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ORIGINAL PAPER

Method for Analysis of Different Oligosacchiride Structures

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Abstract In this study, an improved, rapid, high yield synthesis of *N,N'*-4,4'-bis(benzyl-2-boronic acid)-bipyridinium dibromide (*o*-BBV) is described. The obtained *o*-BVV is applied in a two-component saccharide sensing system (complex) where it serves as a fluorescence quencher and a saccharide receptor. This system was applied to different natural oligosaccharides isolated from molluscan *Rapana venosa* (RvH1-a) and arthropodan *Carcinus aestuarii* (CaeH) hemocyanins (Hcs) and cyclodextrins (CDs). The carbohydrate contents of both Hcs were calculated in our previous work to be 1,6 % and 7 % for CaeH and RvH1-a, respectively. We propose that the difference in fluorescence increase of the native CaeH and RvH1-a when titrating them with the complex is due to the fact that the carbohydrate content of CaeH is lower and the carbohydrate chains are buried in between the structural subunits of the native molecule, while the glycans of the functional unit RvH1-a are exposed on the surface of the molecule leading to a 4-fold fluorescence's intensity change.

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Introduction

The immune system is one of the most commonly studied in which glycans and glycoproteins play an important physiological role. Therefore, saccharide sensing is a current topic of interest in molecular recognition chemistry in determining saccharide concentrations in physiological fluids and clarifying the functions of glycoproteins at biomembranes [1, 2]. There are several methods to identify oligosaccharide structures, as well as glycosylation patterns on cells, tissues and body fluids [3–5]. Although several types of saccharide receptors applying different recognition mechanisms are available, the most well-understood receptors are based on arylboronic acids [5–10] because of their favourable stability in water, and easy detectable UV-VIS and fluorescence signals. In contrast to boronic acid-based saccharide recognition systems consisting of a single detector, we provide in this communication a two-component sensing system [11] comprising an anionic fluorescent dye (1) and a boronic acid-appended cationic viologen (2) (Fig. 1) that serves as fluorescence quencher and saccharide receptor [12–15].

Cyclodextrins (CDs) are a family of cyclic oligosaccharides, composed of α-1,4-linked glucopyranose subunits [16–18]. CDs are produced from starch by enzymatic degradation. α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CDs) are the most common CD species, referred to as first generation parent CDs (six, seven and eight glucosyl units, respectively). Generally, CDs can form host-guest complexes with a large variety of solid, liquid, and gaseous organic compounds by a molecular inclusion phenomenon. This inner inclusion exerts a profound effect on the physicochemical properties of the guest

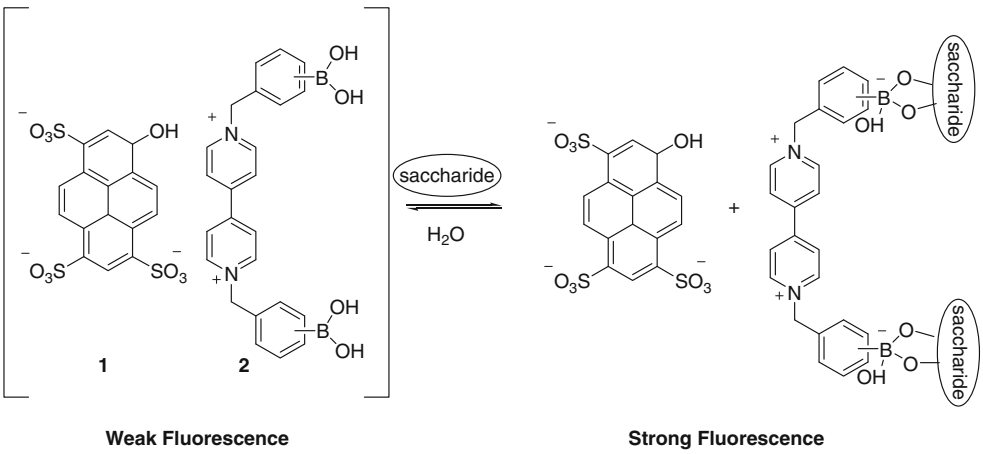


Fig. 1 Proposed mechanism of saccharide detection: saccharide-induced dissociation of ground-state complex results in fluorescence increase

molecules as they are temporarily locked or caged within the host cavity, giving rise to beneficial modifications on the guest molecule's properties (solubility, reactivity, volatility) [19]. Therefore, the native CD modifications are effective templates for generating wide ranges of molecular hosts [20] and are employed as carriers for biologically active substances [21], enzyme models [22], separating agents [23], catalysts [24], mass transfer promoters [25], additives in perfumes, cosmetics, aliments or food [26], environmental protection agents [27], or sensors for organic molecules [28].

Hemocyanins (Hcs) are high molecular mass oxygen-transporting proteins, freely dissolved in the hemolymph of several arthropods and molluscs. Arthropodan Hcs occur as hexamers or multiples of hexamers (up to 8×6-mers) of approximately 75 kDa subunits, each containing a Cu(I) pair able to reversibly bind dioxygen [29, 30]. Molluscan Hcs, in contrast, are decamers or di-decamers of approximately 350–450 kDa subunits, and the (di)decameric structure has the shape of a hollow cylinder [31, 32]. Each structural subunit is organized by seven or eight globular functional units (FUs) of approximately 50 kDa. These FUs contain one dioxygen-binding Cu(I) pair and are assigned by the letters a–g (h) starting from the N-terminus [31–34]. Beside the quaternary structure, there are also large differences in the carbohydrate content and monosaccharide composition of Hcs from arthropods and molluscs. The carbohydrate content of arthropodan Hcs is relatively low (0.1–2 %, w/w) [35], while the molluscan Hcs usually have a higher carbohydrate content (2–9 %, w/w) and may contain unusual monosaccharides [36–39].

Results and Discussion

New methods for analyzing glycans and providing information about the glycosylation patterns on cells, tissues and in body fluids, are needed for diagnostics and treatment, as well as for facilitating studies on the effects of glycosylation. Therefore, we represent herein the application of a complex between an anionic fluorescent dye and a boronic acid-appended cationic viologen, serving as a fluorescence quencher and a saccharide receptor.

A literature survey revealed two main synthetic procedures to obtain *N,N'*-4,4'-bis(benzyl-2-boronic acid)-bipyridinium dibromide, which differ in used solvents and reaction temperatures. The described methods for quaternization of 4,4′-bipyridine with *o*-bromomethylphenylboronic acid were carried out in different solvents—in methanol at room temperature for 15 h [40] or in DMF by heating to 70 °C for 48 h [41]. The most frequently described and cited method applies stirring for 15 h at room temperature in an inert atmosphere, followed by a multistep and laborious isolation procedure [40]. In order to shorten and improve the synthetic procedure as well as to avoid the use of a solvent, we simply melted the starting materials and so the reaction temperature was determined by the melting points of the starting compounds. It was found that if the temperature of the reaction mixture is below 100 °C, this does not allow obtaining of a homogenous melt at low reaction rate. Heating of the reaction mixture to 100–110 °C for 20 min (Fig. 2) was found to be optimal, giving a final product in high purity and excellent yield by TLC monitoring. The structure was confirmed by elemental analysis and ^{1}H NMR spectroscopy.

The absorbance spectrum of pyrinine shows only one maximum at 432 nm and two maxima were identified at 440 nm and 470 nm after titration with *o*-BBV (Fig. 3). For our fluorescence spectroscopic measurements, a solution of pyranine (1.33×10^{-6}) in 50 mM phosphate buffer, pH 7.5, was titrated with increasing amounts of the *o*-BBV and a decrease (4-fold) in fluorescence emission of pyranine (λ_{em}=508 nm, λ_{ex}=460 nm) was observed (Fig. 4). The fluorescence quantum yield (Φ) of pyranine in our instrumental setup in aqueous solution has a value of 0.89. After titration of pyranine with *o*-BBV, a complex formation has

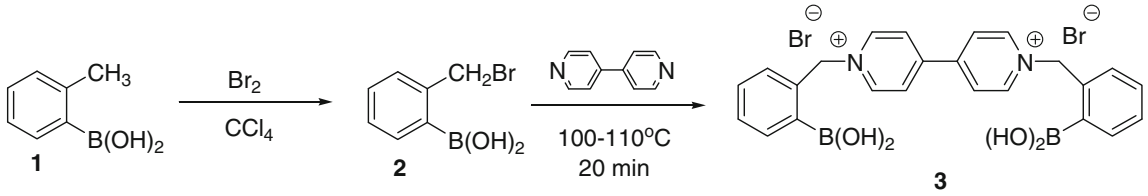


Fig. 2 Synthesis of o-bromomethylphenylboronic acid (2) and N,N'-bis-(benzyl-2-boronic acid)-[4,4′]bipyridinium dibromide (*o*-BBV, 3)

occurred, which cause reduction of the pyranine's quantum yield up to 0.18.

In the proposed mechanism in Fig. 1, the electrostatic association of the pyranine and the quencher results in ground-state complex formation, facilitating electron transfer from the dye to the viologen, which leads to a decrease in fluorescence intensity [42, 43]. When different saccharides are added to the system, formation of two anionic boronate esters effectively neutralize the dicationic viologen, thus greatly diminishing its quenching efficiency, and an increase in the fluorescence intensity of the dye is observed. Fluorescence modulation is therefore directly correlated with saccharide concentration. Verification of this method includes titration of the above mentioned complex with three types of cyclodextrins, namely α-CD, β-CD and acetyl- β-CD, as well as two different types of hemocyanins from molluscs and arthropods. All of the above mentioned components contain carbohydrates with different structures.

The experiment was started with titration of the simplest samples, namely α-CD, β-CD and acetyl-β-CD with the above described complex and the effect was estimated by fluorescence spectroscopy. The interaction of the complex with the hydroxyl groups in α-CD is represented by the change of fluorescence intensity (arbitrary units, a.u.) as it is shown on Fig. 5. Increase in the concentration of the titration agent does not lead to a significant change in the fluorescence intensity (12 a.u.) of the pyranine and the quantum yield has slightly increased from 0.18 to 0.21. The same results were observed for the other two cyclodehtrins, β-CD and acetyl-β-CD (results are not shown). All three tested cyclodextrins have similar behaviour towards the used complex (Fig. 5, insert). This lack of significant changes in the fluorescence intensity could be partially explained with the structure of these cyclic carbohydrates (Fig. 6).

As a consequence of the $^4C_1$ conformation of the glucopyranose units, all secondary hydroxyl groups are situated on one of the two edges of the ring, whereas all the primary ones are placed on the other edge. The ring, in reality, is a conical cylinder, which is frequently characterized as a

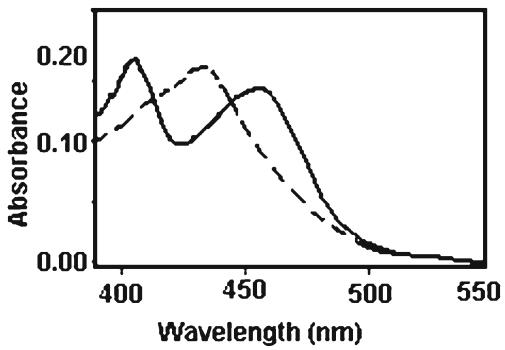


Fig. 3 UV–absorption spectra of pyranine (1×10^{-5} M) (- - - - - - -) and pyranine (1×10^{-5} M) with **o**-BBV (3×10^{-4} M) (—)

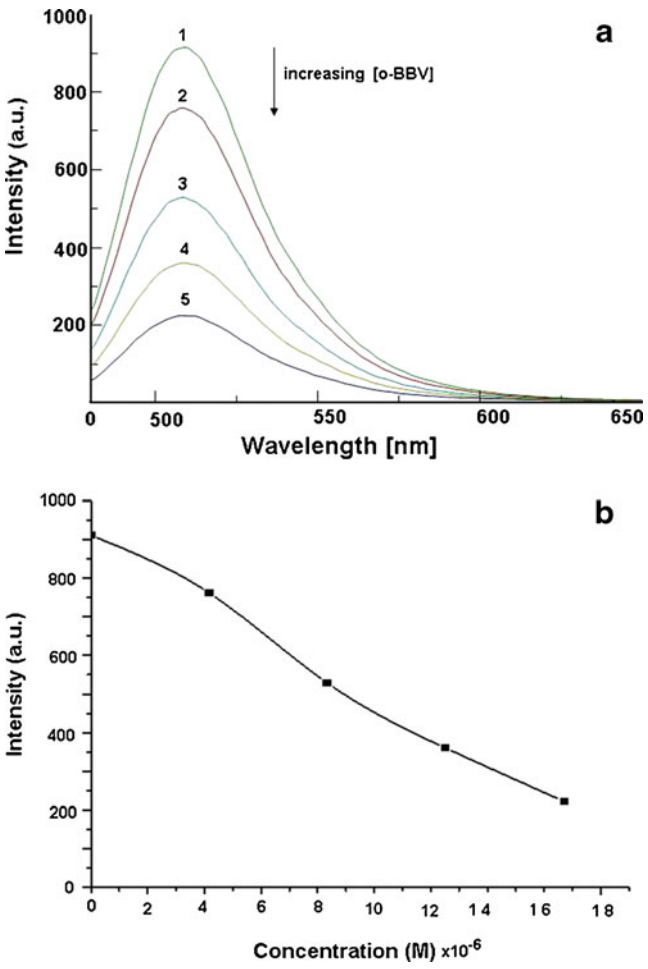


Fig. 4 **a** Fluorescence emission spectra of pyranine (1.33×10^{-6} M in 50 mM phosphate buffer, pH 7.5, λ_{ex}=460 nm and λ_{em}=508 nm) with increasing concentrations (0.0–$1{,}67\times10^{-5}$ M) of *o*-BBV; **b** Relationship between the fluorescence intensity and the concentration of the titration agents

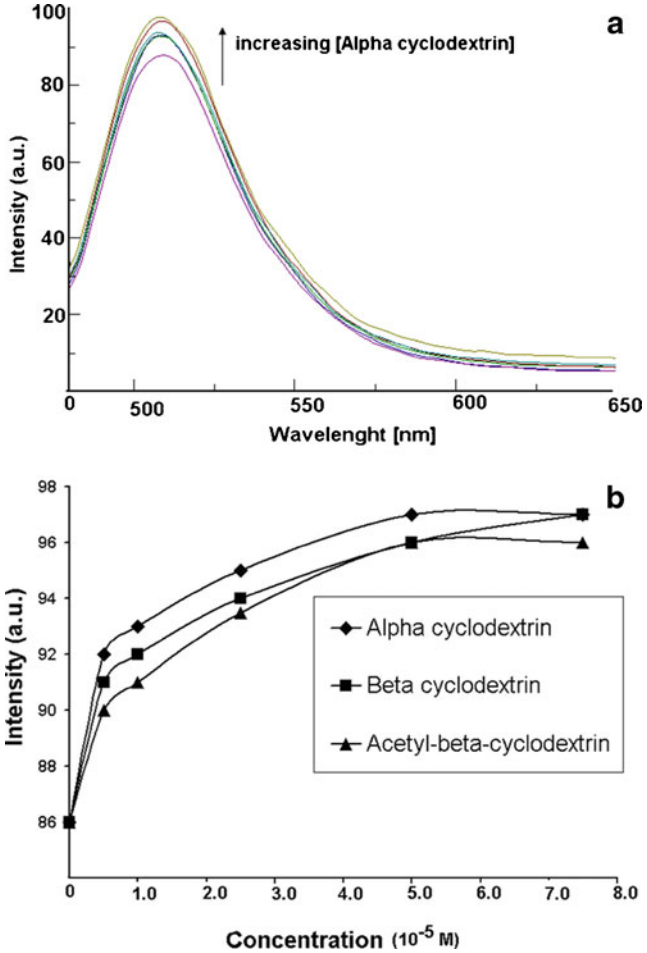


Fig. 5 **a** Fluorescence emission spectra of the complex (pyranine (1.33×10^{-6} M) in presence of *o*-BBV) upon addition of α-CD with increasing concentrations ($0.0–8\times10^{-5}$ M); **b** Relationship between the fluorescence intensity and the concentration of the titration agents: (—•—) α-CD; (—■—) β-CD and (—▲—) acetyl- β-CD

doughnut or wreath-shaped truncated cone. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively. The C-2-OH group of one glucopyranoside unit can form a hydrogen bond with the C-3-OH group of the adjacent glucopyranose unit. In the CD molecule, a complete secondary belt is formed by these hydrogen bonds; therefore the β-CD forms a rather rigid structure. This intramolecular hydrogen bond formation is probably the explanation for the observation of very low fluorescence effect of all CDs. Moreover, the number of free hydroxyl groups is limited effectively neutralizing the dicationic viologen, resulting in very low fluorescence intensity of the pyranine.

Our new carbohydrate determination method was tested with glycoproteins, hemocyanins, with different amount and structure of the glycans. Molluscan and arthropodan hemocyanins are giant oxygen-transporting glycoproteins which have the same function, but quite different structure [32–34]. Arthropodan Hcs exhibit unusual assemblies of up to 48 structural subunits with molecular masses of 75 kDa. Hemocyanin from crab *Carcinus aestuarii* (CaeH) is constituted of three major and two minor structural subunits with molecular masses of 75 kDa [35, 43]. The glycosylation analyses of Carcinus hemocyanin were reported which showed that the carbohydrate of native CaeH represents only 1.6 % of protein mass [35]. Therefore, this hemocyanin was chosen to test the utility and reliability of our new carbohydrate determination method.

A change in the fluorescence intensity with only 30 a.u. and the quantum yield with 0.064 during titration of the complex with different concentrations of CaeH is shown on Fig. 7. These data are comparable with those of the cyclodextrins' fluorescence intensity changes and could be explained with the oligosaccharide's structure of CaeH:

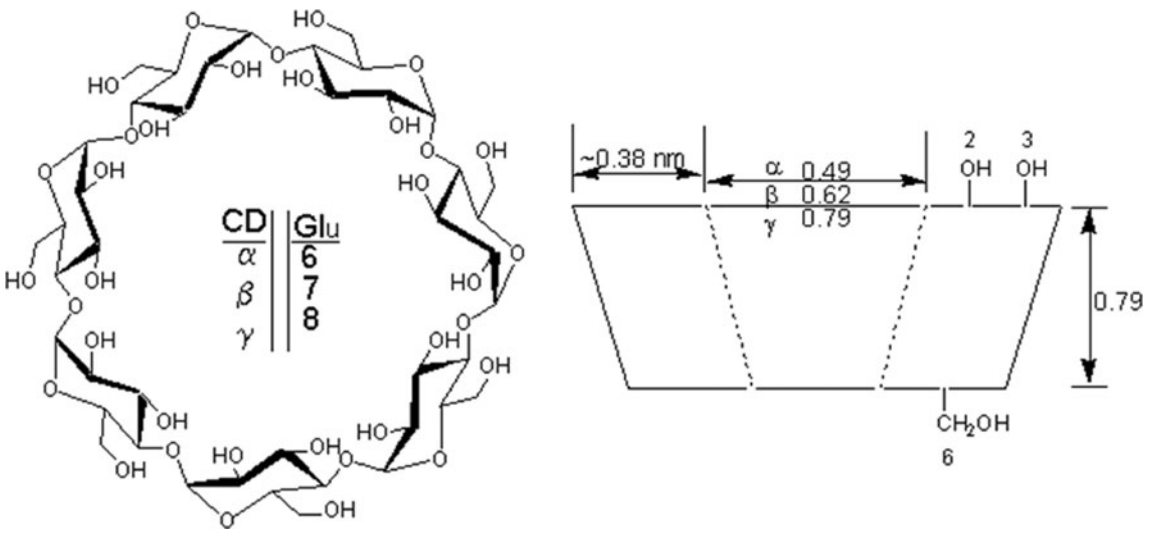


Fig. 6 Common structures of α-CD with 6 Glu, β-CD with 7 Glu and γ-CD with 8 Glu

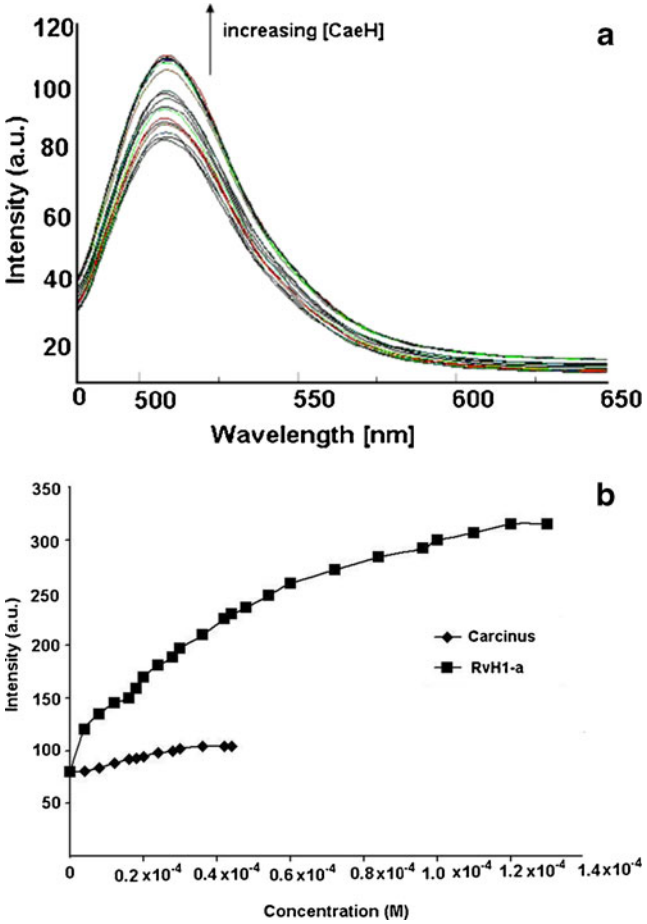


Fig. 7 **a** Fluorescence emission spectra of the complex (pyranine (1.33×10^{-6} M) in presence of *o*-BBV) upon addition of increasing concentrations (0–0.47×10^{-4} M) of CaeH; **b** Relationship between the fluorescence intensity and the concentration of the titration agents: CaeH (—♦—) and RvH1-a (—■—)

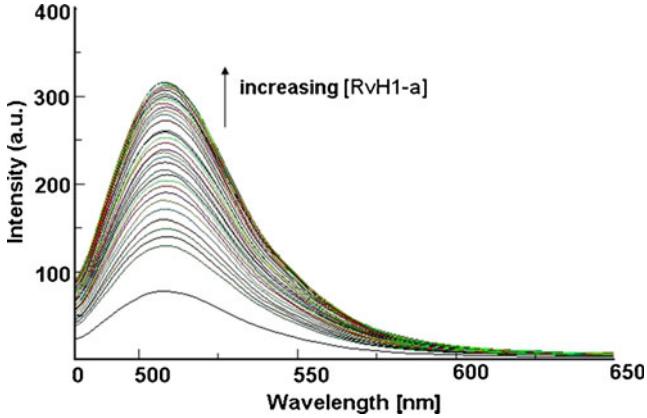
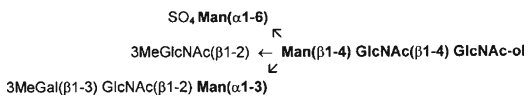


Fig. 8 Fluorescence emission spectra of the complex of pyranine (1.33×10^{-6} M) in presence of *o*-BBV, upon addition of increasing concentrations (0.0–1.4×10^{-4} M) of RvH1-a

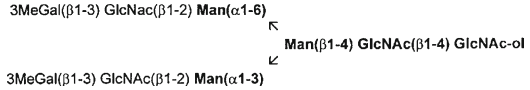
Two short O-glycan sequences with suggested structures (N-Acetyl-O-$NeuAc_2Gal_3GalNAc_2$ and N-Acetyl-O-di-$NeuAc_2$-$Gal_2GalNAc_2$) and one N-glycan sequence ($SO_4Man_4GlcNAc_3$) were identified by MALDI-MS analyses and enzymatic digestions [35]. Carbohydrate groups of these glycans most probably are buried in between the structural subunits of the global protein CaeH and interactions through van der Waals forces or hydrogen bonds may be involved, preventing the access of the complex to the hydroxyl groups.

In contrast to this result, titration of the functional unit RvH1-a of the molluscan hemocyanin *Rapana venosa* with the complex leads to 4-fold increase in arbitrary units (a.u.) of fluorescence intensity and 0.46 units in the quantum yield (Fig. 8). Hemocyanins of molluscs are high molecular mass glycoproteins (9,000 kDa) with a complex quaternary structure organized by ten structural subunits of RvH1 and RvH2, arranged by 8 FUs [31–33]. The oligosaccharide structures of both structural subunits and of some FUs are very well studied [38]. Therefore, the reactivity of the complex pyranine (1.33×10^{-6} in 50 mM phosphate buffer, pH 7.5) and *o*-BBV, was tested using RvH1-a, a functional unit of the structural subunit RvH1, the carbohydrate content of which is known [44]. Several techniques were applied, including capillary electrophoresis, matrix-assisted laser desorption ionization MS, and electrospray ionization MS in combination with glycosidase digestion, to determine a carbohydrate content for RvH1-a of 7 % and structures for its N-glycosidically-linked carbohydrates as follows :

Glycan 1:



Glycan 2:



Based on the models of several FUs from different molluscan Hcs, we suggest that both glycans are exposed on the surface of the molecule. Thus their hydroxyl groups are not involved in any interactions, such as van der Waals forces or hydrogen bonds and as a consequence they can be easily accessed by the complex. This leads to a 4-fold increase in fluorescence intensity.

Conclusion

According to these preliminary results we suggest that our invention provides a new tool to determine accessible free hydroxyl groups of carbohydrates attached to glycoproteins, glycolipids and proteoglycans which are not buried within the macromolecular structure or fixed by hydrogen bridges. This kind of information may be of enormous value studying carbohydrate-based interaction between biomolecules or cells. Studies in our laboratories are under way testing

oligosaccharides and glycoproteins of different structure to develop a more quantitative test system.

Materials and Methods

All starting materials and solvents were commercial products purchased from Sigma-Aldrich (Germany). 2-Bromomethylphenylboronic acid was synthesized by modification of the reported procedure [40]. Analytical samples of the reaction products were obtained by recrystallization in ethanol. All products were characterized and compared with reported data. ^{1}H NMR spectra were obtained on a Bruker Advance II 600 MHz instrument in $CDCl_3$ as solvent. Elemental analyses were performed on a Vario III apparatus. Melting points were determined on a K**o**fler apparatus and are uncorrected.

Synthesis of 2-Bromomethylphenylboronic Acid

Synthesis of 2-Bromomethylphenylboronic acid was carried out as shown in Fig. 2. To a solution of 2-methylphenylboronic acid (**1**) (1 g, 0.0073 mol) in CCl_4 (20 ml) was added dropwise a solution of Br_2 (1.2 g, 0.0075 mol) in CCl_4 (3 ml). The reaction mixture was stirred and irradiated with an UV lamp for 1 h at room temperature and then refluxed for 2 h. The progress of the reaction was monitored by TLC (Merck F 254 silica gel; dichloromethane). After cooling, the resulting precipitate was filtered off from the reaction mixture, the filtrate concentrated under reduced pressure to give a yellowish-brown oil (**2**), which became solid after few days of storage in a refrigerator. The product was used without further purification. Yield 82 %. m.p. 156–159 °C (lit. m.p. 158–162 °C) [40]**.**

Synthesis of N,N'-Bis-(Benzyl-2-Boronic Acid)-[4,4′] bipyridinium Dibromide (o-BBV)

2-Bromomethylphenylboronic acid (2) (1.57 g, 0.0073 mol) and 4,4′-bipyridine (0.38 g, 0.0024 mol) were mixed together and heated at 100–110 °C for 20 min. The progress of the reaction was monitored by TLC (Merck F 254 silica gel; dichloromethane: methanol: acetic acid, 86:13:1). After cooling of the resulting yellow-brown melt to room temperature, 10 ml ethanol was added to dissolve the solid mass and then the solution was diluted with 20–30 ml diethyl ether. The product precipitate**d** as yellow-brown solid, which was filtered off, washed with diethyl ether, and recrystallized in ethanol. Yield 69 %, m.p. (ethanol) 280–283 °C (decomposition).

^{1}H NMR ($CDCl_3$): δ 6.07 (s, 4H, NCH_2), 7.36–7.53 (m, 6H, ArH), 7.78 (d, 2H, ArH), 7.83 (d, 2H, ArH), 8.78 (d, 2H, ArH), 9.38 (d, 2H, ArH), 9.48 (d, 2H, ArH). Elemental analysis calcd for $C_{24}H_{24}B_2Br_2N_2O_4$: C, 49.20; H, 4.13; N, 4.78. Found: C, 48.89; H, 3.92; N, 4.56.

Isolation of FU of Hemocyanins

The glycosylated functional unit RvH1–a was isolated *via* an anion-exchange chromatography column on a FPLC system, as described by Dolashka et al [34]. Native Hc from the crab *C. aestuarii* was prepared from the hemolymph obtained from the dorsal lacuna of living animals collected in the lagoon of Venice as reported by Dolashka et al [35].

Fluorescence Measurements

Absorbance spectra were recorded in a Schimadzu spectrometer of (1×10^{-5} M) pyranine and after titration of pyranine with **o**-BBV (3×10^{-4} M). Fluorescence measurements were carried out on a spectrofluorimeter Jasco FP-6600. The emission spectra of pyranine (1.33×10^{-6}) in 50 mM phosphate buffer, pH 7.5, were measured between 460 and 650 nm, with excitation wavelength at 460 nm in a 1 cm quartz cuvette upon addition of samples with different concentrations. For CaeH and RvH1-a the concentration range was within 0–1×10^{-4} mol/l and for cyclodextrin 0–8×10^{-6} mol/l . All studies were carried out under ambient conditions (25°C, in air).

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