New ligands for boronate affinity chromatography

Synthesis and properties[a]

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ABSTRACT

In order for a boronate ligand to be useful in affinity chromatography for the purification of biomolecules, it must be able to form a stable complex in an environment (pH) in which the affinity molecule is stable. A major limitation of the widely used ligand, 3-aminophenylboronate, is its high ionization constant (pK_a 8.75). To make this complex under more favorable pH conditions, different methods have been explored here in order to introduce an electron-withdrawing (nitro) group in the phenyl ring. Reagents and procedures for the preparation of *ortho-*, *meta-* and *para-*nitro derivatives of succinamidophenylboronic acid using nitronium trifluoromethanesulfonate are described. Preferential substitution of the nitro functionality into the *ortho* position of the boronic acid is exploited by selective use of acetic anhydride for the reaction medium. This method yields mostly an *ortho-*nitro derivative (pK_a 7.4) under selected reaction conditions. The ionization and solute–ligand interaction of several phenylboronates are studied in solution by using ^{11}B NMR and spectrophotometric methods. The results indicate the presence of specific chemical shifts for the neutral (δ 30), the boronate anions (δ 3), and the *cis-*diol-complexed boronate species (δ 7.5). In the presence of a *cis-*diol derivative, the complex formation is favored over anionization of neutral species. Moreover, the complex is formed approximately one pH unit below the ionization constant of the ligand and is stable, *i.e.* fails to break down in boronate anion, even when the solution pH is raised appreciably. Two boronate affinity column matrices were examined for their binding capacity and apparent dissociation constant. The results clearly indicate that the formation and also the breakdown of the complex are greatly enhanced because of the presence of the electron-withdrawing group in the boronate ligand. The results further demonstrate that small structural differences in affinity molecules have significant differences on their binding capacities. A comparison of binding between alkyl-*cis-*diols and aryl-*cis-*diols to different boronate matrices indicates that the aryl affinity molecules not only form a complex but do so very effectively. The *significance* of this work lies in the demonstration that the best environment for the ligand–solute interaction can be established by carrying out studies in solution, without prior immobilization of the ligand. The results derived from in-solution studies and those from the affinity columns are in very good agreement. The new nitrophenylboronate matrix offers enhanced binding of most affinity molecules over those examined with the phenylboronate matrix. In addition, the new matrix offers chromatographic separations of alkali-unstable biomolecules.

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INTRODUCTION

Boronate matrices have been employed successfully for the separation of a wide variety of biomolecules. A few examples of their applications in recent years include: the separation of ribonucleosides, nucleotides, and oligonucleotides from their deoxy derivatives in different situations [1–10]; the separation and assay of modified nucleosides from common nucleosides [11]; nucleotides from 3',5'-cyclic AMP (cAMP) [12]; the assay of benzo[*a*]pyrene-DNA adducts in cells [13]; the isolation of the nucleotidyl-peptides [14]; the assay of catechols [15]; catechol estrogens and other hormones [16]; the separation of different sugars [17]; the isolation of a specific tRNA [aminoacyl–tRNA (AA-tRNA)] from 19 other tRNAs [11,18–20]; the separation of capped from uncapped mRNA [21]; the separation of ADP-ribosyl-protein from common proteins [22]; the separation of γ-interferon and immunoglobulin G (IgG) [23]; the characterization of specific membrane glycoproteins [24]; the separation of glycosylated proteins [25]; and the purification of serine proteases from other enzymes [26].

The phenylboronate matrix has also been useful for several clinical studies. For example, glycosylated hemoglobins are used increasingly for the assessment of glycemia [27]. Immobilized phenylboronates have been used to measure the level of glycosylated hemoglobin [28–30]. The levels of nucleosides and other metabolites have been measured in the gastro-intestinal mucosa of normal individuals and cancer patients in which greater amounts of specific metabolites are claimed to be indicative of gastric cancer. Similarly, hypoxanthine, uridine, and inosine have been linked to other disorders [31,32]. Substituted phenylboronates have even been shown to exhibit significant antimicrobial activity against common pathogens [33]. Thus, the boronate ligands have been employed for a wide variety of applications involving basic biochemistry and clinical medicine.

The presently available boronate matrices are unstable, function only for a few compounds, and do not work under acidic conditions in which most metabolites are relatively more stable, especially catecholamines and AA-tRNAs. The mechanism of boronate complex formation with compounds of different complexities is largely unknown. Except for the pH, very little is known about controlling factors enhancing adsorption and desorption of the desired molecules from the phenylboronate columns. To fill this void, we have synthesized several boronate affinity ligands and studied their interaction with *cis*-diol model compounds.

A boronate ligand should possess several major characteristics in order to be considered useful in affinity chromatography for the purification of biomolecules. A major limitation of the use of phenylboronate ligands for affinity chromatography is their high ionization constant. The most commonly used ligand, 3-Aminophenylboronic acid (abbreviated here as 3aPBA), has a relatively lower ionization constant (pK_a 8.75) because of the amine group in the *meta* position. Several attempts have been made to synthesize boronate ligands having lower pK_a values. In this study an electron-withdrawing (nitro) group has been introduced into the phenyl ring to make boronate more acidic. The reaction of 3aPBA and its nitro derivative N-(6-nitro-3-dihydroxyborylphenyl)succinamic acid (6nsPBA) with a variety of *cis*-diols is studied here in detail because this compound and its derivatives are used extensively for the design of affinity material by making use of the amino functional group for attachment to a spacer arm or directly to an affinity matrix [2,11].

Chemical shifts of ^{11}B NMR of anionic boronate species, neutral boronic acid and complexed-anionic species are expected to change after reaction with *cis*-diols, due to differential electronic shielding of the ^{11}B atom among these molecules. We realize the potential of the ^{11}B NMR for studying the reaction mechanism of the complex formation with different phenylboronates. In this study, ^{11}B NMR spectroscopy and absorption spectrophotometry techniques are employed to study the boronate complex formation in solution. The results of in-solution complex formation are compared with those derived from binding of affinity molecules with boronate column matrices.

EXPERIMENTAL

Materials

Phenylboronic acid (PBA), obtained from Aldrich, was crystallized from water. 3-Aminophenylboronic acid hemisulfate, also from Aldrich, was converted to its free acid by neutralization followed by crystallization from water. *o*-Nitrophenylboronic acid (2nPBA), *m*-nitrophenylboronic acid (3nPBA), and *p*-nitrophenylboronic acid (4nPBA), were prepared by the reported procedures [34]. *o*-Nitrosuccinamidophenyl-boronic acid (4nsPBA), *m*-nitrosuccinamidophenylboronic acid (5nsPBA), and *p*-nitrosuccinamidophenylboronic acid (6nsPBA), were synthesized as described in a section below. *β*-Methylribofuranoside (mRib) and *α*-O-methylglucopyranoside (mGlc) were purchased from Sigma. Not all the ligands used for NMR studies were used for spectrophotometric studies or were immobilized to the matrix. This is because some of these phenylboronate derivatives are only partially soluble in the medium due to the presence of a spacer arm in their structure.

Solutions for spectrophotometry

Stock solutions of phenylboronic acid derivatives were prepared in glass-distilled deionized water. Appropriate dilutions of stock solutions were made in 50 mM buffers of various pH values. The samples were diluted to yield an absorbance of approximately one unit at λ_{max} and pH 4.0. Molar concentrations of mRib and mGlc used were five times greater than those of the phenylboronic acids in order to evaluate the effect of these polyalcohols on pK_a values of the boronate derivatives.

Determination of pK_a by spectrophotometry

A microprocessor-controlled spectrophotometer (Ciba-Corning Gilford Systems, Oberlin, OH, U.S.A., Model Response II) was used for the absorption spectroscopy work. The analytical wavelength for different boronate derivatives was selected so that there was a significant difference in the absorbance between the neutral and the anionic species. The spectrum of the ligand was established for the neutral (A_m) and the ionic (A_i) species, using an identical concentration of the material at pH 4 and pH 10, respectively. The following equations were used for pK_a determinations:

if $A_i > A_m$, then: $pK_a = pH + \log [(A_i - A)/(A - A_m)]$

if $A_m > A_i$, then: $pK_a = pH + \log [(A - A_i)/(A_m - A)]$

where A was the absorbance of the boronate ligand at any given pH value [35]. The concentration of boronate anions (free boronate and those complexed with *cis*-diols) was determined from changes in the absorbance at two selected analytical wavelengths using the following equation:

$$C_{B^-} = \{A_{\lambda_2} - [(\varepsilon_{\lambda_2}^{B^o} \cdot A_{\lambda_1})/\varepsilon_{\lambda_1}^{B^o}]\}/\{\varepsilon_{\lambda_2}^{B^-} - [(\varepsilon_{\lambda_2}^{B^o} \cdot \varepsilon_{\lambda_1}^{B^-})/\varepsilon_{\lambda_1}^{B^o}]\}$$

where C_{B^-} indicates moles of the boronate anion; A_{λ_1} and A_{λ_2} are variable absorptions at the two selected wavelengths, λ_1 and λ_2; $\varepsilon_{\lambda_1}^{B^o}$ and $\varepsilon_{\lambda_2}^{B^o}$ are the molar extinction coefficients of the neutral species of the boronate ligand determined at λ_{max_1} and λ_{max_2} values; and similarly, $\varepsilon_{\lambda_1}^{B^-}$ and $\varepsilon_{\lambda_2}^{B^-}$ are those of the anionic species at the two wavelengths.

^{11}B NMR spectroscopy

NMR spectra were recorded at 22°C, using a Varian XL-300 spectrometer at 96.248 MHz. Boronate derivatives were dissolved in ^2H_2O while maintaining the sample volume to 0.5 ml. The pH was adjusted with NaO^2H in ^2H_2O and measured with a combination micro-pH electrode (Model PHR-146, Lazar, Los Angeles, CA, U.S.A.). Typically, a 50-mM solution of a PBA in ^2H_2O was used for the NMR spectra and the p^{2}H of the solution controlled using ^2HCl and NaO^2H (the solution p^{2}H was carefully measured inside the NMR tube). Chemical shifts were measured using $BF_3O(C_2H_5)_2$ as the external standard. ^{11}B chemical shifts were measured above the boron background present in the NMR probe. The chemical shifts appeared as sharp peaks above the dome-shaped boron background. Results are plotted here as chemical shifts *versus* p^{2}H of the solution in bar graphs, where bar heights bear no significance.

Synthesis of boronate ligands

A nitrating reagent consisting of nitronium trifluoromethanesulfonate and hydronium trifluoromethanesulfonate mixture ($NO_2^+CF_3SO_3^- – H_3O^+CF_3SO_3^-$ in a 1:1 proportion) was used [36,37]. Three reaction conditions of nitration were employed (*Methods A, B* and *C*, see below). Synthesis and recovery of the nitro isomers were influenced by small differences in reaction conditions. The best results were obtained by conducting the reaction in anhydrous conditions, using a freshly-distilled acetic anhydride, and observing the order in which the nitrating reagent and N-(3-dihydroxyborylphenyl)succinamic acid (sPBA) were added to the flask while carefully controlling the reaction temperature. Reaction products were characterized by liquid chromatographic and spectroscopic methods. (Mass spectroscopic and elemental analyses could not be relied on because phenylboronic acid molecules easily transform into anhydrides in variable amounts upon drying.) Isomers were further identified by deboronation. For example, in compound 4nsPBA, the boron–carbon bond was eliminated by treating it with an ammoniacal silver nitrate solution and the product characterized as N-(4-nitrophenyl)succinamic acid, thus confirming the presence of the nitro group in position 4 of 4nsPBA. Ionization constants of different derivatives were determined by spectrophotometric methods. Melting points were determined with a Fisher–Johns apparatus and were not corrected. IR spectra were obtained using a Perkin-Elmer (Model 1330) spectrometer. High-performance liquid

chromatography (HPLC) analyses were performed with the help of a microprocessor-controlled liquid chromatograph linked to a minicomputer and equipped with a multi-step gradient elution pump (Perkin-Elmer, Model series 4), a diode-array detector (LKB-Bromma, Model 2140), and a C_{18} reversed-phase column (25 cm × 4.5 mm I.D., Separation Group, Hesperia, CA, U.S.A. Model Vydac 201HS54) [38–40]. A two-step linear gradient of methanol with 20 mM sodium succinate buffer (pH 5.3) was used for elution and simultaneous characterization of the different isomers.

3aPBA. A stirring suspension of hemisulfate salt of 3aPBA (5.58 g, 30 mmol) in water (50 ml) was adjusted to pH 7 by dropwise addition of 1 M NaOH. The free base was extracted with ethyl acetate (3 × 40 ml), dried over MgSO$_4$, the organic solvent evaporated *in vacuo*, and the crude 3aPBA thus obtained was crystallized from water. Yield: 3.2 g (78%), m.p. 170–171°C (*cf.* 164–165°C [2]). IR (KBr): = 3470, 3450, 3140 cm^{-1}. UV (H$_2$O): λ_{max} = 295 nm. ^{1}H NMR ([^{2}H$_6$]dimethyl sulfoxide, DMSO-d$_6$): δ = 4.98 (Br, 2H, NH$_2$); 6.61 (m, 1H, H$_{arom}$ 5); 6.97 (m, 3H, H$_{arom}$ 2, 4, and 6).

sPBA. This compound was essentially prepared by the procedure of Weith *et al.* [2]. The reaction was carried out in a nitrogen atmosphere and the product crystallized from water. Yield: 75%, m.p. 174–175°C (*cf.* 173–174°C [2]). IR (KBr) 3000–2680 cm^{-1}, 1650 cm^{-1}. UV (H$_2$O) λ_{max} = 243 nm. ^{1}H NMR (DMSO-d$_6$): δ = 2.53 (m, 4H, CH$_2$CH$_2$); 7.24 (m, 1H, H$_{arom}$ 5); 7.45 (d, 1H, J = 8.1 Hz, H$_{arom}$ 4); 7.70 (d, 1H, J = 8.1 Hz, H$_{arom}$ 6); 7.83 (S, 1H, H$_{arom}$ 2); 8.0 [S, 2H, B(OH)$_2$]; 9.88 (S, 1H, CONH); 12.14 (S, 1H, COOH).

Nitration of sPBA

Method A. The nitrating reagent (a mixture of NO$_2^+$CF$_3$SO$_3^-$–H$_3$O$^+$ CF$_3$SO$_3^-$ in a 1:1 proportion; 0.5 g, 1.38 mmol) was added to a three-neck round-bottom flask (50 ml), fitted with a CaCl$_2$ drying tube, nitrogen inlets, and a thermometer, and then cooled to −12°C. While stirring this solution at −12°C, trifluoromethanesulfonic acid (0.2 ml) was added with 100% H$_2$SO$_4$ (1.0 ml) and dry CH$_2$Cl$_2$ (5.0 ml), and then sPBA (0.3 g, 1.3 mmol). After stirring the mixture for 0.5 h, the temperature was gradually raised to 20–22°C and stirred for an additional 1 h. The reaction mixture, free of organic solvent, was poured onto ice. The crude product was extracted with ethyl acetate (4 × 25 ml) and the combined extracts were washed with a saturated NaCl solution (3 × 25 ml), then dried (MgSO$_4$) and the organic solvent evaporated *in vacuo*. The red solid product (0.18 g) obtained showed a spectrum with a λ_{max} at 324.5 nm; it was crystallized from benzene–ethyl acetate (1:1) to yield yellow crystals (0.015 g). IR and NMR analyses confirm the product was 4nsPBA. IR (KBr): 3800–2700, 1690, 1500 cm^{-1}. ^{1}H NMR (DMSO-d$_6$): δ = 2.51 (m, 4H, CH$_2$CH$_2$); 7.83 (S, 1H, H$_{arom}$ 2); 8.17 (S, 1H, H$_{arom}$ 4); 8.34 (S, 1H, H$_{arom}$ 6); 10.4 (S, 1H, CONH); 12.1 (Br, 1H, COOH). After crystallization of 4nsPBA, the filtrate was evaporated to dryness. HPLC analysis of the solid showed three compounds and NMR data also confirmed the presence of all three nitro derivatives. No further attempts were made to isolate the isomers.

Method B. The sPBA (0.5 g, 2.1 mmol) was suspended in dry, distilled acetic anhydride (5.0 ml) and placed into a flask equipped as in *Method A*. To the cold stirring mixture, the nitrating reagent (1.42 g, 4.0 mmol) was added in small portions at such a rate that the reaction temperature remained below 15°C. All additions were completed within 0.5 h, while the mixture gradually turned dark yellow. The reaction

mixture was further stirred at 20–22°C for 3 h, quenched with ice cold water and extracted with ethyl acetate (4 × 40 ml). The combined ethyl extracts were washed with a saturated NaCl solution (4 × 30 ml). Evaporation of the organic solvent *in vacuo* changed the product into a viscous, orange-red oil. The oily substance was purified on an acidic alumina column while eluting it with an ethyl acetate–methanol mixture (97:3). Fractions rich in the compound of interest were pooled, evaporated, and the resultant semisolid substance was triturated with methanol to obtain a solid material. This material was crystallized from methanol and a pure sample of N-(3-nitro-3-dihydroxyborylphenyl)succinamic acid (3nsPBA) was obtained (130 mg, 0.46 mmol); m.p. 146–147°C. IR (KBr) : 3445, 3220, 1520 cm^{-1}. UV (H$_2$O): λ_{max} = 325 nm. ^{1}H NMR (DMSO-d$_6$): δ = 2.57 (m, 4H, CH$_2$CH$_2$); 7.62 (S, 1H, H$_{arom}$ 2); 7.76 (d, 1H, J = 8.4 Hz, H$_{arom}$ 6); 8.10 (d, 1H, J = 8.4 Hz, H$_{arom}$ 5); 8.16 [S, 2H, B(OH)$_2$]; 10.49 (S, 1H, CONH); 12.15 (S, 1H, COOH). The methanol filtrate was evaporated after crystallization of the above compound. A semisolid material obtained upon crystallization from the methanol–ethyl acetate mixture (1:1) was characterized as 6nsPBA (30 mg, 0.11 mmol). 1H NMR (DMSO-d$_6$): δ = 2.6 (m, 4H, CH$_2$CH$_2$); 7.48 (S, 1H, H$_{arom}$ 2); 7.55 (d, 1H, J = 9.9 Hz, H$_{arom}$ 4); 7.96 (d, 1H, J = 9.9 Hz, H$_{arom}$ 5).

Method C. The nitrating reagent (3.63 g, 10 mmol), cooled to −30°C, was placed into a three-neck round-bottom flask (125 ml) equipped as in *Method A*. Dry acetic anhydride (15.0 ml) and then sPBA (0.592 g, 2.5 mmol) were added while stirring the mixture. The reaction temperature was gradually raised to −10°C and maintained for 2 h. The mixture was then poured onto ice and the crude product was extracted with ethyl acetate (4 × 40 ml). The combined ethyl acetate extracts were washed with a saturated NaCl solution (4 × 30 ml), dried (Na$_2$SO$_4$) and the organic solvent removed *in vacuo*. The semisolid material thus obtained was triturated with CH$_2$Cl$_2$ and the yellow solid product was recovered by filtration. This impure product was purified by silica gel chromatography while eluting the column with ethyl acetate. Evaporation of the ethyl acetate fractions gave the *para*-nitro derivative (6nsPBA) (15 mg, 53 μmol). The column, after elution with ethyl acetate, was further eluted with an ethyl acetate–ethanol (95:5) mixture. Evaporation of the pooled fractions gave the *ortho*-nitro derivative (4nsPBA) (160 mg, 57 mmol). HPLC and spectrophotometric analyses confirmed the structure of the two compounds.

Deboronation of 4nsPBA. To a one-neck round-bottom flask (equipped as in *Method A*), 4nsPBA (20 mg, 70 μmol) was added and then a solution of silver nitrate (14 mg, 80 μmol) dissolved in 2% NH$_4$OH (10 ml). The reaction mixture was gently heated to the reflux temperature for 20 min, cooled, and extracted with ethyl acetate (3 x 20 ml). Combined extracts were dried (MgSO$_4$), filtered, and evaporated to dryness to yield a yellow solid of N-(4-nitrophenyl)succinamic acid (15 mg), m.p. 92–93°C (*cf.* 96–97°C [41]). IR (KBr): 3340, 1690, 1535 cm^{-1}. ^{1}H NMR (DMSO-d$_6$): δ = 2.58 (m, 4H, CH$_2$CH$_2$); 7.84 (d, 2H, J = 9.3 Hz, H$_{arom}$ 2); 8.22 (d, 2H, J = 9.3 Hz, H$_{arom}$ 3).

Immobilization of boronate ligands on a hydrophilic vinyl polymer

Toyopearl, a product of TosoHaas (Bioseparation Specialists, Supelco, Bellefonte, PA, U.S.A.), is a porous and semirigid spherical gel of vinyl polymer. The AF-Amino Toyopearl 650 matrix used in this work had the structure of Gel–CH$_2$–CH(OH)–CH$_2$–NH$_2$ and contained *ca.* 100 μmol of amine per ml of gel. The matrix

was coupled with sPBA (using 126 μmol of sPBA per ml of gel) and nsPBA (using 68 μmol of nsPBA per ml of gel). The coupling reaction was carried out according to the method of Weith *et al.* [2], but using a different carbodiimide and with small differences in experimental conditions. A 15-ml sample of the Toyopearl gel suspended in 20 ml of water, was mixed with 1.7 mmol of sPBA, which was dissolved in 50% aqueous tetrahydrofuran (THF) (10 ml). To the mixture, cooled in an ice bath and the pH adjusted to *ca.* 6, 2 mmol of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC) were added and the suspension stirred while maintaining the solution pH $\approx$ 6 by the addition of 2 M HCl. After 4 h, the temperature of the reaction mixture was raised to 22°C and stirred for another 4 h, and then the product was washed with 10 ml each of 50% aqueous solutions of THF followed with dimethylformamide, and finally with water until the washings were free of the reactants. The coupling of nsPBA to the gel matrix was carried out similarly. Each matrix was acetylated in order to block any unreacted amine in the gel. In a typical reaction the gel, suspended in water in an ice bath and adjusted to pH 9, was mixed dropwise with acetic anhydride (140 μmol per ml of gel). The suspension was stirred and pH maintained at *ca.* 9 for 1 h and then the product washed with a 50 mM sodium acetate buffer (pH 4.5) containing 0.1 M NaCl.

Liquid chromatography

The chromatographic system consisted of: (a) a manual injection valve (Rheodyne, Cotati, CA, U.S.A., Model 7125), (b) a programmable mobile phase gradients pump (Perkin-Elmer, Norfolk, CT, U.S.A., gradient pump, Series LC4), and (c) a diode-array effluent monitor (LKB, Bromma, Sweden, Model 2140 Rapid Spectral Detector). A microcomputer (Zenith Data Systems, St. Joseph, MI, U.S.A., Model Z-248, IBM AT-compatible) was used to operate the detection unit and also to collect and store the absorption spectrum of the effluent every 0.3 s (for example, from 220–300 nm) with the aid of a software program (LKB-Produkter, Bromma, Sweden, Model Wavescan-EG). To test the purity of boronate ligands, a silica-based reversed-phase (C_{18}) matrix (5-μm beads) was used and the column eluted with a pH 5.5 buffer and methanol gradient at 40°C.

For boronate affinity chromatography, glass (6.34 mm diameter) columns, fitted with movable pistons on both ends and jacketed for 6°C temperature control were used. The boronate columns were eluted in three different ways. One, the column was equilibrated and the sample was applied in an alkaline buffer to form the boronate complex, and then eluted with an acid buffer to break the complex. Two, the sample was applied in the buffer of a selected pH and eluted with the same buffer, but also containing 5 mM sorbitol. Three, the sample was applied and the column was eluted in the same buffer without any change in the buffer composition. The first method gave inconsistent results and reduced the column life. The second method, although producing sharper peaks, was cumbersome and had little advantage over the third method. The third method was simple to follow and gave the true value of the retention time (k'). The results in this study are reported by using the last procedure.

Determination of binding capacity of affinity columns by frontal analysis. The frontal analysis method was used to determine the binding capacity and binding constant of the affinity matrix [43]. A buffer containing the affinity molecule (*e.g.* adenosine, A) was applied continuously to the column packed with the affinity ma-

trix. This was performed in an environment in which adenosine could freely bind to the boronate matrix, such as at pH 8.5. The application was continued until all binding sites of the matrix were saturated with the affinity molecule and the effluent concentration of the molecule became equal to the concentration of the feed solution. The column was washed until all bound molecules were eluted from the matrix, and then a buffer containing the same concentration of the molecule (A) was applied in a non-binding environment, such as at pH 5.5. The frontal volume (V_f) was derived from differences in the elution volume between a 50%-saturation point of A for binding and non-binding conditions. The binding capacity was calculated from the values of frontal volume (V_f), total column volume (V_t), adenosine concentration in the feed solution [A], and by the following equation:

$$\text{Binding Capacity} = (V_f \, [A])/V_t$$

The apparent dissociation constant was determined from the ligand concentration [L] in the matrix and by using the following equation [44].

$$K_{\text{diss.}} \text{ (apparent)} = (V_t \, [L] - V_f \, [T])/V_f$$

RESULTS

Synthesis of novel boronate ligands

Different methods for nitration of sPBA were examined. First, we followed the published procedure [43]. It involved acylation of 3aPBA to protect the amino group against oxidation, followed by nitration, and then removal of the protecting group. The last step required the use of a hot HCl treatment, therefore it caused extensive deboronation of the nitro isomers. The product contained a very small amount of the desired product and scores of other derivatives [very complex HPLC and gas chromatography (GC) results]. (The deboronation under acidic conditions is well documented [45–50].) The second method involved the use of fuming HNO_3 for nitration. It gave a resinous material even under mild reaction conditions. The third method involved the use of a commercially available nitrating agent [50,51], nitronium tetrafluoroborate ($NO_2{}^+BF_4^-$). This reagent produced only 3% of the nitrophenyl derivatives. Finally, a nitrating reagent consisting of a mixture of nitronium trifluoromethane and hydronium trifluoromethanesulfonate ($NO_2^+CF_3SO_3^- - H_3O^+CF_3SO_3^-$, 1:1) was employed. Prior to nitration, succinylation of the amino group in 3aPBA offered protection and also provided a carboxyl group linked to a small spacer arm needed for immobilization (Fig. 1). Nitration of sPBA with this reagent in different solvents was examined to maximize the yield of *ortho*- and *para*-nitro derivatives. A mixture of all three isomers was produced in each of CH_2Cl_2, CF_3SO_3H and 100% H_2SO_4. However, only the two desired nitro isomers were formed in acetic anhydride medium (4nsPBA and 6nsPBA). In two sets of reaction conditions (*Method B* and *Method C*), the *ortho* derivative was the predominant isomer (Fig. 1a). The use of acetic anhydride had a distinct advantage. For example, the ratio of 4nsPBA to 6nsPBA was 4.3 in CH_2Cl_2 (*Method A*) (Fig. 1b), but it increased to 11 in acetic anhydride.
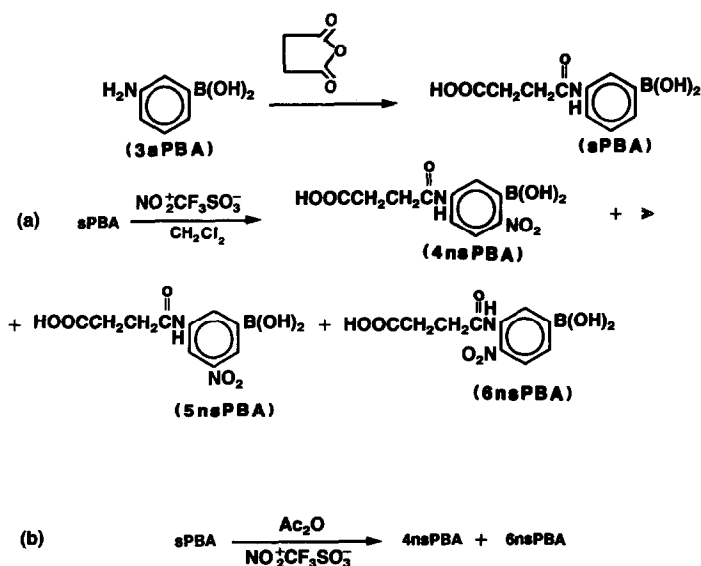
Fig. 1. The nitration of phenylboronate derivatives carried out by (a) *Method A*; and (b) by *Method B* or *Method C*. Ac = Acetyl; 3aPBA = 3-aminophenylboronic acid; sPBA = N-(3-dihydroxyborylphenyl)-succinamic acid; 4nsPBA = N-(4-nitro-3-dihydroxyborylphenyl)succinamic acid; 5nsPBA = N-(5-nitro-3-dihydroxyborylphenyl)succinamic acid; 6nsPBA = N-(6-nitro-3-dihydroxyborylphenyl)succinamic acid (see text for details).

2. Ionization and complex formation of boronate ligands

Studied by spectrophotometry. The boronate complex formation greatly depends on the ionization characteristic of the boronate ligand. A two-wavelength procedure, as described in Experimental, was used to determine the ionization constants of important phenylboronate derivatives. Results in Table I indicate, while PBA and 3aPBA have similar pK_a values, introduction of a nitro group to the phenyl ring substantially lowers the pK_a of the product. A nitro substitution in the *para*-position, as expected, showed a slightly lower pK_a value than in the *meta*-position of the phenyl ring. However, a higher pK_a value for the *ortho* derivatives (2nPBA and 4nsPBA) was observed by this method. (See the next section for explanation of these unexpected results.)

A change in the absorption spectrum of the phenylboronate occurs when it is complexed with a *cis*-diol, because the boron atom acquires an anionic charge in the complexed form. To assess the effect of nitro substitution, this property was used to study the affect of complex formation on the ionization of different ligands. The changes in spectra were examined *before* (boronic acid) and *after* complexing (boronate anion) them with model diols. The latter compounds were selected such that they caused no interference in the analytical wavelength region. Results in Table I indicate the addition of a *cis*-diol compound, *e.g.* mRib, to a 3aPBA solution significantly lowered the pK_a value of the complex by as much as one pH unit. Moreover, the addition of a *trans*.-diol, *e.g.* mGlc, had little effect on the ionization of the PBA derivative. The PBA ionization was also influenced with the presence of other *cis*-diol

TABLE I

IONIZATION CONSTANTS OF PHENYLBORONIC ACID DERIVATIVES AND *cis*-DIOL COMPLEXES DETERMINED BY SPECTROPHOTOMETRY

Phenylboronates (and with *cis*-diol derivatives)	pK_a values (± 0.02)	
3-(Aminophenyl)boronic acid	8.75[a]	
Complexed with α-O-methylglucopyranoside		8.60
Complexed with β-methylribofuranoside		7.85
Phenylboronic acid	8.8[b]	
Complexed with β-methylribofuranoside		8.1
Complexed with catechol[c]		8.5
Complexed with L-dopa[d]		8.4
Complexed with dopamine[e]		7.9 (± 0.47)
2-(Nitrophenyl)boronic acid	9.17[f] (± 0.06)	
Complexed with β-methylribofuranoside		8.95
3-(Nitrophenyl)boronic acid	7.15[g]	
Complexed with β-methylribofuranoside		6.98
Complexed with L-dopa[d]		6.75
4-(Nitrophenyl)boronic acid	7.00[h]	
Complexed with α-O-methylglucopyranoside		7.00 (± 0.09)
Complexed with β-methylribofuranoside		6.71 (± 0.09)
Complexed with L-dopa[d]		6.74
Complexed with dopamine[e]		6.92
N-(4-Nitro-3-dihydroxyborylphenyl)succinamic acid	8.45[i](± 0.05)	
Complexed with β-methylribofuranoside		8.25
N-(6-Nitro-3-dihydroxyborylphenyl)succinamic acid	7.15 (± 0.05)	
N-(5-Nitro-3-dihydroxyborylphenyl)succinamic acid	7.30	

[a] The pK_{a_1} and pK_{a_2} of 3aPBA are reported as 4.47 and *8.81*, respectively [61,62].

[b] The pK_a value of PBA is reported as 8.86 [61,63].

[c] The pK_a value of catechol is reported to be 9.48 [64].

[d] The pK_a values of L-dopa [3(3,4-dihydroxyphenyl)alanine] are reported as 2.32, *8.68*, and 9.88, respectively [65].

[e] The pK_a values of dopamine (3,4-dihydroxyphenethylamine) are reported as *8.68*, and 9.88, respectively [65] and also as 8.9 (amine) and 10.5 (hydroxyls), respectively, elsewhere [66].

[f] The pK_a value of 2nPBA [2(nitrophenyl)boronic acid] is reported as 9.2 [67]. This apparently high pK_a of this compound is apparently due to internal cyclization of the *ortho* nitro group with the vicinyl boronic acid [68].

[g] The pK_a value of 3nPBA [3(nitrophenyl)boronic acid is reported as 7.3 [5].

[h] The pK_a value of 4nPBA [4(nitrophenyl)boronic acid] is reported as 7.15 [69].

[i] This apparently high pK_a of this compound is apparently due to internal cyclization of the *ortho* nitro group with the vicinyl boronic acid [68].

derivatives. For example, the pK_a value of PBA was lowered by 0.3, 0.4, and 0.9 pH units when mixed respectively with catechols, L-dopa and dopamine. A similar decrease in pK_a values of nitro-PBA derivatives was noted when the latter were complexed with *cis*-diols (Table I).

The ionization and complex-formation properties of two ligands (3aPBA and the other with a nitro substitution, 4nPBA) with mRib and mGlc were examined at
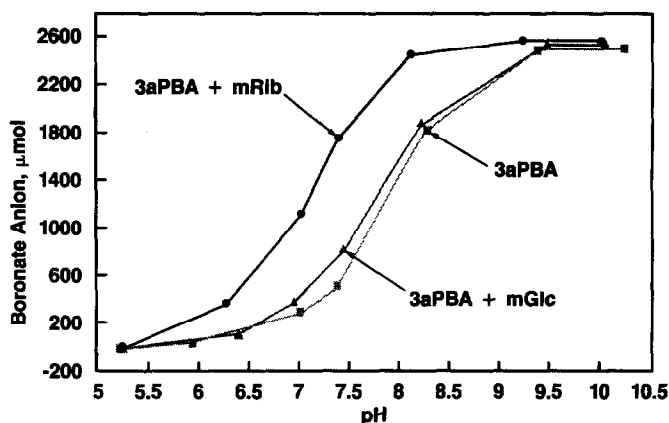
Fig. 2. Boronate complex formation studied by absorption spectroscopy (without ligand immobilization) between a boronate ligand (3aPBA = 3-aminophenylboronic acid) and polyalcohols affinity molecules (mRib = β-methylribofuranoside; mGlc = α-methylglucopyranoside).

different pH values. The boronate–*cis*-diol complex formation with mRib occurred approximately one pH unit below the pK_a of 3aPBA, but 1.8 pH units below the pK_a value of 4nPBA (Figs. 2 and 3). However, the pK_a values of the two ligands remained *unaffected* by the presence of a non-*cis*-diol sugar (mGlc). Thus, the substitution of a nitro group in the phenyl ring significantly lowers the pK_a value of the PBA derivative, and furthermore, it allows the complex formation with a *cis*-diol at one pH unit below the pK_a value of the nitro derivative.

Studied by ^{11}B NMR

Chemical shifts of ^{11}B NMR of boronates ligands were expected to change as a result of complexation with *cis*-diols because of differential electronic shielding in the
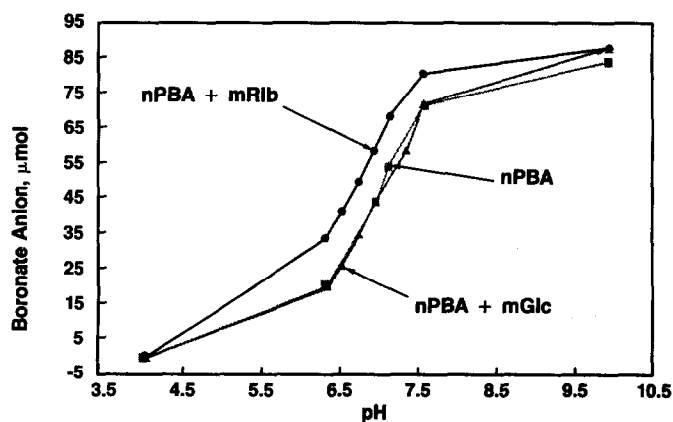

Fig. 3. The ionization of 4-nitrophenylboronic acid (4nPBA) studied at different pH values by using a spectrophotometric method and in buffered solutions. Note boronate complex formation with β-methyl-ribofuranoside (mRib), but none with α-methylglucopyranoside (mGlc).
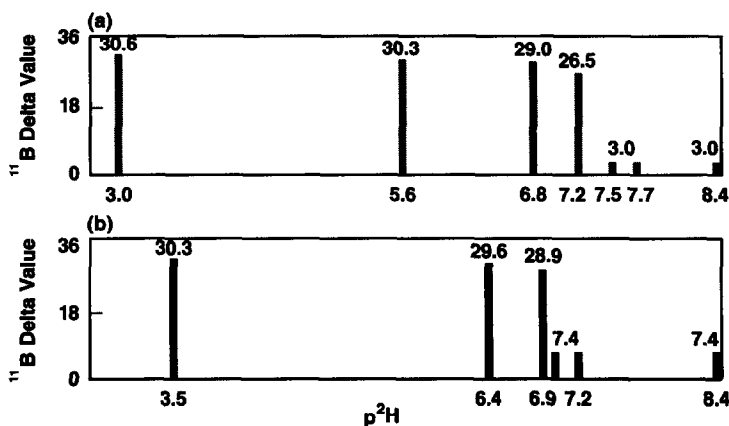
Fig. 4. ^{11}B NMR spectroscopy of ionization of a boronate ligand, (a) phenylboronic acid (PBA); and (b) boronate–cis-diol complex in solution. Chemical shifts: (a) 8.2 mM PBA; (b) 8.2 mM PBA mixed with 50 mM β-methylribofuranoside (mRib).

[11]B atom. Thus, specific signals can be distinguished between the complexed-boronate anions (Bc) and the neutral (B⁰) species. With this technique, we wanted to compare the complex formation in solution to that observed with the ligand linked to the matrix, i.e. in actual chromatography conditions.

In Fig. 4a, the bars represent chemical shifts (δ values) of a ^{11}B NMR spectrum of PBA analyzed at several p^2H values. The neutral molecule, such as that at p^2H 3.0, displayed a single peak at δ 30.6. However, as the solution turned basic by NaO^2H addition, the peak position gradually shifted to a lower δ value. PBA gave chemical shifts of δ 29 and δ 26.5 at p^2H values of 6.8 and 7.2, respectively. Thus, the lower δ value (between 0 and 3) was characteristic of the anionic species and the higher δ value (ca. 30) was specific for the neutral species. This was further confirmed by returning the p^2H of the solution from the alkaline back to the acidic medium, which produced a characteristic peak at δ 30. In the presence of mRib and under acidic conditions (p^2H 3.5 to 6.4), PBA produced the usual shift at δ 30, but two peaks at δ 28.9 and δ 7.3 were observed as the solution p^2H was raised to p^2H 6.85. Moreover, the first peak (δ 28.9) disappeared as soon as the solution p^2H was further raised to 7.0. At this and higher p^2H values, the chemical shift only at δ 7 was observed (Fig. 4b). The results indicate that the addition of mRib to PBA causes: (a) a rapid loss of signal due to neutral species, (b) the presence of two distinct signals, one specific for the neutral and the other for the anionic species, at a p^2H value consistent with the start of boronate–cis-diol complex formation, and (c) absence of the chemical shift specific for pure PBA anion (δ 3), but the presence of a new signal (δ 7.4) characteristic of the boronate–cis-diol anionic complex. These observations were further confirmed by regaining the δ 30 signal after acidification of the basic PBA-mRib solution.

To confirm these conclusions, complexation of different PBA derivatives was examined at various p^2H values. Results with 3aPBA (Fig. 5a) indicate a gradual downfield shift of δ 29 peak (p^2H 4.9) to a lower δ value with an increase in the p^2H of the solution. Above p^2H 8.2, only a signal (δ 0 to δ 3), indicative of the pure anions, was observed. The downfield peak is typical of the neutral boronic acid molecule and
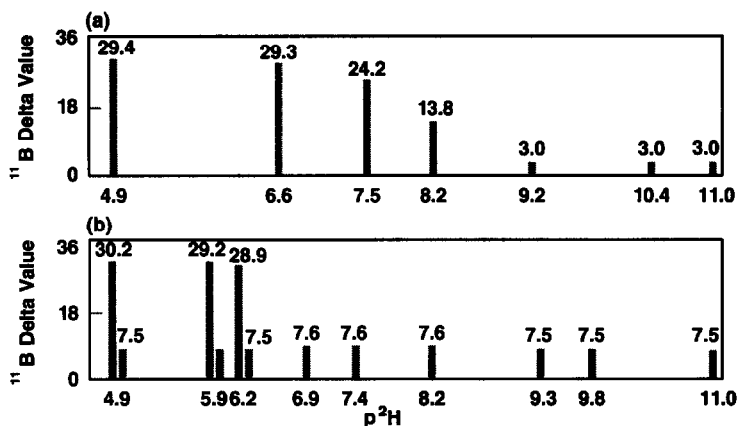
Fig. 5. ^{11}B NMR spectroscopy of ionization of (a) 3-aminophenylboronic acid, 3aPBA; and (b) boronate–*cis*-diol complex. Chemical shifts: (a) 50mM 3aPBA; (b) this 3aPBA mixed with 250 mM β-methylribofuranoside (mRib).

the upfield peak characteristic of the boronate anion. In the presence of mRib, 3aPBA spectrum gave two peaks (δ 30 and δ 7.5) under acidic conditions (p^{2}H 4.9–6.2), but only one peak (δ 7.5) at p^{2}H 7 and above (Fig. 5b). Thus, a chemical shift of δ 3 is specific of the *pure* boronate anion (p^{2}H > 8.2, Fig. 5a), while that at δ 7.0–7.6 is due to the *complexed* boronate anion (with mRib at p^{2}H < 7, Fig. 5b). These results were further confirmed by substituting ribonucleosides, *e.g.* uridine and cytidine for ribose in the reaction mixture. Each nucleoside in the 3aPBA solution produced two peaks (δ 28.42 and δ 7.21) at p^{2}H 6.4, but only one (δ 8) at p^{2}H 6.8 and above (results not shown). Thus, the complex formation with the nucleosides occurs at a p^{2}H value very close to that of ribose (p^{2}H 6.8 *versus* 7), but at a pH significantly *less* than the ionization (pK_a) of the free 3aPBA.
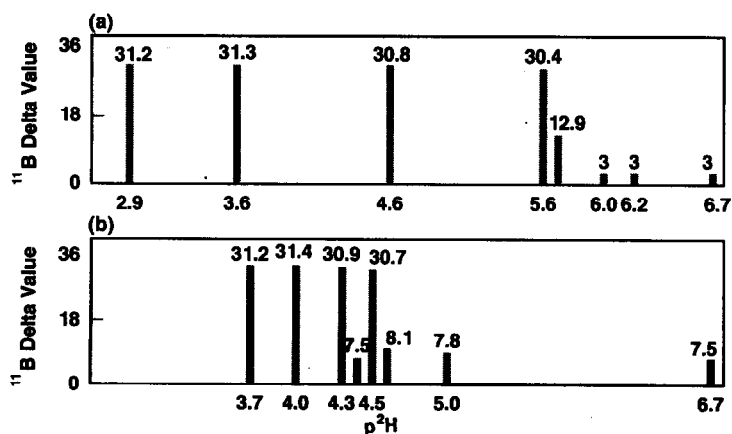

Fig. 6. Ionization of 2-nitrophenylboronic acid (2nPBA) studied by ^{11}B NMR in the presence and absence of a *cis*-diol at different p^{2}H values. Chemical shifts: (a) 8.2 mM 2nPBA and (b) this 2nPBA mixed with 10 mM β-methylribofuranoside (mRib).
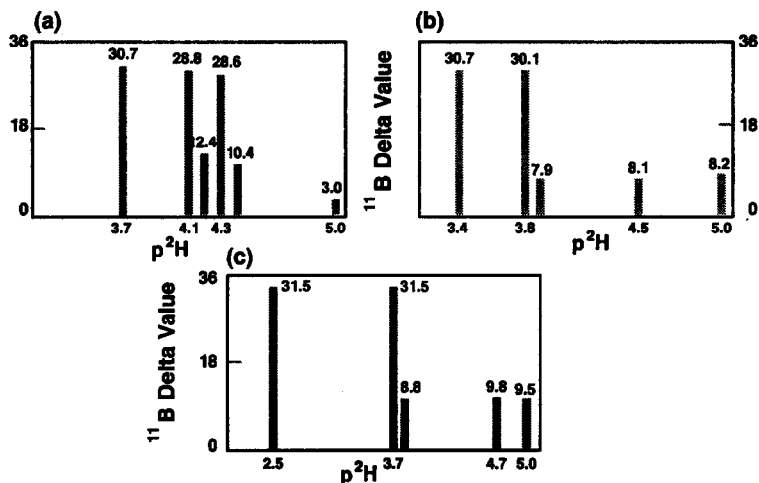
Fig. 7. Ionization of N-(4-nitro-3-dihydroxyborylphenyl)succinamic acid (4nsPBA) observed by ^{11}B NMR in the presence and absence of a *cis*-diol at different p²H values. Chemical shifts: (a) 50 m*M* 4nsPBA, (b) this 4nsPBA mixed with 250 m*M* β-methylribofuranoside (mRib) and (c) 4nsPBA also mixed with 250 m*M* with dopamine.

    The effect of a nitro group, especially one in the *ortho* position of PBA, on the boronate complex formation is shown in Fig. 6. The ionization of free 2nPBA occurred at only p²H 6, but those of PBA and 3aPBA took place at higher p²H values (7.2 and 8.2). This effect was more pronounced in spectra determined in the presence of ribose. For example, in the presence of ribose, 2nPBA gave a single peak at p²H 5 (Fig. 6b), while PBA and 3aPBA produced this chemical shift of the boronate complex at p²H 7.2 and 6.9, respectively (Figs. 4b and 5b). Similar to the other two ligands, the boronate complex with ribose also occurred at one pH unit (pH 4.98) less than the ionization of free 2nPBA (p²H 6.0).

    The ^{11}B NMR spectrum of an affinity ligand with a five-atom spacer arm, 4nsPBA, at different p²H values was examined. Transformation of free 4nsPBAcid to its anionic form, as shown in Fig. 7a, occurs between p²H 4.3 and p²H 5.0. Furthermore, its complex with ribose or an aromatic *cis*-diol, *i.e.* dopamine (3,4-dihydroxyphenethylamine), is observed at approximately p²H 4.5 (Fig. 7b and c). Thus, this ligand can form the boronate complex under fairly acid conditions as determined by ^{11}B NMR spectroscopy (see Discussion).

Evaluation of boronate affinity matrices

    Two boronate affinity column matrices were examined for their binding capacity and apparent dissociation constant. Each column was then evaluated for retention (k') of affinity molecules of different structures, such as alkyl and aryl *cis*-diols. An example of frontal analysis of 4nsPBA column is shown in Fig. 8 and the results from the two matrices are compared in Table II. Though a smaller concentration of the 4nsPBA ligand (27% less) had been used for immobilization, the matrix containing the nitro functionality exhibited a binding capacity almost two times that of the matrix having no such group (sPBA). The apparent dissociation constant of the

TABLE II

CHARACTERISTICS OF BORONATE AFFINITY COLUMNS

Column properties	Boronate ligands	
	sPBA	4nsPBA
Amount of boronate ligand reacted[a] with each ml of the matrix[b] to immobilize (mmol)	220	160
Column bed volume (V_t), ml	2.37	1.42
Frontal volume (V_f), ml	11.01	13.07
Binding capacity: mmol of adenosine[c] bound to each ml of matrix	0.74	1.47
Apparent dissociation constant (K_d), mM	21.4	10.7

[a] A 1-mmol amount of the carbodiimide coupling reagent (EDAC) was used for each immobilization.
[b] The amount of amine present in the matrix (Amino-Toyopearl 650, TosoHaas, Japan) was 100 mmol/ml of the gel.
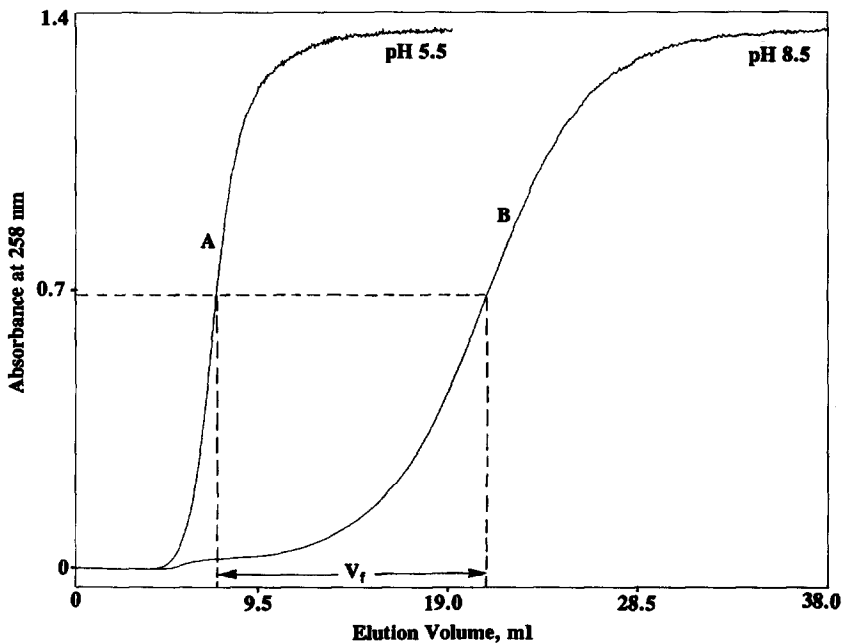[c] A 0.16 mM solution of adenosine [A] was used in the feed solution.

Fig. 8. Binding capacity of a boronate affinity (4nsPBA) column determined by frontal uptake of the affinity molecule (adenosine) by the matrix. The curve A was obtained by passing a 0.16 mM adenosine solution made in a 50 mM phosphate buffer (pH 5.5), *i.e.* in a non-complexing environment. The curve B was similarly obtained using adenosine under complexing conditions (pH 8.5). The frontal volume (V_f), 13.1 ml, was derived from the volume difference between the 50% saturation points (mid-points) of the two curves.

4nsPBA matrix was 50% of the sPBA matrix. Thus, in the nitrophenylboronate matrix, the affinity molecule binds with the matrix to a much greater degree and the boronate complex also breaks down with the same spontaneity.

The retention of adenosine (A) and cytidine (C) is compared in Fig. 9. Though both ribonucleosides have the same sugar, A is a purine while C is a pyrimidine derivative. However, this structural difference should not influence the complex formation. Nevertheless, A is retained three times more than C as evident from their k' values on the two columns. The two matrices also yield appreciably different results. For example, A is retained more strongly on the 4nsPBA matrix than on the sPBA matrix in buffers of pH > 7. However, at pH less than 7, the retention is poor and the difference in their retention is less obvious. Thus, the retention capacity is greatly influenced by both the *nature* of the affinity molecule and also by the *structure* of the boronate ligand.

The two boronate matrices were examined for complexation of aromatic *cis*-diols. Dopamine, in addition to the catechol structure, contains an ethylamine substitution. The results in Fig. 10 clearly indicate that both molecules are retained to approximately the same extent by each boronate matrix. However, the sPBA matrix exhibits a very low retention for both catechol and dopamine. In fact, catechol in buffers of less than pH 7 and dopamine in less than pH 6.5 exhibit very little retention on the sPBA matrix. The 4nsPBA matrix consistently shows high capacity factors, but relatively more for catechol than dopamine. The two aryl molecules exhibit different pK_a values for their hydroxyl groups (catechol, pK_a 9.5; dopamine, pK_a 10.5), dopamine in addition has a cationic charge due to the primary amine group (pK_a 8.9). Though diols exhibit different ionization constants, they make no difference to the structures since they remain unionized under the chromatography conditions. Steric hindrance, possibly caused by the presence of ethylamine substitution, and the cation-
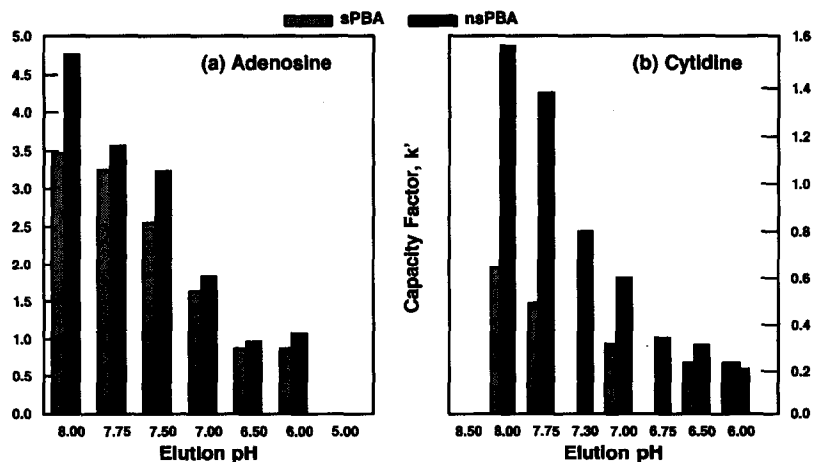

Fig. 9. A comparison of capacity factors of ribonucleosides on two boronate affinity matrices [sPBA = N-(3-dihydroxyborylphenyl)succinamic acid-matrix; nsPBA = N-(6-nitro-3-dihydroxyborylphenyl)succinamic acid-matrix]. Note different scales are used for the k' values. Binding and desorption of the two nucleosides were carried out in identical conditions. (For details, see the Experimental section.)
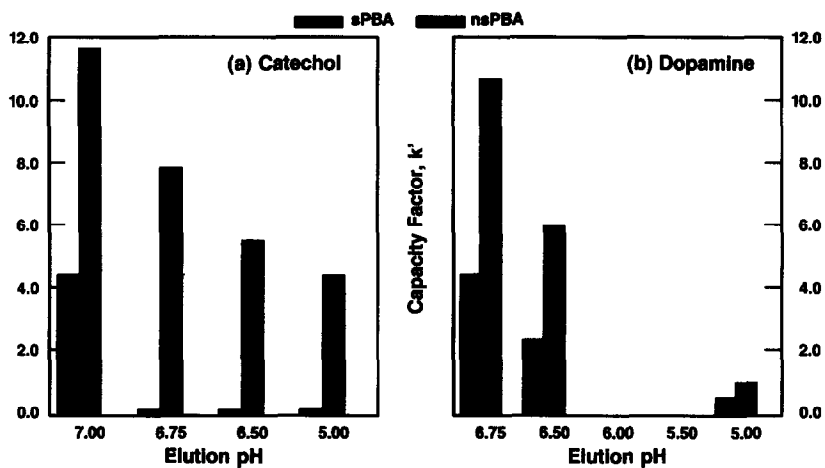
Fig. 10. A comparison of capacity factors of two aryl-*cis*-diols on boronate affinity matrices. Note very little binding of catechol to the sPBA affinity column (see legend to Fig. 9 for details).

ic charge on dopamine are perhaps responsible for the loss of retention on the sPBA matrix.

The separation of three important bioamines was compared on the two boronate matrices (Fig.11). Epinephrine and norepinephrine, with a secondary amine (pK_a 9.9) and a primary amine (pK_a 8.6) respectively, exhibit very similar retentions on each boronate matrix. However, L-dopa (L-3,4-dihydroxyphenylalanine), also
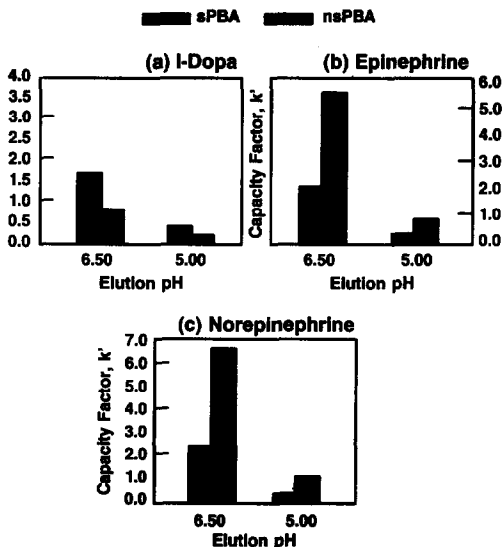

Fig. 11. The retention of three structurally similar compounds at pH 5.0 and 6.5 on two different boronate matrices. Note different scales are used to express the k' values (see legend to Fig. 9 for details).

having a primary amine group (pK_a 8.7), shows very little retention in the pH range examined. This loss of affinity for L-dopa is perhaps caused by the carboxylic group in the structure, although it is not ionized under these conditions.

DISCUSSION

The results indicate that the complex formation of boronate ligands can be studied by absorption spectrophotometry and ^{11}B NMR spectroscopy techniques, without their immobilization to solid supports. The results obtained by the two procedures are in fairly good agreement. For example, the complex formation with *cis*-diols occurs approximately one pH unit below the pK_a value of the boronic acid ligands (compare Table I data with results in Figs. 2–11). The boronate ligands clearly demonstrate the effect of nitro functionality in phenylboronates. The introduction of this electron-withdrawing group to the ring stabilizes the tetrahedral boronate anion in the interaction of the ligand with *cis*-diols, even at lower pH values . For example, a nitro group introduced into the phenyl ring causes boronate to be more acidic [43,53] (Table I). The *ortho*-nitro derivative, 4nsPBA, appears to be the best among the different isomers studied. The nitrophenylboronate ligand forms a complex with *cis*-diols at a much lower pH value than does the phenylboronate (compare Fig. 2 *versus* Fig. 3 and Fig. 4 *versus* Fig. 6). Small differences in the ligand structure apparently cause important differences in complex formation (compare Fig. 3 *versus* Fig. 7a).

The mechanism of boronate complex formation. A typical reaction of phenylboronate with 1,2-diol and 1,3-diol compounds involves cyclic ester formation. One of the proposed mechanisms involves a change of the *trigonal* boron structure in boronic acid into a *tetrahedral* boronate anion upon ionization of the acid and subsequent reaction with 1,2-diols or 1,3-diols to yield a complex [54]. Another proposed mechanism involves the sequential nucleophilic attack of the diol oxygen atoms on the boron atom of the free acid, thus yielding an anionic or neutral complex [55,56]. Our results summarized in Fig. 12 indicate: (1) The [11]B resonance frequency gradually shifts from δ 30 to δ 3 with an increase in the pH of the PBA solution. The signal
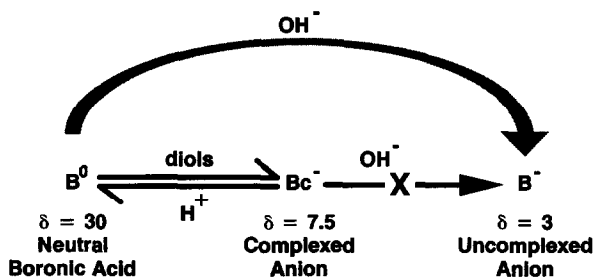

Fig. 12. A reaction mechanism proposed on the basis of ionization and complex formation of ligands studied by ^{11}B NMR and spectroscopic methods. Neutral boronic acid (B[0]) undergoes a facile complex formation in the presence of *cis*-diols, yielding a complexed anion (Bc[-]). The complex is stable and fails to dissociate even under alkaline conditions though the neutral boronic acid ionizes under such basic conditions.

shows an upfield shift with an increase in pH of the solution; the peak is assigned to the equilibrium state, [$B^0 \leftrightarrow B^-$], between the B^0 and B^- species [57]. *The conversion of neutral to anionic species occurs at a very fast rate*, consequently escaping detection of the signal on the ^{11}B NMR time scale. Thus, the change of B^0 to B^- is so rapid that only the average signal of the two species can be recognized. At a higher pH value, *e.g.* pH 9, the δ value of B^- lies between 0 and 3, since this peak remains unresolved from the external standard peak due to broadening of the boron signals. Similar broadening of the ^{11}B NMR signal has also been observed by others while working with phenylboronate compounds [58]. (2) An addition of a *cis*-diol compound to the PBA solution results in different chemical shifts. Specific signals of the complexed-boronate anion (Bc) and the neutral (B^0) molecules can be distinguished. For example, while the complexed species exhibit a characteristic δ of 7.5, the neutral ones exhibit a chemical shift of approximately δ 30. The results indicate all the boronic acid molecules are complexed (δ 7.5) without exhibiting δ 3 signals. From this we conclude that *the forward reaction resulting in complex formation is favored*. (3) *The boronate–cis-diol complex (Bc-) is stable* and fails to break down in free boronate anion, even when the pH of the solution is raised appreciably. In fact, the boronate complex of alkali-unstable compounds, *e.g.* catechols, exhibit their original spectrum upon lowering the pH of the complex from a fairly basic to an acidic pH.

The complex formation of a phenylboronate having a nitro group with *cis*-diols is shown in Fig. 13. The presence of an electron-withdrawing group in the immediate vicinity of boron lowers the ionization constant of phenylboronic acid. For example, phenylboronic acid exhibits a pK_a value of 8.8, but the introduction of a nitro group
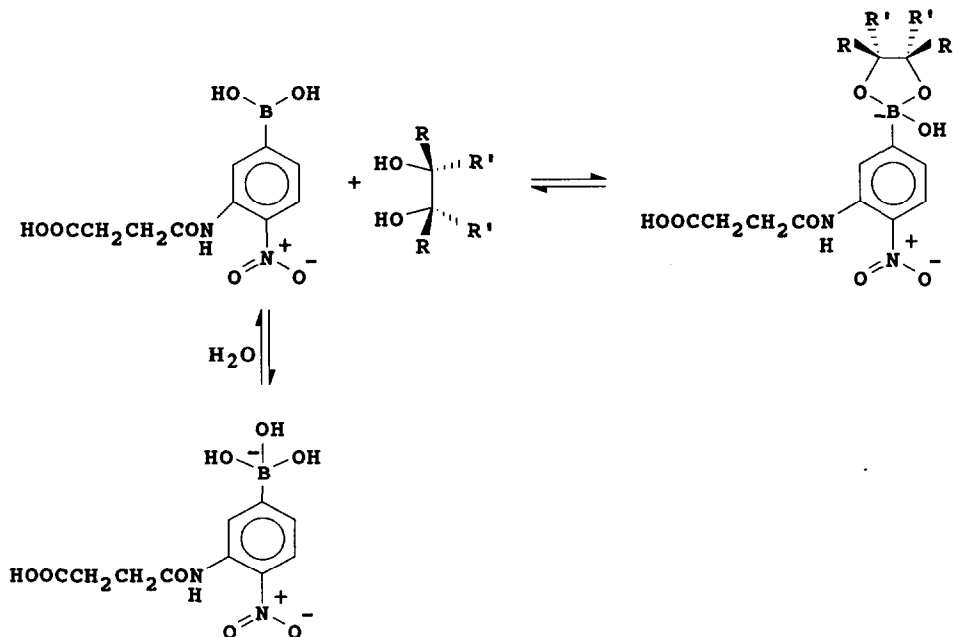

Fig. 13. The mechanism of complex formation between N-(6-nitro-3-dihydroxyborylphenyl)succinamic acid and *cis*-diols. Note the electron-withdrawing effect on the boron atom.

in the *meta-* or *para-*positions of the boron atom lowers the pK_a value to 7.15 and 7.00, respectively. The nitro group in the *ortho-* position causes an internal cyclization with vicinal boronic acid [53].

   Discrepancy between NMR data and UV data. In the NMR spectrum of 4nsPBA, the peak of δ 12.4 is apparently due to an internal nucleophilic attack by the electron from the oxygen atom of the nitro group on the boron atom. The pK_a of 2nPBA was found to be 9.17 and that of 4nsPBA as 8.45 by the spectrophotometric method, but boronate anion signals were observed at a more acidic p^{2}H (p^{2}H 6.0). We believe this difference is due to intramolecular nucleophilic attack, resulting in an internal cyclic and tetragonal boron structure. Its existence tends to decrease the strength of the acid because of the negative charge imposed on the boron atom [59]. This structure, however, easily makes a complex with the *cis*-diols at an acidic p^{2}H, as evident from the NMR and spectrophotometric data. However, in the pK_a determination by UV, as the pH is raised, the OH$^-$ ions of the medium attack the cyclic structure causing it to change to boronate anion; hence a higher pK_a value by this procedure. For this reason, we did not observe a higher pK_a in the NMR experiments for the *ortho* derivatives. Since the complex forms at approximately one p^{2}H unit below the pK_a of the ligand, this *apparent* high pK_a should not interfere with its application to affinity chromatography. Moreover, 6nsPBA (unlike 4nsPBA), having no such internal cyclization and a pK_a of 7.1, should form complex with *cis*-diols under acidic conditions.

   Interaction of cis-diols with different boronate matrices. Frontal analysis of two matrices, containing sPBA ligand and 4nsPBA ligand, indicate significant differences in binding capacities and apparent dissociation constants between the two matrices (Table II). The complex formation and also the breakdown of the complex are greatly enhanced due to the presence of the electron-withdrawing group in the boronate ligand. A greater amount of 4nsPBA is immobilized to a hydrophilic matrix than that of sPBA, perhaps because of greater solubility and reactivity associated with the nitro group in the 4nsPBA structure. The binding capacities determined for the two matrices clearly indicate that although affinity molecules can bind to the matrix under the same acidic conditions as those observed by ^{11}B NMR spectroscopy, the binding is very weak ($k' < 1$). Effective binding can be observed at a slightly higher pH value, approaching a neutral pH. This difference in complex formation can perhaps be caused by greater accessibility of the affinity ligand in the solution than in the solid (immobilized) medium. The results further demonstrate that small structural differences in the affinity molecule —which may not even influence the net charge of the molecule— have significant influence on the binding capacities (compare retention of adenosine *versus* cytidine in Fig. 9). A comparison of binding between alkyl-*cis*-diols and aryl-*cis*-diols to the boronate matrices indicates that the aryl affinity molecules not only form a complex but they do so very effectively (compare results in Fig. 9 to those in Fig. 10). The nervous system amines, such as L-dopa, epinephrine and nore-pinephrine, form a stable complex at pH 5.0. This complex has a far greater binding capacity at pH 5.5 than at pH 5.0 (Fig. 11). Again, the binding capacity of these molecules on sPBA matrix is far less than observed on 4nsPBA (except for L-dopa for some unknown reason). The results with immobilized matrices are in good agreement with those observed derived from studies in solution by ^{11}B NMR spectroscopy (compare Fig. 7 with Fig. 11).

Boronate ligand synthesis. Synthesis and recovery of the nitro isomers are influenced by the reaction conditions. Though the net yield of nitro derivatives remains approximately constant (26 mol% of the starting material, sPBA), three isomers are formed in different proportions, depending on the reaction method used. A preferential substitution of the nitro group in the *ortho* position of the boronic acid is apparently caused by coordination with a pair of electrons of the oxygen atom (acetic anhydride) to the electron-deficient boron atom (boronic acid), thus resulting in an enhanced electrophilicity of the *ortho* carbon [60]. [The ionization constant of the *para*-nitro derivative (pK_a 7.2) is slightly less than that of the *ortho*-nitro derivative (pK_a 7.4–7.6).]

Significance. The significance of this work lies in the demonstration that the best environment for the ligand–solute interaction can be determined by studies in solution, without prior immobilization of the ligand. The results derived from in-solution studies and those from the affinity columns are in very good agreement. The new nitrophenylboronate matrix offers enhanced binding of most affinity molecules over those examined with phenylboronate matrix. In addition, the new matrix offers chromatographic separations of alkali-unstable biomolecules. The results further demonstrate that significant differences in capacity factors can be caused by small differences in the structure of the affinity molecules.

ACKNOWLEDGEMENT

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