Triazole-linked fluorescent bisboronic acid capable of selective recognition of the Lewis Y antigen

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ARTICLE INFO

Article history:

Received

Revised

Accepted

Available online

Keywords:

Lewis sugars

bisboronic acid

Shinkai's anthracene-based fluorescent sensor

biochemical recognition

ABSTRACT

Cell surface carbohydrates of the Lewis blood group antigens, Lewis X (Le^x), Lewis Y (Le^y), Lewis A (Le^a), and their sialylated derivatives, such as sialy Lewis X (sLe^x) and sialy Lewis A (sLe^a), play important roles in various recognition processes. These cell surface carbohydrates have also been associated with the development and progression of many types of cancers. Recently, we synthesized four anthracene-based fluorescent bisboronic acid sensors (compounds **2a-d**) linked by 'click' chemistry with tethers of different lengths to match the epitope of various Lewis group of sugars. Among the four compounds, **2a** appears to have both high sensitivity and selectivity for Le^y among other carbohydrate antigens.

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Cell surface carbohydrates, such as Lewis X (Le^x), Lewis Y (Le^y), Lewis A (Le^a), and their sialylated derivatives, sialy Lewis X (sLe^x) and sialy Lewis A (sLe^a) (Fig 1), play very critical roles in normal physiology and disease development.¹⁻³ Chemosensors that can selectively bind various carbohydrates of biological significance are important research tools and potential diagnostic agents. On appearance, carbohydrates have many hydroxyl groups. One might consider it would be easy to design carbohydrate chemosensors because one could take advantage of hydrogen bond interactions in designing the “binding cavity.” However, in an aqueous environment, the presence of hydroxyl groups does not offer much advantage in chemosensor design because hydroxyl groups are heavily solvated. In reality, hydrophobic interactions have been exploited in designing carbohydrate sensors that function in aqueous solution.^{4,5}

Boronic acids have been known for decades to bind saccharides *via* reversible covalent interactions.⁶⁻¹³ The most common interactions are with linear diols, *cis*-1,2-diols on five-membered rings, or 1,3-diols to form five- or six-membered rings, respectively. Carbohydrate sensing work during the last decade has been focused on using boronic acid as the basic recognition moiety.¹⁴⁻²¹ We have had a long-standing interest in developing boronic acid-based sensors for various carbohydrates and diol-containing compounds including glucose,²² glucarate,²³ sLe^x ,^{24,25} Le^x ,²⁶ and catechols^{27,28} by using the boronic acid functional group with appropriate positioning of the boronic acid moieties for selective recognitions. Some of our designs are based on an anthracene-based fluorophore developed by Shinkai and James, which responds to binding by changing fluorescence.^{6,7,29-31} Continuing our interest in search for selective chemosensors for carbohydrates in the Lewis group antigen including Le^x , Le^y , Le^a , and their sialylated derivatives, such as sLe^x and sLe^a , we are especially interested in searching for chemosensors for Le^y because of its implication in cancer metastasis and disease diagnosis.^{32,33} So far, there are still no sensitive and selective boronic acid-based sensors for Le^y , although binding to Le^y by antibodies and lectins have been extensively studied.^{34,35} Along the lines of earlier work in designing bisboronic acids (typical compound **1**, Fig 1) with varying linkers for spatial separation and arrangements, we used click chemistry to synthesize additional bisboronic acids. Specifically, compounds **2a-d** (Fig 2) linked by ‘click’ chemistry with different length of linker to match the epitope of sub-terminal tetrasaccharides on Lewis sugars have been synthesized (Scheme 1).

Fig 1 Structures of Lewis oligosaccharides and bisboronic acid compound **1**

Fig 2 Structures of bisboronic acid compounds **2a-d**

Briefly, anthracene-based amine **3** was prepared by similar following procedures reported previously.²⁵ Subsequent amidation reaction with **4** containing an alkynyl group gave compound **5**. Compounds **7a-d** were generated from the amidation reaction of **3** and **6a-d** bearing a primary bromide. An azido group was introduced to give compounds **8a-d** by substitution reaction using sodium azide. The click reactions of **5** and **8a-d** were performed in the presence of CuSO_4 to produce the bis-anthracene-based protected amines **9a-d**. After the removal of Boc groups from compounds **9a-d** using TFA, the unprotected free amines were then reacted with boronate **10** under basic conditions to give the aimed bisboronic acid compounds **2a-d**, after deprotection using 10% NaHCO_3 .

Scheme 1. Synthesis of the bisboronic acid compounds **2a-d**

Next we studied the ability for these compounds to bind to various saccharides of interest. The fluorescence spectra of **2** were measured in various 0.1M PBS/methanol mixed solvents (Fig. 3). Using compound **2a** as example (others are presented in Supporting Information), it can be seen from Fig. 3A and 3B (the concentration of **2a** was 1.0×10^{-5} or 10^{-6} M, respectively), that the emission λ_{max} (424nm) and spectral shape remained the same with increasing concentrations of methanol (40%-75%). Such results suggested that the bisboronic acid compound **2a** ($c = 1.0 \times 10^{-5}$ or 10^{-6} M) existed as a solution in 60% methanol with 0.1 M PBS buffer at pH 7.4. This point is further supported by the good linear relationships in the concentration-dependent absorbance (Fig. 3C) and fluorescence plots (Fig. 3D) at 1.0×10^{-5} – 6.0×10^{-7} M. Due to the good solubility in the tested concentration range, we decided to use 1.0×10^{-5} and 10^{-6} M concentrations of **2a** and 2:3 PBS/methanol (v/v) as a standard condition for binding studies with carbohydrates.

Fig.3 The fluorescence spectra curve of (A) **2a** ($c = 1.0 \times 10^{-5}$ M) and (B) **2a** ($c = 1.0 \times 10^{-6}$ M) in various PBS/methanol mixed solvents; (C) UV absorption spectrum of **2a** with the concentration range of 1.0×10^{-5} – 6.0×10^{-7} M in 60% methanol with 0.1M PBS buffer at pH 7.4; (D) the fluorescent intensity of **2a** with the concentration range of 1.0×10^{-5} – 6.0×10^{-7} M in 60% methanol with 0.1M PBS buffer at pH 7.4. $\lambda_{ex} = 375$ nm, $\lambda_{em} = 424$ nm.

The fluorescent binding experiments of the bisboronic acids **2a-d** with several Lewis sugars were conducted in a mixture of MeOH and 0.1 M phosphate buffer (pH=7.4) (3:2, v/v). The sensor (**2**) concentration was fixed at 1.0×10^{-5} M or 1.0×10^{-6} M, and the concentration of target sugars was set at 60 μ M, which was chosen based on literature precedent.²⁵ The fluorescence intensity profiles for these bisboronic acids are shown in Fig 4. Before mixing with a sugar, the bisboronic acids **2a-d** showed rather weak fluorescence. After binding with the various sugars, the fluorescence intensity increased significantly. As shown in Fig 4, compounds **2a** and **2d** exhibited high fluorescence intensity enhancement upon binding with Le^y but almost no change with other Lewis sugars, which indicated that **2a** and **2d** recognize Le^y with selectivity over other Lewis sugars. Furthermore, the fluorescence intensity changes for **2a** are concentration-dependent (Supporting Information). Compound **2c** showed the highest fluorescence intensity enhancement (over 70%) upon binding with Le^y . However, the fluorescence intensity also increased in the presence of sLe^x , sLe^a , and Le^x although to a lesser degree. Compound **2b** did not show significant fluorescent intensity changes, indicating weak binding. All the bisboronic acids **2a-d** have a very similar molecular structure. The only structural difference among them is the length of the linker, which affords difference in flexibility and possibly relative position/orientation of the boronic acid moieties, and thus might be the reason for their selectivity. These results suggest that minor structural changes can significantly affect the selectivity among structurally similar sugars and are consistent with our earlier findings.

Due to the high fluorescent enhancement of **2a** upon binding with Le^y , we were interested in assessing the ability for **2a** to selectively label Le^y -expressing HEP3B cells.^{25,36} The non-expressing GES-1 cells and sLe^x -expressing HEPG2 cells were used as controls. Incubation of **2a** (10 μ M or 50 μ M) with different cells was carried out in six-well plates in darkness at 4 $^{\circ}$ C. After 45 min, the cells were observed using a FV-1000 laser scanning confocal fluorescence microscope.

Fig. 4 The fluorescence intensity change profile of the bisboronic acids (**2a**, **2c**: 1.0×10^{-6} M; **2b**, **2d**: 1.0×10^{-5} M) upon binding with different oligosaccharides (60 μ M), $\lambda_{ex} = 375$ nm, $\lambda_{em} = 424$ nm.

Fig. 5 Representative fluorescent labeling studies of HEP3B, HEPG2, GES-1 cells. HEP3B cells express only Le^y , HEPG2 cells express only sLe^x , GES-1 cells do not express either antigen. (A) Negative controls; (B) Compound **2a** was used in these cells; $\lambda_{ex} = 375$ nm, $\lambda_{em} = 424$ nm.

In the absence of the sensor, none showed any fluorescence (Fig. 5A). As expected, incubation of cells with **2a** (10 μ M) led to fluorescent labeling of HEP3B cells, while even at 50 μ M HEPG2 cells and GES-1 cells were not labeled (Fig. 5B). The results are in agreement with the results from the fluorescent binding experiments (Fig. 4), suggesting that **2a** has selectivity for Le^y over other Lewis sugars and can be used for labeling of cells expressing Le^y.

In order to achieve some initial understanding of structural features required for selective binding with Le^y, we conducted molecular dynamics simulations to sample the distance between the two boronic acid units using method described in earlier studies.²⁶ Specifically, we conducted molecular dynamics simulations and saved frames every 100 ps, and generated 1000 frames for a total 100 ns simulation (Fig. 6). Our results showed that the average distance of **2a**, **b**, **c**, and **d** is $11.44 \pm 2.12 \text{ \AA}$, $12.78 \pm 2.90 \text{ \AA}$, $10.24 \pm 3.23 \text{ \AA}$ and $11.07 \pm 1.61 \text{ \AA}$, respectively. The initial distance-based assessment indeed indicates differences in conformations, which afford selectivity. However, further studies will need to take into consideration of reaction free energies in water, leading to the formation of the charged boronate esters as well as the relative orientations of the boronic acid groups. Such computational work is way beyond the scope of this study.

Fig.6 Distance between boron atoms during molecular dynamics simulation.

In conclusion, fluorescent bisboronic acids **2a-d** have been synthesized to target Lewis oligosaccharides. Compound **2a** recognized Le^y with selectivity over other Lewis sugars, and could significantly stain Le^y-expressing HEP3B cells with selectivity. Because Le^y is implicated in the development of many malignant tumors,^{32,33,36,37} chemosensors for Le^y are very valuable research tools and potential diagnostic agents.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC) (21372060), Hebei Province Natural Science Fund for Distinguished Young Scholars (B2015201005). We gratefully acknowledge the use of Orion that is supported by Georgia State University's Research Solutions. This work used the Extreme Science and Engineering Discovery Environment (XSEDE), which is supported by National Science Foundation grant number ACI-1053575.

Supplementary data

Experimental procedures and characterization data for compounds **4**, **5**, **7a-d**, **8a-d**, **9a-d** and **2a-d** are available. Supplementary data associated with this article can be found, in the online version, at doi:

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Graphical Abstract

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