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FAB-MS MEASUREMENT OF 2-HYDROXYESTRONE AND MONOSACCHARIDES ASSISTED BY 4-PYRIDINEBORONIC ESTER DERIVATIZATION

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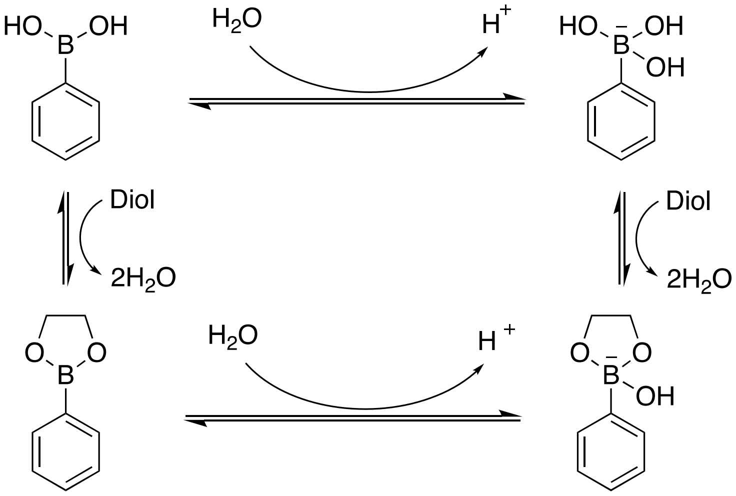
Abstract – Phenylboronic acid (PBA) reacts specifically with diol compounds such as sugars. Compared with general derivatization, boronic ester derivatization has the advantage of being a simple procedure and requires fewer resources for sample pretreatment. To improve the detection sensitivity and selectivity for diol compounds in mass spectrometry, we investigated the effect of boronic ester derivatization of diol compounds on the detection sensitivity. As a result, several proton-affinitive boronic acids were examined, and the peak intensities of the ions associated with 2-hydroxyestrone were observed with 4-pyridineboronic acid as the reagent. The 4-pyridineboronic ester derivatization approach was found to be a useful labeling method for FAB-MS measurements due to the proton and diol affinity from the heterocyclic moiety.

INTRODUCTION

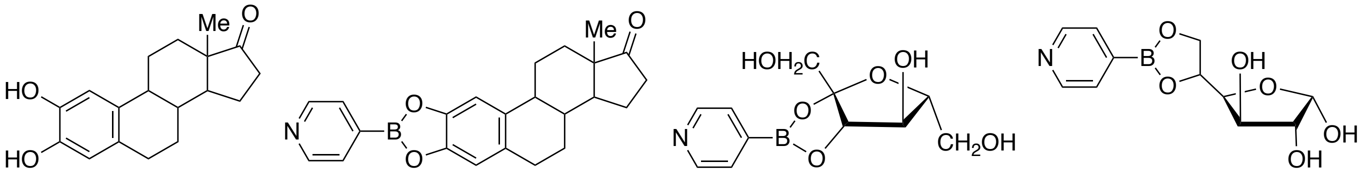
Phenylboronic acid (PBA) forms cyclic esters with diol compounds such as sugars in aqueous solution (Scheme 1).[1] PBA and its analogs have been widely used as sensors to detect diol compounds by colorimetric analysis,[2-4] fluorescence measurements,[5-8] and electrochemical detection.[9-11] In addition, new materials synthesized based on boronic acid have been investigated as biochemical tools for various applications. For example, polymers that function as insulin autoregulation systems,[12,13] glucose-responsive nanofilms,[14,15] nanoparticles for tumor integration,[16] and solid-phase extraction substrates that improve the concentration efficiency of glycoproteins and glycopeptides[17] have been developed. In addition, we have previous synthesized a heterocyclic boron compound and reported the synthesis of primin with antiproliferative activity against M109 murine lung cancer cells (IC_{50} = 10 ug/mL) and A2780 human ovarian cancer cells (IC_{50} = 2.9 ug/mL)[18] via a *B*-alkyl Suzuki-Miyaura cross-coupling reaction.[19a] As a developmental study, we have applied boronic acid esters as protecting groups for the facile total synthesis of violaceoids A and C.[19b]



Scheme 1. Complexation reaction of PBA with diols

In this study, we focused on derivatization using modified PBAs, heterocyclic boronic acids, and arylboronic acids to develop boronic acid reagents for high-sensitivity detection of diols by mass spectrometry. Mass spectrometry has been used alone or in combination with chromatography and electrophoresis for simultaneous analysis of biological samples and quantification of pharmaceuticals. Liquid chromatography-mass spectrometry (LC/MS) is widely used for metabolomics targeting proteins and sugar chains because the method provides high-sensitivity analysis and spectral information useful for identifying molecular structures. We hoped to use boronic acid derivatization with LC/MS for more reliable identification and quantification of substances with low ionization efficiency, such as sugars and lipids. However, because boronic esterification is a reversible reaction, it is not stable in aqueous solution, and there are few examples of pre-column derivatization with boronic acid in LC/MS analysis.[20] To perform high-sensitivity analysis while suppressing the degradation of boronic esters, online derivatization systems, such as post-column derivatization[21] and ambient desorption ionization,[22-24] have been investigated. These methods require a dedicated system, which limits the installation of equipment and the simplicity of the measurement. Therefore, we examined analytical procedures for using boronic ester derivatization with existing mass spectrometry systems while suppressing boronic ester degradation. Boronic esters are often optimized for measuring protonated molecular ions ($[M + H]^+$) or metal adduct ions in positive mode, and deprotonated molecular ions ($[M - H]^-$) in negative mode. Due to the principles of mass spectrometry, negative-mode detection is less sensitive than positive-mode detection, and this lower sensitivity is unfavorable for analysis.[25] Therefore, developing boronic acid reagents that can be detected in positive mode is expected to lead to measurements with high sensitivity and low noise.

In this study, we focused on using boronic acids to derivatize diol compounds and we developed labeling reagents for mass spectrometry. First, we used 2-hydroxyestrone (2-OHE1) (**1**) as a model diol, and we evaluated the FAB-MS spectra of 2-OHE1 (**1**)-PBA ester derivatized with various boronic acid reagents. For the boronic acid with the best performance, the matrix used for the measurement was optimized. The peak intensities of cations associated with 2-OHE1 (**1**) in the FAB-MS spectrum were increased 3.4-fold by 4-pyridineboronic acid (4-PyBA) esterification [2-OHE1 (**1**)-4-PyBA ester **2**]. Finally, the wider applicability of this method was investigated using D-(-)-fructose-4-PyBA ester **3** and D-(+)-glucose-4-PyBA ester **4** (Figure 1).



2-OHE1 (**1**) 2-OHE1 (**1**)-4-PyBA ester **2** D-(-)-Fructose-4-PyBA ester **3** D-(+)-Glucose-4-PyBA ester **4**

Figure 1. Structures of 2-OHE1 (**1**) and its 4-pyridineboronic acid (4-PyBA) ester derivative **2**-**4**

RESULTS AND DISCUSSION

Steroid molecules usually have low ionization efficiencies, so chemical derivatization is widely used to improve the detection sensitivity in mass spectrometry.[26] Chemical derivatization of steroid compounds involves using permanently charged reagents or proton affinity reagents, which are more likely to be detected as ions associated with their respective molecular weights. Usually, chemical derivatization protocols lead to an increase analytical cost, but boronic ester derivatization is performed in one simple step, consisting of mixing reagents in solution. In this study, we used 2-OHE1 (**1**), which has a relatively high ionization efficiency for a steroid, as a model compound to investigate the effect of various boronic acid reagents on the peak intensities of steroid cations.

FAB-MS is a soft ionization method that provides the molecular weight information for a sample in the molecular weight-related peaks. The method is characterized by its short analysis time and ability to measure both solid and liquid samples. Therefore, we used FAB-MS because it is suitable for measuring a sample mixture containing boronic acid reagents directly. Figure 3 shows the FAB-MS spectra of sample solutions containing 2-OHE1 (**1**) and 4-PyBA (1 mM each in methanol) measured with 3-nitrobenzyl alcohol (NBA). The spectrum with 4-PyBA present [Figure 2(a)] contained peaks for the 2-OHE1 (**1**) molecule cation (M^+, *m/z* 286), the protonated molecular ion of the ester derivative with loss of two water molecules from 2-OHE1 (**1**) and 4-PyBA ($[M + H]^+$, *m/z* 374), and the NBA adduct protonated molecular ion of the ester derivative ($[M + H + NBA]^+$, *m/z* 526). The relative intensities of the peaks for 2-OHE1 (**1**) (*m/z* 286) and its 4-PyBA ester **2** (*m/z* 374) to the base peak were 22% and 79%, respectively. In contrast, in the spectrum without 4-PyBA [Figure 2(b)], the relative intensities of *m/z* 286 and 374 were 68% and 1%, respectively. These results suggest that the *m/z* 374 peak originated from the addition of 4-PyBA to 2-OHE1 (**1**). The esterification by 4-PyBA is a reversible reaction, and unreacted 4-PyBA and 2-OHE1 (**1**) coexisted in the sample solutions. As expected, we were not able to isolate and purify **2**, but we succeeded in proving the formation of boronic acid ester in the sample solution from FAB-MS analysis data.

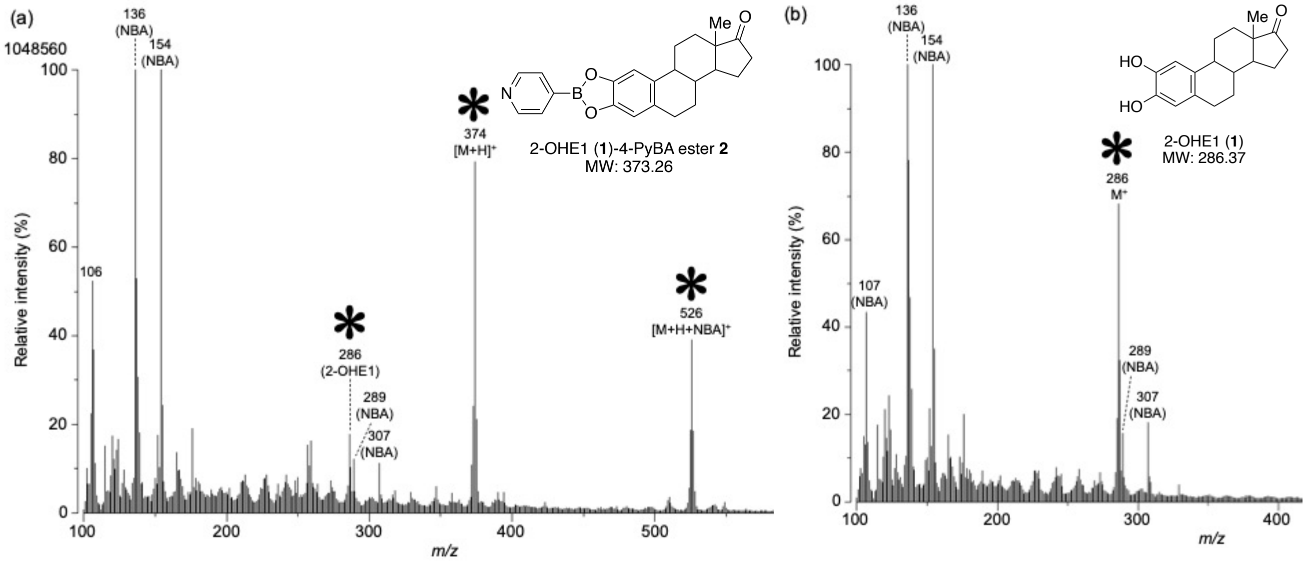
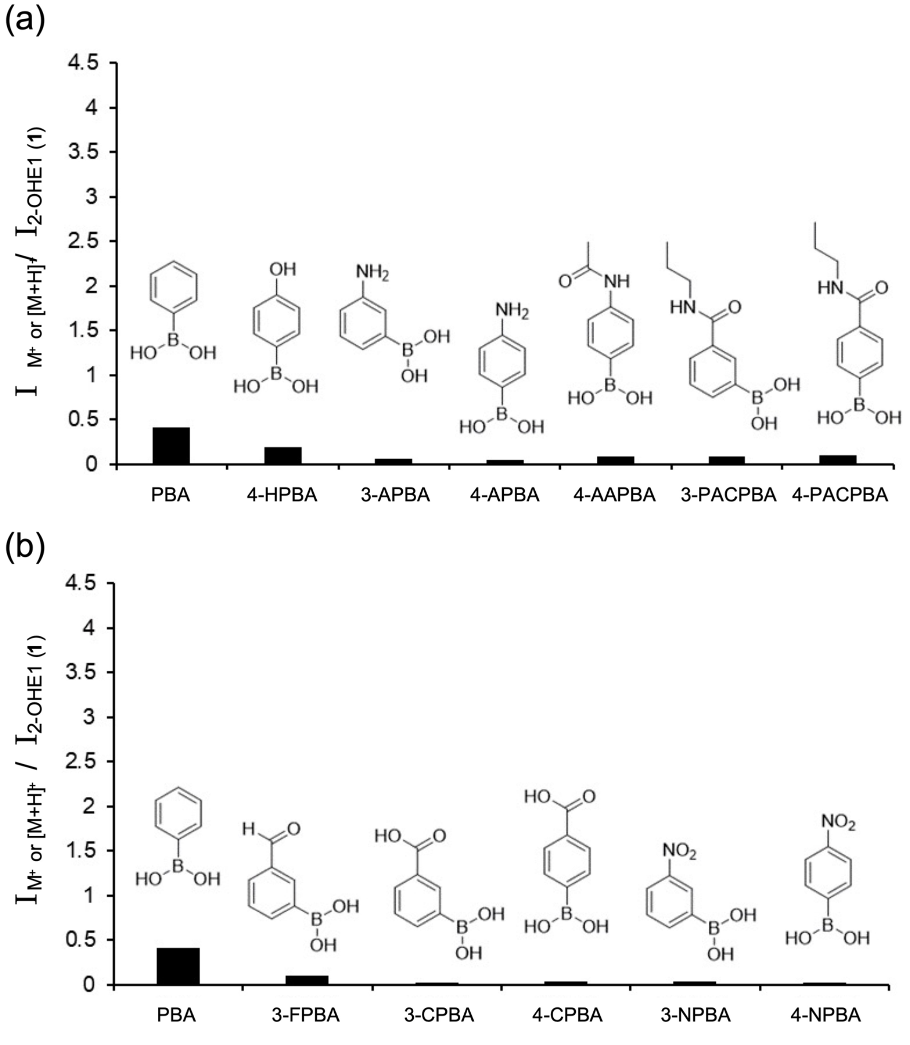


Figure 2. Positive FAB-MS spectra of 2-OHE1 (**1**) in the (a) presence and (b) absence of PBA (matrix: NBA)

Similarly, FAB-MS measurements of 2-OHE1 (**1**) were performed using 17 boronic acid reagents under the same experimental conditions to investigate the effect of each on the peak intensity of esterified 2-OHE1 (**1**). The peak intensities of the boronic ester derivatives were evaluated by the relativdde intensity (%) of the boronic ester molecule cations (M^+) or the protonated molecular ions of the ester ($[M + H]^+$) to the base peak in each mass spectrum. The relative intensity of the ester formed by the reaction of 2-OHE1 (**1**) with unmodified PBA (M^+, *m/z* 372) to the base peak was 33%. Figures 3(a) and 3(b) show that the peak intensities of the esters formed with boronic acids bearing electron-donating groups were larger than with those bearing electron-withdrawing groups. The 4-PyBA ester ($[M + H]^+$, *m/z* 374) had the highest relative intensity of 79% [Figure 3(c)], suggesting that the ionization efficiency of 4-PyBA was higher than that of other modified boronic acid reagents. The proton affinities of benzene, pyridine, pyrimidine, naphthalene, and ferrocene are 179.3, 222.3, 211.7, 191.9, and 206.4 kcal/mol, respectively (NIST Chemistry Webbook, https://webbook.nist.gov/). Therefore, the presence of the pyridine moiety increased the sensitivity of positive-mode detection. However, for the ester derivative with 3-pyridineboronic acid (3-PyBA), a pyridineboronic acid, the relative intensity of the peak was 9%. The rate constant for the complexation reaction of boronic acid with diol compounds can be estimated from the acid dissociation constant (pK_a^B) of the boronic acid moiety.[27] Experiments with hinokitiol showed that the pK_a^B of pyridineboronic acids and PBA were 4-PyBA < 3-PyBA < PBA, suggesting that the binding of 4-PyBA to diols proceeds favorably at neutral pH. In addition, the acid dissociation of pyridineboronic acid was retained even when substituents were introduced on the pyridine nitrogen, suggesting that molecules based on pyridineboronic acid will be useful in developing labeling reagents that are more specific. For example, in desorption electrospray ionization experiments for the sensitive detection of glucose, *N*-methyl-4-pyridineboronic acid with a permanently charged group increases ionization efficiency.[22]

The peak intensities of 2-OHE1 (**1**)-4-PyBA ester **2** derivatives showed a large variation from one measurement to another, and the peak intensity of $[M + H]^+$ (*m/z* 374) measured three times was 52.52 ± 26.26%. The variation in the peak intensities of the ester derivatives may be caused not only by sampling errors, but also by the FAB-MS measurement protocol using a mixed solution without purifying the ester. The absolute amount of the ester derivative introduced into the MS is expected to vary in a sample solution in which unreacted boronic acid and 2-OHE1 (**1**) coexist. In the future, stable isotope-labeled internal standards could be used to make the method quantitative.



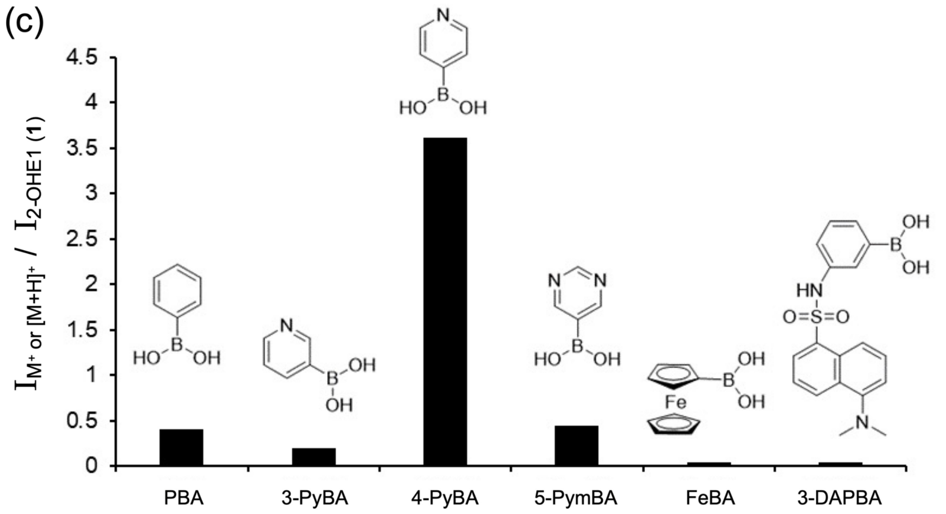


Figure 3. Peak intensities of ions associated with the molecular weight of 2-OHE1 (**1**) boronic esters in positive FAB-MS spectra (matrix: NBA). (a) Boronic acids with electron-withdrawing substituents, (b) boronic acids with electron-donating substituents, and (c) heterocyclic or other arylboronic acids.

Next, we optimized the analytical conditions for 4-PyBA, which was the best-performing boronic acid in this study. Nine matrices were used to measure sample solutions containing 2-OHE1 (**1**) and 4-PyBA (1 mM each in methanol) by FAB-MS. The peak intensities of the 2-OHE1 (**1**)-4-PyBA ester **2** (*m/z* 374) were compared in the mass spectra measured with different matrices (Figure 4). In the spectra using NBA and 2,2′-dithiodiethanol (DTDE), the relative intensities of *m/z* 374 to the base peak were as high as 79% and 73%, respectively. Because NBA is a monoalcohol and the two hydroxyl groups in DTDE are separated, the stability of the ester derivative was higher than for the other matrices.

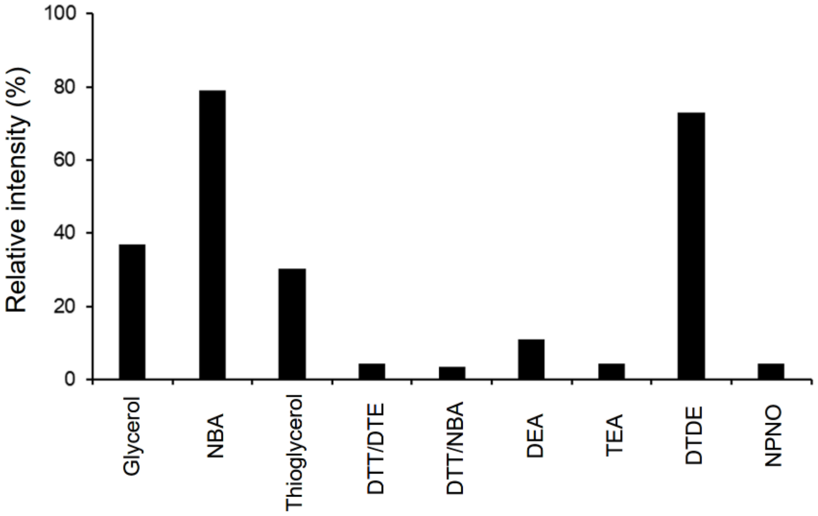


Figure 4. Comparison of peak intensities of the 2-OHE1 (**1**)- 4-PyBA ester **2** derivative (*m/z* 374) in mass spectra using various matrices

To investigate the optimal concentration of boronic acid reagents, FAB-MS measurements were performed on mixtures of 2-OHE1 (**1**) (0.1 mM in methanol) with 4PyBA (0.1, 0.2, 0.5, and 1.0 mM in methanol). We compared the peak intensities of the 2-OHE1 (**1**)-4-PyBA ester **2** (*m/z* 374) in the spectra using NBA and DTDE as matrices. The relative intensities of *m/z* 374 to the base peak with both NBA [Figure 5(a)] and DTDE [Figure 5(b)] spectra did not change substantially with the 4-PyBA concentration. In FAB-MS measurements, it was sufficient to add an equimolar amount of 4-PyBA to 2-OHE1 (**1**). The solubility of 4-PyBA in methanol is poor and the solution becomes a suspension at a concentration of 1 mg/mL.

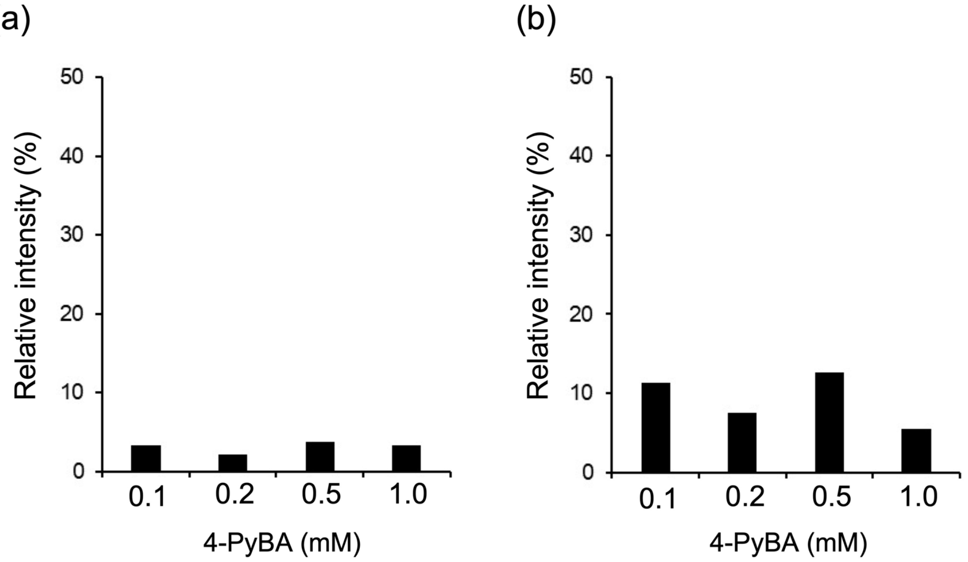


Figure 5. Peak intensities of the 2-OHE1 (**1**)-4-PyBA ester **2** derivative (*m/z* 374) at different 4-PyBA concentrations. Matrix of (a) NBA and (b) DTDE.

Finally, we investigated the suitability of 4-PyBA ester derivatization for D-(-)-fructose and D-(+)-glucose. In general, sugar molecules do not have chromophores and have no easily protonated or deprotonated sites, and thus their detection sensitivity is low for mass spectrometry. Hence, it is necessary to derivatize sugars and glycation products for high-sensitivity mass spectrometry.[25,28] In this study, we investigated whether the addition of 4-PyBA would increase the peak intensities of sugar molecules in FAB-MS measurements. Figure 6 shows the FAB-MS spectra of the mixture containing D-(-)-fructose and 4-PyBA (1 mM each in 90% methanol). The D-(-)-fructose cations (M^+, *m/z* 180) and the protonated molecular ion of the D-(-)-fructose-4-PyBA ester **3** ($[M + H]^+$, *m/z* 268) were observed [Figure 6(a)]. The relative intensities of *m/z* 180 and 268 were 3% and 22%, respectively. In contrast, in the FAB-MS spectrum of the solution containing only D-(-)-fructose [Figure 6(b)], the relative intensity of D-(-)-fructose (M^+, *m/z* 180) was 10%. These results suggest that the addition of 4-PyBA to D-(-)-fructose improves the detection sensitivity in FAB-MS, as in the case of 2-OHE1 (**1**). We conducted similar FAB-MS measurements using solutions containing D-(+)-glucose (Figure 7). In the FAB-MS spectrum of the mixture containing D-(+)-glucose and

4-PyBA (1 mM each in 90% methanol) [Figure 7(a)], the D-(+)-glucose cation (M^+, *m/z* 180) and the protonated molecular ion of D-(+)-glucose-4-PyBA ester **4** ($[M + H]^+$, *m/z* 268) were observed. However, the relative intensities of *m/z* 180 and 268 were 7% and 5%, respectively. In addition, there was no change in the relative intensity of D-(+)-glucose (*m/z* 180) in the absence of 4-PyBA [Figure 7(b)]. Lorand et al.[29] reported that the binding affinity of D-(+)-glucose and PBA is smaller than that of D-(-)-fructose. Therefore, the binding of D-(+)-glucose to 4-PyBA may be weaker than that to D-(-)-fructose, as well as to PBA. The results from the FAB-MS spectra in this study are consistent with the nature of the binding between sugar molecules and boronic acid reagents.

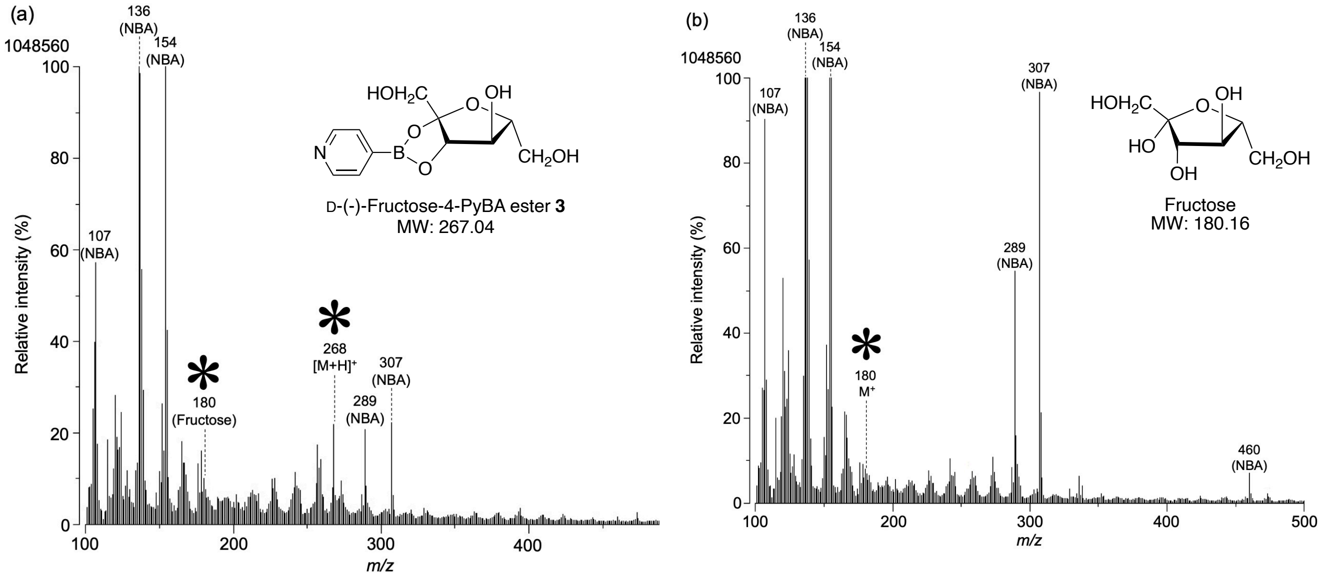


Figure 6. Positive FAB-MS spectra of D-(-)-fructose in the (a) presence and (b) absence of 4-PyBA (matrix: NBA)

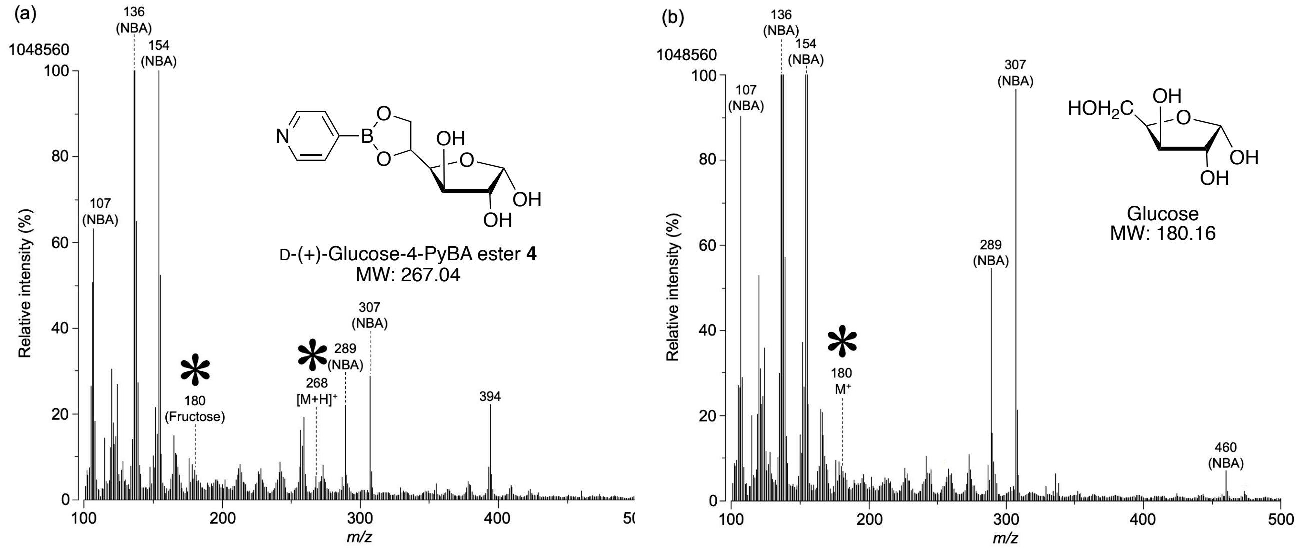


Figure 7. Positive FAB-MS spectra of D-(+)-glucose in the (a) presence and (b) absence of 4-PyBA (matrix: NBA)

To characterize the 4-PyBA ester derivatization in mass spectrometry, we performed similar mass spectrometry measurements using an ionization method other than FAB. Positive electron ionization-mass spectrometry (EI-MS) of a mixture containing D-(-)-fructose and 4-PyBA (1 mM each in 90% methanol) detected the cations of D-(-)-fructose ($[C_6H_{12}O_4]^+$, *m/z* 148) and its 4-PyBA ester **3** (M^+, *m/z* 267), and several fragment ions were identified. However, the peak intensity of the D-(-)-fructose-4-PyBA ester **3** (*m/z* 267) detected by EI-MS was 32% of the intensity of that (*m/z* 268) detected by FAB-MS. In the EI-MS spectrum, the peak intensity of the fragment ion of D-(-)-fructose (*m/z* 148) was large, but unreacted D-(-)-fructose coexisted in the sample solution. Hence, EI-MS is not suitable for detecting 4PyBA esters, and the detection sensitivity is insufficient. In addition, positive chemical ionization-mass spectrometry (CI-MS) using isobutane was used to measure the mixture of D-(-)-fructose and 4-PyBA. In the CI-MS spectrum, the peak intensity of the D-(-)-fructose 4-PyBA ester **3** ($[M + H]^+$, *m/z* 268) was 11% of the intensity of that (*m/z* 268) in the FAB-MS spectrum. These results suggest that FAB-MS is suitable for analyzing 4-PyBA ester derivatives **2**-**4**. To investigate the peak intensity in electrospray ionization (ESI-MS), which is commonly used in LC/MS, positive ESI-MS measurements of a mixed solution containing 2-OHE1 (**1**) and 4-PyBA (in 90% methanol) were performed. However, few peaks associated with the 2-OHE1 (**1**)-4-PyBA ester **2** were detected. To use boronic acid ester derivatization in LC/MS analysis, it is necessary to improve the binding stability of the ester in the mobile phase.

CONCLUSIONS

We showed that 4-PyBA ester derivatization is suitable for FAB-MS measurements of compounds containing diols. PBA and its analogs are organic molecules; thus, the structure of these compounds can be tailored. Our findings support further improvement of the stability and ionization efficiency of boronic ester derivatives. Furthermore, we expect that the detection of specific biomolecules with the 4-PyBA ester derivatives could be achieved by designing the binding configuration of the 4-PyBA ester derivatives for compounds that have multiple diols suitable as boronic acid binding sites.

EXPERIMENTAL

MATERIALS:

PBA, D-(-)-fructose, and D-(+)-glucose were purchased from Nacalai Tesque (Kyoto, Japan). 4-Hydroxyphenylboronic acid (4-HPBA), 3-aminophenylboronic acid monohydrate (3-APBA•H_2O), 4-acetamidephenylboronic acid (4-AAPBA), 3-nitrophenylboronic acid (3-NPBA), 4-nitrophenylboronic acid (4-NPBA), and 5-pyrimidylboronic acid (5-PymBA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 3-Formylphenylboronic acid (3-FPBA), 3-carboxyphenylboronic acid (3-CPBA), 4-carboxyphenylboronic acid (4-CPBA), 3-pyridineboronic acid (3-PyBA), and 4-PyBA were purchased from FUJIFILM Wako Chemicals (Osaka, Japan). 3-[(*N*-Propylamino)carbonyl]phenylboronic acid (3-PACPBA) and 4-[(*N*-propylamino)carbonyl]phenylboronic acid (4-PACPBA) were purchased from Combi-Blocks (San Diego, CA, USA). 4-Aminophenylboronic acid hydrochloride (4-APBA•HCl), ferroceneboronic acid (FeBA), and 3-(dansylamino)phenylboronic acid (3-DAPBA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was produced by a Millipore Milli-Q water purification system (Milli-Q Integral 3, Burlington, MA, USA). 2-OHE1 (**1**) was purchased from Steraloids (Newport, RI, USA).

FAB-MS MEASUREMENT:

FAB-MS spectra were measured using a mass spectrometer (JMS-700V, JEOL Tokyo, Japan). All mass spectra of the sample solutions were measured in positive mode. The matrices were JEOL FABMS Matrix Kit glycerol, 3-nitrobenzyl alcohol (NBA), α-thioglycerol, Magic Bullet (dithiothreitol (DTT)/dithioerythritol (DTE), 3:1), YOKUDEL-FAB-Matrix (DTT/NBA, 1:1), diethanolamine (DEA), triethanolamine (TEA), 2,2′-dithiodiethanol (DTDE), or 2-nitrophenyl *n*-octyl ether (NPNO). The samples were mixed with the boronic acid reagent in methanol (MeOH) for 2-OHE1 (**1**), or 90% MeOH (in distilled water, pH 7) for sugars, and then introduced directly into the mass spectrometry.

ACKNOWLEDGEMENTS

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