Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **43**, 251-260 (2000)

Closo-dodecaborate (2-) anion as a potential prosthetic group for attachment of astatine to proteins. Aspects of the labelling chemistry with Chloramine-T.

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SUMMARY

Closo-dodecaborate (2-) was proposed as a prosthetic group for direct labelling of proteins with ^{211}At for radionuclide therapy. Astatination of *closo*-dodecaborate (2-) anion using Chloramine-T was studied, and the influence of pH, reaction time, amount of substrate and oxidant was determined. A maximum labelling yield of 55 - 75% was found in the pH range 7-8. A spontaneous astatination of *closo*-dodecaborate (2-) in the absence of Chloramine-T was also found, presumably due to generation of oxidising products from water radiolysis. Our results of indicate that derivatives of *closo*-dodecaborate (2-) anion may be used as prosthetic groups for direct labelling of proteins.

Key words: *Closo*-dodecaborate (2-) anion, ^{211}At, Astatination, Chloramine-T

INTRODUCTION

Astatine-211 is a promising radionuclide for systemic therapy (1-3) due to its decay properties. It has a half-life of 7.2 hours and an effective emission of one α-particle per decay. However, the use of astatine-211 in clinical practice was hampered because of the weak astatine-protein bond formed after direct astatination of targeting proteins (1,4). *N*-succinimidyl-3-(trialkylstannyl) benzoate was

CCC 0362–4803/2000/030251–10$17.50
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Received 10 February 1999
Revised 29 March 1999
Accepted 7 October 1999

introduced as an intermediate for the astatination of antibodies using conjugation procedures (5). A general disadvantage of such two-step procedures used for radioiodination is the lower labelling yield obtained in comparison with direct labelling (6,7), and we can expect similar problems with astatination. Indeed the yields reported for astatination *via* benzoate were all within 10-40% the range (5, 8-10).

An attractive alternative would be to attach a prosthetic group to the targeting protein, which could form a stable bond with astatine in a direct labelling procedure. Candidates are e.g. the polyhedral boron compounds like *closo*-dodecaborate, *closo*-decaborate, and *closo*- and *nido*-carboranes. They are three-dimensional inorganic aromatic structures that are easily halogenated (11-13) and form thermodynamically very stable boron-halogen bonds. The *nido*-carborane has already been used for two-step radioiodination of proteins (14-17) and the *nido*-carboranyl propionate was used as a pendant group for astatination of biotin for multi-step targeting (18). Recently, 7-(3-amino-propyl)-7,8-dicarba-*nido*-undecaborate (-) (ANC-1) was successfully applied as a prosthetic group for direct labelling of proteins (19).

Figure 1. Structure of *closo*-dodecaborate anion

In this paper we suggest to use derivatives of *closo*-dodecaborate (2-) anion (Fig.1) as prosthetic group for astatination. These boron cages differ from *closo*- and *nido*-carboranes in charge, chemical properties and lipophilicity, which may be of advantage in the labelling process and the subsequent handling of the labelled proteins. Thus, Wilbur et al. (17) indicated that *closo*-borates are more avidly halogenated than carboranes and are less sticky which simplifies labelling and injection procedures. These differences may also be used to modify the biochemical properties of the halogen-labelled compound, including aspects of non-specific binding to normal cells, intracellular retention and excretion of the label after catabolism of targeting compound.

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In the development of targeting compound for boron neutron capture therapy (BNCT), basic knowledge has been gained concerning methods for conjugation of polyhedral boron compounds (15). Carboranes have obtained most attention since they contain carbon atoms that can be used for further attachment to tumour-seeking molecules. Because *closo*-dodecaborate does not contain carbon atoms in the boron cage, there are some difficulties with their attachment to proteins. However, several methods have been reported for the synthesis of *closo*-dodecaborate derivatives containing organic side-chains. Thus, sulfhydryl boron hydride (BSH) was covalently coupled to an allylated 70-kDa dextran chain for use in BNCT (20). Further, the boronated dextrin was coupled to tumour-seeking protein, epidermal growth factor (EGF)The resulting conjugate demonstrated specific binding to cultured glioma cells (20). Recently, syntheses of derived dodecaborate anions, $[B_{12}H_{11}OC(O)R]^{2-}$ (21), $[B_{12}H_{11}NH_2CH_2R]^-$ (22), $[B_{12}H_{11}OR]^{2-}$ (23) and $[B_{12}H_{11}NH_2C(O)(CH_2)_2Br]^-$ (24) have been reported. These derivatives are candidates as prosthetic groups for astatination since they can be used to attach *closo*-dodecaborate to proteins and peptides.

This study was aimed to estimate the *closo*-dodecaborate (2-) astatination yield dependence on different reaction parameters so that this anion can be used as a prosthetic group for astatination of protein.

RESULTS AND DISCUSSION

Typical results from labelling experiments during different conditions are shown in Figure 2 as four thin layer chromatograms. Figure 2a represents the results of a blank experiment without NaI as carrier. A narrow peak is seen at the starting place of the TLC, most likely representing oxidised astatine, i.e. oxoanions, followed by a broad radioactivity distribution, probably reflecting a continuous astatine oxidation during the TLC development. Blank experiment with NaI as carrier (Figure 2b) clearly shows the positive role of iodide anion as non-isotopic carrier for astatine analysis. Astatine is probably stabilised in the form of AtI or AtI_2^- compounds (25, 26), which enables good resolution of the two present astatine forms. In the chromatogram of a standard labelling procedure (Figure 2c), the central peak

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of R_f=0.5-0.6 represents the astatinated dodecaborate anion which is well resolved both from highly oxidised astatine and the reduced rest of astatine. This peak has the same R_f as monoiodinated dodecaborate anion in this TLC system. Figure 2d shows the result of spontaneous labelling in the absence of oxidising agent.

The labelling yield under the same conditions was found to decrease as a function of time elapsed from the astatine separation. For this reason, the labelling dependence on each parameter was always measured at the same time in relation to the production time in order to exclude the influence of the astatine aging effect.

The radiochemical yields of the astatination of *closo*-dodecaborate (2-) at different reaction conditions (buffer pH, reaction time, and amount of substrate) are

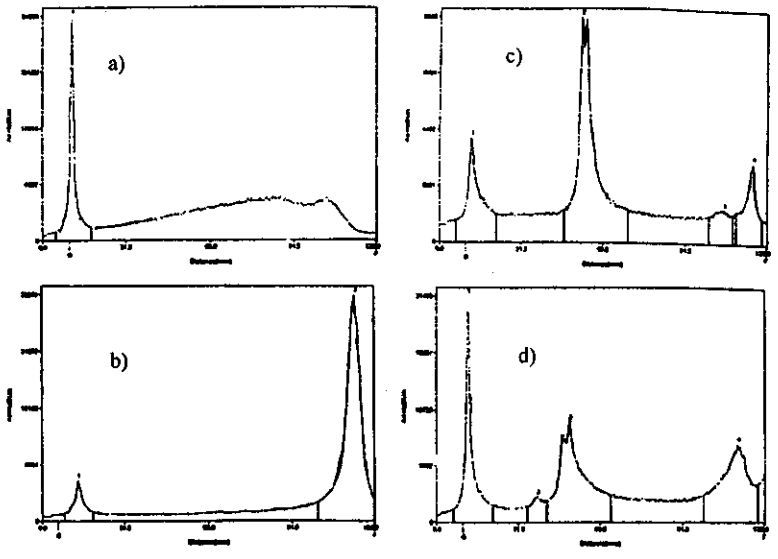

Figure 2. Radiochromatograms obtained using TLC: a) ^{211}At distribution after oxidation and subsequent reduction of astatine in the absenceof *closo*-dodecaborate and sodium iodide; b) ^{211}At distribution after oxidation and subsequent reduction of astatine in the absence of *closo*-dodecaborate; c) ^{211}At distribution after oxidation and subsequent reduction of astatine in the presenceof *closo*-dodecaborate; d) ^{211}At distribution after a 20-minute incubation of astatine in the presence of *closo*-dodecaborate. No oxidant was added (spontaneous labelling). Sodium iodide carrier was added to the quenched reaction mixture before analysis in samples b), c), and d).

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shown in Figures 3-4. The description of the labelling conditions of each experiment is given in the figure legends. Error bars were calculated as maximal errors according to formula: $Er_{max} = (Y_{max} - Y_{min})/2$.

The reaction seemed to be essentially completed after 5 minutes of incubation at room temperature (Figure 3a). The astatination yield reached its maximum in the pH range of 7.0-8.0 (Figure 3b). Thus, the experiments below were carried out at pH 7.4 and the incubation time was kept at 5 min.

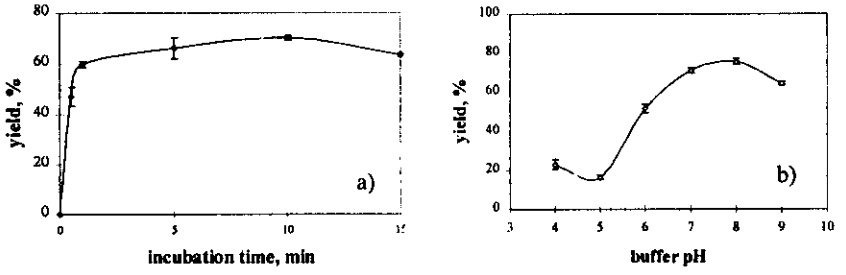

Figure 3. a) *Closo*-dodecaborate labelling yield dependence on reaction time. The reaction mixture, which contained 35 nmol of precursor and 50 µg Chloramine-T, was left to react for various times at room temperature in PBS (pH 7.4); b) *Closo*-dodecaborate labelling yield dependence on pH. The reaction mixture contained 35 nmol of *closo*-dodecaborate and 50 µg Chloramine-T, was left to react for 5 min at room temperature at various pH.

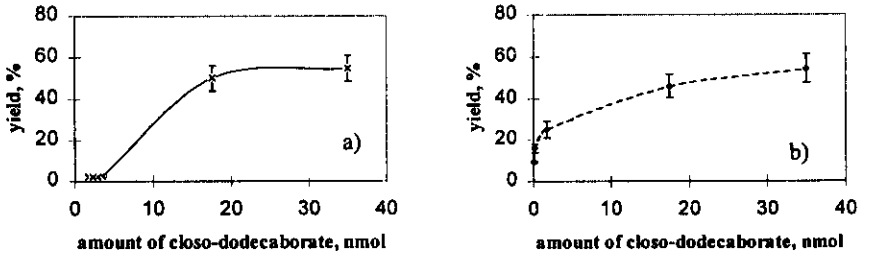

Figure 4. *Closo*-dodecaborate labelling yield dependence on precursor concentration. The reaction mixture contained various amounts of *closo*-dodecaborate, 50 µg of Chloramine-T, it was left to react for 5 min at room temperature at pH 7.4 in phosphate buffer (a) or in phosphate buffered saline (b).

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The astatination yield increased steadily as a function of the amount of *closo*-dodecaborate in the reaction as shown in Figure 4a and 4b. A plateau was reached at about 25-30 nmol of *closo*-dodecaborate anion in the reaction mixture. This amount of *closo*-dodecaborate corresponds to 2-3 prosthetic groups per molecule when labelling (e.g. 1 mg of antibodies). The labelling yield was reduced marginally when the precursor amount was halved. A marked change in labelling efficiency in different buffers and at low concentrations of *closo*-dodecaborate was found. The use of chloride-containing buffer (PBS) affected positively the labelling yield, thus enabling a more effective labelling of smaller substrate amounts.

Different Chloramine-T (CAT) concentrations gave widely varying labelling yields. The amount of CAT (μg) and corresponding yields (%) were: 0.05, 49 ± 10; 0.275, 56 ± 7; 0.5, 43 ± 2.5; 2.75, 45 ± 5; 5, 50 ± 2; 27,5, 64 ± 1.5. The precursor is labelled efficiently even at very low amounts of oxidant. Since the alpha-radiation of ^{211}At may generate chemical agents with high oxidative ability, e.g. hydrogen peroxide, the labelling yield of astatinated *closo*-dodecaborate in the absence of the Chloramine-T, i.e. spontaneous labelling was checked. In total absence of CAT the labelling yield was greatly decreased but was still about 15%. If sodium metabisulfite was added before astatine in the solution, no labelling occurred. This observation supports the hypothesis concerning spontaneous labelling of *closo*-dodecaborate. For this reason, it is difficult to distinguish what part of the labelling is due to the addition of CAT and which is caused by self-oxidation. The use of CAT enables, however, more controlled labelling conditions.

The spontaneous labelling phenomenon can explain the loss of the reactivity of astatine with time, i.e. the aging effect, which is due to oxidation of astatine to non-reactive high oxidation states by oxidants generated during radiolysis of solvent.

The stability of boron-astatine bond was studied both at room temperature and at 37°C. A sample of the labelling reaction mixture was stored at the respective temperature and analysed on TLC at 0, 3, 5 and 7 h post reaction termination. In both cases no significant change was seen in the reaction mixture composition.

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Radiation induced halogenation of *closo*-dodecaborate anion is studied now in details in our laboratories. The results of this study will be reported separately.

MATERIAL AND METHODS

Materials: Sodium iodide (NaI), methanol (MeOH), aqueous ammonia (25% solution), acetone, and chemicals for a set of citrate-phosphate, phosphate and borate buffers were of p.a. grade, and used as supplied by E. Merck, Darmstadt, Germany. High quality Elga© water (resistance higher than 18 MΩ/cm) was used for preparation of solutions and buffers. Di(triethylamonium) salts of dodecahydro-*closo*-dodecaborate(2-), $(Et_3NH)_2B_{12}H_{12}$, and monoiodo-undecahydro-*closo*-dodecaborate(2-), $(Et_3NH)_2B_{12}H_{11}I$, were synthesised by known methods (11, 27). $(Et_3NH)_2B_{12}H_{11}I$ was characterised by IR and ^{11}B NMR analysis. The salts were dissolved in water; in a typical experiment a concentrations of 1mg/ml were used. *N*-Chloro-*p*-toluenesulfonamide sodium salt, Chloramine T, CAT, (SIGMA Chemical Company, St. Louis, MO, USA) was prepared as a 5 mg/ml solution in water. Sodium metabisulfite, $Na_2S_2O_5$, (SIGMA Chemical Company, St. Louis, MO, USA) was used as a 4 mg/ml solution in water. Sodium iodide was used as a20 mg/ml solution in water. The solutions of CAT and sodium metabisulfite were always prepared freshly, within 5 min before each experiment. The solutions of $B_{12}H_{12}^{2-}$, NaI and buffers were prepared 5 h before the astatine delivery and stored in the refrigerator in plastic vessels.

TLC system: Alumina TLC (neutral type, 0.2 mm thick layer on aluminium, E. Merck, Darmstadt, Germany) were always pre-washed and developed by acetone : 0.1 *M* ammonia (9:1) mixture. For the analysis, 1-2 µl of the reaction mixture was immediately applied per TLC plate, left to evaporate spontaneously and developed in the fresh eluent. The TLC strips (15×140 mm, elution path 120 mm) were measured on the Cyclone™ Storage Phosphor System and analysed on the OptiQuant™ Image Analysis Software.

All labelling experiments were performed in Eppendorf's tubes at room temperature. Experiments were performed in duplicates.

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Astatine production and separation from the target: ^{211}At was produced with the U-120M Cyclotron, Nuclear Physics Institute, Rez near Prague, using the ^{209}Bi(α,2n)^{211}At nuclear reaction. Separation of astatine from the target was performed by dry distillation technique with cryogenic trapping, and astatine was obtained as a solution in 100-200 μl of methanol (19).

Labelling experiments. *Closo*-dodecaborate solution (10 μl) and astatine solution in methanol (5 μl) were added to 30 μl buffer. Labelling was started by addition of 10 μl CAT solution. The mixture was vortexed for a few secondsand left to react for the pre-determined time. The reaction was quenched with20 μl sodium metabisulfite solution. NaI (5 μl) was added as carrier before analysis. Blank experiments were performed according the same protocol but without adding *closo*-dodecaborate. Some experiments were performed without adding iodide carrier.

Spontaneous labelling. *Closo*-dodecaborate solution (10 μl, 1 mg/ml) and astatine solution in methanol (5 μl) were added to 30 μl phosphate buffer (pH 7.2). No Chloramine-T was added. After 20 min of incubation, the reaction was quenched with 20 μl sodium metabisulfite solution and 5 μl NaI solution as carrier was added. To determine the role of oxidation in the spontaneous labelling, the same experiments were performed in presence of sodium metabisulfite solution. $B_{12}H_{12}^{2-}$ solution (10 μl, 1mg/ml) in 30 μl phosphate buffer (pH 7.2) were mixed with 20 μl of sodium metabisulfite solution. ^{211}At solution (5 μl) was added to the mixture. After 20 minutes of incubation, 5 μl of NaI as carrier was added and the sample was taken for TLC analysis.

CONCLUSION

An efficient analytical procedure for resolving the labelled boron compound from other chemical forms of astatine was found using TLC. Labelling of dodecahydro-*closo*-dodecaborate(2-) anion with^{211}At using Chloramine-T as oxidant resulted in radiochemical yield up to 75% at pH 7.4. The B-Atbond is stable *in*

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vitro for at least one half-life of ^{211}At. Derivatives based on dodecaborate(2-) anion are potential candidates as prosthetic groups in direct labelling of proteins.

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