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A fluorescent bisboronic acid compound that selectively labels cells expressing oligosaccharide Lewis X

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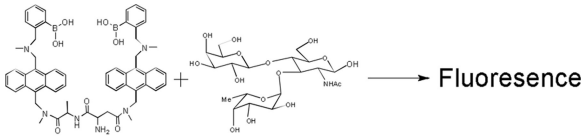
Abstract

Two fluorescent diboronic acid compounds (**6a** and **6b**) with a dipeptide linker were synthesized as potential sensors for cell surface saccharide Lewis X (Le^X). Compound **6a** with a dipeptide (H-Asp-Ala-) as the linker was found to selectively label CHOFUT4 cells, which express Le^x, at micromolar concentrations, while non-Le^x-expressing control cells were not labeled.

Graphical Abstract



6a

Keywords

Biomarker; Boronolectin; Optical imaging

The cell surface carbohydrates of Lewis blood group antigens, Lewis X (Le^x), Lewis Y (Le^y), Lewis A (Le^a), Lewis B (Le^b), and their sialylated derivatives (e.g. sialy Lewis X (sLe^x)) that contain the fucose moiety play important roles in various types of biochemical recognition processes. These cell surface carbohydrates have also been associated with the development and progression of many types of cancers.[1–4]

The Le^x structure (Figure 1), a trisaacharide Galβ(1–4)[Fucα(1–3)]GlcNAcβ-1, is present in a variety of normal and malignant human tissues.[5] While the precise physiological role of Le^x is still unknown, some insight has been gained regarding its biological activity. It has

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been shown that the expresion of the Le^x antigen varies greatly during the maturation of normal human cell.[6] Therefore, Le^x is believed to play a role in normal cell development and differentiation.[7] The Le^x antigen is also presumably associated with the growth of malignant cells because many human cancer tissues accumulate this antigen.[8] Furthermore, Le^x determinant may function as a recognition structure for endogenous receptors and adhesion molecules such as lectins that are a class of proteins of non-immune origin and bind carbohydrates specifically and noncovalently.[9] Additionally, it was suggested that Le^x may be related to learning dysfunction in some patients with mental retardation.[10] Finally, there is evidence that the Le^x determinant may play a role in inflammatory responses.[11] Since the Le^x determinant is believed to be very pathologically important, the development of compounds (sensors) that selectively recognize Le^x could help the diagnosis and early detection of various diseases. Such compounds could also aid in interpreting the binding of Le^x with different receptors and therefore aid in the development drugs that act as inhibitors of the binding and activity of Le^x.

Boronic acids have been known for decades to bind saccharides via reversible covalent interactions.[12–14] 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. Therefore, carbohydrate sensing work during the last decade has been focused on using boronic acid as the basic recognition moiety.[15–17] Our laboratory has been particularly interested in the development of fluorescent sensors for biologically important carbohydrates.[18–21] For the construction of fluorescent sensors for cell surface carbohydrates, we envision that bisboronic acid compounds with a proper spatial arrangement of the two boronic acid moieties, which are complementary to the multiple pairs of diols, have the potential for selective recognition of the target carbohydrate. In doing so, we have selected Shinkai's anthracene-based fluorescent boronic acid as the binding and reporting unit[22] and have sampled a series of linkers with different length, rigidity, and spatial orientation in search of an optimal arrangement of the two boronic acid units. In our previous work, we reported a boronic acid-based fluorescent sensor that could selectively label sLe^x.[18–20] The same sensor was found to be useful in mass spectrometric imaging work for cancer tissues.[21] Herein, we report a fluorescent boronic acid compound (**6a**) that selectively label Le^x, which was derived from screening a large number of analogs with a range of boron-boron distance considered as appropriate for recognition of Le^x. The computational aspect of this study is addressed later.

Compounds **6a** and **6b** contain the dipeptides H-Asp-Ala and H-Glu-Ala, respectively, as dicarboxylic acid linkers. The synthesis of these two compounds is shown in Scheme 1. The anthracence-based amine **1** was prepared by following a procedure reported previously.19 Compounds **3a** and **3b** were obtained through a coupling reaction of the anthracence-based amine **1** with Fmoc protected dipeptide acids **2a** and **2b**, respectively.[19] After Boc deprotection of compound **3** (**3a** and **3b**) with trifuoroacetic acid (TFA), the unprotected free amines were then reacted with boronate **4** in the presence of potassium carbonate to give the diboronic acid compound **5** (**5a** and **5b**).[20] The final product **6** (**6a** and **6b**) was obtained through deprotection of the Fmoc group with diethylamine in CH_2Cl_2.[21]

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Flow cytometry analysis of hepatocellular carcinoma (HCC) lines with anti-carbohydrate monoclonal antibodies was performed to characterize surface glycan expression. Cell lines were prepared and stained with monoclonal anti-carbohydrate antibodies at saturating concentrations as described.[23,24] Cells were analyzed on a Becton-Dickinson FACS as previously described.[24] The results indicated that CHOFUT4 expresses Le^x only; Chinese hamster ovary (CHO) cell line does not express any glycans; HEP3B predominantly express Le^y; B16FUT3 expresses sialyl Lewis a (sLe^a) only; COLO205 expresses sLe^x and sLe^a, but not Le^x.

Compounds **6a** and **6b** were examined for their ability to label cells that express different carbohydrates. The fluorescent labeling studies were carried out in a cell culture system using these cells following previous described procedures.[19] The plates containing the labeled cells were examined with phase contrast microscopy followed by fluorescent microscopy (blue cube wavelengths 370 nm excitation, 426 nm emission; 20 × lens). Plates were photographed and images were captured with the Nikon ACT-1 program (v 2.10). The phase contrast and fluorescent images were then overlaid, organized and labeled using Adobe Photoshop. The fluorescence intensity is displayed with mean gray value: black represents low signal intensity and white represents high signal intensity. The units (mean gray value) were subtracted from background, where there are no cells. The fluorescent signal was stable for at least 96 h when cells were maintained in darkness.

Figure 2 shows the mean gray value of different cells after incubation with fluorescent boronic acid compound **6a**. As discussed above, larger mean gray values mean stronger fluorescence intensity. It is well known that the fluorescence intensity of boronic acid compounds such as **6a** would increase after binding with a sugar. It is clear from Figure 2 that CHO that does not express the target carbohydrates shows almost no fluorescence; HEP3B, B16FUT3 and COLO205 that express Le^y, sLe^a, and sLe^x (and sLe^a), respectively, show weak fluorescence; CHOFUT4 that express Le^x shows the highest fluorescence. These results indicated that compound **6a** recognize Le^x with selectivity over Le^y, sLe^x, and sLe^a.

Figure 3 shows representative images of cell-labeling studies. Compared with the control cell CHO that does not express any glycans, the fluorescence of CHOFUT4 that express only Le^x increased significantly after incubatation with compound **6a**. Compound **6a** selectively stained CHOFUT4 at the concentrations of both 2 and 8 μM. Furthermore, compound **6a** only slightly stained HEP3B, B16FUT3, and COLO205 that expresses other carbohydrates such as Le^y, sLe^x, and sLe^a (Figure 4).

The labeling of these cells using compound **6b** was also studied. The results indicated that compound **6b** did not show significant labeling of these antigens (data not shown). Compound **6a** and **6b** have a very similar molecular structure. The only structural difference between **6a** and **6b** is in their linker: compound **6a** has H-Asp-Ala as the linker, but **6b** has H-Glu-Ala as the linker. These results suggest that a small structural change can significantly affect the ability of a sensor to bind a specific carbohydrate.

In order to understand the conformational features of compounds **6a** and **6b** that contribute to binding, we performed molecular dynamics simulations of compound **6a**,**b** and Le^x in an

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explicit water model with Desmond3.6.[25] As can be seen from Figure 5, the distance between the two boron atoms in compound **6a** settled quickly to 5 Å in only 1 ns and stayed there for the duration of the entire simulation process (20 ns). However, the distance between the two boron atoms in compound **6b** took quite a long time (5 ns) to reach 5 Å, which is similar to the boron distance in compound **6a**. Furthermore, this distance fluctuated in **6b,** but not in **6a**. These boron atoms even went away from each other for the last 2 ns of simulation in **6b**. The results suggested that **6a** was able to maintain a stable conformaiton, which is probably favorable for binding. On the other hand, **6b** was not able to stay in a conformation favorable for binding. However, because we have no structural evidence to indicate which way the boronic acids might bind with Le^X. Therefore, no further computational work was conducted. Thus the computational work only gives hints as to why **6a** exibits selectivity.

In conclusion, a fluorescent bisboronic acid compound **6a** with a dipeptide linker has been developed to target Le^x. Compound **6a** was found to stain CHOFUT4 cells expressing Le^x at micromolar concentrations. To the best of our knowledge, this is the first such synthetic receptor that has been found to fluorescently and selectively label cell expressing Le^x.

Acknowledgement

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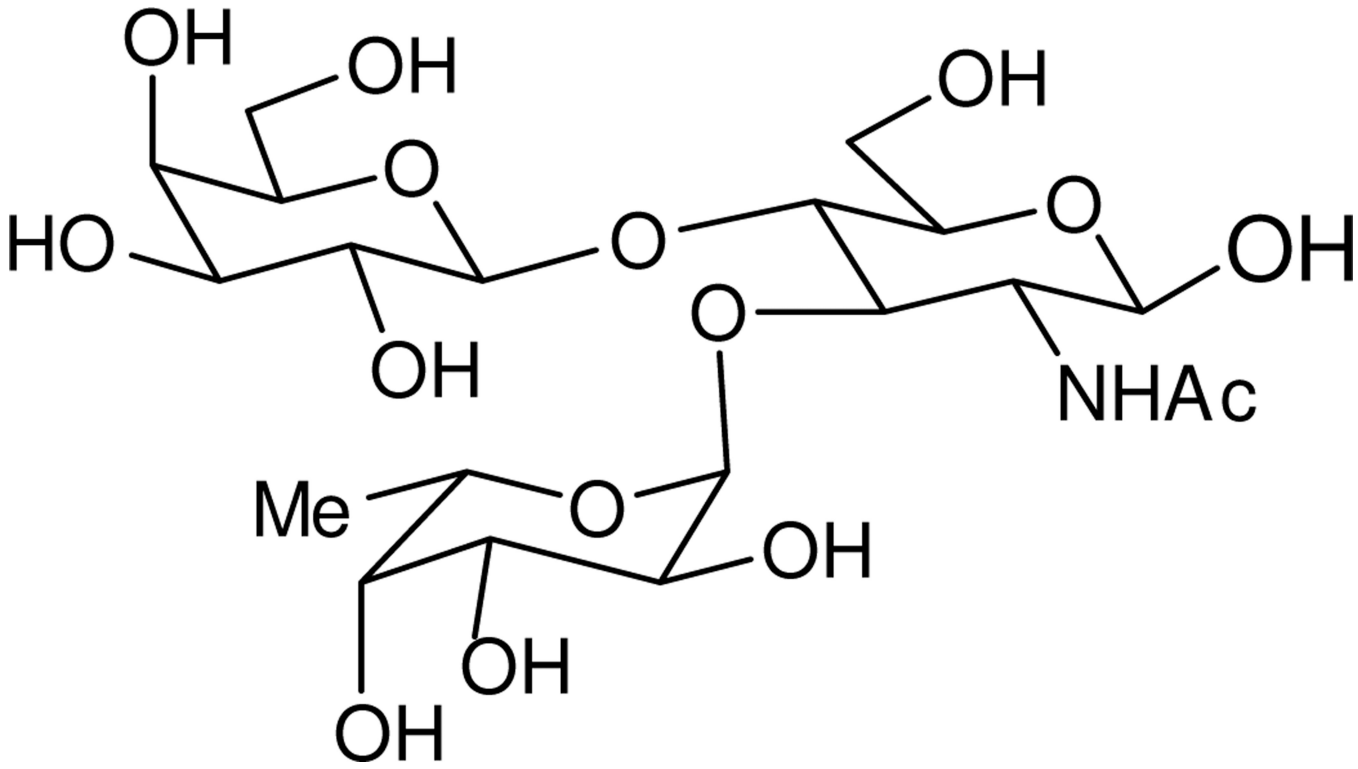

Figure 1.
The structures of Lewis X (Le^x) trisaccharide.

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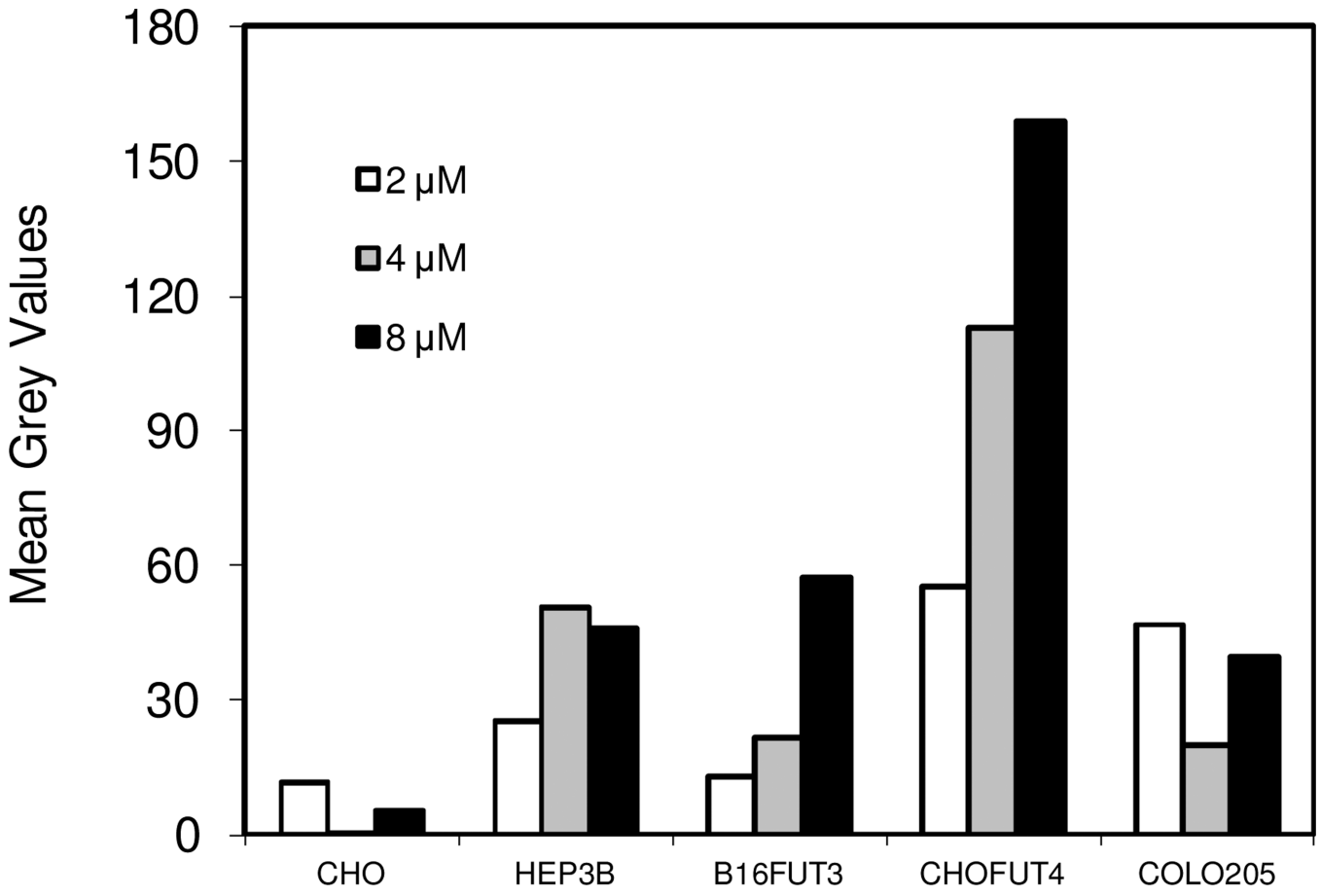

Figure 2.

Densitometry quantification of fluorescent intensity of cells upon incubation with compound **6a**. Cells were incubated with 2, 4, and 8 μM of **6a** for 30 min. Mean gray values (y-axis) were determined after subtraction of cell-free background. CHOFUT4: Le^x only; CHO: none of the glycans; HEP3B: predominantly Le^y; B16FUT3: sLe^a only; COLO205: sLe^x and sLe^a, no Le^x.

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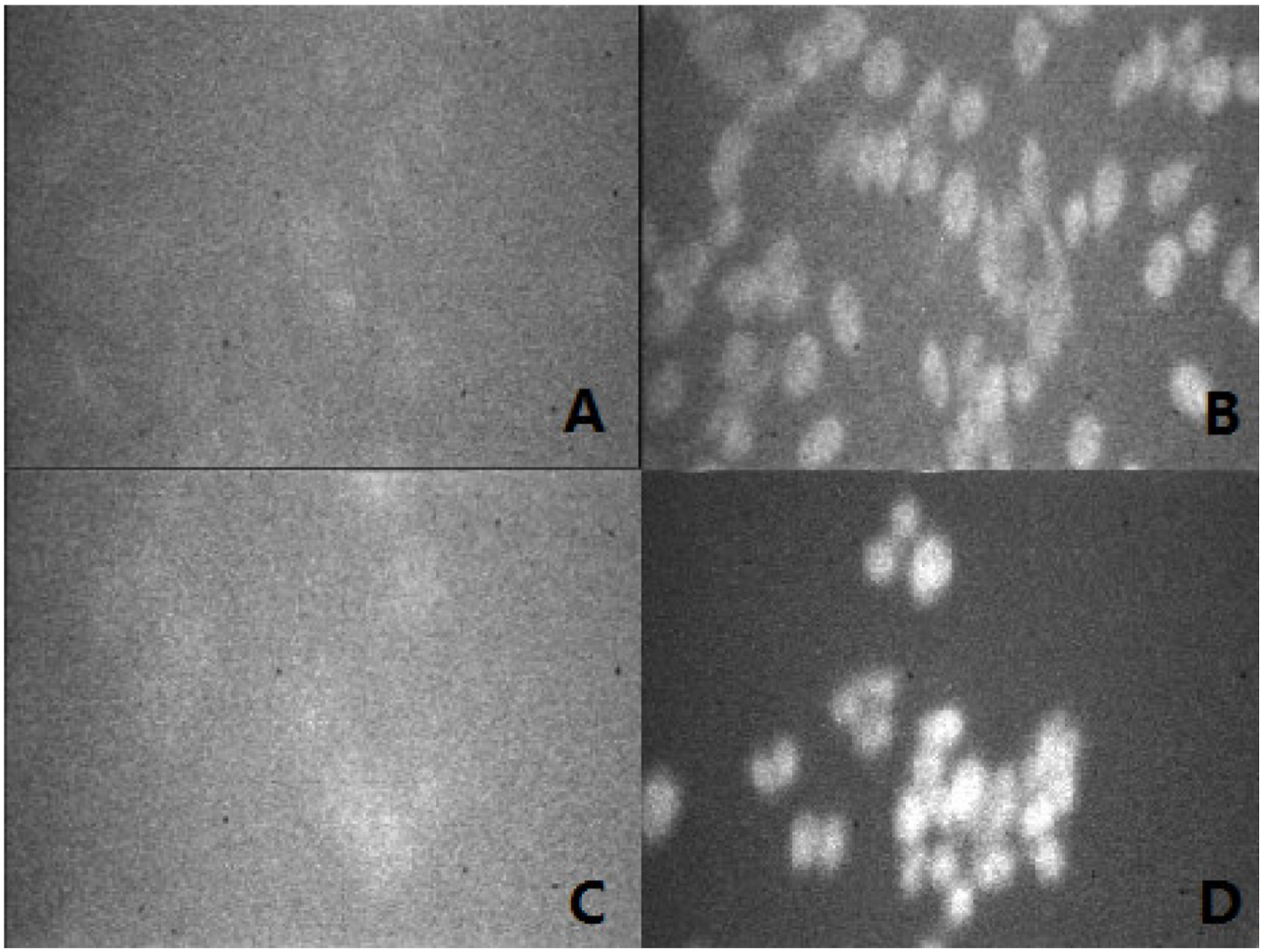

Figure 3.

Representative fluorescent labeling studies of compound **6a** for CHOFUT4 and CHO cells. A: **6a** at 2 μM for CHO; B: **6a** at 2 μM for CHOFUT4. C: **6a** at 8 μM for CHO; D: **6a** at 8 μM for CHOFUT4. λ_{ex} = 370 nm, λ_{em} = 426 nm.

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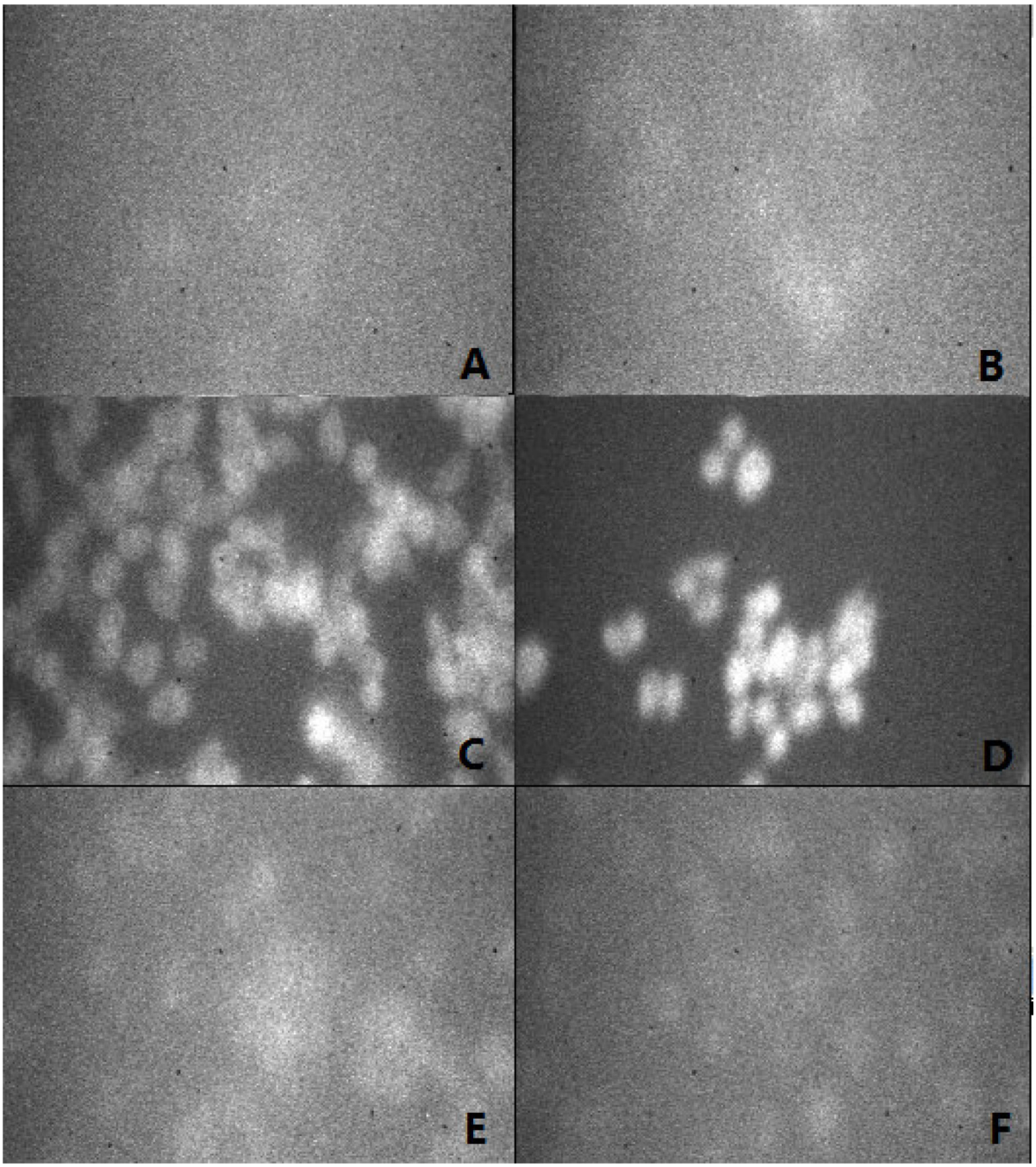

Figure 4.

Selectivity labeling studies of compound **6a**. A: CHO (negative control) at 4 uM; B: CHO at 8 uM; C: CHO-FUT4 (Lex-expressing) at 4 uM; D: CHO-FUT4 at 8 uM; E: COLO205 (sLe^x and Sle^a; no Le^x) at 4 uM; F: COLO205 at 8 uM. The bar graph is the same data in pixels.

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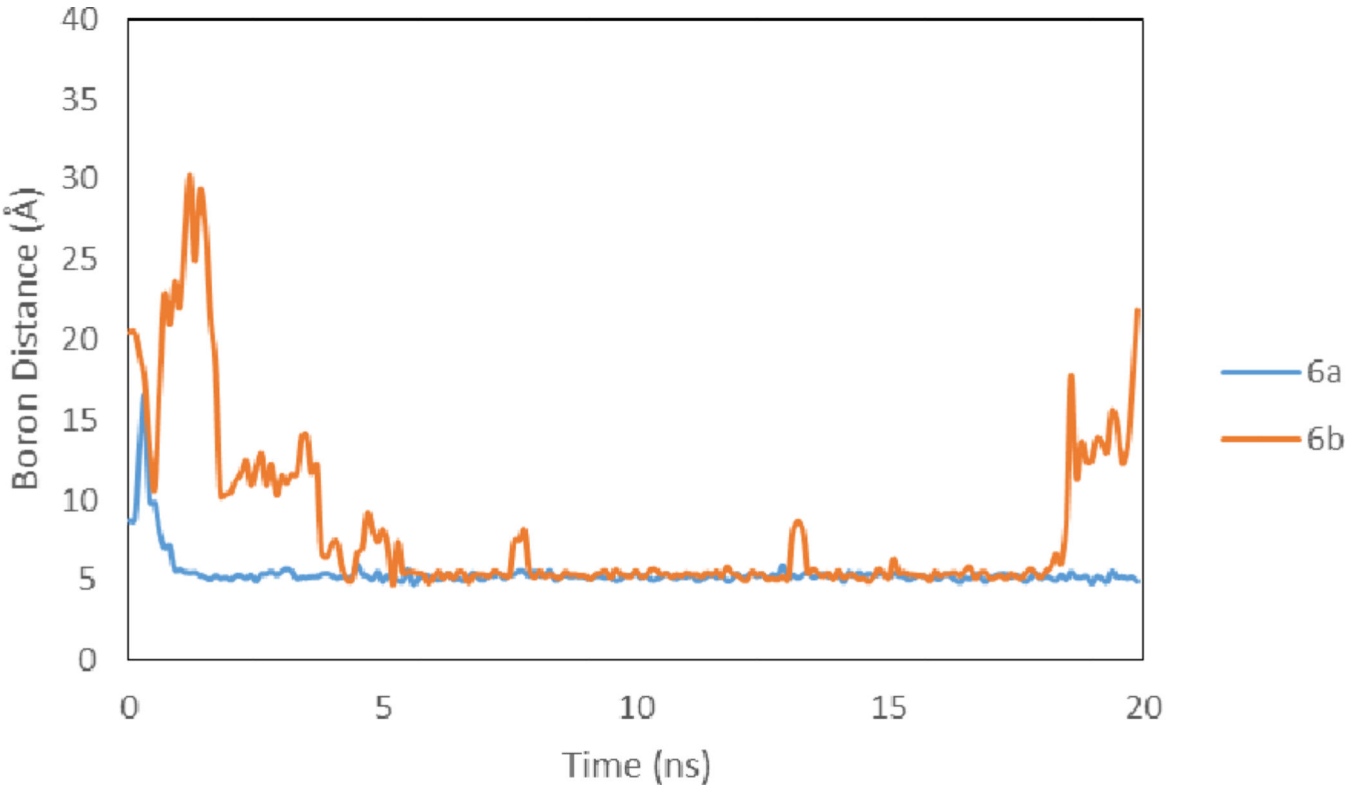

Figure 5.
Boron distances of compound 6a and compound 6b.

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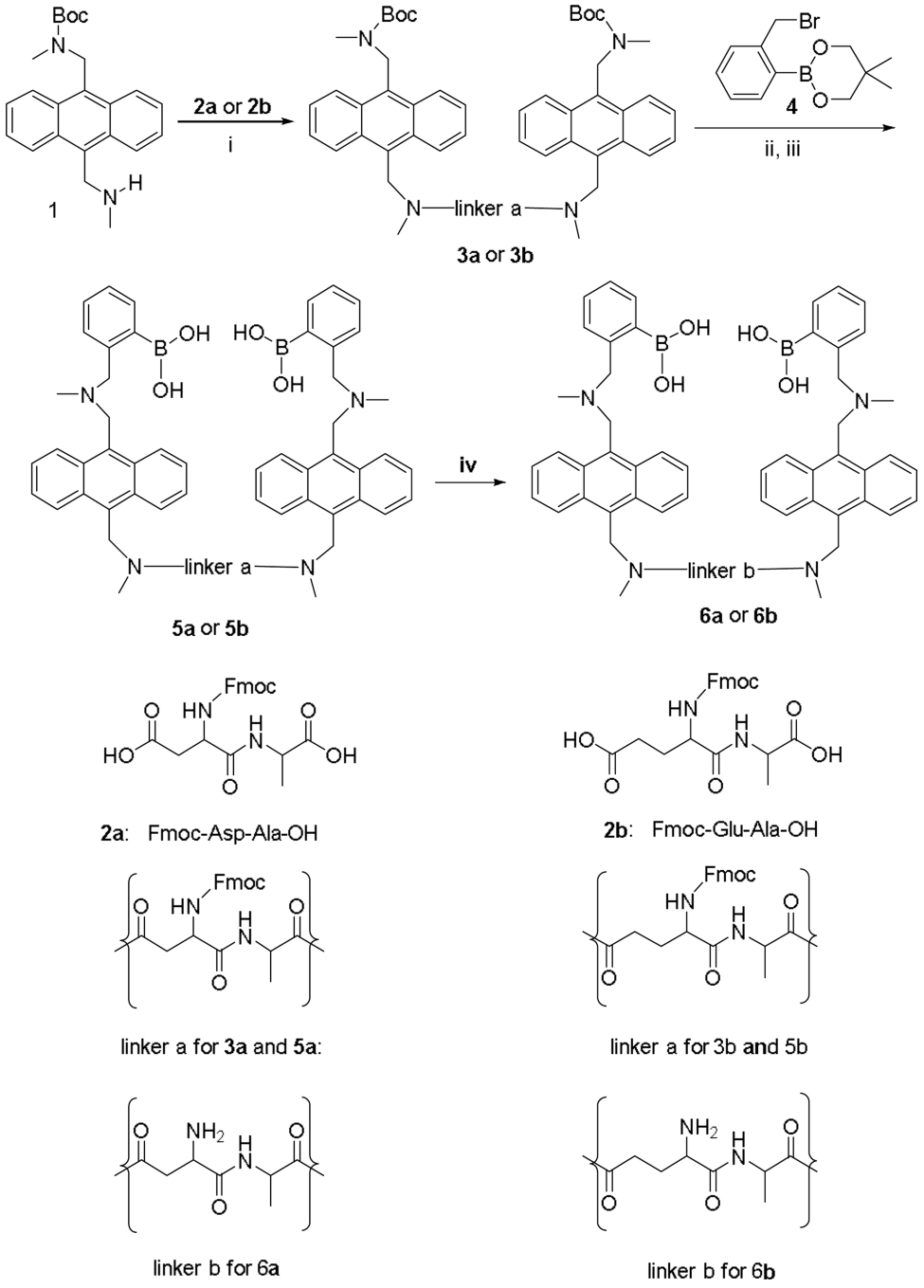

Scheme 1.

Synthesis of boronic acid compounds **6a** and **6b**: i) **2a** or **2b**, EDCI, HOBt, DIEA, CH_2Cl_2; ii) TFA/ CH_2Cl_2 (1:2, v/v); iii) 4, K_2CO_3, CH_3CN; iv) $(CH_3CH_2)_2NH$/ CH_2Cl_2 (1:1, v/v).