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Reaction of Chloroacetyl-Modified Peptides with Mercaptoundecahydrododecaborate (BSH) Is Accelerated by Basic Amino Acid Residues in the Peptide

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Abstract: We assessed a reactivity of chloroacetyl-modified tripeptides consisting of various amino acid residues (Cl-3X) and mercaptoundecahydrododecaborate (BSH) by converting Cl-3X to its reactant (BS-3X). We showed that the Cl-3X consisting of basic amino acid residues (e.g., Arg) reacted with BSH effectively and its conversion decreased as the number of Arg residues in the Cl-3X decreased. Furthermore, a reactivity of the peptides with introduction of an alkyl linker between the triarginine and the chloroacetyl group (Cl-Cn-3R) with BSH decreased with increasing alkyl linker length. These results indicate that an electrostatic attraction of positively charged amino acid residues in the tripeptides and negatively charged BSH causes BSH to gather in a vicinity of the chloroacetyl group, resulting in an accelerated reaction. This work should aid a development of new boron agents using BSH in boron neutron capture therapy.

Keywords: BSH; peptide; boron neutron capture therapy (BNCT)

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1. Introduction

Boron neutron capture therapy (BNCT) is a cancer treatment method involving the killing of only nearby cancer cells by α and ^{7}Li particles generated via an irradiation of epithermal neutrons to agents containing a boron isotope ^{10}B delivered to tumor tissue [1,2]. Many researchers have developed boron agents derived from 4-borono-L-phenylalanine (BPA) or mercaptoundecahydrododecaborate (BSH; a thiol derivative composed of 12 ^{10}B atoms) for BNCT [3–5]. With boron agents, it is expected that the cell killing effect will be higher as they are closer to the target cell nucleus [6]. Therefore, we [6,7] and Nakase's group [8] conjugated BSH with cell-penetrating peptide (CPP), and reported that the BSH-CPPs delivered into cells showed effective BSH-induced cell killing. In previous studies [7,9], we used a maleimide group for the conjugation of BSH and CPP, while other researchers used the conjugation of BSH and carborane with functionalized molecules by a Michael addition reaction via the maleimide group [8,10–14]. However, the use of the maleimide group produces enantiomers, and the enantiomers may show different carcinogenicity and teratogenicity [15]. Therefore, in this study, we used a chloroacetyl group instead of the maleimide group to achieve achiral BSH-CPP conjugation.

Gabel's protocol is well known as a general synthetic method for conjugating BSH and functional molecules using a protected BSH and an alkyl halide [16–30]. This protocol is completed by reacting BSH once protected with a cyanoethyl group with the alkyl halide (alkyl bromide and alkyl iodide, etc.) at a relatively high temperature and/or for a long time, and then deprotecting the protecting group. On the other hand, our compounds, BSH-CPPs, completed the reaction without protection of BSH at room temperature and in a short time, as described in results below. Therefore, we were interested in the high reactivity between peptides modified with the chloroacetyl group and BSH. In other words, we were interested in why the BSH-CPP reaction proceeds under mild conditions. In this

study, we hypothesized that a progression of this reaction might be related to the sequence of peptides in the vicinity of the reaction site.

We synthesized tripeptides consisting of various amino acid residues with the chloroacetyl group modification at the N-terminus, Cl-X, Cl-2R, Cl-1R and Cl-C*n*-3R, as shown in Figure 1A. Using these peptides, we investigated an effect of amino acid residues in the tripeptide on the reactivity of BSH and the chloroacetyl group. We also investigated an effect of the number of arginines contained in the tripeptide, that is, the number of positive charges, on the reactivity of BSH and the chloroacetyl group. Furthermore, we evaluated an effect of an alkyl linker inserted between the chloroacetyl group and the tripeptide, that is, a distance between the positive charge and the reactive site, on the reactivity of the BSH and the chloroacetyl group. Based on the obtained results, we discussed a mechanism by which this reaction proceeds.

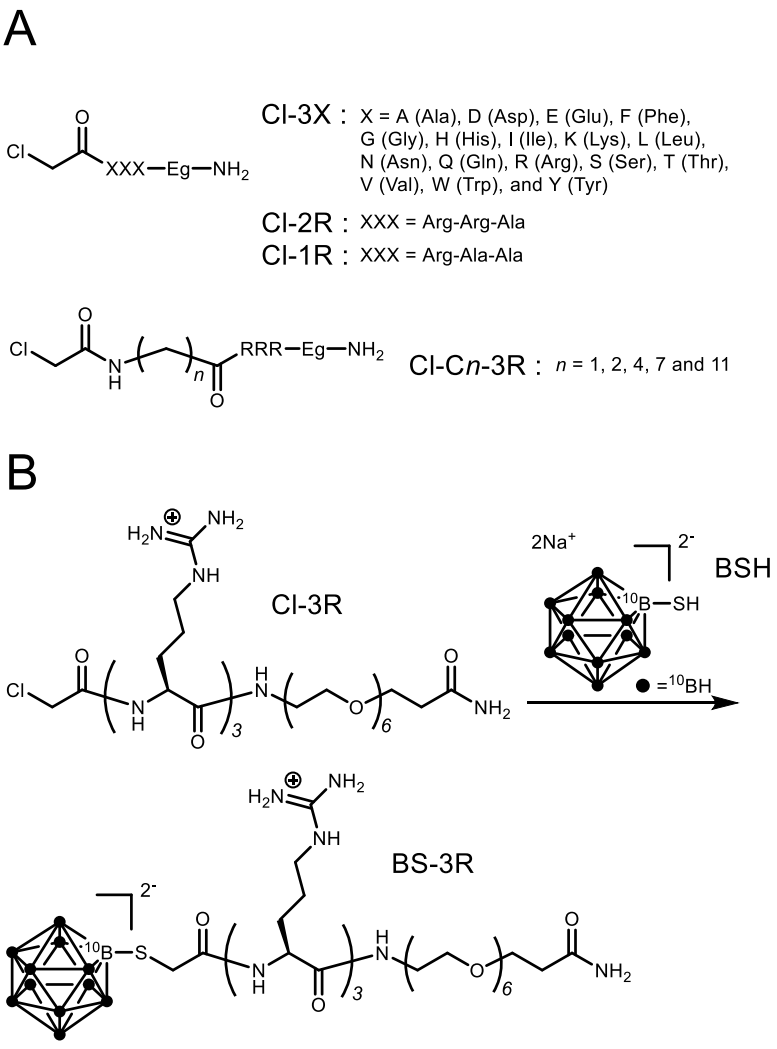


Figure 1. (**A**) Chemical structures of chloroacetyl-modified tripeptides (Cl-3X, Cl-2R and Cl-1R) and chloroacetyl-modified tripeptides into which an alkyl linker (Cl-Cn-3R) was introduced. Eg indicates a water-soluble linker consisting of six ethylene glycol residues. (**B**) Representative route of synthesis of BSH-modified tripeptides (BS-3R) from Cl-3R.

2. Materials and Methods

Undecahydro-mercaptocloso-dodecaborate ($Na_2B_{12}H_{11}SH$; BSH) was purchased from Katchem (Prague, Czechia). 9-Fluorenylmethyloxycarbonyl group (Fmoc)-derivatized super acid labile-poly(ethylene)glycol (Fmoc-NH-SAL-PEG) resin, Fmoc-derived amino acids, Fmoc-derived alkyl linkers, piperidine, *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium

hexafluorophosphate (HBTU), *N*-methylmorpholine (NMM), trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) were purchased from Watanabe Chemicals (Hiroshima, Japan). *N*,*N*′-Dimethylformamide (DMF), *N*-methyl-2-pyrrolidinone (NMP), diethyl ether, acetonitrile, triethylamine (TEA), dimethyl sulfoxide (DMSO) and dichloromethane (DCM) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Chloromethylcarbonyloxysuccinimide (ClAc-OSu) was synthesized using a procedure described in literatures (Figures S1 and S2) [31,32].

Twenty-four tripeptides consisting of a chloroacetyl group on the N-terminal, Cl-3X, Cl-2R, Cl-1R and Cl-C*n*-3R, were synthesized on Fmoc-NH-SAL-PEG resin by conventional solid-phase peptide synthesis. Fmoc deprotection was performed with 20% piperidine in DMF for 7 min at room temperature. After washing seven times with DMF, Fmoc-derived amino acids and Fmoc-derived alkyl linkers were coupled with HBTU/NMM reagents for 50 min at room temperature. No capping step was performed. After the final step, ClAc-OSu was bound with the elongated peptide chain in NMP on the resin and the resin was washed with DCM and treated with 95% TFA, 2.5% water and 2.5% TIPS for 1.5 h at room temperature. Crude peptides were precipitated in diethyl ether and washed with diethyl ether until neutral pH was reached. The crude peptides were purified by reverse-phase high-pressure liquid chromatography (HPLC) on a C18 preparative column (Cadenza 5CD-C18; Imtakt, Japan) with buffer A (0.1% TFA in water) and buffer B (acetonitrile). Identification of the peptides was performed using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu AXIMA Confidence) (see Supporting Information: Figures S3–S26) and HPLC using a C18 analytical column (Cadenza CD-C18; Imtakt) and a linear gradient from 0% to 100% of B solvent. The detection was carried out at 230 nm and the flow rate was 1.0 mL/min (see Supporting Information: Figures S27–S50). Obtained chloroacetyl-modified tripeptides were reacted with BSH as shown in Figure 1B. These tripeptides were dissolved in DMSO/water (=1/4 *v/v*) before 1.5 eq. BSH aqueous solution was added to the peptide solution. TEA was further added to the mixture, the pH in the solution was adjusted to 8 and the reaction mixture was stirred at room temperature. A final concentration of the peptides in the reaction solution was 5.0 mM. Part of the solution was taken out and HPLC was performed using a C18 analytical column (see Supporting Information: Figures S51–S74). For peptides whose reaction progress was confirmed, the reaction was stopped by adding 0.5% TFA to the reaction solution until the pH reached 4. The reaction mixtures were purified by preparative HPLC and identified by MALDI-TOF mass spectrometry (Figures S75–S84) and HPLC (Figures S85–S94) in the same manner as above.

3. Results and Discussion

3.1. Reactivity of BSH with Cl-3X

First, we performed HPLC on the Cl-3R/BSH reaction mixture and Cl-3A/BSH reaction mixture (Figure 2). In HPLC of Cl-3R before the reaction without adding BSH (0 min), a peak derived from Cl-3R was confirmed at a retention time of 8.8 min (Figure 2A). In the HPLC at 30 min after adding BSH, the peak at 8.8 min disappeared and a new peak appeared at 10.3 min. It was confirmed by MALDI-TOF mass spectrometry that the peak at 10.3 min represented a reactant BS-3R (Figures S77 and S87). We also confirmed that the obtained BS-3R was successfully delivered in cells (Figure S95). On the other hand, in HPLC of Cl-3A before the reaction, a peak at 9.3 min was observed, which was also observed after 30 min; the other peak was not observed (Figure 2B). These results indicate that the reaction of the chloroacetyl group with BSH is dramatically dependent on the amino acid residues in the tripeptide. Therefore, we next investigated a reactivity of BSH with chloroacetyl-modified tripeptides containing other amino acid residues.

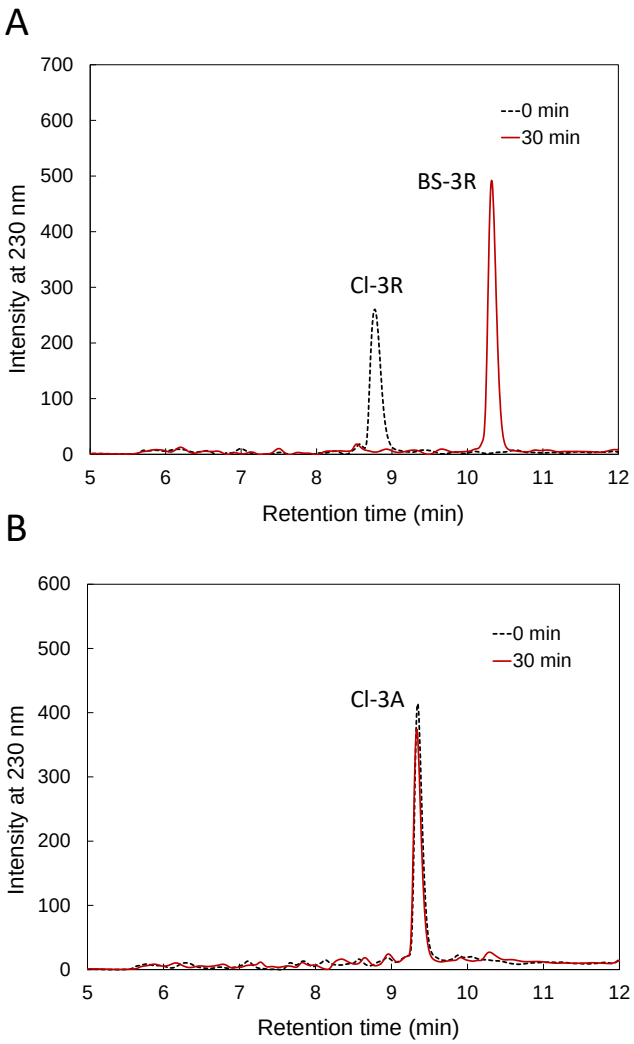


Figure 2. HPLC chromatograms of reaction mixtures of (**A**) Cl-3R and BSH, and (**B**) Cl-3A and BSH at 0 min (black broken lines) and after 30 min (red solid lines).

To investigate whether chloroacetyl group-modified tripeptides consisting of 17 standard amino acids (Cl-3X; three standard amino acids, Cys, Met and Pro, were excluded from this assay) react with BSH, we performed HPLC on each reaction mixture of tripeptides and BSH. The reactivity of tripeptides with BSH clearly differed depending on the amino acid residues (Table 1 and Figures S51–S67). Of the 17 amino acid residues, the reaction proceeded with tripeptides containing basic amino acids Arg, Lys and His, but the reaction did not proceed at all with the other 14 amino acid residues. These results indicate that tripeptides containing basic amino acids play an important role in the progress of the reaction with BSH. In other words, the results suggest that positively charged amino acid residues electrostatically interact with negatively charged BSH to accelerate the reaction between the chloroacetyl group and BSH. Therefore, we further investigated a relationship between the number of positively charged amino acid residues in tripeptides and the reaction of BSH with chloroacetyl groups.

Table 1. Amino acid residues contained in chloroacetyl-modified tropeptides and whether the reaction with BSH proceeded with them.

Tripeptides for Which the Reaction Proceeded	Tripeptides for Which the Reaction Did Not Proceed
BS-3X	BSH/Cl-3X
X = His, Lys, Arg	X = Ala, Asp, Glu, Phe, Gly, Ile, Leu, Asn, Gln, Ser, Thr, Val, Trp, Tyr

3.2. Reactivity of BSH with Cl-3R, Cl-2R and Cl-1R

If the progress of the reaction is caused by an electrostatic interaction between positively charged amino acid residues and negatively charged BSH, the reactivity is expected to depend on the number of positively charged amino acid residues. Therefore, we performed HPLC on reaction mixtures of tripeptides consisting of Arg/Ala mixed sequences and BSH and plotted the conversion of the chloroacetyl-modified tripeptides to BSH-modified tripeptides to assess the reactivities (Figure 3, Figures S68 and S69). Cl-3R, a chloroacetyl-modified tripeptide consisting of three sequential Arg residues, reached 100% conversion after 30 min (black filled circles), whereas the conversion of Cl-2R, a chloroacetyl-modified tripeptide containing two Arg residues with BSH, was 69%, showing a clear reduction. Moreover, the conversions of Cl-2R were 84% and 90% after 90 and 150 min, respectively (red filled circles). Furthermore, the conversion of Cl-1R, a chloroacetyl-modified tripeptide containing an Arg residue, was 29% after 30 min, 45% after 90 min and 54% after 150 min (blue filled circles). These results show that the reactivity decreases as the number of positively charged amino acid residues (Arg) in the tripeptide decreases. In other words, the results show that the chloroacetyl group in the tripeptide reacts with BSH via electrostatic interaction of the amino acid residues with BSH.

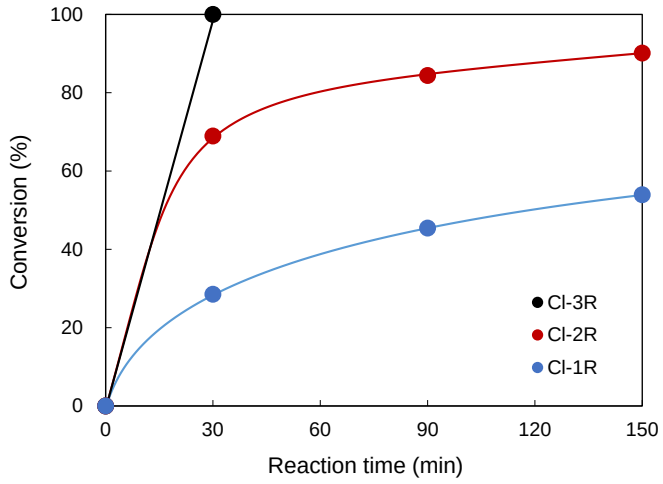


Figure 3. Time dependence of the conversion of Cl-3R (black filled circles), Cl-2R (red filled circles) and Cl-1R (blue filled circles) to corresponding BSH-modified tripeptides.

3.3. Reactivity of BSH with Cl-Cn-3R

If the reaction of BSH with the chloroacetyl group involves electrostatic interaction of an adjacent basic peptide with BSH, the reaction is expected to depend on a distance between the chloroacetyl group and the basic amino acid residues in the peptides. Therefore, we assessed the conversion of Cl-C*n*-3R, in which an alkyl linker was introduced between the chloroacetyl group and triarginine, to BS-C*n*-3R (Figure 4 and Figures S70–S74). The longer the carbon chain in the alkyl linker, the greater the distance between the chloroacetyl group and triarginine is expected to be. Peptides with a glycine linker (C1) inserted between triarginine and the chloroacetyl group, Cl-C1-3R, underwent a rapid reaction similar to that of chloroacetyl-modified triarginine without a linker, Cl-3R, and after 30 min of reaction,

the conversion reached 100%. However, a peptide with insertion of a β-alanine linker (C2), Cl-C2-3R, had conversion of only 27% after 30 min of reaction and 45% and 56% after 90 and 150 min, respectively. These results indicate that the insertion of the C2 linker dramatically reduced the promotion of the tripeptide's reactivity with BSH, resulting in reactivity similar to that of tripeptide containing one arginine. Cl-Cn-3R introduced with C4, C7 and C11 linkers showed further reductions in reactivity with BSH. The rates of conversion to reactant, BS-Cn-3R, after 150 min were 34%, 27% and 8%, respectively. These results indicate that the reactivity decreases as the distance between the amino acid residues and the reactive site increases. They also indicate that the reaction is promoted by the gathering of BSH in the vicinity of the reactive site via electrostatic interaction between BSH and basic amino acid residues.

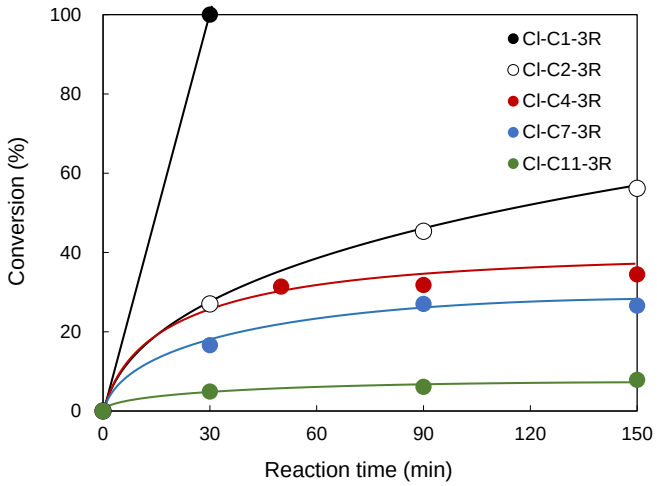


Figure 4. Time dependence of conversion of Cl-Cn-3R to BS-Cn-3R. n = 1 (black filled circles), n = 2 (black open circles), n = 4 (red filled circles), n = 7 (blue filled circles) and n = 11 (green filled circles).

4. Conclusions

To assess the reactivity of Cl-3X to BSH, we evaluated the conversion of Cl-3Xs consisting of various amino acid residues. The results showed that, when the basic tripeptide is modified with the chloroacetyl group, the desired reaction proceeds. The reactivity of Cl-3X to BSH depended on the number of basic amino acid residues in the tripeptide, and the results suggested that the electrostatic interaction between the positively charged amino acids in Cl-3X and the negatively charged BSH accelerates the reaction of Cl-3X with BSH. Furthermore, we found that the reaction proceeds depending on the distance created due to the introduction of the alkyl linker. In other words, these results revealed that arginine is involved in the reaction of BSH with the chloroacetyl group, indicating that an increasing number of cationic groups (Arg) and the proximity of the cationic group to the chloroacetyl group accelerate this reaction. Although it is necessary to investigate the effects of counter cations and solvents in order to reach detailed conclusions, based on these findings, we considered that the reaction achieved by our method applied here proceeds as follows (Figure 5). BSH has a negative charge of −2, and it is considered that the reaction accelerates because of easier access to the chloroacetyl group (reactive site) in the vicinity of the positively charged basic amino acid residue by electrostatic interaction with the residues. In this study, unprotected BSH was used, unlike in Gabel's protocol. It is known that two functional molecules bind to BSH using the unprotected BSH. However, we obtained no evidence that two Cl-3Xs bound to BSH. Perhaps electrostatic repulsion by the first positively charged tripeptide bound to BSH prevents the addition of another tripeptide. This method requires the attachment of positive and negative charges to two molecules to be conjugated, respectively, and could be applied to conjugating relatively large molecules such as peptides. This work should help the development of new boron

agents for BNCT, such as CPP and BSH conjugates. In the future, we plan to investigate the cell killing effect of BSH-peptides obtained by this method.

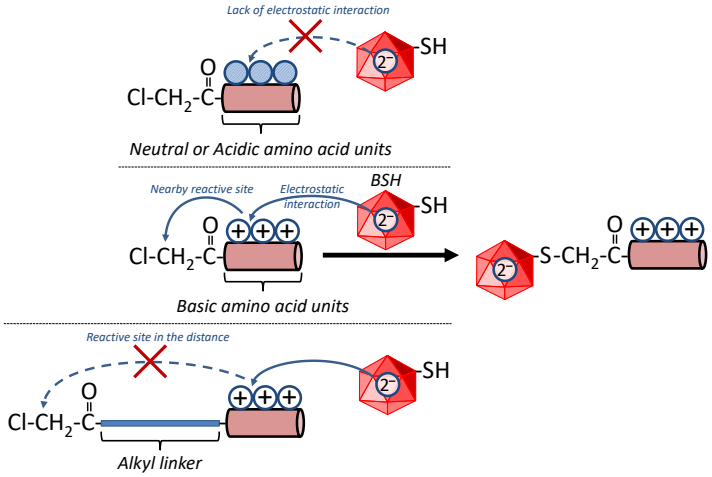


Figure 5. Proposed schematic of the promotion of reactivity of chloroacetyl-modified peptide with BSH by the basic amino acid residue close to the chloroacetyl group.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10112200/s1,: Figure S1: ^{1}H NMR spectrum of ClAc-OSu, Figure S2: ^{13}C NMR spectrum of ClAc-OSu, Figures S3–S26: MALDI-TOF mass spectra of tripeptides, Figures S27–S50: RP-HPLC charts of tripeptides, Figure S51–S74: RP-HPLC charts of reaction mixture of BSH and tripeptide, Figure S75–S84: MALDI-TOF mass spectra of BSH peptides, Figure S85–S94: RP-HPLC charts of BSH peptides.

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