Chemical Communications (communication article)

Supplementary Information

Title:

Intracellular target delivery of cell-penetrating peptide-conjugated dodecaborate for boron neutron capture therapy (BNCT)

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1. Materials and Methods

General

^{10}BSH was provided by Stella Pharma Corporation (Osaka, Japan) (Fig. S1). Flash ODS column chromatography was carried out using an Isolera Spektra (Biotage Sweden AB, Uppsala, Sweden) with a SNAP UltraC18 Cartridge (60 g, Biotage Sweden AB). ^{1}H and ^{13}C NMR spectra were measured on a JNM-ECZR (500 MHz, JEOL Ltd., Tokyo, Japan). ESI-MS measurements were performed on a JMS-T100LC AccuTOF™ (JEOL Ltd., Tokyo, Japan)

Synthesis of S-(2-hydroxycarbonylethyl)thioundecahydro-closo-dodecaborate disodium salt (DB-COOH)

A solution of BSH·2Cs (200 mg, 0.464 mmol) in H_2O (5 mL) was added to 1 N NaOH (0.46 mL) and methyl acrylate (46.2 μL, 0.510 mmol). After stirring for 1 hr, 2 N NaOH (5 mL) was added to the reaction mixture and stirred for 12 hr at 60 °C. The reaction mixture was acidified with Amberlite IR-120(H^+). After filtration, the solution was neutralized with 1 N NaOH. The mixture was concentrated *in vacuo*, and the residual oil was purified by flash ODS column chromatography to give DB-COOH (120 mg, 92%) as a colourless oil. ^{1}H-NMR: (500 MHz, D_2O) δ 2.67 (t, J = 7.7 Hz, 2H), 2.50 (t, J = 7.7 Hz, 2H), 1.76-0.83 (11H) (Fig. S8). ^{13}C-NMR: (126 MHz, D_2O) δ 181.76, 39.66, 28.45 (Fig. S9). MS: (neg. ESI, m/z) 281.2140 $[M]^-$ (Fig S10).

Peptide synthesis

All peptides were chemically synthesized on a Rink amide resin with a coupling system using 1-hydroxybenzotriazole (HOBt) / 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (Peptide Institute, Osaka, Japan) / N,N-diisopropylethylamine (DIEA) in 9-fluorenylmethyloxycarbonyl (Fmoc) using a solid-phase peptide synthesis method, as previously described.[1,2] The Rink amide resin and the Fmoc-amino acid derivatives were purchased from Shimadzu Biotech (Kyoto, Japan) and the Peptide Institute (Osaka, Japan), respectively. Deprotection of the protected peptide and cleavage from the resin was performed by treatment with a trifluoroacetic acid (TFA) / ethanedithiol (EDT) mixture (95:5) for 3 hr at 20 °C, followed by reverse-phase high-performance liquid chromatography (HPLC) for peptide purification. The purity of each peptide was estimated to be >97% on the basis of the analytical HPLC. The structures of the synthesized peptides were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) (Microflex, Bruker, Billerica, MA, USA).

RLA (NH_2-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-amide): MALDI-TOF MS: 1691.0 [calculated for $(M+H)^+$: 1691.1]. HPLC retention time, 12.6 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 20.0%.

RLA-GC (NH_2-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-Gly-Cys-amide): MALDI-TOF MS: 1850.6 [calculated for $(M+H)^+$: 1851.3]. HPLC retention time, 13.5 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin 8.6%.

r8

(NH_2-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-amide): MALDI-TOF MS: 1266.8 [calculated for $(M+H)^+$: 1266.5]. HPLC retention time, 7.6 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 23.7%.

r8-GC

(NH_2-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-Gly-Cys-amide): MALDI-TOF MS: 1426.8 [calculated for $(M+H)^+$: 1426.7]. HPLC retention time, 8.1 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 22.4%.

Fluorescently labelled peptides

Fluorescent labelling of the purified peptides, *RLA*-GC and r8-GC, was performed by treatment with 1.1 equivalents of BODIPY FL N-(2-aminoethyl))maleimide (ThermoFisher Scientific Inc., Rockford, IL, USA) in a dimethyl formamide / methanol mixture (1:1) for 1.5 hr at room temperature, followed by HPLC purification, as previously reported.[2]

RLA (BODIPY)

(NH_2-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-Gly-Cys(BODIPY)-amide): MALDI-TOF MS: 2264.6 [calculated for $(M+H)^+$: 2265.5]. HPLC retention time, 15.5 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 4.9%.

r8 (BODIPY)

(NH_2-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-Gly-Cys(BODIPY)-amide): MALDI-TOF MS: 1840.5 [calculated for $(M+H)^+$: 1840.9]. HPLC retention time, 13.3 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 13.7%.

Conjugation of DB-COOH and cell-penetrating peptide

Conjugation of DB-COOH to the N-terminal of each purified peptide (RLA, r8, RLA (BODIPY), or r8 (BODIPY)) was performed by treatment with 4 equivalents of DB-COOH in dimethyl formamide with the following coupling reagents: HOBt, HBTU, and DIEA (each 4 equivalent) for 1.5 hr at 50 °C, followed by HPLC purification (Fig. S1).

DB-*RLA* (Dodecaborate-S-C_2H_4-CO-NH-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-amide): MALDI-TOF MS: 1911.2 [calculated for $(M+H)^+$: 1910.3]. HPLC retention time, 16.5 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 1.0%.

DB-r8

(Dodecaborate-S-C_2H_4-CO-NH-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-amide): MALDI-TOF MS: 1487.1 [calculated for $(M+H)^+$: 1485.7]. HPLC retention time, 9.5 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 3.4%.

DB-*RLA* (BODIPY)

(Dodecaborate-S-C_2H_4-CO-NH-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-$_D$Leu-$_D$Ala-$_D$Arg-Gly-Cys(BODIPY)-amide): MALDI-TOF MS: 2484.8 [calculated for $(M+H)^+$: 2483.8]. HPLC retention time, 19.9 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 0.4%.

DB-r8 (BODIPY)

(Dodecaborate-S-C_2H_4-CO-NH-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-$_D$Arg-Gly-Cys(BODIPY)-amide): MALDI-TOF MS: 2059.9 [calculated for $(M+H)^+$: 2060.1]. HPLC retention time, 15.1 min (column: Cosmosil 5C18-AR-II (4.6 × 150 mm); gradient: 5-95% B in A (A = H_2O containing 0.1% CF_3COOH, B = CH_3CN containing 0.1% CF_3COOH) over 30 min; flow: 1 mL/min; detection: 220 nm). Yield from the starting resin: 0.4%.

Cell cultures

C6 glioma (rat brain glioma-derived) cells were kindly provided by Dr. Minoru Suzuki (Kyoto University). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (D-MEM) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated FBS (Gibco, Life Technologies Corporation). The cells were grown in 100-mm dishes and incubated at 37 °C under 5% CO_2.

Confocal microscopy (live cells)

C6 glioma cells (each 4.7×10^4 cells/200 μL) were seeded into a μ-Slide 8 well chamber (ibidi, Martinsried, Germany) and incubated in D-MEM containing 10% FBS for 24 hr at 37 °C under 5% CO_2. After complete adhesion, the cells were treated with each sample (200 μl/well) at 37 °C under 5% CO_2. The cells were stained with MitoTracker Red CMXRos (500 nM) (ThermoFisher Scientific) and Hoechst 33342 dye (5 μg/mL) (ThermoFisher Scientific) for 15 min at 37 °C. The cells were then washed with fresh cell culture medium and analysed using an FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40× or 60× objective without cell fixation.

Confocal microscopy (antibody stain)

C6 glioma cells (4.7×10^4 cells/200 μL) were seeded into a μ-Slide 8 well chamber (ibidi,

Martinsried, Germany) and incubated in D-MEM containing 10% FBS for 24 hr at 37 °C under 5% CO_2. After complete adhesion, the cells were treated with each sample (200 μL/well) at 37 °C under 5% CO_2. After removal of the medium, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min and washed with PBS. The cells were then treated with 0.1% Triton X-100 (200 μL/well in PBS) at room temperature for 5 min and again washed with PBS. Cellular dodecaborate uptake was visualized by treatment with anti-BSH antibody A9H3[3] and fluorescently labelled secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L), ThermoFisher Scientific) (each 1 hr at room temperature) prior to analysis using an FV1200 confocal laser scanning microscope (Olympus) equipped with a 40× objective.

Cell viability (WST-1 assay)

Cell viability was analysed using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay, as previously reported.[4,5] C6 glioma cells (1×10^4 cells/100 μL) were incubated in 96-well microplates in MEM containing 10% FBS for 24 h at 37 °C under 5% CO_2. The cells were then treated with each sample (50 μL) at 37 °C under 5% CO_2. After the sample treatment, WST-1 reagents (10 μL) were added to each well, and the samples were incubated for 40 min at 37 °C. The absorbencies at 450 nm (A450) and 620 nm (A620) were measured, and the value obtained by subtracting A620 from A450 corresponded to the viable cell number.

ELISA

C6 glioma cells (1.5×10^6 cells/well) were pre-cultured in a 24-well microplate for 24 hr in 10% FBS-containing D-MEM. The medium was replaced with an equivalent medium containing each boron compound (the final concentration was 1.0 mM BSH and 25 μM CPP-conjugated dodecaborate in 10% FBS-containing D-MEM (900 μL)). After incubating for 30 min at 37 °C under 5% CO_2, the cells were harvested with 0.05% trypsin/0.02% EDTA in PBS and suspended in 5 mL of D-MEM. After cell counting, the cells were collected by centrifugation (194 × g) for 5 min. The pelleted cells were resuspended in 5 mL of 0.05% Tween-20 and allowed to stand for 10 min. The boron concentration of the obtained cell lysate was determined by competitive ELISA using anti-BSH antibody A9H3.[3]

Thermal neutron irradiation and assessment for cell-killing effect (surviving fraction)

C6 glioma cells (1.0×10^5 cells/well) were seeded into a 6-well microplate and grown for

24 hr in 10% FBS-containing D-MEM. The medium was replaced with an equivalent medium containing each boron compound (the final concentration was 1.0 mM BSH, 5, 15, or 25 μM CPP-conjugated dodecaborate in 10% FBS-containing D-MEM (850 μL/well)). The cells were cultured for 30 min at 37 °C under 5% CO_2, and the medium was removed by aspiration. The cells were then washed with PBS, harvested with 0.05% trypsin/0.02% EDTA in PBS, and then counted. After centrifugation, trypsin was removed by aspiration, and 10% FBS-containing D-MEM was added to the residual cells. The cell suspension in 10% FBS-containing D-MEM (5.0×10^3 cells/mL, 1 mL) was irradiated with thermal neutrons (Institute for Integrated Radiation and Nuclear Science, Kyoto University, Osaka, Japan) for 90 min in a column-shaped tube. The thermal neutron fluence was determined by averaging two gold foils that were symmetrically attached to the surface of the column-shaped tube along the direction of incidence of the thermal neutrons. After thermal neutron exposure, 1,000 cells were placed in 6-well microplates with 10% FBS-containing D-MEM (3 mL) 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.

Caspase 3/7 assay

After the thermal neutron irradiation described in "*Thermal neutron irradiation and assessment for cell-killing effect (surviving fraction)*", the cells were added to lysis buffer (50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). The protein concentration was then determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). Each cell lysate was added to caspase 3/7 substrate solution (Caspase-3/7 Fluorescence Assay Kit, Cayman Chemical, Ann Arbor, MI, USA) and incubated in accordance with the instructions prior to reading the fluorescence intensity (485 nm (excitation), 535 nm (emission)).

ATP assay

After the thermal neutron irradiation described in "*Thermal neutron irradiation and assessment for cell-killing effect (surviving fraction)*", the cells were added to lysis buffer (50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate). The protein concentration was then determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc.). Each cell lysate was added to ATP assay probe solution (ATP Assay Kit, Abcam, Cambridge, UK) and incubated in accordance with the instructions prior to detecting the

optimal density (OD 570 nm).

Annexin V assay

After the thermal neutron irradiation described in "*Thermal neutron irradiation and assessment for cell-killing effect (surviving fraction)*", the cells were seeded into glass-bottom dishes with 10% FBS-containing D-MEM and incubated for 24 hr at 37 °C under 5% CO_2. After pre-incubation for 30 min at 4 °C, the cells were treated with FITC-annexin V (0.5 μg/mL) and propidium iodide (2 μg/mL) in 10% FBS-containing D-MEM (total 100 μL) for 30 min at 4 °C. The cells were washed with fresh cell culture medium and analysed using an FV1200 confocal laser microscope equipped with a 40× objective.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (ver. 5.00; GraphPad, San Diego, CA, USA). For comparisons of two groups, unpaired Student's t-test was used after verification of the equal variances with an F-test. For multiple comparison analyses, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. Differences were considered significant when the calculated p-value was < 0.05.

References

[1] S. Futaki, W. Ohashi, T. Suzuki, M. Niwa, S. Tanaka, K. Ueda, H. Harashima, Y. Sugiura, *Bioconjug Chem.,* 2001, **12**, 1005–1011.

[2] I. Nakase, I. A. Tadokoro, N. Kawabata, T. Takeuchi, H. Katoh, K. Hiramoto, M. Negishi, M. Nomizu, Y. Sugiura, S. Futaki, *Biochemistry,* 2007, **46**, 492–501.

[3] M. Kirihata, K. Uehara, T. Asano, Preparation of borocaptate(10B) (BSH) derivatives as hapten compound for production of antibody against BSH. *PCT Int. Appl*. 2007, WO2007097065 A1 20070830.

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[5] I. Nakase, S. Futaki, *Sci. Rep.,* 2015, **5**, 10112.

2. Supporting Figures

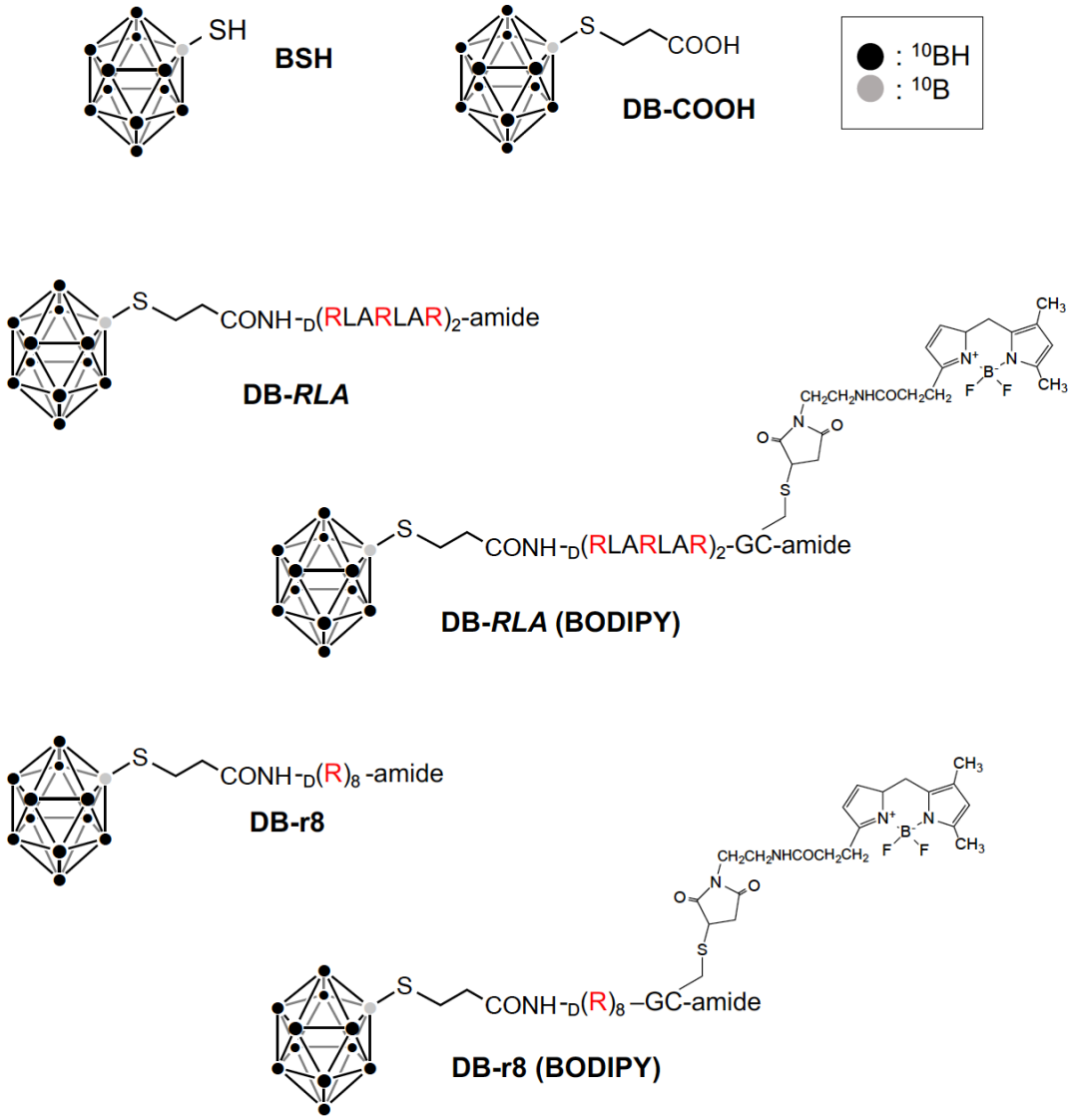


Fig. S1. Chemical structures of cell-penetrating peptide-conjugated dodecaborates.

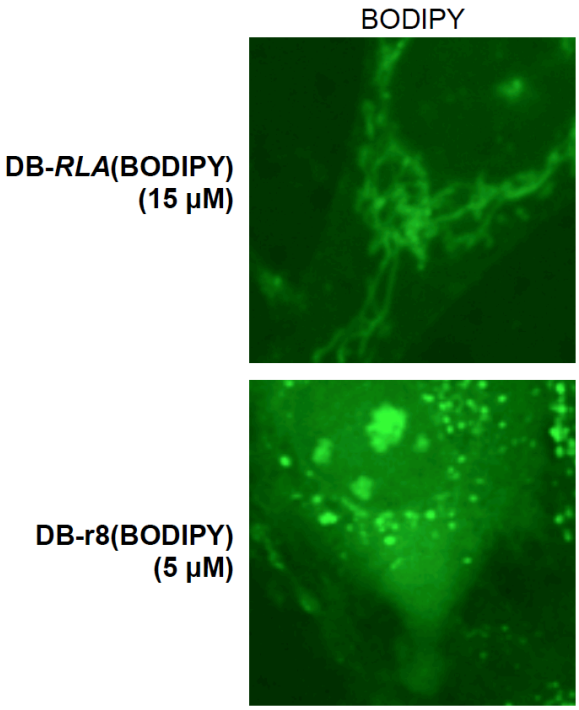


Fig. S2. Mitochondria and cytosolic delivery of dodecaborates by conjugation with mitochondria or cytosol-targeted CPPs. Enlarged pictures of Fig. 2a. Confocal microscopic observation of C6 glioma cells treated with each BODIPY-labelled dodecaborate-CPP conjugate in 10% FBS-containing D-MEM for 30 min at 37 °C. Green signal: BODIPY.

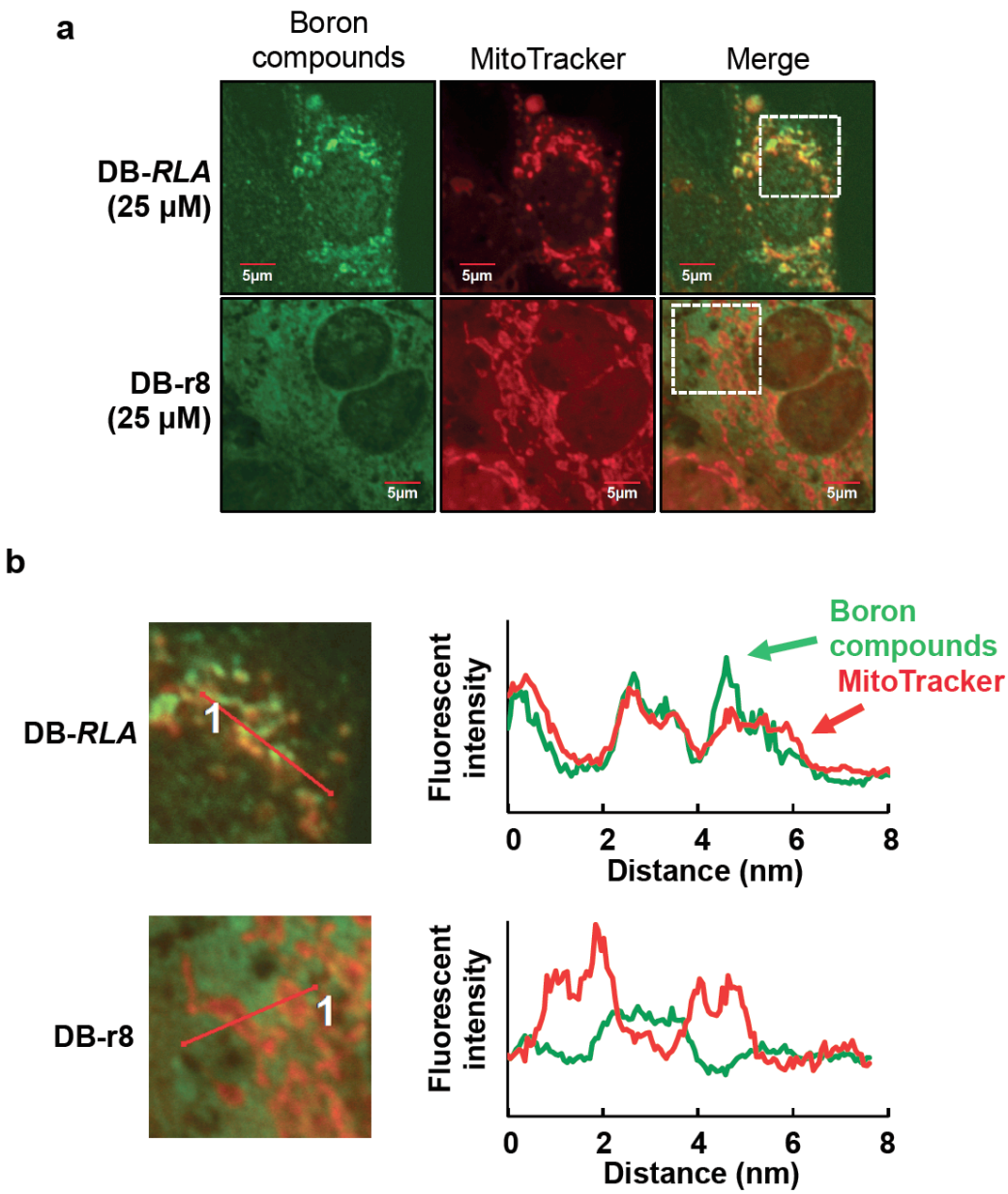


Fig. S3. Mitochondria- and cytosol-targeted CPPs can carry dodecaborates to targeted intracellular locations. (a) Confocal microscopic observation of C6 glioma cells stained with anti-dodecaborate antibody after treatment with each dodecaborate-CPP conjugate in 10% FBS-containing D-MEM for 30 min at 37 °C. Green signal: boron compounds, red signal: MitoTracker. Scale bars: 5 μm. (b) Fluorescent intensity of red lines in the merged images of (a) (dotted squares in (a)). Green: boron compounds, red: MitoTracker.

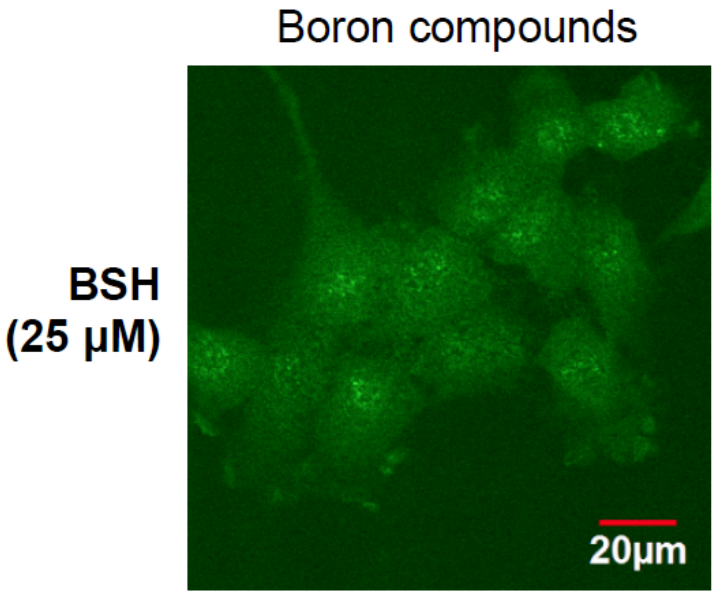


Fig. S4. Intracellular location of BSH after cellular uptake. Confocal microscope observation of C6 glioma cells stained with anti-dodecaborate antibody after treatment with BSH (25 μM) in 10% FBS-containing D-MEM for 30 min at 37 °C. Green signal: boron compounds. Scale bars: 20 μm.

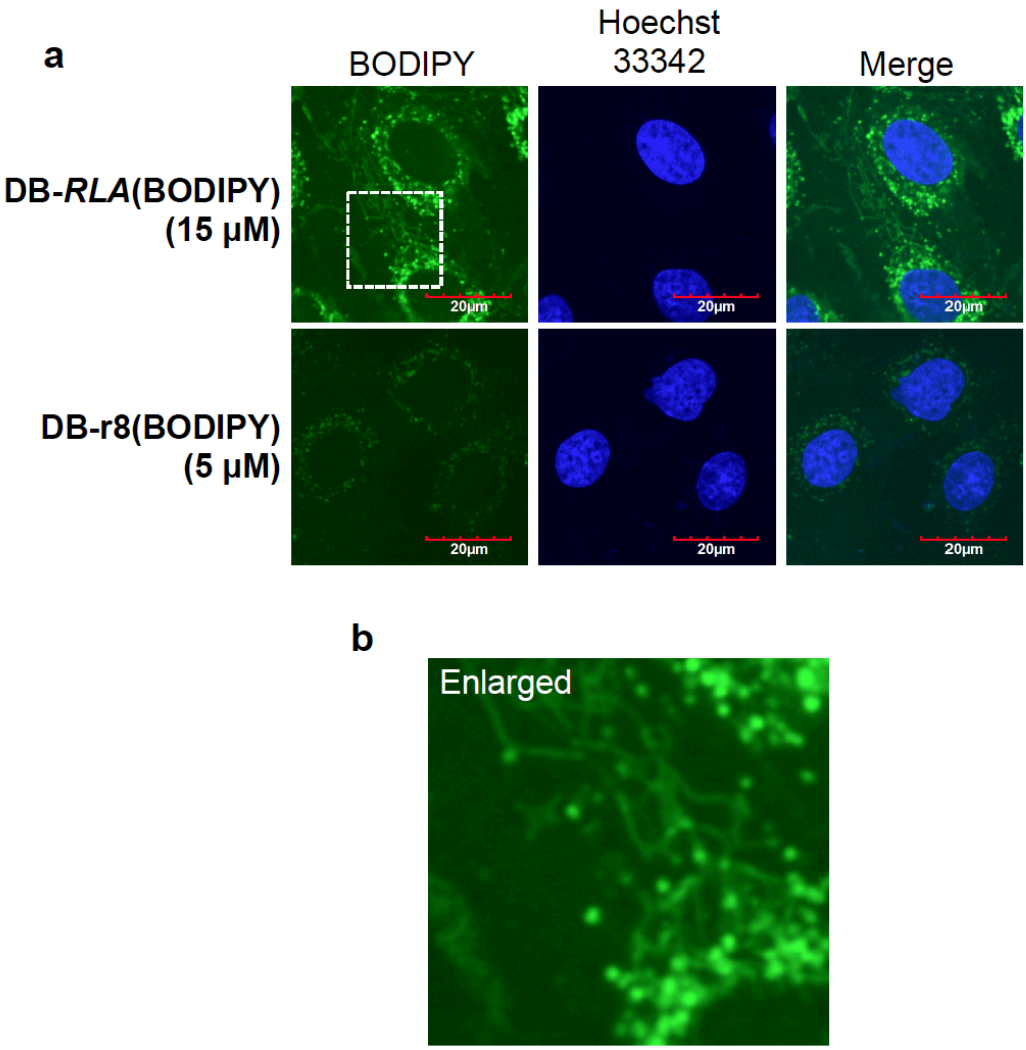


Fig. S5. High retention of *RLA* peptide-conjugated dodecaborate in mitochondria. (a) Confocal microscope observation of C6 glioma cells incubated for 24 hr at 37 °C after treatment with BODIPY-labelled DB-*RLA* or DB-r8 for 30 min at 37 °C in 10% FBS-containing D-MEM, followed by cellular washing with fresh cell culture medium. Green signal: BODIPY, blue signal: Hoechst 33342. Scale bars: 20 μm. (b) Enlarged picture of (a) (dotted square in (a)).

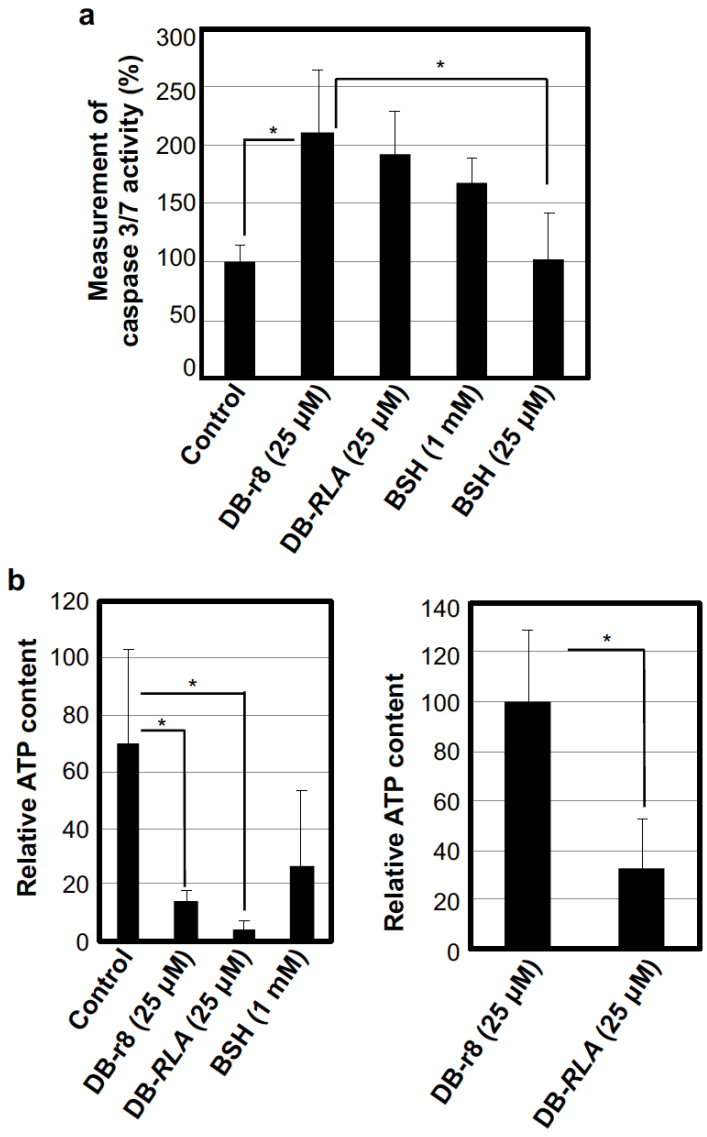


Fig. S6. *RLA* peptide-conjugated dodecaborates decreased ATP production after thermal neutron irradiation. (a, b) Measurement of caspase 3/7 activity (a) or ATP production (b) of C6 glioma cells after treatment with each dodecaborate, with or without CPP conjugation, for 30 min at 37 °C in 10% FBS-containing D-MEM and neutron irradiation (thermal neutron fluence: 1.1×10^{13} cm^{-2}, 90 min), and the amount of cell lysis. The data are expressed as the mean (± SD) of three experiments. *$p < 0.05$.

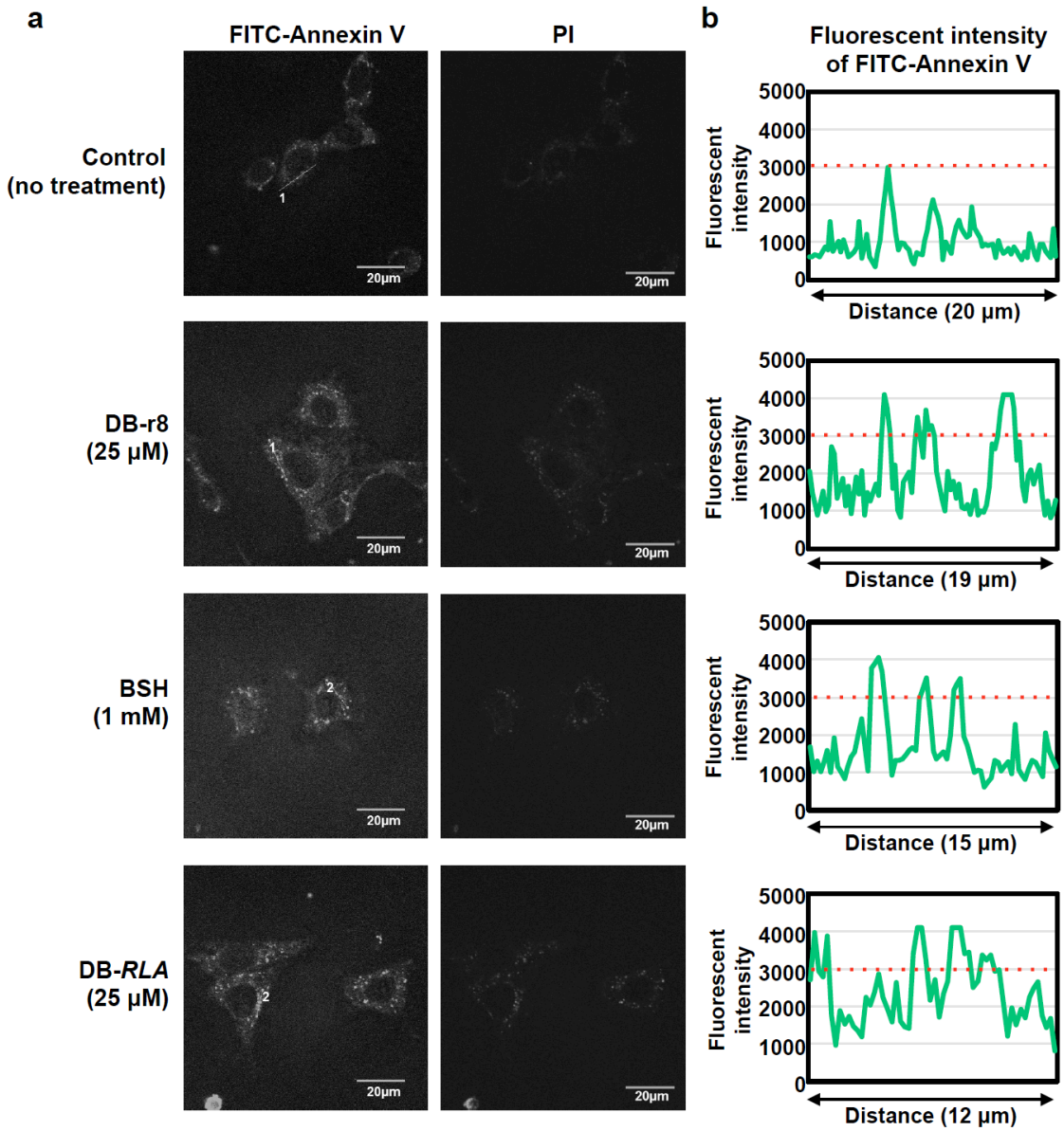


Fig. S7. Increased binding of Annexin V after treatment of CPP-conjugated dodecaborate and neutron irradiation. (a) Confocal microscope observation of C6 glioma cells after treatment with each dodecaborate, with or without conjugation to CPPs, for 30 min at 37 °C in 10% FBS-containing D-MEM, neutron irradiation (11×10^{13} cm^{-2}, 90 min), and 24 hr of incubation. (b) Fluorescence intensity along the lines in each picture of FITC-Annexin V (a).

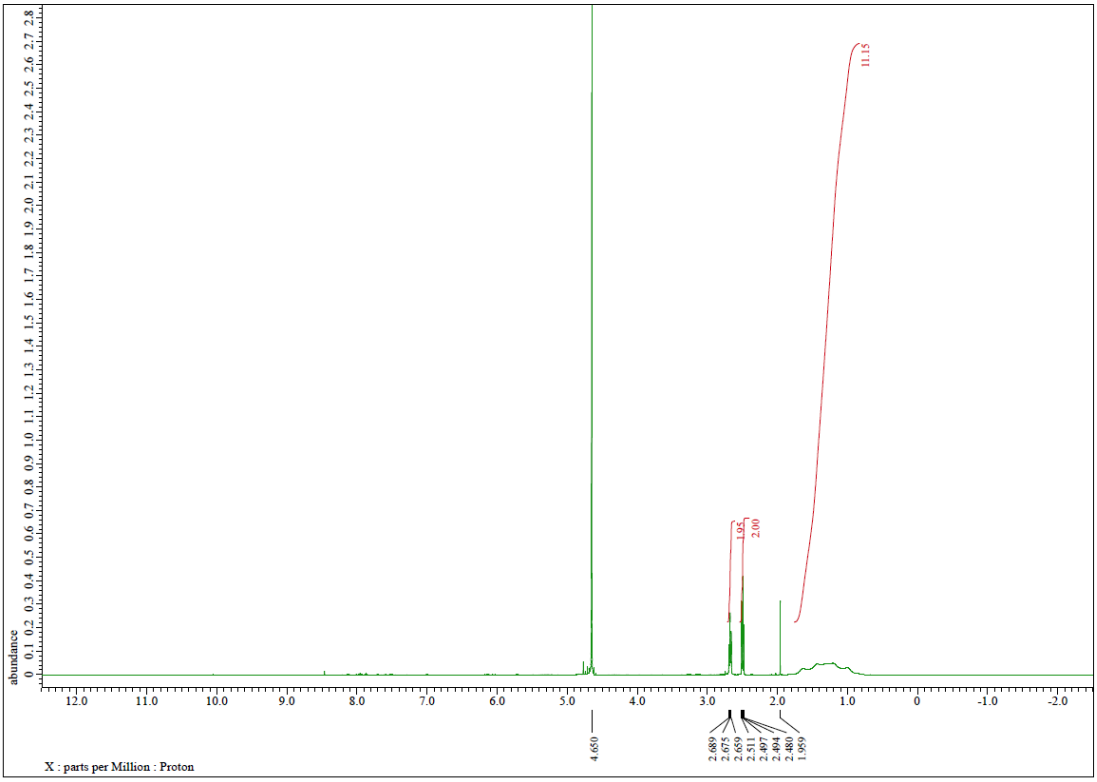


Fig. S8. ^{1}H NMR (D_2O) of *S*-(2-hydroxycarbonylethyl)thioundecahydro-*closo*-dodecaborate disodium salt.

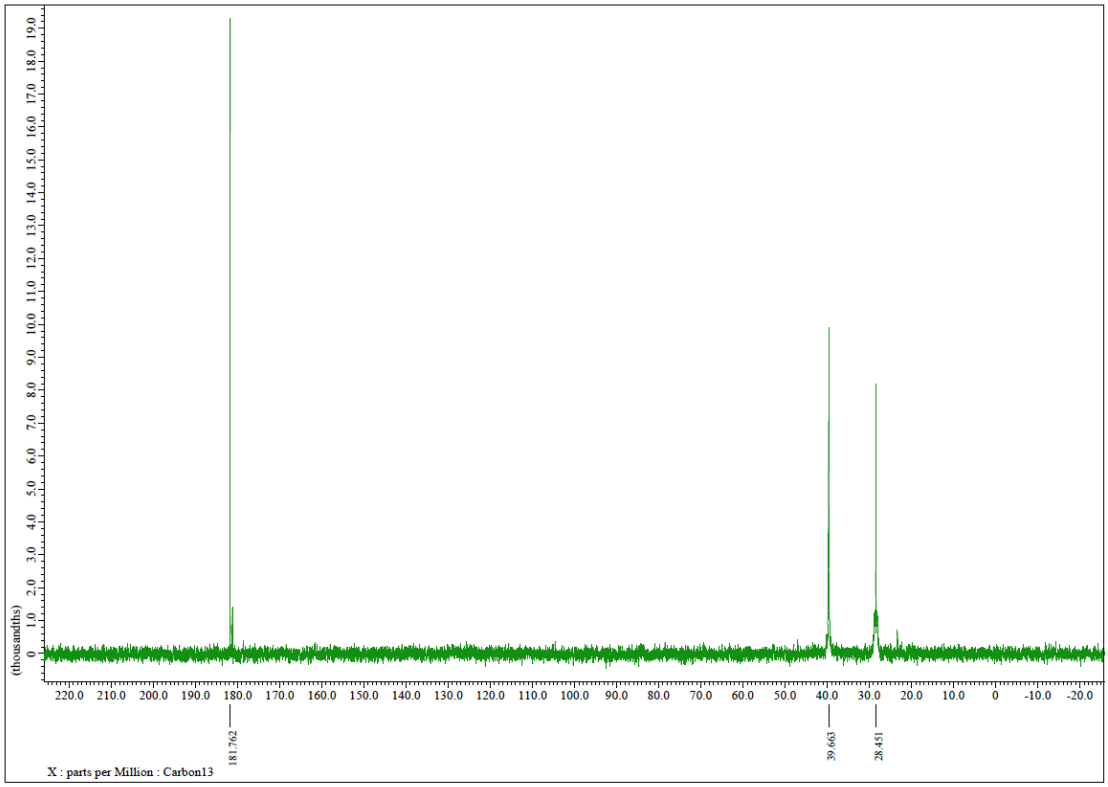
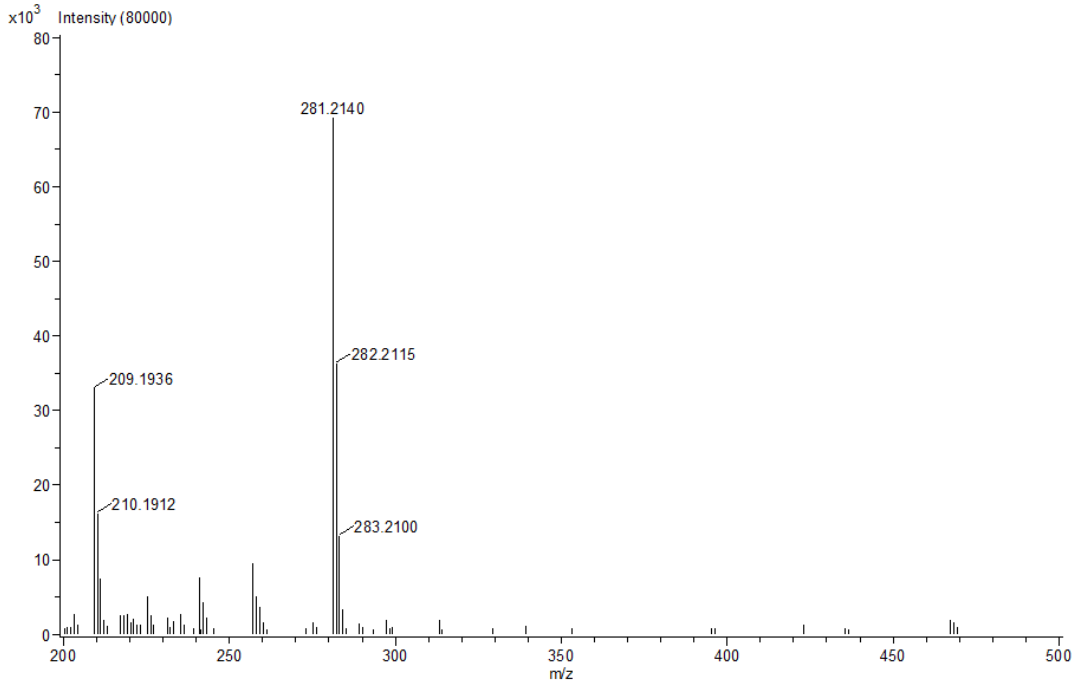
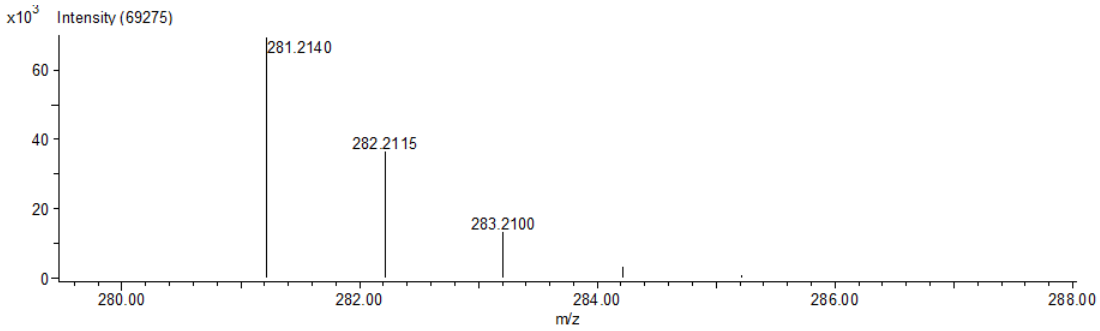


Fig. S9. ^{13}C NMR (D_2O) of *S*-(2-hydroxycarbonylethyl)thioundecahydro-*closo*-dodecaborate disodium salt.

ESI-MS (neg.)



HRMS



MS: 281.21404

Intensity: 69275.09

MS (calculated): 281.21406

Formula: $^{12}C_3{}^{1}H_{15}{}^{10}B_{12}{}^{23}Na_2{}^{16}O_2{}^{32}S_1$

Fig. S10. ESI-MS (D_2O) of *S*-(2-hydroxycarbonylethyl)thioundecahydro- *closo*-dodecaborate disodium salt.