Applications of Radiolabeled Boron Clusters to the Diagnosis and Treatment of Cancer

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I. Introduction

The use of boron compounds for the boron neutron capture therapy (BNCT) of cancer was first proposed by Locher in 1936.[1] It is a binary radiation therapy that entails the capture of thermal neutrons by boron-10 (^{10}B) nuclei that have been selectively delivered to tumor cells. The neutron capture event results in the formation of excited ^{11}B nuclei that fission to yield highly energetic $^4He^{2+}$ and $^7Li^{3+}$ ions. Cell death is triggered by the release of these charged particles that create ionization tracks along their trajectories, resulting in cellular damage. It has been estimated that approximately 10−30 μg of boron-10/g of tumor mass is needed to attain an acceptable therapeutic advantage.[2,3]

At the present time, clinical trials of BNCT are underway in the United States, Japan, and the European Union using L-4-dihydroxyborylphenylalanine hydrochloride as its water-soluble fructose complex (BPA, discovered in 1958) and $Na_2B_{12}H_{11}SH$ (BSH, developed in about 1965). These compounds are now regarded as "first-generation" agents that exhibit indefinite selectivity toward tumor cells at therapeutic concentrations. The current clinical trials are intended only to demonstrate that the procedures employed are safe and not deleterious to patients (phase I).

In the past three decades, many different substituted boron clusters, predominantly derivatives of *closo*-$B_{12}H_{12}^{2-}$, *closo*-$B_{10}H_{10}^{2-}$, and the three isomeric dicarba-*closo*-dodecaborane(12) species,[4] were synthesized with the hope of achieving therapeutic ^{10}B concentrations and high tumor selectivity.[5,6] The most promising approaches for this task involve porphyrins, monoclonal antibodies, epidermal growth factors, nucleosides, amino acids, and liposomes among others. Furthermore, it has been recently demonstrated that therapeutic quantities of noncytotoxic boron-rich oligomeric phosphate diesters are taken up and persistently held by the cell nucleus following their delivery into the cytoplasm of TC7 cells by microinjection, thus providing a potential new tool for nuclear targeting.[7]

Several methodologies have been developed to determine the boron content of biological research samples taken at biopsy and in tissues obtained from animal biodistribution experiments. Such samples are often available in macroscopic quantities of 50−100 mg or more. Following the oxidative dissolution of weighed tissue samples, boron analysis of the aqueous sample is accomplished with inductively coupled or direct current plasma-atomic emission spectroscopy (ICP-AES[8] and DCP-AES,[9] respectively) methods. These AES methods determine boron in tissue reliably at a level of 1 ppm. Extension of this method to microscopic tissue samples obtained by needle biopsy is feasible with a modified sample preparation procedure.

For in vivo imaging of boron compounds, radiolabeled derivatives are of particular interest since their biodistribution can be easily monitored by using single-photon emission computed tomography (SPECT) and positron emission tomography (PET), depending on the radionuclide employed. Although the amount of radioactivity used in radioimmunoimaging experiments is extremely small in comparison to the large number of boron atoms required for efficient BNCT, the labeling of boron compounds with radionuclides enables their direct detection in biological systems during studies of biodistribution and pharmokinetics. This is important in the evaluation

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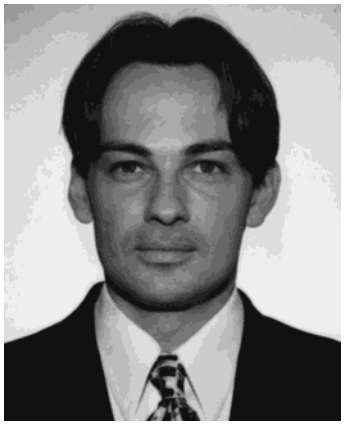
M. Frederick Hawthorne was born in 1928 in Fort Scott, KS. He completed his B.A. degree in Chemistry from Pomona College in 1949. That same year, he began graduate work at UCLA where he joined the research group of Donald J. Cram. He became an Atomic Energy Commission Predoctoral Fellow in 1951, and in 1953, Dr. Hawthorne began postdoctoral work in physical–organic chemistry with George S. Hammond at Iowa State University. In 1954, he took a position with the Redstone Arsenal Research Division of the Rohm and Haas Co., Huntsville, AL, where he later founded an exploratory research group (1956) that was concerned with the use of boron hydride derivatives as energetic rocket fuels. This enterprise led to a new world of chemistry in which boron largely replaced carbon and the "aromatic" polyhedral ions and carboranes led to the development of families of novel molecules for application throughout chemistry. Among these contributions was his discovery, in 1965, of borane and carborane cages that contain vertices comprised of metals from throughout the periodic table. This work, which wedded borane chemistry to metal coordination chemistry, originated following his appointment as Professor of Chemistry at the University of California, Riverside, in 1962. In 1969, Dr. Hawthorne returned to UCLA as a Professor of Inorganic Chemistry. Also in 1969 he became Editor-in-Chief of *Inorganic Chemistry,* published by the American Chemical Society. Dr. Hawthorne was appointed to the highest academic rank of University Professor by the Regents of the University of California in 1998. Dr. Hawthorne is a member of the U.S. National Academy of Sciences (1974), the American Academy of Arts and Sciences (1975), and a Corresponding Member of the Göttingen Academy of Sciences. A large proportion of Dr. Hawthorne's research program is concerned with the merging of polyhedral borane and organic chemistries and the applications of the resulting hybrid field to important problems facing mankind. A unique example of this effort concerns a binary noninvasive radiological therapy for cancer known as boron neutron capture therapy. He served as President of the International Society for Neutron Capture Therapy of Cancer from 1996 to 1998.



Andreas Maderna was born in Darmstadt, Germany, in 1966. From 1987 to 1994, he studied chemistry at the Ruprecht-Karls-Universität in Heidelberg, Germany. In 1994, he obtained a Diplom Chemiker degree after work with Prof. Dr. Walter Siebert on the reactivity of alkynylboranes. From 1994 to 1997, his research focused on the synthesis of hexaborylbenzene and tetraborylethene derivatives. For this, he was granted his Ph.D. from the Ruprecht-Karls-Universität, Heidelberg. Since 1998, he has held a postdoctoral position at the University of California, Los Angeles, in the research laboratories of Prof. M. Frederick Hawthorne. Dr. Maderna's research interests include the synthesis of new boron cluster derivatives and their evaluation for medical applications.

of boron systems especially designed for enhanced tumor selectivity with regard to BNCT, and it also offers the synthesis of new inorganic tumor imaging agents that display high chemical stability of the radiolabel. This is of special importance since enzymatic cleavage in vivo resulting in loss of the radiolabel is often a problem.

This paper surveys the synthesis and use of radiolabeled boron clusters in view of their possible application both to tumor imaging for general diagnosis and to the analytical support of BNCT. Initially, labeling reactions of boron clusters with iodine-131, iodine-125, astatine-211, tritium, and cobalt-57 are presented. The use of fluorine-18,[10,11] a positron emitter detectable with PET, is only reported for studies with BPA and is not treated further in this paper since BPA is not a boron cluster. The subsequent portion of this paper describes in vitro and in vivo results obtained with radiolabeled conjugates composed of boron cluster derivatives and biomolecules, principally antibodies or antibody fragments.

II. Boron Clusters Available for Radiolabeling

The discovery of polyhedral boranes and polyhedral heteroboranes, which contain at least one atom other than boron in the cage, initiated a new era in boron chemistry.[12−17,4] Since the parent species may contain up to 12 boron atoms, such clusters are of particular interest in BNCT, which requires a high boron concentration. Furthermore, it is possible to synthesize dimeric and oligomeric cluster chains with a much higher boron content. Most commonly, of the three commercially available isomeric dicarba-*closo*-dodecaborane(12) carboranes (1,2-(*ortho*)-, 1,7-(*meta*), and 1,12-(*para*)-), the 1,2-(*ortho*) isomer has been used for functionalization and connection to organic molecules. These icosahedral carboranes are isoelectronic and geometrically identical to the *closo*-$B_{12}H_{12}^{2-}$ dianion (Figure 1). The highly delocalized three-dimensional cage-bonding that characterizes these carboranes and *closo*-$B_{12}H_{12}^{2-}$ provides extensive thermal and kinetic stabilization as well as photochemical stability in the ultraviolet and visible regions. The unusual icosahedral geometry of these species not found in hydrocarbon chemistry provides precise directional control of all exopolyhedral bonds. Bonds originating at the 1,2-, 1,7-, and 1,12-vertex pairs of the *closo*-$C_2B_{10}H_{12}$ isomers subtend angles of 63°, 117°, and 180°; values closely corresponding to those of the *ortho*-, *meta*-, and *para*-phenylene groups, respectively.

In terms of chemical reactivity, the carbon and boron vertexes of all three icosahedral carborane isomers are essentially orthogonal to one another while demonstrating electrophilic substitution reminiscent of aromatic hydrocarbon reactions at BH vertexes. The CH vertexes are most easily modified by prior conversion to a CLi or similar nucleophilic vertex commonly followed by reaction with an electrophilic organic substrate. On the other hand, *closo*-$B_{12}H_{12}^{2-}$ and *closo*-$B_{10}H_{10}^{2-}$ are extremely reactive in

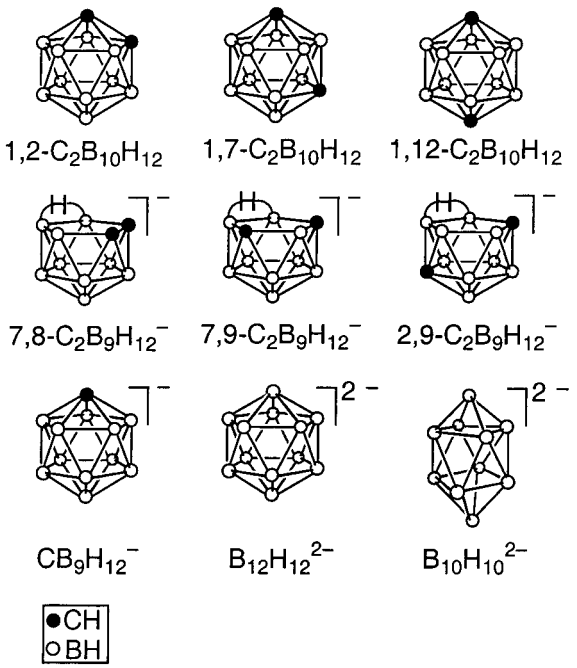


Figure 1. Boron clusters commonly employed in biomedical applications.

electrophilic substitution reactions such as halogenation.[18]

Biomedical applications of the icosahedral carboranes make use of their extraordinary hydrophobicities when employed as substituents in biomolecules, their apparent invisibility to known enzyme systems, and their high boron content suitable for BNCT. All three icosahedral carborane isomers are easily converted to the corresponding hydrophilic *nido*-$C_2B_9H_{12}^-$ anions by reaction with strong bases (deboronation reaction) under a variety of conditions[19,20] (Figure 1). These anions are water-soluble in the form of their lithium, sodium, and potassium salts and exhibit the same remarkable stability as the *closo* counterparts. Due to their water solubility and strong electrolyte character, the three isomeric *nido*-$C_2B_9H_{12}^-$ ions may be effectively utilized in buffered solutions. To the present time, boron clusters and their derivatives employed for radiolabeling include *nido*-7,8- and *nido*-7,9-$C_2B_9H_{12}^-$, *closo*-$CB_{10}H_{11}^-$, *closo*-$B_{12}H_{12}^{2-}$, and *closo*-$B_{10}H_{10}^{2-}$.

III. Methods of Radiolabeling

A. Radioiodinated Boron Clusters

1. General Remarks

The labeling of proteins with radioisotopes of iodine is a very important tool in biomedical research.[21] Because of their relatively low cost, commercial availability, and facile detection with conventional γ-cameras, the isotopes iodine-131 and iodine-125 are commonly encountered. Iodine-125 has a half-life of 59.41 d and decays by electron capture.[22] Since the emitted γ-photons are of relatively low energy (35.5 keV), this isotope is attractive for in vitro labeling experiments due to the fact that collateral radiation damage suffered by biomolecules upon storage is minimal. Another advantage is its high specific activity (81 PBq/mol), which permits the labeling of ligands only available in short supply. Due to the effective attenuation of the low-energy photons in tissue, in vivo studies with iodine-125 are restricted to small animals, such as mice. Iodine-125 is a nuclide mainly used for endoradiotherapy.[23] Iodine-131 is a β^- and γ emitter with a half-life of 8.02 d.[22] Because the emitted γ-photons are of high energy (364 keV), this radionuclide can be detected with single-photon emission computed tomography (SPECT) and is often used for in vivo studies with larger animals and humans. On the other hand, γ-emissions are not optimally counted due to their high energy, and the β^--particle emission results in a greater radiation dose to tissue than is desirable. Iodine-123, which has a short half-life of 13.2 h,[24] exhibits an easily detectable γ-emission (159 keV) in high abundance. This isotope presents an interesting alternative. Unfortunately, its generation free of radionuclide impurities is expensive, and its general use is impractical. Also of interest is the positron-emitting iodine-124 with a half-life of 4.14 d.[25]

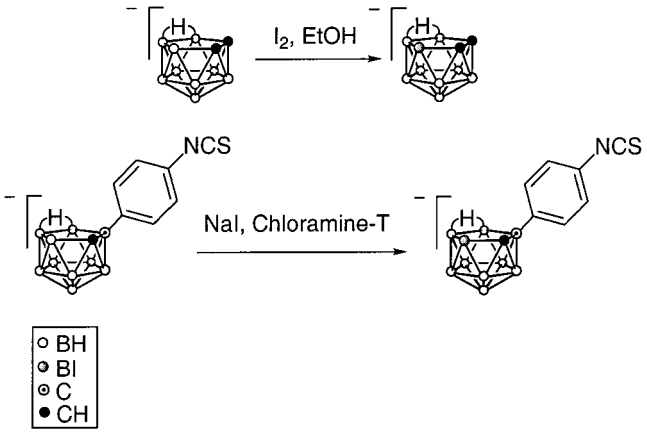
Independent of the identity of the iodine isotope employed, the iodine label is usually introduced to the targeted peptide or protein molecule by oxidation of iodide ion with a mild oxidizing agent, such as chloramine-T. The incipient I^+ formed in situ reacts with functional groups present on the biomolecule (direct labeling), e.g., the aromatic ring systems of tyrosine, histidine, and phenylalanine.[21] Other methods employed to label proteins include the use of iodine monochloride, the commercially available reagents Iodogen and Iodo Beads, and enzymatic and electrolytic methods.[26] In a different methodology, radioiodinated small molecules are synthesized first and then linked to the protein (conjugate labeling).[21] The benefits of the latter method are the mild conditions employed without exposure of the proteins to oxidation agents, the possible application of dual labeling by using two different radioisotopes, and the possible stabilization of the iodine radiolabel with respect to in vivo dehalogenation by enzymes.

2. Iodination of *nido*-7,8-$C_2B_9H_{12}^-$ and *closo*-$B_{12}H_{12}^{2-}$

The iodination of *nido*-7,8-$C_2B_9H_{12}^-$ was first reported by Hawthorne et al.[27] in 1965. The reaction of the potassium salt of this anion with elemental iodine in absolute ethanol afforded the monoiodinated product in 75% yield (Scheme 1). Characterization was accomplished by elemental analyses and ^{11}B NMR. The reaction should be regarded as an electrophilic substitution at the open face of the *nido*-7,8-$C_2B_9H_{12}^-$ ion. Because of the pattern of the ^{11}B NMR spectrum, the authors assigned the position of the boron bearing the iodine substituent as adjacent to one of the carbon atoms. In a later study, the same authors reinvestigated the iodination reaction of 7-(4-C_6H_4NCS)-*nido*-7,8-$C_2B_9H_{11}^-$ employing sodium iodide and chloramine-T as an oxidizing agent. The *p*-isothiocyanatophenyl substituent was present for later linkage of the iodinated *nido* anion to a protein amino group, thus accomplishing conjugate labeling.

The reaction conditions employed corresponded to those commonly used for the radioiodination of

Scheme 1. Iodination of a *nido*-Carborane Anion Places the Iodine on the Open Face of the Cluster



proteins, where incipient I^+ formed in situ functions as the electrophile. An X-ray diffraction analysis[28] of the product revealed the iodine in a position adjacent to the unsubstituted CH vertex, as expected.[27]

Employing the chloramine-T method with $Na^{125}I$, Hawthorne et al.[29] reported the first radioiodinated carborane species with the radioiodine attached to a carborane cage and investigated its use as an inorganic carrier for radioiodine in biodistribution studies. Since the B−I bond (bond dissociation energy = 381 ± 21 kJ/mol) is substantially stronger than the C−I bond (209 ± 21 kJ/mol),[30] it is not unreasonable to expect that this relative stability would be maintained in vivo and that the boron-iodinated derivative would be less prone to undergo the in vivo dehalogenation process encountered with commonly used carbon-iodinated biomolecules. This thermodynamic effect would be enhanced by the fact that the carborane iodine carrier is inorganic in nature and is most assuredly not recognized by the enzymes required for the C−I dehalogenation.

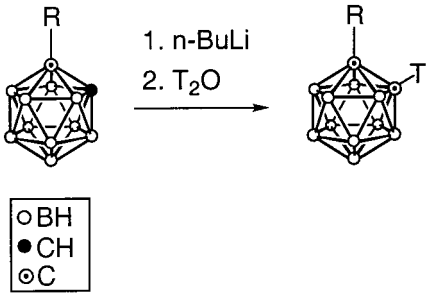
Somewhat later, Wilbur et al.[31,32] repeated the iodination of a substituted *nido*-carborane anion using incipient I^+ generated in situ from sodium iodide and *N*-chlorosuccinimide and described the synthesis of *nido*-carborane anions linked with 2-nitroimidazoles along with other derivatives.

Other *nido*-carborane species that could be successfully radioiodinated for labeling studies include a derivative bearing an amide substituent[33] as well as oligomeric phosphate diesters[34] and *nido*-carboranyl peptides.[35] In all cases, the radioiodine is substituted on the open face of the cluster, as shown in Scheme 1.

The *closo*-carboranes are readily B-iodinated[36] under electrophilic conditions, and subsequent palladium-catalyzed coupling reactions with Grignard reagents lead to carbon−boron bond formation. This sequence provides an important method for attaching carbon substituents to boron vertexes of the *closo*-carborane cage.[37] However, electrophilic radioiodination reactions have not yet been reported.

Additionally, *closo*-$B_{12}H_{12}^{2-}$ is rapidly iodinated with elemental iodine[18] or with I^+ formed in situ. Radioiodinated derivatives of *closo*-$B_{12}H_{12}^{2-}$ were prepared by reaction with radioactive iodide in the presence of chloramine-T or Iodogen[38] and by reaction

Scheme 2. Tritium Is Introduced to *closo*-Carboranes by Deprotonation of a CH-Vertex with Base Followed by Addition of T_2O



with aqueous ^{131}ICl. The latter was prepared by iodine exchange between nonradioactive ICl and $Na^{131}I$.[39]

Other boron clusters that were successfully radioiodinated include *closo*-$CB_{11}H_{12}^-$ and *closo*-$B_{10}H_{10}^{2-}$.[40] Likewise, the labeling of *nido*-7,8-$C_2B_9H_{12}^-$ and *closo*-$B_{12}H_{12}^{2-}$ with the α-emitting astatine-211 (half-life 7.2 h)[41] has been recently described.[42,43]

B. Tritiation of Carboranes

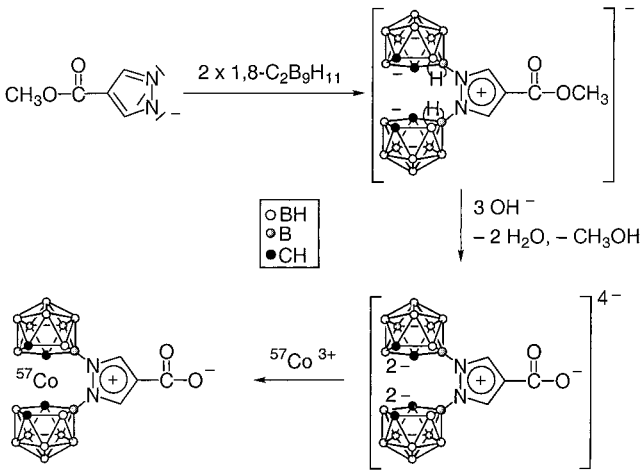
Tritium is a β^- emitter with a half-life of 12.323 years.[44] Because of its long half-life, this radionuclide is not suited for clinical applications. Nevertheless, it is used for in vitro and in vivo studies with animals, since tritium is easily introduced into molecules (e.g., via hydrogen or proton exchange reactions using tritium gas, HTO or T_2O).[45] Carboranes can be tritiated by prior deprotonation of the CH vertex with n-BuLi, followed by quenching the resulting anion with T_2O (Scheme 2).[46,47]

C. Radiometallacarboranes

The most common procedure employed for radiolabeling proteins in the past has been radioiodination. An alternative approach is radiolabeling proteins that have been covalently modified with metal-chelating groups. Immunoproteins radiolabeled using radioactive metal ions chelated in this manner with small molecules and peptides have been used clinically for the diagnosis of cancer and other purposes.[48] Ligand systems used for metal complexation include derivatives of diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), and cryptands.[49] Other important metal-chelating ligands are TETA (1,4,8,11-tetraazacyclo-tetradecane-1,4,8,11-tetrayltetraacetic acid),[50,51] which complexes ^{67}Cu with improved in vitro and in vivo stability, and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayltetraacetic acid),[52] which has been employed as a ligand system for ^{90}Y. Stable ^{111}In and ^{67}Ga complexes are formed with 1,4,7-triazacyclononane-1,4,7-triyltriacetic acid (NOTA), and the stability of these parent complexes with respect to acid-catalyzed dissociation has been demonstrated both in vitro and in vivo.[53,54]

Methods for direct covalent linkage of chelators to protein amino groups include acylation with active esters, aromatic diazonium ion coupling, and coupling with *p*-isothiocyanatophenyl groups.[49] Commonly

Scheme 3. Synthesis of the Venus Flytrap Complex (VFC) with Radiocobalt[a]



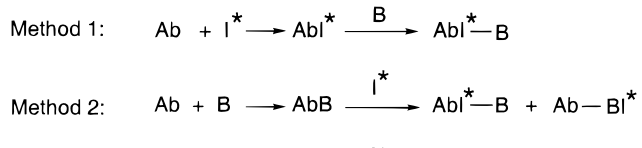
[a] The pyrazole ring is substituted with a carboxylate function for subsequent coupling to a monoclonal antibody.

used metals are for example ^{111}In, ^{99m}Tc, ^{67}Ga, and ^{68}Ga. It is essential for effective imaging that the radioactive metal ions remain complexed by the protein−chelator conjugate. A particular concern in this regard is the competition for metal binding from serum transferrin, which is known to remove metal ions from chelates in vivo.[55] Of utmost importance is the magnitude of the radionuclide−chelator dissociation rate, which must be miniscule. Most desired would be an especially robust chelation system that is promptly excreted along with tightly-held radiometal even if the antibody or the chelate−antibody linker molecule suffered catabolic degradation.

One possible solution of this problem was envisioned using an extraordinarily stable metal cluster system readily prepared in aqueous solution and bearing organic substituents for conjugation purposes. This concept led to the so-called Venus flytrap ligand[56] comprised of two *nido*-7,9-$C_2B_9H_{11}^{2-}$ η^5-dicarbollide ions hinged together through a bifunctional pyrazole molecule. Hawthorne and co-workers[57] first described *commo*-bisdicarbollide transition metal complexes in which the metal ion is held between two $\eta^5-\pi$-bonding $C_2B_9H_{11}^{2-}$ dianion ligands. With d^6 metal ions, these complexes conform to both 18-electron and the cluster electron-counting rules that followed. They display extraordinary stability due to the delocalized cluster bonding of the transition metal with ligand orbitals of appropriate symmetry. Scheme 3 shows the synthesis of a so-called Venus flytrap complex (VFC),[56] in which radioactive ^{57}Co (half-life = 271.79 d) is tightly bound in a metallacarborane structure by two *nido*-carboranyl ligands, which are covalently bridged by a pyrazole ring.

The VFC is generated in both the diastereomeric *dl* and *meso* forms, which can be separated by HPLC. The preorganization given the two *nido* ligands by the pyrazole hinge enhances the ease of radiometal complexation, stabilizes the resulting cluster product, and provides an attachment point for the carboxyl group employed to bond the radioactive cluster to an amino group of the desired immunoprotein.

Scheme 4. Principal Methods for the Radioiodination of Boron Cluster−Protein Conjugates Exemplified with an Antibody Conjugate[a]



[a] Ab, antibody; B, boron cluster.

Despite the long half-life of ^{57}Co (271.79 d),[58] it is useful for in vitro and in vivo studies in animals. Interesting for in vivo studies is the analogous positron-emitting ^{55}Co, since its half-life is only 17.54 h and its fate can be precisely monitored using PET.

IV. In Vitro and In Vivo Studies with Radiolabeled Boron Clusters

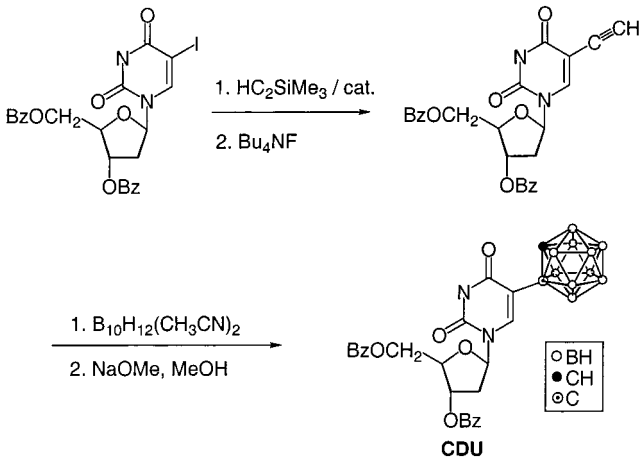
The in vitro and in vivo studies described in the following discussion use radioactive iodine, tritium, and cobalt as radionuclides. In many studies, boron clusters are conjugated to biomolecules, such as epidermal growth factors or immunoproteins. The corresponding radioiodinated conjugates can be synthesized by employing three different methodologies that are outlined in Scheme 4:

By using the first method the biomolecule, for instance a monoclonal antibody, is radioiodinated and then conjugated with the boron cluster. Here the label is exclusively placed on the antibody molecule. In the second method, the antibody is first conjugated with the boron cluster derivative, and the subsequent radioiodination of the whole conjugate places the radioiodine on the boron cluster and on the aromatic moieties of the protein. In this case, the position of iodination is dependent upon the nature of the boron cluster employed as well as the identitiy of the oxidizing agent and the reaction conditions. Generally, in the case of nucleophilic *nido*-carborane anions, the iodine preferentially bonds to the boron cage. In the third method, the boron cluster derivative is first radioiodinated and then conjugated with the antibody. The latter method provides conjugates in which the label is placed only on the boron cage.

A. Carborane-Substituted Deoxyuridine

One of the strategies to deliver boron-containing compounds to the interior of cells and their nuclei is the design and synthesis of carboranyl nucleosides and carboranyl oligonucleotides.[5] Consequently, Schinazi et al.[47] tested a carborane-substituted deoxyuridine (CDU) previously synthesized by Yamamoto et al.[59] on the premise that this compound would be subject to phosphorylation by cellular kinases resulting in its incorporation into DNA as an analogue of natural 2′-deoxypyrimidine nucleosides (Scheme 5). Cell selectivity of CDU would be obtained in brain tumors following its penetration of the blood−brain barrier, since normal central nervous system cells do not synthesize significant amounts of DNA in contrast to tumor cells.

Scheme 5. Synthesis of a *closo*-Carborane-Substituted Deoxyuridine (CDU)



CDU

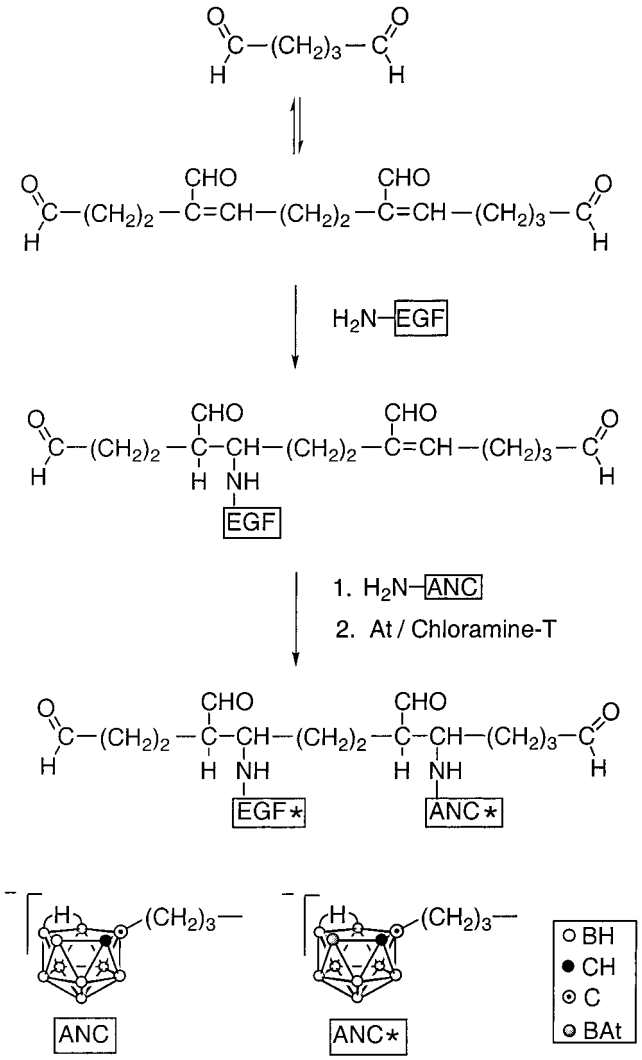
The intracellular uptake and metabolism of CDU was investigated in primary lymphocytes and in a T lymphoblastoid cell line using unlabeled and tritium-labeled compound. The label was introduced by deprotonation of the bisbenzyl-protected CDU with 2 equiv of *n*-butyllithium followed by the subsequent addition of carrier-free T_2O. Besides the tritiation of the CH cluster vertexes, the authors observed tritiation of the benzyl groups as well since the subsequent debenzylation of CDU decreased its overall radioactivity. The in vitro studies that followed revealed that CDU is rapidly taken up by the cells employed and efficiently phosphorylated to produce CDU monophosphate. Significantly, the CDU monophosphate is retained in the cells for up to 48 h. On the basis of these uptake studies, the authors estimated that CDU incorporation in the cells was 5.5 times greater than required for effective BNCT. The in vivo cytotoxicity and pharmokinetics of CDU was investigated in mice and rats, respectively.[60] The results of these studies suggest that it is possible to achieve significant levels of CDU in the brain, and a high degree of plasma protein binding restricted extensive distribution of this lipophilic compound. For a review about nucleic acids and nucleosides containing carboranes, see ref 60.

B. Boron Cluster Conjugates of Epidermal Growth Factor

One possible approach to the selective transport of therapeutic agents such as toxins, radionuclides, or boron compounds to tumor cells employs conjugation of the human epidermal growth factor (EGF). EGF is a fairly small polypeptide that binds specifically to its receptor, a transmembrane glycoprotein with tyrosine kinase activity. This receptor may be overexpressed in tumor cells. Many studies, reviewed elsewhere,[5,6] have been carried out with the aim to generate highly boronated EGFs for BNCT.

Recently, Sjöberg et al.[42] described the synthesis of a *nido*-carborane anion conjugated to EGF and reported the labeling of the conjugate with ^{211}At (method 2 in Scheme 4). The objective of that study was the exploration of a method for the indirect labeling of proteins with astatine using EGF as a

Scheme 6. Synthetic Route for the Conjugation of EGF with a Propylamino-Substituted *nido*-Carborane Anion[a]



[a] EGF, epidermal growth factor; ANC, 7-(3-$NH_2(CH_2)_3$)-*nido*-7,8-$C_2B_9H_{11}^-$.

model substrate. It was proposed that a compound bearing a B−^{211}At bond could be useful as a radioimmunotherapy agent, since ^{211}At is a short-lived α-emitter (half-life = 7.22 h). The possible employment of At for the radioimmunotherapy of cancer was investigated in preclinical studies by Link et al.[61] For this purpose, the boron cage was intended to be a stable carrier of the cytotoxic moiety. This is of importance since the direct astatination of biomolecules fails presumably due to unstable C−At bonds involved in this method. Scheme 6 illustrates the synthetic route for the conjugation of the EGF with the *nido*-carborane anion using trimerized glutaraldehyde as a cross-linking agent and 7-(3-$NH_2(CH_2)_3$)-*nido*-7,8-$C_2B_9H_{11}^-$ (ANC) as the *nido*-carborane anion derivative.

Subsequent astatination of the whole conjugate with ^{211}At was conducted using chloramine-T and resulted in 71% labeling efficiency. In a comparative experiment, the native unconjugated EGF molecule was astatinated providing only 4% labeling. This result can be associated with unstable At−C bonds present in the labeled EGF molecule. Due to this

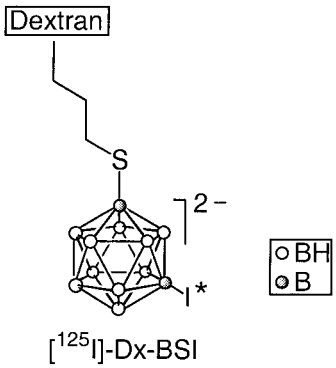


Figure 2. Radioiodinated BSH conjugated to allyl-substituted dextran.

observation, the authors concluded that astatination of the whole ANC−EGF conjugate places the label preferably on the *nido*-carborane cage. Preliminary studies indicated an appreciable in vitro stability of the conjugate.[42]

In another study, 7-(3-$NH_2(CH_2)_3$)-*nido*-7,8-$C_2B_9H_{12}^-$ (ANC) was coupled to dextran by reacting the free amino group of ANC with oxidized residues of the glucose polymer.[62] Subsequent reduction of the initially formed imine with cyanoborhydride afforded the stable conjugate. The concept of using dextran as the linker between a radionuclide carrier and a tumor-seeking protein (e.g., EGF) is currently under investigation by Carlsson et al.[38,62,63] The purpose of employing dextran is to create a spacer to attach radionuclides or other cytotoxic substances at a suitable distance from the antigen- or receptor-binding site of the protein. The authors anticipate that dextran, along with its attached radionuclide carrier, remains incorporated within the cell if the conjugated protein undergoes degradation. Thus, the rapid loss of cell-bound radioactivity usually encountered with directly radioiodinated proteins would be suppressed.[38]

The radioiodination of the dextran−ANC conjugate was carried out using Iodogen and $Na^{125}I$. Subsequent in vitro studies in rat liver homogenates revealed that the radioiodinated dextran−ANC conjugate was about four times more stable toward degradation than radioiodinated albumin.[59]

Detailed in vivo investigations with radioiodinated *closo*-$B_{12}H_{11}SH^{2-}$ (BSH) conjugated to dextran were conducted recently by Carlsson and Sjöberg et al.[38] BSH was attached to dextran by addition of the thiol group to the double bound present in allyl-fuctionalized dextran. Subsequent radioiodination using the chloramine-T method gave the radioiodinated conjugate ([^{125}I]Dx−BSI) with the iodine attached to the boron cage (Figure 2). In biodistribution studies with rats, high radioactivity uptake was found in liver and kidneys reaching a maximum at 3−6 h after injection. At this time the blood pool was cleared of ([^{125}I]-Dx−BSI), and thereafter only a small decrease in kidney and liver activities was observed. The thyroid uptake of radioactivity increased over time but was found to be very low. These data illuminate the in vivo stability of the radiolabeled conjugate since kidney and liver are the organs in which the highest rate of radioiodine loss is expected.[64]

C. Boron-Rich Immunoconjugates

1. General Remarks

The search for molecules that are expressed on the surface of human cancer cells, but that are not expressed to the same degree on normal or nonmalignant cells, continues to be of great importance. Substantial data now prove that such molecules can be detected using a variety of immunologic methods. As such, these molecules are referred to as tumor-associated antigens. Antibodies are biological macromolecules with specific affinity for their corresponding antigens and consequently represent a natural choice as targeting moieties for drug delivery. With the availability of monoclonal antibodies, an increasing number of immunoconjugates (antibody−drug conjugates with tumor selectivity) were reported. Most immunoconjugates are derived from intact IgG antibodies or antibody fragments such as Fab or $F(ab')_2$ as the targeting moiety. Cytotoxic species for the immunotherapy of cancer, which have been conjugated with immunoproteins are toxins, low molecular weight drugs, biological response modifiers, and radionuclides (radioimmunotherapy).[65]

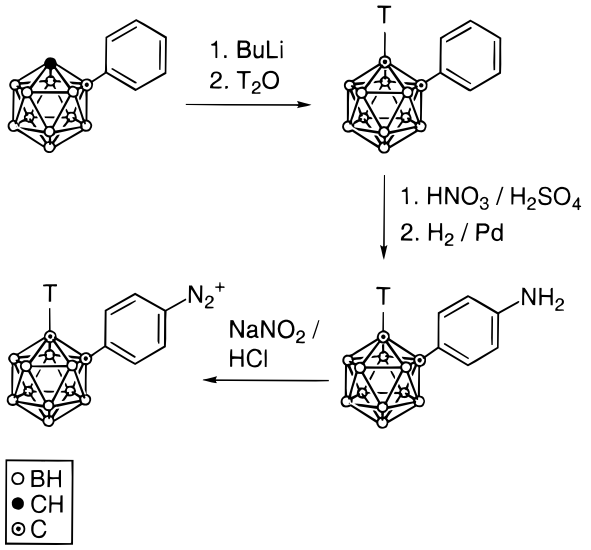
The idea of coupling ^{10}B-containing species to an antibody was first suggested by Bale in 1952.[66] Later, Mallinger et al.[67] and Hawthorne et al.[68] reported encouraging initial results involving the conjugation of boron-containing reagents to polyclonal antibodies. The latter group was able to demonstrate selective cytotoxicity using a simple conjugation method and thermal neutrons in vitro. With the development of tumor-associated monoclonal antibodies, several studies concerned with the possible use of boron−antibody conjugates for BNCT were published.

In the two sections that follow, studies with boron cluster derivatives conjugated to immunoproteins will be described.

2. Radiolabeled Immunoconjugates with Small Carborane Derivatives

One possible way to link compounds to a protein employs a substituted benzene diazonium ion reagent.[68] Scheme 7 shows the synthesis of a tritium-labeled 1-(4-$C_6H_4N_2$)-1,2-*closo*-$C_2B_{10}H_{11}^+$ ion.[46] This compound was then coupled with a purified polyclonal antibody against the carcinoembryonic antigen (CEA). This antigen, first described by Gold and Freedman,[69] belongs to a family of closely related glycoproteins present in a number of different organs and tissues and found in increased amounts in tumors derived therefrom. The basic molecule is a typical glycoprotein with a molecular weight of about 200 000. The coupling of the diazonium ion reagent with polyclonal anti-CEA IgG was conducted using different molar ratios of the diazonium reagent to antibody molecules at varying pH. The aim was to attach as many cages as possible to the antibody without affecting its immunoreactivity. The average number of carborane moieties per antibody molecule was determined by measuring the ^{3}H activity associated with a known protein concentration. The results show that up to eight carboranes could be coupled to the antibody without loss of its immunoreactivity,

Scheme 7. Synthesis of a Tritium-Labeled *closo*-Carborane-Substituted Benzene Diazonium Ion

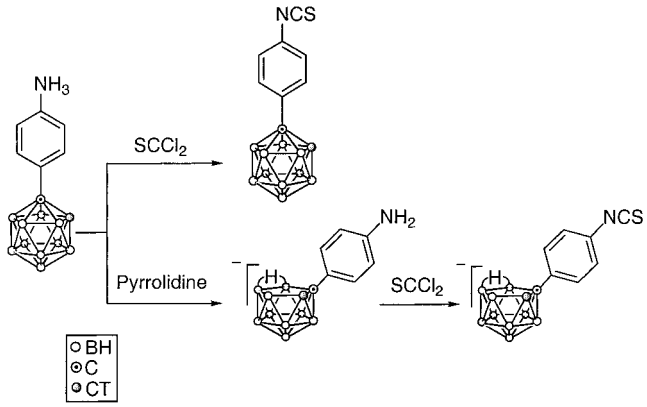


measured by determining the percentage of binding to a CEA affinity column. Any attempts to increase the population of coupled boron cages beyond this point resulted in the precipitation of the conjugated antibody protein. Whether this loss occurred due to denaturation or due to a decrease in the solubility of the *closo*-carborane-containing antibody conjugate could not be ascertained.

The objective of the in vivo studies that followed employing hamsters bearing CEA-containing human colonic carcinoma (GW-39 xenografts) was to ascertain the tumor-localizing capabilities of the antibody conjugated to the boron cluster derivatives.[70] For this purpose, the carborane−antibody conjugate was radiolabeled with ^{131}I using the chloramine-T method, by which the radioiodine was placed on the boron cluster and on the aromatic moieties of the antibody molecule (method 2 in Scheme 4). The data showed that the conjugation of the *closo*-carborane-containing reagent to the anti-CEA antibody (carborane:IgG ratio = 3) did not appreciably alter the specific tumor localization properties of the antibody, nor was there any significant difference in the antibody distribution in nontumor tissues. The conjugate was stable in normal hamster plasma and retained its immunoreactivity.

To further investigate the boronation of anti-CEA IgG, hydrophobic 1-(4-C_6H_4NCS)-1,2-*closo*-$C_2B_{10}H_{11}$, described above, and its water-soluble *nido* anion derivative were synthesized and used in antibody conjugation studies (Scheme 8).[28] The specific activities of the tritium radiolabeled *nido* species were only about 10% of the specific activity of the corresponding *closo* precursor. This loss of radioactivity was the result of base-catalyzed hydrogen exchange of the carborane C−T label during the carborane cage deboronation step using ethanolic KOH. By employing pyrrolidine as the degradation reagent, 50% of the original radioactivity could be retained. As in the previously described study, the introduction of tritium was used to determine the average number of carborane moieties per antibody molecule in the

Scheme 8. Synthesis of Carborane-Substituted Phenylisothiocyanates



conjugation experiments. The utility of the isothiocyanate group for antibody protein labeling has been extensively studied with respect to fluorescence-labeled antibody techniques,[71] and it has been the functional group of choice for the conjugation of metal chelates, boron compounds, and other reagents to antibody molecules. When using the anionic water-insoluble *closo*-isothiocyanate derivative, *N*,*N*-dimethylformamide/water mixtures were employed during the conjugation reaction to attain reagent solubility. When using the water-soluble *nido*-isothiocyanate derivative, up to nine carborane cages could be bound to a single murine monoclonal antibody Mu-9 molecule with 49% recovery of the antibody.[28] If the conjugation reaction was carried out at higher protein concentration or at high proportions of the *nido*-carborane reagent to the antibody, the formation of high molecular weight species was observed and the protein precipitated. In the case of the corresponding *closo*-isothiocyanate derivative, only one cage could be coupled to Mu-9. Both conjugates retained their immunoreactivity.

For the subsequent biodistribution studies,[29] the carborane−antibody conjugates were radioiodinated with ^{131}I using the chloramine-T method, by which the label was placed on the carborane as well as on the antibody molecule (method 2 in Scheme 4).

Two conjugates were investigated, one bearing a single *closo*-carborane cage and one bearing four *nido*-carborane anions attached to the murine monoclonal antibody Mu-9. The data revealed an early enhanced uptake of the *nido*-carborane conjugate in the liver, with a 2−3-fold decrease in the level of radioactivity in the blood and other organs. The biodistribution and tumor uptake for the *closo*-carborane conjugate were similar to those observed for the unconjugated antibody, whereas the tumor uptake was significantly decreased in the case of the *nido*-carborane conjugate.

The authors concluded that the coupling of multiple boron cages to a single monoclonal antibody without altering the apparent immunoreactivity of the antibody is possible, but that the in vivo tumor uptake may be reduced and the rate of clearance from the blood and normal tissues increased if the conjugation with the carborane reagent alters the physical properties of the antibody. This alteration is significant

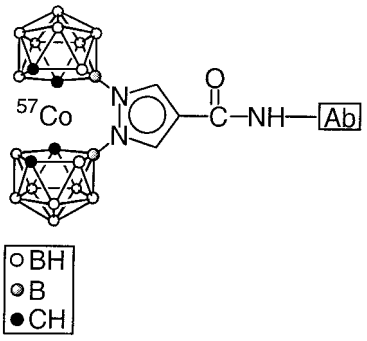


Figure 3. Venus flytrap complex with radiocobalt, conjugated to a monoclonal antibody.

if more than one carborane cage is attached to the antibody.

In addition to radiolabeling using radioiodine, *nido*-carborane dianions form extremely stable complexes with cobalt and other transition metals and are potentially of great interest as carriers for radiometals. Figure 3 shows the above-mentioned Venus flytrap complex (VFC) with ^{57}Co as the γ-photon-emitting radiometal. The structure of this complex was determined in an X-ray diffraction study using isotopically normal cobalt.

Conjugation of the mixture of ^{57}Co-labeled VFC isomers (*dl* and *meso*) to the anti-CEA monoclonal antibody, T84.66, could be accomplished via its *N*-hydroxysulfosuccinimide ester. In this manner an average of 0.03−0.05 molecules of VFC reagent/molecule of antibody was conjugated in accord with the VFC concept of providing an extraordinarily stable imaging agent rather than a therapeutic agent for BNCT.[72] The VFC conjugate retained >90% immunoreactivity, was stable in serum (more than 7 days), and demonstrated excellent localization in LS174T tumor xenografts during in vivo studies with nude mice.[72] In the latter study, the pharmacokinetics of ^{57}Co VFC-T84.66 were compared to those of T84.66 MAb conjugated with either DTPA or its benzylisothiocyanate derivative (BzDTPA) labeled with ^{111}In. The whole-body half-life for VFC-T84.66 was less than for either DTPA-T84.66 or BzDTPA-T84.66. The blood clearance rate was similar for all three radioimmunoconjugates. Hepatic uptake of the radiolabel was rapid and remained constant for 7 d for both DTPA radioimmunoconjugates. For VFC, however, the initially observed liver radioactivity decreased rapidly to about 10% of its original value, suggesting a possible role for VFC radioimmunoconjugates in the imaging and β^- therapy of liver metastases.

3. Radiolabeled Immunoconjugates with Oligomeric Carborane Derivatives

A requirement for BNCT is the delivery of therapeutic amounts of boron to tumor cells. The main problem seen in all previously reported studies with boron-rich immunoconjugates was the precipitation and loss of antibody during attempts to conjugate a large number of small boron cluster derivatives to immunoprotein. It was anticipated that a possible means to circumvent this problem would involve the synthesis of a boron-rich oligomer that could then be

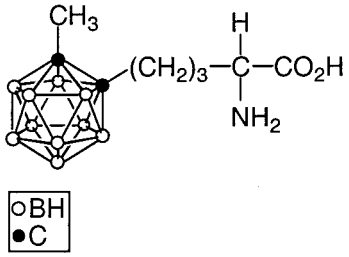


Figure 4. Structure of a carboranyl amino acid employed for the resin-supported synthesis of oligomeric boron-containing peptides.

conjugated to the biomolecule (e.g., antibody) at a minimal number of sites. It was hoped that by pursuing this methodology the conjugation of the biomolecule with the small number of oligomeric reagents required would not significantly alter tumor localization in vivo. For this purpose, several different strategies have been developed, including those based upon boron derivatives of polylysines, polyornithines, and dextrans.[5] One of the original limitations with such structures is that the precursor oligomers were not discrete and homogeneous.

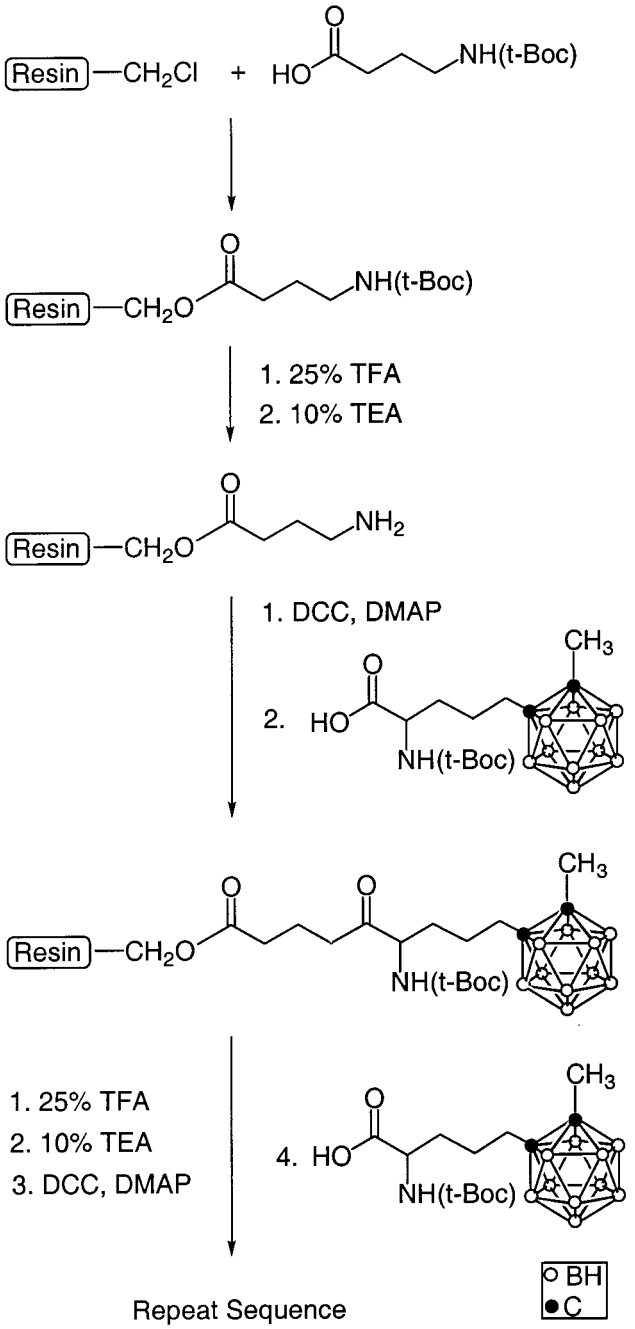
Hawthorne et al.[73] were the first to pursue the idea of assembling a series of discrete, precisely synthesized oligomeric reagents (trailers), each of which contains a fixed number of boron atoms up to 200. These oligomeric reagents would contain a free carboxylic acid group for conjugation with lysine ϵ-NH_2 groups of the antibody. Attachment of an average of five of these oligomeric species would provide the desired population of 10^3 boron atoms per antibody molecule and not critically alter the overall IgG performance as in the case of a large number of small boron cluster conjugations.

In the following studies, trailer-conjugated antibodies will be described. One way to synthesize well-defined biocompatible oligomers is available through Merrifield peptide syntheses. Consequently, this methodology was used for the synthesis of boron-containing peptides.[74] Figure 4 shows the structure of the carboranyl amino acid[74,75] that was employed for the resin-supported peptide synthesis.

It was found that only the amino acid containing the *closo*-carborane cage gave peptides in good yield, whereas the *nido*-amino acid derivative was not suited for peptide synthesis. Therefore, the peptides were initially prepared with the *closo*-amino acid, and the isolated peptides were then converted to the corresponding water-soluble *nido* oligomers by the cage deboronation reaction with pyrrolidine. Scheme 9 shows a typical example of the stepwise peptide synthesis.

For in vitro and in vivo studies,[35] a *nido*-carborane dimer ($[CB]_2$) and a *nido*-carborane decamer ($[CB]_{10}$) were employed (Figure 5). In the synthesis of $(CB)_2$, the monomeric amino acid was directly loaded on the resin. For the synthesis of longer peptide sequences, like $(CB)_{10}$, common amino acids such as lysine, glycine, or γ-aminobutyric acid were employed as the initial amino acid of the peptide sequence. Interestingly, these amino acids led to a considerably higher degree of loading on the Merifield resin.

Scheme 9. Principle of Resin-Supported Merrifield Peptide Synthesis Using Carboranyl Amino Acids



For in vitro assays, the free amino groups of $(CB)_2$ and $(CB)_{10}$ were labeled with the fluorescent dansyl group, whereas their free carboxylic acid groups were used for subsequent coupling to the anti-CEA monoclonal antibody (MAb) T84.66 employing *N*-hydroxysulfosuccinimide and diisopropylcarbodiimide as coupling reagents. On the basis of the measured fluorescence intensity and protein concentration, T84.66-$(CB)_2$ contained an average of 3.5 molecules of $(CB)_2$/antibody molecule, whereas T84.66-$(CB)_{10}$ contained 2 molecules of $(CB)_{10}$/antibody molecule. Only during the conjugation of $(CB)_{10}$ to the antibody was the undesirable formation high molecular weight (HMW) species observed. This was explained as being due to the nonspecific binding of the unconjugated

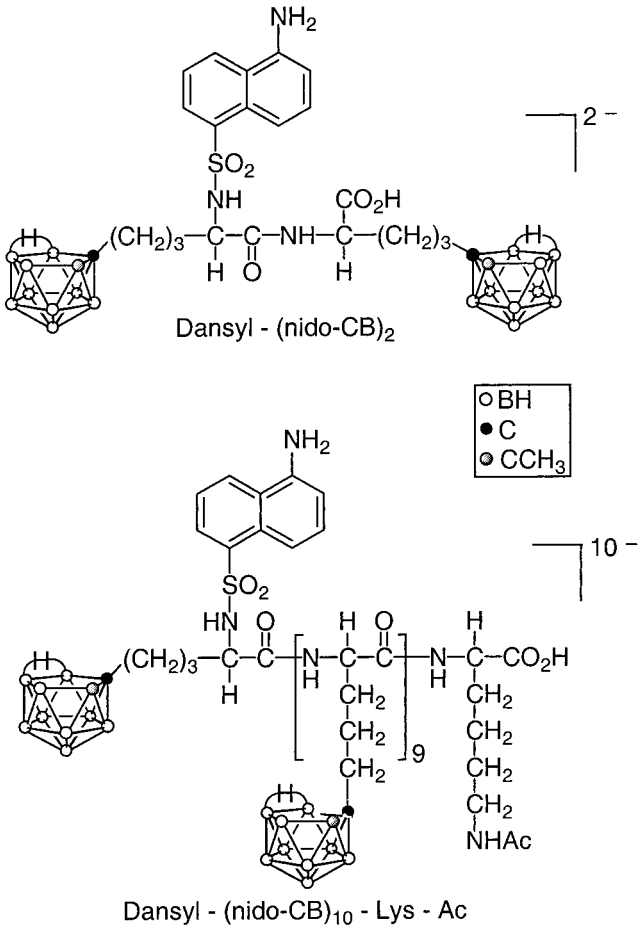


Figure 5. Structures of a *nido*-carborane dimer ($[CB]_2$) and a *nido*-carborane decamer ($[CB]_{10}$) used for in vivo and in vitro studies.

trailer to the antibody.[35] Both conjugates retained their immunoreactivity. Biodistribution data were collected using nude mice bearing LS174T xenografts and carborane−antibody conjugates that were radioiodinated using $Na^{125}I$ and chloramine-T (method 2 in Scheme 4).

While the lightly B-loaded dipeptide conjugate gave biodistribution results that closely resembled those of native T84.66 MAb, the decapeptide conjugate displayed greatly enhanced liver uptake and decreased tumor accretion. These results suggest that, as the boron-containing burden on the supporting immunoprotein is markedly increased, loss of circulating conjugate to liver accretion effectively competes with the desired tumor localization.

It was anticipated that with an increasing number of boron cages the peptide becomes more hydrophobic, despite the fact that each *nido* cage bears a negative charge. This hydrophobicity in the peptides was believed to adversely affect the biodistribution of the conjugate.

To increase the hydrophilicity of the boron-rich conjugation reagents, *nido*-carborane cages were coupled using the phosphate diester function as the linking group (Figure 6).[76] Homogeneous oligomeric phosphate diesters (OPDs) containing up to 400 boron atoms have been synthesized with a variety of structures, and these highly charged species are water-soluble. The phosphate diester trailers were

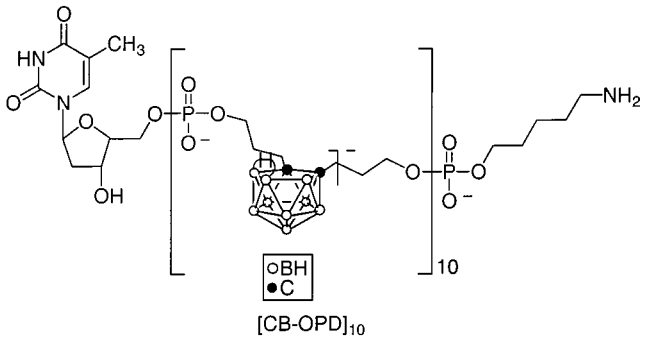


Figure 6. Example of an oligomeric *nido*-carborane phosphate diester derivative.

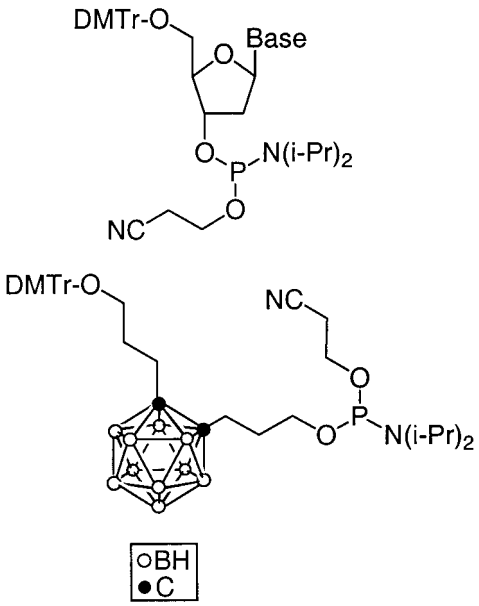
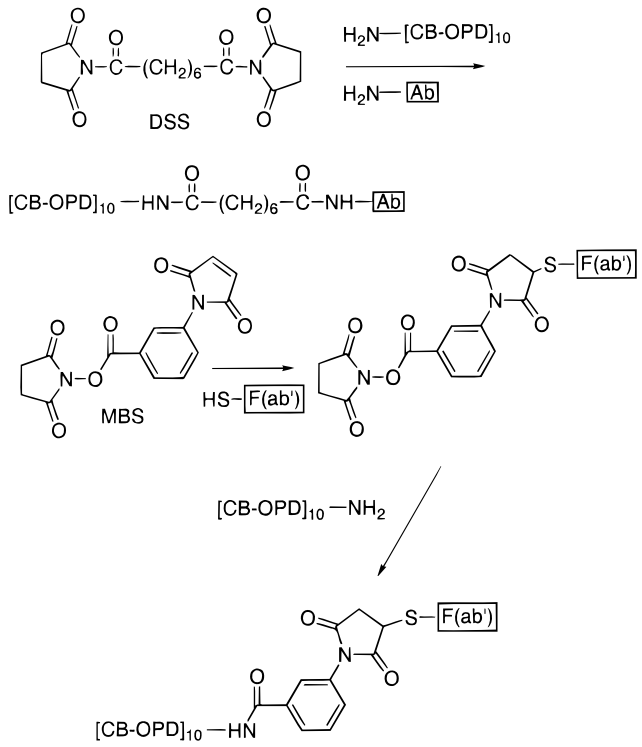


Figure 7. *closo*-Carborane derivative bearing a protected hydroxyl group and a phosphoramidite moiety is functionally equivalent to nucleoside monomers and can be employed in supporting oligophosphate synthesis.

prepared by using automated DNA synthesizer technology[77] with no modifications to the standard types of reagents or procedures. The most common method for automated DNA synthesis involves the stepwise coupling of 5′-*O*-(dimethoxytrityl)-3′-(*N*,*N*-diisopropylamino)(*β*-cyanoethyl)-phosphoramidite nucleoside derivatives (Figure 7). It was found that a disubstituted *closo*-1,2-$C_2B_{10}H_{12}$ cage bearing a protected hydroxyl group and the same phosphoramidite moiety[77] is functionally equivalent to the nucleoside monomers in supporting oligophosphate synthesis. The synthesized oligomeric chains begin with a 3′-thymidine residue, while the 5′-terminal group is an aminohexyl moiety to allow protein conjugation. Extended ammonium hydroxide treatment cleanly and quantitatively converted the oligophosphates containing *closo*-carborane cages to their anionic and highly hydrophilic *nido*-carborane derivatives.

The trailer with 10 anionic *nido*-carborane residues ([CB-OPD]$_{10}$, shown in Figure 6) was coupled to the anti-CEA antibody T84.66 and its F(ab′) fragment.[34] The coupling to the proteins was achieved with disuccinimidyl suberate (DSS) and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) as coupling reagents (Scheme 10).

Scheme 10. Coupling of an Oligomeric Phosphate Diester to an Antibody F(ab′) Fragment[a]



[a] DSS, disuccinimidyl suberate; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; [CB-OPD], carborane-containing oligomeric phosphate diester.

Scheme 11. Synthesis of a Dual-Labeled Immunoconjugate Using Two Different Iodine Isotopes[a]

[a] Mab, monoclonal antibody; [CB-OPD], carborane-containing oligomeric phosphate diester.

To determine the content of [CB-OPD]$_{10}$ in the conjugates, the proteins were labeled with ^{125}I (Iodogen method), and the [CB-OPD]$_{10}$ was labeled with ^{131}I prior to their conjugation, as shown in Scheme 11 (dual labeling). In the case of the [CB-OPD]$_{10}$-T84.66 conjugate, the moles of [CB-OPD]$_{10}$ covalently incorporated into the conjugate ranged from 1.2 to 6.2, depending on the molar ratios used. Immunoreactivity of these conjugates was retained even for the highest level of [CB-OPD]$_{10}$ incorporation. As in other similar studies, the formation of high molecular weight (HMW) species was significant. Since the yield of the Fab′ conjugates was low, biodistribution studies were carried out only with [CB-OPD]$_{10}$—T84.66 IgG conjugates. When the purified conjugate was injected into animals bearing a CEA-positive tumor xenograft, a large portion of the conjugate accumulated in liver, and the tumor uptake decreased relative to that of the native antibody. Interestingly, the data reported indicate a significant dehalogenation of ^{131}I from the antibody, whereas the ^{125}I-labeled carborane remains intact. This observa-

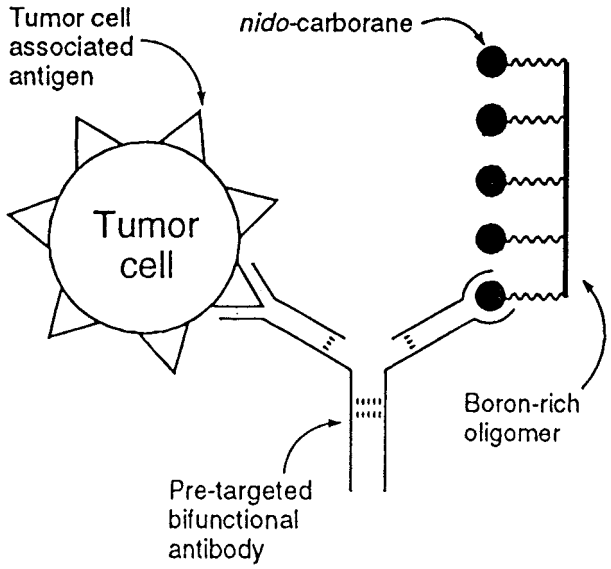


Figure 8. Schematic diagram demonstrating the principle of pretargeting with BsMAbs. The antibody is first pretargeted to the tumor by its reaction with a tumor cell surface antigen. The remaining antigen-combining site is then available for binding a boron-rich haptenic macromolecule that is subsequently administered.

tion further supports the possible use of carboranes as stable carrier systems for radioiodine. The biodistribution results are almost identical to those obtained for the carborane-containing peptides and reveal that the loading of antibodies with either small or oligomeric boron cluster derivatives significantly alters their biodistributions leading to increased liver and decreased tumor uptakes. Similar results emerged in a study where boron-rich oligophosphates were conjugated to a genetically engineered anti-dansyl IgG immunoprotein equipped with two unnatural cysteine residues exposed for site-specific conjugation.[78]

In another approach to resolve this problem, bispecific (Fab'_{CEA})−(Fab'_{Nido}) antibodies (BsMAbs) were engineered that simultaneously target the tumor antigen as well as the boron compound (a hapten), thus avoiding chemical conjugation of the immunoprotein (Figure 8).[79,80] In practice, the bispecific antibody would be injected into the tumor-bearing animal and bind to the tumor cell antigen with the Fab'_{CEA} portion of its structure. After maximum tumor loading has been achieved and the blood cleared, the animal would then be inoculated with the hapten-bearing boron carrier. Binding of this large molecule to the free and uncomplexed (Fab'_{Nido}) arm of the already cell-bound (Fab'_{CEA})−(Fab'_{Nido}) should provide each cellular antigenic site involved with 10^3 B-atoms without the use of conventional covalent bonds.

In one study, CEA was selected for the tumor-specific component of the BsMAbs also possessing reactivity for a *nido*-carborane hapten bearing an amide substituent (Figure 9).[33] For localization experiments in vivo, radioiodinated BsMAb and anti-CEA MAb were separately injected into athymic nude mice bearing LS-174T colon carcinoma xenografts. After 24 h, the uptake of the BsMAb in tumor was similar to that of the parental anti-CEA MAb.

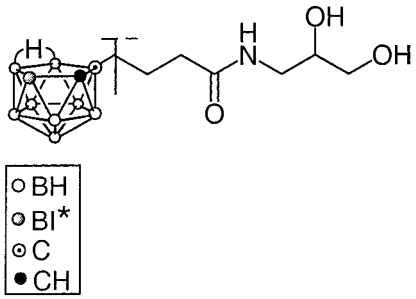


Figure 9. Radioiodinated *nido*-carborane hapten bearing an amide substituent.

Subsequent experiments were performed to determine if the BsMAb could target the *nido*-carborane derivative shown in Figure 9 to cancer cells expressing CEA. CEA-positive human colon carcinoma cells (LS-174T) were sequentially exposed to BsMAb followed by the amide-substituted *nido*-carborane hapten, which had previously been radioiodinated using $Na^{125}I$ and chloramine-T. In this case, only the *nido*-carborane derivative was labeled with radioiodine. The carcinoma cells showed enhanced binding of the amide-substituted *nido*-carborane hapten when previously exposed to the BsMAb as compared to the binding obtained after exposure of the cells to a physical mixture of the respective parental antibodies.

These studies demonstrate that BsMAbs prepared with an anti-*nido*-carborane anion specificity can selectively localize a *nido*-carborane anion structure to a tumor cell surface. This is of particular importance, since the binding of a boron-rich oligomeric phosphate diester (trailer) comprised of 10 haptenic *nido* cages to an anti-*nido*-MAb could be demonstrated.[79]

Using a different concept, Soloway et al.[81] investigated commercially available dendritic macromolecules consisting of repetitive monomeric units and peripheral functional groups that can be modified using a variety of reagents and ligands. Such species are referred to as Starbust Dendrimers (SDs) because of their branching, treelike pattern. They are composed of repetitive poly(amidoamino) groups and are classified as zero-, first-, second-, third- and fourth-generation SDs (SD-1, SD-2, ...), depending upon the numbers of N−N bonds formed in the polymer (Figure 10). In the described study,[81] an SD-4 was directly coupled with an isothiocyanato-substituted *closo*-$B_{10}H_{10}^{2-}$ affording boronated SDs (BSDs) with a boron content of 250−1000 boron atoms/polymer molecule. The subsequent coupling of the BSD to MoAb IB16−6, a monoclonal antibody against the murine B16 melanoma, was conducted using *m*-maleimidobenzoyl *N*-hydroxysulfosuccinimide ester (sulfo-MBS) and *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), as outlined in Scheme 12.

Subsequent biodistribution studies were performed in C57Bl/6 mice carrying subcutaneous implants of B16 melanoma. The immunoconjugate, which was stable and retained its immunoreactivity, was radiolabeled with $Na^{125}I$ using Iodogen as oxidizing agent. Under these conditions, the radiolabel was most likely attached at the boron cage as well as the

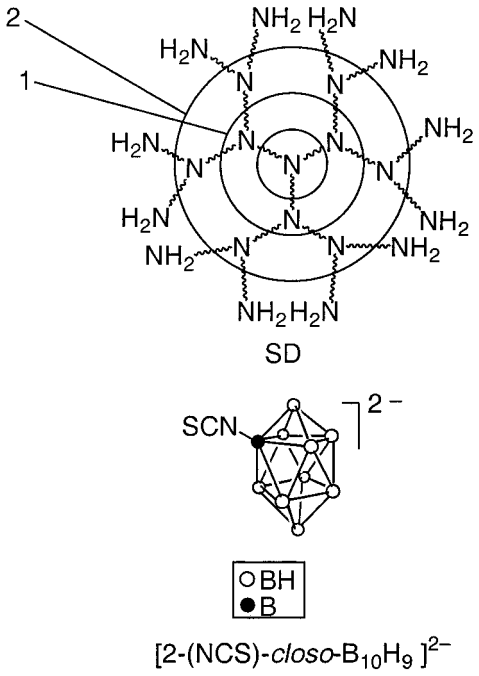
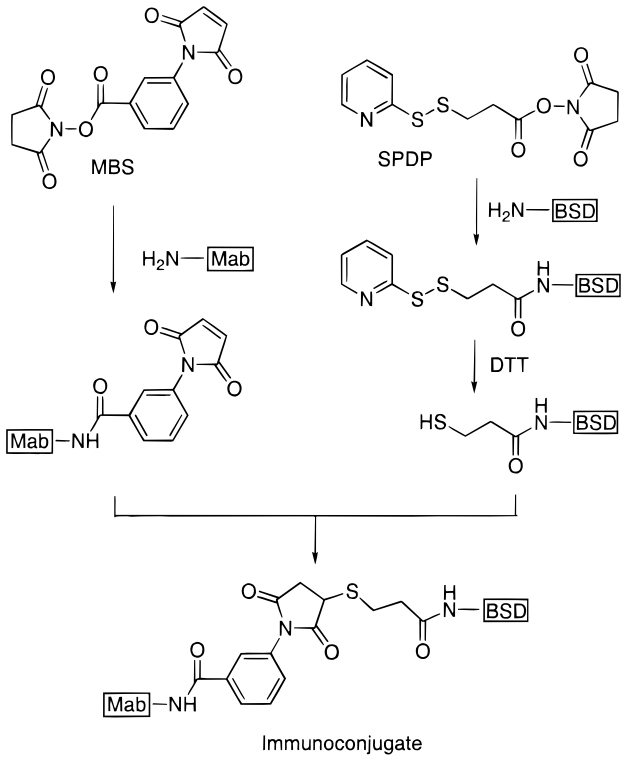


Figure 10. Starbust dendrimers (SD) composed of repetitive poly(amidoamino) groups can be coupled with an isothiocyanato-substituted *closo*-$B_{10}H_{10}^{2-}$ dianion.

Scheme 12. Coupling of a Boronated Starbust Dendrimer (BSD) to a Monoclonal Antibody[a]



[a] MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; DTT, *threo*-1,4-dimercapto-2,3-butanediol.

aromatic moieties of the protein (method 2 in Scheme 4). The data obtained demonstrated that SDs have a propensity to localize in the liver and spleen and that the absolute amount appeared to be directly related to the molecular weight and number of reactive terminal amino groups present on the dendimer.

V. Summary

The aforementioned studies reveal that boron cluster anion derivatives can be successfully employed as carrier systems for radioiodine, astatine, tritium, and radiocobalt. The radioiodination of the targeted boron cluster anions is simple and provides stable radiolabeled products that are attractive due to the enhanced stability of the B−I bond with respect to in vivo dehalogenation. This appreciable in vivo stability can be attributed to the inorganic nature of the boron clusters and their apparent invisibility to enzyme systems responsible for dehalogenation. The conjugation of boron cluster derivatives to biomolecules, such as antibodies and growth factors, is possible using standard conjugation techniques. A general problem with the performance of all these conjugates is the increased liver uptake and decreased tumor uptake relative to the native antibody. The undesired liver uptake becomes significant as the number of boron cluster derivatives attached to the tumor-targeting molecule is increased. It remains questionable whether it will be possible to deliver the large amounts of boron required for BNCT to tumor cells employing this methodology. On the other hand, the conjugation of just one boron cage to the investigated antibodies did not lead to alteration of their in vivo properties. This observation is important and indicates that radiolabeled boron cluster immunoconjugates can be used as excellent tumor-imaging agents (method 3 in Scheme 4). This could be demonstrated in the case of the Venus flytrap complex with ^{57}Co, which is probably the most stable π-complex of cobalt known today. Currently under investigation are the clinically important positron-emitting ^{55}Co isotope and other radiometals, such as ^{99m}Tc, complexed by the Venus flytrap. Effective tumor-imaging and radioimmunotherapy require the boron cluster to be radiolabeled prior to its conjugation to the immunoprotein. This is exemplified in the cases of radioiodination and astatination by method 3 in Scheme 4, since only by employing this strategy will the radiohalogen be exclusively placed on the boron cage.

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VII. References

(1) Locher, G. L. *Am. J. Roentgenol. Radium Ther.* **1936**, *36*, 1.
(2) Javid, M.; Brownell, G. L.; Sweet, W. H. *J. Clin. Invest.* **1952**, *31*, 603.
(3) Hawthorne, M. F. *Mol. Med. Today* **1998**, *April*, 174.
(4) Grimes, R. N. *Carboranes*; Academic Press: New York, 1970.
(5) Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F.-G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998**, *98*, 1515.
(6) Hawthorne, M. F. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 950.
(7) Nakanishi, A.; Guan, L.; Kane, R. R.; Kasamatsu, H.; Hawthorne, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 238.
(8) Rupprecht, M.; Probst, T. *Anal. Chim. Acta* **1998**, *358* (3), 205.
(9) Barth, R. F.; Adams, D. M.; Soloway, A. H.; Mechetner, E. B.; Alam, F.; Anisuzzaman, A. K. M. *Anal. Chem.* **1991**, *63*, 890.
(10) Imahori, Y.; Ueda, S.; Ohmori, Y.; Sakae, K.; Kusuki, T.; Kobayashi, T.; Takagaki, M.; Ono, K.; Ido, T.; Fujii, R. *Jpn. Clin. Cancer Res.* **1998**, *4* (8), 1833.

(11) Kabalka, G. W. *Book of Abstracts*, 217th ACS National Meeting, Anaheim, CA, March 21−25, 1999; NUCL-153; American Chemical Society: Washington, DC, 1999.
(12) Hawthorne, M. F.; Pitochelli, A. R. *J. Am. Chem. Soc.* **1959**, *81*, 5519.
(13) Lipscomb, W. N.; Pitochelli, A. R.; Hawthorne, M. F. *J. Am. Chem. Soc.* **1959**, *81*, 5833.
(14) Pitochelli, A. R.; Hawthorne, M. F. *J. Am. Chem. Soc.* **1960**, *82*, 3228.
(15) Miller, H. C.; Miller, N. E.; Muetterties, E. L. *J. Am. Chem. Soc.* **1963**, *85*, 3885.
(16) Hawthorne, M. F.; Pilling, R. L. *Inorg. Synth.* **1967**, *9*, 16.
(17) Muetterties, E. L.; Knoth, W. H. In *Polyhedral Boranes*; Dekker: New York, 1968.
(18) Knoth, W. H.; Miller, H. C.; Sauer, J. C.; Balthis, J. H.; Chia, Y. T.; Muetterties, E. L. *Inorg. Chem.* **1964**, *3*, 159.
(19) Busby, D. C.; Hawthorne, M. F. *Inorg. Chem.* **1982**, *21*, 4101.
(20) Hawthorne, M. F.; Young, D. C.; Garrett, P. M.; Owen, D. A.; Schwerin, S. G.; Tebbe, F. N.; Wegner, P. A. *J. Am. Chem. Soc.* **1968**, *90*, 862.
(21) Wilbur, D. S. *Bioconjugate Chem.* **1992**, *3*, 433.
(22) Adloff, J. P.; Lieser, K. H.; Stöcklin, G. *Radiochim. Acta* **1995**, *70/71*, 412.
(23) Stöcklin, G. *Radiochim. Acta* **1995**, *70/71*, 249.
(24) Coursey, B. M.; Calhoun, J. M.; Cessna, J.; Golas, D. B.; Schima, F. J.; Unterweger, M. P. *Nucl. Instrum. Methods Phys. Res.* **1994**, *A339*, 26.
(25) Warr, N.; Drissi, S.; Garret, P. E.; Jolie, J.; Kern, J.; Lehmann, H.; Mannanal, S. J. *Nucl. Phys.* **1998**, *A636*, 379.
(26) Saha, G. B. In *Radioimmunoimaging and Radioimmunotherapy*; Burchiel, S. W., Rhodes, B. A., Eds.; Elsevier: New York, 1983; pp 171−185.
(27) Olsen, F. P.; Hawthorne, M. F. *Inorg. Chem.* **1965**, *4*, 1839.
(28) Mizusawa, E. A.; Thompson, M. R.; Hawthorne, M. F. *Inorg. Chem.* **1985**, *24*, 1911.
(29) Varadarajan, A.; Sharkey, R. M.; Goldenberg, D. M.; Hawthorne, M. F. *Bioconjugate Chem.* **1991**, *2*, 102.
(30) Kerr, J. A. In *Handbook of Chemistry and Physics*, 72nd ed.; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 1991; pp 9−113.
(31) Wilbur, D. S.; Hamlin, D. K.; Liversey, J. C.; Srivastava, R. R.; Laramore, G. E.; Griffin, T. W. *Nucl. Med. Biol.* **1994**, *21*, 601.
(32) Wilbur, D. S.; Hamlin, D. K.; Srivastava, R. R. *J. Labelled Compd. Radiopharm.* **1994**, *35*, 199.
(33) Primus, F. J.; Pak, R. H.; Rickard-Dickson, K. J.; Szalai, G.; Bolen, J. L.; Kane, R. R.; Hawthorne, M. F. *Bioconjugate Chem.* **1996**, *7*, 532.
(34) Chen, C.-J.; Kane, R. R.; Primus, F. J.; Szalai, G.; Hawthorne, M. F.; Shively, J. E. *Bioconjugate Chem.* **1994**, *5*, 557.
(35) Paxton, R. J.; Beatty, B. G.; Varadarajan, A.; Hawthorne, M. F. *Bioconjugate Chem.* **1992**, *3*, 241.
(36) Jiang, W.; Knobler, C. B.; Curtis, C. E.; Mortimer, M. D.; Hawthorne, M. F. *Inorg. Chem.* **1995**, *34*, 3491 and references therein.
(37) Zheng, Z.; Jiang, W.; Zinn, A. A.; Knobler, C. B.; Hawthorne, M. F. *Inorg. Chem.* **1995**, *34*, 2095 and references therein.
(38) Tolmachev, V.; Koziorowski, J.; Sivaev, I.; Lundqvist, H.; Carlsson, J.; Orlova, A.; Gedda, L.; Olsson, P.; Sjöberg, S.; Sundin, A. *Bioconjugate Chem.* **1999**, *10*, 338.
(39) Brattsev, V. A.; Yadrovskaya, V. A.; Savina, E. P.; Oulianenko, S. E.; Morris; J. H. *Abstracts of Papers*, Eight International Symposium on Neutron Capture Therapy for Cancer; Poster Session, Chemistry and Physics; La Jolla; September, 1998; p 85.
(40) Wilbur, D. S.; Hamlin, D. K.; Buhler, K. R.; Srivastava, R. R.; Stray, J. E.; Daniel, J.; Vessela, R. L. *Q. J. Nucl. Med.* **1995**, *39*, 51.
(41) Schuurmans, P.; Will, B.; Berkes, I.; Camps, J.; De Jesus, M.; De Moor, P.; Herzog, P.; Lindroos, M.; Paulsen, R.; Severijns, N.; Van Geert, A.; Van Duppen, P.; Vanneste, L. Nicole and Isolde Collaboration. *Z. Phys.* **1997**, *A358*, 239.
(42) Koziorowski, J. Dissertation, Uppsala University, Sweden, 1998.
(43) Orlova, A.; Lebeda, O.; Tolmachev, V.; Sjöberg, S.; Carlsson, J.; Lundqvist, H. Submitted for publication.
(44) Stephenson, G. J., Jr.; Goldman, T. *Phys. Lett.* **1998**, *440B*, 89.
(45) Filer, C.; Hurt, S.; Wan, Y.-P. In *Receptor Pharmacology and Function*; Williams, M., Glennon, R. A., Timmermans, P. B. M. W. M., Eds.; Marcel Dekker: New York, 1989; p 105.
(46) Mizusawa, E.; Dahlman, H. L.; Bennet, S. J.; Goldenberg, D. M.; Hawthorne, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 3011.
(47) Schinazi, R. F.; Goudgaon, N. M.; Fulcrand, G.; El Kattan, Y.; Lesnikowski, Z.; Ullas, G.; Moravek, J.; Liotta, D. *Int. J. Radiat. Oncol. Biol. Phys.* **1994**, *28*, 1113.
(48) Meares, C. F.; Wensel, T. G. *Acc. Chem. Res.* **1984**, *17*, 202 and references therein.
(49) Brechbiel, M. W.; Gansow, O. A.; Atcher, R. W.; Schlom, J.; Esteban, J.; Simpson, D. E.; Colcher, D. *Inorg. Chem.* **1986**, *25*, 2772 and references therein.
(50) Deshpande, S. V.; De Nardo, S. J.; Meares, C. F.; McCall, M. J.; Adams, G. P.; Moi, M. K.; De Nardo, G. L. *J. Nucl. Chem.* **1988**, *29*, 217.
(51) Moi, M. K.; Meares, C. F.; McCall, M. J.; Cole, W. C.; De Nardo, S. J. *Anal. Biochem.* **1985**, *148*, 249.
(52) Cox, J. P. L.; Jankowski, J. K.; Kataky, R.; Parker, D.; Beeley, N. R. A.; Boyce, B. A.; Eaton, M. A. W.; Miller, K.; Millican, A. T.; Harrison, A.; Walker, C. *J. Chem. Soc., Chem. Commun.* **1989**, 797.
(53) Craig, A. S.; Helps, I. M.; Jankowski, K. J.; Parker, D.; Beeley, N. R. A., Boyce, B. A.; Eaton, M. A. W.; Millican, A. T.; Millar, K.; Rhind, S. K.; Harrison, A.; Walker, C. *J. Chem. Soc., Chem. Commun.* **1989**, 794.
(54) Craig, A. S.; Parker, D.; Adams, H.; Bailey, N. R. *J. Chem. Soc., Chem. Commun.* **1989**, 1792.
(55) Moerlein, S. M.; Welch, M. *J. Int. J. Nucl. Med. Biol.* **1981**, *8*, 277.
(56) Hawthorne, M. F.; Varadarajan, A.; Knobler, C. B.; Chakrabarti, S. *J. Am. Chem. Soc.* **1990**, *112*, 5365.
(57) Hawthorne, M. F.; Young, D. C.; Andrews, T. D.; Howe, D. V.; Pilling, R. L.; Pitts, A. D.; Reintjes, M.; Warren, L. F.; Wegner, P. A. *J. Am. Chem. Soc.* **1968**, *90*, 879.
(58) Martin, R. H.; Burns, K. I. W.; Taylor, J. G. V. *Nucl. Instrum. Methods Phys. Res.* **1997**, *A390*, 267.
(59) Yamamoto, Y.; Seko, T.; Nakamura, H.; Nemoto, H.; Hojo, H.; Mukai, N.; Hashimoto, Y *J. Chem. Soc., Chem. Commun.* **1992**, 157.
(60) Lesnikowski, Z. J.; Shi, J.; Schinazi, R. F. *J. Organomet. Chem.* **1999**, *581*, 156 and references therein.
(61) Link, E. M.; Michalowski, A. S.; Rosch, F. *Eur. J. Cancer* **1996**, *11*, 1986.
(62) Tolmachev, V.; Bennarsten, J.; Bruskin, A.; Carlson, J.; Lundqvist, H. Submitted for publication.
(63) Gedda, L.; Olsson, P.; Carlsson, J. *Bioconjugate Chem.* **1996**, *7*, 584.
(64) Khaw, B. A.; Cooney, J.; Edingtom, T.; Strauss, H. W. *J. Nucl. Med.* **1986**, *27*, 1293.
(65) Vogel, C.-W. In *Immunoconjugates. Antibody Conjugates in Radioimmaging and Therapy of Cancer*; Vogel, C.-W., Ed.; Oxford University Press: New York, 1987; pp 3−7.
(66) Bale, W. F. *Proc. Natl. Cancer Conf.* **1952**, *2*, 967.
(67) Mallinger, A. G.; Jozwiak, E. L., Jr.; Carter, J. C. *Cancer Res.* **1972**, *32*, 1947.
(68) Hawthorne, M. F.; Wiersema, R. J.; Tagasuki, M. *J. Med. Chem.* **1972**, *15*, 449.
(69) Gold, P.; Freedman, S. O. *J. Exp. Med.* **1965**, *122*, 462.
(70) Goldenberg, D. M.; Sharkey, R. M.; Primus, F. J.; Mizusawa, E.; Hawthorne, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 560.
(71) Kawamura, A. In *Fluorescent Antibody Techniques and Their Applications*; University of Tokyo Pess: Tokyo, 1969; Chapter 3.
(72) Beatty, B. G.; Paxton, R. J.; Hawthorne, M. F.; Williams, L. E.; Rickard-Dickson, K. J.; Do, T.; Shively, J. E.; Beatty, J. D. *J. Nucl. Med.* **1993**, *34*, 1294.
(73) Hawthorne, M. F. *Pure Appl. Chem.* **1991**, *63*, 327.
(74) Varadarajan, A.; Hawthorne, M. F. *Bioconjugate Chem.* **1991**, *2*, 242.
(75) Leukart, O.; Caviezel, M.; Eberle, A.; Escher, E.; Tun-Kyi. A.; Schwyzer, R.; *Helv. Chim. Acta* **1986**, *59*, 2184.
(76) Kane, R. R.; Drechsel, K.; Hawthorne, M. F. *J. Am. Chem. Soc.* **1993**, *115*, 8853.
(77) Gait, M. J., Ed. *Oligonucleotide Synthesis: A Practical Approach*; IRL, Ltd.: Oxford, 1984.
(78) Guan, L.; Wims, L. A.; Kane, R. R.; Smuckler, M. B.; Morrison, S. L.; Hawthorne, M. F. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13206.
(79) Pak, R. H.; Primus, F. J.; Rickard-Dickson, K. J.; Ling Ng, L.; Kane, R. R.; Hawthorne, M. F. *Proc. Natl. Acad. U.S.A.* **1995**, *92*, 6986.
(80) Liu, L.; Barth, R. F.; Adams, D. M.; Soloway, A. H.; Reisfeld, R. A. *J. Hematother.* **1995**, *4*, 477.
(81) Barth, R. F.; Adams, D. M.; Soloway, A. H.; Alam, F.; Darby, M. V. *Bioconjugate Chem.* **1994**, *5*, 58.

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