Recognition of saccharides in the NIR region with a novel fluorogenic boronolectin: *in vitro* and live cells labeling

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1. **General Information**

The solvents used were dried and purified by standard methods prior to use. All reactants were purchased from Sigma-Aldrich and used as received. Boroxole (2-Hydroxymethylphenylboronic acid) and 4-(4-BOC-piperazine)phenylboronic acid were purchased from Combi-Blocks, Inc. (San Diego, USA). ER-Tracker™ Blue-White DPX was purchased from Invitrogen.

Corrected fluorescence emission spectra were obtained on a Cary Eclipse spectrophotometer equipped with two Czerny-Turner monochromators and a 15 W Xenon pulse lamp (pulse width: 2-3 us, power: 60-75 kW). The probe was excited at 720 nm for in solution experiments. When temperature control was necessary, the cell was thermostated using the Thermal Application of the ADL software.

Confocal laser scanning microscopy was performed with an Olympus Fluoview FV 1000 microscope with a UPLSAPO 60x 1.2 NA water immersion objective. Excitation and emission filters were as follows: excitation DAPI, 405 nm; emission DAPI, band pass (BP): 430–470 nm; excitation Probe, 637 nm; emission Probe, BP: 655-755 nm; excitation ER tracker, 405 nm; emission ER tracker, BP: 505-525 nm. We always used the sequential mode for image acquisition.

$^1$H-NMR spectra were measured with a Varian Bruker AC-200 and Bruker Avance II-500 spectrometers. Mass spectra were measured with a Bruker Daltonik microTOF mass spectrometer (ESI+, ESI-).
2. Synthesis of Boronolectin 1

\[ \text{N-[5-Anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadiene-1-ylidene]anilinium Chloride (S1)}^{1,2} \]
At 0 °C, phosphorus oxychloride (2 mL, 22 mmol) was added dropwise from a pressure-equalizing addition funnel to anhydrous DMF (2.4 mL, 31 mmol). After 30 min, cyclohexanone (1 mL, 9.6 mmol) was added and the mixture was refluxed for 1 h. Next, an aniline/EtOH [1:1 (v/v), 3.3 mL] mixture was added dropwise with constant cooling at 20 °C. Reaction was continued for an additional 30 min after aniline addition, and then the deep purple mixture was poured into ice cold H$_2$O/concentrated HCl (10:1, 20 mL). Crystals were allowed to form for 2 h in an ice bath, then filtered, washed with cold H$_2$O and Et$_2$O, and then dried in vacuo: yield 2.95 g (84%).

$^1$H NMR (200 MHz, MeOD-d$_4$) $\delta$ 8.68 (s, 2H), 7.58 – 7.17 (m, 10H), 2.74 (t, J = 6.2 Hz, 4H), 2.11 – 1.75 (m, 2H). HRMS (ESI+): calcd (M+H)$^+$ 323.1310; found, 323.1308.

\[ \text{1,1,2-Trimethyl-3-(3-sulfopropyl)-1H-benzo[e]indol-3-ium-, Inner Salt (S2)}^{1,2} \]
Toluene (5 mL), 1,1,2-trimethylbenzo[e]indol (1.3 g, 0.062 mol), and 1,3-propane sultone (1.1 mL, 0.093 mol) were heated under reflux for 18 h. The reaction mixture was allowed to cool to room temperature. The resulting bluish crystals were filtered and washed with ether (3 x 10 mL). The filtered product was crystalized from MeOH and Et$_2$O. The crystals were collected and dried in vacuo to yield 1.68 g (82%). $^1$H NMR (200 MHz, MeOD-d$_4$) $\delta$ 8.30 (d, J = 8.4 Hz, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.11 (d, J = 8.9 Hz, 2H), 7.78 (t, J = 7.1 Hz,
1H), 7.68 (t, J = 7.5 Hz, 1H), 4.75 (t, J = 7.2 Hz, 2H), 3.30 (dd, J = 3.2, 1.6 Hz, 2H), 2.43 (dt, J = 14.5, 7.7 Hz, 2H), 1.82 (d, J = 3.6 Hz, 6H. HRMS (ESI+): calcd (M+H)\(^+\) 332.1315; found, 332.1315.

**Compound S3**\(^{1,2}\): A solution of 1,1,2-trimethyl-3-(3-sulfopropyl)-1H-benzo[e]indol-3-ium-, inner salt (1.69 g, 6 mmol), N-[5-anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadiene-1-ylidene]anilinium chloride (1.079 g, 3 mmol), and anhydrous sodium acetate (600 mg, 7 mmol) in absolute EtOH (60 mL) was heated under reflux for 3.5 h under a N\(_2\) atmosphere. The EtOH was removed under reduced pressure, the residue was washed with Et\(_2\)O and the crude was purified by recrystallization from MeOH: Et\(_2\)O. The crystals obtained were filtered and dried under vacuum, yielding a pure crystalline material (1.35 g, 93%). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 8.32 (d, J = 14.1 Hz, 2H), 8.25 (d, J = 8.5 Hz, 2H), 8.05 (d, J = 8.9 Hz, 2H), 8.01 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 9.0 Hz, 2H), 7.60 (td, J = 6.8, 1.2 Hz, 2H), 7.47 (td, J = 6.8, 1.2 Hz, 2H), 6.52 (d, J = 14.3 Hz, 2H), 4.47 (t, 4H), 2.74 (t, J = 5.6 Hz, 4H), 2.60 (t, J = 6.7 Hz, 4H), 2.05 (quin, J = 7.2 Hz, 4H), 1.90 (s, 12H), 1.82 (quin, J = 5.8 Hz, 3H). HRMS (ESI-): calcd 797.2486; found, 797.2493.

**Boronolectin 1**: Compound S3 (120 mg) and 4-piperazine phenylboronic acid (58 mg) were dissolved in 1 mL of anhydrous DMF, then triethylamine was added (540 \(\mu\)L, 3.9 mmol. The resulting solution was stirred overnight protected from light. The solvent was removed yielding an intense blue residue which was purified by column chromatography (Sephadex LH-20, MeOH) to afford 65.3 mg of the desired product as a blue solid (yield 45%). \(^1\)H NMR R\(_f\) (MeOH/H\(_2\)O 8:2)=0.49, R\(_f\) (MeOH/H\(_2\)O 9:1)=0.76
\(^1\)H NMR (500 MHz, MeOD) \(\delta\): 8.10 (m, 4H), 7.95 (t, J = 9.3 Hz, 4H), 7.82 (d, J = 8.5 Hz, 1H), 7.73 (d, J = 8.7 Hz, 1H), 7.62 (d, J = 8.85 Hz, 2H), 7.58 (m, 2H), 7.41 (t, J = 7.6 Hz, 2H), 7.14 (d, J = 9 Hz, 1H), 7.11 (d, J = 9 Hz, 1H), 6.24 (d, J = 13.7 Hz, 2H), 4.38 (m, 4H), 3.77 (m, 8H), 2.99 (t, J = 6.9 Hz, 4H), 2.66 (t, J = 6.3 Hz, 4H), 2.29 (m, 4H), 1.96 (s, 6H), 1.93 (s, 4H), 1.90 (m, 2H). HRMS (ESI-): Calcd, 967.3929; Found, 967.3951

**4-piperazine phenylboronic acid (S4)**: 4-(4-BOC-piperazine)phenylboronic acid (101 mg, 0.33 mmol) was suspended in 2 mL of anhydrous DCM. A volume of 1.5 mL of trifluoroacetic acid (TFA) was added dropwise at the resulting suspension with stirring at 0\(^\circ\)C. Reaction was complete 60 min after TFA addition. The solvent was removed \textit{in vacuo} yielding a colorless oil (58 mg, 100%).
3. Determination of pKa of boronolectin 1 and its complexes

The value of pKa for the probe and the complexes were determined by the change of fluorescence intensity with pH, at the maximum of the spectrum (Figure S2). We used quartz cuvettes of size 5 × 5 mm and the pH was determined at each point (InLab® Micro Mettler Toledo). The curves were fitted with the Henderson-Hasselbach equation were \( x \), \( xA \) and \( xB \) corresponds to the signal obtained for the simple and the acid and basic species.

\[
pH = pKa + \log \left( \frac{[B]}{[A]} \right) = pKa + \log\left(\frac{(x - xA)/(xB - x)}{1} \right) \quad \text{equation 1}
\]

Fig. S1 Absorption (left) and emission (right) spectra of boronolectin 1 2.8 μM in methanol (black) and buffer (red). \( \lambda_{exc} = 720 \) nm

Fig. S2 Change of the emission of boronolectin 1 2.8 μM (absorbance < 0.05) within the pH range 4-12
4. Calculation of binding constants

Solutions of 2.8 µM of boronolectin 1 in buffer PBS pH 7.4 in 5x5 mm quartz cuvettes were titrated with increasing amounts of monosaccharides, with continuous stirring. Plots of Intensity (820 nm) versus sugar concentration were fitted directly with equation (2)^3.

\[
I_F = \frac{(I_{Fmin} + I_{Fmax} \times K \times [sugar])}{(1 + K \times [sugar])}
\]

\textit{equation 2}
Fig. S3 Changes of the emission at 820 nm by cumulative additions of sugar to a 2.8 µM solution of 1 and fit with equation 2
5. Binding of model proteins

Solutions of 2.8 μM of boronolectin 1 in buffer PBS pH 7.4 in 5 × 5 mm quartz cuvettes were titrated with increasing amounts of each model protein, with continuous stirring. The change in the emission spectrum exciting at 720 nm was monitored for each titration solution.

6. Live and fixed cell labeling with boronolectin 1

Two days before the experiment, MCF10 cells were seeded on glass coverslips in complete medium. The day of the experiment cells were washed three times with PBS and, in the corresponding case, pre-incubated with Boroxole 50 mM in modified Tyrode’s buffer (135 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 50 mM Tris pH 7.5) at 37°C for 15 min. After three washes with PBS, cells were incubated with 50 μM probe in modified Tyrode’s buffer for 20 min at 37 °C. After that, cells were fixed in 4% PFA at RT during 20 min, and stained with DAPI before visualization. The former labeling procedure was established after exploring different incubation conditions (Fig. S5).

In case of ER-tracker staining, incubation with 1 μM ER-Tracker™ Blue-White DPX in PBS at 37°C for 30 min was performed following probe labeling. After washing, live visualization was performed. In the case of acid labeling, cells were incubated with 50 μM probe 1 in Acetate Buffer (135 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.1 M Acetate Buffer pH 4) instead of modified Tyrode’s buffer. For fixed cells labeling, cells were fixed with methanol at -20 °C for 20 min. Then, incubation with 50 μM probe in PBS for 30 min at RT was performed (Fig. S6).

When the effect of dopamine was assayed, a complex of 50 μM probe-50 mM dopamine in modified Tyrode’s buffer was pre-incubated for 30 min at 37 °C. Then, cells were incubated with this complex for 20 min at 37 °C (Fig. S7 and S8).
Fig. S4 MCF-10 cells were incubated alive in different conditions with probe 1 and fixed. Representative images are shown.
**Fig. S5** Image of MCF-10 cells incubated with probe 1 after fixation.

**Fig. S6** MCF-10 cells were incubated alive with the complex boronolectin 1-dopamine and fixed. Representative images are shown.
Fig. S7 Chemical structure of boroxole (left) and binding of dopamine with the boronic acid residue (right)

References

Qualitative Compound Report

Analysis Info
Analysis Name: D:\Data\gc\CSL295\d
Method: tune_high Formato negativo.m
Sample Name: CSL295 (en modo negativo)
Comment: Srv. metanol
Carla Spagnuolo

Acquisition Date: 1/2/2014 11:01:54 AM
Operator: Gabriel Cases
Instrument: microCTOF-Q II

Acquisition Parameter
Source Type: ESI
Focus: Not active
Scan Begin: 600 m/z
Scan End: 1600 m/z
Ion Polarity: Negative
Set Capillary: 3000 V
Set End Plate Offset: -500 V
Set Collision Cell RF: 1600.0 Vpp
Set Neulizer: 0.4 Bar
Set Dry Gas: 4.0 l/min
Set Dry Heater: 180°C
Set Divert Valve: Source

Intensity

m/z  967.3929

966  967  968  969  970  971  972

Meas. m/z 967.3929

#  Formula  nVz  err [ppm]  Mean err [ppm]  rdb  N-Rule  e'' Conf  mSigma
1  C54H60B4N4O8S2  2.2  2.9  27.5  ch  even  14.59

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