3-[(1-[[4-(5,6-Dimethoxy-1-oxoisindolin-2-yl)-2-methoxyphenyl]sulfonyl]-pyrrolidin-2-yl)carbonylamino]phenylboronic Acid as a Fluorescent Labeling Reagent for Determination of Diol Compounds by HPLC

Isao TERADO, Hirofumi INOUE, Yoshie TAO, and Yasuto TSURUTA[†]

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University,
Fukuyama, Hiroshima 729-0292, Japan

(Received April 20, 2000; Accepted May 31, 2000)

Boronic acids react with some diol compounds to form cyclic boronates.^{1,2} Various labeling reagents containing boronic acid have been used for measuring diol compounds by HPLC. These include naphthaleneboronic acid (NTB),³ ferroceneboronic acid (FBA),⁴ dansylaminophenylboronic acid (Dansyl-APB),^{5,6} 9-phenanthreneboronic acid (PTB)⁷ and 1-cyanoisindole-2-*m*-phenylboronic acid (CIPB).⁸ The derivatives of diol compounds labeled with NTB were detected by a UV detector, those with FBA by an electrochemical detector, and those with Dansyl-APB, PTB and CIPB by a fluorescence detector. However, the sensitivities of these reagents were too low to measure low concentrations of diol compounds.

Some phenylphthalimides (2-phenylisindoline-1-one derivatives) which are highly fluorescent are useful as fluorophores in fluorescent labeling reagents.⁹⁻¹² Recently, we developed a fluorescent labeling reagent, 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl),¹¹ for determination of amino acids, and showed that the derivative of proline labeled with DMS-Cl was highly fluorescent. In this study, as a fluorescent labeling reagent for diol compounds, we developed 3-[(1-[[4-(5,6-dimethoxy-1-oxoisindolin-2-yl)-2-methoxyphenyl]sulfonyl]pyrrolidin-2-yl)carbonylamino]phenylboronic acid (DMPB) which is a boronic acid derivative incorporating the derivative of proline with DMS-Cl as a fluorophore.

Experimental

Chemicals and solvents

All chemicals were of analytical-reagent grade, unless stated otherwise. DMS-Cl was prepared as described previously.¹¹ *m*-Aminophenylboronic acid, 2,4-dimethyl-2,4-pentanediol, 1,3-butanediol, 1,4-butanediol, 2,3-butanediol and 2-methyl-2,4-pentanediol were purchased from Tokyo Kasei (Tokyo, Japan). 2,3-Dimethyl-2,3-butanediol, ethylene glycol, cyclohexanediol, 2,4-pentanediol, DL-tartaric acid and acetonitrile (HPLC-grade) were obtained from Wako (Osaka, Japan). Mephensin and pinane-2,3-diol were from Sigma (St. Louis, MO, USA). Organic solvents except acetonitrile were of the highest purity available. Benzene was purified by distillation prior to use. Deionized-distilled water was purified with the Milli-QII

system (Yamato, Tokyo, Japan) prior to use.

Synthesis of and analytical data for 3-[(1-[[4-(5,6-dimethoxy-1-oxoisindolin-2-yl)-2-methoxyphenyl]sulfonyl]pyrrolidin-2-yl)carbonylamino]phenylboronic acid (DMPB)

L-Proline (222 mg, 1.92 mmol) in borate buffer (pH 9.0, 100 mM, 30 ml) and DMS-Cl (383 mg, 0.96 mmol) in acetone (100 ml) were mixed and stirred at 50°C for 30 min. After evaporation of acetone under reduced pressure, the mixture was acidified with concentrated hydrochloric acid (200 µl) to precipitate the derivative of proline with DMS-Cl. The derivative was recrystallized from methanol. To the solution of the derivative (216 mg) in chloroform (100 ml) was added thionyl chloride (9 ml), and the mixture was refluxed for 2 h. After evaporation of the solvent, distilled benzene (40 ml) was added to the residue to obtain the precipitates. The precipitates were filtered with a glass filter and washed with distilled benzene. The product (213 mg, 0.43 mmol) in acetone (100 ml) and *m*-aminophenylboronic acid (73 mg, 0.47 mmol) in acetone (30 ml) were mixed and stirred at room temperature for 2 h. After evaporation to dryness, the residue was recrystallized from methanol to obtain DMPB as white needles (yield 84 mg, m.p. 203–208°C). Analysis: calculated for C₂₈H₃₀N₃O₉SB (1/2·H₂O), C 55.91, H 5.14, N 6.98; found, C 55.98, H 5.11, N 6.89%. EI-MS (*m/z*): 551 ([M-B(OH)₂]⁺). ¹H NMR (δ, ppm, in CDCl₃): 1.72–4.53 (7H, m, H of pyrrolidine ring), 3.86, 3.90 and 4.00 (3H each, s each, OCH₃ each), 4.97 (2H, s, CH₂ of isindoline-1-one), 7.26 (2H, s, aromatic H of isindoline-1-one), 7.27 (1H, t, H of phenylboronic acid, *J* = 7.34 Hz), 7.49 and 7.76 (1H each, d each, H of phenylboronic acid, *J* = 7.34 Hz each), 7.52 and 7.83 (1H each, d each, H of benzene ring, *J* = 8.80 Hz each), 7.85 (1H, s, H of phenylboronic acid), 7.93 (1H, s, H of benzene ring), 8.21 (2H, s, -B(OH)₂), 9.81 (1H, s, -CONH-).

Labeling procedure for diol compounds

A test solution of diol compounds (in acetonitrile, 20 µl) and DMPB (1 mM in 1% pyridine/acetonitrile, 100 µl) was mixed well and allowed to stand for 40 min at 120°C. After cooling, the reaction mixture was subjected to TLC. For HPLC analysis, the reaction mixture was diluted 10-fold with acetonitrile and an aliquot (20 µl) of the resulting mixture was subjected to HPLC.

TLC conditions

A silica gel plate was used with benzene-acetone (70:30, v/v) as a solvent and the spot was detected by irradiation of UV (254

[†] To whom correspondence should be addressed.
E-mail: tsuruta@fupharm.fukuyama-u.ac.jp

Fig. 1 The structure of DMPB.

nm and 365 nm).

HPLC conditions

The HPLC system consisted of two BIP-I HPLC pumps (Jasco, Tokyo, Japan), an GP-A50 gradient programmer (Jasco), a 880-51 2-line degasser (Jasco), a Rheodyne Model 7125 injector (20 μ l loop), an FS-8010 fluorescence detector (Tosoh, Tokyo, Japan) and a C-R6A recorder (Shimadzu, Kyoto, Japan). A TSK-gel ODS-80Ts (4.6 $\times$ 150 mm, i.d., 5 μ m, Tosoh, Tokyo, Japan) connected with a TSK-guardgel ODS-80Ts (3.2 $\times$ 15 mm, i.d., Tosoh) as a guard column was used at 10°C. The column temperatures were controlled using a water bath, an ECS-1 eyela cooler (Eyela, Tokyo, Japan) and a Dx-80 thermo minder (Titec, Tokyo, Japan). An isocratic elution with water-acetonitrile (35:65, v/v) was used at a flow rate of 1.0 ml/min. The fluorescence intensities were monitored at 388 nm with an excitation wavelength of 314 nm.

Results and Discussion

Synthesis of DMPB

DMS-Cl reacted with amino compounds to produce fluorescent derivatives. However, the derivatives of aromatic amines (e.g., *p*-aminophenylboronic acid) labeled with DMS-Cl fluoresce weakly, while the derivatives of amino acids are highly fluorescent. Therefore, we decided to employ the derivative of proline labeled with DMS-Cl as a fluorophore in the labeling reagent for determination of diol compounds. The labeling reagent, DMPB, was synthesized by the reaction with *p*-aminophenylboronic acid after the chlorination of the derivative of proline with DMS-Cl. The structure of DMPB is shown in Fig. 1.

Reaction of DMPB with diol compounds and fluorescence spectra

The reactions of DMPB with some diol compounds (5 mM, in acetonitrile) were examined by TLC. The fluorescent derivatives of 1,3-butanediol (dihydroxyl component: primary/secondary), 2,3-butanediol, 2,4-pentanediol, DL-tartaric acid (secondary/secondary), pinane-2,3-diol, 2-methyl-2,4-pentanediol (secondary/tertiary), 2,3-dimethyl-2,3-butanediol and 2,4-dimethyl-2,4-pentanediol (tertiary/tertiary) were detected by TLC at a *R_f* value of 0.27, 0.26, 0.31, 0.5, 0.62, 0.58, 0.54 and 0.62, respectively, although those of ethylene glycol, 1,4-butanediol (primary/primary), mephensin (primary/secondary) and cyclohexanediol (secondary/secondary) were not detected. Fluorescence spectra of the derivative of 2,4-dimethyl-2,4-pentanediol labeled with DMPB were measured after extraction with acetonitrile from the spot on the TLC plate. Maximum fluorescence wavelengths were 314 nm for excitation and 388 nm for emission.

HPLC chromatogram

When the reaction mixture was analyzed by a reversed-phase HPLC under the conditions described in the Experimental section, the reaction of the diol compounds having

Fig. 2 Chromatograms of the reaction mixture and reagent blank.

secondary/tertiary and tertiary/tertiary dihydroxyl components with DMPB were confirmed, but the derivatives of other diol compounds were not. These results suggested that the derivatives of both secondary/tertiary and tertiary/tertiary dihydroxyl components were stable, but those of primary/secondary and secondary/secondary dihydroxyl components were hydrolyzed during the separation under the reversed-phase conditions. A typical HPLC chromatogram of the diluted reaction mixture using a standard solution of 2,3-dimethyl-2,3-butanediol, 2-methyl-2,4-pentanediol, 2,4-dimethyl-2,4-pentanediol and pinane-2,3-diol (10 μ M, in acetonitrile) as model compounds is shown in Fig. 2. The peaks due to diol compounds eluted at 8.4, 10.6, 13.5 and 20.5 min, respectively, and completely separated from the peaks due to reagent blank.

The column temperature was considered to affect the stability of some derivatives. The peak due to 2-methyl-2,4-pentanediol decreased to 75% at 25°C, although peaks due to other diol compounds were little affected.

Labeling reaction conditions

The labeling reaction conditions were studied by a reversed phase HPLC using a standard solution of 2,3-dimethyl-2,3-butanediol, 2-methyl-2,4-pentanediol, 2,4-dimethyl-2,4-pentanediol and pinane-2,3-diol. In general, the formation of cyclic boronate proceeded in the presence of a basic catalyst such as pyridine. When the effect of the pyridine concentration on the labeling of diol compounds with DMPB was examined, the maximum peak areas were obtained at concentrations above 1%. The effect of reaction time on the labeling was also tested at various temperatures. As shown in Fig. 3, the labeling reaction for 2,4-dimethyl-2,4-pentanediol proceeded at 120°C and was completed within 40 min. Similar results were obtained with other diol compounds. The reaction mixture was stable for at least a week in the refrigerator.

Precision, calibration curves and detection limits

The precision was tested using a standard solution of 2,3-dimethyl-2,3-butanediol, 2-methyl-2,4-pentanediol, 2,4-dimethyl-2,4-pentanediol and pinane-2,3-diol (10 μ M each). The relative standard deviations ($n = 8$) of peak area were 4.1, 4.5, 3.7 and 4.1%, respectively. The relationships between the peak area and the concentration of diol compounds were linear in the concentration range from 0.5 to 100 μ M. The detection limits (signal-to-noise = 3) were 31, 37, 40 and 50 fmol/injection, respectively.

Fig. 3 Effect of reaction time on the labeling of 2,4-dimethyl-2,4-pentanediol with DMPB at various temperatures. Curves: 1, 70°C; 2, 100°C; 3, 120°C; 4, 150°C.

Comparison with Dansyl-APB

The sensitivity of DMPB was compared with that of Dansyl-APB using a standard solution of 2,3-dimethyl-2,3-butanediol, 2-methyl-2,4-pentanediol, 2,4-dimethyl-2,4-pentanediol and pinane-2,3-diol. Although the labeling reaction conditions of diol compounds with Dansyl-APB employed in the previous paper⁶ was at 70°C for 10 min, the reaction of the above diol compounds with Dansyl-APB were incomplete under the conditions. Therefore, the labeling reaction with Dansyl-APB were carried out at 120°C for 40 min and the derivatives were separated under the present HPLC conditions except for the detection wavelength (excitation: 338 nm; emission: 527 nm). The peak areas of DMPB derivatives of 2,3-dimethyl-2,3-butanediol, 2,4-dimethyl-2,4-pentanediol and pinane-2,3-diol were greater by about 22-, 20- and 17-fold than those of Dansyl-APB derivatives, respectively (retention time: 9.9, 15.5 and 24.5 min, respectively). The derivative of 2-methyl-2,4-pentanediol

with Dansyl-APB could not be measured because the peak overlapped with a peak of reagent blank appeared at 12.5 min. DMPB was stable in a crystalline state for at least a year in a stoppered glass vial at room temperature, while dansyl moiety of Dansyl-APB is said to be light-sensitive.

DMPB is a highly sensitive fluorescent labeling reagent for determination of diol compounds having secondary/tertiary and tertiary/tertiary dihydroxyl components by HPLC. Therefore, DMPB will be useful for the determination of certain physiologically important diol compounds in biological fluids.

References

1. C. F. Poole and A. Zlatkis, *J. Chromatogr.*, **1980**, 184, 99.
2. J. W. Brooks and W. J. Cole, *J. Chromatogr.*, **1987**, 399, 207.
3. K. Gamoh, T. Kitsawa, S. Takatsuto, Y. Fujimoto, and N. Ikekawa, *Anal. Sci.*, **1988**, 4, 533.
4. K. Gamoh, H. Sawamoto, S. Takatsuto, Y. Watanabe, and H. Arimoto, *J. Chromatogr.*, **1990**, 515, 227.
5. T. J. Burnett, H. C. Peebles, and J. H. Hageman, *Biochem. Biophys. Res. Comm.*, **1980**, 96, 157.
6. K. Gamoh, N. Okamoto, S. Takatsuto, and I. Tejima, *Anal. Chim. Acta*, **1990**, 228, 101.
7. K. Gamoh, K. Omote, N. Okamoto, and S. Takatsuto, *J. Chromatogr.*, **1989**, 469, 424.
8. K. Gamoh and S. Takatsuto, *Anal. Chim. Acta*, **1989**, 222, 201.
9. Y. Tsuruta, T. Teranishi, Y. Date, and K. Kohashi, *J. Chromatogr.*, **1993**, 617, 213.
10. Y. Tsuruta, Y. Date, H. Tonogaito, N. Sugihara, K. Furuno, and K. Kohashi, *Analyst* [London], **1994**, 119, 1047.
11. Y. Tsuruta and H. Inoue, *Anal. Biochem.*, **1998**, 265, 15.
12. Y. Tsuruta, S. Kitai, S. Watanabe, and H. Inoue, *Anal. Biochem.*, **2000**, 280, 36.