quite precipitously by 48 h to values that were one-third of their peak value, while the normal brain and blood values were low at the three time points (0.2–3.3 μg/g) except for those animals that received the 1.0 mg dose. Their normal brain values were higher at 0, 24 and 48 h (6.5, 10.1 and 3.0 μg/g, respectively). Although the highest T:Br ratio was seen with the dose of 0.5 mg at 48 h after administration, the tumor boron concentration was low (16.8 μg/g). Therefore, the most favorable values of tumor boron (102.9 μg/g) and T:Br ratios for H_2TCP were seen with this dose at 24 h. In contrast, the tumor boron values for H_2TBP and H_2DCP at the same time and dose were 61.9 ± 16.4 and 35.6 ± 9.0 μg/g, respectively (Table 3). The highest tumor boron concentration was seen 24 h following short term (30 min) CED of H_2TBP (140.3 ± 70.9 μg), but as indicated by the large SD, there was considerable animal to animal variability. It should be noted that the T:Br boron concentration ratios of the carboranylporphyrins were markedly increased over those that we have observed following either i.v. or intracarotid (i.c.) administration of BPA and BSH [43].

Boron neutron capture therapy

The carboranylporphyrins were administered 14 days following tumor implantation and BNCT was carried out 24 h after termination of delivery. This was well tolerated and weight loss in the first week was less than 20%, following which the animals regained their weight. The estimated physical radiation doses delivered to tumor, brain and blood were calculated according to boron concentrations summarized in Table 3. In contrast, following i.c. administration of the carboranylporphyrins no boron was detected in samples of liver, spleen, kidneys, lungs and heart (data not shown). The highest physical radiation doses delivered to the tumor were 34.0 Gy for H_2TBP, administered by CED, and 25.4 Gy for H_2TCP, administered by Alzet pumps. The corresponding normal brain doses were 1.9 and 2.5 Gy, respectively (Table 3). The survival data following BNCT are summarized in Table 4, and Kaplan–Meier survival plots are shown in Figs. 2, 3 and 4. The MSTs were 35.0 ± 3.7 and 43.8 ± 10.0 days, respectively, for rats that received H_2TCP and H_2TBP by Alzet pump (Table 4). Animals that received H_2TBP by Alzet pumps had a significantly longer MST than those that received H_2DCP ($P < 0.017$). Further studies were carried out using H_2TBP at a dose of 0.2 mg, administered by CED, either alone or in combination with i.v. BPA. The corresponding MSTs were 33.8 ± 3.1 and 42.8 ± 9.0 days, respectively (Table 4 and Fig. 3). As shown in Fig. 4, there were more long term survivors among rats that received the combination of i.v. BPA and H_2TBP, compared to those that

Table 4 Survival times of F98 glioma bearing rats following i.c. delivery of H_2TCP and H_2TBP by CED or osmotic pumps

Agent/route	Survival time				%ILS[b]	
	N[a]	Range	Mean ± SD	Median	Mean	Median
H_2TCP/pump	18	30–41	35.0 ± 3.7	36	49.6	60.0
H_2TBP/pump	10	32–59	43.8 ± 10.0	40.5	87.2	80.0
H_2TBP/CED	10	30–39	33.8 ± 3.1	33.5	44.4	48.9
H_2TBP/CED + i.v. BPA	9	33–61	42.8 ± 9.0	41	70.0	75.5
BPA/i.v.	10	33–48	39.8 ± 1.6	39.5	59	58
Irradiated controls	9	24–31	27.4 ± 2.7	27	17.3	20.0
Untreated controls	10	21–29	23.4 ± 2.5	22.5	–	–

[a] A total of either 0.2 mg of the compound was administered by CED for 30 min or 0.5 mg by Alzet osmotic pumps for 24 h. BNCT was initiated 24 h after termination of either CED for Alzet osmotic pump infusion or 2.5 h after i.v. administration of BPA

[b] N is the number of animals per group

[c] Percent increased life span (%ILS) was defined relative to the mean and median survival times of untreated controls

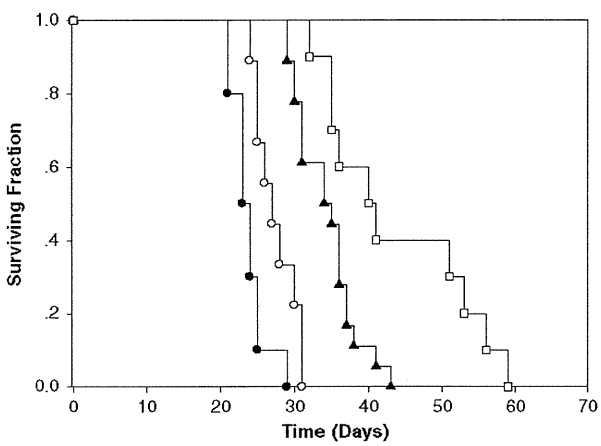

Fig. 2 Kaplan–Meier survival plots for F98 glioma bearing rats following Alzet pump delivery of H_2TCP or H_2TBP followed by BNCT. Survival times in days after implantation have been plotted for untreated animals (*filled circle*), radiation controls (*open circle*), H_2TCP (*filled triangle*) or H_2TBP (*open square*)

received H_2TBP alone ($P < 0.001$). The animals that received of H_2TBP by Alzet pump (Table 4 and Fig. 5) had longer MSTs than those that received it by CED (43.8 vs. 33.8 days), demonstrating that Alzet pump delivery was more effective than CED ($P < 0.013$). If the MSTs of animals that received i.v. BPA are compared to those of rats received H_2TBP by either CED or Alzet pump using a log-rank test, they were not significantly different from one another ($P = 0.38$ and 0.16, respectively). The highest %ILS (87.2%) was observed among those animals that received H_2TBP by osmotic pumps and this was equivalent to the %ILS of animals that received H_2TBP by CED and i.v. BPA (82.8%).

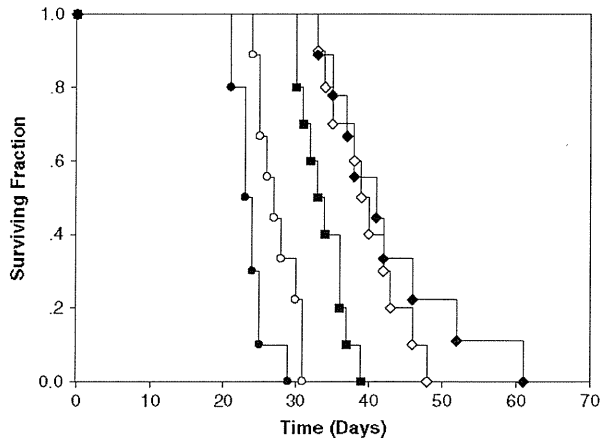

Fig. 3 Kaplan–Meier survival plots for F98 glioma bearing following CED of H_2TBP followed by BNCT. Survival times in days after implantation have been plotted for untreated animals (*filled circle*), irradiation controls (*open circle*), H_2TBP (*filled square*), i.v. BPA (*open diamond*) and H_2TBP plus BPA (*filled diamond*)

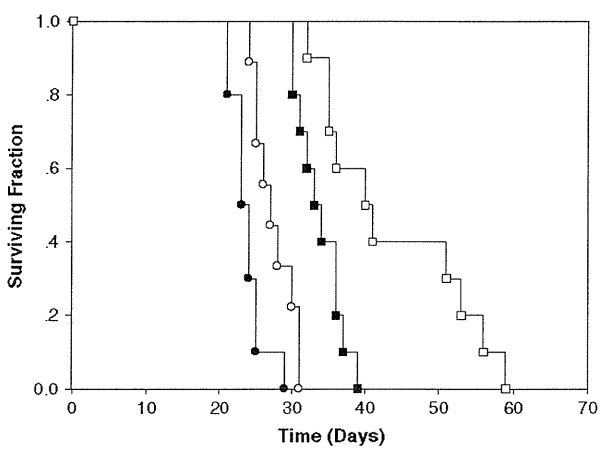

Fig. 4 Kaplan–Meier survival plots for F98 glioma bearing rats following either CED or Alzet pump delivery of H_2TBP followed by BNCT. Survival times in days after implantation have been plotted for untreated animals (*filled circle*), radiation controls (*open circle*), CED delivery of H_2TBP (*filled square*), Alzet pump delivery of H_2TBP (*open square*)

Neuropathologic evaluation

The most notable histopathologic finding was the presence of porphyrin laden macrophages and extracellular deposits of porphyrins in the tumors of many of the rats that received either H_2TCP or H_2TBP by either CED or Alzet pump infusion (Fig. 5A and B). As shown in Fig. 5B, this material was bright orange in color and was easily discernible on H&E stained sections of the tumor. In most instances, the appearance of the tumor in both treated and untreated animals was similar to that previously described by us [39]. As previously described by one of us (RFB), the tumor was composed of cells that varied in size and shape from ovoid to fusiform, sometimes displaying a whorled pattern of growth [42]. Frequently, there were microscopic deposits of tumor cells invading the surrounding white matter and a central zone of necrosis.

Discussion

Biodistribution studies demonstrated that high tumor boron concentrations could be achieved by either short term (30 min) CED or a 24 h infusion via Alzet osmotic pumps. Based on these observations, therapy studies were carried out using H_2TCP and H_2TBP as boron delivery agents. A MST of 43.8 days was obtained using H_2TBP, compared to 35.0 days for H_2TCP, both delivered by Alzet pumps, and 39.8 days for i.v. BPA. Our results are in agreement with those recently reported by Jori et al. [24, 48] who observed that the tumor boron concentrations following i.t. administration of H_2TCP to C57B1/6 mice bearing s.c. implants of the B16 melanoma were 10× greater than those observed following i.v. injection (60 vs. 6 μg/g). However, following BNCT the tumor growth delay was practically identical for both groups. Although the tumor boron concentrations for the latter animals was not determined, published data would suggest that it could have been in the range of 10 μg/g [49]. Similarly, Shibata et al. [50] have reported a very modest increase in the MST of 9L gliosarcoma bearing rats that received a BSH-porphyrin compound designated STA-BX900 (16.2 vs. 14.8 days and 12.8 days for irradiated and untreated control animals, respectively).

However, since the tumor boron concentrations were so much higher following either CED or infusion by Alzet pumps than those obtained with other boron compounds, it was puzzling why the survival data were similar to those obtained with BPA, which had a much lower tumor boron concentration. Histopathologic examination of brains from tumor bearing, BNCT treated rats revealed that in most animals there were large numbers of porphyrin containing macrophages (Fig. 5A and B) indicating that in reality the tumor cell uptake was much lower than would have been predicted, based on the tumor boron values determined by DCP-AES. This provides an explanation as to why the survival data were similar to those obtained with BPA, despite the seemingly high "tumor" boron concentrations. One possible explanation for the high uptake of the carboranylporphyrins by macrophages and the relatively low uptake by tumor cells could be related to their propensity to form aggregates when high concentrations are solubilized in water. This problem could be obviated by initially dissolving them in dimethylsulfoxide (DMSO) and then

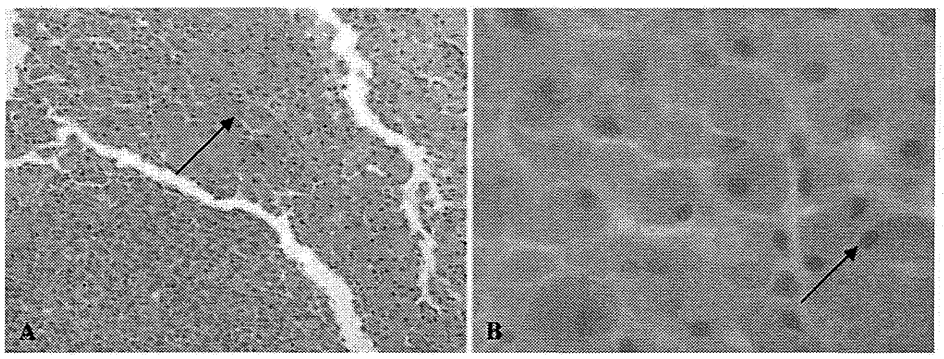

Fig. 5 **A** Low power photomicrograph from the brain of a BNCT treated, F98 glioma bearing rat following administration of H_2TBP by CED. There are large numbers of porphyrin laden macrophages (*arrows*). **B** High power photomicrograph showing porphyrin laden macrophages. These photos were taken at the time of death of the animal at which time they had progressively growing brain tumors

diluting it down to 1% DMSO. Theoretically, bystander killing might occur if the ^{10}B containing macrophages were adjacent to tumor cells. However, the potential lethality of the alpha particles produced as a result of the $^{10}B(n,\alpha)^7Li$ capture reaction would be much less than if they were produced within the tumor cells. Although CED has been effective in improving the distribution of a variety of agents in rats with brain tumors, its effectiveness in humans has been much more problematic [35, 36]. However, as demonstrated in the present study, direct i.c. delivery of therapeutic agents, which bypass the BBB, results in much higher concentrations in the brain tumor and concomitantly lower concentrations in extracranial sites thereby reducing systemic toxicity.

Ozawa et al. [30, 31], as well as we [37], have observed that CED and Alzet pump infusion resulted in significantly higher tumor and lower normal brain boron concentrations than those obtained following systemic administration. They were the first to report that CED significantly increased the tumor uptake of two boronated porphyrins, designated TABP-1 and BOPP, although no BNCT studies were carried out. As shown by us in the present studies, although CED of the carboranylporphyrins has solved the problem of high extracranial tissue uptake by the liver and spleen, the seemingly high tumor boron values did not accurately reflect the true intracellular uptake of the compounds. Direct i.c. administration appears to be the preferred route of administration for the presently available carboranylporphyrins. However, increasing their intracellular localization and homogeneous distribution depend on their physico-chemical properties and mechanism of delivery, which could improve their therapeutic efficacy. Therefore, we currently are synthesizing compounds with enhanced tumor cell uptake. In addition, it would be highly advantageous to have tumor selective compounds that readily cross the BBB, and that could be administered systemically and attain high tumor and low normal brain and extracranial tissue concentrations. It is noteworthy that in vitro studies on the cellular uptake of H_2TCP studies with the murine B16 melanoma [24, 48] cells and H_2TBP with human T98 glioblastoma cells [28] demonstrated intracellular fluorescence of cells that had been incubated with these compounds. Despite their tetra-anionic nature, they were able to penetrate plasma membranes to a certain extent, and may not have formed aggregates, thereby producing intense cellular fluorescence.

Our data provide a cautionary note that high "tumor" boron concentrations do not necessarily mean that the boron delivery agent is localized *within* tumor cells. In the future, we plan to carry out studies using secondary ion mass spectrometry (SIMS) [51] to obtain quantitative data on the boron concentrations of individual tumor cells in tissue sections. This method has been used to determine the cellular and subcellular localization of BPA [52], BSH [53] and carboranyl nucleosides [54]. The challenge will be synthesize and evaluate non-toxic carboranylporphyrins with improved water solubility, which attain high in vivo tumor cell uptake following either systemic injection or direct i.c. administration. Based on the studies of Ozawa et al. [30, 31], Jori, et al. [48] and ourselves, it can be concluded that these compounds are a class of boron delivery agents that warrant further investigation.

Acknowledgments This paper is dedicated to Professor Otto Harling in recognition of his outstanding contributions to the field of BNCT research, and more specifically to his vision and foresight that made the Massachusetts Institute of Technology Research Reactor one of the leading facilities in the world to carry out BNCT studies. Sadly, such studies are no longer being carried out at this facility. We thank Ms. Michelle Van Fossen for expert secretarial assistance in the preparation of this manuscript and Dr. Michael Pennell, Division of Biostatistics, OSU, College of Public Health, for his helpful comments relating to statistical evaluation of the data. The studies described in this report were supported by N.I.H. grants R01 CA098902 (M.G.H.V.) and R01 CA098945 (R.F.B.), and the United States Department of Energy through the program of Innovations in

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Conflicts of interest There are no conflicts of interest.

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Synthesis of optically active dodecaborate-containing L-amino acids for BNCT

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ABSTRACT

A convenient and simple synthetic method of dodecaboratethio-L-amino acid, a new class of tumor-seeking boron carrier for BNCT, was accomplished from *S*-cyanoethylthioundecahydro-*closo*-dodecaborate (*S*-cyanoethyl-^{10}BSH, $[^{10}B_{12}H_{11}]^{2-}SCH_2CH_2CN$) and bromo-L-α-amino acids by nearly one step *S*-alkylation. An improved synthesis of *S*-cyanoethyl-^{10}BSH, a key starting compound for *S*-alkylation, was also performed by Michael addition of ^{10}BSH with acryronitrile in high yield. Four kinds of new dodecaboratethio-L-amino acids were obtained in optically pure form without the need for any optical resolution.

1. Introduction

Boron-containing L-amino acids are worthwhile synthetic targets due to their potential biological activities, particularly with respect to the boron-neutron capture therapy (BNCT). In many tumor tissues, L-amino acid transport is enhanced to guarantee the multiplication of tumor cells compared with normal tissues (Endou and Kanai, 1999). Therefore, various boron-containing α-amino acids that are closely similar in structure to the usual amino acid such as *p*-boronophenylalanine (BPA) and *o*-carboranyl-glycine, have been synthesized and evaluated (Varadarajan and Hawthorne, 1991; Srivastsva et al., 1997). However, such boron-containing amino acids have low water-solubility associated with poor bioavailability as disadvantages.

Recently, Gabel et al. reported that the synthesis of a new class of water soluble α-amino acids in racemic states, which contained the dianionic dodecaboratethio ($[^{10}B_{12}H_{11}]^{2-}$-S-) unit from undecahydro-*closo*-dodecaborate (BSH) by stepwise alkylation using bromoalkyl-*N*-aceto-amidomalonate derivatives followed by decarboxylation and hydrolytic deprotection (Slepukhina and Gabel, 2006). However, these methods have not been entirely satisfactory, particularly for large amount preparation owing to multiple steps, and for racemic form of the target amino acids.

Here, we describe an efficient route for the simple synthesis of optically pure dodecaboratethio-L-amino acids **1–4** (Fig. 1) as illustrated in the schemes.

2. Material and method

2.1. General

^{1}H NMR spectra were measured on a JMTC-400/54/SS (400 MHz, JEOL Ltd., Tokyo, Japan) spectrometer. The chemical shifts in ^{1}H NMR are given in δ values from TMS used as internal standard. Optical rotations were measured on a Jasco P-2200 polarimeter (JASCO Co., Tokyo, Japan). Electron spray ionization time of flight mass spectra (ESI-TOF MS) was obtained on a Nanofrontier LD (Hitachi High-Technologies Corporation, Tokyo, Japan). ^{10}BSH was provided by Stella Pharma Corporation (Osaka, Japan).

2.2. Synthesis of bis-tetramethylammmonium S-(cyanoethyl)-thioundecahydro-closo-dodecaborate (2) by Michael addition

To a solution of ^{10}BSH.2NMe$_4$ (1.00 g, 3.20 mmol) and 1N NaOH aq. (3.20 mL, 3.20 mmol) in H_2O (20 mL) was added acrylonitrile (255 mg, 4.80 mmol) at room temperature. After stirring for 3 h, the reaction mixture was washed with EtOAc (20 mL × 3), and the aqueous layer was concentrated in vacuo. The residual solid was recrystallized from H_2O to give **2** as colorless crystal (1.08 g, 92%): mp 280–285 °C, ^{1}H NMR (400 MHz, D_2O) δ 0.7–1.5 (11H, m, $^{10}B_{12}H_{11}$), 2.56 (2H, m, $CNCH_2CH_2S$-), 2.65 (2H, m, $CNCH_2CH_2S$-), 3.10 (24H, s, $-N^+(CH_3)_4$).

2.3. Synthesis of dodecaboratethio-L-amino acids (1a–d)

The mixture of bis-tetramethylammmonium *S*-(cyanoethyl)thio-undecahydro-*closo*-dodecaborate (**2**, 0.27 mmol) and ω-bromo-L-amino acids **3a–d** (0.41 mmol) in dry MeCN (7 mL) under argon atmosphere was refluxed for 12 h, and the reaction mixture was

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concentrated in vacuo. The residual solid was suspended in acetone (30 mL), and the suspension was filtrated by suction to remove the insoluble solid. To the filtrate was added 10% tetramethylammonium hydroxide in MeOH (0.28 mmol) at 0 °C, and the mixture was stirred for 30 min at the same temperature. The collected precipitate by filtration was washed quickly with acetone (30 mL). After dissolving with water, the aqueous solution was passed through an ion-exchange column (Amberlite IR-120, H^+ form). The neutralized filtrate with NaOH was chromatographed using of ODS column to give pure dodecaboratelthio-L-amino acids **1a–d**.

*2.3.1. (R)-2-Amino-3-(dodecaboranylthio)pro-panoic acid disodium salt (**1a**)*

^{1}H NMR (D_2O); 0.75–1.80 (11H, m, $^{10}B_{12}H_{11}$), 2.52–2.66 (2H, m, 3-CH_2), 3.80 (1H, m, 2-CH); ESI-TOF MS (neg.): found m/z 274.5 $[M+Na]^-$ (calcd. for $C_3H_{17}{}^{10}B_{12}NO_2S$+Na: 274.2).

*2.3.2. (S)-2-Amino-4-(dodecaboranylthio)butyric acid disodium salt (**1b**)*

^{1}H NMR (D_2O); 0.75–1.60 (11H, m, $^{10}B_{12}H_{11}$), 1.91–2.03 (2H, m, 3-CH_2), 2.43 (2H, m, 4-CH_2), 3.62 (1H, m, 2-CH); $[\alpha]_D^{25}$ −1.93 (*c* 0.505, H_2O); ESI-TOF MS (neg.): found m/z 288.2 $[M+Na]^-$ (calcd. for $C_4H_{19}{}^{10}B_{12}NO_2S$+Na: 288.3).

*2.3.3. (S)-2-Amino-5-(dodecaboranylthio)pentanoic acid disodium salt (**1c**)*

^{1}H NMR (D_2O); 0.75–1.50 (11H, m, $^{10}B_{12}H_{11}$), 1.50 (2H, m, 4-CH_2), 1.60–1.80 (2H, m, 3-CH_2), 2.37 (2H, m, 5-CH_2), 3.30 (1H, m, 2-CH); $[\alpha]_D^{25}$ −2.06 (*c* 0.515, H_2O); ESI-TOF MS (neg.): found m/z 302.6 $[M+Na]^-$ (calcd. for $C_5H_{21}{}^{10}B_{12}NO_2S$+Na: 302.3).

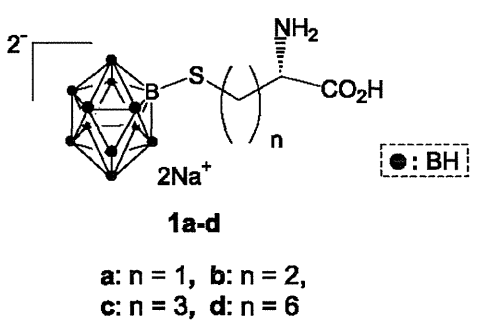


Fig. 1. Dodecaboratethio-L-amino acids.

*2.3.4. (S)-2-Amino-5-(dodecaboranylthio)oc-tanoic acid disodiumsalt (**1d**)*

^{1}H NMR (D_2O); 0.75–1.60 (11H, m, $^{10}B_{12}H_{11}$), 1.21–1.41(4H, m, 4-CH_2, 5-CH_2), 1.41 (2H, m, -6-CH_2), 1.70(4H, m, 3-CH_2-, 7-CH_2), 2.34 (2H, t, J=7.3 Hz, 8-CH_2), 3.57(1H, m, 2-CH); $[\alpha]_D^{25}$ −1.96 (*c* 0.515, H_2O); ESI-TOF MS (neg.): found m/z 344.5 $[M+Na]^-$ (calcd. for $C_8H_{27}{}^{10}B_{12}NO_2S$+Na: 344.3).

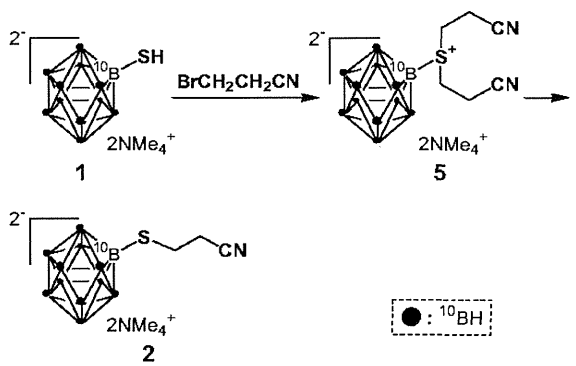
3. Results and discussion

In our initial attempt we employed direct alkylation of ^{10}BSH with ω-bromo-L-amino acid to prepare mono-*S*-alkyl^{10}BSH, however, the inseparable mixture of mono- and di-*S*-alkyl adducts were invariably formed. After several unsuccessful trials, we employed stepwise alkylation method using *S*-cyanoethyl-^{10}BSH (**2**), a key intermediate in this synthesis, according to the reported method (Gabel et al., 1993).

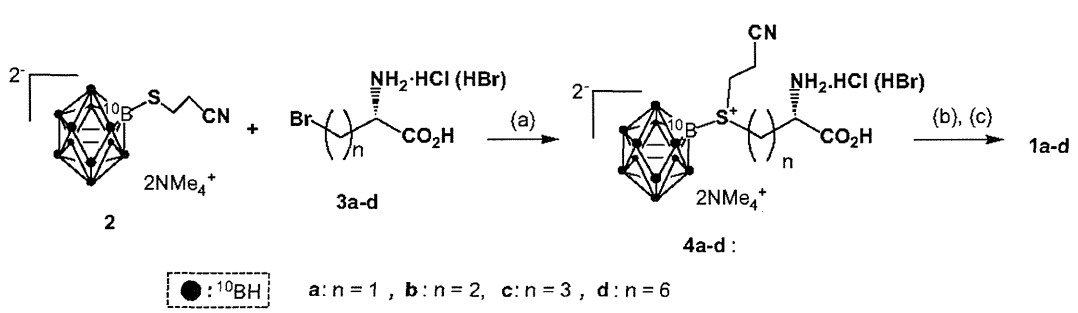
Gabel et al. have reported the stepwise synthesis of *S*-cyanoethyl-^{10}BSH (**2**), a useful intermediate for alkylation, stating from ^{10}BSH and bromo-propionitrile by two steps sequence. However, the overall yields were unsatisfactory.

We devised more efficient synthetic route based on hetero Michael reaction as shown in Scheme 1–3. Thus, ^{10}BSH was treated with acrylonitrile in aqueous solution using sodium hydroxide as a base to give pure *S*-cyanoethyl-^{10}BSH (**2**) as solid in 88% yields.

On the other hand, ω-bromo-L-amino acids (**3a–d**), represented as Br-(CH_2)n-CH(NH_2)COOH (n=1, 2, 3, 6), were prepared as hydrochloric or hydrobromic salts. Among them, (*S*)-2-amino-4-bromobutyric acid (**3b**, n=2) was commercially purchased, and other ω-bromo-L-amino acids bearing (*L*)-configuration were obtained

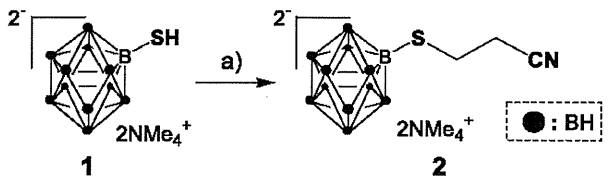


Scheme 2. Stepwise synthesis of *S*-cyanoethyl BSH by alkylation.



Reagents and conditions: a) MeCN, reflux, 24h, b) Me_4NOH, $MeNH_2$, acetone, r.t., 30 min, c) amberlite IR-120 (Na+)

Scheme 1. Simple and efficient synthesis of dodecaboratethio -L-amino acids (**1a–d**).



Reagents and conditions: a) acrylonitrile, NaOH / H_2O

Scheme 3. One step synthesis of *S*-cyanoethyl BSH by hetero Michael reaction.

according to the modified literature methods (Phadnis and Mugesh, 2005; Kanai et al., 1985; Watanabe et al., 2004), respectively.

General synthetic procedure for alkylation of **2** with bromo-L-amino acids (**3**) is very simple as follows; a mixture of **2** and **3** in acetonitrile was refluxed for one day, followed by condensation to give conjugates (**4**), which was used to the next step without further purification. Treatment of **4** in acetone with tetramethylammonium hydroxide (Me_4NOH) in the presence of methylamine furnished the target amino acid (**1**) in moderate yields. In the case of **1a**, the overall yields were poor (21%) due to its lability. The purity and chemical structure of **1** were analyzed by NMR, ESI-MS and capillary electrophoresis.

The biological activities of synthesized L-amino acids **1b–d** are currently examined using cultivated tumor cells and animals bearing B16 cancer cells.

4. Conclusions

We have accomplished the effective and simple synthesis of dodecaboratethio-L-amino acid by nearly one-step alkylation of *S*-cyanoethyl BSH, with non-protected bromo-L-amino acids in moderate yields. In the present synthesis, an absolute configuration of the starting bromo-L-amino acid is to be introduced to the final amino acids in retention. We believe that this synthetic method could be applied to another boron cluster containing optically active amino acids, such studies being currently progress. Biological study of the compounds obtained here is also now under investigation.

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