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Synthesis and Biological Data of *ortho*-Carborane Analogs from 4-Aminobenzoic Acid and 4-Hydroxybenzoic Acid as a Potential Boron Neutron Capture Therapy Agent

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Boron neutron capture therapy (BNCT) has recently received considerable attention due to a highly selective therapy for treating cancer in clinical trial.[1] BNCT is binary therapy based on the combination of two components: non-radioactive boron (^{10}B) atom and low energy thermal neutron. As the thermal neutron irradiates ^{10}B atom, alpha (α)-particle and lithium ion (^{7}Li) are generated as well as substantial sufficient energy to damage a cell is produced.[2] Since the α-particle and ^{7}Li ion traverse within confined distance (i.e. ~5-10 μm) corresponding diameter of tumor cell, BNCT can be utilized to selectively kill tumor cells but minimize irradiation damage on normal cells.

Major challenge in BNCT is to find a safe and selective boron delivery agent. BNCT agents for successful therapy have been required to accumulate high ^{10}B concentration in tumor (i.e., 10-30 μg of ^{10}B per gram of tumor) with high selectivity (i.e., tumor to normal cell ratio is 5:1) and with low toxicity.[3] Currently successful BNCT agents are sodium borocaptate (BSH) and boronophenylalanine (BPA), which are ongoing for clinical trial (phase I/II), but they have also some limitations such as insufficient selectivity, low retention time in tumor, low chemical stability due to air-oxidation in BSH, and need of large amount of drug administration due to low boron percentage (5%) in BPA.[4] Although a number of new BNCT agents are developing to address these problems, demands of novel boron carrier in BNCT still remain.

Folate receptor (FR) has been an important therapeutic target for cancer treatment, because many cancer cells exhibit high levels of FR on their surface while FR expression in most normal cells is highly restricted.[5] Also, folic acid is able to bind to FR with extremely high affinity ($K_D \approx 10^{-10}$ M) and transport a drug in cell *via* an endocytic process.[6] Folate-conjugated drugs could be selectively delivered to highly FR-expressing cancer cell. Therefore, folic acid natural compound might be potentially a non-toxic and selective boron delivery agent in BNCT.

On the other hands, dicarba-*closo*-dodecaborane ($C_2B_{10}H_{12}$; commonly referred to as carborane; exists *ortho*, *meta*, and *para* isomers) has been widely considered for developing new BNCT agents, because use of carborane has benefits such as high boron content, stability to catabolism, hydrophobic interaction enhancement, and extraordinary chemical and thermal stability. Specifically, the volume of carborane has not only topologically similar with that of a phenyl ring rotating about its C1-C4 axis, but also the diameter of carborane is 5.25 Å in comparison with that of phenyl ring is 4.72 Å.[7] Therefore, typical strategy has been to prepare carborane-conjugated biomolecules or to introduce carborane cluster in place of aryl group.

In the past, we have reported the synthesis and testing of new class of *ortho*-carborane-conjugated aminobenzoylglutamate as a BNCT agent.[8] The carborane analog of folate showed several advantages such as sufficient water solubility for *in vitro* test, low toxicity towards melanoma cells (IC_{50} 6.9×10^{-4} M), and boron uptake of 0.37 μg B per 10^6 cells. Especially, the aminobenzoylglutamate moiety in the carborane seems to be very important natural carrier for water solubility, and cell-uptake mechanism in the biological system. Therefore, we hypothesized that the new class of folate compounds, introduced *ortho*-carborane in place of heteroaromatics, could be potentially promising BNCT agent to deliver on FR or internalize in cell nucleus as well as to minimize damage to normal cell for successful cancer therapy. On the other hands, a computational design quite recently showed that *ortho*-carborane could fit in the active site of human dihydrofolate reductase (hDHFR).[9]

In this paper, as our continuous work, we present the

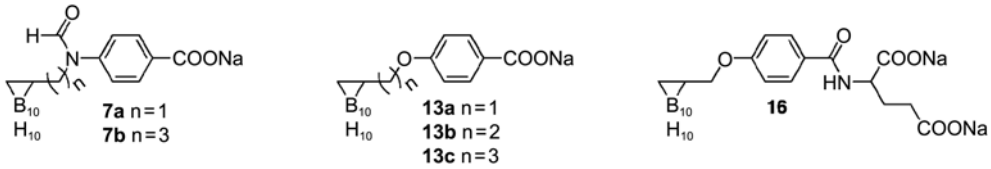


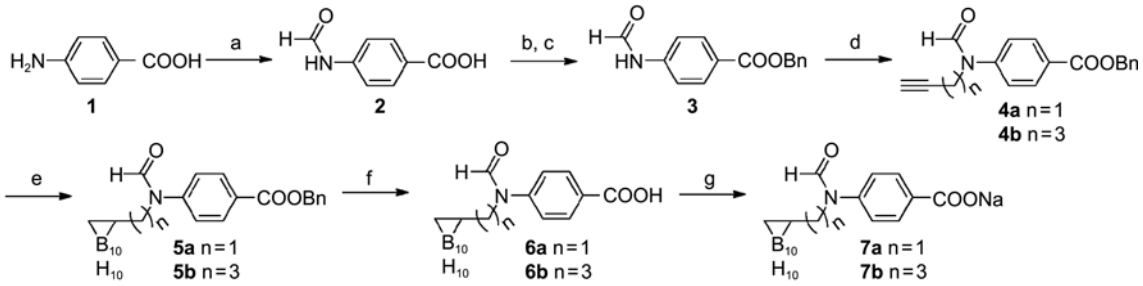
Figure 1. Structures of the designed *ortho*-carborane compounds (**7**, **13** and **16**).

synthetic and biological data of new class of *ortho*-carborane analog of folate sodium salt as a potential BNCT agent. The *ortho*-carboranes as shown in Figure 1 were synthesized from 4-aminobenzoic acid and 4-hydroxybenzoic acid. All carborane compounds were tested as mono- or disodium salt forms to confer water solubility.

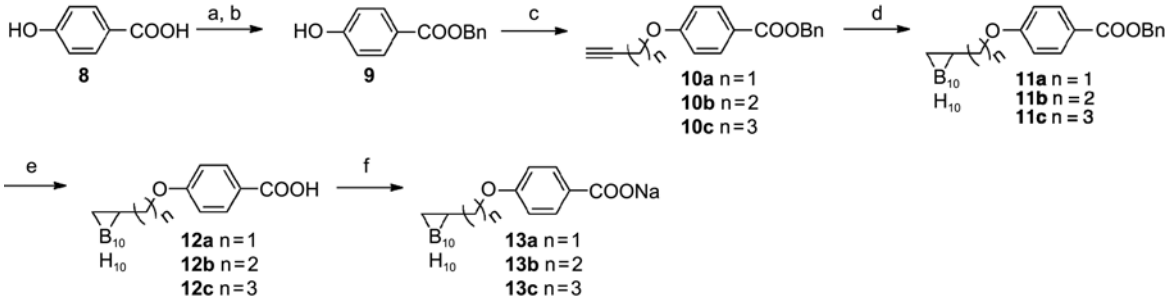
Results and Discussion

The first carborane compounds (**7a** and **7b**) have been synthesized as shown in Scheme 1 (also as shown in supplementary materials). The formylation reaction of 4-aminobenzoic acid in the presence of acetic anhydride and formic acid at room temperature (rt) gave 4-(*N*-formylamino)-benzoic acid (**2**) in 92% yield. The *in situ* cesium salt of **2** easily transformed benzylated compound (**3**) in 88% yield. The *N*-alkylation reaction with propargyl bromide in acetone in the presence of tetra-*n*-butylammonium bromide under reflux for 12 h gave the propargyl product (**4a**) in 87% yield. Also, the same reaction of **3b** with 4-pentynyl *p*-toluenesulfonate (tosylate) leaded to **4b** in 63% yield. The *ortho*-carboranes readily formed in 77% and 72% yields, respectively, by refluxing the solution of propargylation products and decaborane ($B_{10}H_{14}$) in toluene and acetonitrile cosolvent. Benzyl protecting groups of **5a** and **5b** were easily deprotected *via* catalytic transfer hydrogenation in the presence of 10% Pd/C and 4.5% formic acid in ethanol and the **6a** and **6b** were obtained in quantitative yields. The two acids were quantitatively transformed into sodium salts (**7a** and **7b**) to increase the water solubility for *in vitro* toxicity and boron uptake tests.[10,11] In the end, the *ortho*-carborane analogs of folate sodium salts (**7a** and **7b**) were prepared from 4-aminobenzoic acid through 7 steps in 54% and 36% overall yields, respectively.

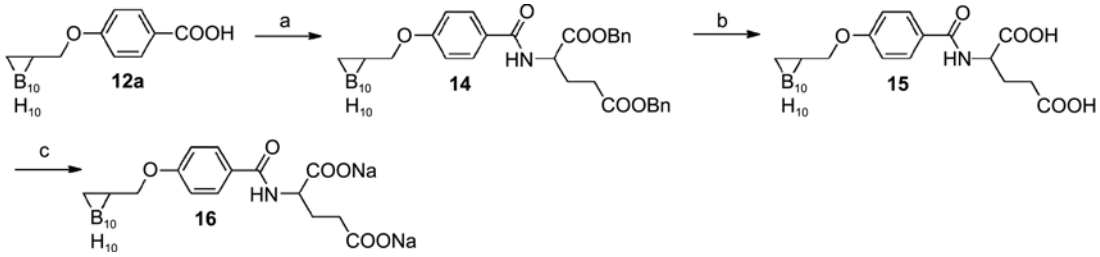
The second target compounds of *ortho*-carborane sodium salts (**13a-c**) were prepared from the 4-hydroxybenzoic acid (**8**) as shown in Scheme 2. The cesium salt of 4-hydroxybenzoic acid was treated with benzyl bromide in DMF at rt and the benzyl (4-hydroxy)benzoate (**9**) formed in 93% yield. The *O*-alkylation with propargyl bromide (n = 1), 4-pentynyl tosylate (n = 2) or 5-butynyl tosylate (n = 3) gave the propargylated compounds (**10a-c**) in 96% (n = 1), 68% (n = 2), and 61% (n = 3) yields, respectively. The carboranization of the esters (**10a-c**) with decaborane in refluxing toluene and acetonitrile produced the *ortho*-carborane (**11a-c**) in 84% (n = 1), 68% (n = 2), and 72% (n = 3) yields, respectively. The carboranyl benzoic acids (**12a-c**) were quantitatively created *via* catalytic transfer hydrogenation in the presence of 10% Pd/C and 4.5% formic acid in ethanol. Also, the resulting three acids (**12a-c**) were quantitatively transformed into the sodium salts (**13a-c**) to confer the sufficient water solubility for the *in vitro* tests.[12-14] In fact, the *ortho*-carborane sodium salts (**13a-c**) were prepared from 4-hydroxybenzoic acid through 6 steps in 68%, 39%,



Scheme 1. Synthesis of *ortho*-carborane sodium salts (**7**) from 4-aminobenzoic acid: (a) Ac_2O in HCOOH at rt for 6 h, 92%; (b) Cs_2CO_3 in MeOH at rt for 30 min, quantitative; (c) BnBr in DMF at rt for 48 h, 88%; (d) $(n\text{-}Bu)_4NBr$, propargyl bromide (n = 1) or 4-pentynyl *p*-toluenesulfonate (n = 3), K_2CO_3 in acetone at reflux for 12 h, 87% (n = 1), 63% (n = 3); (e) $B_{10}H_{14}$ in CH_3CN and toluene at reflux for 7 h, 77% (n = 1), 72% (n = 3); (f) Pd/C and 4.5% HCOOH in MeOH at rt for 3 h, 99% (n = 1) and 99% (n = 3); (g) $NaHCO_3$ in MeOH and H_2O at rt for 0.5 h, 97% (n = 1) and 98% (n = 3).



Scheme 2. Synthesis of *ortho*-carborane sodium salts (**13**) from 4-hydroxybenzoic acid: (a) Cs_2CO_3 in MeOH at rt for 30 min, quantitative; (b) BnBr in DMF at rt for 18 h, 93%; (c) Propargyl bromide (n = 1) or 3-butynyl *p*-toluenesulfonate (n = 2) or 4-pentynyl *p*-toluenesulfonate (n = 3), K_2CO_3 in acetone at reflux for 12 h, 96% (n = 1), 68% (n = 2), 61% (n = 3); (d) $B_{10}H_{14}$ in acetonitrile and toluene at reflux for 7 h, 84% (n = 1), 68% (n = 2), 72% (n = 3); (e) Pd/C and 4.5% HCOOH in MeOH at rt for 3 h, 99%; (f) $NaHCO_3$ in MeOH and H_2O at rt for 30 min, quantitative.



Scheme 3. Synthesis of *ortho*-carboranyl hydroxybenzoylglutamate sodium salt (**16**): (a) Dibenzyl glutamate, EEDQ in chloroform at rt for 48 h, 98%; (b) Pd/C and 4.5% HCOOH in MeOH at rt for 3 h, 99%; (c) $NaHCO_3$ in MeOH and H_2O at rt for 30 min, quantitative.

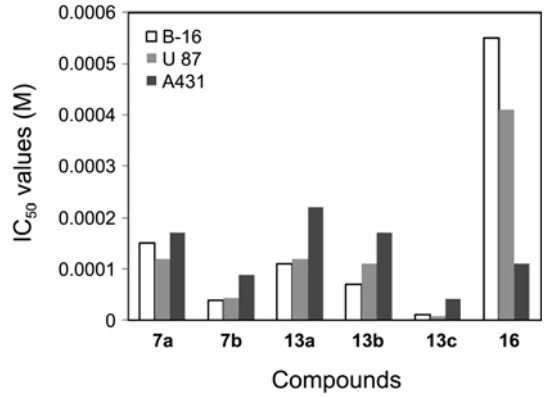


Figure 2. Biological activities of the *ortho*-carborane compounds (**7**, **13** and **16**).

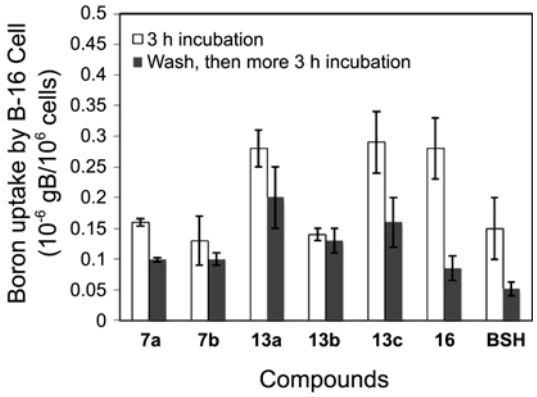


Figure 3. Cellular uptake of the *ortho*-carborane compounds (**7**, **13** and **16**).

and 37% overall yields, respectively.

Finally, the third target molecule of *ortho*-carborane analog of folate (**16**) was quantitatively prepared from **12a** as shown in Scheme 3. The L-glutamic acid moiety was conjugated with the carborane compound (**12a**) through 2-ethoxy-l-ethoxy-carbonyl-l,2-dihydroxyquinoline (EEDQ)-mediated amide formation reaction. Benzyl group of **14** was selectively deprotected *via* catalytic transfer hydrogenation in the excellent yield. **15** was also transformed as the sodium salt before *in vitro* tests.[15] Eventually, the *ortho*-carborane analog of folate sodium salt (**16**) was synthesized from the carboranyl benzoic acid (**12a**) through 3 steps in 96% overall yield (from 4-hydroxybenzoic acid (**8**) through 8 steps in 66 % overall yield).

All *ortho*-carboranes as sodium salt (**7a**, **7b**, **13a**, **13b**, **13c**, **16**) for BNCT were used for testing *in vitro* cytotoxicity in three kinds of tumor cells such as B-16 mouse melanoma cell line (i.e., FR positive malignant tumor), U 87 brain tumor cell, and A 431 epidermoid carcinoma cell line (i.e., epidermal growth factor receptor (EGFR) positive malignant tumor), as shown in Figure 2 (also as shown in supplementary materials Table 1). Especially, the cytotoxicity of sodium salt (n = 1) (**16**) was relatively lower than that of the other salts on B-16 and U 87, and that of sodium salt among them, **13a** showed relatively low cytotoxicity on A 431. However, toxicities of elongated carboranes (n = 2 and 3) (**7b** and **13c**) were relatively high. It might be the reason that the carborane salts are structurally associated with potent retinoid agonists and antagonist.[16]

On the other hands, in comparison with BSH, all carboranes were efficiently accumulated into FR receptor positive tumor cell. Especially, **13a** was stably incorporated into the cell after additional incubation as shown in Figure 3 (also as shown in supplementary materials Table 2). It was considered to be a good indication of the boron compound as a boron carrier for BNCT.

Conclusion

In summary, all six *ortho*-carborane analogs of folate were synthesized and characterized for developing a new BNCT agent. *Ortho*-carborane-conjugated benzoic acid sodium salts (**7** and **13**) were prepared from 4-aminobenzoic acid in 7 steps in 54% (n = 1) and 36% (n = 3) overall yields, respectively, and from 4-hydroxybenzoic acid in 6 steps in 68% (n = 1), 39% (n = 2), and 37% (n = 3) overall yields, respectively. Furthermore, the *ortho*-carborane analog of folate sodium salt (**16**) was efficiently synthesized from the carboranyl acid (**12a**) in 96% overall yield (66% overall yield from 4-hydroxybenzoic acid, **9**). In particular, the carboranes (n = 1) (**7a**, **13a** and **16**) have relatively low toxicity and high level of accumulation in FR positive cell. It could be potentially promising BNCT agent to deliver on FR target or internalize in cell nucleus for successful cancer therapy.

Acknowledgments. This work was supported by Korea University.

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10. **Characterization of 6a and 7a. (6a):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.49 (s, 1H, formyl H), 8.23 (d, 2H, J = 8.4 Hz, aromatic H), 7.29 (d, 2H, J = 8.4 Hz, aromatic H), 4.57 (s, 2H, -NCH_2-), 4.13 (s, br, 1H, CH in carborane cage). *Anal Calcd*: C, 41.11; H, 5.96. Found: C, 41.17; H, 6.04. **(7a)**: Anal Calcd: C, 38.48; H, 5.28. Found: C, 38.53; H, 5.29.
11. **Characterization of 6b and 7b. (6b):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.51 (s, 1H, formyl H), 8.25 (d, 2H, J = 8.7 Hz, aromatic H), 7.34 (d, 2H, J = 8.7 Hz, aromatic H), 4.53 (t, 2H, J = 7.2 Hz, -CH_2-), 4.17 (s, br, 1H, CH in carborane cage), 4.05 (t, 2H, J = 7.2 Hz, -NCH_2, 1.80-1.89 (m, 2H, -CH_2-). *Anal Calcd*: C, 44.68; H, 6.63. Found: C, 44.51; H, 6.65. **(7b)**: *Anal Calcd*: C, 42.04; H, 5.92. Found: C, 42.21; H, 5.99.
12. **Characterization of 12a and 13a. (12a):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.08 (d, 2H, J = 9.3 Hz, aromatic H), 6.91 (d, 2H, J = 8.7 Hz, aromatic H), 4.49 (s, 2H, -OCH_2-), 4.07 (s, br, 1H, CH in carborane cage). *Anal Calcd*: C, 40.80; H, 6.16. Found: C, 41.86; H, 6.33. **(13a)**: *Anal Calcd*: C, 37.97; H, 5.42. Found: C, 38.05; H, 5.48.
13. **Characterization of 12b and 13b. (12b):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.06 (d, 2H, J = 9.0 Hz, aromatic H), 6.88 (d, 2H, J = 9.0 Hz, aromatic H), 4.14 (t, 2H, J = 5.4 Hz, -OCH_2-), 3.83 (s, br, 1H, CH in carborane cage), 2.77 (t, 2H, J = 5.4 Hz, -CH_2-). *Anal Calcd*: C, 42.84; H, 6.54. Found: C, 42.89; H, 6.45. **(13b)**: *Anal Calcd*: C, 39.99; H, 5.80. Found: C, 39.87; H, 5.84.
14. **Characterization of 12c and 13c. (12c):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.03 (d, 2H, J = 8.7 Hz, aromatic H), 6.89 (d, 2H, J = 8.7 Hz, aromatic H), 4.00 (t, 2H, J = 6.0 Hz, -OCH_2-), 3.62 (s, br, 1H, CH in carborane cage), 2.43 (t, 2H, J = 6.0 Hz, -CH_2-), 1.95-2.08 (m, 2H, -CH_2-). *Anal Calcd*: C, 44.70; H, 6.88. Found: C, 44.65; H, 6.89. **(13c)**: *Anal Calcd*: C, 41.85; H, 6.15. Found: C, 41.77; H, 6.19.
15. **Characterization of 15 and 16. (15):** ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.07 (d, 2H, J = 8.7 Hz, aromatic H), 6.88 (d, 2H, J = 8.7 Hz, aromatic H), 4.43 (s, 2H, -OCH_2-), 4.09-4.17 (m, 2H, CH in carborane cage, CH in glutamate), 1.96-2.49 (m, 4H, 2 × CH_2 in glutamate). *Anal Calcd*: C, 42.54; H, 5.95. Found: C, 42.68; H, 5.99. **(16)**: *Anal Calcd*: C, 38.54; H, 4.96. Found: C, 38.65; H, 4.93.
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