Glycopolymer Binding

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Probing Bacterial-Toxin Inhibition with Synthetic Glycopolymers Prepared by Tandem Post-Polymerization Modification: Role of Linker Length and Carbohydrate Density**

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Protein-carbohydrate interactions mediate many critical biological recognition processes, such as those involved in cell signaling, fertilization, and inflammation, as well as the adhesion of viruses and bacterial toxins.[1] The proteins responsible for deciphering this information are termed lectins, which specifically (and noncovalently) bind carbohydrates based on their branching pattern, stereochemistry, and chemical functionality.[2] The protein–saccharide interactions are usually weak, but are amplified by clustered saccharides, resulting in a binding constant which is greater than the simple sum of the total number of ligands. This observation is referred to as the "cluster glycoside" effect.[3] Considering this information, glycopolymers are attractive materials to interact with lectins and have been shown to display binding affinities several orders of magnitude greater than a single carbohydrate molecule.[4] One of the most attractive applications of glycopolymers is to interfere with the binding of lectins from a pathogen to the host organism and therefore prevent infection from occurring; this is known as anti-adhesion therapy.[5]

Anti-adhesion therapy is not limited to polymeric glycoconjugates, and many high-affinity, small-molecule inhibitors of lectin binding are known.[6] The increased affinity of these small-molecule inhibitors is often because of favorable interactions within the sugar-binding pocket, obtained by structural biology studies.[6–7] Despite the wealth of structural information available, this design approach has rarely been applied to the design of polymeric inhibitors.[8] The toxin Ctx secreted by *Vibrio cholerae*, which is the causative agent of cholera, is a multimeric AB_5 lectin-like complex. The five B subunits bind GM1 gangliosides (Galβ1-3GalNAcβ1-4(Neu5Acα2-3)-Gal-β1-4Glc ceramide) present on the surface of the intestinal epithelium cells, initiating a series of events that results in the cholera symptoms. Multivalent, glycosylated STARFISH dendrimers successfully protected cells from similar toxins.[9] Polizzotti and Kiick have demonstrated that galactose-functionalized poly(L-glutamic acid) (PLG) is an effective inhibitor of Ctx.[10] Interestingly, lowering the density of the galactose residues on the PLG backbone from approximately 50% to 10% gave a dramatic increase in activity. The inhibitory activity was further enhanced when the spacing between PLG and galactose was increased, making the binding site more accessible, and was also related to the depth of the Ctx galactose binding pocket, which was estimated as being 16 Å. Others have speculated that longer linkers could enhance binding affinity, but these studies were not systematic or relied on a simple turbidimetry-based assay. The thermodynamics of this process have been evaluated in detail by Kane.[11]

A challenge associated with studying multivalent interactions is the synthesis of libraries of glycopolymers with precise control over the chain length, carbohydrate density, and nature of the linker. Direct polymerization of two glycosylated monomers is unlikely to lead to polymers with identical degrees of polymerization, which considering the importance of valency in carbohydrate-lectin interactions, is a major challenge.[12] The synthesis of glycopolymers by post-polymerization modification has attracted much interest, especially with the development of "click"-type reactions.[13] We have synthesized several classes of glycopolymers through cycloaddition of glycosyl azides with poly(propargylmethacrylate) scaffolds[4a,14] and used these to inhibit the binding of gp120 (derived from HIV) to DC-SIGN (one of its targets in humans)[15] The direct polymerization of alkyne (or alkene) monomers by radical methods is generally not possible because of competing side reactions,[13b] and the incorporation of co-monomers has the problem of obtaining identical length polymers. To address this, we introduced tandem post-polymerization modification to introduce incompatible functionality and control functional group density.[16] Using poly(pentafluorophenyl methacrylate) as the template scaffold, allylamine was introduced as a reactive, orthogonal "handle" and the remaining ester groups reacted with 2-hydroxypropylamine to give a water soluble backbone. This powerful method allows precise control over of a wide range of chemical space.

Herein, we use tandem post-polymerization modification to obtain glycopolymers with precisely controlled chain length, carbohydrate density, and crucially, defined back-

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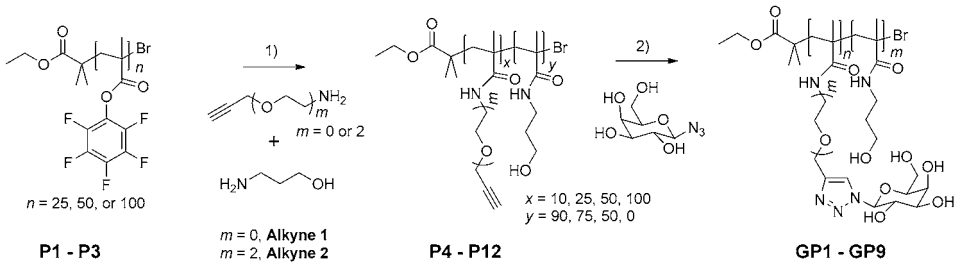
bone–carbohydrate linker lengths. This series of polymers was used to study the multivalent interactions between cholera toxin and peanut agglutinin, to probe the impact of modulating the binding site complementarity on the inhibitory activity of the glycopolymers. This unique combination of structural biology with materials science gives insights into the cluster glycoside effect and will allow design of active inhibitors.

The tandem post-polymerization modification strategy is outlined in Scheme 1. This approach is advantageous because it allows us to explore a large area of chemical space (through side-chain modification) while ensuring that the average degree of polymerization is identical within each series.



Scheme 1. Synthesis of glycopolymer libraries. 1) amine (variable amounts)/triethylamine (TEA; 1 equiv)/dimethylformamide (DMF), 5 h.; 2) GalN3 (1.5 equiv)/CuBr/TBTA, DMSO.

Two different amino-functional alkynes were selected as the polymer-carbohydrate spacers. Propargyl amine (**Alkyne 1**) acts as a short linker and a diethylene glycol group acts as a longer spacer (**Alkyne 2**). The reactive polymeric precursor, poly(pentafluorophenyl methacrylate), PPFMA, was synthesized by copper-mediated controlled-radical polymerization to give a series of polymers with varying degrees of polymerization, Table 1. As previously reported,[17] size-exclusion chromatographic (SEC) analysis of PPFMA gave values of M_n in disagreement with the theoretical M_n. PPFMA reacts readily with sterically unhindered amines, such as those used here.[17]

Homopolymers were synthesized by addition of a three-fold molar excess of **Alkyne 1** or **2**, as well as several copolymers (see below for rationale) by addition of 10, 25, and 50 mol% of **Alkyne 2**, followed by a three molar excess of 3-aminopropanol. Conversion of the pentafluorophenyl (PFP) groups into amides was confirmed by IR spectroscopy as the desired amide product (1675 cm^{-1}) and PFP ester (1786 cm^{-1}) have unique vibrational frequencies. In all cases, a small amount (< 5%) of carboxylic acid units can be seen at 1730 cm^{-1}, Figure 1. Fluorine NMR spectroscopy is often used to determine conversion of PFP groups, but does not discriminate between functionalization and hydrolysis, and therefore can give false positive results. The alkyne functional (co)polymers were subsequently functionalized with 1-azido-β-D-galactose ($GalN_3$) using the Cu^I/tris(benzyltriazolylmethyl)amine (TBTA) catalyst system, in dimethyl sulfoxide (DMSO)[4a] to yield homogenous glycopolymers, with no detectable alkyne or azide groups by NMR or IR spectroscopy. Over the course of the tandem post-polymerization functionalization, there was no evidence of fractionation, and all the polymers obtained were well-defined with polydispersity indexes (PDIs) below 1.3 (SEC data are included in the Supporting Information). Table 2 summarizes the diverse glycopolymer library that was synthesized and used for biological evaluation, below.

The aim of this investigation was to probe the effect of carbohydrate-binding site accessibility on the measured affinity between multivalent glycopolymers and their target lectins. The B subunit domain of cholera toxin was chosen because it is nontoxic,

Table 1: PPFMA precursor polymers.

Polymer	[M]:[I][a]	Conv. [%][b]	$M_{n(theo)}$[c]	$M_{n(SEC)}$[d]	M_w/M_n[d]	DP[e]
P1	25	71	4500	7800	1.19	18
P2	50	65	8200	8800	1.20	33
P3	100	70	17640	11400	1.16	70

[a] Feed ratio of monomer to initiator. [b] Determined by ^{1}H NMR spectroscopy. [c] Theoretical M_n, calculated from the feed ratio and percent conversion. [d] Determined by SEC in tetrahydrofuran (THF) using polystyrene standards. [e] Theoretical number-average degree of polymerization.

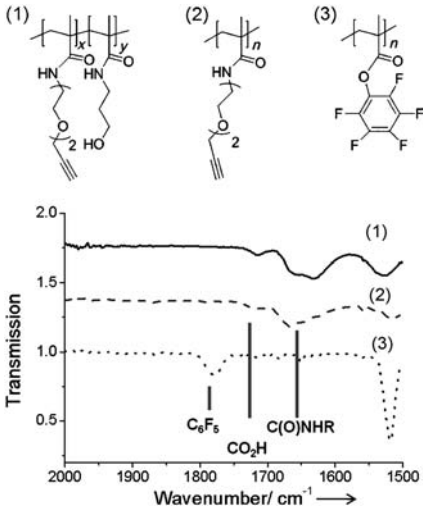


Figure 1. Infrared spectra showing conversion of the PFP groups (1786 cm^{-1}) into amides (1675 cm^{-1}). Carboxylic acid impurities are also indicated. Copolymer (solid line) has two distinct amide peaks.

Table 2: Glycopolymers obtained by tandem modification.

Polymer	DP[a]	Linker[b]	Density[c]	M_n[d]	M_w/M_n[d]
GP1	18	**Alkyne 1**	100	5100	1.29
GP2	33	**Alkyne 1**	100	5340	1.27
GP3	70	**Alkyne 1**	100	6000	1.26
GP4	18	**Alkyne 2**	100	7250	1.32
GP5	33	**Alkyne 2**	100	10000	1.28
GP6	70	**Alkyne 2**	100	11900	1.27
GP7	33	**Alkyne 2**	50	10800	1.23
GP8	33	**Alkyne 2**	25	10700	1.21
GP9	33	**Alkyne 2**	10	10500	1.20

[a] Theoretical number-average degree of polymerization. [b] Alkyne used to modify PPFMA scaffold. [c] Percent of repeat units functionalized with a galactose unit. [d] Determined by SEC in DMF.

and a report by Polizzotti and Kiick showed that longer linkers resulted in increased inhibitory activity of cholera toxin.[10] Peanut agglutinin (PNA) was used as a control as it also binds β-galactose, but its binding sites are surface exposed.[18] It is critical to include this second lectin, because it allows us to separate the effect of binding-site accessibility from increased side chain flexibility; the latter has been suggested to alter the binding of glycopolymers by allowing access to a larger number of possible conformations and leading to increased clustering. A recent study found that longer polymer–carbohydrate linkers showed increased affinity for the lectin, but the valency of each structure was not identical and there was no consideration of the depth of the lectin binding site.[19] Figure 2 shows schematic depictions of the galactose-binding domains of Ctx and PNA in comparison to the two linker systems used herein, which were selected to mimic these distances.

Fluorescence-linked sorbent assays were used to measure the inhibitory activity of the polymers. Briefly, microtitre plates were functionalized with GM1 ganglioside, which binds strongly to both lectins.[20] Fluorescein-labeled lectins (0.5 μM) were then incubated with a dilution series of the polymers for 30 minutes at 37 °C, and the unbound lectins were washed away. The total fluorescence was measured and inhibitory curves constructed. Data is presented as the minimum concentration required to inhibit 50 % binding of the lectin (MIC_{50}), Figure 3.

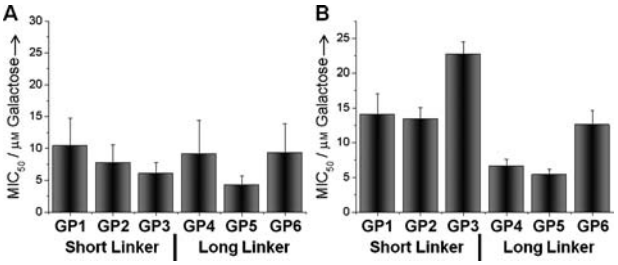


Figure 3. Inhibitory activity of glycopolymers **GP1**–**GP6** with A) PNA and B) Ctx. MIC_{50} values are expressed as total galactose concentration.

For PNA (Figure 3 A), there was a very weak effect of polymer chain length on inhibitory activity, but the differences were not statistically significant. The inhibitory activity of all the polymers is greatly increased relative to monovalent galactose (circa 100-fold). Although there are many reports of longer polymers having increased association constants with lectins, we recently demonstrated that this does not necessarily translate into improved inhibitory activity, in agreement with the data presented herein.[21] Our previous work also showed that above a certain chain length, no improved inhibition (based on carbohydrate concentration) was measured, as seen with the current results. For Ctx inhibition (Figure 3 B), there was a similarly weak dependence on polymer chain length, and no statistically significant differ-

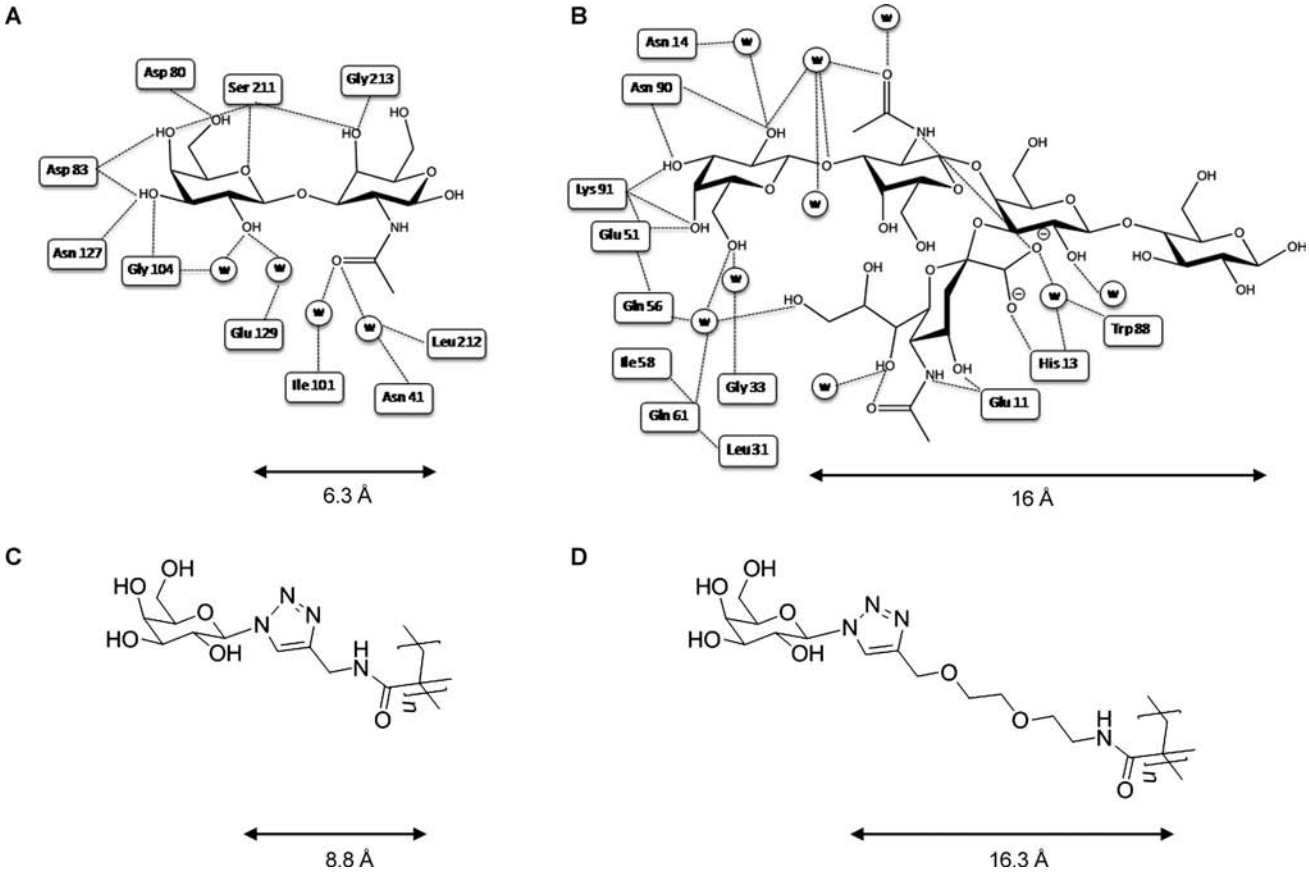


Figure 2. Binding sites of PNA (A) and Ctx (B). W = water ligand. C, D) Polymer side-chain linkers. Arrows indicate the distance from the terminal β-Gal residue to the far end of the native ligand. Distances for the protein-binding site of Ctx are taken from literature values.[10]

ences were recorded. However, there was a significant decrease in the MIC_{50} values upon increasing the length of the linker. This result agrees with the hypothesis that the longer linker better mimics the native ligand, GM1. The use of a hydrophilic ethylene glycol linker allows us to rule out any hydrophobic interactions in the binding pocket. Because we used the B subunit of Ctx that has only a single binding site, the differences in inhibition from linker length cannot be attributed to differences in cross-linking or intra-lectin binding-site spanning. These carefully chosen conditions and the use of a control lectin (PNA) ensure that binding-pocket depth is the only parameter contributing to increased activity. Control experiments with an α-manno polymer showed no inhibition in the concentration range tested (less than 100 μM carbohydrate), highlighting the specificity of the interaction.

Previous research has shown that a decrease in galactose density on the polymer backbone is accompanied by an increase in Ctx inhibition.[10,22] However, rigid α-helical polypeptide scaffolds were used, which cannot easily be reconfigured, and they also had a net-negative charge, which might promote electrostatic interactions. **P2** was functionalized with 10, 25, or 50 mol% of the longer linker (because of its higher binding affinity) followed by an excess of 3-aminopropanol to give variable density, and uncharged glycopolymers (**GP7–9**). Inhibition data are shown in Figure 4.

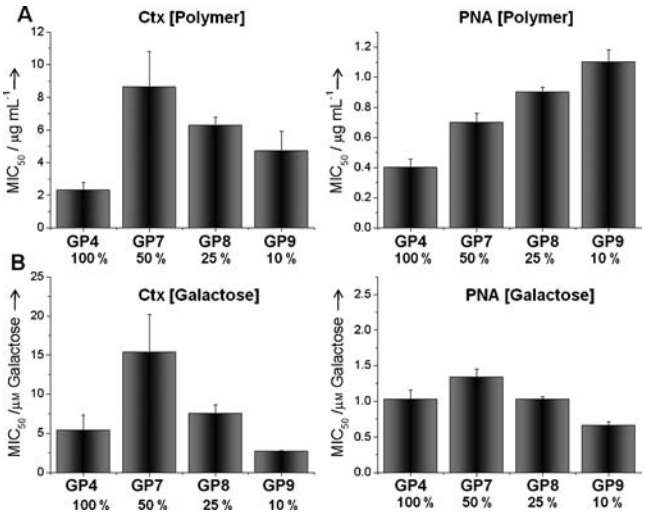


Figure 4. Inhibitory activity of variable density glycopolymers expressed in terms of A) polymer mass concentration and B) galactose concentration. Percentages on the *x*-axis indicate the percentage of repeat units on the polymer chain that have a galactose moiety.

Figure 4A shows inhibition data in terms of polymer mass concentration. For PNA, decreasing the saccharide density gives a concurrent increase in MIC_{50} (i.e. less activity), which can be interpreted as lower galactose densities leading to a relative decrease in binding affinity/inhibitory activity. For Ctx, the 50% functionalized polymer was the least active. However, this analysis is oversimplified, and it is necessary to consider the data in terms of relative activity per saccharide unit (Figure 4B). In this manner, the 10% and 100% functionalized polymers are the most active on a per-carbohydrate basis for both lectins, suggesting several features of the macromolecules contribute to inhibitory activity. The Ctx used in this study has a single binding site (compared to five sites in the native toxin), which excludes the contribution from spanning multiple sites. The control experiments with PNA, which has multiple binding sites, also showed a clear decrease in affinity as the galactose density decreased, implying that spanning of multiple sites is not the most important feature for inhibition. This result agrees with our previous findings using the ConA/Mannose pairing that indicated the spanning of multiple sites contributed to higher association constants, but not to increased inhibition.[21] A second component of this result could be steric hindrance of adjacent galactose residues; crowding may reduce rebinding of galactose into the deep binding pocket of Ctx resulting in lower inhibitory activity for 50% polymers compared to the 10% functionalized polymers. These data fit with the hypothesis that the binding site in Ctx limits accessibility, relative to the shallow binding site on PNA, for which the polymers with the highest valency showed highest inhibitory activity. The high activity (on a per-sugar basis) of the 100% versus 50% functionalized polymers would seem to contradict the above hypothesis. However, in this case the density of the 100% functionalized polymers might be sufficiently high to overcome the limitations of steric crowding and benefit from a higher rate of statistical rebinding, or slower rate of dissociation. Detailed studies into these binding events are currently underway using a range of biophysical techniques, and these findings are being applied to the rational design of highly active inhibitors of infection.

In summary, a series of glycopolymers with varying saccharide density, linker length, and chain length were synthesized by tandem post-polymerization modification. Longer linkers were shown to result in increased inhibition of the B subunit of cholera toxin, which is attributed to the depth of the binding pocket. Comparison with peanut agglutinin, which has a shallower binding pocket, revealed no difference in inhibitory activity as a function of linker length. The tandem post-polymerization modification strategy also allowed the effect of carbohydrate density to be studied. A nonlinear relationship was measured, in which the highest and lowest density polymers tested (100% and 10%) were most active, on a per-sugar basis, highlighting the complexity of these interactions. These measurements demonstrate that in the design of biomimetic macromolecules for anti-adhesion or other therapeutic applications, structural biological information must be considered, in conjunction with using the relevant assays. Furthermore, the best polymer structure for a particular lectin is not necessarily the optimum structure for other lectins, even those with the same carbohydrate specificity. Future work will be focused on the development of highly active inhibitors, diagnostics, and gaining a thorough understanding of the cluster glycoside effect with glycopolymers through the use of complimentary biophysical techniques.

Experimental Section

Functionalization of PPFMA with amino-functional alkynes: Poly(pentafluorophenyl methacrylate) (100 mg, 42 mmol PFP), alkyne linker (propargyl amine or 2-[2-(prop-2-ynyloxy)ethoxy]ethanamine (varying amounts)), and TEA (80 mg, 84 mmol) in DMF (5 mL) were stirred at 50 °C for 16 h. After this time a three molar excess (relative to PFP groups) of 3-aminopropanol was added, when copolymers were desired, and stirred for a further 16 h at 50 °C. The solution was twice precipitated into diethyl ether, then centrifuged and dried under vacuum to afford the alkynyl-functionalized polymers.

Alkyne 1: ^{1}H NMR (400 MHz, MeOD): $\delta = 1.2$ (CH_3 backbone), 1.8 (CH_2 backbone), 3.2 (C≡CH), 4.4 ppm (s, NH−CH_2); IR: $\tilde{\nu} = 1650\ cm^{-1}$ (amide C=O). **Alkyne 2**: ^{1}H NMR (400 MHz, MeOD): $\delta = 1.2$ (CH_3 backbone), 1.8 (CH_2 backbone), 3.1 (CH_2), 3.2 (C≡CH), 3.5 (CH_2), 3.7 (CH_2), 4.2 ppm (s, NH−CH_2); IR: $\tilde{\nu} = 1720$ (CO_2H), 1650 cm^{-1} (amide C=O).

Copper-catalyzed [3+2] cycloaddition of $GalN_3$ with alkyne-functionalized polymers: Alkynyl functionalized polymer (20 mg, 0.18 mmol), 1-azido-β-D-galactose (80 mg, 0.36 mmol), TEA (10 mg, 0.072 mmol), and CuBr (2 mg) dissolved in [D_6]DMSO (4 mL) were charged in an ampoule and deoxygenated through three freeze–pump–thaw cycles before being placed under nitrogen. TBTA (8 mg, 0.14 mmol) was added and the reaction was degassed and left under nitrogen for 48 h. The glycopolymer was purified by dialysis against water using 1000 Da MWCO tubing and freeze-dried. **GP1**: ^{1}H NMR (400 MHz, MeOD): $\delta = 1.2$ (CH_3 backbone), 1.8 (CH_2 backbone), 4.4 (s, NH−CH_2), 3.4–3.5 (2H, H-2 + H-3), 3.60–3.80 (3H, CH_2 and CH, H-6a, H-6b, H-5, H-4), 4.7 ppm (1H, H-1); IR: $\tilde{\nu}$ =3100–3300 (OH), 3050 (C−H), 1650 cm^{-1} (amide C=O); SEC (DMF): $M_n = 5100$, $M_w/M_n = 1.29$.

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