Recent Progress in the Field of β-(1,3)-Glucans and New Applications

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Abstract: β-(1,3)-Glucans are widely distributed within microorganisms or seaweeds in which they act as membrane components or for energy storage, respectively. Since these glucans are not biosynthesized by mammals, they are likely to activate the immune system of their host. Since the discovery of their positive involvement as immunomodulator agents, numerous studies were published all around the glycosciences. These works deal with purification procedures, analytical chemistry, synthetic processes, chemical modification of the natural polysaccharides, determination of their physicochemical properties, and assessment of their biological and medicinal effects through *in vitro* and *in vivo* studies. This article aims at presenting some recent results linked to β-(1,3)-glucans through two closely connected points of view, i.e. biology and chemistry. Biological aspects will be focused more particularly on discovery of some receptors present on immunocompetent cells and scope and limitations of chemical synthesis and/or modifications will be described. Moreover, this paper will also introduce some new chemo-enzymatic synthetic methods using wild-type or mutant glycosidases and will be extended to novel opportunities of applications of β-(1,3)-glucans in nanotechnology resulting from a better understanding of their self-assembling propensity in aqueous media.

Key Words: β-(1,3)-Glucans, receptors of β-(1,3)-glucans, immunostimulation, chemical modification, synthesis.

INTRODUCTION

Carbohydrate-based molecules widely occur in living organisms and are generally found as polymeric substances and/or as conjugates with lipids, peptides or proteins. The resulting substrates present a large panel of more or less complex chemical structures that require to be subtly determined in order to increase knowledge on how carbohydrate domains interact with their receptors to trigger biological activities. As a consequence, the natural abundance of polysaccharides and glycoconjugates together with their structural diversity undoubtedly give numerous opportunities to discover new involvements in biological mechanisms and to develop new therapeutic topics.

Since the establishment of the clear correlation between benefits originated in folk remedy of some mushrooms and β-(1,3)-glucans, those biopolymers of glucose aim at finding an increasing place within the "glycoworld". Although absorption of β-(1,3)-glucans by plant can elicit natural defence mechanisms causing the production of reactive oxidizing species [1] and phytoallexins [2,3], it was also highlighted the stimulation of macrophages by zymosan [4,5] a particle extracted from *Saccharomyces cerevisiae* and notably consisting of glucans and mannans. Few years later, Chihara *et al*. [6] prepared four polysaccharidic extracts from *Lentinus edodes* and determined *in vivo* antitumor activities for β-(1,3)-glucans. Subsequent to these observations over the past forty years, thousands of publications have emerged, describing for instance sources, purification processes, chemical structures, physicochemical properties, biological activities, therapeutic uses, related genes [7-12] and also chemical modifications, the properties of the resulting non natural derivatives, the synthesis of oligo-β-(1,3)-glucans.

This article provides an overview of two important topics which have to be closely considered in order to ensure a better understanding of the biological roles of poly-, oligo- and small β-(1,3)-glucans. Indeed, recent advances in the field of β-(1,3)-glucan receptors in mammals and the current increasing interest for the development of new approaches towards small oligoglucans are described. Moreover, the use of these biopolymers as hosts for nucleic acids or synthetic organic or inorganic polymers is also included in this article. Such findings also contribute to open interesting new pharmaceutical applications for β-(1,3)-glucans.

1. IMMUNOMODULATING β-(1,3)-GLUCANS AND THEIR RECEPTORS

β-(1,3)-Glucans, also called laminari-polysaccharides, are a family of homopolysaccharides of glucose which are commonly found in fungi [11], yeasts [11], plants [13,14] and seaweeds [15]. They can serve as storage carbohydrates, can be useful for their structural behaviour, when located within the inner part of the cell wall, or can interact with higher plants and animals after presentation of extracellular membrane components of some fungi. β-(1,3)-Glucans differ from each others depending on the presence of side chain (at O-4 for plants [14] at O-6 for microorganisms and seaweeds [15]), the degree of branching [curdlan is strictly linear while laminarans present one β-(1,6)-glucopyranosyl unit for every 20-30 glucose residues per chain, schizophyllan one branching glucose at every third main-chain glucose, lentinan two β-(1,6)-linkages for every five residues], chain length (Fig. **1**). These differences involve significant variations on phys-

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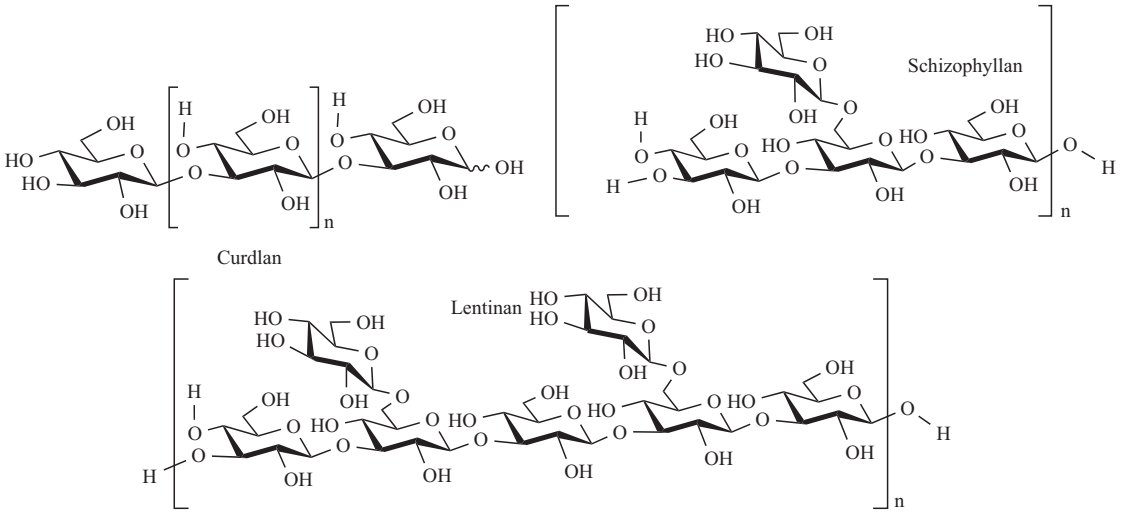


Fig. (1). Chemical structures of the main β-(1,3)-glucans.

icochemical parameters such as molecular weigh, temperature-dependant solubility in water, spatial supramolecular structures (random coils or more ordered conformations, i.e. mono- *vs*. triple helice).

On a biological point of view, it has been recognized for a long time that β-(1,3)-glucans belong to the group of Biological Response Modifiers (BRM), that means that they do not have direct cytotoxic activities but that they are able to boost the natural defence mechanisms of the host. Since the action of β-(1,3)-glucans is mediated through own immune system, they are generally correlated with a low level of harmfulness [16]. Therefore, this results in an increased resistance against bacterial, viral, mycotic and microparasitic [11] infections as well as malignant cell growth. The pioneer position of Asiatic countries in this field has to be underlined with respect to the number of patents applied in this continent and also because schizophyllan and lentinan are commercialized in Japan as medicines for instance for the treatment of uterine cancer [17]. The biological effects of β-(1,3)-glucans are measured through the evaluation of activation of natural killer (NK) cells, T-cells and nuclear factor-κB (NF-κB), phagocytic activity, stimulation of secretion of cytokines such as different interleukins (IL), tumour necrosis factor (TNF-α) or interferon-γ, production of reactive oxygen species, for instance superoxide anion and hydrogen peroxide. Among others, studies describing cellular recognition events have identified pattern-recognition receptors (PRR), i.e. a set of molecules of the innate immune system that does not undergo mutation, able to interact with a variety of antigenic substances, here β-glucans. These PRR are composed of at least four receptors for β-glucans: Dectin-1, complement receptor 3 (CR3; CD11b/CD18), lactosylceramide and scavenger receptors.

Dectin-1

In the course for the research of β-(1,3)-glucan receptors, the role of Dectin-1 was determined only at the end of the 1990s [18]. It was first identified as a dendritic cell-associated molecule by Ariizumi *et al*. [19] and, one year later, as a new receptor for β-glucans by Gordon and Brown [18]. It is expressed at the surface of many organs, mainly on liver, lung and thymus but also on heart, stomach, small intestine and kidney. More precisely, it is found on cell surfaces of dendritic cells and leukocytes, with the highest levels of surface expression on monocytes, macrophages and neutrophils [20]. It was shown on a murine model that Dectin-1 receptor is a type II membrane receptor consisting of four main parts: (i) a C-type lectin-like domain with two potent *N*-glycosylation sites, (ii) a stalk, (iii) a transmembrane domain, and (iv) a cytoplasmic unpaired immunoreceptor tyrosine-based activation motif (ITAM) [21]. Subsequently, Herre and co-workers fully characterized eight isoforms (A-F) for human Dectin-1 [20]. The main isoforms A and B closely resemble to the murine receptor, except the number of *N*-glycosylation sites in A and the absence of the stalk in B. Moreover, some heterogeneity was observed regarding the cell distribution of both A and B isoforms. More interestingly, Herre *et al*. [20] highlighted that Dectin-1 is indeed the major β-glucan receptor on primary macrophages capable for mediating the non-opsonic recognition of zymosan and that Dectin-1 presents at least two ligand-binding sites, one that interacts with exogenous β-glucans and the second that recognizes an endogenous ligand on T cells inducing the proliferation of the latter. As a consequence, Dectin-1 mediates not only β-glucan binding but also cell activation, as could be expected through the presence of an intracellular immunoreceptor. This phenomenon was also reported by Ahrén *et al*. who demonstrated that the respiratory tract pathogen nontypeable *Haemophilus influenzae* (NTHi) can activate eosinophils in a β-glucan-dependent manner [22] to produce radical anion superoxide, signal transduction molecules and proinflammatory mediators.

It is also noteworthy that binding of β-glucans to Dectin-1 could cooperate with other molecular interactions to produce a noticeable effect such as production of TNF-α. Taylor and coworkers emphasized that this response, observed

through the nonopsonic recognition of yeast by specific macrophages, was dependant on the presence of the mannose receptor SIGNR1. These studies yielded to the conclusion that Dectin-1 isoforms are able to induce this response, not essential when an alternative receptor is present but contribute to the magnitude of the biological response [23]. This effect could also require signals generated by the Toll-like receptors (TLRs) [24,25]. Very promising results were described for a chemically modified β-glucans in the domain of the cardioprotection [26].

Complement Receptor 3 (CR3)

The complement system is a set of approximately twenty proteins which are present but inactive in bloodstream. Once activated by interaction between antigen-antibody complexes with protein C1 or according to a pathway non dependant on antibodies, a cascade of reactions gives active enzymatic proteins and pro-inflammatory factors. The ultimate stage of the all mechanism provides the membrane attack complex (MAC) that is able to kill cells which present non-self structures. It was shown that low immunogenic, but essential for microbial pathogen survival β-(1,3)-glucans interact with the complement receptor type 3 (CR3). This heterodimeric integrin receptor (CD11b/CD18) is expressed on numerous cells such as macrophages, neutrophils, monocytes, NK and myeloid cells. With respect to their molecular size, β-glucans could present two activation pathways. The first one involves (i) adhesion of pathogenic cell with the I-domain of iC3b fragment present on immunocompetent cells of the host and (ii) specific interaction between small soluble polysaccharides and a lectin binding site also present on the α-chain (CD11b/α_M) of CR3 [27-30]. This phenomenon results in phagocytosis and degranulation of cells or particles and in the production of various cytokines and chemokines, more precisely tumor-necrosis factor (TNF)-α, interleukins (IL-1, IL-6, IL-8,…), interferons (IFN-α, IFN-γ) and nuclear transcription factors such as NF-κB and NF-IL-6 [31]. For the second route, it is hypothesized that poly-β-(1,3)-glucans with higher molecular weigh, and so generally characterized by low solubility in aqueous media, do not need assistance of opsonization and interact directly with CR3 of leukocytes to trigger production of reactive mediators.

Although knowledge dealing with the mechanisms of action of β-(1,3)-glucans is regularly improved, it is obvious that recruitment of effectors of the innate immune system initiated by β-(1,3)-glucans is not efficient enough for the development of novel therapies. Emerging strategies often require specific antibodies (Abs) and more particularly monoclonal antibodies (mAbs). Such Abs and mAbs are used to target tumor cells, which are however known for their low antigenic power. This is one of the major reason why synergetic effects are searched by coupling targeting activity of mAbs against tumors and immunostimulating ability of β-(1,3)-glucans. Amongst interesting publications, Gordon and his coworkers first demonstrated a 57-90% reduction of tumor weigh caused by interaction between β-glucans and CR3, the desired interaction being clearly established by the use of tumor-specific Abs that deposit iC3b onto surface of tumor cells that however lack the receptor required by the polysaccharides [32]. More recently, the same team showed that activation of complement by mAbs, such as Herceptin, Rituxan, or Erbitux, was significantly enhanced by combination with β-(1,3)-glucans intra-venous injected [33,34] or orally administered [34,35], that opens very interesting opportunities for further immunotherapies.

Lactosylceramides and Scavenger Receptors

Although research activities were mainly turned towards effects of β-(1,3)-glucans on complement and towards their interactions with Dectin-1, two additional specific receptors were identified. In 1998, Zimmerman *et al*. reported the binding of PGG-glucan, also known as Betafectin®, in a multivalent fashion, with lactosylceramide present on the surface of human leukocytes [36]. This preliminary work was further improved by an electrophoretic study which established that the homopolysaccharide was able to induce activation of an NFκB-like nuclear transcription factor in human neutrophils [37].

The last specific receptor is indeed a family of compounds which recognize acetylated or oxidized low density lipoproteins (LDL). The so called scavenger receptors could interact with macromolecules generally presenting negative charges. Nevertheless, neutral β-(1,3)-glucans indeed interact with acetylated LDL (AcLDL) which present at least two binding sites on human U937 cells [38]. Within this study, the authors have shown that a glucan phosphate was able to completely inhibit binding of U937 membranes to AcLDL. Moreover, while laminarin could partially inhibit the same interaction, the branched schizophyllan had no ligation affinity. Consequently, this study showed the requirement of negative charge on the polyglucans to improve interactions with AcLDL but also the influence of primary and/or secondary structures to form the desired complex.

Structure – Activity Relationships

Mechanisms of interaction between β-(1,3)-glucans and their receptors were widely described but are still interest of investigation [39] Numerous parameters were proposed to explain observed immunostimulating effects of these glucans: (i) their molecular weight, (ii) their solubility in water, (iii) their conformation (random coiled, simple or triple helix), (iv) the degree of branching of the main β-(1,3)-chain, (v) their sources and the purification procedures [40-44]. On the assumption of such findings, it seems nowadays really perilous to predict with confidence what kind of parameters could positively or negatively modulate the biological potential of glucans. As an example, it has been demonstrated that helical conformation is required to elicit stimulating answer [45]. In this context, laminariheptaose has indeed a stimulatory effect on the production of reactive oxidizing species (ROS) greater than that observed for laminarihexaose. This finding was correlated with the ability of the former to complete a rotation in its secondary structure [46]. On the contrary, Ohno and coworkers concluded to a conformation independent production of IL8 by β-glucans and platelets [47]. Nevertheless, the precise role of conformation and thus the competition between poly- and oligo-β-(1,3)-glucans was recently one more time discussed since Vetvicka *et al*. [48] showed that action of linear laminaritetraose and laminaripentaose, two small glucans structurally unable to adopt a helical conformation in water, is similar to that produce by Phycarine® on a murine model. In conclusion, if receptors

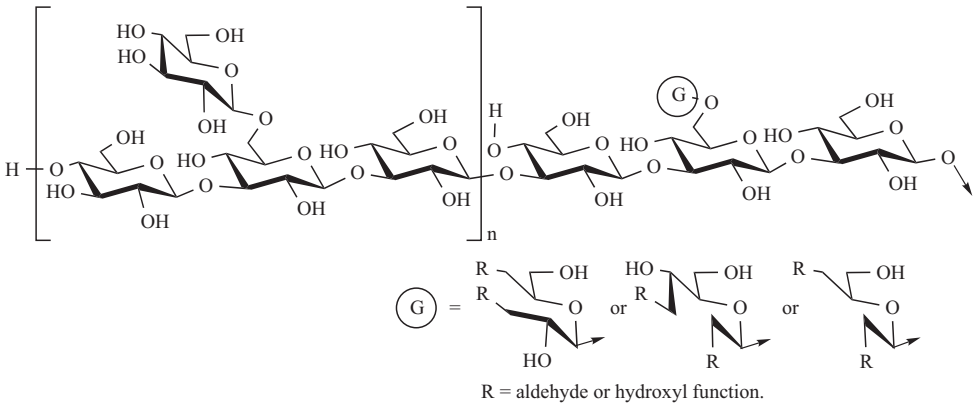


Fig. (2). Potential derivatization of schizophyllan according to an oxidative process to design new hosts for oligo- and polynucleotides.

are of great importance in their molecular interactions with β-(1,3)-glucans, it seems that anticancer potential of β-(1,3)-glucans is dependant on a set of data including at least fine chemical structure, global conformation and studied biological targets.

2. SYNTHESIS OF β-(1,3)-GLUCANS AND DERIVATIVES

Chemical Modulations of Poly-β-(1,3)-glucans

Poly-β-(1,3)-glucans are generally isolated by extraction from fungi or seaweeds [49-53]. Although the resulting polysaccharides present some structural heterogeneousness (number of glucose units and molecular weight, number and length of branching can not be roughly determined), they were directly involved in biological studied. Nevertheless, some side effects were associated with particulates glucans. Consequently, a number of chemical modifications were proposed and resulted in new families of charged (sulfates [54], phosphates [55], glucuronic acids [56,57], carboxymethylated [56,58]) or neutral (oxidizing cleavage of vicinal diols from non reducing terminal glucose units followed by reduction affords new hydroxyl groups [59]; Fig. **2**) β-(1,3)-glucan derivatives.

Many of these compounds are interesting intermediates for further conjugation with various linkers themselves connected with molecules of biological interest such as immunostimulants (Fig. **3**) [57], oligopeptides or cholesteryl derivatives [57,60].

On the assumption that β-(1,3)-glucans are able to exist as a triple helix in water, which can be denatured by adding dimethylsulfoxyde (DMSO), and that the resulting random coil can be reformed by adding water [61], it has been hypothesized that β-(1,3)-glucans could be very interesting candidates for complexing helical nucleic acids. Such a concept was demonstrated by the teams of Shinkai and Sakurai. They have shown how to incorporate a nucleotidic oligomer within the triple helix of schizophyllan [59,60]. This process leads to macromolecular complexes, stabilized by hydrogen bonds between glucose units and the base moieties, which were used for antisense oligonucleotides delivery. Another family of appended β-(1,3)-glucan derivatives was very recently proposed by the same group [62,63]. The strategy was based on "click chemistry" performed between 6-azido-6-deoxy-curdlan and terminal alkyne group of lactosyl or porphyrin derivatives, chosen for their ability to interact with asialo-glycoprotein receptors on hepatocytes or with oxygen, respectively.

It is finally interesting to note that β-(1,3)-glucans have recently gained increasing interest in a domain independent on the health field. Indeed, Shinkai *et al*. have extensively

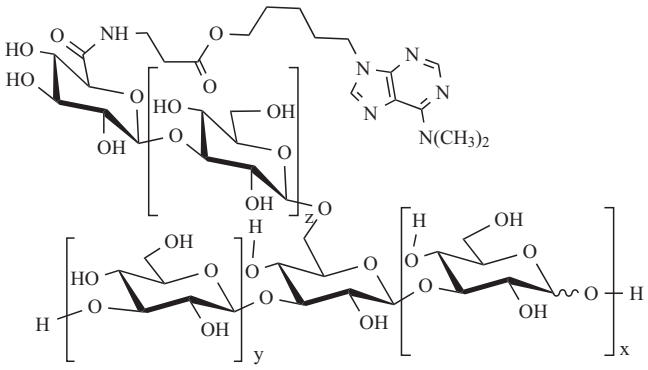


Fig. (3). β-(1,3)-glucan conjugates with small immunostimulant molecule.

published on how β-(1,3)-glucans, and more especially schizophyllan, are able to act as one-dimensional host to create new nanofibers based on silica [64], poly(diacetylene) [65] or chiral polythiophene [66]. The design of this approach is also based on self-assembly ability of β-(1,3)-glucans in water. The newly formed materials could find applications as memories or conductive wires in nanoscale electric circuits, but also, because of interactions of polymer with polysaccharide that allow solubility in water, as biosensors. Such an approach thus opens new opportunities for further involvement of β-(1,3)-glucans in bio- and nanotechnologies.

Chemical Approaches

Considering the biological potential of poly-β-(1,3)-glucans, increasing attention has been turned towards the preparation of oligomeric structures found in natural β-glucans according to synthetic processes. Such studies are of high interest because (i) chemistry affords glucans with well defined structures and high degree of purity, indispensable data to calculate the active dose for a therapeutic treatment, (ii) the resulting oligosaccharides are required to study the structure-activity relationships and more particularly to determine the smallest repeating unit likely to induce biological activities, and (iii) non natural derivatives inspired from natural models could be easily designed and further prepared. Various conventional strategies were applied to the chemical preparation of β-glucans, and to linear and 3-branched β-(1,6)-glucans [67-71]. Amongst these studies, innovative approaches in glycochemistry were proposed, and notably supported synthesis [72], methodology using rearrangement of intermediate orthoesters [73,74], one-pot glycosylation methodology [75-78] and iterative methods based on orthogonal activation of β-bromoglycosides derived from β-selenoglycosides (Fig. **4**) [68].

More recently, another iterative procedure was developed by Vetvicka *et al*. [48] It was based on a glycosylation reaction followed by a selective 3-*O*-deprotection step starting from a unique thioethyl glycoside as a glycosyl donor (Fig. **5**) bearing an orthogonal 3-*O*-naphthyl protecting group. This strategy thus lead to pure linear oligo-β-(1,3)-glucans which could not be obtained with similar purity by enzymatic hydrolysis.

The success of the methods proposed for the synthesis of *a priori* simple β-(1,3)-glucosides should however not hide the fact that some limitations were observed. It is obvious that new β-glycosidic linkages are synthetically obtained with high efficiency owing to the anchimeric assistance of a

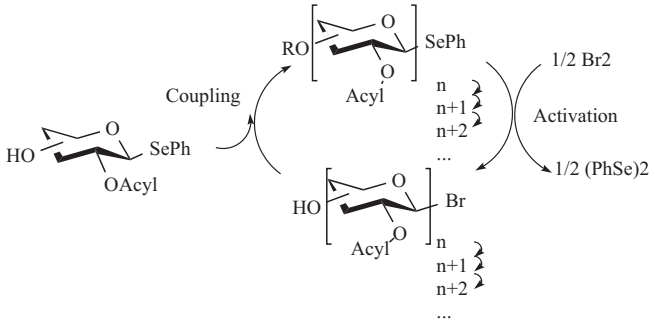


Fig. (4). Iterative synthesis based on orthogonal reactivity of two glycosyl donors.

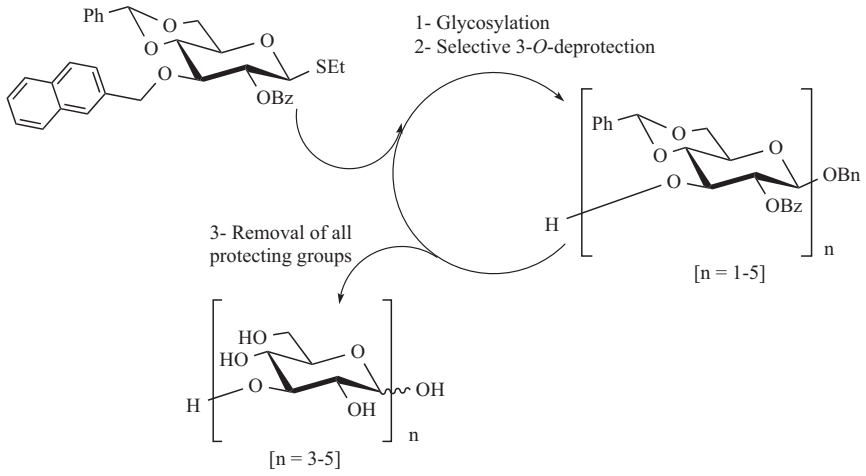


Fig. (5). Iterative synthesis based on a unique glucosyl donor.

neighboring participating protecting group. Nevertheless, it was published some unexpected impossible coupling reactions [79] or α-glucosylation, even in the presence of a 2-ester group (Scheme **1**) [79,80]. The fundamental reasons of these striking results are still unexplained but it seems that stereoselectivity of the glycosidic couplings is highly dependent on (i) the choice of protecting groups on both glycosyl donor and acceptor, (ii) the number of glucosyl units characterizing eletrophilic and nucleophilic species, and (iii) experimental conditions (temperature, dilution, nature of the catalyst or promoter). However, it could be mentioned that the more reproducible results were obtained from donors and/or acceptors bearing 2-*O*-benzoyl and 4,6-*O*-benzylidene protecting groups [48,81,82].

These studies underline the difficult generalization of the main concepts of glycosidic synthesis for the general formation of selected β-(1,3)-glucans. As a consequence, novel synthetic designs of linear or branched β-(1,3)-glucoconjugates still require procedures adapted to each particular target.

Nevertheless, chemical approaches prove to be really relevant for the synthesis of non natural β-(1,3)-glucan derivatives. To illustrate this purpose, Huang *et al*. proposed to add an epoxy group at the reducing end of the laminaripentaose in order to increase the stability of the elicitor-active parent pentasaccharide towards glucanases (Fig. **6**) [83].

Moreover, it is well known that neutral oligo- and polysaccharides bind weakly to their protein receptors. In order to overcome such low affinity constants, it was proposed by several research groups to create new glycodendrimers with significant higher bioactivities compared to the corresponding monomers (Fig. **7**) [67,84].

Finally, Ning *et al*. [85] have also described the synthesis of an amphiphilic schyzopyllan derivative, characterized by the presence of a lactosyl residue, and the preliminary biological effects on Sarcoma-180 cell line. The authors expected synergetic effects between immunostimulant β-(1,3)-glucan and inhibiting action of lactose again the aggregation of certain types of tumor cells (Fig. **8**).

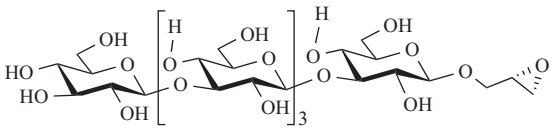


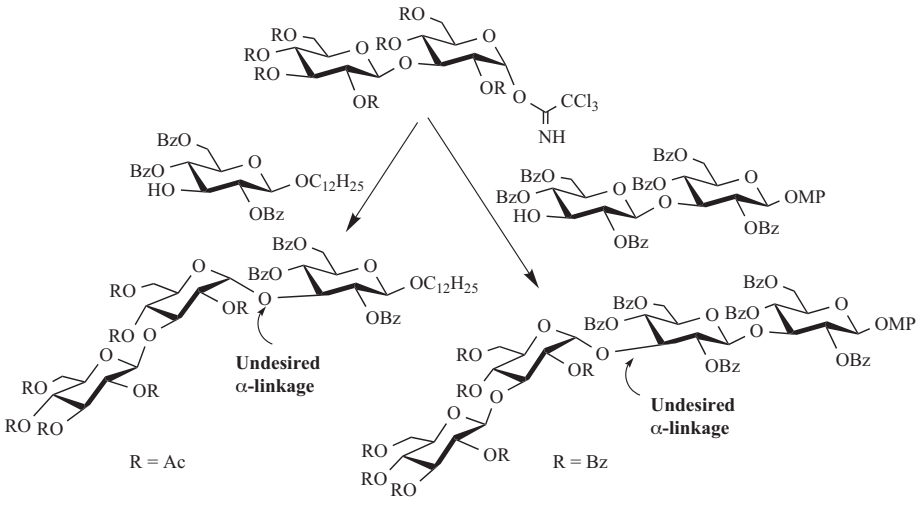
Fig. (6). Epoxy laminaripentaose.

This list of examples is obviously not exhaustive and there is no doubt that numerous neoglucan derivatives will be proposed in a near future.

Chemo-Enzymatic Synthesis

Although conventional glycosidic synthesis are quite efficient to produce number of β-(1,3)-glucans and related but non natural conjugates with high purity and well-defined structures, they still require protecting group manipulations. Therefore, these approaches are sometimes not suitable for providing target oligosaccharides in a timely fashion. To overcome such a difficulty, methods combining chemical synthesis and enzymatic approaches were developed. The former are suitable for the preparation of glycosyl donors and acceptors and the latter are useful to create new glycosidic linkages because biocatalysts allow regio- and stereoselectivities within only one step, generally starting from unprotected building blocks. Therefore, no deprotection reaction is required at the ultimate stage of the all process, thus avoiding time consuming purification step on targeted substrates. Moreover, such strategies are compatible with current environmental requirements.

In this context, various mixed β-(1,3)/β-(1,n)-glucans (with n = 4 or 6) were prepared using appropriate wild glucanases [86,87]. Transglycosylation reactions catalyzed by β-(1,3)-D-glucanases (laminarinases) were also performed to afford a number of β-(1,3)-oligosaccharides conjugated with coumarin derivatives [88].



Scheme (1). Examples of unexpected α-glycosylation reactions.

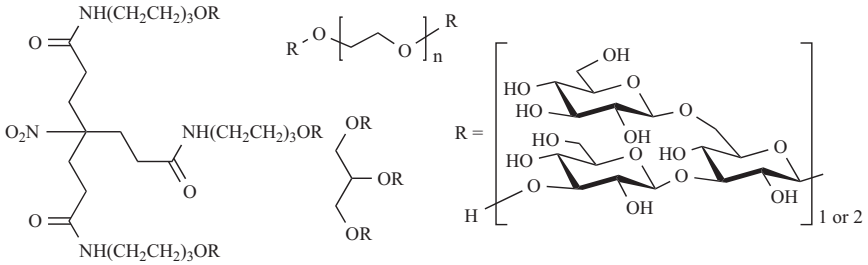


Fig. (7). Neo-oligo-β-(1,3)-glucoside-based dendrimers.

Nevertheless, the most promising approaches developed during the last ten years are based on molecular biology for the design of new biocatalysts. The concept relies on the fine modification of the active site since the nucleophilic carboxylic residue initially present in the wild-type enzyme is able to be replaced by a chemically inert function. Consequently, the mutant, also called glycosynthase, presents an alanine residue instead of the crucial aspartate or a glutamate one, and has lost any hydrolyzing capability (Fig. **9**).

Since the best results were obtained by mutation of retaining enzymes, the glycosynthases now function as inverting enzymes and require glycosyl donors able to mimic the covalent glycosyl-enzyme complex generally proposed as an intermediate in the hydrolysis mechanism. The corresponding donors generally used are glycosyl fluorides characterized by a α-anomeric configuration. For instance, the β-(1,3)-β-(1,4)-endoglucanase from *Bacillus* was first inactivated by site-directed mutagenesis of the catalytic nucleophile and its ability for transfer α-laminaribiosyl fluoride was indeed established to prepare crystalline tailor-made oligosaccharides [89]. In their side, Fincher and Driguez showed that glucosynthases could derived from barley β-(1,3)-endoglucanases and were further used for the preparation of polyglucans in yields up to 75%, such polymers adopting a parallel triple helical conformation [90]. Moreover, the Glu231Gly mutant showed some interesting versatility towards a variety of hexopyranosidic acceptors [91].

CONCLUSIONS

All fundamental results obtained during the last ten years showed that specific interactions of β-(1,3)-glucans with PRRs, including more particularly Dectin-1 and CR3, trigger

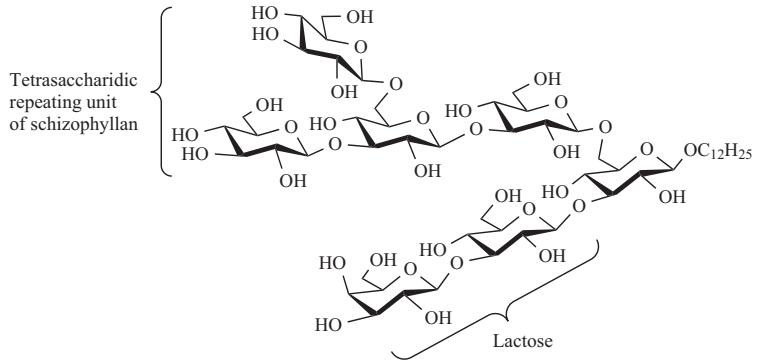


Fig. (8). Heptasaccharide bearing a lactosyl residue.

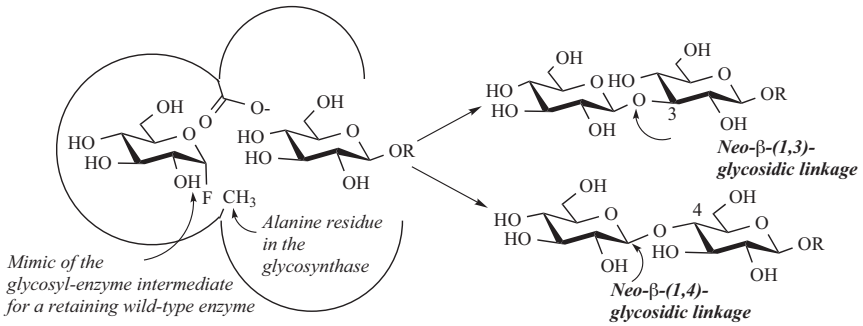


Fig. (9). Principle of chemo-enzymatic glycosylation using glycosyl fluoride as a donor and a glycosynthase as a biocatalyst.

a cascade of reactions notably generating cytokins, interleukins, tumor necrosis factor. The potential of β-(1,3)-glucans as therapeutic agents was complemented by preclinical and clinical studies [92]. For the treatment of infectious diseases caused by antibiotic-resistant bacteria, it was established that β-(1,3)-glucans can efficiently enhance the effect of antimicrobial agents [93,94]. It was also underlined the positive effects of β-(1,3)-glucans on the reduction of tumor-size and growth, resulting in increased survival in animals. The current increasing involvement of β-(1,3)-glucans in innovative therapies has to answer to several important questions: (i) the frequency, length and mode of administration, (ii) how actions of β-(1,3)-glucans could be extended to other types of cancer, (iii) how every patient reacts to administration of glucans, (iv) the need for a precise determination of the proper dose, that requires the reproducible production of sets of β-(1,3)-glucans with high degree of purity, and (v) what kinds of structure is really efficient, and in what case. Consequently, efforts have continuously to be made to increase knowledge on *in vivo* interactions of β-(1,3)-glucans with their receptors and have also to be accompanied by the design of still new derivatives for improving both their activities and their applicative potential in combination with other therapeutics agents.

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