A fluorescent water-soluble naphthalimide-based receptor for saccharides with highest sensitivity in the physiological pH range

Sabine Trupp, Annika Schweitzer and Gerhard J. Mohr*

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A new water-soluble fluororeactand for the optical detection of saccharides is presented. We have combined the functional naphthalimide fluorophore with the well-known ability of boronic acids to bind to the diol moiety of saccharides. The fluororeactand exhibits sensitivity in the mM range, absorbance and emission in the visible spectral range, large Stokes' shift and fluorescence increase in the physiological pH range.

Introduction

The last 10 years have seen significant progress in the synthesis and investigation of selective receptors using fluorescence for detection.[1–7] Nowadays the quantification of saccharides belongs to the most important fields in medical diagnostics, where the main objective is continuous, noninvasive saccharide monitoring, for example for controlling blood glucose levels to detect diseases such as hypoglycaemia or Diabetes mellitus.[8–10] Saccharides also are highly important feed materials for enzymatic syntheses and their continuous monitoring would help in process optimisation.

Thus there is still a need for new indicators in this field, if several requirements are considered. The indicator dyes have to absorb and emit in the visible spectral range and should have large Stokes' shifts, in order to avoid problems caused by sample autoluminescence, and to simplify the set-up of the analytical device. The dyes should be easily functionalised to either make them water soluble for application in medical research, to make them polymer soluble for optical sensing applications or to covalently link them to support materials (again mainly for sensing purposes but also for chromatographic applications). Finally, there should be a significant and preferably selective response to the analyte, and, especially in the case of fluorescence, an increase in signal upon interaction with the analyte is preferred over a decrease.

Naphthalimide derivatives are well suited for optical sensing because their absorption and emission are in the visible spectral range, they have a large Stokes' shift and allow the step-wise introduction of functional groups. In 1998 de Silva and coworkers[11] applied a naphthalimide dye to detect thiols *via* irreversible Michael addition and in 2002 Lakowicz and coworkers introduced *N*-phenylboronic acid derivatives of 1,8-naphthalimide for glucose detection.[12] The latest work in the field of naphthalimide dyes has been reported by Qian and coworkers, who developed water-soluble fluorescent pH sensors.[13]

Being aware of the more than decennial work of researchers such as Shinkai, James or Lakowicz,[14–24] who used boronic acid derivatives to detect saccharides, and taking into consideration our experience with indicators, which can perform reversible chemical reactions with the analyte,[25–28] our present work aims at providing a further step in the improvement of saccharide indicators.

With FR-530 (Fig. 1) we introduce a new fluorogenic structure including a boronic acid moiety, with the well known ability to covalently react with 1,2- or 1,3-diols in aqueous solutions by forming five- or six-membered cyclic esters. Because these are reversible chemical reactions the indicator dye is called a "fluororeactand".

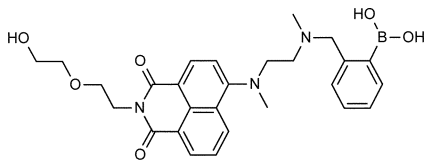

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Fig. 1 Structure of compound **4**, fluororeactand FR-530.

Usually the nitrogen in the vicinity of the phenylboronic acid moiety causes PET fluorescence quenching by transferring its lone-pair electrons to the naphthalimide chromophore. Protonation of this nitrogen leads to signal increases in fluorescence because the PET mechanism is modulated (Fig. 6, ● without D-fructose; see later). The boronic acid moiety in the 2-position, close to the nitrogen, promotes dative B–N bond formation, which lowers the pK_a of the boronic acid and because of this the binding affinity to saccharides increases. When FR-530 forms the ester with the diol moiety of saccharides, the strengthening of the B–N bond reduces PET fluorescence quenching and consequently an increase in fluorescence is detected (Fig. 2).[29,30] The derivatives with the boronic acid moiety in the 3- or 4-position did not show any signal increases in contact with saccharides because there is no interaction between the boronic acid moiety and the nitrogen atom.

Results and discussion

The dye was synthesised in a three step procedure shown in Fig. 3. First **1** was reacted with 2-(2-aminoethoxy)ethanol to give **2**. In the second step **2** was reacted with *N*,*N*′-dimethylethylenediamine to give **3**, which finally was reacted with

Friedrich-Schiller University, Institute of Physical Chemistry, Lessingstrasse 10, D-07743, Jena, Germany. E-mail: gerhard.mohr@uni-jena.de; Fax: +49 3641 948302; Tel: +49 3641 948379

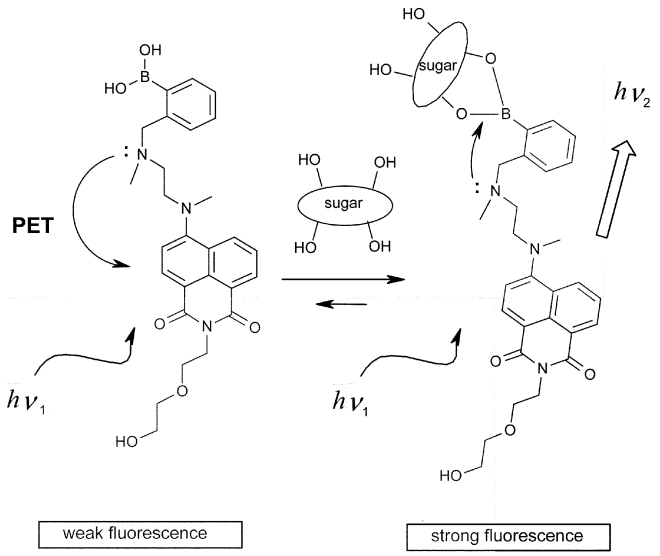

Fig. 2 Esterification causes an increase in fluorescence.

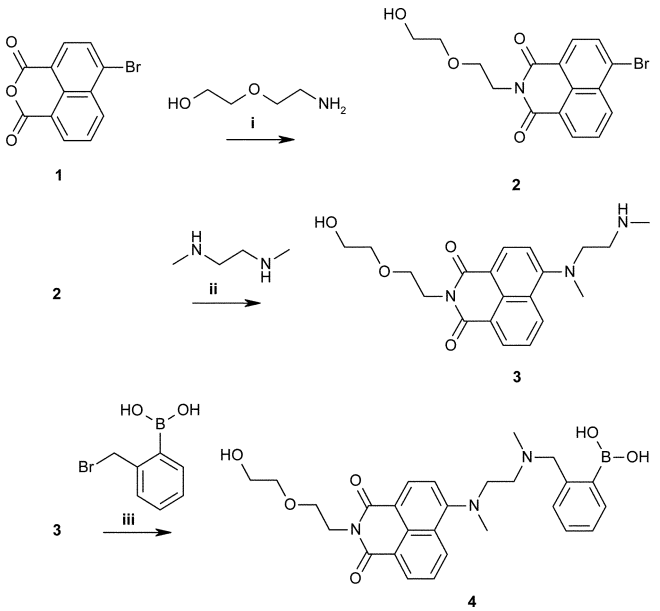

Fig. 3 Synthesis of compound **4**. *Reagents and conditions* (yield): i, **1**, 2-(2-aminoethoxy)ethanol, ethanol, reflux (90%); ii, **2**, 1 equiv. of *N*,*N*′-dimethyl-ethylenediamine, 2-methoxyethanol, reflux (80%); iii, **3**, (2-bromomethylphenyl)boronic acid, triethylamine, dry tetrahydrofuran, reflux (65%).

(2-bromomethylphenyl)boronic acid to give compound **4** as the product, denoted fluororeactand FR-530.†

FR-530 has the advantage that it is water soluble and its preparation is facile. Because of the hydroxy group the dye can be derivatised, for example with functional groups that can polymerise, or with long alkyl chains to change the solubility.

When the boronic acid moiety in FR-530 and the diol moiety of saccharides are forming a covalent bond, the dye changes its fluorescence. FR-530 responds to increasing concentrations of D-fructose by a significant increase in fluorescence at λ_{em} = 530 nm, while there is no significant change in absorbance at λ_{max} = 410 nm. The maximum signal increase of FR-530 in contact with D-fructose at pH 7.15 was found to be 130% (Fig. 4).

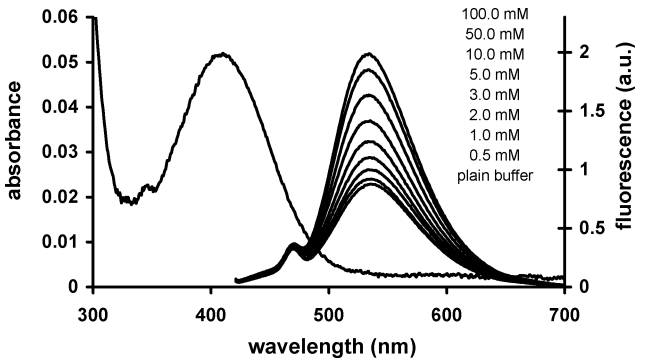

Fig. 4 Absorbance spectrum with λ_{max} = 410 nm, fluorescence spectra of FR-530 in contact with plain buffer and buffered solutions of D-fructose, all at pH 7.15 with λ_{exc} = 410 nm.

The sensitivity of FR-530 for D-fructose is highest in the range from 1.0–20.0 mM with a limit of detection of 0.1 mM (Fig. 5). The fluorescence quantum yield of the pH = 7.15 buffered solution of FR-530 (Φ = 0.99%) increases accordingly when 100 mM D-fructose is added (Φ = 2.60%).

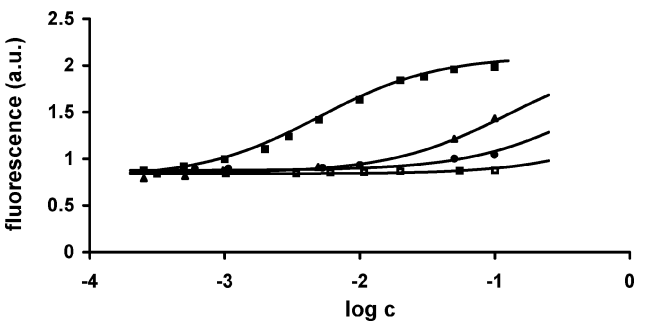

Fig. 5 Fluorescence intensity *vs.* log [saccharide], calibration graph of FR-530 upon exposure to buffered solutions of ■ D-fructose; ▲ D-galactose; ● D-glucose; □ ethylene glycol, all measured at pH 7.15.

The behaviour of FR-530 against saccharides follows a trend that is typical for aromatic boronic acids, in that the affinity to D-fructose > D-galactose > D-glucose > ethylene glycol (Table 1). Our findings are in accordance to the data found in the literature[31–34]

† Physical characteristics of 2-({[2-({2-[2-(2-hydroxyethoxy)ethyl]-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-yl}methylamino)ethyl]-methylamino}methyl)boronic acid **4**, denoted FR-530. Yield: 47%. Melting point: 174 °C. Elemental analysis: Found: C, 64.22; H, 6.73; N, 8.49. $C_{27}H_{32}BN_3O_6$ requires C, 64.17; H, 6.38; N, 8.31%. UV absorptions: $\lambda_{max}(H_2O)$/nm 410. IR absorptions: ν_{max}/cm^{-1}: 1448 (B–O), 1379, 1279, 1247, 1114, 760. NMR data: δ_H (250 MHz; MeOD; Me_4Si) 2.43 (s, 3 H, -CH_3); 2.95 (t, 2 H, N-CH_2); 3.62 (t, 2 H, N-CH_2); 3.02 (s, 3 H, -CH_3); 3.61 (m, 4 H, HO-CH_2-CH_2-OR); 3.75 (s, 2 H, N-CH_2-aryl); 3.78 (t, 2 H, CH_2-OR); 4.29 (t, 2 H, N-CH_2); 6.98–8.33 (m, 5 H, naphthalene-); 7.14–8.18 (m, 4 H, aryl-). δ_C (250 MHz; MeOD; Me_4Si) 39.14 (1 C, N-CH_2); 41.05 (1 C, -CH_3); 42.76 (1 C, -CH_3); 51.26 (1 C, N-CH_2); 51.33 (1 C, N-CH_2); 60.63 (1 C, CH_2OH); 62.51 (1 C, N-CH_2-aryl); 67.72 (1 C, CH_2-OR); 71.85 (1 C, CH_2-OR); 114.97, 121.72, 125.03, 125.26, 126.82, 131.41, 131.85, 132.42, 132.79, 156.34 (10 C, naphthalene-); 127.01, 128.18, 128.50, 129.38, 129.73, 140.32 (6 C, aryl-); 164.77, 165.41 (2 C, N-C=O). Mass spectrometry data: (FAB in 3-nitrobenzyl alcohol) *m*/*z* (%): 506 [M + 1].

Table 1 Association constants of FR-530

Sugar	K_a/M^{-1}
D-Fructose	170
D-Galactose	8
D-Glucose	2
Ethylene glycol	0.5

for K_a, where phenylboronic acid has a binding constant of 162 M^{-1} for fructose and 5 M^{-1} for glucose. The selectivity pattern represents (a) the preorganisation of the diol (*cis* or *trans* configuration) and (b) the sterical hindrance *via e.g.* the hydroxymethyl group of the saccharide.[35]

The relative signal changes in going from zero to 100 mM D-fructose are highly dependent on buffer pH. Thus, virtually no changes in fluorescence are observed at highly acidic or basic pH, while significant signal changes are within the pH 5.0 to 10.0 region. To study cross-sensitivity to pH we determined the fluorescence in the absence and presence of D-fructose by changing the pH value from 1 to 13. Maximum signal changes were found at around pH 6.3 which is appropriate for physiological measurements (Fig. 6).

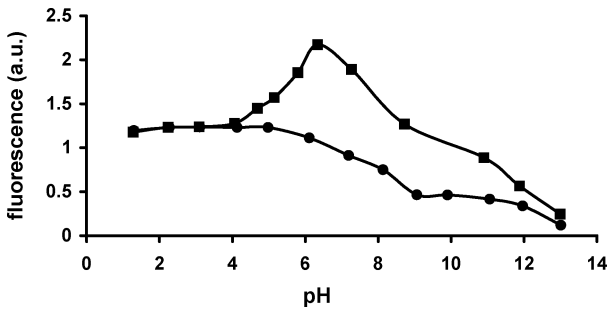

Fig. 6 Fluorescence intensity *vs.* pH range, graph shows relative signal change of FR-530 in contact with plain phosphate buffer and buffered solutions of 100 mM D-fructose. ● without D-fructose; ■ with D-fructose.

Furthermore, the two analogues of FR-530 with the boronic acid moiety in 3- and 4-position and a reference substance without the boronic acid moiety have been sythesised to control the results. The reference substance did not show any response to D-fructose, indicating that the specific interaction of saccharides with boronic acids is responsible for the observed changes in fluorescence.

Experimental

Synthesis of FR-530

(i) 4-Bromo-1,8-naphthalic acid anhydride **1** was suspended in ethanol, the suspension was warmed to get a clear solution and then an equimolar amount of 2-(2-aminoethoxy)ethanol was added dropwise to the solution. After refluxing for 10 hours the pale yellow solid was washed with ethanol and then recrystallized from ethanol to give 6-bromo-2-[2-(2-hydroxy-ethoxy)-ethyl]-benzo[de]-isoquinoline-1,3-dione **2**.

(ii) In the second step **2** was refluxed with the equimolar amount of *N*,*N*′-dimethylethylene-diamine in 2-methoxyethanol to give 2-[2-(2-hydroxy-ethoxy)-ethyl]-6-[methyl-(2-methylamino-ethyl)-amino]-benzo[de]iso-quinoline-1,3-dione **3**. The product **3** was purified on silica gel using dichloromethane/methanol 3:1 to give a yellow solid.

(iii) The 2-({[2-({2-[2-(2-hydroxy-ethoxy)-ethyl]-1,3-dioxo-2,3-dihydro-1H-benzo[de]iso-quinolin-6-yl}-methyl-amino)-ethyl]-methyl-amino}-methyl)-boronic acid **4** was synthesized by refluxing **3** with (2-bromomethylphenyl) boronic acid, ratio 1:2, in dry tetrahydrofuran for 6 hours. The product was purified on silica gel, using dichloromethane/methanol 5:1 to give **4** (FR-530) as a yellow solid.

Measurements

The fluorescence spectra were recorded on a Spex Fluorolog 3 (Jobin Yvon) spectrometer, while absorbance spectra were recorded on a Lambda 16 UV-VIS spectrometer (Perkin Elmer), both at 25 ± 2 °C.

The measurements at different pH values (1–13) were performed by using an universal buffer composed of 40 mM acetic acid, 40 mM sodium dihydrogen phosphate and 40 mM sodium borate and adjusting to the required pH by adding 1.0 M sodium hydroxide or hydrochloric acid solution. The effect of pH on the fluorescence of FR-530 was measured by preparing 13 solutions, each of 0.5 ml FR-530 solution + 0. 5 ml distilled water + 2 ml buffer solution (with the adequate buffer from 1 to 13). The measurements on the effect of pH in the presence of saccharides and ethylene glycol followed this procedure, in that 0.5 ml FR-530 + 0.5 ml saccharide solution (concentration 0.1 mol l^{-1}) + 2 ml buffer solution (from 1 to 13) were analysed in the fluorescence spectrometer after a response time of half an hour. At the end of all measurements the pH value was determined again and the results were evaluated as shown in Fig. 6 for D-fructose.

For the concentration series of D-fructose FR-530 was dissolved in aqueous solution, filtered and buffered to pH 7.15 by the use of 88 mM phosphate buffer. Different solutions were prepared consisting of 0.5 ml FR-530 solution + 0.5 ml D-fructose solution (with the required amount of D-fructose to obtain concentrations ranging from 0.0005 mol l^{-1} to 0.1 mol l^{-1}) + 2 ml buffer solution. The results of the measurements are represented in Fig. 4.

Conclusions

In summary, our fluororeactand is useful for detecting saccharides because it exhibits absorbance and fluorescence in the visible spectral range, shows increases in fluorescence upon exposure to the analyte and has a Stokes' shift of 120 nm. While FR-530 can be used for quantifying saccharides in aqueous solution at physiological pH, the facile introduction of a long alkyl chain, instead of the water-soluble hydroxy-ethoxy-ethyl group that was used here, will make the dye more lipophilic and also allow the dye's use in polymer layers. This approach is currently under investigation in our laboratory.

The evaluation of FR-530 for fructose detection is relevant for medical research because of hereditary fructose intolerance (HFI). It is complicated to diagnose this kind of metabolic dysfunction caused by an aldolase-B insufficiency where the decomposition of fructose is disturbed, because the symptoms are quite complex (aversion to sweets, diarrhoea, adephagia) and often are mixed with symptoms of a fatty liver. Today this disease is detected by the H_2-exhalation test, which is dangerous and is connected to

adverse effects, because the assumed HFI patient has to consume a fructose solution. Only a few clinical laboratories routinely perform analysis of HFI in blood samples on the basis of molecular biological diagnostics. Therefore, a continuous monitoring of blood samples, detecting blood fructose levels, is of significant relevance.

Acknowledgements

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