was treated with a saturated solution of $KMnO_4$ until a pink coloration persisted for 3–5 min. The mixture was adjusted to pH with $NaHCO_3$ to coagulate the MnO_2. The mixture was then filtered and the filtrate was adjusted to pH 5 with concentrated HCl. The solution was evaporated to a volume of 25 mL (32 °C (15 mm)) and adjusted to pH 1.5 with concentrated HCl. The sulfone was extracted with ether, dried, dried over anhydrous $MgSO_4$, and evaporated to an oil, which was solidified by stirring with Skellysolve B. The solid was then dissolved in 15 mL of acetone and treated with solid potassium 2-ethylhexanoate. The white crystals were collected and weighed 142 mg after air-drying: mp >140 °C slow dec; IR 2960 (s), 1795 (s), 1615 (s), 1310 (m), 1140 (s) cm^{-1}; ^1H NMR (D_2O) δ 5.05 (m, 1), 4.4 (s, 1), 3.9–4.3 (m, 2), 3.2–4.0 (m, 2), 3.67 (s, 3). Anal. ($C_8H_9BrKNO_5S$) C, H, N.

Dicesium *N*-Succinimidyl 3-(Undecahydro-*closo*-dodecaboranyldithio)propionate, a Novel Heterobifunctional Boronating Agent

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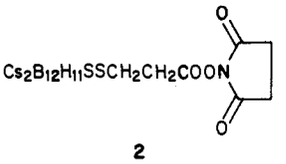
The synthesis of a novel heterobifunctional agent, dicesium *N*-succinimidyl 3-(undecahydro-*closo*-dodecaboranyldithio)propionate, is described. This structure contains an active ester component known to react rapidly under very mild conditions with amino groups of proteins, resulting in covalent linkage. With use of this boronating agent, approximately 480 boron atoms have been incorporated per molecule of a polyclonal antibody directed against human thymocytes and 1300 boron atoms per molecule were incorporated into a monoclonal antibody, 17-1A, directed against human colorectal carcinoma cells. Binding of the boronated antibodies to the corresponding target cells was demonstrated by means of membrane immunofluorescence. There was some loss in reactivity, as determined by fluorescent end point titers, but specificity remained unchanged. The data suggest that boronated antibodies potentially could be used to selectively deliver boron-10 to tumor cells in order to achieve their destruction by neutron capture.

Since Locher[1] first proposed the use of boron-10 for neutron capture therapy (BNCT) in 1936, the key limitation of this therapeutic approach has stemmed from an inability to deliver a sufficient concentration of the neutron absorber specifically and uniformly throughout the tumor.[2] The development of hybridoma technology[3] has offered the possibility of selectively delivering the capture agent to tumors by linking boron-10-containing compounds to monoclonal antibodies directed against tumor associated antigens. Toward this end, a number of protein-binding boron compounds have been synthesized and covalently incorporated into proteins.[4-11] In this study, we have utilized an established protein-linking heterobifunctional reagent,[12] *N*-succinimidyl 3-(2-pyridyldithio)propionate, **1**, as the coupler and have synthesized a polyhedral borane analogue containing an active ester function that would permit covalent incorporation of this boronating agent. The compound synthesized, **2**, is dicesium *N*-succinimidyl 3-(undecahydro-*closo*-dodecaboranyldithio)propionate.[13]



2

Reactions of structure **2** (abbreviated as SBDP) with both monoclonal and polyclonal antibodies were undertaken to evaluate the boronating potential of this agent. The reactions were carried out under very mild conditions, by allowing the reagent to react with the antibody in PBS (phosphate buffered saline, pH 7.2) for 1 h at ambient temperature. Following overnight storage at 4 °C, the boronated antibody was separated from excess reagent and byproducts by sequential passage through Sephadex G-25 columns (Pharmacia Fine Chemicals, Piscataway, NJ). The purified, conjugated antibody was analyzed for boron content by prompt-γ analysis[14] and for protein concentration by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). With use of these data, the number of

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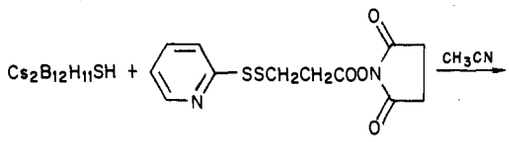
boron atoms incorporated per molecule of antibody was calculated.

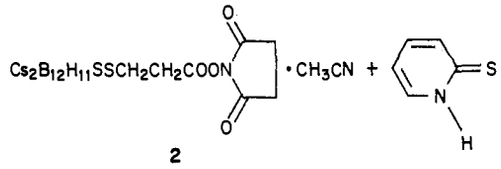
The effect of boronation on the ability of the antibodies to bind to the corresponding target cells was determined by means of membrane immunofluorescence, by comparing the end point titers of the boronated antibody with those of the unboronated antibody.

Results and Discussion

The reaction of dicesium mercaptoundecahydro-*closo*-dodecaborate[15] with an excess of the heterobifunctional agent *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) yields the boronated ester **2**, solvated with 1 mol of acetonitrile. Moisture must be excluded from the reaction system because the ester moiety is susceptible to hydrolysis.



1 (SPDP)

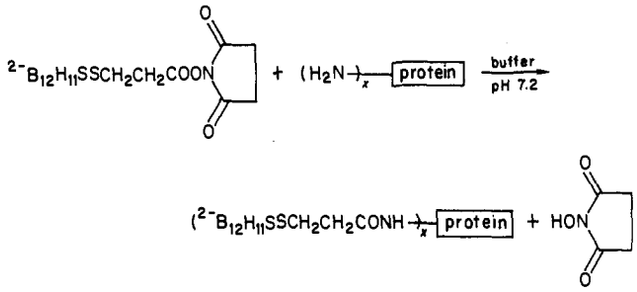


2

TLC of the purified product on DEAE-cellulose (developed with 3 M NH_4NO_3, visualized with 1% aqueous $PdCl_2$ spray) indicates the absence of any unreacted $B_{12}H_{11}SH^{2-}$. The R_f of the product is 0.25, whereas the R_f of $B_{12}H_{11}SH^{2-}$ is 0.34. The product hydrolyzes slowly in water at ambient temperature at pH 7 with a half-life of approximately 3–4 h as observed by TLC. The results are similar to those reported for SPDP.[12] The proton NMR, boron-11 NMR, and IR spectra of the product are consistent with structure **2**. When formic acid is added to a solution of the product in Me_2SO, a deep blue color is formed, providing evidence of a disulfide linkage to the boron cage. The blue color is due to the formation of an unusually stable free radical by homolytic cleavage of the disulfide bond linked to the dodecahydrododecaborate cage.[16]

The boron-10 isotope is potentially the most important nuclide for boron neutron capture therapy because of its high thermal neutron capture cross section, which is 3850 barns. Natural boron contains less than 20% by weight of boron-10 and therefore is less effective as a capture agent than boron-10-enriched material. Boron-10-enriched **2** was prepared with use of 95% boron-10-enriched disodium mercaptoundecahydro-*closo*-dodecaborate,[17] $Na_2{}^{10}B_{12}H_{11}SH$.

The *N*-hydroxysuccinimide ester end group is known to react rapidly with amino groups on proteins under very mild conditions.[12] In aqueous solutions, at neutral or slightly acidic pH, the reaction of the active ester end group is much faster than the hydrolysis reaction.[12] The interaction of **2** with proteins would, therefore, result in their boronation as shown below.



Boronation reactions with this novel reagent, **2**, were successful. Its reaction with a polyclonal antibody directed against human thymocytes (ATGAM, Upjohn, Kalamazoo, MI) at a molar ratio of 200:1 resulted in the incorporation of 4.8×10^2 boron atoms per molecule of ATG. A similar reaction of **2** with the monoclonal antibody 17-1A directed against colorectal carcinoma cells[18] at a molar ratio of 1300:1 resulted in the incorporation of 1.3×10^3 boron atoms per antibody molecule. With use of *p*-[1,2-dicarba-*closo*-[1-^{3}H]dodecaboran(12)-2-yl]benzenediazonium chloride, it has been reported that approximately 30–50 boron atoms were incorporated per molecule of polyclonal antibody directed against carcinoembryonic antigen (CEA).[10,11] In contrast to these results, boronation levels in the range of 480–1300 atoms per molecule of antibody were achieved with **2**. Such numbers are essential if antibodies are to be used for the delivery of a sufficient quantity of boron-10 to sustain a lethal n,α reaction at the cellular level.[19] Since the active ester end group on **2** reacts rapidly with amino groups in aqueous media and hydrolyzes relatively slowly, it has been possible to achieve a high degree of boronation under very mild conditions. There was no observed precipitation of the antibody, even though a large molar excess of the reagent was used.

The membrane immunofluorescence end point titer of the boronated polyclonal antibody, ATG, with human peripheral blood lymphocytes was determined to be 1:600 after correcting for protein concentration, compared to 1:3200 for the native antibody. Similarly, the end point titer of the boronated monoclonal antibody 17-1A with SW1116 colorectal cancer cells was determined to be 1:640, compared to 1:6400 for the unmodified antibody. On a percentage basis, this represented 80% and 90% reductions in activity. This was not surprising in view of the fact that approximately 40 boron cages were conjugated per molecule of ATG and over 100 boron cages were conjugated per molecule of 17-1A. Mizusawa et al.[10] have reported that the linkage of approximately five carborane cages (50 boron atoms) per molecule of anti-CEA antibody did not result in a loss of antibody reactivity. This level of boronation, however, falls far short of what would be required, i.e., 10^9 boron-10 atoms per target cell.[20] Thus, even with tumor cells that have high antigen-site densities (10^6 per target cell), over 1000 boron-10 atoms per antibody molecule would be required to reach this critical number.

A major limitation of using an agent containing a single boron cage for boronation is that a large number of sites on the antibody molecule must be modified in order to attain 10^3 boron atoms. Conversely, the fewer the number

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of sites that are modified by covalent attachment of the boron moiety to the antibody, the greater the probability that antibody avidity and affinity will not be adversely affected. Pursuing this line of reasoning, we have undertaken the synthesis of oligomers or small boron polymers that could be attached covalently to antibodies. In this way, a fewer number of sites on the antibody molecule would need to be modified in order to achieve the boron concentration needed for effective BNCT.

In summary, this new heterobifunctional reagent, **2**, is a convenient and effective boronating agent for antibodies and other proteins. The levels of boron incorporation that are required for BNCT have been achieved by using this reagent under very mild conditions. However, this degree of boronation resulted in some loss of antibody reactivity. For this reason we have undertaken the synthesis of protein-binding boron polymers that can be linked to antibody molecules at a single reactive site.

Experimental Section

The melting point was determined with a Thomas-Hoover apparatus. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and Schwarzkopf Microanalytical Laboratories, Woodside, NY. Infrared spectra were obtained (KBr pellets of samples) on a Beckman IR-4230 spectrometer. Proton NMR spectra of samples were recorded on a Bruker HX90E spectrometer with Me_2SO-d_6 as solvent and internal Me_4Si as reference. Boron-11 NMR spectra of samples were recorded at 96.3 MHz on a Bruker WM-300 spectrometer with Me_2SO-d_6 as solvent and are referenced to external $BF_3{\cdot}OEt_2$. The preparation and purifications of **2** were carried out in an argon atmosphere.

Synthesis of Dicesium *N*-Succinimidyl 3-(2-Undecahydro-*closo*-dodecaboranyldithio)propionate (2). To a stirred solution of 137 mg (0.44 mmol) of SPDP in dry acetonitrile was added 137 mg (0.30 mmol) of dicesium mercaptoundecahydro-*closo*-dodecaborate. The boron compound dissolved slowly. After 1 h, the solution was filtered to remove suspended particles. The solvent of the filtrate was removed under reduced pressure; the resulting solid was washed several times with dry dichloromethane. This product was dried overnight under vacuum to yield 128 mg (63% yield) of a colorless solid; a sample of this solid, when heated in a sealed tube in a Thomas-Hoover apparatus, darkens at 210 °C but does not melt up to 260 °C. Anal. ($Cs_2B_{12}H_{19}S_2C_7NO_4{\cdot}CH_3CN$) C, H, N, B. This compound should be stored below 5 °C in a dessicator.

NMR. The proton NMR of the product in Me_2SO-d_6 exhibits a singlet at δ 2.8 ($OCCH_2CH_2CON$), overlapping multiplets at δ 2.83, partially buried under the singlet at δ 2.80 ppm (SCH_2CH_2COO), and a singlet at δ 2.06 (CH_3CN solvated). For comparison, a proton NMR spectrum of SPDP in Me_2SO-d_6 was also recorded; it exhibits a singlet at δ 2.84 ($OCCH_2CH_2CON$), another singlet δ 3.10 (SCH_2CH_2COO), and several multiplets in the aromatic region for the pyridyl protons: δ 7.12 (1 H), 7.70 (2 H), 8.50 (1 H). The proton NMR of the product exhibits no peaks in the aromatic region, indicating complete loss of the pyridyl group. The broad singlet at δ 3.10 for the four propionate protons in the proton NMR of SPDP is changed to overlapping multiplets centered at δ 2.83 in the proton NMR of the product due to replacement of the pyridyl group of SPDP by the strongly electron-donating boron cage in the product.

The ^{11}B NMR of the product exhibits a singlet (5.9 ppm, 1 B) corresponding to the sulfur-bound boron and three doublets (14.2 ppm, 5 B; 15.2 ppm, 5 B; 17.0 ppm, 1 B), all of which appear as singlets on hydrogen decoupling. This spectrum compares with that of $Na_2B_{12}H_{11}SH$ reported earlier,[16] which exhibits a singlet (10.7 ppm, 1 B) and three doublets (14.8 ppm, 5 B; 17.0 ppm, 5 B; 20.5 ppm, 1 B).

IR. The IR spectrum of the product is also consistent with structure **2**. There is a strong, broad absorption at 2485 cm^{-1} due to B–H stretches and another strong absorption at 1728 cm^{-1} with a shoulder at 1700 cm^{-1} due to carbonyl stretches. In the fingerprint region a strong absorption at 1214 cm^{-1} and three overlapping peaks at 1083, 1065, and 1051 cm^{-1} are similar to those found in the IR spectrum of SPDP. The most significant differences in the IR spectrum of the product from the IR spectrum of SPDP are the appearance of a strong absorbance at 2485 cm^{-1} due to B–H stretch and disappearance of the peak at 1570 cm^{-1} due to loss of the pyridyl group.

Preparation of Boron-10-Enriched 2. In preparing the isotopically enriched **2**, the procedure described above for the synthesis of **2** was modified to increase the yield. A sample of 95% boron-10-enriched disodium mercaptoundecahydro-*closo*-dodecaborate (kindly supplied by Dr. H. Hatanaka, Department of Neurosurgery, Teikyo University, Tokyo, Japan) is converted to the cesium salt by precipitation with cesium chloride from aqueous solution. The product was recrystallized from hot water and dried under vacuum over P_4O_{10}. To 110 mg (0.24 mmol) of the cesium salt was added an excess of **1** (100 mg, 0.32 mmol) in dry actonitrile. The mixture is allowed to stir overnight at ambient temperature. TLC of the reaction mixture at this time indicates complete reaction of the mercaptododecaborate anion. The product is precipitated with dry dichloromethane, filtered, and washed several times with dry dichloromethane to yield 139 mg (90%) of the desired product, 95% boron-10-enriched **2**.

Boronation of Antithymocyte Globulin (ATG) with 2. To 10 mg (1.0 mL) of ATG (ATGAM), kindly provided by the Upjohn Co., Kalamazoo, MI, in PBS buffer, was added slowly a solution of 8 mg of **2** in 0.5 mL PBS over a period of several minutes (molar ratio 200:1). The mixture was gently stirred for 1 h at ambient temperatures and then stored at 4 °C overnight. Subsequently, the reaction mixture was passed through a Sephadex PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ) and the protein collected in a 2.5-mL void volume. This protein fraction was concentrated to 0.5 mL on a Minicon (Amicon Corp., Danvers, MA) and fractionated on a Sephadex G-25 column (0.8 cm × 20 cm). The protein fraction was then collected in the first 2.0-mL void volume. This purified fraction was analyzed for boron and protein content. From calibration experiments, the standard deviation for protein determination by Bio-Rad protein assay was found to be less than ±2%. The standard deviation for boron determination by prompt-γ method[14] at the High Flux Beam Reactor (HFBR) of the Brookhaven National Laboratory (BNL) was determined to be ±0.2 μg of ^{10}B (personal communication from Dr. Ralph G. Fairchild at BNL). Since our boronated antibodies contained more than 10 μg of ^{10}B in each analyzed sample, the standard deviation becomes less than ±2%.

As a blank, a solution of 10 mg of tritium-labeled $Na_2B_{12}H_{12}$ was fractionated on Sephadex gel filtration columns in the same manner. No significant quantity of the polyhedral borane was present in the protein fraction after the two gel filtration steps.

Boronation of Monoclonal Antibody 17-1A with Boron-10-Enriched 2. Monoclonal antibody 17-1A generously provided by Dr. Z. Steplewski, The Wistar Institute, Philadelphia, PA, was boronated with 95% boron-10-enriched **2** by the procedure described above. A much larger molar ratio (1300:1) of the reagent to the antibody was used, however.

Determination of Boron Content. The boron content of the boronated antibodies were determined by the prompt-γ method[14] by Dr. Ralph Fairchild at Brookhaven National Laboratories. In a nuclear reactor, 1.0 mL of the boronated antibody solution in a boron-free glass tube was subjected to a thermal neutron flux of 3×10^7 $n{\cdot}cm^{-2}{\cdot}s^{-1}$ for 200 s. The nuclear reaction of ^{10}B with thermal neutrons results in the prompt emission of a 480 keV γ-ray. The boron content was determined from the measurement of the 480-keV peak area in the γ-ray spectrum. For calibration, NBS standard boron-10 enriched boric acid was used with the appropriate buffer as the blank.

Determination of the in Vitro Binding of Boronated Antibody by Membrane Immunofluorescence. Serial twofold dilutions of the nonboronated and boronated antibodies were prepared by adding 50 μL of antibody to 50-μL aliquots of PBS. Human peripheral blood lymphocytes were separated out from whole blood by means of Ficoll-hypaque density gradient centrifugation,[21] and SW1116 colon cancer cells were disaggregated from monolayer cultures by the addition 1 mmol ethylenedi-

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aminetetraacetic acid. Cells were washed in PBS and resuspended, and 50-μL volumes containing 1.5×10^6 cells were added to each tube. They were incubated for 30 min at ambient temperature, washed two times, and then resuspended in 100-μL volumes of PBS. An equal volume of fluorescein isothiocyanate (FITC) conjugated rabbit anti-horse IgG (Miles-Yeda, Ltd, Israel) at a dilution of 1:30 was added to the lymphocytes or FITC rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA) at a dilution of 1:25 to the SW1116 cells. They were allowed to incubate for an additional 30 min at ambient temperature, washed two times with PBS, and resuspended in a 1:1 mixture of 50% glycerol and PBS. Fluorescence was scored 0–4+ with a Zeiss fluorescence microscope with epiillumination and a halogen light source. End point titers were recorded at the dilution of antibody that gave 0 (i.e., background) fluorescence.

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Synthesis and Evaluation of *N,N'*-Bis(arylsulfonyl)hydrazines as Antineoplastic Agents

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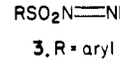
Several *N,N'*-bis(arylsulfonyl)hydrazines, with the potential to function as biological methylating agents, were synthesized and evaluated for antineoplastic activity against the L1210 leukemia and other transplanted rodent tumors. In general, the *N*-methyl-*N,N'*-bis(arylsulfonyl)hydrazines that possess the capacity to generate an alkylating species under physiological conditions showed significant antineoplastic activity, while *N,N'*-bis(phenylsulfonyl)-hydrazine and *N*-methyl-*N,N'*-dibenzoylhydrazine were inactive.

N-Acyl-*N'*-(arylsulfonyl)hydrazines (1) are decomposed by bases to form aldehydes in moderate yields (Scheme I);[1] this reaction often proceeds at high temperatures.[2] Replacement of the acyl group in compound 1 by an arylsulfonyl moiety, to form an *N,N'*-bis(arylsulfonyl)-hydrazine, enhances the acidity of the proton β to the leaving group and a reaction analogous to the one depicted in Scheme I can occur with greater facility. The reaction intermediate in this case would be 3 and the product, an arenesulfinic acid. While species 3 would be expected to

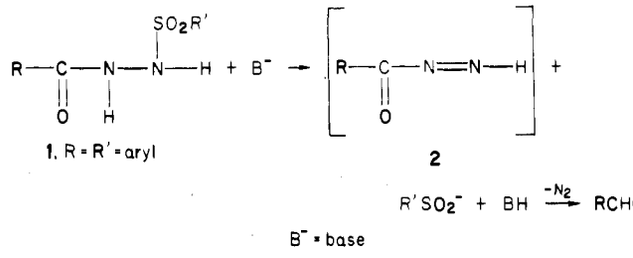


3, R = aryl

lose a molecule of nitrogen readily to give the corresponding arenesulfinic acid, a compound or intermediate such as 5, generated from the precursor molecule 4, would be less prone to such decomposition. Furthermore, since arenesulfinate is a good leaving group, compound 4 may function as an alkylating agent in a manner analogous to the *N*-alkyl-*N*-nitrosoureas, which generate as biological alkylating agents alkanediazohydroxides[3] (Scheme II).

Methylating agents form a useful group of antineoplastic agents, with procarbazine, streptozotocin, and dacarbazine being clinically active methylating agents. To exploit

Scheme I



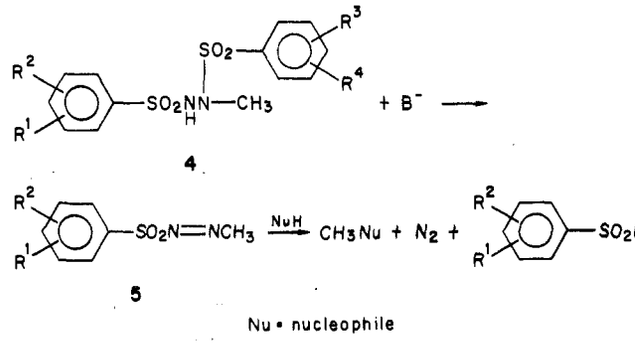
1, R = R' = aryl

2

$R'SO_2^- + BH \xrightarrow{-N_2} RCHO$

B^- = base

Scheme II



4

5

Nu = nucleophile

further the impact of methylation in cancer chemotherapy, we have synthesized a new class of potential methylating agents and have tested them for antineoplastic activity against the L1210 leukemia and other transplanted rodent tumors.

Chemistry. Bis(arylsulfonyl)hydrazines (6–11) were prepared by reacting the appropriate arenesulfonyl chloride with hydrazine or methylhydrazine in a 2:1 molar ratio

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