Journal of Medicinal Chemistry

Brief Article

pubs.acs.org/jmc

# Biological Evaluation of Dodecaborate-Containing L-Amino Acids for Boron Neutron Capture Therapy

Yoshihide Hattori,*[,†] Shintaro Kusaka,[‡] Mari Mukumoto,[§] Kouki Uehara,[§] Tomoyuki Asano,[§] Minoru Suzuki,[∥] Shin-ichiro Masunaga,[∥] Koji Ono,[∥] Shinji Tanimori,[‡] and Mitsunori Kirihata[†]

[†]Research Center of Boron Neutron Capture Therapy, Research Organization for the 21st Century, Osaka Prefecture University, 1-1 Gakuen-cho, Nakaku 599-8531, Sakai, Japan

[‡]Department of Bioscience and Informatics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Nakaku 599-8531, Sakai, Japan

[§]Stella Pharma Co., ORIX Kouraibashi Building 5F, 3-2-7 Kouraibashi, Chuo-ku, Osaka, Japan

[∥]Kyoto University Research Reactor Institute, 2 Asashiro-Nishi, Kumatori-cho, Sennan-gun, Osaka, Japan

**ABSTRACT:** To develop a boron carrier for practical purposes, new boron-containing amino acids with an undecahydro-*closo*-dodecaboranylthio ($[^{10}B_{12}H_{11}S]^{2-}$−) unit in the side chain of the $\alpha$-amino acid have already been designed and synthesized. In the present paper, cytotoxicity, the incorporation amounts into tumor cells, and the tumor cell killing effects of these compounds were elucidated to evaluate their usefulness as boron carriers. Furthermore, the microdistribution of the amino acids in tumor cells was established.

## INTRODUCTION

Recently, boron neutron capture therapy (BNCT) has become recognized as an essential therapy for brain cancer, head and neck cancer, and melanoma.[1] BNCT is based on the nuclear capture and fission reactions of the $^{10}B$ atom with low energy thermal/epithermal neutrons to yield high linear energy transfer $\alpha$ particles and recoiling $^{7}Li$ nuclei. Because the path lengths of the articles are approximately 9−10 $\mu$m, equal to the dimensions of a single cell, $^{10}B$-containing cells are selectively destroyed by BNCT.

Although many kinds of boron compounds, including amino acids, nucleic acids, and liposomes, have been reported as boron delivery agents (boron carriers) for BNCT,[2−6] only two compounds, *p*-borono-L-phenylalanine (BPA) and disodium mercapto-*closo*-undecahydrododecaborate ($[B_{12}H_{11}SH]^{2-}2Na^{+}$, BSH), are clinically used in treatment of cancer with BNCT.[7] For a boron delivery agent to be successful in BNCT, the compound must have following properties: (i) high tumor targeting selectivity ($T/N$ > 3−4:1), (ii) low systemic toxicity, (iii) concentrations of ∼20 $\mu$g $^{10}B$/g tumor tissues, especially the boron compound, is preferred to accumulate into tumor cell and/or cell nuclei, and (iv) high water-solubility. On the other hand, the L-amino acid transport system in tumor cells is enhanced to ensure cell multiplication compared with normal tissues. Therefore, there has been a long-standing interest in the design, synthesis, and biological evaluation of boron-containing $\alpha$-amino acids with tumor-seeking and tumor-localizing properties.

L-BPA has been used as an excellent boron delivery agent for BNCT. Because L-BPA is selectively uptaken into tumor cells, particularly accumulating in the cell nuclei, L-BPA is clinically used in BNCT.

BSH and its derivatives are of increasing interest as boron carriers for BNCT with the aim to deliver large amounts of $^{10}B$ atoms to tumor cells.[8] BSH is a class of water-soluble boron cluster compound and has low toxicity compared with another boron cluster compounds. Owing to these properties, BSH is clinically used for the treatment of brain tumors with BNCT as a $^{10}B$ carrier,[9] although tumor selectivity of BSH is slightly low.

To develop practical materials utilizing $^{10}B$ carriers, we previously designed and synthesized several new boron-containing amino acids, including C4-BSH-AA ($n$ = 2) (**1a**), C5-BSH-AA ($n$ = 3) (**1b**), and C8-BSH-AA ($n$ = 6) (**1c**), which include the undecahydro-*closo*-dodecaboranylthio ($[^{10}B_{12}H_{11}S]^{2-}$−) unit through a boron−sulfur−carbon bond connection to the side chain of $\alpha$-amino acid[10] (Figure 1).

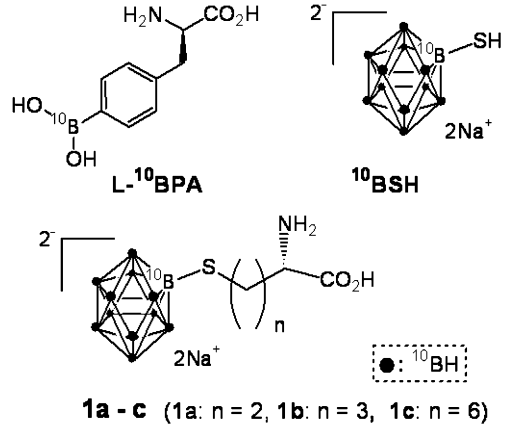



**Figure 1.** Boron-containing compounds.

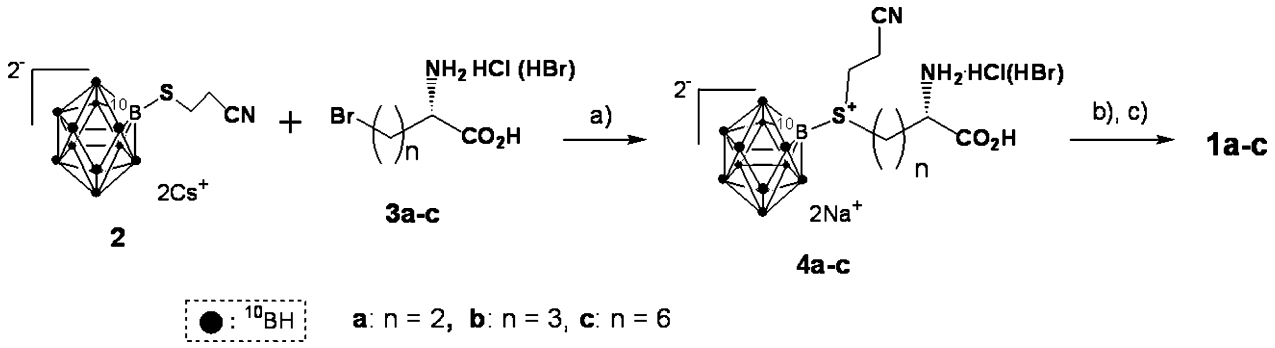
**Received:** May 30, 2012
**Published:** July 12, 2012





dx.doi.org/10.1021/jm300749q | J. Med. Chem. 2012, 55, 6980−6984

**Scheme 1. Synthesis of BSH-Amino Acids (1a−c)**[a]




[a]Reagents and conditions: (a) MeCN, reflux, 24 h; (b) $Me_4NOH$, $MeNH_2$, acetone, rt, 30 min; (c) amberlite IR-120($Na^+$).

Here, we report the distribution of the BSH-amino acids **1a−c** in tumor cells and their cytotoxicities, the incorporated amount into cancer cells, and the tumor cell killing effects.

## MATERIALS AND METHODS

**General.** L-$^{10}$BPA and $^{10}$BSH were provided by Stella Pharma Corporation (Osaka, Japan). Capillary electrophoresis analysis was carried out using Photal CAPI-3300 (Otsuka Electronics Co. Ltd., Osaka, Japan) equipped with a fused silica capillary of 75 $\mu$m i.d. and a total length of 50 cm (effective length of 38 cm). A new capillary was conditioned with 0.1 M NaOH for 30 min followed by 15 min of distilled water and 15 min of electrolyte buffer (5 mM sodium phosphate buffer, pH 9.0). The applied potential was 20 kV and detection at 200 nm. Inductively coupled plasma optical emission spectrometry (ICP-OES) was obtained on a VISTA-MPX ICP-OES spectrometer (Seiko Instruments, Chiba, Japan). Fluor 488 goat anti-mouse IgG was purchased from Life Technologies (Carlsbad, CA, US). Permafluor was purchased from Immunotech (Marseille, France).

**Synthesis of BSH-Amino Acids (1a, 1b, and 1c).** The synthesis of BSH-amino acids **1a−c** was carried out according to previously described method.[10] The purity of **1a−c** was analyzed by capillary electrophoresis, and the analysis of **1a−c** was revealed the purity to be >95%.

**Cells and Cell Culture.** SAS (human oral squamous cell carcinoma), B16 (mouse melanoma), and C6 (rat glioma) cell lines, used in cytotoxicity analyses, boron incorporation, tumor cell killing effect, and immunostaining, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 24 mM sodium hydrogen carbonate at 37 °C in a 5% $CO_2$ atmosphere. Cells in the monolayer were harvested with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in $Ca^{2+}$-free phosphate-buffered saline (PBS). Matrigel (growth factor reduced type) was purchased from BD Science (San Jose, CA, US).

**Water-Soluble Tetrazolium (WST)-8 Assay.** The cytotoxicity of each amino acid was assayed using the WST-8 assay with a cell counting kit (Wako Pure Chemicals, Osaka, Japan) following the manufacturer's protocols with some modifications. Briefly, a 96-well microplate was seeded with $5 \times 10^3$ cells suspended in 100 $\mu$L of cell growth media and allowed to settle for 16 h at 37 °C. The medium was removed by aspiration, and 100 $\mu$L of growth medium (DMEM with 10% FBS containing each compound at different concentrations) was added to each well. After incubation for 48 h at 37 °C, the medium was removed by aspiration and 100 $\mu$L of WST-8 solution [5 mM WST-8, 20 mM HEPES (pH 7.4) and 0.2 mM 1-methoxy PMS dissolved in PBS] were added to each well, followed by incubation for 4 h at 37 °C. The absorbance at 450 nm (reference: 655 nm) was read on a microwell plate reader. A well without cells was used as the blank. Relative cell survival was calculated as follows;

**Table 1. Cytotoxicity of Boron Amino Acids against Tumor Cells**

| | $IC_{50}$ (mM) | | |
|---|---|---|---|
| compd | C6 | SAS | B16 |
| L-$^{10}$BPA | >2 | >2 | >2 |
| C4-BSH-AA (**1**) | 6.6 | 7.8 | 5.6 |
| C5-BSH-AA (**2**) | 2.4 | 4.9 | 6.6 |
| C8-BSH-AA (**3**) | 4.7 | 5.6 | 5.1 |

relative cell survival (%)
= (absorbance value of compound treated well
/absorbance value of untreated well) × 100

The relative cell survival was plotted against the compound concentration, and $IC_{50}$ values for each compound were graphically determined.

**Boron Incorporation into Cultured Tumor Cells.** Cultures were inoculated with $1.0 \times 10^6$ cells/dish, and cells were grown for 24 h in DMEM. The medium was replaced with an equivalent medium containing each boron-containing amino acid (the final concentration was 2.0 mM in each case). The cells were cultured for 24 h, and the medium was then removed by aspiration. The cells were washed thrice with PBS, harvested by trypsinization, and then counted. Each sample, containing $1 \times 10^6$ cells, was added to a mixture of $HClO_4$ (60%, 0.3 mL) and $H_2O_2$ (31%, 0.6 mL) and then heated at 75 °C for 1 h. The mixture was filtered through a membrane filter (Millipore, 0.45 $\mu$m), and the boron concentration was measured by ICP-OES.

**Immunostaining of C6 Cells.** Immunostaining was performed to detemine the incorporation of boron-containing amino acids into C6 cells, according a previously described method with some modifications.[11] Glass coverslips coated with Matrigel (3.5 $\mu$g/cm$^2$ protein) were seeded with C6 cells ($0.8 \times 10^5$ cells suspended in 3 mL of DMEM) and allowed to settle for 1 h at 37 °C. The medium was replaced with an equivalent medium containing compound **1a** (the final concentration was 2.0 mM in each case), and the cells were cultured for 24 h at 37 °C. After being washed with DMEM, C6 cells were fixed with 10% paraformaldehyde in PBS for 10 min at room temperature. The cells were rinsed with PBS and treated with 0.05% Triton X-100 for 10 min at room temperature. Further, the cells were washed with PBS and preincubated in a humid chamber with 1.0% BSA/0.02% $NaN_3$ in PBS at room temperature, followed by incubation with the anti-BSH monoclonal antibody A9H3 in PBS containing 1.0% BSA/0.02% $NaN_3$ (0.2 $\mu$g/mL) for 60 min at 32 °C. The cells were rinsed with PBS and then incubated with Alexa-Fluor 488 goat anti-mouse IgG in PBS containing 1.0% BSA/0.02% $NaN_3$ (0.2 $\mu$g/mL) for 30 min at 32 °C. After washing with PBS, the cells were mounted with Permafluor and then photographed with a microscope (IX-70, Olympus, Tokyo) equipped with a cooled charge-coupled device


dx.doi.org/10.1021/jm300749q | J. Med. Chem. 2012, 55, 6980−6984

camera (UIC-QE, Molecular Devices Co., Sunnyvale, CA, U.S.) controlled by MetaMorph software (Molecular Devices Co.).

**Tumor Cells Killing Effect Study.** Cultures were inoculated with $1.0 \times 10^6$ cells/dish, and cells were grown for 24 h in DMEM. The medium was replaced with an equivalent medium containing each boron-containing amino acid (the final concentration was 2.0 mM in each case). They were cultured for 24 h, and the medium was removed by aspiration. The cells were washed with PBS, harvested by trypsinization, and then counted. After centrifugation, trypsin was removed by aspiration, and DMEM was added to the residual cells. The cell suspension in DMEM ($5.0 \times 10^3$ cells/mL, 1 mL) was irradiated with thermal neutrons for 0–90 min in a column-shape tube. The thermal neutron fluence was determined by averaging two gold foils, symmetrically attached to the surface of the column-shape tube along the direction of incidence of the thermal neutrons. After thermal neutron exposure, 600 cells were placed in three Corning 60 mm tissue culture dishes containing 3 mL of DMEM to examine colony formation. Seven days later, the colonies were fixed with ethanol and stained with 0.1% crystal violet for quantitative visualization by the naked eye.

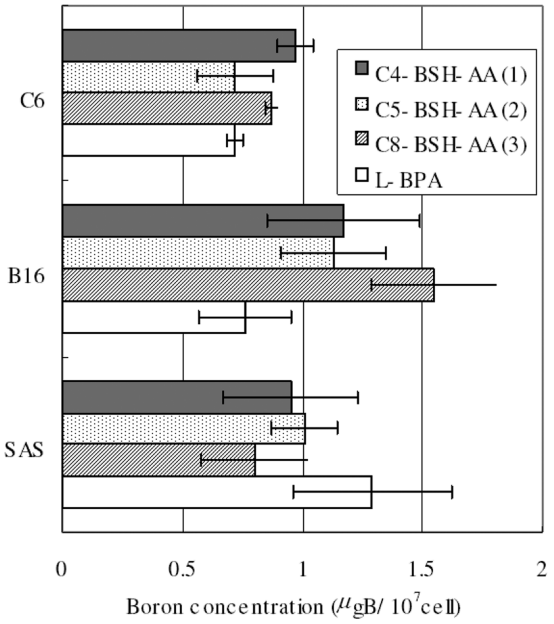



**Figure 2.** Incorporated amount of boron amino acids.

## RESULTS AND DISCUSSION

An alternative route of synthesis of the three BSH-amino acids (**1a–c**) is illustrated in Scheme 1. As shown, the starting compound, *S*-(cyanoethyl)-BSH tetramethylammonium salt (**2**),[12] was prepared with a high yield by hetero-Michael reaction of BSH dicesium salt with acrylonitrile, using sodium hydroxide as a base.[13] On the other hand, the *ω*-bromo-L-amino acids (**3a–c**), represented as Br-$(CH_2)n$-$CH(NH_2)$-COOH ($n$ = 2, 3, and 6, respectively), were prepared as hydrochloric or hydrobromic salts. Among these, (*S*)-2-amino-4-bromobutyric acid (**3a**, $n$ = 2) was commercially purchased, and the others (**3b** and, **3c**) bearing the (*S*)-configuration were synthesized according to modified versions of published methods.[14,15]

The alkylation reaction of 2 with *ω*-bromo-L-amino acids (**3**) was completed by a simple procedure. The purity and chemical structure of **1** were analyzed by $^1$H and $^{13}$C NMRs, ESI-MS, and capillary electrophoresis. In the present synthesis, absolute configuration of the starting *ω*-bromo-L-amino acid was introduced to the final amino acids in retention without any racemization.

The cytotoxicities of the BSH-amino acids **1a–c** and L-BPA toward the C6, SAS, and B16 cell lines were determined using the WST-8 test (Table 1). As shown in Table 1, the cytotoxicity of each compound was very low. However, the cytotoxicity of the three BSH-amino acids was higher than that of L-BPA.

To elucidate the incorporation amounts of BSH-amino acids **1a–c** into cancer cells, we measured the boron concentrations in three kinds of cancer cells, C6, B16, and SAS by ICP-OES (Figure 1). The compounds **1a**, **1b**, and **1c** were up-taken into cancer cells. In particular, the amounts of **1a–c** that were incorporated ware higher than those of L-$^{10}$BPA for C6 and B16 cells. These results suggest that the BSH-amino acids **1a–c** are as useful as L-$^{10}$BPA as $^{10}$B carrier.

To determine the distribution of each BSH-amino acid in tumor cells, we stained C6 cells that incorporated compound **1a** with the anti-BSH antibody A9H3[16] (Figure 2). The results showed the distribution of BSH-amino acid **1a** was very different from that of L-BPA. L-BPA was reported that widely distributed in the cytoplasm and the cell nuclei there are no regions within the cells in which the concentration of L-BPA is especially high.[17,18] In contrast, the compound **1a** was

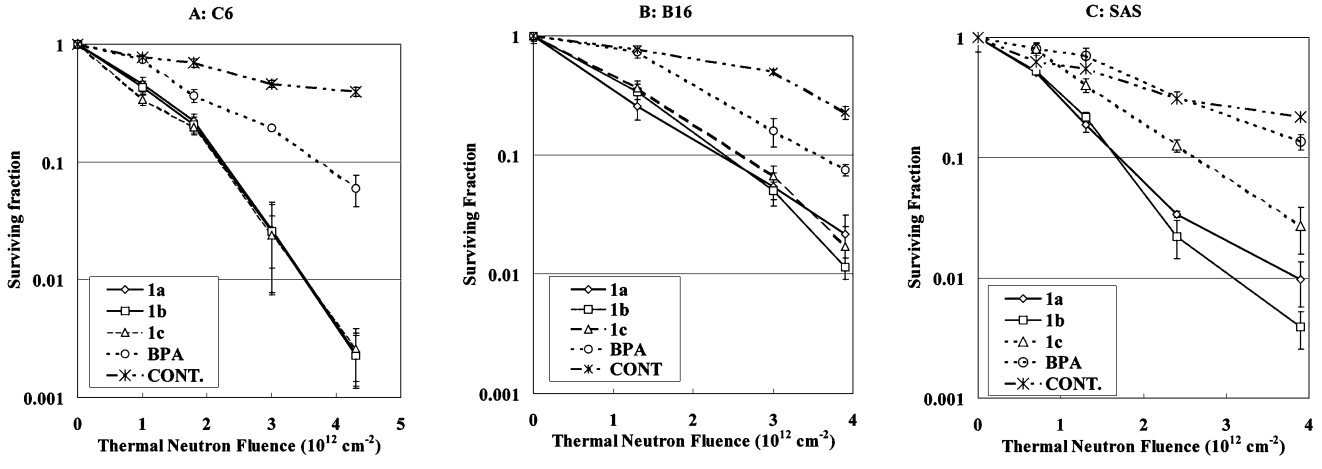



**Figure 3.** The tumor cell killing effects of boron-containing amino acids (A) against C6 cells, (B) against B16 cells, and (C) against SAS cells.


dx.doi.org/10.1021/jm300749q | J. Med. Chem. 2012, 55, 6980–6984

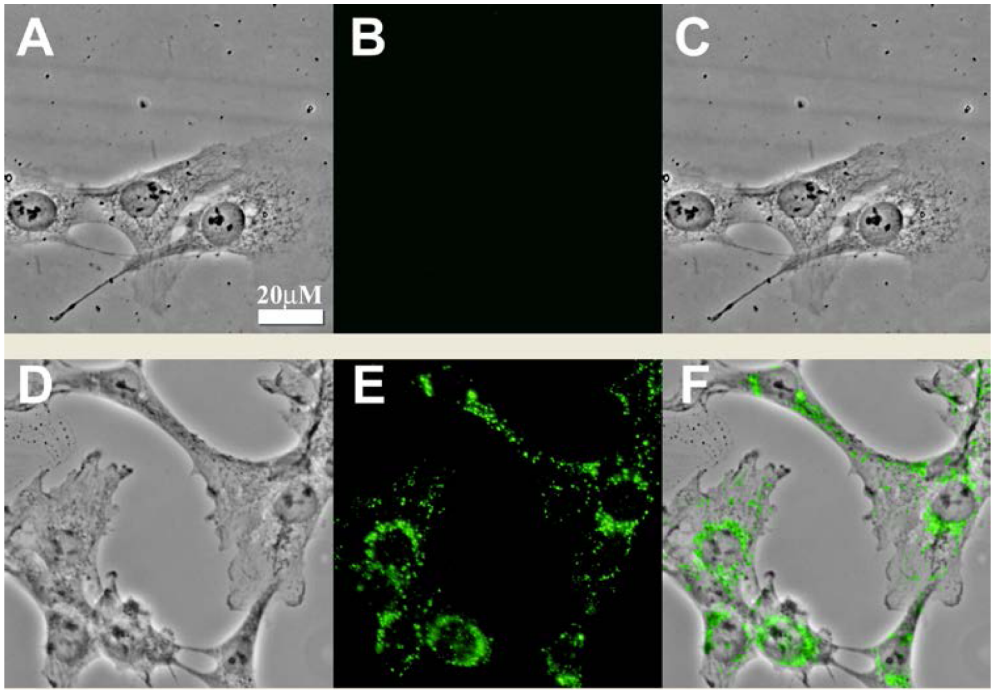



**Figure 4.** The microdistribution of compound **1a** in C6 cells. (A) A phase-contrast micrograph of C6 cells that were cultured in DMEM. (B) A fluorescence micrograph of C6 cells that were cultured in DMEM stained with the anti-BSH antibody A9H3. (C) A merged image of A and B. (D) A phase-contrast micrograph of C6 cells that were cultured in DMEM containing compound **1a**. (E) A fluorescence micrograph of C6 cells that were cultured in DMEM containing compound **1a** stained with the anti-BSH antibody A9H3. (F) Merged image of D and E.

incorporated into the cell membrane of the C6 cells and aggregated on the fringe of the cell nuclei (Figure 4).

To confirm the usefulness of the BSH-amino acids **1a–c** for BNCT, we examined the tumor cell killing effects of L-$^{10}$BPA and compounds **1a–c** against tumor cells in vitro using neutron irradiation (Figure 3). Despite the finding that the amounts of the three amino acids incorporated were closely equivalent to that of L-$^{10}$BPA, **1a–c** showed higher killing effects than L-$^{10}$BPA for all types of tumor cells tested.

## CONCLUSION

From these results presented, we conclude that the undecahydro-*closo*-dodecaboranylthio unit is well suited as a boron source, and the BSH-amino acids **1a–c** are useful as $^{10}$B carriers because they aggregated on the fringe of the cell nuclei and showed higher cell-killing effects than L-BPA toward several tumor cells types. In vivo evaluation of compounds **1a–c** is ongoing, and the results will be reported soon.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +81-72-254-8447. E-mail: y0shi_hattori@riast.osakafu-u.ac.jp.

### Author Contributions

These authors contributed equally.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Professor K. Ogawa for his technical support with immunostaining.

## ABBREVIATIONS USED

BNCT, boron neutron capture therapy; L-$^{10}$BPA, *p*-($^{10}$B)-borono-L-phenylalanine; $^{10}$BSH, mercapto-undeca-hydro-*closo*-dodeca($^{10}$B)borate; ICP-OES, inductively coupled plasma optical emission spectrometry; WST, water-soluble tetrazolium; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

## REFERENCES

(1) Barth, R. F.; Coderre, J. A.; Vicente, M. G.; Blue, T. E. Boron neutron capture therapy of cancer: current status and future prospects. *Clin. Cancer Res.* **2005**, *11*, 3987–4002.

(2) Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F. G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. The chemistry of neutron capture therapy. *Chem. Rev.* **1998**, *98*, 1515–1562.

(3) Endou, H.; Kanai, Y. Amino acid transporter molecule as a drug target. *Nippon Yakugaku Zasshi* **1999**, *114*, 11–16.

(4) Kabalka, G. W.; Yao, M. The synthesis and use of boronated amino acids for boron neutron capture therapy. *Anti-Cacncer Agents Med. Chem.* **2006**, *6*, 111–125.

(5) Srivastsva, R. R.; Shinghaus, R. R.; Kabalka, G. W. Synthesis of 1-amino-3-[2-(1,7-dicarba-*closo*-dodecaboran (12)-1-yl)ethyl]cyclo-butanecarboxylic acid: a potential BNCT agent. *J. Org. Chem.* **1997**, *62*, 4476–4478.

(6) Varadarajan, A.; Hawthorne, M. F. Novel carboranyl amino acids and peptides: reagents for antibody modification and subsequent neutron-capture studies. *Bioconjugate Chem.* **1991**, *2*, 242–253.

(7) Miyatake, S.; Tamura, Y.; Kawabata, S.; Iida, K; Kuroiwa, T.; Ono, K. Boron neutron capture therapy for malignant tumors related to meningiomas. *Neurosurgery* **2007**, *61*, 82–90.

(8) Slepukhina, I.; Gabel, D. Synthesis and in vitro toxicity of new dodecaborate-containing amino acids. Proceedings of 12th International Congress on Neutron Capture Therapy, Kagawa, Japan, October 9–13, 2006, pp 247–250.

(9) Kageji, T.; Mizubuci, Y.; Nagahiro, S.; Nakagawa, Y.; Kumada, H. Clinical results of boron neutron capture therapy (BNCT) for glioblastoma. *Appl. Radiat. Isot.* **2011**, *69*, 1823−1825.
(10) Kusaka, S.; Hattori, Y.; Uehara, K.; Asano, T.; Tanimori, S.; Kirihata, M. Synthesis of optically active dodecaborate-containing L-amino acids for BNCT. *Appl. Radiat. Isot.* **2011**, *69*, 1768−1770.
(11) Ogawa, K.; Wada, H.; Okada, N.; Harada, I.; Nakajima, T.; Pasquale, B. E.; Tsuyama, S. EphB2 and ephrin-B1 expressed in the adult kidney regulate the cytoarchitecture of medullay tubule cells through Rho family GTPases. *J. Cell Sci.* **2005**, *119*, 559−570.
(12) Gabel, D.; Moller, D.; Harfst, S.; Rosler, J.; Ketz, H. Synthesis of *S*-alkyl and *S*-acyl derivatives of mercaptoundeca-hydrododeca-borate, a possible boron carrier for neutron capture therapy. *Inorg. Chem.* **1993**, *32*, 2276−2278.
(13) Hattori, Y.; Uehara, K.; Asano, T.; Tanimori, S.; Kirihata, M. Synthesis of new derivatives by hetero-Michael addition in water. Proceedings of 14th International Congress on Neutron Capture Therapy, Buenos Aires, Argentina, October 25-29, 2010, pp 343−344.
(14) Kanai, F.; Isshiki, K.; Umezawa, Y.; Morishima, H.; Naganawa, H.; Takita, T.; Takeuchi, T.; Umezawa, H. Vanoxonin, A new inhibitor of thymidylate symyjetase. *J. Antibiot.* **1985**, *38*, 31−38.
(15) Watanabe, L. A.; Jose, B.; Kato, T.; Nishino, N.; Yoshida, M. Synthesis of L-$\alpha$-amino-$\omega$-bromoalkanoic acid for side chain modification. *Tetrahedron Lett.* **2004**, *45*, 491−494.
(16) Kirihata, M.; Uehara, K.; Asano, T.; Preparation of borocaptate-(10B) (BSH) derivatives as hapten compound for production of antibody against BSH. PCT Int. Appl. WO2007097065 A1 20070830, 2007.
(17) Chandra, S.; Kabalka, G. W.; Lorey, D. R.; Smith, D. R.; Coderre, J. A. Imaging of fluorine and boron from fuorinated boronophenylalanine in the same cell at organelle resolution by correlative ion microscopy and confocal laser scanning microscopy. *Clin. Cancer Res.* **2002**, *8*, 2675−2683.
(18) Wittig, A.; Arlinghaus, H. F.; Kriegeskotte, C.; Moss, R. L.; Appelman, K.; Schmid, K. W.; Sauerwein, W. A. G. Laser postionization secondary neutral mass spectrometry in tissue: a powerful tool for elemental and molecular imaging in the development of targeted drugs. *Mol. Cancer Ther.* **2008**, *7*, 1763−1771.