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INVESTIGATION OF A ONCHOCERCIASIS BIOMARKER DERIVATIVE (GLUCURONIC ACID) VIA A TWO COMPONENT SYSTEM BASED ON BORONIC ACID APPENDED VIOLOGENS RECEPTORS

A thesis submitted by

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ABSTRACT

Sugar acids are monosaccharides with carboxyl groups. The main classes of sugar acids include Aldonic acids, Ulosonic acids, Uronic acids and Aldaric acids. Glucuronic acid is an example of a uronic acid that was first isolated from urine and is important for the metabolism of microorganisms. Glucuronic acid is also a component of the Onchocerciasis (riverblindness), a neglected disease biomarker N-acetyltyramine-O, β-glucuronide (NATOG). NATOG is a neurotransmitter- derived secreted metabolite from filarial parasitic nematode *Onchocerca volvulus* (Globisch et al 2013). These parasites are transmitted during a blood meal by a black fly vector (*Simulium* sp.) carrying larvae, and affect more than 37 million people worldwide. Most infections occur in areas south of the Sahara Desert in Africa, Middle East, Central and now South America where there are limited resources for early detection and monitoring of disease. Current methods for early detection of the NATOG metabolite require specialized instrumentation, such as high performance liquid chromatography and mass spectrometry, which limit its use as a diagnostic biomarker. To circumvent this, an alternative rapid and inexpensive analytical tool is needed that would allow operation in these low resource settings. A two-component fluorescent system has been developed that utilizes a series of boronic acid receptors coupled to a fluorophore for quantification of sugar acids. The binding capabilities of the NATOG derivative glucuronic acid to six boronic acid receptors and the detection and quantification limits have been determined.

INTRODUCTION

Onchocerciasis is a neglected tropical disease commonly known as river blindness, a condition that affects millions of people worldwide. Filarial parasitic nematode *Onchocerca volvulus*, causes severe inflammatory response that leave many blind. These parasites are transmitted via black fly (*Simulium* sp.) bites. Once inside a human host, larvae develop into adults forming a bundle of worms under the skin. In Onchocerciasis, microfilariae migrate through nodular tissue to the skin and eyes, resulting in inflammation. Eventually, invasion of the cornea by filarial worms leads to blindness, which is the common pathology associated with this neglected disease (Globisch et al 2013). Resources for detection and monitoring of the disease are limited in the affected areas, leaving individuals to depend on insect repellent and proper clothing to avoid getting bitten by black flies.

The discovery of (NATOG) N-Acetyltyramine-O, β-glucuronide (Figure 1), a unique derived metabolite form *O. volvulus* as a biomarker for river blindness can facilitate the prevention and monitoring of the disease. An important component of the NATOG biomarker is a sugar acid motif, glucuronic acid. An investigation using a two component system based on boronic acid- appended viologens receptors (BBV) and a fluorophore can provide a rapid alternative approach to quantifying the NATOG biomarker.

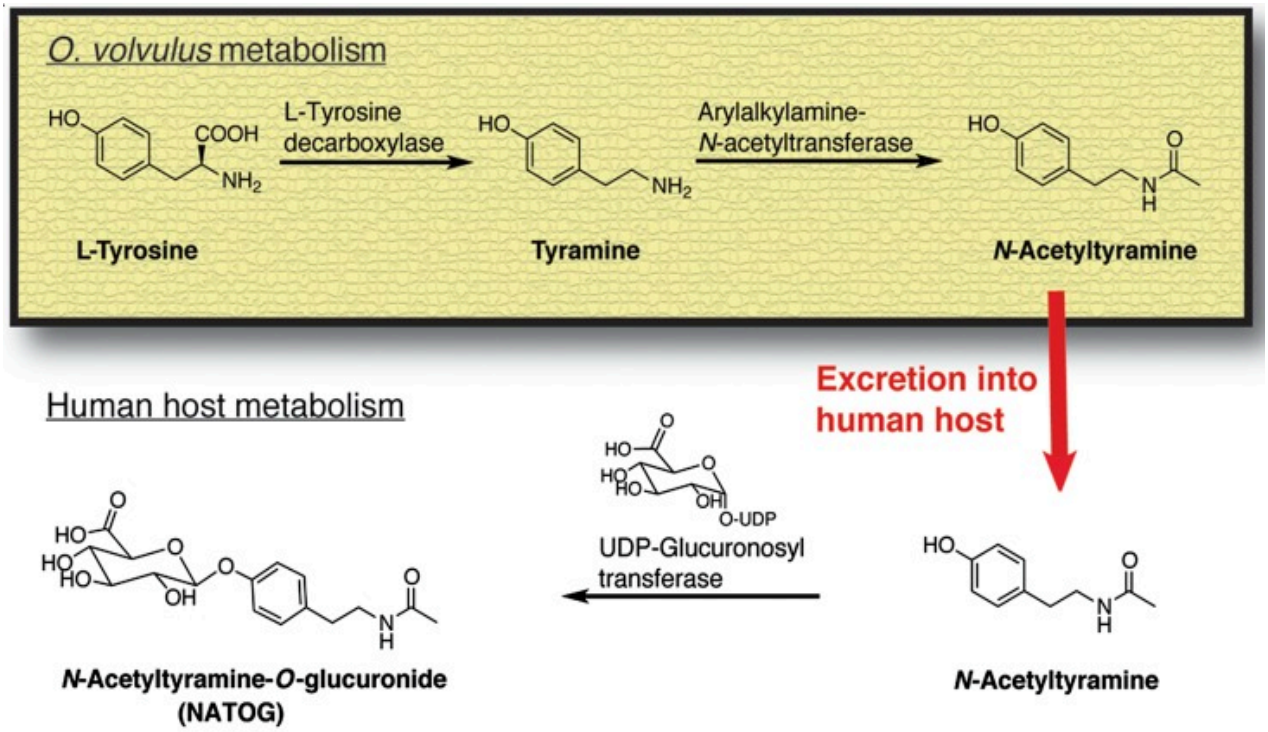

Figure 1. Metabolism of *Onchocerca volvulus* L-tyrosine to N-Acetyltyramine. Once parasite excretes N-Acetyltyramine into the human host, it is metabolized into N-Acetyltyramine-O-glucuronide (NATOG) by UDP-Glucuronosyl transferase enzyme (1).

Our laboratory has developed a two-component system that can recognize molecules such as sugars or sugar acids. This system utilizes an anionic fluorescent dye 8-hydroxypyrene-1, 3, 6-trisulfonic acid trisodium salt (HTPS) and cationic BBV receptor, which interact to form a non-fluorescent ground state complex (Fig. 1). In the presence of sugar acids, the dye no longer interact with the BBV receptor resulting in an increase in fluorescent signal, which is directly proportional to the concentration of sugar acids. A series of six cationic boronic acid appended benzyl viologens were used to determine binding affinity of the receptors to glucuronic acid and other sugar acids, such as gluconic acid, galacturonic acid, malic acid, D-tartaric acid, and L-tartaric acid.

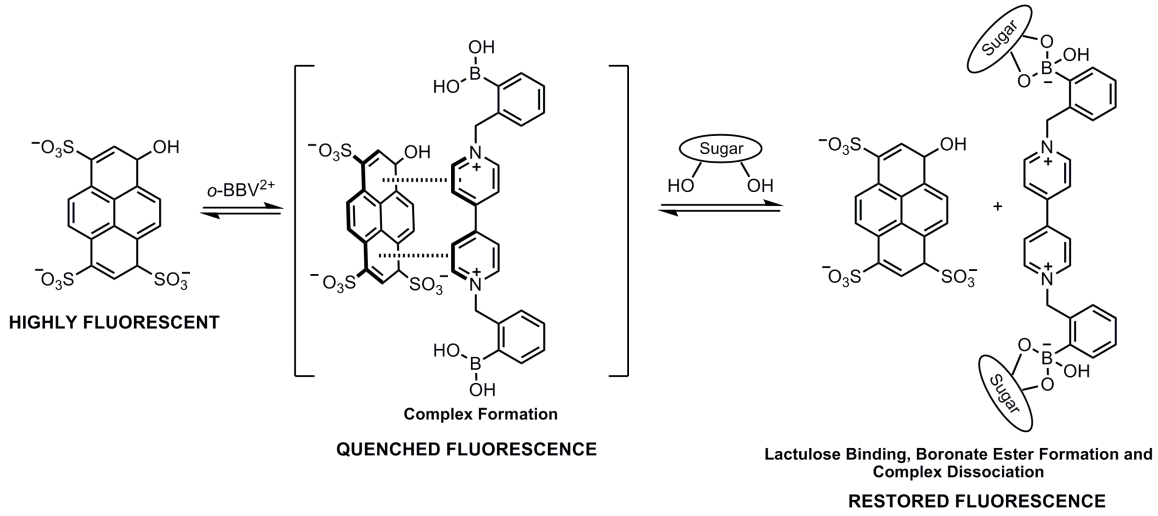

Figure 2: illustrates when the anionic fluorescent dye and the cationic BBV receptor interact they are said to be in ground state and form a weak fluorescent complex (Schiller et al 2007).

In the presence of saccharides, such as sugar acids, the dye will no longer interact with the BBV receptor resulting in a fluorescent signal increase which can be used for quantification of sugar acids. The established chemistry can be further used for specific clinically relevant saccharide biomarkers such as the NATOG. Current methods for early detection of NATOG metabolites require specialized instrumentation such as high performance liquid chromatography and mass spectrometry, which limit its use as a diagnostic biomarker. To circumvent this, we will develop a low-cost, user-friendly high throughput platform to measure and monitor these biomarkers in urine during disease progression in areas of limited resources. Our objective is to investigate the NATOG biomarker derivatives and to determine which boronic acid receptor has the best binding affinity for sugar acids including glucuronic acid.

MATERIALS AND METHODS

The affinity of boronic acid viologen receptors to sugar acids was determined by measuring the fluorescence of the HTPS reporter dye bound to receptors in a 96 well plate.

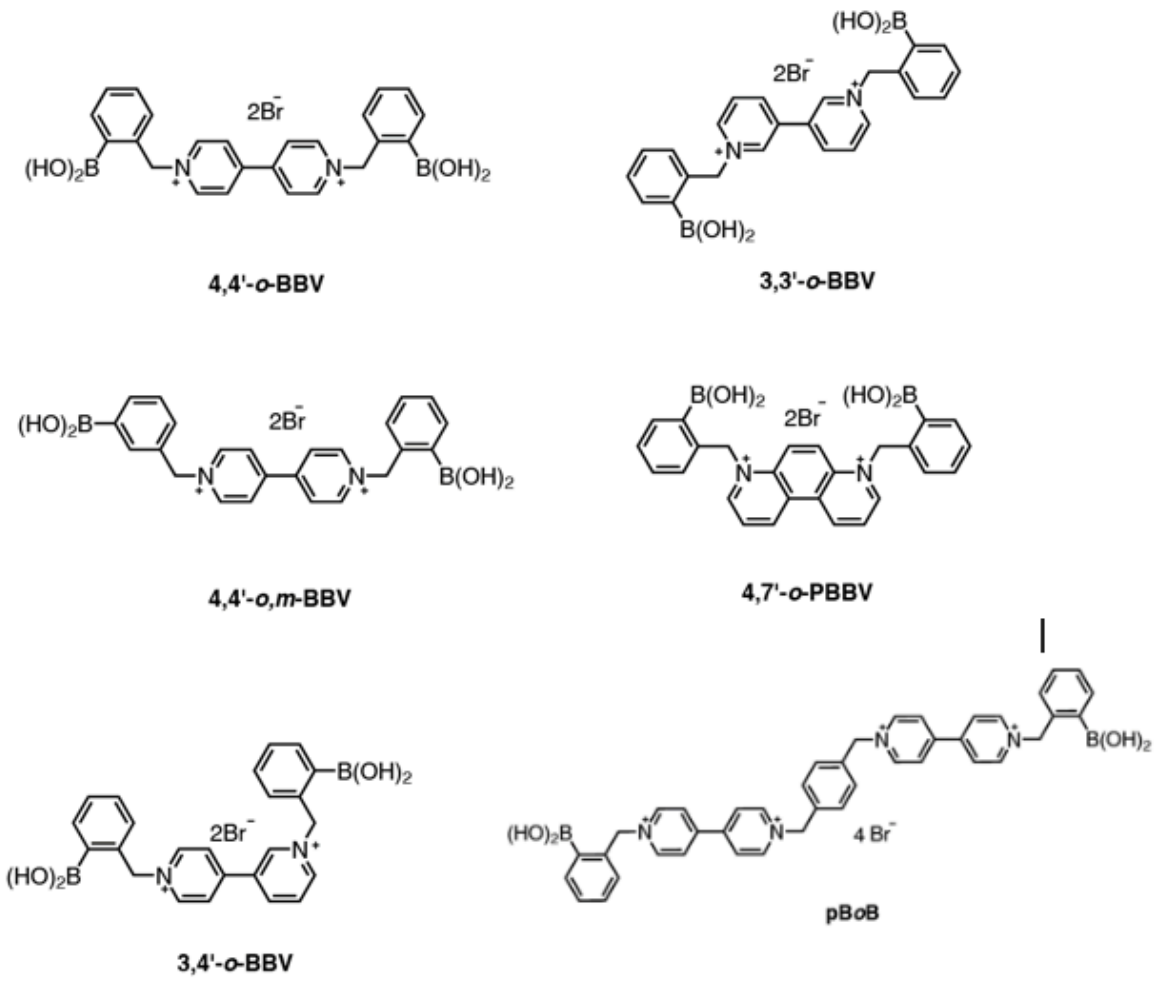

Figure 3: Six different forms of Boronic acid benzyl viologens (BBV). Boronic acid violegens- 4,4-o-BBV, 3,4-o-BBV, 3,3-o-BBV, 4,4-o, m-BBV, 4,7-o-BBV and pBOB were tested against six independent sugar acids at pH 7.4.

Preparation of stock sugar solutions

$1x10^{-4}$ moles of sugar were necessary to produce a 20mM solution (5 mL). 0.024g of Glucuronic acid was mixed with 5mL of sodium phosphate (NaP) buffer solution pH 7.4. Solution was then vortexed and sonicated to ensure sugar was completely dissolved.

Preparation of Probe

Table 1: Preparation of Boronic acid Benzyl Violgens

Boronic acid Benzyl Violgen	Amount of BBV (g)	Amount of buffer NaP (µl)	Amount of HTPS dye (µl)
4,4'-o-BBV	0.003	5,038	82
3,3'-o-BBV	0.003	5,038	82
4,7-o-PBBV	0.001	7,872	182
3,4'-o-BBV	0.003	5,038	82
4,4' o,m-BBV	0.003	5,038	82
pBoB	0.003	5,038	82

$5.12x10^{-6}$ mol (0.003g 4,4'-o-BBV) of quencher were necessary to pepare 5.12 ml of 1mM solution. 0.003g of 4,4'-o-BBV quencher was measured and mixed with 5.038 ml of pH 7.4 NaP buffer in an eppendorf tube. The quencher was then vortexed and followed by sonication for 30 minutes to allow quencher to completely dissolve. After sonication 82 µl of HTPS dye was added to probe solution giving it 20 minutes to allow boronic acid benzyl violegen and reporter dye complex formation. This process was repeated for the 3,3'-o-BBV and 4,7-o-PBBV, 3,4'-o-BBV and pBoB quenchers with respective amounts of BBV and buffer shown on Table 1.

Preparation of Sugar Dilution

Table 2: Sugar dilutions

Dilution tube number	Glucuronic Acid Volume	Buffer (NaP) Volume (µl)
8	1ml	---
7	800ul of tube 8	200ul
6	500ul of tube 7	500ul
5	500ul of tube 6	500ul
4	500ul of tube 5	500ul
3	500ul of tube 4	500ul
2	500ul of tube 3	500ul
1	500ul of tube 2	500ul

Dilution tube number 8 corresponds to the stock solution of glucuronic acid made. Glucuronic acid was then diluted with Sodium Phosphate (NaP) buffer in tubes 7-1. Tube 1 corresponds to the concentration of sugar acid most diluted. Same procedure was followed for other sugar acids in this investigation.

Preparation of 96 Well Plate

As shown in Figure 4, to well A1-B12, 20 µl of probe 4,4 BBV was added and 20µl of the glucuronic acid corresponding sugar dilution. To well C1-D12, 20µl of probe 3,3 BBV was added and 20µl of the glucuronic acid corresponding sugar dilution. To well E1-F12, 20µl of probe 4,7 BBV was added and 20µl of the glucuronic acid corresponding sugar dilution. Well G1-G12 were left blank and to well H1-H3, 40µl of NaP buffer was

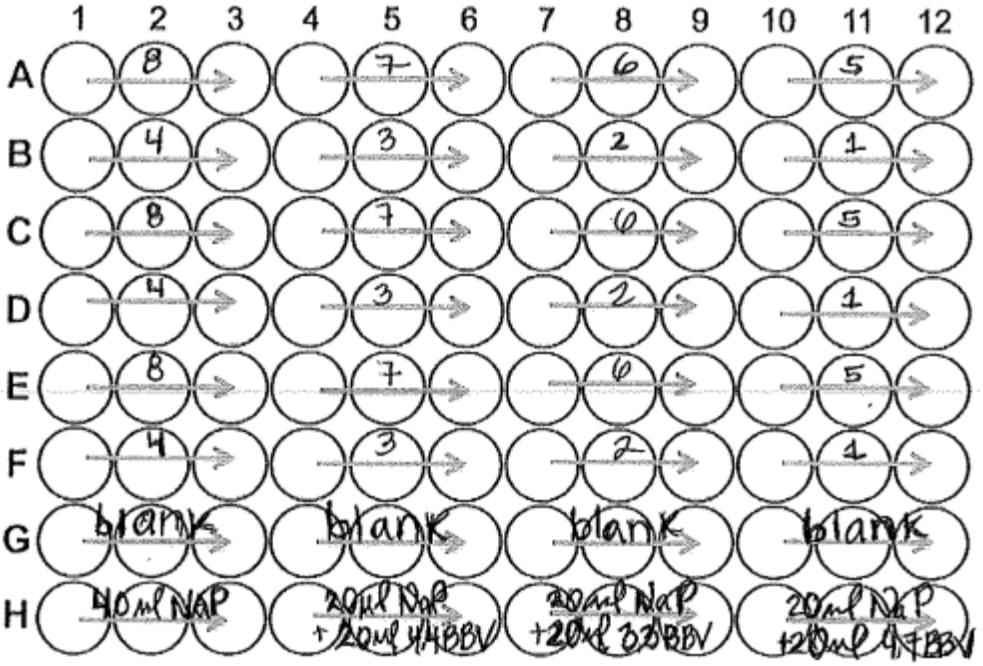

Figure 4: 96 well plate is shown with corresponding dilutions of glucuronic acid and boronic acid appended viologens.

added. To well H4-H6, 20µl of NaP + 20µl of 4,4 BBV was added, to well H7-H9 20µl of NaP + 20µl of 3,3 BBV was added and to well H10-H12, 20µl of NaP + 20µl of 4,7 BBV was added. The same logic was followed for the plating of glucuronic acid and the boronic acid viologen receptors 4,3 BBV, 4,4 o-m BBV and pBPOB on a separate plate. Next, plates were sealed, centrifuged, and fluorescence was measured with a fluorecence plate reader (405 excitaction, 535 emission filters).

RESULTS

The 4,7-o-BBV form of boronic acid form demonstrated to have the best binding affinity for glucuronic acid as shown in Figure 2.

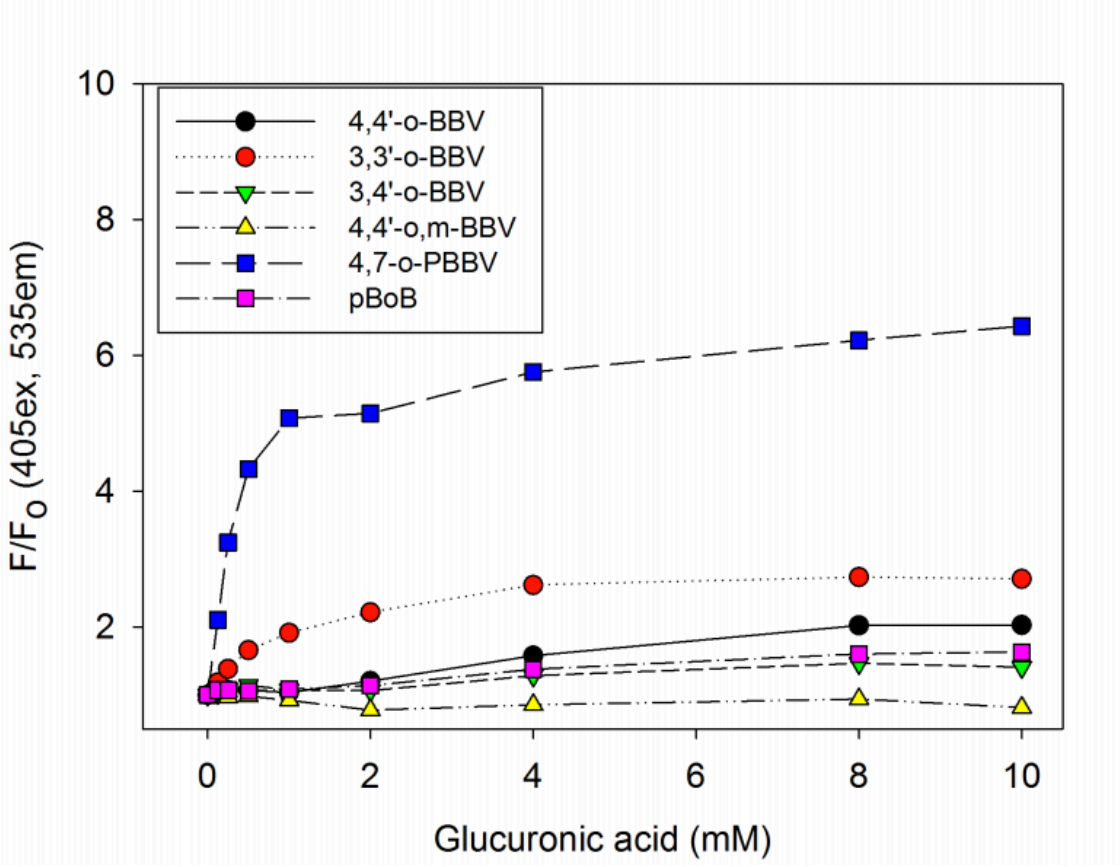

Figure 5: Binding of glucuronic acid against six different forms of Boronic acid benzyl viologens (4,4-o-BBV, 3,4-o-BBV, 3,3-o-BBV, 4,4-o, m-BBV, 4,7-o-BBV and pBOB).

Glucuronic acid was examined with the corresponding boronic acid receptors to determine their effectiveness. The 4,7-o-PBBV receptor demonstrated to be the most responsive with a 6-fold recovery at 8mM glucuronic acid and 3,3'-o-BBV being the second best. The other (4,4'-o-BBV, 3,4'-o-BBV and pBoB) gave 1.5-fold recovery. The boronic acid viologen with the weakest affinity was 4,4-o-BBV.

DISCUSSION

Further studies will involve synthesizing the NATOG biomarker will be produced enzymatically or synthesized chemically to monitor the binding of 4,7-o-BBV receptor to glucuronic acid. Glucuronic acid will be characterized further to determine what part of the compound is responsible for the high binding affinity to 4,7-o-BBV. The C-1 hydroxyl group and the terminal group will be investigated in glucuronic acid to determine if the hydroxyl or carboxyl group is responsible for the binding event.

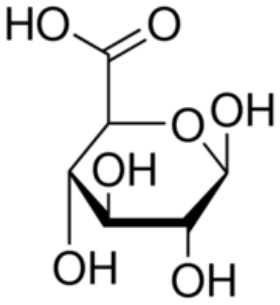

Figure 6: Glucuronic acid (4)

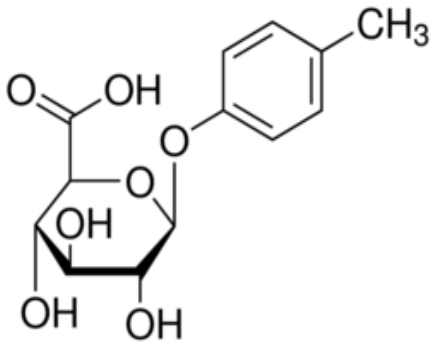

Figure 7: p-Tolyl- β-D-glucuronide (4)

A form of the NATOG derivative that is a mimic (Fig. 6) compound (glucoronide) is also being investigated. This compounds has the hydroxyl group substituted for a phenyl group. (Fig. 7). If the hydroxyl group is imperative for 4,7-o-PBBV binding, then the binding affinity will be lowered.

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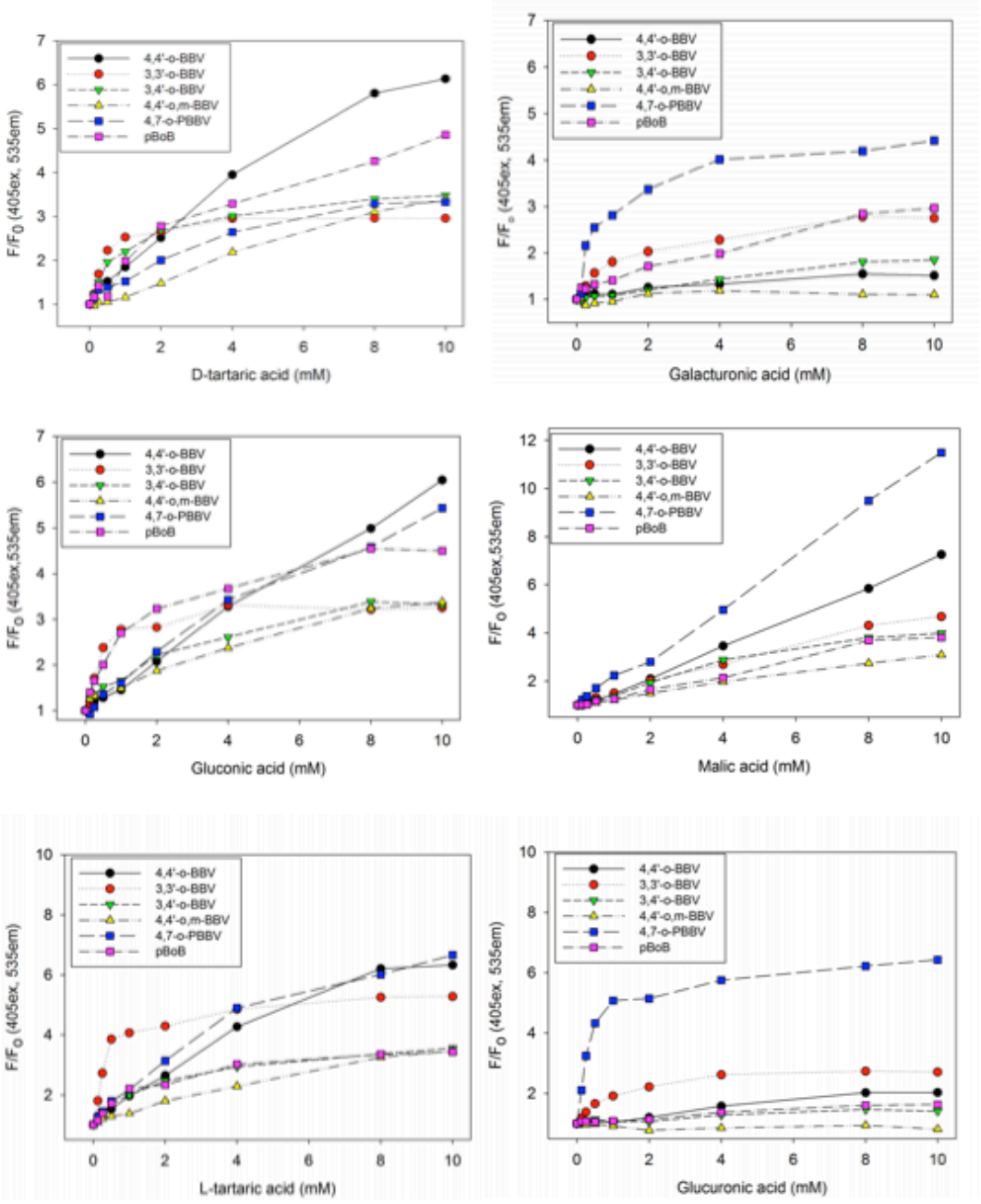
SUPPLEMENTAL FIGURES



Supplemental Figure 1: BBV receptor affinity graphs for different sugar acids. Six Boronic acid Benzyl Viologens (4,4-o-BBV, 3,3-o-BBV, 3,4-o-BBV, 4,4-o, m-BBV, 4,7-o-BBV and pBOB) receptor affinities were compared towards the six sugar acids (D-tartaric acid, Galacturonic acid, Gluconic acid, Malic acid, L-tartaric acid and Glucuronic acid).

SUPPLEMENTAL RESULTS

The results indicated that boronic acid receptors have a high binding affinity for some sugar acids (Supplemental Fig. 1). For example, D-Tartaric acid had a high affinity for the 3,3-o-BBV and 3,4-o-BBV viologens. On the other hand, Glucuronic acid, Galacturonic acid and Malic acid exhibited good binding affinity to the boronic acid viologens 4,7-o-BBV and 3,3-o-BBV. Gluconic acid showed high affinity for the 3,3-o-BBV and pBOB receptors. The boronic acid viologen with the weakest affinity for all six sugar acids was 4,4-o-BBV.