Dynamic Combinatorial Carbohydrate Libraries: Probing the Binding Site of the Concanavalin A Lectin

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Abstract: Dynamic combinatorial chemistry (DCC) has emerged as an efficient approach to receptor/ligand identification based on the generation of combinatorial libraries by reversible interconversion of the library constituents. In this study, the implementation of such libraries on carbohydrate–lectin interactions was examined with the plant lectin Concanavalin A as a target species. Dynamic carbohydrate libraries were generated from a pool of carbohydrate aldehydes and hydrazide linker/scaffold components through reversible acylhydrazone exchange, resulting in libraries containing up to 474 constituents. Dynamic deconvolution allowed the efficient identification of the structural features required for binding to Concanavalin A and the selection of a strong binder, a tritopic mannoside, showing an IC_{50}-value of 22 μM.

Keywords: carbohydrates · combinatorial chemistry · Concanavalin A · glycoconjugates · receptors

Introduction

Dynamic combinatorial chemistry (DCC) has emerged as a new, potent approach to rapid ligand or receptor identification based on the implementation of dynamic assembly and recognition processes.[1–6] The concept is based on reversible connections between suitable building blocks, leading to spontaneous assembly of all their possible combinations and allowing for the simple one-step generation of extended libraries. Because of their dynamic nature, such libraries allow for target-driven and self-screening processes that may lead to the preferential expression of the active species presenting the strongest binding to the target entity.

Libraries emanating from this process, dynamic combinatorial libraries (DCLs), have been applied in a number of cases both in the generation and identification of ligands targeting a certain receptor, as well as the converse situation where receptors are selected for given ligands or templates. In our laboratory, strand self-recognition in helicate formation[7] and anion-dependent generation of circular helicates[8] led to the formulation of the DCC concept. Inorganic[9–12] and organic model systems,[13–18] in addition to studies involving various biological target molecules,[19–25] have further demonstrated the potential of this technique.

Molecular recognition of carbohydrates represents a research area with strong potential bearing on drug discovery, as well as various biotech applications.[26,27] Carbohydrates play central roles in many biological processes, such as cell–cell interactions and cell communication, and numerous enzymes are involved in various carbohydrate-mediated processes associated with cell proliferation and cell death, for example. Carbohydrates are therefore highly attractive tools for generating mimics and analogues of such recognition processes and many attempts have been made to evaluate the possibility of designing ligands, based on naturally occurring carbohydrates, for direct or indirect inhibition of carbohydrate-recognising enzymes or as potential agonists/antagonists of carbohydrate receptors.

However, the synthesis of combinatorial carbohydrate libraries is a challenging task[28–30] in which DCC may offer a complementary route, especially in forming libraries of dynamically interchanging carbohydrate clusters. Examples in which DCC has been applied in glycoscience include the generation of prototype dynamic combinatorial libraries using metal coordination in generating tritopic clusters[25,31] and disulfide interchange to produce ditopic carbohydrate structures.[22]

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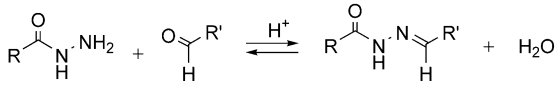
To widen the scope of the use of DCC in biological systems, and especially to further explore the potential of DCC in glycobiology, we have developed dynamic libraries of constituents that are susceptible to binding to the plant lectin Concanavalin A (Con A) using the hydrazidecarbonyl/acylhydrazone interconversion as reversible chemistry. In addition to demonstrating the use of this chemistry for carbohydrate applications, the results also provide initial steps towards the generation of efficient substrates for other carbohydrate binding targets.

Results and Discussion

Design of the dynamic libraries: Imine generation and exchange, especially imine formation from hydrazides (acylhydrazones, Scheme 1), has proven highly suitable in designing dynamic combinatorial libraries in aqueous media.[24,32]

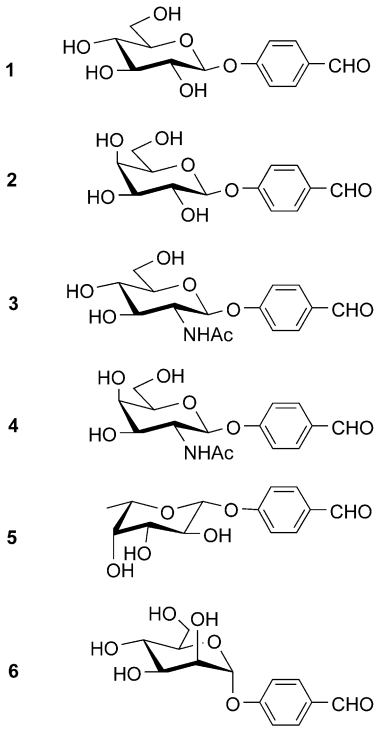


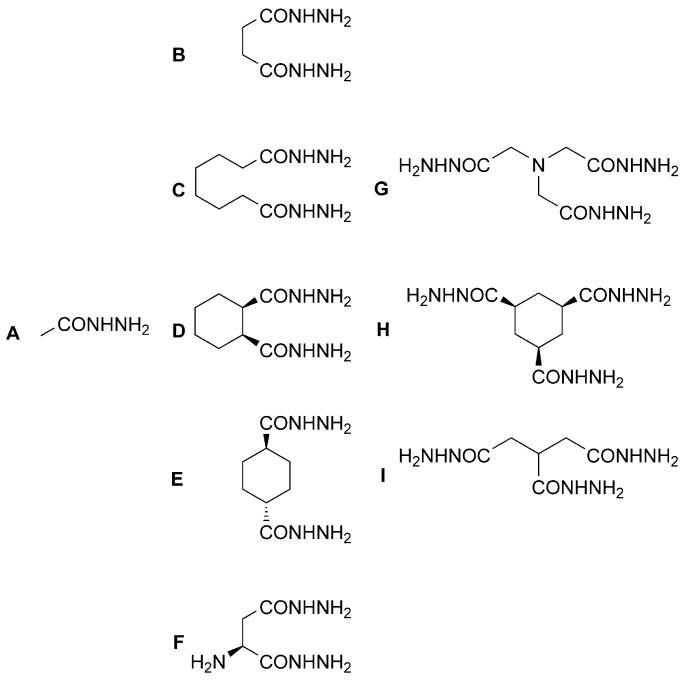
Scheme 1. Reversible acylhydrazone formation.

Acylhydrazone interconversion enables rapid library generation in which the dynamic properties can be easily controlled by pH; that is, the formation and exchange reactions take place at a lower pH (3–7) and can essentially be stopped at a higher pH (>7). Acylhydrazone libraries are also amenable to high structural complexity due to the orthogonal nature of the different components. The aldehyde functionality can be reserved for the interactional components and the hydrazide functionality for the structural components, or vice versa, allowing for branching and substitution of multifunctional cores.

In the present study, acylhydrazone libraries were generated from the dynamic assembly of a series of oligohydrazide core building blocks used to arrange the interactional components in a given geometry, with a set of aldehyde counterparts potentially capable of interacting with the binding site of the target species. The interactional components (**1**–**6**) were based on the six common, naturally occurring carbohydrates: β-D-glucopyranose (Glc), β-D-galactopyranose (Gal), 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc), 2-acetamido-2-deoxy-β-D-galactopyranose (GalNAc), α-L-fucopyranose (Fuc) and α-D-mannopyranose (Man). The structural core components (**A**–**I**) were chosen to contain one, two or three attachment groups in order to probe the effect of multivalency through the presence of several interacting groups in the same library constituent.

Hydrazides were chosen as structural core components, since they represent linkers/scaffolds of high solubility in the aqueous buffer system used. For the same reason, the aldehyde counterpart was attached to the carbohydrate moieties, solubilising the otherwise nonpolar benzaldehyde fragments. All components were easily soluble in the buffers used.





Generation of the dynamic combinatorial libraries (DCLs): Generation of the dynamic libraries was easily accomplished at a moderately acidic pH (ammonium formate buffer, pH 4), upon gentle agitation overnight. Scrambling of an acylhydrazone library of this type is, however, normally completed within a few minutes to a couple of hours depending on hydrazide/aldehyde combination and the pH of the solution.[33] A complete acylhydrazone library resulting from these 15 components amounts to at least 474 different constituents, not counting partially formed species, but ac-

counting for symmetry effects. The final concentration of the libraries amounted to 15 mM in total for both hydrazide and aldehyde, resulting in a theoretical yield of 32 μM per library member.

Screening/deconvolution procedure: In order to screen the libraries against the lectin Con A, an enzyme-linked lectin assay (ELLA) in a standard 96-well format was adopted based on yeast mannan as the immobilised ligand.[34] When testing the complete 474-membered library in the assay, it became clear that it contained one or more active species that were potent inhibitors of the binding. Thus, addition of the complete library to the binding assay resulted in strong inhibition of the binding signal up to a concentration of 1/50 of the stock solutions, corresponding to approximately 0.15–2.4 μM of each library member.

In order to identify the active compounds of the libraries, a previously described dynamic deconvolution protocol relying on the dynamic features of the libraries was utilised.[24] This method is based on the removal of single building blocks from the complete library, resulting in redistribution of the remaining components and suppression from the equilibrating pool of all constituents containing the removed components. For each component making up the DCL, a sublibrary is prepared from which all library constituents based on this element are removed. Thus, when using this dynamic deconvolution strategy a decrease in inhibitory effect reveals the importance of the removed component in the generation of active compounds in the dynamic library. This protocol leads to a limited number of samples (one for each building block plus references), but is nevertheless highly efficient in targeting active species.

The complete library (CL) pool was generated by simultaneously adding all the building blocks (**1**–**6**, **A**–**I**) together under pre-equilibrating conditions in acidic buffer at ambient temperature. At the same time, 15 sublibraries were formed by mixing all components except one specific hydrazide or aldehyde building block under the same conditions. The respective libraries obtained were composed of all possible condensation products in proportion to their relative thermodynamic stability. Together with a reference sample (buffer) containing no building blocks, this series of 17 samples was sufficient for screening the entire 474-member library. Following equilibration, the libraries were subsequently subjected to the lectin assay in which the inhibitory potency of the library constituents towards Con A in competition with the immobilised ligand was monitored.

The results obtained from this library generation and screening process are presented in Figure 1a in which the inhibitory effects of the sublibraries have been related to the activity of the complete library. The inhibition of the Con A binding activity by a library indicated the presence of one or several active condensation products in a given equilibrated mixture. On removal of individual building blocks from the complete library in the collection of sublibraries, an increase in activity indicated that the omitted component contributed significantly to the inhibitory effect, while a decrease in activity indicated that the component hampered the effect of the more active compounds. The data in Figure 1a show that

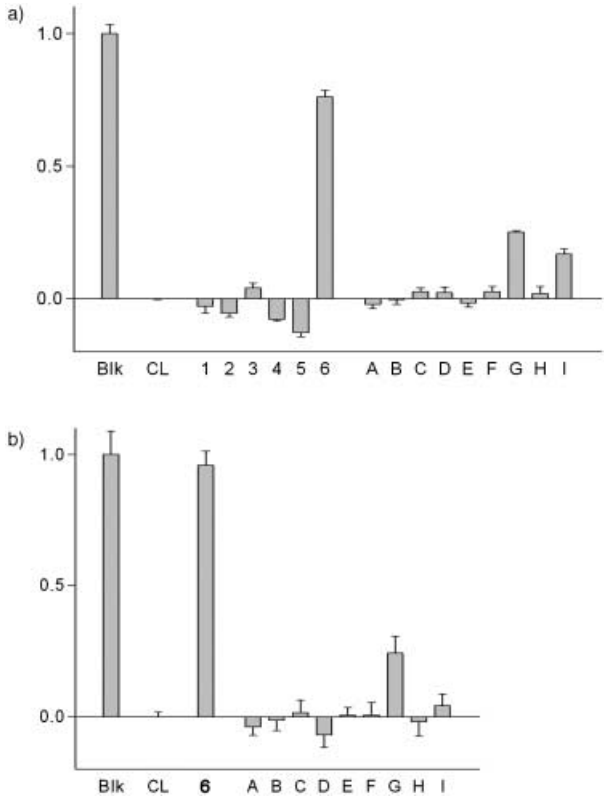


Figure 1. Dynamic deconvolution of dynamic combinatorial libraries of carbohydrates demonstrating the detection of the potency of compound **6** (Man derivative) and compound **G** (tritopic hydrazide). a) DCL containing all carbohydrate aldehydes and all hydrazide components used. b) DCL containing one carbohydrate aldehyde (compound **6**) and all hydrazide components. Blk denotes blank sample containing buffer only, CL denotes complete library comprising all components. Abscissa figures and letters denote sublibraries in which the indicated component is removed.

several components proved active in the present study with the largest effect arising on removal of the mannose unit (**6**) from the complete DCL. Clearly component **6** is necessary for inhibition to occur, whereas the other aldehyde building blocks are less important, much in accordance with the binding selectivity of this lectin. Similarly, of the core building blocks hydrazide **G** was most active, followed by **I**, while smaller effects were observed for the other structural components. Consequently, the most active constituent is likely to come from the assembly of fragments **6** and **G**.

As a reference, a reduced library containing only one aldehyde unit (the most active component **6**) was prepared to further probe the individual effects of the structural components. When omitting five out of six interactional groups, the size of this library is reduced to nine different acylhydrazone constituents (not counting partially formed species). Ten sublibraries were simultaneously prepared and all libraries screened in the lectin assay. The results from this campaign are presented in Figure 1b, accentuating the effects found with the 474-member library. Clearly, components **6** (Man) and **G** are the most active components in this library.

Three main conclusions may be drawn from the results obtained:

1) By far the most active carbohydrate component is that containing mannose, as expected.
2) The two trivalent library constituents derived from the trihydrazides **G** and **I** are more active than the divalent ones overall, in agreement with the fact that the natural substrate of Con A is a trimannoside.
3) Of the three trivalent components (**G**, **H** and **I**), the more flexible **G** and **I** correspond to the higher activity of all the core units (**A**–**I**), whereas the more rigid component **H** is very inefficient and comparable to the divalent hydrazides **B**–**F**, despite the similar distance between the three hydrazide functions in **G**, **H** and **I**. This result may mean that these three components are probably too large, but that **G** and **I** can adjust, whereas **H** cannot, pointing to the importance of flexibility.

Evaluation of the best binder of the DCL: From both libraries tested, the components building up constituent $\mathbf{6_3}$**–G** were identified as most important. This constituent was synthesised separately from the peracetylated derivative **7** of compound **6** and structural component **G**, as shown in Scheme 2.

The effect of this constituent was subsequently estimated in a binding assay (Figure 2), resulting in an IC_{50} value in the micromolar range (22 μM). For comparison, methyl-α-D-mannoside was used as a reference ($IC_{50} = 0.8$ mM), resulting

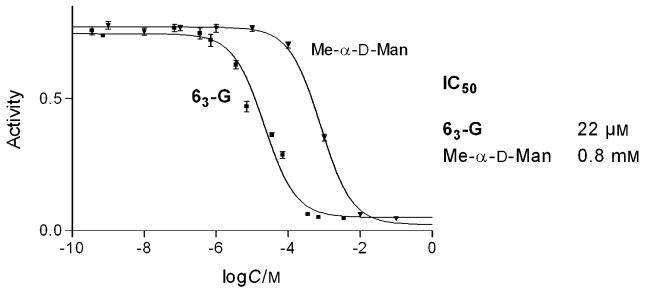
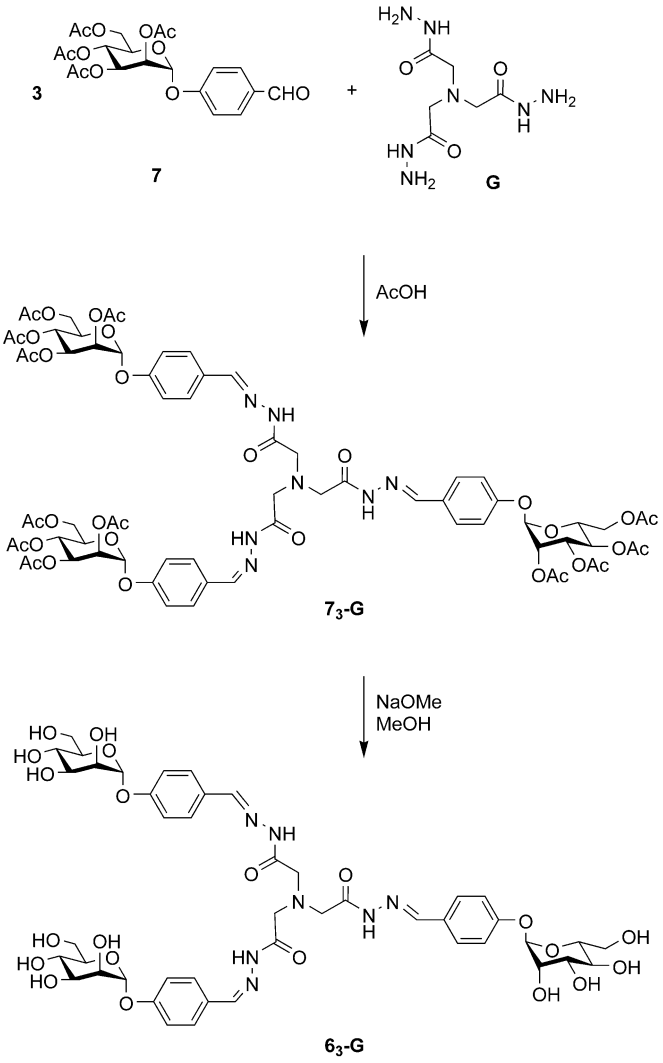


Figure 2. Estimation of the relative binding potency (IC_{50}-values) of library hit $\mathbf{6_3}$**–G** and a reference compound, Me-α-D-mannoside, in the interaction with Concanavalin A.

in a 36-fold binding difference. This is comparable to the natural trimannoside ligand, which shows a 60-fold higher affinity than methyl-α-D-mannoside at pH 7.2.[35]

Conclusion

In this study it has been shown that lectin ligands can be generated by acylhydrazone formation and exchange, allowing the efficient generation of dynamic combinatorial libraries in aqueous media. A set of 15 initial building blocks yielded a library containing at least 474 different species. Among all possible acylhydrazones formed, active compounds of the appropriate geometry-containing potent recognition groups could be rapidly identified using a dynamic deconvolution process. This method enabled the efficient identification of a novel tritopic mannoside, showing potent



Scheme 2. Synthesis of compound $\mathbf{6_3}$**–G**.

binding to Concanavalin A. The data also point to the fact that in the first stage of component selection for setting up DCLs, it is beneficial to introduce flexible components to allow adaptation to the target of the DCL constituent generated. Once a preliminary consituent has been found, more precisely defined structural components can be implemented in the next stage.

Experimental Section

General: All reagents were purchased from commercial sources and used after appropriate purification. Compounds **1** and **2**,[36] **3** and **4**,[37] **5**–**7**[38] and **D**–**I**[38] were synthesised according to literature procedures. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AC200 spectrometer at 298 K. Mass spectra were determined by the Service de Spectrométrie de Masse at the Institut de Chimie, Université Louis Pasteur. Microanalyses were performed at Service de Microanalyse at the Institut de Chimie, Université Louis Pasteur, or at the Institut Universitaire de Technologie, Strasbourg-sud, France. Binding assays were monitored using a Victor^2 plate reader (Perkin Elmer).

Synthesis of dynamic combinatorial libraries: Stock solutions of individual aldehyde and hydrazide components (2–50 mM) were prepared in water. The libraries were subsequently generated by combining the solutions assuming equal concentrations of all potential acylhydrazones

(32 μM each), the pH was adjusted to 4.0 by addition of ammonium formate buffer (AFB, 1 M) and the resulting mixture was allowed to equilibrate at ambient temperature overnight to ensure a full reaction. Aliquots of the equilibrated solution were then tested in the binding assay. Sublibraries were prepared in the same way using water instead of excluded library components.

Analysis of binding to Concanavalin A: Binding of the library constituents to Concanavalin A was monitored by an enzyme-linked assay[39] in a 96-well microtiter plate format. The wells were first incubated with yeast mannan (Sigma M7504, 10 $\mu g L^{-1}$ in 10 mM phosphate buffered saline (PBS) pH 7.4, 100 μL per well) at room temperature overnight. Following washing three times with washing buffer (PBS containing 0.05% (v/v) Tween 20 (PBST), 300 μL per well), the wells were blocked with bovine serum albumin (BSA, 1% in PBS, 150 μL per well) for 1 h at 37 °C. After a new wash with PBST (3×300 μL per well), the wells were incubated with library solutions containing horse-radish peroxidase-labelled Concanavalin A (Con A-HRP, ICN 153246, 0.9 $mg mL^{-1}$ in PBS, 100 μL per well) at room temperature for 2 h. The plates were washed with PBST (2×300 μL per well), and developed by addition of the diammonium salt of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 0.25 $mg mL^{-1}$ in 0.2 M citrate/phosphate buffer containing 0.015% H_2O_2, pH 4.0, 50 μL per well). The reaction was finally stopped after 20 min by addition of dilute sulfuric acid (1 M, 50 μL per well), and the optical density measured at 405 nm.

Synthesis of 7_3–G: Compound **G** (50 mg, 0.2 mmol) was dissolved in acetic acid (2 mL). Compound **7** (300 mg, 0.65 mmol) was added, and the reaction allowed to proceed for 30 min at ambient temperature under argon. After the reaction mixture had been concentrated, the product was purified by flash chromatography (SiO_2, hexane/methanol/ethyl acetate 30:60:10–20:70:10, v/v) and dried in vacuo. $[\alpha]_D = +92°$ ($c = 0.8$ in $CDCl_3$); ^1H NMR ($CDCl_3$): $\delta = 11.9$ (br s, 1.3H; NH), 11.1 (br s, 1.7H; NH), 8.76 (br s, 1.3H; CH=N), 8.20 (d, $J = 4.0$ Hz, 1.7H; CH=N), 7.75–7.52 (m, 6H; H-*meta*), 7.09 (d, $J_{o,m} = 8.7$ Hz, 6H; H-*ortho*), 5.55 (d, $J_{1,2} = 1.5$ Hz, 3H; H-1), 5.54 (dd, $J_{2,3} = 9.3$ Hz, $J_{3,4} = 3.3$ Hz, 3H; H-3), 5.44 (m, 3H; H-2), 5.36 (dt, $J_{4,5} = 10.0$ Hz, 3H; H-4), 4.27 (dd, $J_{5,6a} = 5.6$ Hz, $J_{6a,6e} = 12.7$ Hz, 3H; H-6a), 4.18–3.99 (m, 9H; H-5, H-6e, CH_2), 3.81–3.60 (m, 3H; CH_2), 2.20 (s, 9H; Ac), 2.05 (s, 9H; Ac), 2.03 (s, 9H; Ac), 2.02 ppm (s, 9H; Ac); ^{13}C NMR ($CDCl_3$): $\delta = 170.5$, 170.0, 157.3, 143.8, 129.4, 128.9, 116.7, 95.7, 69.4, 68.9, 65.9, 62.1, 20.7 ppm; elemental analysis calcd (%) for $C_{69}H_{81}N_7O_{33}$: C 53.94, H 5.31, N 6.38; found: C 53.61, H 5.44, N 6.12; MS (FAB positive mode): m/z calcd: 1535.5; found: 1536.5 [M+H].

Compound 6_3–G: Compound 7_3–**G** (27 mg, 0.018 mmol) was dissolved in methanol (1 mL). NaOMe (0.5 mg) was added, and the reaction allowed to proceed at ambient temperature overnight. Evaporation and drying of the reaction mixture yielded 18 mg of white solid (quant.). $[\alpha]_D = +115°$ ($c = 0.8$ in H_2O/MeOH 1:1, v/v); ^1H NMR (CD_3OD/D_2O 1:1, v/v): $\delta =$ 8.16 (s, 1.2H; CHN), 8.05 (s, 0.9H; CHN), 7.90 (s, 0.6H; CHN), 7.79 (s, 0.3H; CHN), 7.70–7.44 (m, 6H; H-*meta*), 7.17–6.92 (m, 6H; H-*ortho*), 5.62–5.38 (m, 3H; H-1), 4.12–3.45 ppm (m, 24H; H-2, H-3, H-4, H-5, H-6a, H-6e, CH_2); ^{13}C NMR (CD_3OD/D_2O 1:1, v/v): $\delta = 170.8$, 170.5, 159.5, 159.1, 151.8, 151.5, 130.8, 130.2, 129.1, 118.2, 99.6, 75.2, 72.2, 71.6, 68.0, 62.2, 59.4 ppm; elemental analysis calcd (%) for $C_{45}H_{57}N_7O_{21}\cdot 2\,CH_3OH/H_2O$: C 50.67, H 6.06, N 8.80; found: C 50.49, H 5.93, N 8.66.

Estimation of IC_{50} values: The IC_{50} (50% inhibition of Concanavalin A binding) curves were based on consecutively diluted concentrations of tested inhibitors included in the assay with HRP-labelled Concanavalin A. Typically, each condition was tested in triplicate. The program GraphPad Prism (GraphPad Software) was adopted for nonlinear regression analysis determining IC_{50} values using the following equation: $A = A_{min} + (A_{max} - A_{min})/(1 + 10^{(C - \log IC50)})$, in which A is the inhibitory activity and C is the inhibitor concentration.

Acknowledgement

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