Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 6960

www.rsc.org/obc

PAPER

Schiff base formation and recognition of amino sugars, aminoglycosides and biological polyamines by 2-formyl phenylboronic acid in aqueous solution†

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Received 10th April 2012, Accepted 10th July 2012
DOI: 10.1039/c2ob26290h

Interactions of 2-, 3- and 4-formyl phenylboronic acids (FPBAs) with sugars, amino sugars, aminoglycosides and various poly- and monoamines have been studied by UV-vis, ^{1}H and ^{11}B NMR titrations in water at variable pH. Behavior of 2-FPBA was anomalous in several aspects. Transformation of the acid into its conjugate base was slow in NMR time scale and was accompanied by intramolecular cyclization affording the respective benzoboroxole. The equilibrium constants for imine formation (K_{imine}) between 2-FPBA and simple monoamines including amino sugars were *ca.* 2 orders of magnitude larger than those with other isomers. Still one order of magnitude larger K_{imine} values were observed for 2-FPBA with aminoglycosides (kanamycin, amikacin, gentamicin, neomycin) and polyamines (spermine, spermidine). The examination of UV-vis and ^{11}B NMR spectra of imines formed with 2-FPBA showed that formally neutral Schiff bases in fact were zwitterionic species containing a protonated imine group and an anionic $B(OH)_3^-$ group. The enhanced stability of imines with monoamines can therefore be attributed to the electrostatic stabilization provided by the zwitterionic structure and further increased stability of imines with antibiotics and polyamines is explicable by additional stabilization of the borate anionic group by ion paring with ammonium groups not involved in Schiff base formation. Thanks to high molar absorptivity of protonated imines interaction of 2-FPBA with aminoglycosides allows detecting them spectrophotometrically in a μM concentration range in neutral aqueous solutions in the presence of sugars, amino sugars and amino acids.

Introduction

Formyl phenylboronic acids (FPBA, Chart 1) have found numerous applications as bifunctional building blocks for construction of self-assembling macrocyclic, cage and polymeric supramolecular structures owing to their capacity of reversible formation of iminoboronate esters.[1] In area of molecular recognition a convenient and efficient procedure for determination of enantiomeric excess of chiral amines and chiral diols has been developed on the basis of self-assembly of 2-FPBA with formation of diastereomeric iminoboronate esters of general type **1**.[2]

These reactions are performed in low polar media, typically in chloroform, in order to shift the equilibria of imine and boronate ester formation to the respective products. The initial purpose of this paper was to explore a possibility of recognition of amino polyols *via* iminoboronate formation in water. Although formation of structures of type **1** is unlikely in polar media,[3] one may expect simultaneous formation of an imine and a tetrahedral boronate ester bonds between an amino polyol and a suitable isomer of formyl phenylboronic acid with geometrically matching carbonyl and boronic acid functionalities in a manner similar to recognition of catecholamines by a boronic acid-containing coumarin aldehyde.[4] In the course of this study we did not find, however, any convincing evidences of such binding mode, but we did observe significantly improved imine formation with 2-FPBA, particularly strong with polyamines including aminoglycosides. This effect is of interest because the imine formation generates a new chromophore and can be employed for optical detection of amines, but the reaction suffers from very low stability of Schiff bases in water.[5,6]

The target amino polyols employed in this study are amino sugars and aminoglycosides, which serve as biomarkers[7] and antibiotics[8] respectively (Chart 2). Recognition and sensing of these compounds is an important analytical problem. They do

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†Electronic supplementary information (ESI) available: Spectrophotometric titrations of isomeric formyl phenylboronic acids by glucose; ^{1}H NMR titrations of 3-FPBA and 4-FPBA by $MeOCH_2CH_2NH_2$; observed imine formation constants of 2-FPBA with different amines at variable pH; the species distribution diagram for 2-FPBA in the presence of kanamycin A; spectrophotometric titrations plots at 282 nm of 2-FPBA by some aminoglycoside antibiotics and amino sugars in μM concentration range. See DOI: 10.1039/c2ob26290h

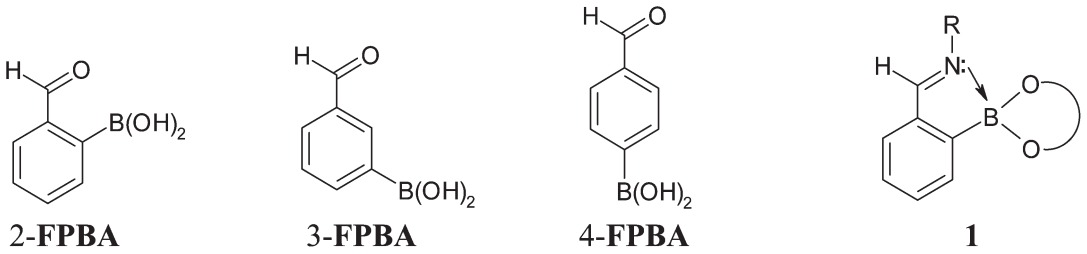


Chart 1

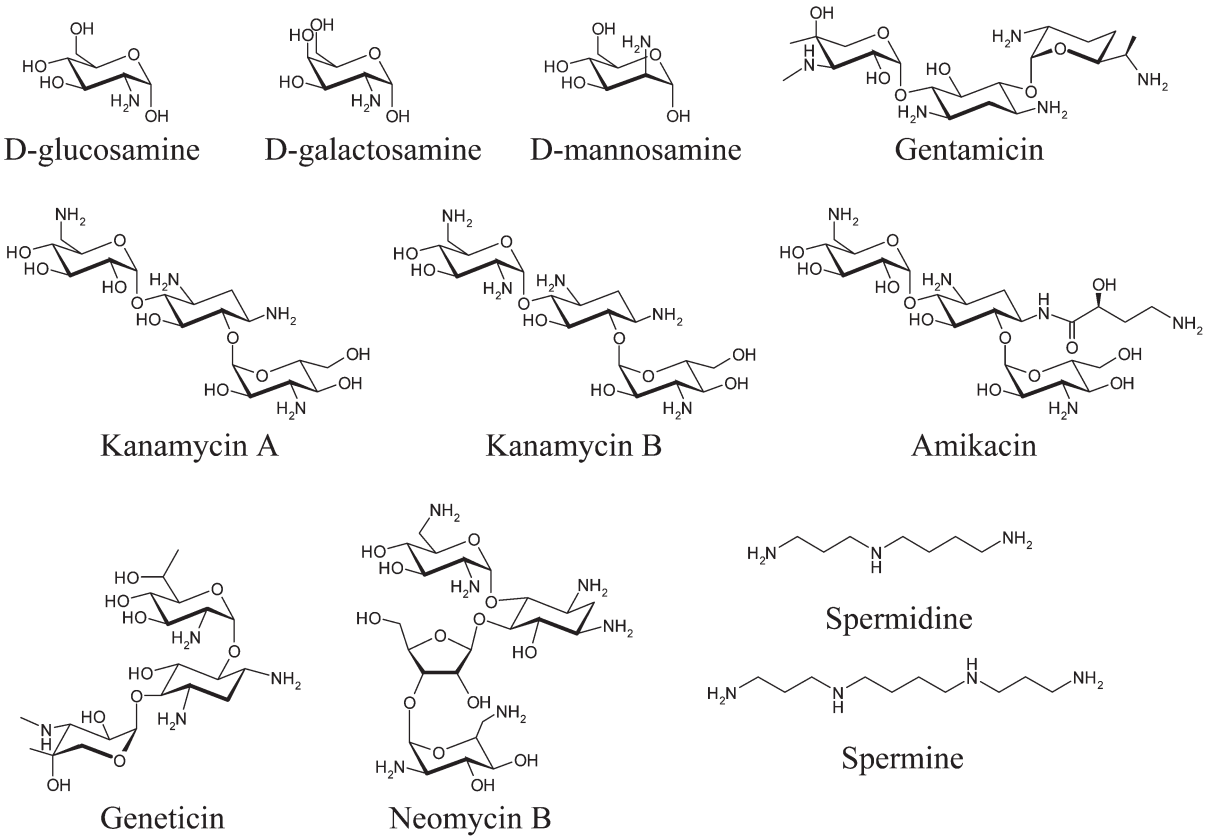


Chart 2 Amino sugars, aminoglycosides and polyamines employed in this study.

not absorb light and are not electroactive. In addition, due to close similarity in their structures both groups of compounds interfere with each other in their analysis.[7] Their determination is achieved mostly by chromatographic techniques with pre-column or post-column derivatization.[9] Recently an interest to direct sensing of amino sugars and aminoglycosides has been awoken. Sophisticated bifunctional receptors combining a crown-ether and phenylboronic acid as recognition elements were proposed for fluorometric sensing of glucosamine.[10] The receptors discriminate D-glucose and D-glucosamine in the range of concentrations 10–100 mM. An indicator-replacement assay based on the organometallic complex $[\{Cp^*RhCl_2\}_2]$ as the receptor was proposed for detection of amino sugars and aminoglycosides in the range 50–120 μM.[11] A "mini-array" version based on comparison of optical signals at three different pH values allowed to discriminate analytes of different structures. An impedimetric assay for neomycin in μM range using an aptamer-based sensor was recently proposed.[12] Highly sensitive surface plasmon resonance analysis of aminoglycoside antibiotics using imprinted boronic acid-functionalized Au nanoparticle composites was developed.[13] As will be shown below, 2-FPBA acts as a simple yet highly selective receptor for aminoglycosides, which allows detecting them spectrophotometrically in a μM concentration range in the presence of sugars, amino sugars and biological monoamines. It also allows one to detect polyamines such as spermine in the presence of monoamines.

Results and discussion

At the first step the acid dissociation constants and interactions of all three isomeric formyl phenylboronic acids with simple sugars were determined by spectrophotometric titrations. Fig. 1a and b show the spectral course of pH-titrations with insets

illustrating the fitting of absorbance *vs.* pH plots to the eqn (1), where ε_{obs} is the measured molar absorptivity at a given wavelength, ε_{B^-} and ε_B are the molar absorptivities of anionic and neutral forms of the boronic acid and K_a^B is the acid dissociation constant.

$$\varepsilon_{obs} = \frac{\varepsilon_{B^-} + \varepsilon_B[H^+]/K_a^B}{(1 + [H^+]/K_a^B)} \quad (1)$$

The spectral changes observed for 2-FPBA are similar to those reported for phenylboronic acid (PBA).[14] The decrease in absorbance is attributed to the transformation of planar neutral $-B(OH)_2$ group into tetrahedral anionic $-B(OH)_3^-$ group. The red shift observed for 3- and 4-isomers probably reflects some sort of conjugation between the carbonyl group and more electron-rich phenyl group of the anionic form of acid. The values of pK_a are given in Table 1.

The ^{11}B NMR spectra of 2-FPBA in neutral solutions show two signals, one at 29.3 and another one at 8.6 ppm (Fig. 2a). The first signal disappears in more basic solutions when pH is significantly higher than pK_a and the second signal disappears in more acid solutions when pH is significantly lower than pK_a indicating that they belong to neutral $R-B(OH)_2$ and anionic $R-B(OH)_3^-$ forms of 2-FPBA respectively. Thus in contrast to what is typically observed for boronic acids in aqueous solutions[15] the ionization of 2-FPBA is a slow process in the NMR time scale. This happens because the process is accompanied by cyclization with an intramolecular covalent bond formation as follows from the ^{1}H NMR study of the system.

The transformation of 2-FPBA into the anionic form affects also the aldehyde and aromatic signals in ^{1}H NMR spectrum (Fig. 2b). On increase in pH from 6.5 to 9.0 the signal of aldehyde –C**H**(=O) proton at 9.96 ppm gradually disappears and is substituted with the signal at 6.09 ppm characteristic of –C**H**-$(OR)_2$ group. Both signals co-exist at intermediate pH values around pK_a of 2-FPBA indicating slow interconversion of species in the NMR time scale. In this range of pH one also observes a superposition of two sets of signals of aromatic protons, one observed at higher pH shifted up-field in respect to another one observed at lower pH. A type of spectrum similar to those in the pH range from 7 to 8 was reported previously for halogen substituted 2-FPBA derivatives in organic solvents and was interpreted in terms of their reversible cyclization to a benzoboroxole (Scheme 1a).[16] Such process was not reported for

Table 1 Values of pK_a (relative error ±0.05) and observed binding constants (K_{obs}, M^{-1}, relative error ±10%) of carbohydrates to isomeric formyl phenylboronic acids at pH 7.0 (0.1 M phosphate buffer)

	2-FPBA	3-FPBA	4-FPBA	$PhB(OH)_2$
pK_a	7.30	7.84	7.43	8.8[17]
		K_{obs}, M^{-1}		
Glucose	10.9	9.5	36	2.6
Fructose	270	460	900	155

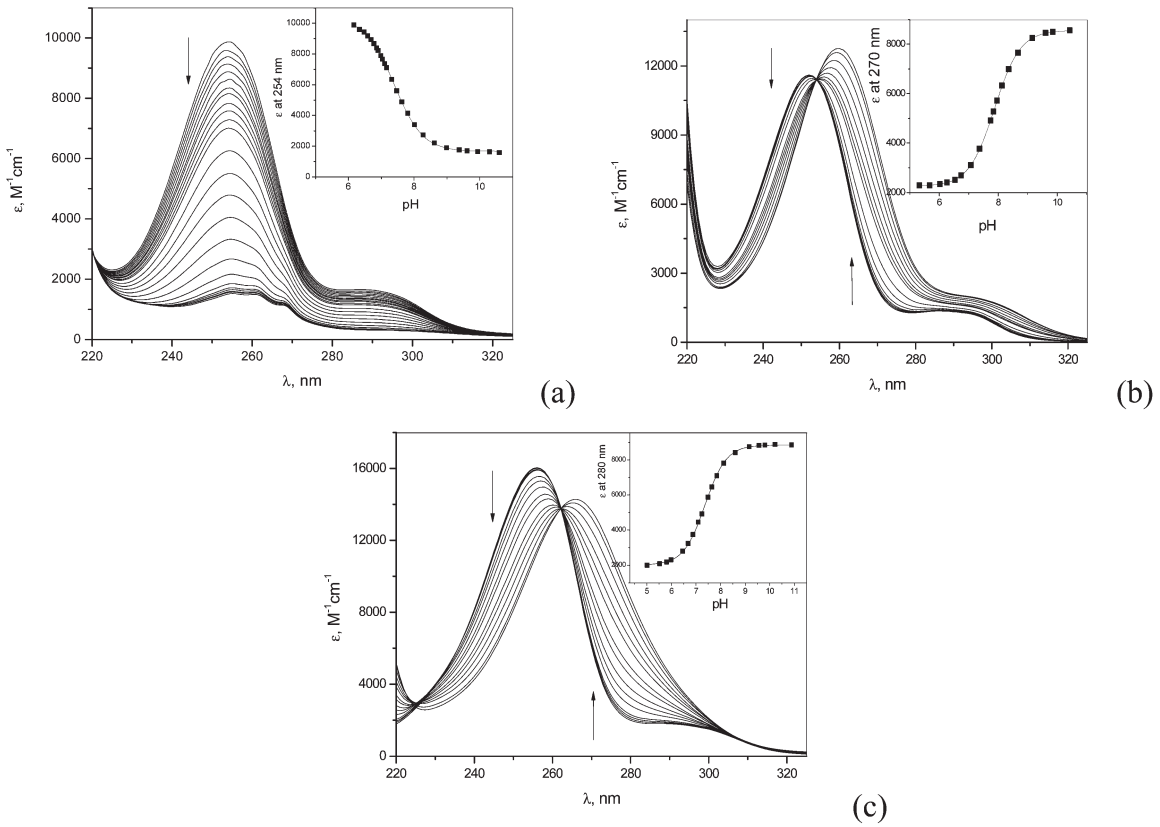


Fig. 1 The course of spectrophotometric pH-titrations of 2-FPBA (a), 3-FPBA (b) and 4-FPBA (c) in water. Insets show the absorbance *vs.* pH profiles at selected wavelengths fitted to eqn (1).

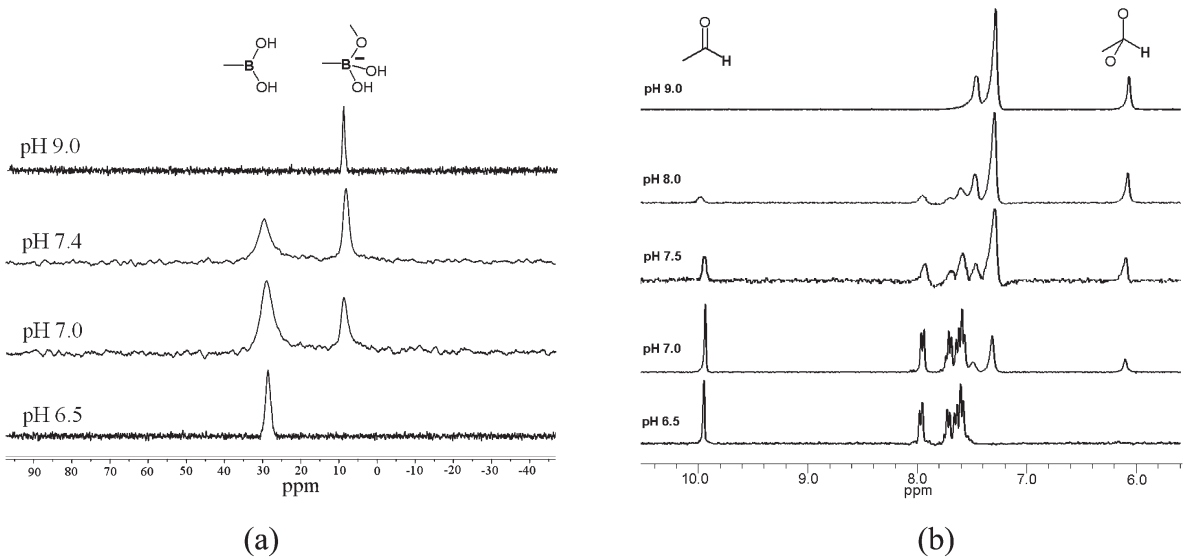
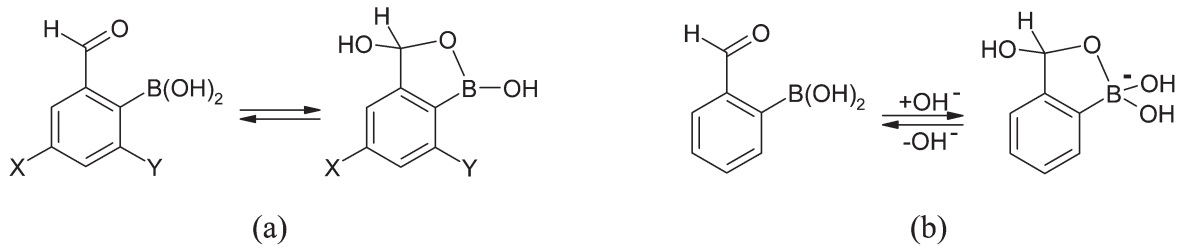


Fig. 2 The ^{11}B (a) and ^{1}H (b) NMR spectra of 2-FPBA at variable pH in D_2O.



Scheme 1

non-substituted 2-FPBA and for substituted compounds it was suppressed by water; however it seems that basic conditions favor the cyclization as shown in Scheme 1b. The reaction can be viewed either as nucleophilic addition of hydroxide anion to the carbonyl group assisted by intramolecular coordination of emerging oxo-anion to the boronic acid group or as a shift of the equilibrium of boroxole formation (Scheme 1a) by addition of hydroxide to the trigonal boron atom and converting it into the tetrahedral state in a way similar to classical stabilization of boronate diol esters.

The binding constants of glucose and fructose to all three isomers of FPBA were determined by direct UV-titrations and in some cases also by indicator-displacement method with alizarin red S.[17] Both methods gave the same binding constants within the limits of experimental errors. Typical results of a spectrophotometric titration are shown in Fig. 1S (ESI†) for glucose. The spectral changes are similar to those observed for addition of hydroxo anions to the respective isomers of FBPA in agreement with formation of anionic tetrahedral boronate esters. In all cases the titration plots were satisfactorily fitted to a simple 1 : 1 binding isotherm; the examples of fittings are shown in insets in Fig. 1S.† The values of observed binding constants (K_{obs}) at pH 7.0 in 0.1 M phosphate buffer solution are given in Table 1 together with the respective data for phenylboronic acid for comparison. All isomeric FPBAs are stronger acids than phenylboronic acid and as a result they have larger K_{obs} at pH 7, which is below pK_a values for all acids. However, among them the most acidic 2-FPBA does not form the most stable complexes apparently because of steric hindrance from the *ortho*-carbonyl group.

The spectral course of titrations of FPBAs by compounds containing amino groups is completely different. A typical behavior is illustrated in Fig. 3 with D-glucosamine as a substrate. Titration of 2-FPBA induces a decrease in absorption maximum at 254 nm and a strong increase in absorption above 260 nm with appearance of a new maximum at 282 nm (Fig. 3a). Titrations of 3- and 4-isomers induce very small spectral changes in the same concentration range of D-glucosamine as exemplified in Fig. 3b for the titration of 3-FPBA. The differential spectrum shown in the inset has a weakly absorbing maximum near 265 nm.

Similar spectral changes were observed for all compounds containing amino groups. Fig. 4 illustrates typical titration profiles of 2-FPBA for a simple mono amine $MeOCH_2CH_2NH_2$ and an aminoglycoside antibiotic neomycin B. In both cases like in the case of D-glucosamine one observes an isosbestic point at 262 nm and a strong increase in absorbance with maximum at 282 nm. Titrations of 3- and 4-isomers by these compounds induced minor spectral changes like those in Fig. 3b. No interaction of these compounds with phenylboronic acid was detected under similar conditions.

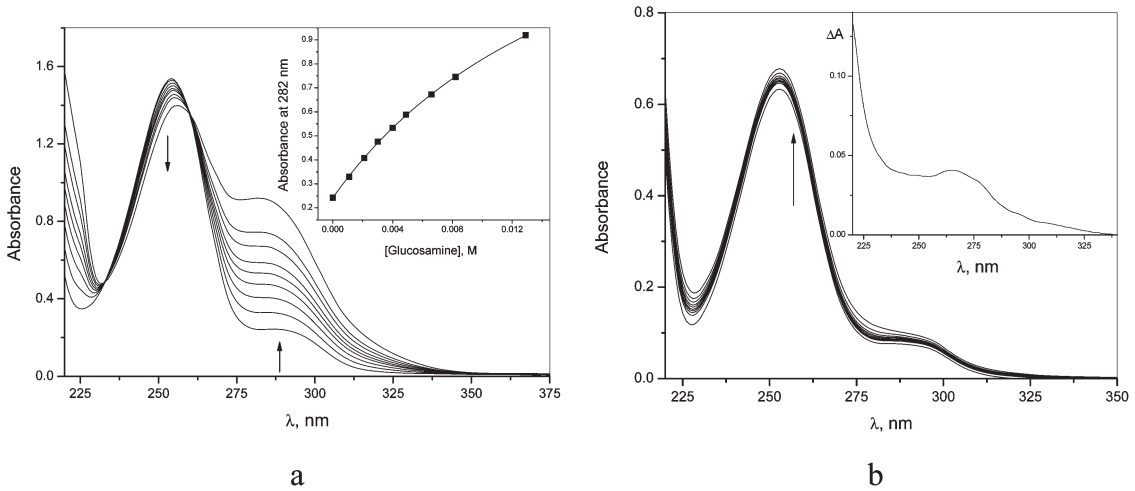


Fig. 3 Spectrophotometric titrations of 0.25 mM 2-FPBA (a) and 0.046 mM 3-FPBA (b) by D-glucosamine (0–13 mM) at pH 7.0. Inset in (a) shows the fitting of the titration plot at 282 nm to the 1 : 1 binding isotherm; inset in (b) shows the differential spectrum at maximum concentration of D-glucosamine.

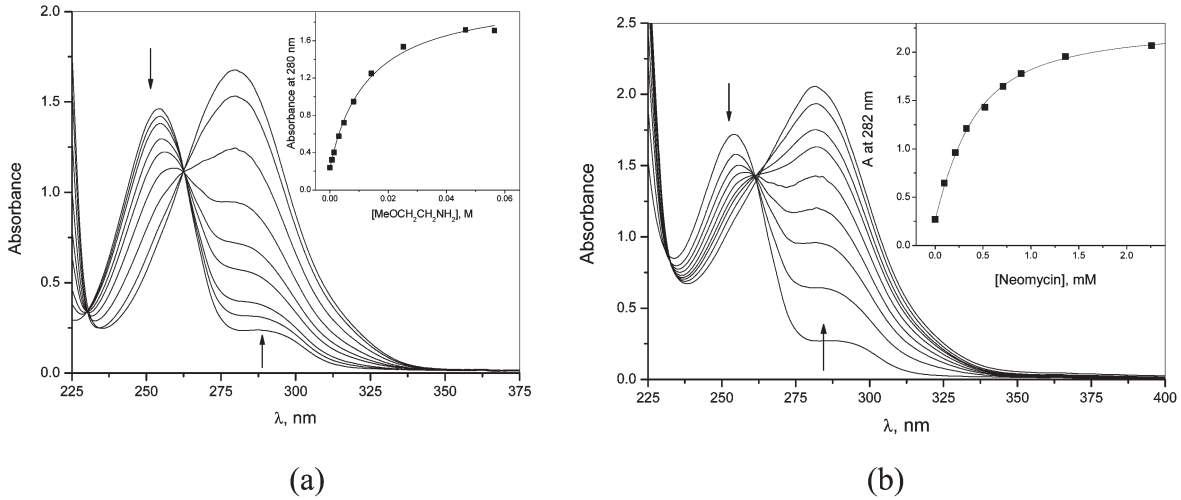


Fig. 4 Spectrophotometric titrations of 0.25 mM 2-FPBA by $MeOCH_2CH_2NH_2$ (a) and neomycin B (b) at pH 7.0. Insets show the fittings of titration plots at selected wavelengths.

The observed spectral changes can be attributed to imine formation, which proceeds much more efficiently with 2-FPBA than with 3- and 4-isomers. Further evidences supporting imine formation come from NMR studies. Fig. 5a shows the ^{1}H NMR spectra of 2-FPBA recorded at increasing amounts of added D-glucosamine. The signal of aldehyde proton at 9.96 ppm gradually disappears and is substituted by two new growing signals at 8.78 and 8.58 ppm. Such up-field shift is typically observed on transformation of the aldehyde group into an imine.[18] Like with other aldehydes[18] imine formation with 2-FBPA is a slow process in the NMR time scale. The existence of two signals of imine C–H proton was observed previously for the Schiff base of D-glucosamine and salicylaldehyde and was attributed to the anomeric equilibrium.[19] The upper spectrum in Fig. 5a corresponds to the Schiff base of 2-FPBA with $MeOCH_2CH_2NH_2$ obtained in the presence of a high excess of the amine and it contains only one signal of the imine proton at 8.6 ppm. The low field portions of ^{1}H spectra of 2-FPBA in the presence of an excess of kanamycin A at variable pH are shown in Fig. 5b. Under these conditions the signal of aldehyde proton disappears completely because of complete transformation of 2-FPBA into the Schiff base. The presence of more than one signal of the imine proton indicates in this case interactions with different amino groups of kanamycin (see below). Spectral changes on titrations of 3- and 4-FPBA by D-glucosamine, $MeOCH_2CH_2NH_2$ and kanamycin A were of the same type, but with imine peak appearing at somewhat higher field at 8.4 ppm for both isomers and detectable only at much higher concentrations of amines (Fig. 2S in ESI† shows as an example titration data for 3- and 4-FPBA with $MeOCH_2CH_2NH_2$).

The interaction of 2-FPBA with aminoalcohols in non-aqueous medium proceeds somewhat differently with formation of binuclear cyclic products where the amino group of amino-alcohol forms the Schiff base with the aldehyde group of one

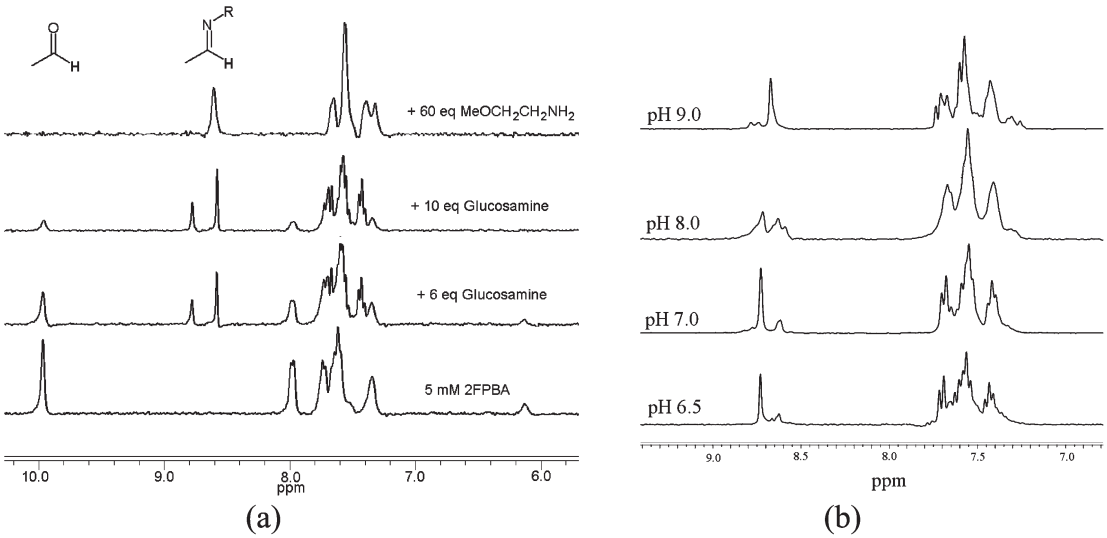


Fig. 5 ^{1}H NMR spectra of 5 mM 2-FPBA in D_2O: (a) at pH 7.0 at increasing amounts of added D-glucosamine and in the presence of saturating concentration of $MeOCH_2CH_2NH_2$ (upper spectrum); (b) at different pH in the presence of 2–3 equivalents of kanamycin A.

Table 2 Observed imine formation constants (K_{obs}, M^{-1}, relative error less then ±10%) of amines, amino sugars and aminoglycosides with isomeric formyl phenylboronic acids at pH 7.0 (0.05 M MOPS)

	2-FPBA	3-FPBA	4-FPBA
n-$BuNH_2$	27		
$MeOCH_2CH_2NH_2$	81	0.21[a]	0.66[a]
Gly	16.1		
GlyGly	130[b]	0.8[a,b]	
D-Glucosamine	83	3.7[a]	4.0[a]
D-Galactosamine	140		
D-Mannosamine	8.3		
Kanamycin A	2790	170[a]	43[a]
Kanamycin B	1350		
Amikacin	1900		
Geneticin	850		
Gentamicin	1340		
Neomycin B	4900		
Spermine	1420		
Spermidine	1500		

[a] 1H NMR titration. [b] pH 7.5.

2-FPBA molecule and the hydroxyl group of aminoalcohol forms the ester bond with $B(OH)_2$ group of the other 2-FPBA molecule.[20] Formation of simple 1 : 1 Schiff bases in water may be attributed to reduced stability of boronic acid esters in aqueous solutions.

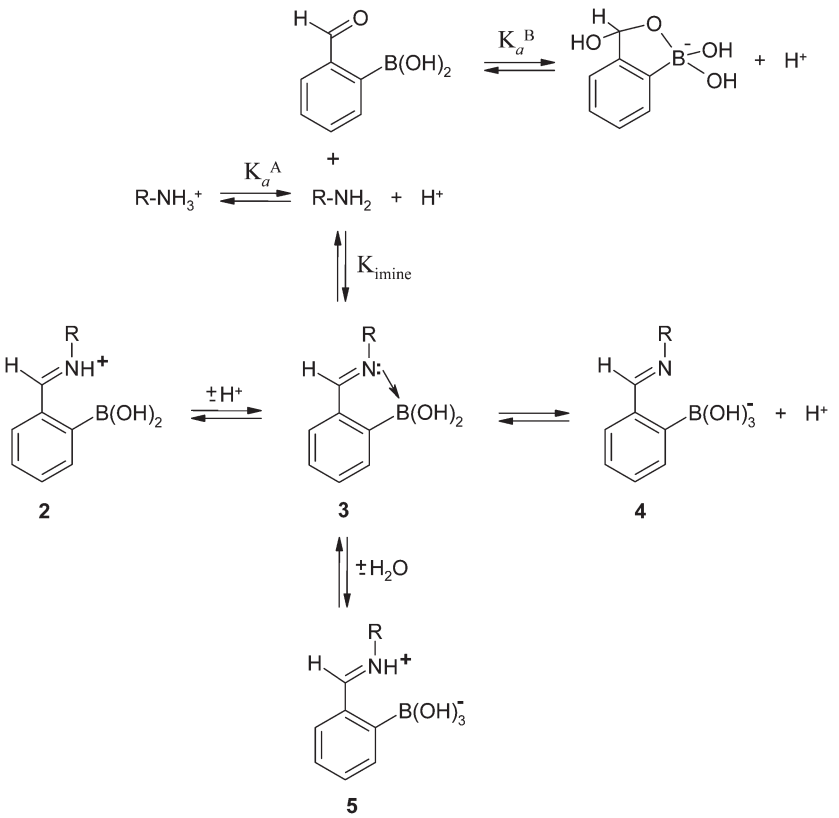
Quantitatively the stability of imines was characterized by the respective formation constant. The titration profiles for 2-FPBA, examples of which are shown in insets in Fig. 3a and 4a,b, followed very well the simple 1 : 1 binding isotherm. The equilibrium constants calculated from the fitting of these results at pH 7 are given in Table 2. The equilibrium constants for 3- and 4-FPBA cannot be calculated from similar data because of too small spectral changes. They were determined by ^{1}H NMR titrations for several systems and also are shown in Table 2. The stabilizing effect of the *ortho*-boronate group is evident from much weaker interactions of amines with 3- and 4-isomers of FPBA.

In the course of this study we noticed that the phosphate buffer solution exerted strong inhibitory effect on interaction of 2-FPBA with aminoglycosides apparently caused by rather strong association of phosphate anions with aminoglycoside polycations.[21] For this reason all further measurements were performed in 0.05 M MES, MOPS or CHES buffers.

In order to evaluate possible selectivity of imine formation with 2-FPBA the equilibrium constants were determined for a wide range of mono- and polyamines of different structures under similar conditions at pH 7 in 0.05 M MOPS buffer, Table 2. As follows from Tables 1 and 2 the affinity of 2-FPBA to aminoglycosides, particularly to kanamycin A and neomycin B, surpasses that to sugars, amino sugars and monoamines by more than one order of magnitude. Also high imine formation constants were obtained for biological polyamines, spermine and spermidine. To obtain a deeper insight into this effect more detailed studies were performed with $MeOCH_2CH_2NH_2$, D-glucosamine, kanamycin A and spermine.

The general scheme of acid–base and condensation equilibria involved in imine formation with 2-FPBA is shown in Scheme 2. Contributions of cationic form **2** and anionic form **4** should be small because they are lacking the stabilizing intramolecular interactions (see below). It is worth noting that in recently reported crystal structure of the Schiff base of 2-FPBA with aniline the expected N → B dative bond shown in **3** is absent.[22] Instead the imine is stabilized by an intramolecular BOH···N hydrogen bond. The zwitterionic (hydrated) form **5** was found to be the predominant form of boronate esters containing the *ortho*-methylamino group in protic solvents.[23,24] Recent high level computational investigation of *o*-(*N*,*N*-dialkylaminomethyl)arylboronate systems demonstrated that hydration is favorable both for esters and for free boronic acids.[25] Since imines are weaker bases than amines, a significant contribution of this form seems less probable here, but as it will be shown below this is the predominant form for imines too.

In accordance with Scheme 2 and ignoring forms **2** and **4** one obtains for the observed equilibrium constant of imine formation



Scheme 2 General scheme of acid–base and condensation equilibria involved in imine formation with 2-FPBA.

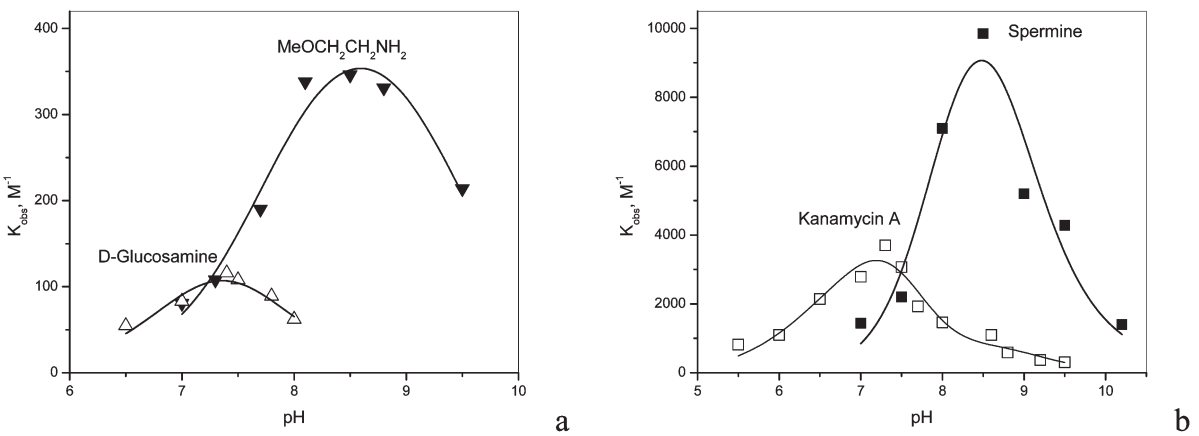


Fig. 6 Observed imine formation constants of 2-FPBA with (a) D-glucosamine and $MeOCH_2CH_2NH_2$; (b) kanamycin A and spermine at variable pH. The solid lines are the theoretical fits to eqn (2) or (3).

(K_{obs}) the expression (2), which predicts the bell-shaped K_{obs} *vs.* pH profile with the maximum at pH = ($pK_a^A + pK_a^B$)/2.

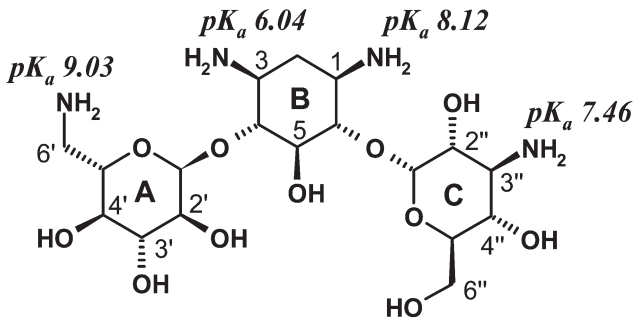
$$K_{obs} = \frac{K_{imine}}{(1 + [H^+]/K_a^A)(1 + K_a^B/[H^+])} \quad (2)$$

The values of K_{obs} for different compounds at variable pH are collected in Table 1S (ESI†) and the pH profiles for interaction of 2-FPBA with D-glucosamine, $MeOCH_2CH_2NH_2$, spermine and kanamycin A are shown in Fig. 6a and b. The fitting of profiles for D-glucosamine and $MeOCH_2CH_2NH_2$ to the eqn (2) shown as solid lines in Fig. 6a were satisfactorily good and allowed us to calculate the imine formation constants K_{imine} given in Table 3. Additional K_{imine} values also shown in Table 3 were obtained for several other mono amines, which cover a wide range of basicity of amino groups (*n*-butylamine, glycine, glycilglycine, 2,2,2-trifluoroethylamine) by using the eqn (2) from data collected in Table 1S†.

The analysis of results with polyamines is more complex. Kanamycin A has four non-equivalent primary amino groups with pK_a values indicated on Scheme 3.[26] The observed optimum in affinity at pH about 7.3, which coincides with pK_a

Table 3 Imine formation constants for 2-FPBA and different amines

Compound	pK_a	K_{imine}, M^{-1}
n-$BuNH_2$	10.77	6.0×10^5
Gly	9.8	1.4×10^4
$MeOCH_2CH_2NH_2$	9.4	5.1×10^4
GlyGly	8.3	2.9×10^3
D-glucosamine	7.4	510
$CF_3CH_2NH_2$	5.6	29.5
Kanamycin A	9.03	6.3×10^4
	7.46	1.4×10^4
	6.04	2.1×10^3
Spermine	10.8	9.8×10^5
	7.96	4.3×10^4



Scheme 3 Numbering of hydroxyl and amino groups in kanamycin A with respective pK_a values at 25 °C and ionic strength 0.05 M.

of 2-FPBA, qualitatively indicates in accordance with eqn (2) the principal contribution of an amino group with matching pK_a value, that is the 3″ amino group in the ring C with pK_a 7.45. The quantitative analysis of the pH-profile for kanamycin A can be performed with eqn (3) derived assuming independent imine formation of each amino group with the neutral form of 2-FPBA.

In eqn (3) K_{a1}, K_{a2}, *etc.* are the acid dissociation constants of ammonium groups of kanamycin A in decreasing order, that is from less to most acidic group and K_{im1}, K_{im2} *etc.* are the equilibrium constants of imine formation with neutral amino groups in the same order.

Also the least basic 3-NH_2 group (ring B) still has the K_{imine} value much larger than glucosamine. The calculated species distribution diagram (Fig. 3S, ESI†) shows that below pH 7 the predominant species is the imine formed with 3-NH_2 group, between pH 7 and 9 – the imine formed with 3″-NH_2 group and at pH 9 and higher – the imine formed with 6′-NH_2 group. The shift from the most intense signal at 8.74 ppm at pH 6.5 to that at 8.68 ppm at pH 9.0 in Fig. 5b most probably reflects this shift in the species distribution as a function of pH.

Interpretation of the results with spermine is more complicated because it contains both primary and secondary amino groups. Formally there are also 4 protonation states of the polyamine each with its own affinity to 2-FPBA and therefore the eqn (3) still can be applied for the fitting. Indeed, the fitting illustrated with the solid line in Fig. 6b is acceptably good. Shifted to a higher value pH-optimum for spermine as compared to kanamycin A reflects larger basicity of the former (first pK_a 7.96 of spermine *vs.* first pK_a 6.05 of kanamycin A). However, one cannot attribute the individual K_{imine} values calculated from the fitting procedure to amino groups with corresponding pK_a values in this case. In contrast to kanamycin A, which possesses four distant and practically independent primary amino groups, spermine has two pairs of symmetrically positioned primary and secondary amino groups with a complex distribution of protonated and free amino groups within each macroscopic protonation state.[27] The only certain attribution is for the first K_{imine} in the eqn (3) which corresponds to the completely deprotonated form of spermine. One also may estimate the last K_{imine} corresponding to the interaction with triprotonated spermine from results at pH below 8 where this form is the only one deprotonated form of the polyamine, which is present in a significant amount. The respective parameters are given in Table 3 and as one can see the stability of imine formed with the neutral polyamine is practically the same as that of imine formed with a monoamine of similar basicity *n*-$BuNH_2$, but the triprotonated polyamine forms much more stable imine than expected from its basicity. We conclude therefore that in both cases (with kanamycin A and spermine) the strongly enhanced stability of imines formed with 2-FPBA is observed when the polyamines are partly protonated.

To interpret this effect one needs to know the actual structure of the imine, which may be either a neutral species **3** with intra-

$$K_{obs} = \frac{K_{im1} + K_{im2}[H^+]/K_{a1} + K_{im3}[H^+]^2/K_{a1}K_{a2} + K_{im4}[H^+]^3/K_{a1}K_{a2}K_{a3}}{(1 + K_a^B/[H^+])\ (1 + [H^+]/K_{a1} + [H^+]^2/K_{a1}K_{a2} + [H^+]^3/K_{a1}K_{a2}K_{a3} + [H^+]^4/K_{a1}K_{a2}K_{a3}K_{a4})} \quad (3)$$

The solid line in Fig. 6b shows the best fit to the eqn (3) and the respective imine formation constants for each amino group are given in Table 3. From these results one can see that the most basic amino group of kanamycin A (6′-NH_2, ring A) actually forms the most stable imine with K_{imine} close to that for $MeOCH_2CH_2NH_2$, a simple monoamine of similar basicity. This interaction contributes however very little to K_{obs} at pH 7 where the 6′ amino group is completely protonated. The contribution of second in order of decreasing basicity amino group 1-NH_2 (ring B) is surprisingly small and the respective K_{imine} can not be estimated reliably from the fitting. On the other hand K_{imine} for interaction with 3″-NH_2 (ring C) is very large as compared with K_{imine} for D-glucosamine possessing similar basicity (Table 3). molecular coordination bond stabilizing the imine formation or the respective zwitterion **5** with the protonated imine bond and tetrahedral anionic boronate, which has an increased stability due to formation of an intramolecular ionic hydrogen bond (see above). The zwitterion is produced by addition of a water molecule to **3** and therefore cannot be discriminated with **3** in aqueous solution simply from titration results. Similar ambiguity exists also in the case of boronate esters containing an *ortho*-methylamino group and it was demonstrated that conclusive evidence regarding their structure can be obtained analyzing the ^{11}B NMR spectra.[23] In the neutral structure of the type **3** the ^{11}B signal should be observed around 14 ppm, but in the zwitterionic structure of the type **5** it appears at the same position as in the

boronate anion below 10 ppm. Following the interaction of 2-FPBA with amines by ^{11}B NMR we observed a strong broadening of the spectrum in the presence of added amine, nevertheless it was possible to see clearly that imine formation leads to disappearance of the signal at 29.3 ppm, which belongs to the neutral boronic acid and to appearance of a single signal at 8.6 ppm, which belongs to the boronate anion $-B(OH)_3^-$, as illustrated in Fig. 7a and b with titration of 2-FPBA by D-glucosamine and kanamycin A.

To further prove the formation of protonated imine in the case of 2-FPBA, the UV-vis spectra of the products were analyzed. As follows from results shown in Fig. 3a–c the imine absorption band appears at longer wavelength in the case of 2-FPBA (maximum at 280 nm) than with other two isomers (shoulder at 265 nm). A red shift is typically observed on protonation of Schiff bases[28] and therefore this observation may reflect the protonation of the imine bond in the Schiff base with 2-FPBA. To observe the effect more clearly the Schiff base of 2-FPBA and *n*-$BuNH_2$ was prepared in two organic solvents: protic ethanol and aprotic acetonitrile. In both solvents the equilibrium was completely shifted to the Schiff base, which was quantitatively generated by mixing the equimolar amounts of reactants. As one can see from Fig. 8 the spectrum in ethanol is essentially the same as in water with the maximum at 282 nm, but in aprotic acetonitrile the maximum is observed at 254 nm with weakly absorbing shoulders at 290–300 nm. Obviously, in ethanol the addition of EtOH molecule can produce the zwitterionic structure similar to **5** containing a protonated imine bond and a $-B(OH)_2(OEt)^-$ anionic group, but this cannot happen in aprotic acetonitrile where the Schiff base should have the structure **3**. However, when 1 equivalent of strong methanesulfonic acid was added to the acetonitrile solution the maximum at 282 nm appeared also in this solvent confirming that this band indeed belongs to the protonated Schiff base.

The stabilizing effect of the internal ion pairing in the structure **5** is reminiscent of well known intramolecular imine stabilization by *ortho*-OH group of salicylaldehyde, operating also in pyridoxal Schiff bases (Scheme 4). Although the details of the nature of the stabilizing effect in the zwitterionic structure **6**, such as the real contribution of resonance stabilization and the degree of hydrogen bonding inside the ionic pair, are still a matter of discussion, the fact of predominant formation of this structure is well established.[29]

Interestingly, the stability of Schiff bases with 2-FPBA is even higher than that with salicylaldehyde. Thus, reported K_{imine} values for Schiff bases of salicylaldehyde with *n*-$BuNH_2$, $MeOCH_2CH_2NH_2$ and Gly are 5.5×10^4, 6.0×10^3 and 3.6×10^3 M^{-1} respectively,[18,30] which are one order of magnitude smaller than those for 2-FPBA (see Table 3).

Other relevant systems are complexes of salicylaldehyde imines with boric or boronic acids studied as intermediates in their catalytic hydrolysis.[30,31] Both complexes of type **7**, similar to **3**, and of type **8**, similar to **5**, were proposed on basis of general considerations. One may notice, however, that the reported UV-vis spectrum of the complex of salicylaldehyde 2-methoxyethylamine Schiff base and boric acid has the absorption band at 345 nm, typical of the protonated imine, while the neutral imine has the absorption band at 305 nm.[30]

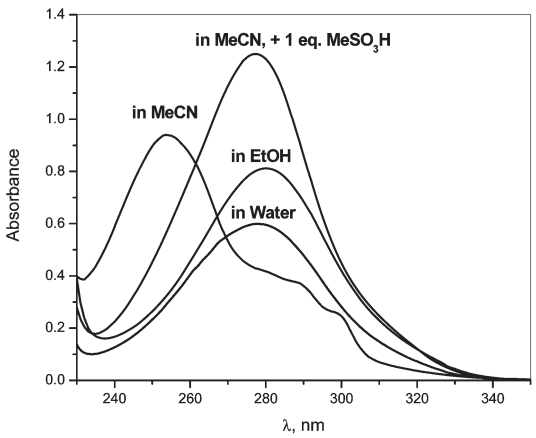


Fig. 8 UV-vis absorption spectra of 0.1 mM Schiff bases prepared from 2-FPBA and *n*-$BuNH_2$ in ethanol, acetonitrile and water (at saturation concentration of *n*-$BuNH_2$ (0.1 M) at pH 7) as well as the spectrum in acetonitrile after addition of 1 equivalent of $MeSO_3H$.

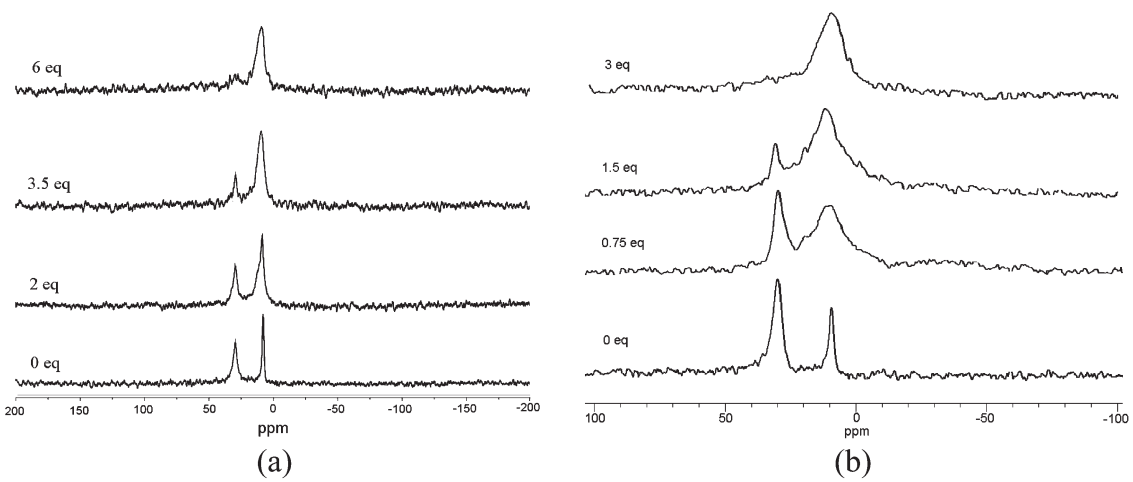
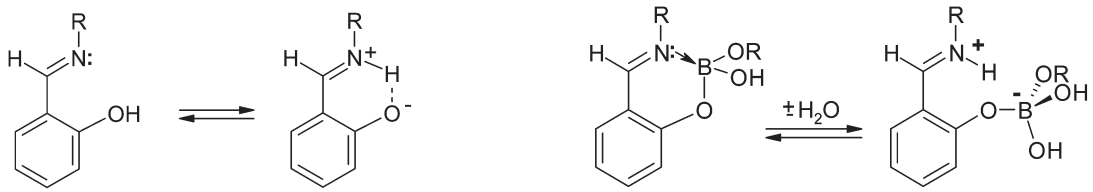


Fig. 7 The ^{11}B NMR spectra of 5 mM 2-FPBA recorded at increased amounts of added D-glucosamine at pH 7.5 (a) or kanamycin A at pH 7.0 (b) D_2O. Amounts of added amines are shown as number of equivalents on the spectra.



Scheme 4 A salicylaldehyde imine and its complex with boric or boronic acid.

The studies at variable pH like those shown in Fig. 6 demonstrate that imines with 2-FPBA remain protonated at pH above pK_a of a parent amine. The protonated forms of Schiff bases of benzaldehyde typically have pK_a values by *ca.* 3 units lower than the protonated forms of parent amines.[28a,32] Therefore the presence of an anionic boronate group must at least compensate this effect. The phenolate group in the *ortho*-position of salicylaldehyde increases the pK_a of a protonated imine group in structure **6** by 6 units,[29] but a large part of this effect comes from the resonance stabilization of the iminium cation, which is impossible with a $B(OH)_3^-$ group. However, the inductive effect of $B(OH)_3^-$ is larger than that of O^- ($F = -0.42$ and -0.26 respectively)[33] and together with electrostatic stabilization this may be sufficient to provide the required shift in pK_a.

The whole set of data for imine formation constants can be discussed in terms of the Brönsted type correlation between log K_{imine} and pK_a of protonated amines. Such correlation with a slope of 0.64 was reported previously for aldehydes of different structures.[18] The results in Table 3 are plotted in Brönsted coordinates in Fig. 9. Data for simple monoamines (black squares) follow a reasonably good linear dependence with a slope of 0.8 ± 0.1. The point for D-glucosamine fits to this line indicating the absence of any additional interaction of 2-FPBA with hydroxyl groups of amino sugar. The point for the most basic 6′-NH_2 group of kanamycin A is also close to this line indicating that this amino group behaves as a "normal" amine of the corresponding basicity, but 3-NH_2 and 3″-NH_2 groups have anomalously high affinities to 2-FPBA manifested in large positive deviations of the corresponding points from values expected for amino groups of such low basicity. Similarly the point for the neutral form of spermine (pK_a 10.8) is on the line for monoamines, but the point for spermine trication (pK_a 7.96) shows a large positive deviation. The open squares show for comparison results for 3- and 4-FPBA.

In case of spermine, which does not have hydroxyl groups, the positive deviation for the protonated form may be attributed to stabilization of the anionic boronate group of the zwitterionic imine by interactions with additional positive charges in the protonated polycation. The conformation of fully protonated spermine is practically all-*trans*, which makes impossible contacts of boronate anion even with the next ammonium group in the chain. However, it has been shown by NMR studies of specifically ^{2}H-labeled spermines that contact with an anion induces strong increase in fraction of *gauche* rotamers around C2–C3, C3–C4 and C6–C7 bonds so that the polycation can essentially wrap the anionic species.[34] To confirm this possibility we performed a M06-HF/6-31G(**)[35] level quantum mechanical

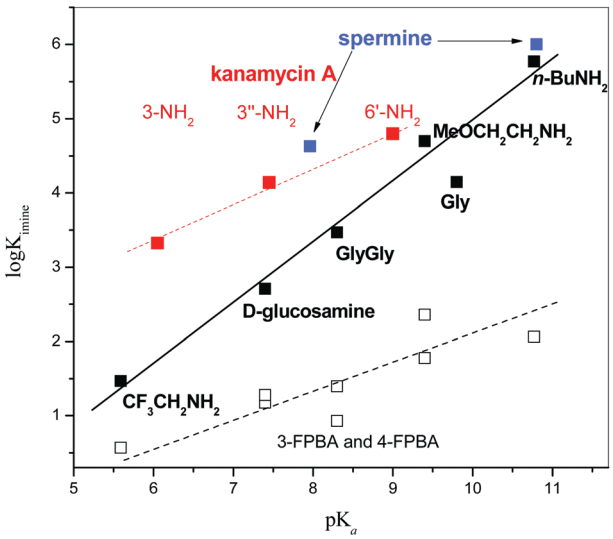


Fig. 9 The Brönsted plot for imine formation constants for 2-FPBA (solid squares) and 3- and 4-FPBA (open squares).

calculation taking into account solvation in water of the structure of imine formed with the terminal amino group of the triply protonated spermine, Fig. 10. In the minimized structure one finds exactly three expected C–C bonds in *gauche* conformation, which allows formation of three short ionic NH···O hydrogen bonds with angles and N–O distances in the range 162.3–177° and 2.52–2.75 Å respectively, in good agreement with respective parameters reported for crystal structures of arylboronate esters containing a protonated amino group in the *ortho* position.[23,36] Also the calculated B–N distances ranging from 3.0 to 3.9 Å are in agreement with reported for these structures values. Thus the increased stability of Schiff bases with partially protonated forms of spermine can be satisfactorily explained by multiple hydrogen bonding provided by ammonium groups in addition to that provided by the protonated imine group.

In case of kanamycin A similar explanation seems also possible. Indeed, the most basic 6′-NH_2 group becomes neutral and starts to form the Schiff base with 2-FPBA when all other amino groups of kanamycin A are already neutral and cannot provide any additional stabilizing interaction, but imine formation with less basic 3-NH_2 and 3″-NH_2 groups occurs when other amino groups are still protonated and a suitably positioned ammonium group can form an additional ion pair with the boronate anion. Similar effect can be observed to a larger or smaller extent for

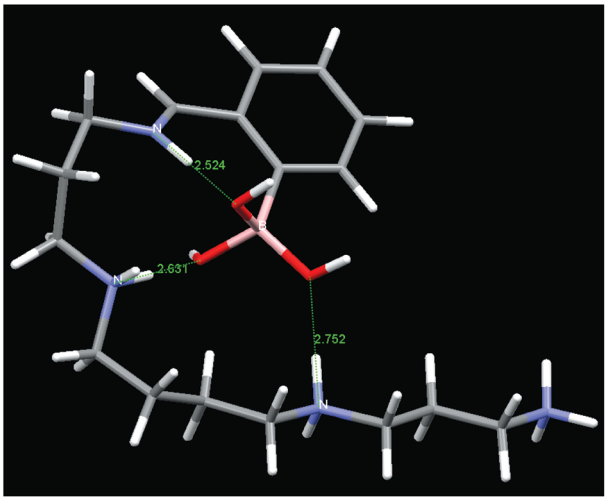


Fig. 10 Simulated M06-HF/6-31G(**) structure of the Schiff base formed between 2-FPBA and triprotonated form of spermine.

other aminoglycosides. However, due to the presence of hydroxyl groups in aminoglycosides one cannot exclude additional stabilization of the Schiff base through formation of boronate ester bonds.

In general, hydroxyl groups of aminoglycosides are poorly positioned for boronate ester formation. Thus, in kanamycin A three hydroxyl groups in the ring A are all in synclinal conformation, while boronate ester formation needs hydroxyls to be synperiplanar;[37] 4″,6″-diol structure in ring C generally provides rather weak binding,[10,37] and kanamycin lacks a free anomeric hydroxyl, which is the principal site of boronate ester formation with carbohydrates, but is involved here in glycoside bond formation. In line with these features we did not observe any interaction of kanamycin A with phenylboronic acid even at high 0.01 M concentration and pH up to 10. The ester formation can be manifested in ^{11}B NMR spectra because the signals of free $-B(OH)_3^-$ group and also tetrahedral anionic, but ester group should be at least slightly different. However strong broadening of the spectra in the presence of kanamycin does not allow detecting this difference. To get a better insight in the mode of interaction of 2-FPBA with kanamycin including possible formation of an iminoboronate ester we performed a M06-HF/6-31G(**) level quantum mechanical simulation of the structures of imines formed with 3-NH_2 and 3″-NH_2 groups of the antibiotic. The simulated structures are shown in Fig. 11a and b.

In the imine formed with 3-NH_2 group (Fig. 11a) the anionic $B(OH)_3^-$ group is stabilized by hydrogen bonding to the 6′ ammonium group in addition to hydrogen bonding to the iminium group and is positioned very far from all hydroxyls. The bonds are shorter than those found in the structure of the Schiff base with spermine with N–O distances 2.47 and 2.60 Å and N–H–O angles 167.5 and 172.4° (imine and ammonium groups respectively), which probably occurs because of larger rigidity of the aminoglycoside skeleton. The boronate group can hardly reach even a single OH group of the antibiotic and increased stability in this case should be attributed to electrostatic/hydrogen bonding stabilization by ammonium groups.

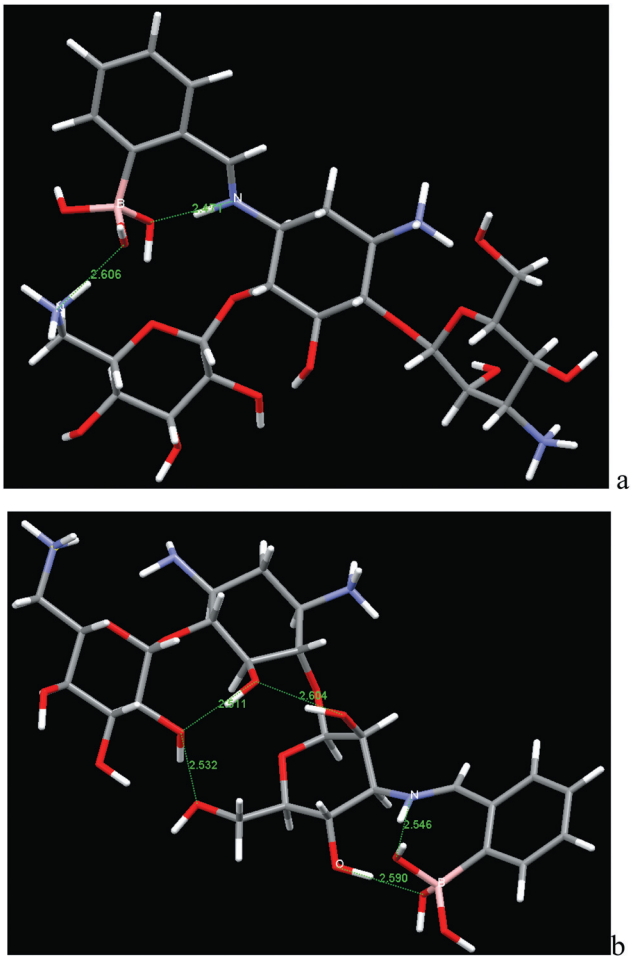


Fig. 11 Simulated M06-HF/6-31G(**) structures of Schiff bases formed by 2-FPBA with 3-NH_2 (a) and 3″-NH_2 (b) groups of kanamycin A.

Such stabilization is less probable for the imine formed with a 3″-NH_2 group in ring C because this group is positioned far from other amino groups as is evident from examination of the minimized structure in Fig. 11b. An attempt to approach the anionic boronate group to the 1-NH_3^+ group in ring B by rotation of the imine fragment around the 3″C–N single bond produced a structure of higher energy. The anionic boronate group is stabilized by hydrogen bonding to the 4″ hydroxyl of ring C, however similar interaction is observed in the minimized structure of the imine of D-glucosamine (See Fig. 4S in ESI†) and it does not provide any increase in the Schiff base stability in this case. This closely positioned hydroxyl group may be involved in formation of an ester boronate bond instead of hydrogen bonding. The respective minimized structure is shown in Fig. 12. The transformation of the hydrogen bonded structure in Fig. 11b into iminoboronate ester in Fig. 12 involves the elimination of a water molecule. The calculated energy change for this reaction is +13.55 kcal mol^{-1} indicating unfavorable formation of the iminoboronate ester because of induced strain and loss of hydrogen bonding to the iminium NH^+ group, which becomes turned outside the boronate group.

The calculated energies of reactions of 2-FPBA with 3″-NH_2 group of kanamycin A and with D-glucosamine in aqueous

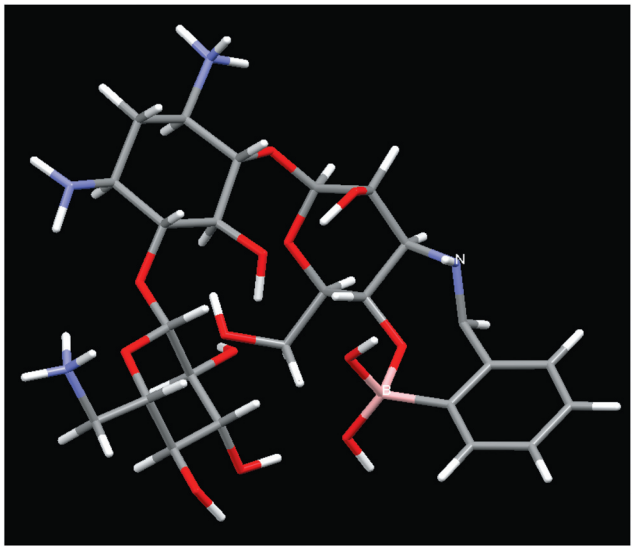


Fig. 12 Simulated M06-HF/6-31G(**) structure of iminoboronate ester formed by elimination of water molecule from boronate OH and 4″-OH of kanamycin A.

environment are −41.0435 kcal mol^{-1} and −33.4045 kcal mol^{-1} respectively, which means that the Schiff base with kanamycin indeed is significantly more exothermic. The origin of its higher stability is not immediately clear, however. One possible explanation is that imine formation induces a noticeable change in the aminoglycoside conformation moving ring C closer to ring A and making the whole molecule more compact. This allows formation of an array of three intramolecular hydrogen bonds between four OH groups (2″OH-5OH-2′OH-6″OH) of the aminoglycoside, which may have increased stability due to the so-called "cooperative effect".[38] Thus we come to the conclusion that increased stability of Schiff bases between 2-FPBA and partly protonated forms of kanamycin A is most probably the result of additional hydrogen bonding of the anionic boronate group to suitably positioned ammonium groups of the antibiotic or to conformational changes rather than to boronate ester formation.

Considering again the results at pH 7 (Table 2) one can notice that observed imine formation constants for all monoamines are below or close to 10^2 M^{-1}, but for all polyamines they are above or close to 10^3 M^{-1}. The rather narrow range of variation in K_{obs} within each group may be explained as follows. The principal factor increasing the imine stability is an increase in amine basicity in accordance with the correlation shown in Fig. 9. However, the fraction of free amine at pH 7 will be smaller for more basic amines. Since the slope of the Brönsted correlation is close to unity these effects nearly compensate each other. The difference in one order of magnitude between formation constants for polyamines and monoamines attributed to a stabilizing effect of an additional ion pair between ammonium and $B(OH)_3^-$ groups precisely corresponds to the average free energy increment −6.5 kJ mol^{-1} of an ion pairing interaction in supramolecular complexes.[39]

Finally, owing to formation of protonated strongly absorbing imines the spectral response to the interaction of aminoglycosides with 2-FPBA is fairly large and allows one detection of as little as 10 μM of kanamycin A or neomycin B in the presence of equivalent amounts of sugars, amino sugars or amino acids (See Fig. 5S in ESI†). Further improvement of analytical procedure is possible by using a variant of an indicator displacement assay. In preliminary tests we found that chromotropic acid can serve as a suitable fluorescence indicator for detection of kanamycin A in neutral solutions.

Experimental

Materials

2-Formyl phenylboronic acid (Aldrich) was purified by crystallization from 10% aqueous methanol. Other reagents and components of buffer solutions MES, MOPS and CHES all from Aldrich, were used as supplied. Buffer solutions were prepared by adjusting the pH of 0.05 M free acids with concentrated NaOH to desired values.

Spectrophotometric titrations

Spectrophotometric titrations were performed on a Hewlett-Packard 8453 or Evolution diode array spectrophotometers equipped with a thermostated cell compartment at 25 ± 0.1 °C. Typically to a 0.05–0.3 mM solution of an isomeric formyl phenylboronic acid in an appropriate buffer portion of concentrated solution of the second component (sugar, amino sugar, amine, *etc.*) in the same buffer were added and the mixture was incubated for 5 min after each addition before recording the spectrum. In an independent experiment it was established that the system equilibrates completely during this incubation period. The observed equilibrium constant of the Schiff base formation (K_{obs}) was calculated from the absorbance (A) *vs.* concentration of the second component (X) profiles at several wavelengths in the interval 260–310 nm by non-linear least-squares fitting to eqn (4) and the results were averaged. In eqn (4) subscript T stands for total concentration, A_0 is the initial absorbance of a formyl phenylboronic acid (R) measured in the absence of X, $\Delta\varepsilon$ is the difference in molar absorptivities between the Schiff base and free R.

$$A = A_0 + 0.5\Delta\varepsilon\{[R]_T + [X]_T + 1/K_{obs} - (([R]_T + [X]_T + 1/K_{obs})^2 - 4[R]_T[X]_T)^{0.5}\} \quad (4)$$

The observed equilibrium constants of the formation of boronic acid–sugar complexes were calculated from similar absorbance *vs.* sugar concentration plots, but by using of a more simple eqn (5) valid under conditions of a large excess of X over R. In the eqn (5) A_C is the absorbance of the complex and other symbols have the same meaning as in the eqn (4).

$$A = (A_0 + A_C K_{obs}[X]_T)/(1 + K_{obs}[X]_T) \quad (5)$$

NMR spectroscopy

^{1}H NMR spectra were recorded on a Varian Gemini 300 NMR spectrometer and ^{11}B NMR spectra were recorded on a JEOL GXS-270 spectrometer in D_2O. Solution pH was adjusted by

additions of NaOD to MOPS free acid. The observed equilibrium constants of Schiff base formation were calculated from integral intensities of signals corresponding to C**H**(=O) and C**H**(=N) protons.

Calculation method

Electronic structure calculations were performed with the Jaguar quantum chemistry software.[40] DFT calculations were carried out using Zhao and Truhlar functional with full HF exchange and M06 local functionals that eliminates long-range self-interaction (M06-HF).[35] The geometries of all compounds were optimized using the standard 6-31G(**) basis set. Solvation in water was accounted for by applying the continuum-solvation approach by numerically solving the Poisson-Boltzmann equation.[41] Reported crystal structures of kanamycin A[42] and D-glucosamine[43] were used as starting structures in calculations.

Acknowledgements

The financial support of CONACyT (project 101699) is gratefully acknowledged. N. J. Gutiérrez-Moreno thanks CONACyT for a Ph.D. Fellowship. We thank M.Sc. M. E. Ochoa and Dr R. Santillán at CINVESTAV-IPN for their help with recording of ^{11}B NMR spectra.

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