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Probe molecule equipped with boronic acid moiety as a reversible cross-linking group improves its binding affinity

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ABSTRACT

Syntheses of biotinylated probe molecules of L-glutathione (GSH) equipped with boronic acid moiety and evaluation of their binding affinities against glutathione-*S*-transferase (GST) were described. It revealed that the presence of boronic acid moiety in an appropriate position enhances binding affinity of GSH probe toward GST probably by forming a reversible cross-link. Among prepared, the boronate-containing probe **8b** exhibited the highest recovering ability of GST from *Escherichia coli* cell lysate.

Identification of target proteins of bioactive compounds is one of the most important and attractive issues in chemical genetics study and drug discovery, and affinity purification using synthetic probe molecule designed from the bioactive compound is a powerful method in identifying target protein.[1] Probe molecule having biotin-tag is effective in some cases, but not in the other case in which the binding affinity between the active molecule and their target protein is relatively weak. While, use of photoreactive cross-linking groups[2] such as benzophenone[3] or aryl diazirine[4] sometimes overcome this affinity problem. Thus, UV irradiation to the probe molecule generates a highly reactive species that could react with amino acid residues to form covalent bonding between the probe molecule and its binding protein. However, cross-linking methods are often problematic because of low yielding and unselective binding, and also cross-linked probe molecule increases complexity in mass spectrometric analysis.[5] Here we present synthesis and evaluation of a novel type of probe molecule equipped with boronic acid moiety as a reversible cross-linking group.

In recent years, boronic acid compounds have attracted much attention, especially in the field of medicinal chemistry, because of its characteristic electronic properties.[6] Boron has sp^2-hybridized orbital and strong Lewis acidity because of an unoccupied p-orbital, and it can form a covalent bond with many Lewis bases such as alcohol, amine, thiol, etc. to form an anionic sp^3 boron center. This process resembles the transition state of cleavage of amide bond through hydrolysis, in which sp^2 carbonyl carbon is converted to sp^3 tetrahedral carbon.

We speculated that the boronic acid moiety introduced in probe molecule would form a reversible cross-linking with the neighboring amino acid residue of its binding protein to enhance its affinity (Fig. 1). This would be helpful in detecting and/or purifying target

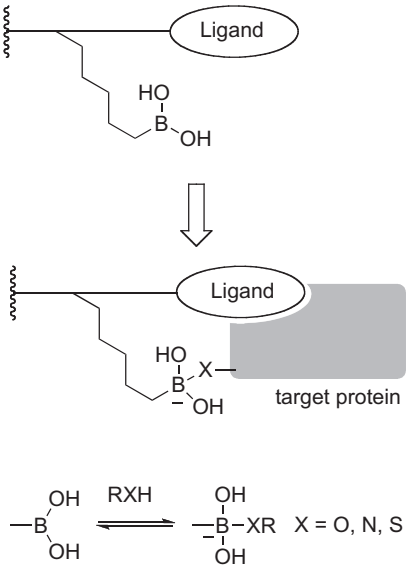

Figure 1. Concept of this work.

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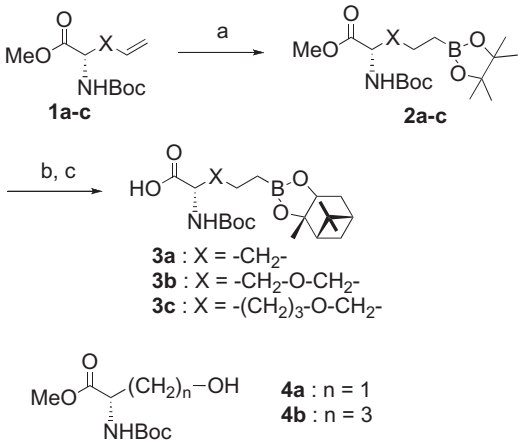
protein, and probe molecules would be easily prepared by using boronic acid-containing building block. As a proof-of-concept experiment, the L-glutathione (GSH)-derived probe molecules equipped with boronic acid moiety were prepared, and their binding affinity to glutathione-*S*-transferase (GST) was evaluated.[7]

Boronic acid-containing building blocks were synthesized as shown in Scheme 1. Iridium-catalyzed hydroboration using pinacolborane[8] against the terminal olefin of the protected allylglycine (**1a**),[9] commercially available unnatural olefinic amino acid, gave a corresponding boronate **2a** in good yield. The methyl ester of **2a** was hydrolyzed by the treatment with 3 equivalent of K_2CO_3, and the concomitantly liberated boronic acid moiety was again protected using (+)-pinanediol[9b,10] to give a desired boronic acid-containing building block **3a** in easy steps. Building blocks having varied chain lengths (**3b** and **3c**) were also synthesized in a similar manner using allyl ethers **1b** and **1c** as starting materials, which were prepared from protected L-serine (**4a**) and 5-hydroxynorvaline (**4b**),[11] respectively, through palladium-catalyzed allylation.[12]

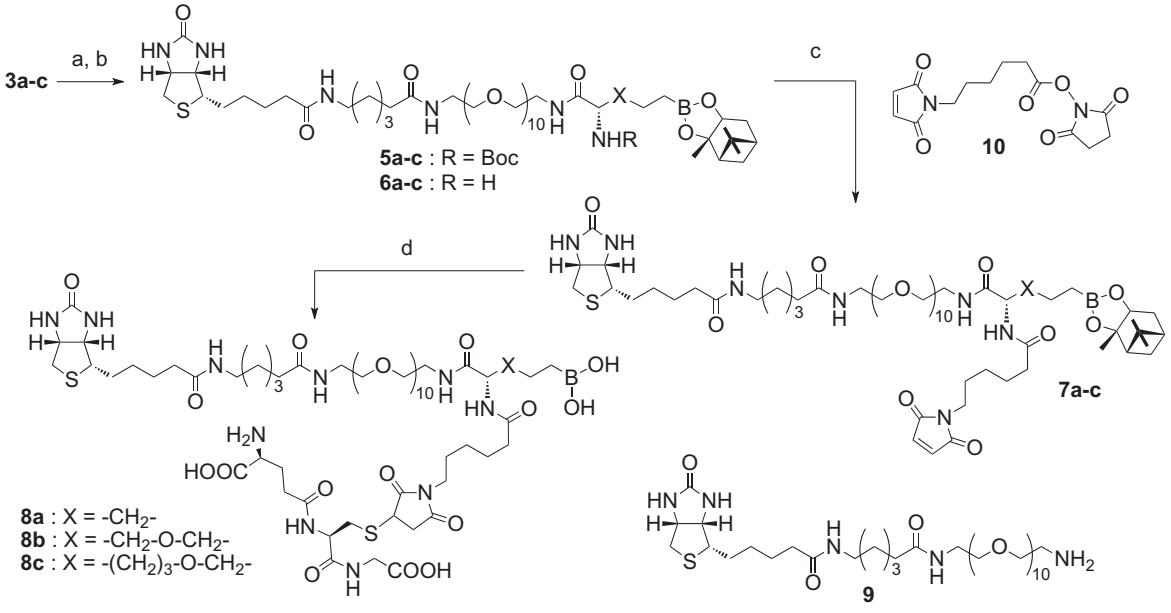


Scheme 1. Reagents: (a) pinacolborane, $[Ir(cod)Cl]_2$, 1,2-bis(diphenylphosphino)ethane, CH_2Cl_2, 55% for **2a**, 76% for **2b**, 49% for **2c**; (b) K_2CO_3 (3 equiv), $MeOH/H_2O$; (c) (+)-pinanediol, $MgSO_4$, Et_2O, two steps 55% for **3a**, 64% for **3b**, 70% for **3c**.

Using these building blocks, biotinylated glutathione probes equipped with boronic acid moiety were prepared by straightforward condensation (Scheme 2). Thus, condensation between the biotinylated PEG linker **9** and **3a–c** using EDCI gave compounds **5a–c** in good yield, respectively. Removal of the Boc group from **5a–c** and subsequent treatment with the maleimide-containing activated ester **10** provided compounds **7a–c**. Then GSH was introduced by Michael addition against the maleimide moiety in **7a–c**, and final deprotection of the boronic acid moiety by transesterification with phenylboronic acid[13] in acidic condition (pH 3) afforded desired boronic acid-containing probe molecules **8a–c**, respectively.[14]

Next, we carried out affinity purification experiment with the synthesized probe molecules.[15] The probes equipped with various length of boronic acid-containing branch were incubated with the cell lysates of *Escherichia coli* overexpressing GST. After further incubation with streptavidin-agarose beads, the beads were washed by lysis buffer with indicated times, and the bound proteins eluted with GSH-containing buffer were analyzed by SDS–PAGE. Figure 2 shows the comparison of the recovering abilities of GST between the probes **11** and **8b**. In the case of using the boronate-lacking probe **11**, substantial amount of the bound GST was leached out with the repeated washing of the agarose gel (lanes 2, 4, and 6). On the other hand, GST was retained by the use of the boronate-containing probe **8b** (lanes 3, 5, and 7). After washing twice, the 1.7-fold recovery of GST by the probe **8b** was observed in comparison with that by **11**, without non-specific binding of other proteins. This result indicates that boronate-containing probe molecule actually showed higher binding affinity with target protein.

Further comparisons between the probe molecules (**8a**, **8b**, **8c**, and **11**) were examined using almost the same method as above, in which the beads were washed twice before eluting bound GST (Fig. 3). It revealed that the boronate-containing probes **8a** (lane 3) and **8b** (lane 4) exhibited 1.4- and 1.7-fold higher recovering abilities of GST from the lysate than the boronate-lacking probe **11** (lane 2), respectively. On the other hand, the probe **8c** (lane



Scheme 2. Reagents: (a) **9**, EDCI·HCl, HOBt, THF/DMF, 68% for **5a**, 69% for **5b**, 61% for **5c**; (b) TFA, CH_2Cl_2, 95% for **6a**, 82% for **6b**, 80% for **6c**; (c) **10**, DMF, 71% for **7a**, 70% for **7b**, 68% for **7c**; (d) L-glutathione, DMF/AcOH; phenylboronic acid, *n*-hexane/H_2O (pH 3), 90% for **8a**, 86% for **8b**, 87% for **8c**.

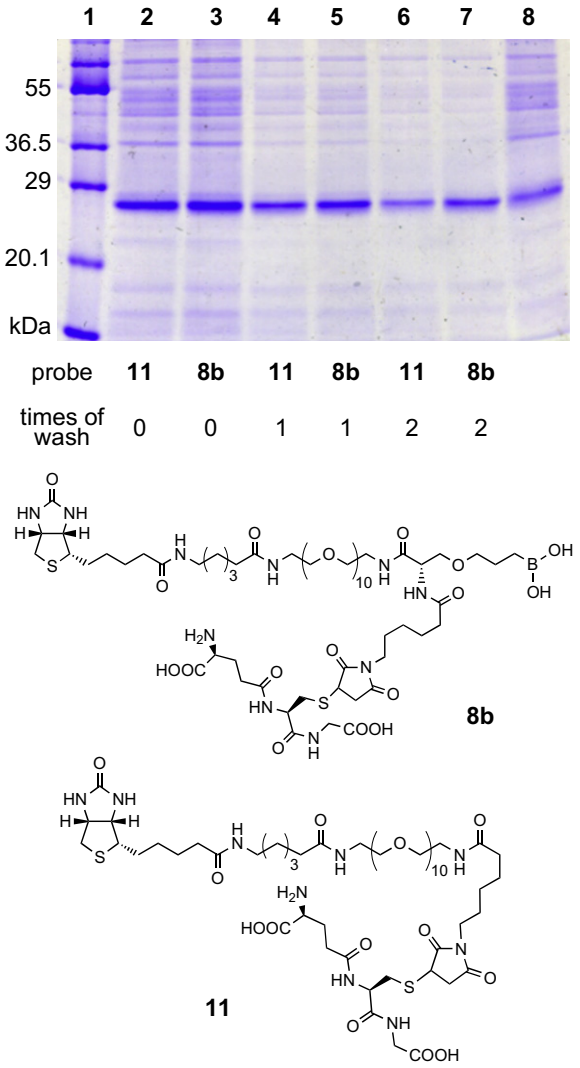


Figure 2. Affinity purification of glutathione-*S*-transferase (GST) using biotinylated probes **11** and **8b**. Lane 1, marker; lane 8, lysate of transformed *E. coli* overexpressing GST.

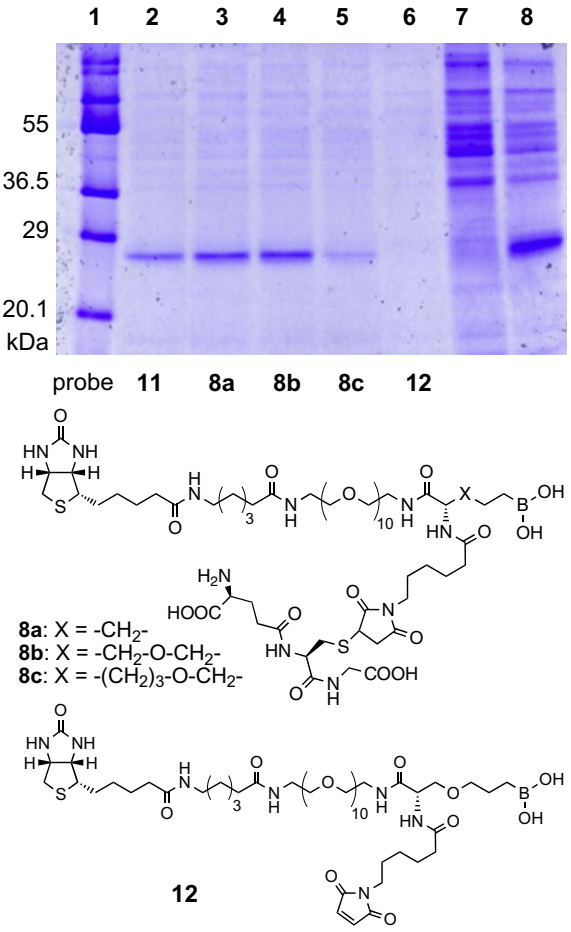


Figure 3. Affinity purification of glutathione-*S*-transferase (GST) using various biotinylated probes. Lane 1, marker; lane 7, lysate of wild-type *E. coli*; lane 8, lysate of transformed *E. coli* overexpressing GST.

5) showed quite less recovering ability (0.4-fold) than **11**. Considering these results, boronic acid moiety enhances binding affinity between probe molecule and its binding protein, when located in appropriate position. Furthermore, almost no GST was recovered by the use of the probe **12** lacking GSH (lane 6), which shows that the higher recovering abilities of the probes (**8a** and **8b**) were not derived from non-specific binding to GST. Although further investigation should be needed, this 'boronate probe' method would be an effective tool for affinity purification.

In summary, we found that introduction of boronic acid moiety enhances binding affinity of the probe molecule toward its target protein. Application of this method to some other bioactive compounds to find their unknown target proteins is in progress.

Acknowledgments

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14. Physical data of the biotinylated probe molecule: **8a**: ^{1}H NMR (500 MHz, in D_2O): δ 4.53–4.50 (1H, m, H-α of Cys), 4.44 (1H, dd, J = 7.5, 4.9 Hz, H-6a of biotin), 4.25 (1H, dd, J = 7.5, 4.1 Hz, H-3a of biotin), 4.05 (1H, t, J = 7.3 Hz, H-α of **3a**), 3.93–3.87 (1H, m, –S–C*H*–), 3.62 (3H, m), 3.54 (36H, m), 3.46 (4H, t, J = 4.3 Hz, –O–C*H*$_2$–CH$_2$–NH–), 3.34 (2H, t, J = 7.0 Hz), 3.30–3.07 (6H, m), 3.02–2.98 (3H, m, –NH–C*H*$_2$–; Hb-β of Cys), 2.86–2.81 (2H, m, Hb-6 of biotin; Ha-β of Cys), 2.61 (1H, d, J = 12.8 Hz, Ha-6 of biotin), 2.58–2.50 (1H, m, Ha of –S–CH–C*H*$_2$–C(O)–), 2.37 (2H, t, J = 6.5 Hz, H-γ of Glu), 2.16–2.09 (6H, m, –C(O)–C*H*$_2$–), 2.00 (2H, q, J = 6.5 Hz, H-β of Glu), 1.58–1.09 (22H, m), 0.63 (2H, dd, J = 12.5, 6.4 Hz, –C*H*$_2$–B(OH)$_2$ of **3a**). FABMS: *m/z* 1562 (M+glycerol+Na−2H$_2$O)$^+$, quasi-molecular ion generated by esterification with glycerol matrix.[16] HR-FABMS:

m/z 1561.7415, calcd for $C_{66}H_{115}{}^{11}BO_{26}N_{10}S_2Na$. Found: 1561.7413. **8b**: ^{1}H NMR (500 MHz, in D_2O): δ 4.56–4.52 (1H, m, H-α of Cys), 4.47 (1H, dd, *J* = 7.9, 4.8 Hz, H-6a of biotin), 4.36 (1H, t, *J* = 5.2 Hz, H-α of **3b**), 4.28 (1H, dd, *J* = 7.9, 4.5 Hz, H-3a of biotin), 3.95–3.88 (1H, m, –S–C*H*–), 3.68–3.47 (39H, m), 3.40–3.34 (4H, m), 3.30–3.11 (12H, m), 3.06–2.99 (3H, m, –NH–CH_2–; Hb-β of Cys), 2.89–2.83 (2H, m, Hb-6 of biotin; Ha-β of Cys), 2.64 (1H, d, *J* = 12.8 Hz, Ha-6 of biotin), 2.59–2.52 (1H, m, Ha of –S–CH–CH_2–C(O)–), 2.42–2.38 (2H, m, H-γ of Glu), 2.18–2.09 (6H, m, –C(O)–CH_2–), 2.03 (2H, q, *J* = 7.0 Hz, H-β of Glu), 1.57–1.36 (14H, m), 1.28–1.14 (6H, m), 0.62 (2H, t, *J* = 7.6 Hz, –CH_2–B(OH)$_2$ of **3b**). FABMS: *m/z* 1570 (M+glycerol+H−2H_2O)$^+$. HR-FABMS: *m/z* 1569.7936, calcd for $C_{67}H_{118}{}^{11}BO_{27}N_{10}S_2$. Found: 1569.8169. **8c**: ^{1}H NMR (500 MHz, in D_2O): δ 4.56–4.52 (1H, m, H-α of Cys), 4.46 (1H, dd, *J* = 7.6, 5.2 Hz, H-6a of biotin), 4.27 (1H, dd, *J* = 7.0, 4.5 Hz, H-3a of biotin), 4.09 (1H, dd, *J* = 8.9, 5.8 Hz, H-α of **3c**), 3.92 (1H, ddd, *J* = 15.0, 8.5, 4.3 Hz, –S–C*H*–), 3.67–3.46 (43H, m), 3.38–3.09 (12H, m), 3.05–3.00 (3H, m, –NH–CH_2–; Hb-β of Cys), 2.89–2.83 (2H, m, Hb-6 of biotin; Ha-β of Cys), 2.63 (1H, d, *J* = 12.8 Hz, Ha-6 of biotin), 2.54 (1H, ddd, *J* = 19.0, 12.0, 4.0 Hz, Ha of –S–CH–CH_2–C(O)–), 2.40 (2H, q, *J* = 6.7 Hz, H-γ of Glu), 2.15–2.08 (6H, m, –C(O)–CH_2–), 2.02 (2H, q, *J* = 7.3 Hz, H-β of Glu), 1.69–1.12 (24H, m), 0.63 (2H, t, *J* = 7.9 Hz, –CH_2–B(OH)$_2$ of **3c**). FABMS: *m/z* 1598 (M+glycerol+H−2H_2O)$^+$. HR-FABMS: *m/z* 1597.8562, calcd for $C_{69}H_{122}{}^{11}BO_{27}N_{10}S_2$. Found: 1597.9109.

15. Binding analysis of biotinylated glutathione (GSH) probes: a 5 μL of the bacterial lysate, prepared from a culture of *E. coli* overexpressing glutathione-*S*-transferase (GST), was diluted with 185 μL of the lysis buffer [20 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.5% NP-40, and 1 mM PMSF], and then incubated with 10 μL of each biotinylated probe solution (25 μM in a final concentration) at 4 °C for 16 h. Slurry of Streptavidin–agarose beads (100 μL, suspended with 100 μL of lysis buffer) was added and incubated at 4 °C for another 2 h. Then, the beads were washed twice with lysis buffer. The bound proteins were eluted with a 50 μL of 20 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 10 mM reduced L-glutathione, followed by SDS–PAGE. The gels were stained with Coomassie brilliant blue (CBB), and the scanned images were analyzed using Photoshop (Adobe) to estimate relative quantities of the recovered GST.
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