cyclized using PyAOP in dimethyl formamide (DMF). The incorporation of sugars **6** to the cyclic core **8** was realized using a stepwise procedure involving successive orthogonal deprotection and amide coupling steps using HATU as activator. Whereas the synthesis of divalent glycopeptides has occurred with satisfactory overall yields and purity, the formation of trivalent glycopeptides was found to be closely dependant on the order of deprotection and sugar coupling and also required high excess of reagents. The carbamate coupling with sugar units **7** was performed following a similar procedure with the exception of coupling reagent. After cleavage from the support by treatment with TFA and acyl deprotection, ten glycoclusters such as **9** combining different sugars were purified by reverse phase HPLC and analysed by mass spectrometry.

It is noteworthy that a chemoenzymatic procedure using both transglutaminase and glycosyltranferase was also reported to conjugate oligosaccharide moieties on glutamine residues [24]. The biological effects of the resulting glycoclusters will be discussed in the next part of this manuscript.

3.2. Utilization of Glycosyl Amino Acid Building Blocks

In another approach, Meldal *et al.* have reported the synthesis of multivalent cycloglycopeptides using pre-synthesized glycosyl amino acid building blocks and a solid-phase cyclization protocol [25]. On the basis of previous studies on short linear peptides glycosylated with mannose 6-phosphate moieties, the authors were interested in constraining the conformation of their derivatives by cyclization to optimize the arrangement of the carbohydrate units. The binding efficacy to a mannose 6-phosphate receptor was then investigated to evaluate the influence of this structural modification. As for the TASP concept, their cyclopeptidic scaffold alternate D- and L- amino acid, not only to facilitate the further cyclization but also to ensure a suitable spatial orientation of the anchoring sites (Scheme **3**).



Scheme 3.

The solid-phase cyclization requires the initial incorporation of Fmoc-Asp-Oallyl to afford a pre-loaded resin **10** that contains an easily removable allyl protecting group to the α-carboxylic function. Fmoc-protected threonine residue **11** activated as pentafluorophenol ester (Pfp) and glycosylated with α-(1-2) linked mannose disaccharide were prepared from glycosylation between the glycosyl donor bromide and Fmoc-Thr-OPfp using silver triflate as promotor. This building block was then incorporated twice during the SPPS of **12** under basic conditions without coupling reagent, whereas other D-amino acids were coupled using TBTU (*O*-(Benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluo-roborate) activation. After terminal Fmoc deprotection and ally removal by treatment with Pd^0, the solid-phase cyclization of linear glycopeptide was performed using repeated treatments with TBTU /DIPEA. As expected, it is noteworthy that the cyclic glycocluster **13** was obtained with high efficiency as a low level of oligomerization was reported owing to the pseudo-dilution effect [26]. After release of the glycocluster from the support by acidolysis, sugar deprotection and preparative reverse-phase HPLC purification, the expected divalent glycosyl cyclopeptides were obtained with acceptable overall yields (20-30%).

3.3. Chemoselective Condensation on Cyclopeptide Scaffold

To overcome the synthetic limitations inerrant to the synthesis of glycoconjugate mentioned above, chemoselective ligation strategies have proved a very attractive alternative because they do not require neither protection nor activation steps for the assembly of complex biomacromolecules [27]. Among these methods, oxime bond formation first developed by Rose for the preparation of homogeneous artificial proteins [28], has found increasing applications over the last decade. This linkage results from the highly chemoselective and quantitative condensation of two molecules bearing either an aldehyde or a keto group and a super nucleophile aminooxy function under mild acidic conditions, despite of the presence of other reactive chemical functions. For the preparation of glycoconjugate, several synthetic procedures based on Mitsunobu coupling [29] or phase transfer catalysis [30] were developed to introduce an aminooxy function to the anomeric position of sugars. More recently, another efficient route using glycosyl fluoride donors was described by our group. *N*-hydroxyphthalimide was introduced by a glycosylation reaction as masked aminooxy group in the presence of $BF_3.Et_2O$ as promoter [31]. This strategy was used successfully to prepare a large variety of biologically relevant carbohydrates (e.g., mannose **14** and fucose **15** [32]) or tumor-associated carbohydrate antigens (e.g., Tn **16** and Thomsen-Friedenreich **17** antigens analogues [33]) with either α or β anomer configuration (Fig. **3**). Such aminooxy carbohydrates can be efficiently incorporated into peptides containing aldehydes or ketone to afford glycoconjugates presenting a well-defined anomer configuration [34].

In our laboratory, we have taken advantage of the topological features of RAFT scaffold to prepare glycoclusters using a combined solid-phase/solution strategy and oxime ligation. We have designed and synthesized several cyclodecapeptides containing a various number of protected lysine (Lys) residues dedicated to the assembly of bioactive molecules in well-defined, independent spatial orientation (Fig. **3**). When incorporated at positions 3, 5, 8 and 10 of the backbone **18**, it was shown that Lys side chains are preferentially oriented on the lower face of the template, whereas the incorporation of one or two more, orthogonally protected Lys at positions 4 and 9 provide additional anchoring sites pointing on the lower face [35]. Hence, this design confers two topological addressable domains that can be decorated, depending on the expected biological effect, by multiple copies of carbohydrate (upper domain) and other bioactive molecules (lower domain) to provide both recognition and effector properties in a modular manner.

Typically, a linear orthogonally protected decapeptide **19**, precursor of the RAFT molecule, was prepared using automated peptide synthesis on acido-labile support such as SASRIN or *o*-chlorotrityl chloride resin (Scheme **4**). In the case of RAFT molecule exhibiting one addressable domain, four Lys residues protected with Alloc were used [36]. After removal of Alloc with Pd^0, protected serine residues were incorporated to the Lys side chain as masked aldehyde functions [37]. The linear peptide was then cleaved from the support and the head-to-tail cyclization achieved by treatment with PyBOP/DIPEA (benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate) at 0.5 mM in DMF.

It is noteworthy that glycine residue was chosen as the first amino acid to secure the cyclization step from epimerization. The subsequent serine deprotection has afforded **20** which was successively treated with sodium periodate generated four glyoxo-aldehyde functions by oxidative cleavage [37]. The final oxime coupling from **21** with fully deprotected aminooxy sugars was achieved in sodium acetate buffer pH 4 to give various glycoclusters such as **22** in excellent yield (80% after semi-preparative HPLC) and purities as proved by the crude HPLC profile [33, 36]. As an alternative to the glyoxo-aldehyde formation by periodate oxidation, the direct introduction of a methyl ketone moiety was proposed [38]. To this end, a levulinic acid building block was coupled as Pfp ester to the Lys under basic conditions with DIPEA in DMF to obtain **23** (Scheme **5**). However, the efficiency of oxime ligation between aminooxy sugars and methyl ketone was proved deceptive (40-60% yield) in comparison with the previous glyoxo-aldehyde method.

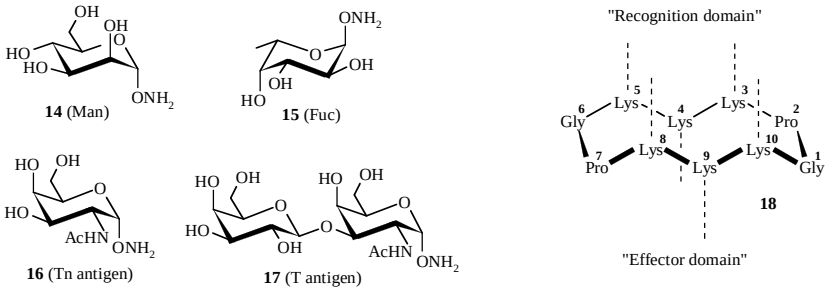
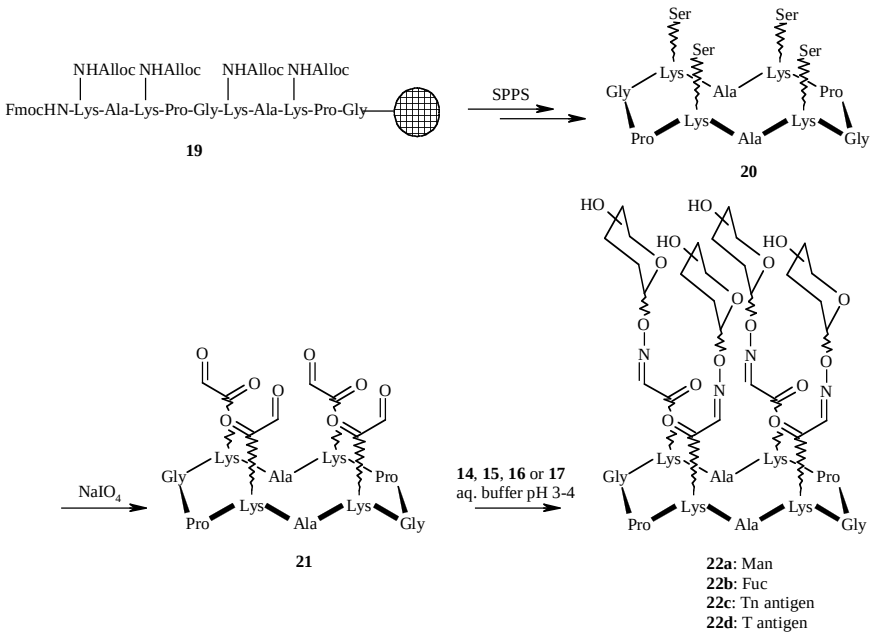


Fig. (3). Chemical structure of a few aminooxy sugars and a RAFT template.



Scheme 4.

The incorporation of other partners oriented through the second addressable domain can be achieved by several strategies. For example, molecular probes such as biotin or fluorescein were grafted respectively by either amide or isothiocyanate bond on the side chain of two supplementary Lys to afford the labelled glycoclusters **24** that are suitable for biological investigations [38]. This synthetic route was recently improved by the development of a fully solid-phase protocol in which D-glutamic acid was used a first amino acid (Scheme **5**) [39].

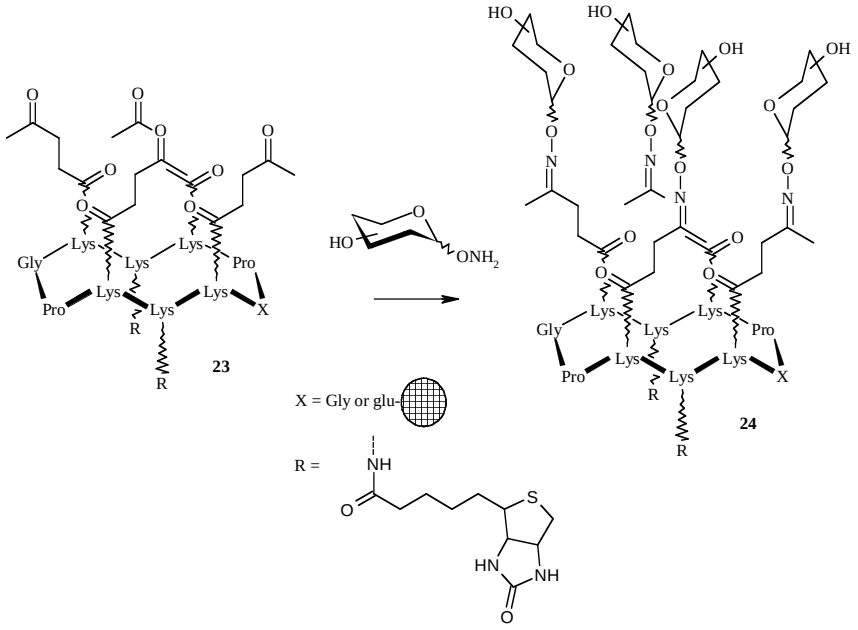
Another strategy using a sequential oxime bond formation was developed [40]. This procedure has lead to the synthesis of an original glycocluster-oligonucleotide conjugate [41]. In this study, a lactosyl cluster was first prepared by chemoselective condensation between cyclopeptide **25** and aminooxy lactose **26** (Scheme **6**). A protected aminooxy function was then incorporated to the resulting glycocluster to obtain **27**. After Boc removal by treatment with TFA, an oligonucleotide **28** bearing a 5' aldehyde-containing linker was subsequently coupled by oxime ligation. The conjugate **29** was found to retain both glycocluster binding affinity and recognition specificity for the complementary oligonucleotide sequence, confirming the absence of steric hindrance between both addressable domains.

3.4. Combinatorial Chemistry

Combinatorial technology comprises a convenient tool to successively prepare and identify in a short time, new biologically active compounds from libraries of structurally diverse molecules. Numbers of approaches were notably adapted for the chemistry of glycoconjugates [42], for example to highlight the binding properties of carbohydrate-binding proteins (CBP). To this end, two different strategies allowing the generation of combinatorial libraries of cyclopeptide-based glycoclusters were described either on solid-support or in solution.

3.4.1. Split and Mix Solid-Phase Synthesis

An interesting study reported by Wittmann and Seeberger has described a combinatorial procedure to synthesize large libraries of conformationally restricted glycoclusters presenting sugars with various valency and density [43]. Their method relies on a "split and mix" synthesis of cyclopeptide scaffolds alternating different amino acids and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)iso-valeryl (Ddv)-protec-ted residues with variable side chain length at different positions of the peptide framework (Scheme **7**). The synthesis was performed using the standard Fmoc strategy on the acido labile Sieber-functionalized Tentagel resin **30** to allow the HPLC analysis and mass spectrometry of each intermediate after treatment of an aliquot of resin under mild acidic conditions. After having optimized the experimental conditions with a model cyclooctapeptide, the procedure were extended to the split and mix method. Linear octapeptide precursors **31** were first prepared then allyl ester and Alloc of both *C*- and *N*-terminal ends were removed by treatment with Pd^0. The peptides of the library were cyclized using HBTU/HOBt/DIPEA to obtain **32**. Interestingly, a new urethane-type linker was developed to graft quantitatively the sugar moiety to the peptide side chain and to facilitate the single-bead analysis of the library by microsequencing under standard Edman degradation [44]. *N*-acetylglucosamine (GlcNAc) **33** bearing the above linker and activated as *p*-nitrophenyl carbamate was thus



Scheme 5.

introduced after Ddv removal with hydrazine. A 18-members library of mono-, di and trivalent glycoclusters **34** was thus prepared successfully.

3.4.2. Mixture-Based Randomized Combinatorial Library

Dumy and co-worker have proposed an original and robust strategy to generate randomized mixture-based combinatorial libraries of glycoclusters presenting various carbohydrates and/or amino acids using an oxime-based strategy [45]. By contrast with classical glycoclusters, these so-called heteroglycoclusters (hGC) were expected to reflect the heterogenic composition of the cell surface glycocalix. Because carbohydrates and proteins interact mainly by forming hydrogen bond network and hydrophobic contacts, the authors reasoned that the structural diversity provided by varying the nature of ligands associated in the glycocluster might emphasize secondary binding sites in close proximity of the sugar binding pocket. For this reason, different aminooxy-modified carbohydrates **14**, **15**, **16**, **26** were first prepared from the corresponding fluoride glycosyl donors following the previously reported procedure [31, 32]. In addition, various charged **35**-**36** or hydrophobic **37** amino acid building blocks were synthesized by incorporation of ethoxyethylidene-protected aminooxy acetic acid linker (Scheme **8**) [46].

The hGC were finally prepared by using an equimolar solution of different aminooxy modified ligands with a cyclodecapeptide scaffold containing aldehyde functions **21**, the excess of unreacted aminooxy building block being remove from the crude mixture by incubation with an aldehyde-functionalized resin. As hypothesized, the randomized distribution each building block on the template was achieved, leading to the formation of all expected library members as illustrated with compound **38**. Various hGC libraries combining either, up to four carbohydrates, or carbohydrate together with amino acid were thus generated following this strategy. Besides the synthetic procedure efficiency, this new generation of glycoclusters was found to provide an excellent tool to explore the impact of a heterogenic cluster display in protein binding processes [45].

4. BIOLOGICAL APPLICATIONS OF CYCLOPEPTIDE-BASED GLYCOCLUSTERS

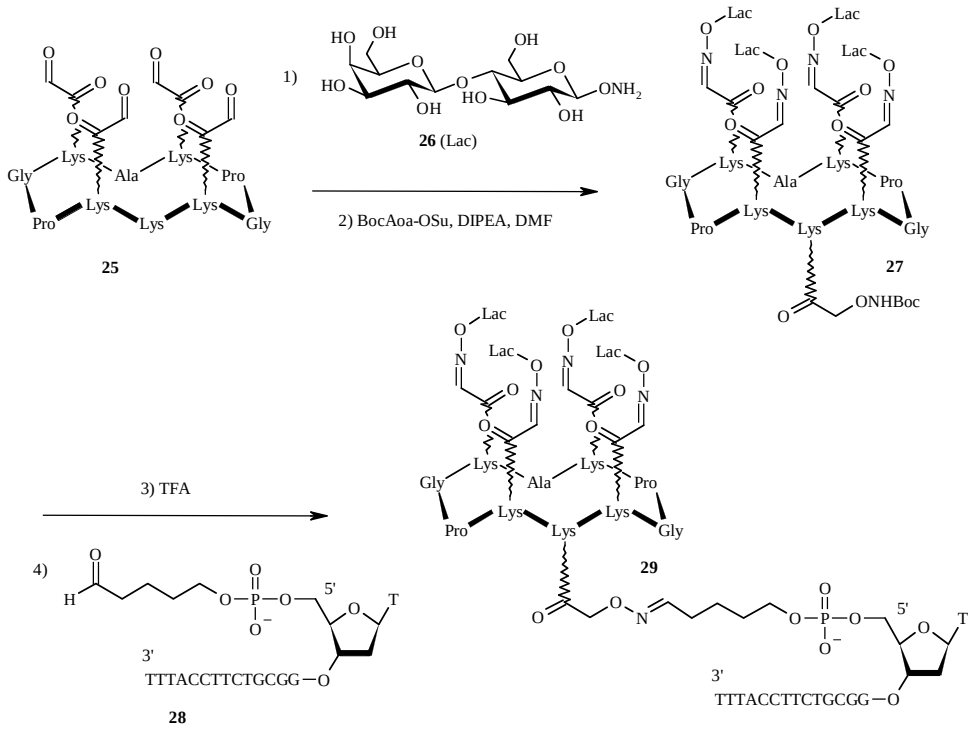
As mentioned in introduction, the biological potentials of glycoclusters are extremely broad, particularly for cyclopeptide-based structures. These templates are advantageous in that their locked conformation of the cyclic framework and their synthetic versatility offer the possibility to explore the spatial arrangement required for a strong binding affinity to biological targets. In addition, by contrast with linear peptides, the cyclic structure of the peptide was proved to prevent *in vivo* proteolytic degradation.

4.1. Inhibitors of Biological Interactions

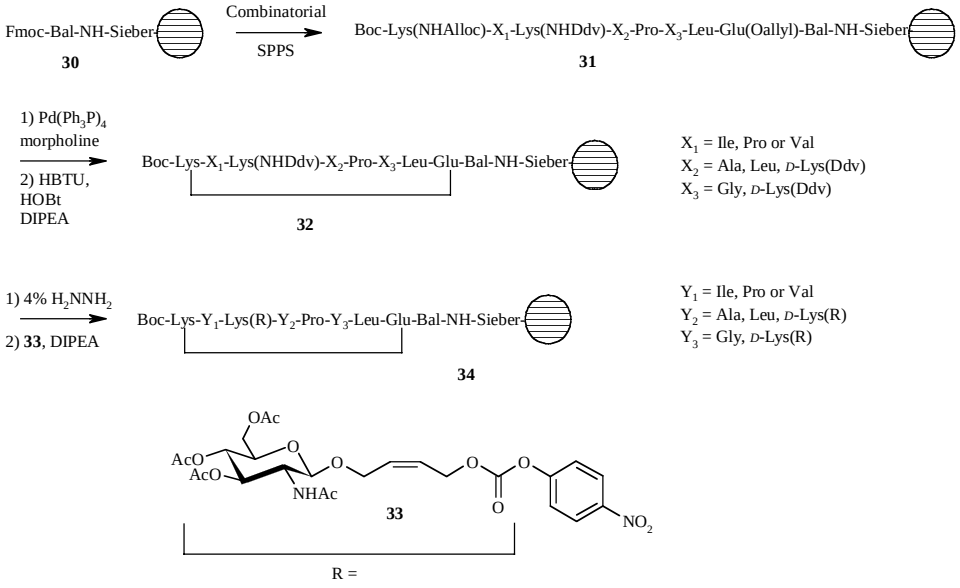
4.1.1. Bacterial Toxin

Number of diseases caused by bacteria, viruses or toxins are initiated by multivalent interactions between CBP expressed on the pathogen membrane and a cluster of oligosaccharides attached to the host cell surface [7]. Consequently, the rational design of multivalent molecules capable of blocking the early stage of adhesion represents a strategy of choice to inhibit these pathogenic processes, as shown previously with the Shiga-like toxins [47]. This family of bacterial toxins consists of an enzymatic A subunit which is responsible of the lethal activity and five identical carbohydrate-recognition B subunits, each presenting well-characterized binding site for galactoside-containing ligands. In a previous study, Fan and co-workers have proposed an elegant modular design of pentavalent inhibitors based on a symmetric pentacyclen core presenting galactose moieties, then they studied the influence of flexible spacer on the binding potency to bacterial enterotoxin pentamer [48]. On the basis of this report, these authors extended their investigations on cyclopeptide scaffolds. Using a convergent approach, several large cyclic decapeptides alternating flexible amino acids without side chain (e.g. glycine, γ-aminobutyric acid, ε-aminohexanoic acid) and L-lysine residues were synthesized to explore the geometric requirement of galactose units for binding optimization (Fig. **4**) [49].

These templates are expected to achieve ring size variations and to ensure a predictable expanded conformation in solution. A set of 12 pentavalent glycoclusters containing linker of various length were tested as inhibitors of the binding of cholera toxin to ganglioside. In comparison with pentacyclen-based ligands, they observed a significant inhibitory improvement for large cyclopeptide scaffolds containing shorter linkers such as **39**, with a 100,000-fold increase over the monovalent galactose unit. Consequently, their experiments have confirmed that the binding optimization is closely related to both size and geometry of the glycocluster in that the sugar cluster should fit with each binding site of the receptor.



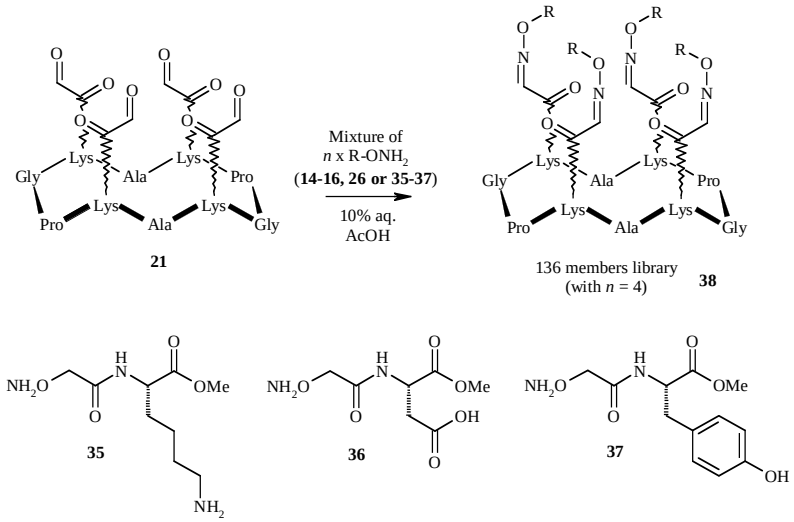
Scheme 6.



Scheme 7.

4.1.2. Virus

A similar approach can be achieved with other biological targets. For example, the influenza virus invasion is mediated by multiple interactions between the viral receptor hemagglutinin (HA) and sialic acid ligands such as GM3 sialotrisaccharide (Fig. **4**) of the cell surface. The knowledge of the three-dimensional HA structure together with molecular modelling studies have allowed Nishimura and co-workers to design potent inhibitors against this viral infection [24]. As the HA trimer contains in each subunit one sialotrisaccharide GM3 binding site separated by 40-50 Å, the authors have considered that the presentation of three copies of GM3 onto cyclopeptide containing 18-21 amino acids may provide glycoclusters fitted to interact simultaneously in each binding site of HA. Thus, several glycoclusters such as **40**, displaying up to three sialotrisaccharides onto a peptide framework composed of either charged or neutral amino acid residues were synthesized using a combined chemical and enzymatic procedure (Fig. **4**). Their inhibitory properties on the hemagglutination of chicken erythrocytes induced by influenza virus H1N1 were subsequently investigated. As confirmed by surface plasmon resonance (SPR) experiments, they first observed that a divalent and trivalent presentation of the trisaccharide is required to ensure a potent inhibitory effect, suggesting the expected multivalent interaction with HA. More interestingly, the fact that no inhibition was detected using scaffolds having identical size, but rather containing neutral amino acids



Scheme 8.

instead of histidine and aspartic acid, indicates that not only the multivalent display of sugar, but also the nature of amino acid residues is crucial for the biological activity. To confirm this hypothesis, NMR and molecular modelling experiments were performed, showing that the nature of amino acid into the peptide framework greatly influence the spatial orientation of grafted sugars without the template, and thereby alters the recognition properties.

4.1.3. Selectin

Among other CBP, selectins represent relevant targets for the design of multivalent inhibitors. These transmembrane glycoproteins, which are involved in cell-adhesion processes such as tumor cell metastasis or inflammatory cascade, bind selectively to Lewis-based ligands [50]. Kunz and co-workers have found that synthetic sialyl Le^X-based trivalent glycopeptide **5** (Scheme **1**) inhibit the adhesion of HL_{60} tumor cells to a surface coated with E-selectin 3-fold more effectively than the monomeric sialyl Le^X [21]. The same research group has further attempted to optimize the binding affinity by using a flexible linker between the carbohydrate moiety and the peptide core [51]. They have designed a divalent cyclopeptide **41** containing two asparagine residues bearing tetraethyleneglycol spacer as anchoring site for sialyl Le^X ligand (Fig. **4**). However, the biological evaluation did not show the expected exponential inhibition effect on adhesion of murine neutrophiles to E-selectin as the divalent glycopeptide was only twice more active than sialyl Le^X itself.

4.2. Synthetic Vaccines

4.2.1. Against Cancer

The surface of cancer cells differs from normal cells by the over-expression of tumor-associated carbohydrate antigens (TACA) which represent attractive targets for cancer immunotherapy. To overcome their poor immunogenicity and promote a strong and selective immune response against tumor cells, it was demonstrated among other attractive approaches, that a cluster of TACA together with a T-cell peptide epitope on a carrier molecule provides potent anticancer vaccine candidates [52, 53]. On the model of the multiple antigen glycopeptides developed by Leclerc and co-workers [54], we have recently investigated the use the cyclopeptide RAFT for the construction of multiepitopic frameworks **42** (Fig. **5**) [55].

Following the sequential oxime-based strategy described earlier [41], we have synthesized well-defined compounds containing a cluster of mucin-associated Tn antigen analogue which are antigenic markers of epithelial tumors [56]. On the second addressable domain of the template, $CD4^+$ helper T-cell peptide epitope from the type I-poliovirus protein was introduced in order to stimulate $CD4^+$ T-cells and activate the production of specific antibodies (Abs) for the Tn antigen. Biological investigations have clearly shown the potency of this multiepitopic construction to elicit a specific immune response towards tumors expressing the human form of Tn antigen, confirming that the RAFT core represents an effective non-immunological carrier molecule.

Further researches prompted us to design a new generation of TACA-based vaccine prototype **43** (Fig. **5**) [57]. This molecule contains indeed four essential components that were assembled on the RAFT core using a combined oxime and disulfide bond formation. First, the vaccine prototype display a cluster of Tn analogue to elicit a B cell response and raise high affinity tumor-specific Abs. Moreover, several studies have suggested that an ideal cancer vaccine formulation must also comprise $CD4^+$ and $CD8^+$ T cell epitopes to ensure both humoral and cellular eradication of cancer cells. Therefore, we introduced a chimeric CTL-Th peptide made of a $CD8^+$ T-cell epitope from ovalbumin ($OVA_{257-264}$) in line with a universal $CD4^+$ T helper (Th) epitope (PADRE). Finally, a palmitic acid moiety was coupled to the N-terminal end of the chimeric peptide to avoid the use of potentially toxic external immuno-adjuvants, providing thus self-adjuvanting properties to the resulting glycolipopeptide vaccine prototype **43**. As expected, the immunization of OVA-expressing mouse B16 melanoma using **43**, without administration of external immuno-adjuvant, has shown a robust induction of IgG/IgM that recognized Tn-positive human tumor cell lines and a stimulation of $OVA_{257-264}$-specific $CD8^+$ T cells and PADRE-specific $CD4^+$ T cells. More interestingly, we observed a significant reduction of tumor size in mice inoculated with MO5 carcinoma cells, as well as an improved survival. Altogether, the safety and strong immunogenicity highlight the high potential of such four-component vaccine constructions for human immunotherapy against cancers.

4.2.2. Against HIV

The discovery that human monoclonal antibodies such as 2G12 bind to heavily glycosylated regions of viral envelop gp120 and is able to neutralize a broad range of HIV strains has opened new perspective on the development of HIV vaccines [58]. Particularly, the identification of a clustered carbohydrate epitope of $Man_9GlcNAc_2$ (Fig. **6**) which is involved in the binding to human HIV-neutralizing antibody 2G12 constitute the molecular basis for the design of new carbohydrate-based vaccine prototypes against HIV.

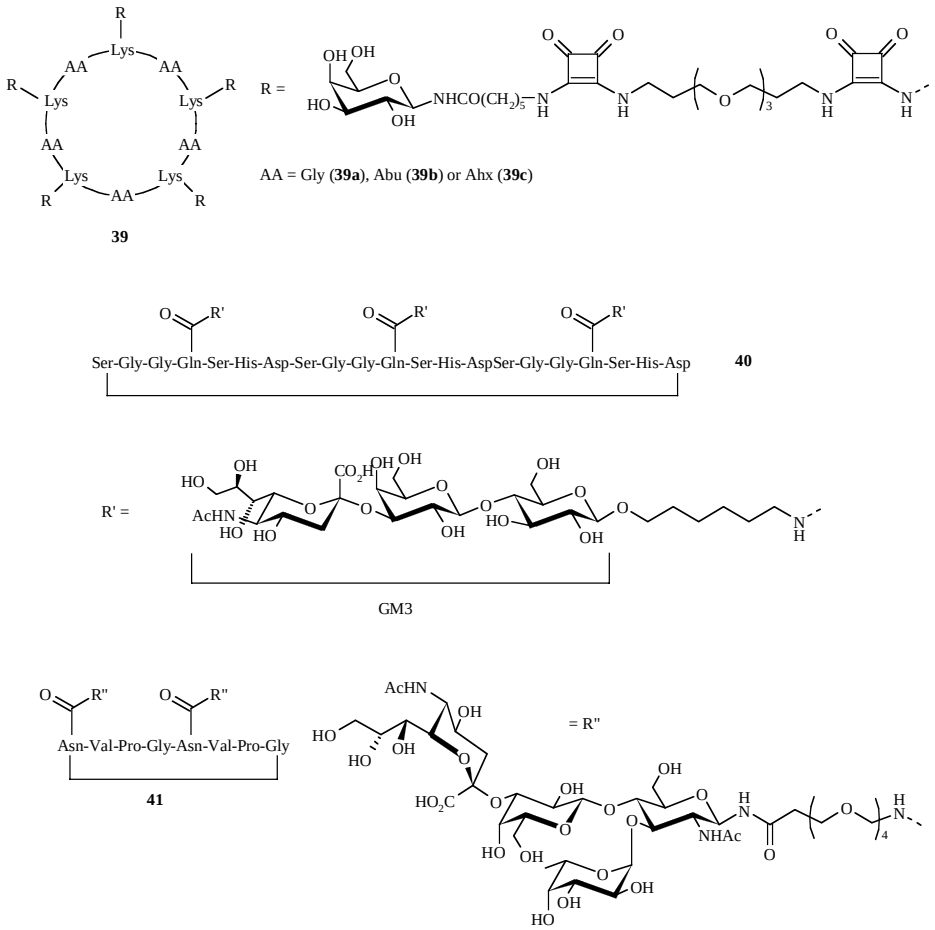


Fig. (4). Different inhibitors of biological interactions.

In a first study, the synthetic versatility and modularity of cyclopeptide scaffold was exploited to prepare RAFT-based glycoclusters using Click chemistry (Fig. **6**) [59]. Tetravalent molecules such as **44** displaying either natural or fluorinated derivative of the simplified tetrasaccharide Man_4 (D1 arm) were synthesized and studied by SPR with 2G12 coated surfaces. The binding experiment has confirmed the expected cluster effect as a high binding affinity was achieved whereas only a weak interaction was observed with the monomeric D1 arm. It was finally presumed that the incorporation of two copies of T-helper antigen from tetanus toxoid on the second domain of the template **45** may provide immunogenic properties against HIV-1 without disturbing the recognition properties of the glycoluster.

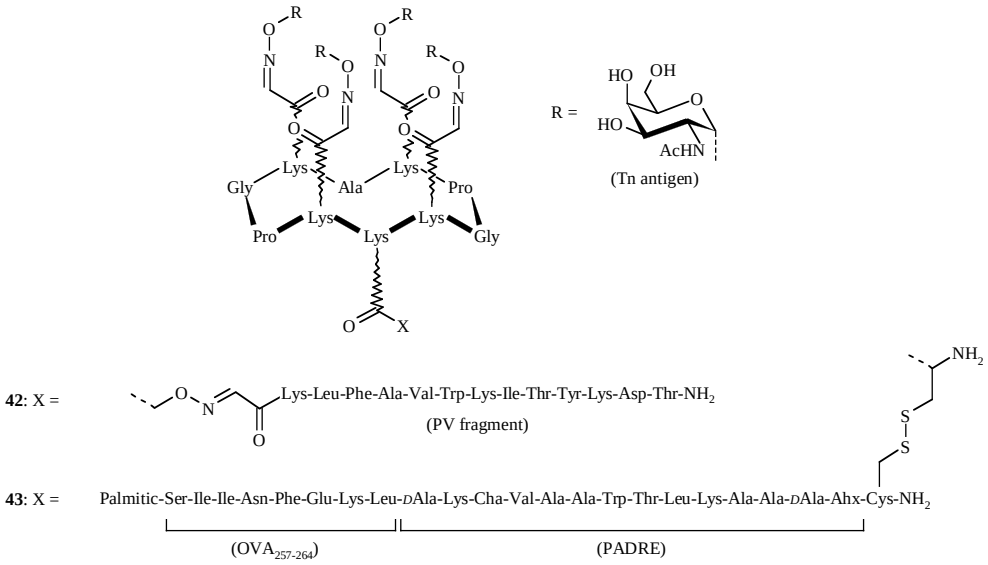


Fig. (5). Multiepitopic anticancer synthetic vaccines against tumors.

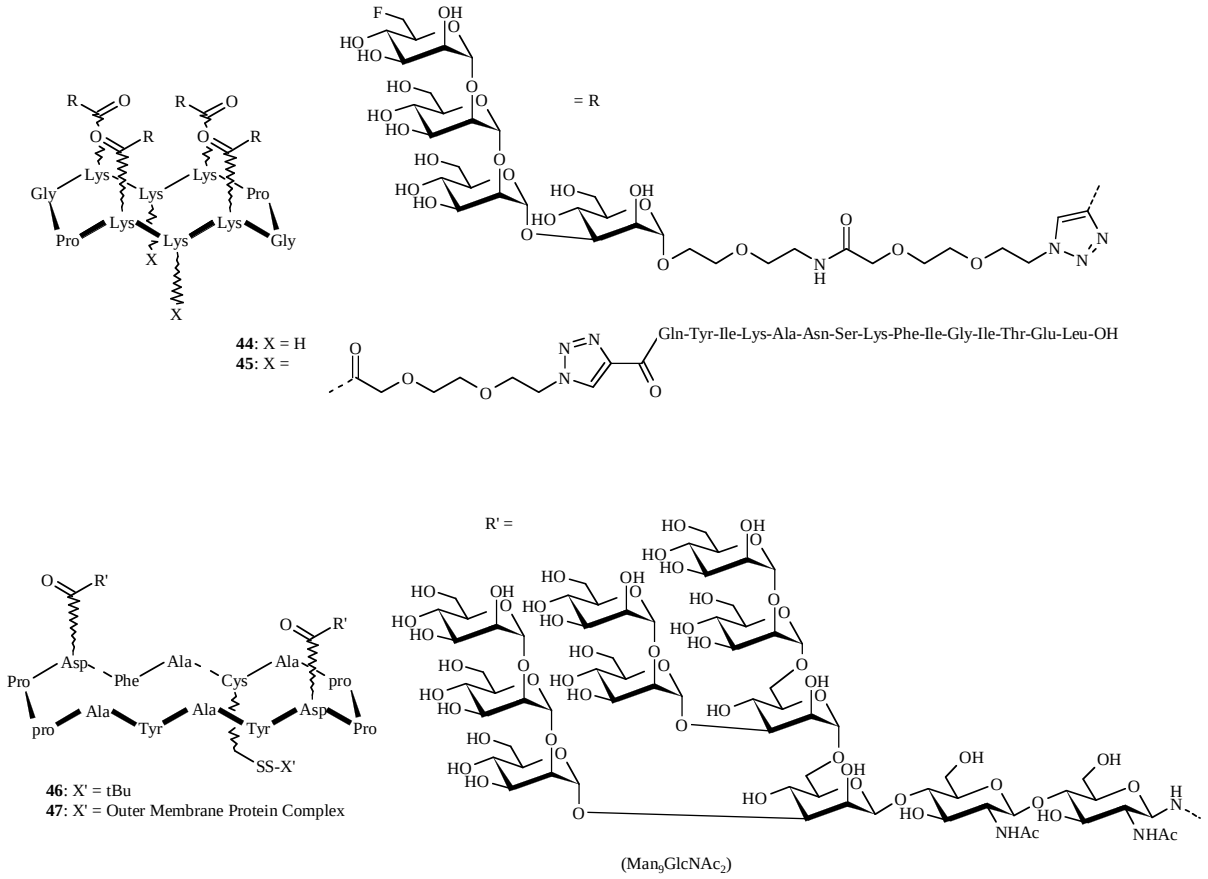


Fig. (6). Anticancer synthetic vaccines against HIV.

On the basis of previous studies, Danishefsky and co-workers have also reasoned that the presentation of the whole $Man_9GlcNAc_2$ epitope in a controlled clustered and spatial orientation might serve as 2G12 epitope mimics, and therefore favour a enhance binding affinity with 2G12 [60]. They have selected a 14-residues cyclic peptide containing aspartate moieties to branch up to three sugar epitope by Lansbury aspartylation [22] and one cysteine residue (Fig. **6**). The latter amino acid was used to conjugate the resulting glycoclusters **46** on a carrier protein for immune adjuvation to obtain **47**. Here again, SPR binding experiments have confirmed that a di- or trivalent presentation of the sugar is required for a strong binding with 2G12, suggesting a structural homology to the natural gp120 epitope. A comparable effect was observed by ELISA assay after conjugation to the immunogenic carrier.

4.3. Immobilization of Glycoclusters on Various Surfaces

Glycomic researches have lead to the development of new analytical tools for probing the functional and structural features of carbohydrate-protein interactions [61]. Particularly, the immobilization of multivalent glycoclusters on surfaces represents an attractive approach to study these complex biological events, to detect carbohydrate binding proteins with weak binding affinity, to analyse the protein glycol-patterning or even to discover new selective ligands. Extensive researches are currently performed in this field.

4.3.1. On-Bead Binding Assay with Lectins

Polyvalent ligands of a GlcNAc-binding plant lectin were identified using a combinatorial approach. Glycoclusters libraries **48** of 19440 compounds displaying up to six GlcNAc moieties on cycloheptapeptide cores were prepared using the split and mix procedure described earlier (Fig. **7**) [43]. The library was designed so that a two dimensional spatial diversity may be generated. For the screening of the binding properties of the library, an efficient on-bead enzyme-immuno lectin assay was developed with the dimeric lectin wheat-germ agglutinin (WGA) [62]. Resin beads bearing the library were successively incubated with the biotinylated WGA, in the presence of GlcNAc to ensure the selection of the best ligands, and monoclonal anti-biotin Ab conjugated with alkaline phosphatase enzyme. After several washings, the presence of WGA was visualized by adding the enzyme substrate which delivers a water-insoluble dye on the bead surface. The more coloured beads were finally pick up and analysed by microsequencing. Interestingly, only a few tetra-, penta- and hexavalent ligands, rather than all hexavalent ligands were thus identified, suggesting that the spatial organisation of the sugars play a crucial role for recognition. The binding enhancement was finally confirmed by an enzyme-linked lectin assay since selected, re-synthesized molecules have shown, as for **49**, up to 600-fold higher affinity than the monomeric reference.

Another on-bead procedure was reported for the synthesis of glycoclusters and binding assay with peroxydase-labelled lectins [63]. Chemoselectively assembled RAFT-based glycoclusters were synthesized following a fully support protocol on NovaSyn® Tentagel resin. Various tetravalent β-lactosyl, *N*-Acetyl-α-galactosyl and α-mannosyl glycoclusters **50** were thus prepared, providing immobilized glycoclusters that were tested for recognition with model peroxydase-labelled lectins (Fig. **7**). After incubation of the beads of resin with either lectins PNA (peanut *Arachis hypogaea agglutinin*), ConA (ConcanavalinA from *Canavalia ensiformis*) or HPA (*Helix pomatia agglutinin*) and visualisation of the binding by adding a peroxydase substrate, the optical density of the supernatant was measured. It was first observed that the clustered presentation of sugars ensures a selective recognition, confirming thus that neither the solid support nor the RAFT core alters the binding. In addition, by contrast with the monovalent cyclopeptide control **51**, an

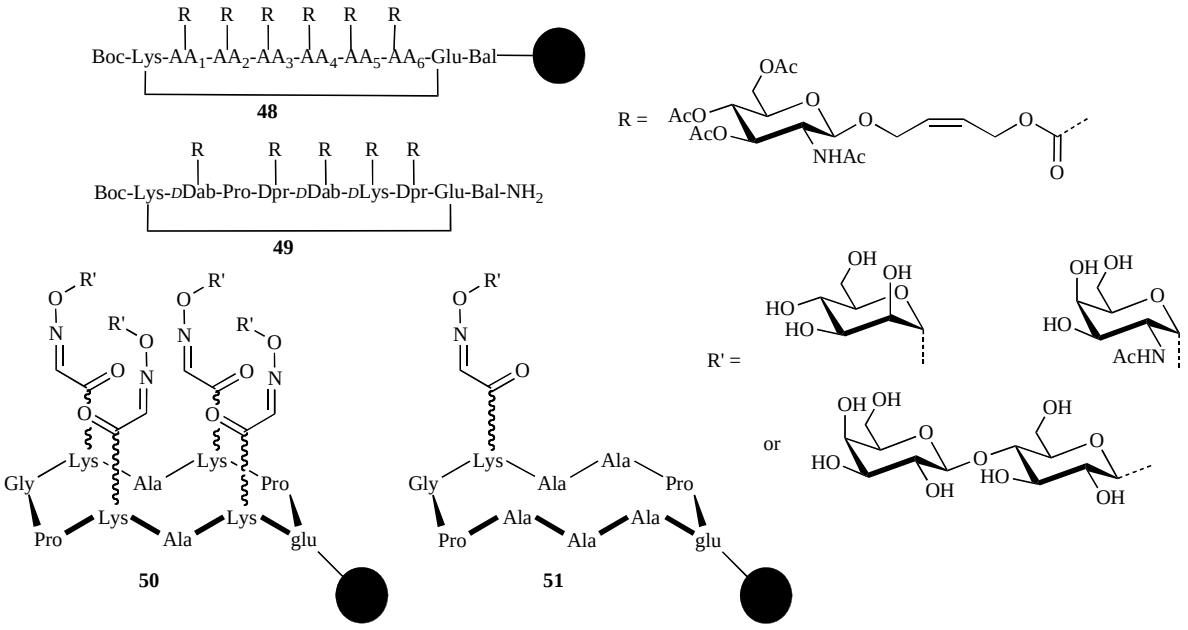


Fig. (7). Immobilized glycoclusters on beads of resin.

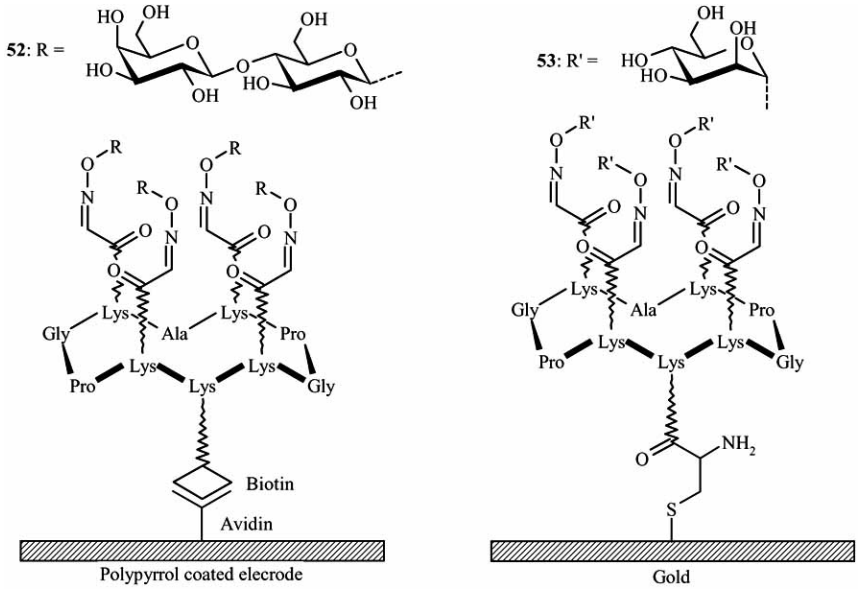


Fig. (8). Immobilized glycoclusters on electrode (left) or as self-assembled monolayer on gold surface (right).

improved recognition was achieved with multivalent ligands **50**. Since the presentation at the resin surface is formally multivalent for all systems, this enhancement emphasizes that not only the clustered presentation on the bead is important but also that the control of its local density is crucial to improve recognition.

4.3.2. Electrochemical Detection

Dumy and co-workers have previously reported a solution [38] and a fully solid phase [39] synthesis of RAFT-based glycolusters displaying four copies of carbohydrates to provide recognition properties and biotins to ensure its direct immobilization on streptavidin-coated surfaces. Particularly, tetravalent lactosyl cluster was studied for the electrochemical detection of PNA (Fig. **8**) [64]. A copolymer conducting film poly(pyrrole-biotin) and poly(pyrrole-ammonium tetrafluoroborate) was first electrogenerated on electrodes and successively coated with an avidin monolayer. The biotinylated lactosyl cluster was subsequently immobilized on the polypyrrole-coated electrode and the construction **52** incubated with unlabeled PNA. The binding was then evaluated by diverse electrochemical measurements in comparison with similar constructions with monovalent lactose. As expected, it was first observed that a higher amount of lectin bind to the RAFT-lactosyl coated electrodes, suggesting a stronger interaction due to cooperative multivalent effect. In addition, the binding strength was evaluated with a peroxidase-labeled PNA by amperometric detection. A 15-fold enhanced affinity per lactosyl unit was measured with the PNA lectin, which is in good agreement with the previously reported effect observed between mannosyl cluster and the model lectin ConA using fluorescence polarization [36]. This study represents the first demonstration that a full electrochemical sensor can be applied for the direct detection of carbohydrate/protein interactions without additional labeling step.

4.3.3. Investigation of the Binding Mechanisms

Both above studies fit in showing that immobilized RAFT-based glycoclusters on different surfaces mediate comparable enhanced recognition with lectins, even though the distance between each sugars moiety precludes contacts in multiple binding sites. These interesting results prompted Dumy and co-workers to investigate these binding mechanisms [65]. For this purpose, tetravalent and monovalent mannosyl compounds were prepared as specific ligand for ConA, whereas the corresponding lactosyl derivatives were used as negative controls. Either a cysteine or a biotin was introduced in each molecule to allow their immobilization respectively on gold, as illustrated with the construction **53,** or streptavidin-coated surfaces (Fig. **8**). Two real time techniques, Quartz Crystal Microbalance with energy Dissipation monitoring (QCM-D) and SPR were employed to quantify the interaction to ConA. In order to dissociate the chelating effect due to the lectin from the multivalent effect induced by the glycocluster, the binding affinity was measured as a function of the grafting ratio of the RAFT glycoconjugate on the surface. First at high surface density, the multiple presentation of mannose on the RAFT template does not improve the binding to ConA since a similar affinity was observed with its monovalent counterpart, suggesting in both cases the close proximity between the sugar moieties. By contrast, different behaviours have appeared between multivalent and monovalent ligands after immobilization at lower surface density. When various amounts were immobilized, a 3 to 7 fold higher affinity was obtained for the tetravalent ligands. In addition, at comparable immobilization ratio, tetravalent ligands can bind about 3-fold higher amounts of Con A due to their ability to form clusters of lectins. This clustering process could be at the origin of the higher affinity exhibited by those immobilized ligands, although it could be postulated that the presence of a high local concentration also participate through a proximity/statistical effect.

5. CONCLUSION

Among other addressable templates, cyclopeptides are subjected to growing interests for the design of multivalent structures. The studies reported in this mini-review emphasize that their conformationally restricted core provides a useful tool for the construction of oligosaccharide clusters in a well-defined spatial orientation. Besides this structural feature, the preparation of cyclopeptide-based glycoclusters can be achieved in a controlled manner, by a large panel of synthetic procedures going from solution or solid-phase strategies, to combinatorial chemistry and oxime ligation. This synthetic modularity enables the rapid optimization of the ligand affinity by tuning both size and geometry so that the sugar moieties match with the binding site distribution on the target proteins. In addition, their potential can be exploited in a large variety of relevant biological applications such as synthetic vaccines or biosensors. Altogether, the cyclopeptide-based glycoclusters have undoubtedly opened new trends in Glycoscience.

ACKNOWLEDGEMENTS

The author thank the Association pour la Recherche contre le Cancer (ARC), the Centre National pour la Recherche Scientifique (CNRS), the Université Joseph Fourier (UJF) and COST D34 that supported financially the results obtained by our group reported herein. The author thanks also Prof. Pascal Dumy, Prof. Julian Garcia and Dr Nicolas Spinelli for their advices and help in the preparation of this review.

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Received: April 14, 2008 **Revised: May 12, 2008** **Accepted: May 16, 2008**