Synthesis and Evaluation of Novel Boron-Containing Complexes of Potential Use for the Selective Treatment of Malignant Melanoma

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Boron-containing complexes that have the potential to irreversibly accumulate in melanoma cells have been prepared by reaction of amino acids with 9-methoxy-9-borabicyclo[3.3.1]nonane. The ability of each complex to act as a substrate for tyrosinase has been probed by oximetry. Increased uptake of the lead candidate in a tyrosinase-rich cell line, compared with a tyrosinase-absent cell line, is reported, with results correlating well with that for a drug currently approved for BNCT.

Boron neutron capture therapy (BNCT)[a] is a two-step radiochemotherapy approach of use for treating cancer.[1] In the first step, a tumor-targeting compound containing a nonradioactive ^{10}B isotope is accumulated in the tumor cells. In the second step, the boron atoms are irradiated with a beam of low energy neutrons to produce high energy α-particles and lithium-7 nuclei. The particles produced are highly cytotoxic and have a combined path length of approximately the diameter of a cell. For BNCT to afford selective treatment, it is essential that targeted delivery of boron is achieved; this has been realized using strategies that involve synthetic chemical approaches as well as biochemical and biophysical approaches.[2] Recently, attention has focused on the use of *p*-boronophenylalanine (BPA) for the treatment of malignant melanoma;[3−6] it was reasoned that, as phenylalanine is an indirect precursor of melanin, its uptake by melanin producing cells should be increased compared with healthy cells, resulting in selective accumulation in melanoma cells. Animal models have illustrated that BPA does indeed concentrate in melanoma tumors, however, accumulation is only temporary. The work described herein aims to widen the utility of BNCT for the treatment of melanoma by utilizing boron-containing therapeutics that can potentially be irreversibly located within the melanoma cells.

When melanocytes become malignant, the genes expressing tyrosinase become up-regulated, resulting in a marked increase in the levels of tyrosinase within the cancerous cells. In addition, the receptors for L-tyrosine are increased.[7] In this work, we aim to exploit these specific properties of malignant melanoma cells in order to both selectively deliver and biologically process boron complexes of L-tyrosine and related amino acids. It is hypothesized that this could result in the synthesis of boron-containing melanin derivatives, thus allowing irreversible location and permanent accumulation of the boron reagents within the cells (Schemes 1 and 2). We have previously investigated tyrosinase mediated prodrug strategies for the selective treatment of melanoma and have illustrated that a range of sterically bulky prodrugs can be biochemically manipulated by tyrosinase.[8] In this work, we have extended this study to synthesize and analyze amino acid−9BBN complexes of use as precursors to boron-containing melanin derivatives. Initial work sought to investigate whether amino acids such as L-tyrosine and L-dopa, which are precursors to melanin and natural substrates of the tyrosinase enzyme, could be chemically manipulated to afford boron-containing targets **1** and **2**. 9-BBN derivative **3** was also considered of interest because the indolic structure is contained within precursors of melanin, and hence complex **3** may also be processed and incorporated within the tumors. Complex **4** was selected for synthesis mainly due to the fact that the structurally related BPA has already proved valuable as a successful drug for melanoma treatment.[9]

Initial attempts to form the complexes involved treating the amino acids with one equivalent of 9-methoxy-9-borabicyclo[3.3.1]nonane (9-OMe-BBN) in DCM for 24 h.[10,11] However, no products were formed, probably due to the insolubility of the amino acids in DCM. After several attempts of performing the reactions at elevated temperatures in different solvents, namely THF, DMF, toluene, and xylene, the reaction was conveniently carried out in pyridine in a closed reactor at 100 °C for 24 h. This afforded complexes **1**, **2**, **3**, and **4** in >90% yields after crystallization from hot MeCN. The synthesis was also carried out successfully using a microwave reactor, where the reaction was complete in 15 min and the products were obtained in >90% yields after crystallization from hot MeCN, as before. The complexes were characterized by ^{1}H and ^{13}C NMR spectroscopic analysis and, for complex **4**, by single-crystal X-ray analysis.

For the complexes to irreversibly accumulate in the melanoma cells, by the mechanism illustrated in Scheme 1, they must be substrates for tyrosinase. When tyrosinase substrates are oxidized, molecular oxygen is absorbed from the surrounding solution. The resulting oxygen depletion can be measured using an oxygen sensor, thereby oxygen uptake is a measure of the rate of tyrosinase oxidation of the prodrugs. Using this technique, we were able to examine the oxidation of the complexes by tyrosinase (EC 1.14.18.1) (Figure 1). In addition, bioprocessing of L-tyrosine and L-dopa were examined for comparative purposes. Complexes **1** and **2** were found to be

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[a] Abbreviations: 9-BBN, 9-borabicyclo[3.3.1]nonane; BNCT, boron neutron capture therapy; BPA, *p*-boronophenylalanine; BPA-F, *p*-boronophenylalanine-fructose complex.

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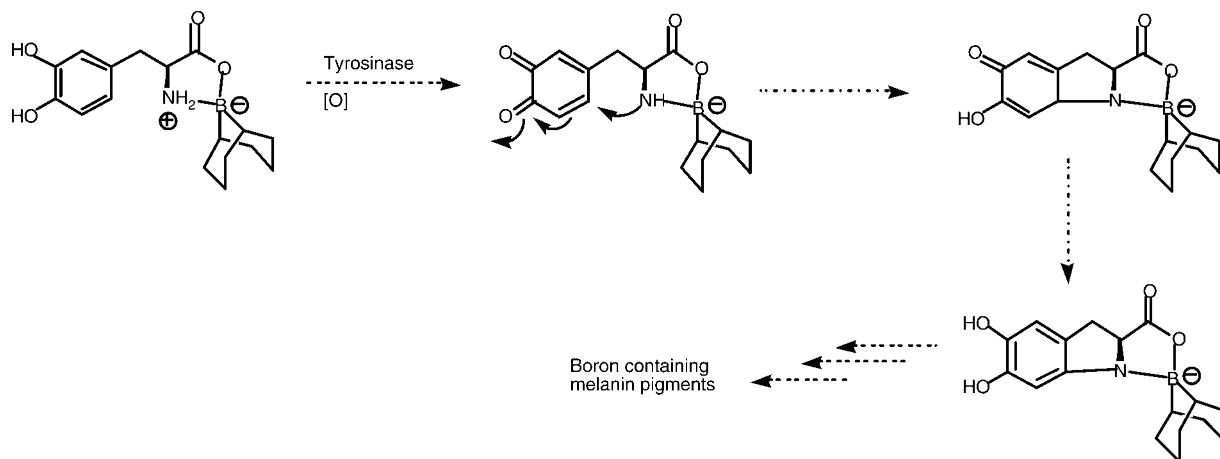
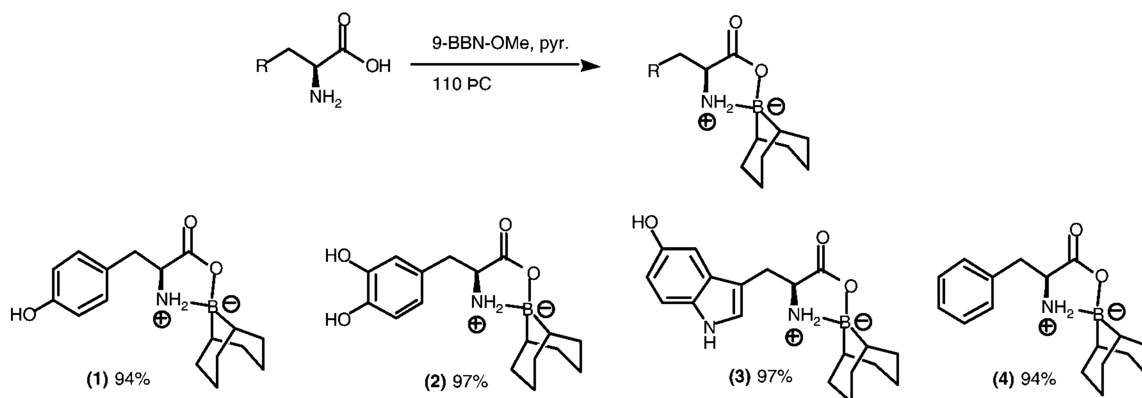
Scheme 1. Tyrosinase Mediated Oxidation of Amino Acid−9-BBN Complexes



Scheme 2. Synthesis of Amino Acid−9BBN Complexes



substrates for tyrosinase and were oxidized at similar rates to the natural L-tyrosine and L-dopa substrates. Importantly, it therefore appears that any steric hindrance conferred by the 9-BBN framework does not detrimentally affect the enzyme processing mechanism. Oximetry suggested that neither complex **3** nor **4** were substrates for tyrosinase and were therefore

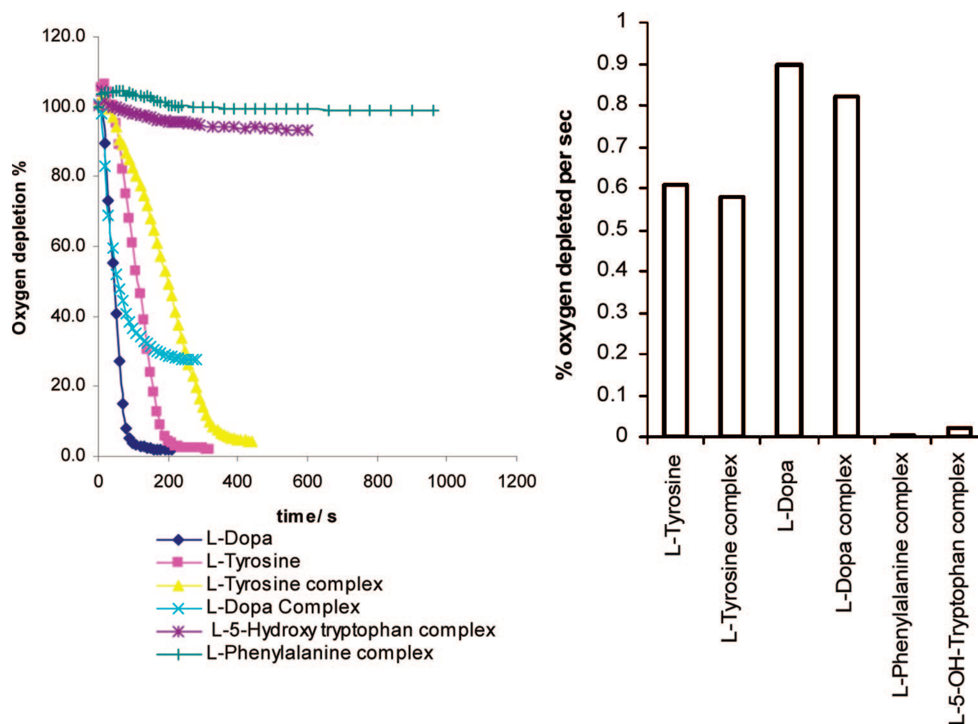


Figure 1. Tyrosinase mediated oxidation of complexes **1**−**4**, L-tyrosine, and L-dopa.

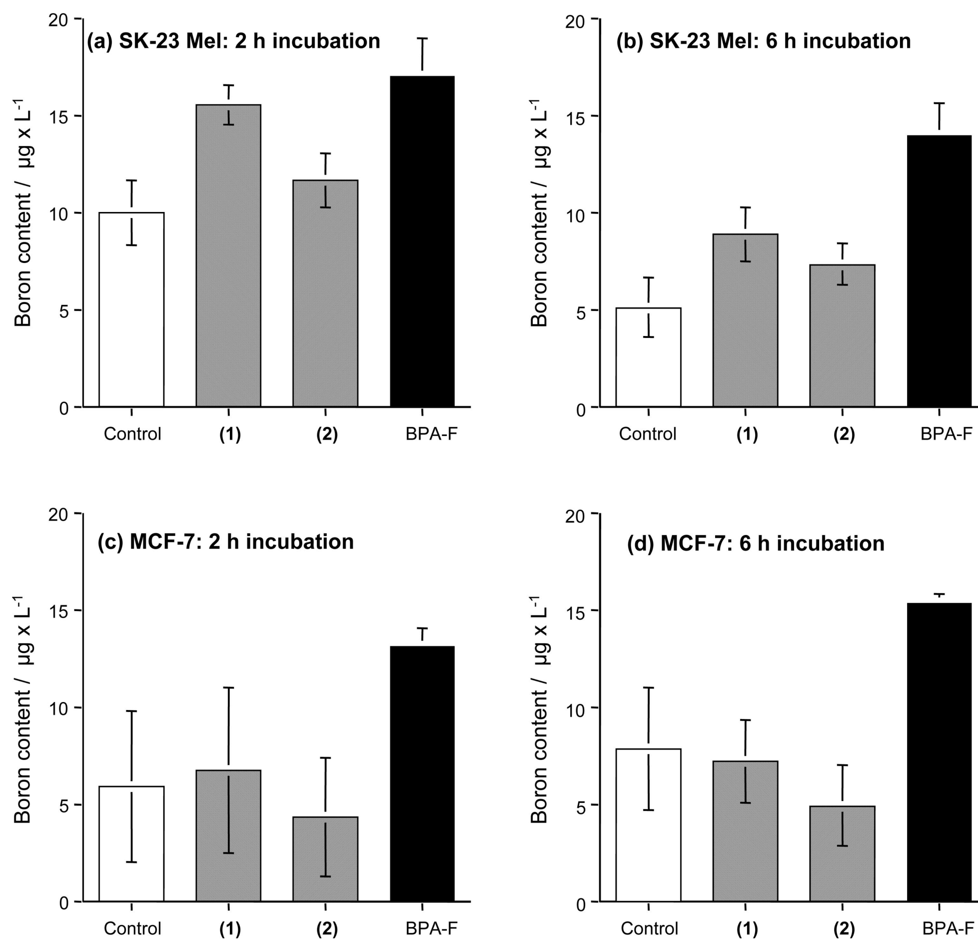


Figure 2. Uptake of **1**, **2**, and BPA-F by SK-23 Mel (a, b) and MCF-7 cells (c, d). Boron content was measured after 2 h (a, c) or 6 h (b, d) incubation with the compounds. Data represent mean ± SEM; $n \geq 6$.

unlikely to be bioprocessed by tyrosinase to allow irreversible accumulation of boron within tyrosinase-rich cells.

As a result of the oximetry studies, it is proposed that complexes **1** and **2** could be processed by tyrosinase to afford boron-containing melanin analogues. To prove that reaction with tyrosinase led to accumulation in tyrosinase-containing cells, uptake studies of **1** and **2** were carried out in SK-23 Mel cells (a melanoma cell line expressing high levels of tyrosinase) and in MCF-7 cells (a breast cancer cell line, in which tyrosinase is absent). Pleasingly, one of the two candidates, **1**, accumulated in SK-23 Mel cells after only 2 h incubation (approximately 50% increase in boron content compared to the control, see Figure 2a). In addition, the boron content also remained high after the 6 h incubation (boron content >50% compared to control, see Figure 2b). Unfortunately, compound **2** did not achieve a similar extent of internalization (Figure 2a, b). It is important to note that although compound **1** did not achieve the same degree of accumulation as BPA-F, the clinical candidate, its selectivity toward tyrosinase-rich cells was much more marked. Indeed, while BPA-F displayed a similar uptake level in SK-23 Mel and in MCF-7 cells (approximately 100% increased uptake compared to the control in all cell lines, Figure 2a−d), **1** selectively accumulated only in SK-23 Mel cells and indeed no uptake was observed in MCF-7 cells for this compound (Figure 2c, d). This therefore supports our hypothesis that substrates of tyrosinase are good candidates for selective delivery and uptake of boron to tyrosinase-rich melanoma cells.

For BNCT to be effective, however, the boron-containing complexes must also have no cytotoxicity or therapeutic activity

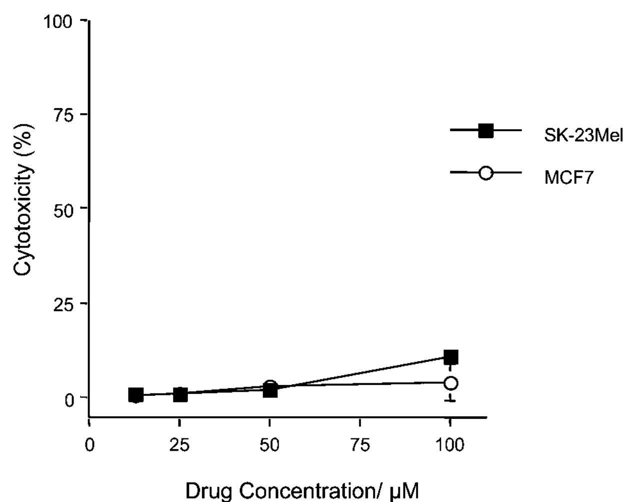


Figure 3. Cytotoxicity of compound **1** against MCF-7 and SK-23 Mel cells. Data represent mean ± SEM ($n = 3$) when not visible, error bars are hidden by the symbol.

until irradiation is performed. Compound **1** was therefore examined further to probe its cytotoxicity in cell lines ahead of irradiation. Thus, the cytotoxicity of compound **1** was evaluated in SK-23 Mel and MCF-7 cells and, pleasingly, very little toxicity (<5% cell death) was evident in both cell lines at all concentrations tested (up to 100 μM) (Figure 3).

To conclude, compound **1** selectively accumulated within melanoma cells, which we hypothesize is based on its ability

to act as a substrate for tyrosinase. It was also observed that, between 2 and 6 h, there was no leakage of the complex from the melanoma cells, which would have resulted in a loss in concentration of the compound in the melanoma cells. The lack of toxicity observed for complex **1**, in the cell lines studied, shows that **1** can be regarded as a promising lead compound for further studies. Future work to synthesize the ^{10}B-enriched derivatives is now being planned to further probe the therapeutic potential of this novel class of BNCT agents.

Experimental Section

Chemistry: General Methods for Synthesis of Complexes. a. Microwave Method. A mixture of 9-methoxy-9-borabicyclo [3.3.1]nonane (1 equiv) and the amino acid (1 equiv) in dry pyridine (6 mL) was placed in a glass tube with a stir bar. The mixture was irradiated using a microwave reactor, at 140 °C, for 15 min. Removal of the solvent in vacuo afforded a thick colorless oil, which was flashed through a short pad of silica (ethyl acetate). The filtrate was concentrated in vacuo and the residue crystallized from hot acetonitrile to afford the complexes.

b. Sealed Tube Method. A mixture of 9-methoxy-9-borabicyclo[3.3.1]nonane (1 equiv) and the amino acid (1 equiv) in dry pyridine under an argon atmosphere was stirred at 100 °C for 24 h in a sealed tube. Removal of the solvent in vacuo afforded a thick colorless oil, which was flashed through a short pad of silica (ethyl acetate). The filtrate was concentrated in vacuo and the residue crystallized from hot acetonitrile to afford the complexes.

The chemical and physical properties of complexes **1**−**4** are reported in the Supporting Information. Data for **1** (fine white crystals) mp 112−116 °C; R_f 0.65 (100% acetone); $[\alpha]_D^{20}$ −45.4, (*c* 1, acetone). δ_H (250 MHz; DMSO-d_6) 9.25 (1H, br s, O$\underline{H}$), 7.15 (2H, d, *J* 9.0, Ar$\underline{H}$), 6.69 (2H, d, *J* 9.0, Ar$\underline{H}$), 6.45 (1H, t, *J* 12.0, one of N$\underline{H}_2$), 5.55 (1H, t, *J* 12.0, one of N$\underline{H}_2$), 3.74−3.80 (1H, m, C$\underline{H}$), 3.07 (1H, dd, *J* 5.0, 15.0, one of C$\underline{H}_2$), 2.85 (1H, dd, *J* 5.0, 15.0, one of C$\underline{H}_2$), 1.06−1.78 (12H, m, 6× BC$\underline{H}_2$), 0.46 (1H, br s, BC$\underline{H}$), 0.37 (1H, br s, BC$\underline{H}$). δ_C (63 MHz; DMSO-d_6) 173.5 ($\underline{C}$=O), 156.4 (Ar$\underline{C}$OH), 130.6 (2× Ar$\underline{C}$H), 127.5 (Ar$\underline{C}$CH$_2$), 115.5 (2× Ar$\underline{C}$H), 56.4 ($\underline{C}$H), 35.6 ($\underline{C}$H$_2$), 31.6 (6× B$\underline{C}$H$_2$), 27.0 (2× B$\underline{C}$H). V_{max}/cm^{-1} 3250, 2920, 1707, 1656. *m/z* (CI) 302 (M + H, 100%), $[C_{17}H_{24}BNO_3 + H]^+$ requires 302.1928, found 302.1932. Anal. ($C_{17}H_{24}BNO_3 \cdot 0.75H_2O$) C, H, N: calcd. for C (64.88%), H (8.17%), N (4.45%); found C (65.12%), H (8.11%), N (4.10%).

Purity data is available in the Supporting Information for complex **1**: Anal. ($C_{17}H_{24}BNO_3 \cdot 0.75H_2O$) C, H, N; for complex **2**: Anal. ($C_{17}H_{24}BNO_4$) C, H, N; and for complex **3**: Anal. ($C_{19}H_{25}BN_2O_3 \cdot 0.4H_2O$) C, H, N.

Oximetry Studies. To a vigorously stirred solution of mushroom tyrosinase (2.2 mL, 300 units, Sigma mushroom tyrosinase 2060 units/mg) in phosphate buffer (pH 7.2) was added 100 μL of a 10 mM solution of the compound under investigation. Oxygen uptake was monitored by means of a YSI 5300 biological oxygen monitor. Experiments were carried out at 37 °C in triplicate.

Biological Studies. Biological experiments were carried out on SK-23 Mel, a melanoma cell line with high levels of tyrosinase, and on MCF-7, a breast cancer cell line not expressing tyrosinase.[9] The latter constituted the negative control. MCF-7 and SK-23 Mel were cultured in standard tissue culture conditions (37 °C and 5% CO_2). RPMI 1640 supplemented with 5% of fetal bovine serum and DMEM supplemented with 10% of fetal bovine serum were used as medium for MCF-7 and SK-23 Mel, respectively.

Cytotoxicity. SK-23 Mel and MCF-7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and allowed to adhere for 24 h. Then the medium was removed and new medium containing the appropriate concentration (12.5−100 μM) of either compound **1** or **2** (100 μL per well) was added. Cells were incubated with the compounds for 6 h. After incubation, the medium was removed and each well washed three times with fresh medium. The plate was placed in a centrifuge for 5 min at 1000 rpm, and 100 μL from each well was transferred to a clean plate, keeping the same well order. The color reagent was made up according to the standard procedure for a colorimetric cytotoxicity assay, used to measure lactate dehydrogenase (LDH). This freshly prepared color reagent (100 μL) was added quickly to each well, and the plate was incubated at room temperature and protected from light until a purple color developed (10−60 min). The reaction was stopped by adding 1N HCl (50 μL) to each well, and the plate was then read on a microtiter plate reader at a wavelength of 490−492 nm.

Uptake Studies and Quantification of Boron Content. SK-23 Mel and MCF-7 cells were harvested, seeded in 92 mm Petri dishes at a density of 2.5×10^6 cells/dish, and allowed to adhere for 48 h. Then the old medium was removed and replaced with fresh medium that contained either compound **1** or **2** or BPA-F at a concentration of 50 μM, and the cells were incubated for either 2 or 6 h. After this interval, the medium was removed and cells were washed three times with PBS (2 mL × 3). After the last wash, 3 mL of purite water was added and the cells were scraped using a cell scraper and collected in falcon tubes. Another 2 mL of purite water was added to each Petri dish to ensure collection of all material and added to the falcon tube. The suspension was filtered using a 0.2 mm filter and the filtrate analyzed by ICP-OES.

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Supporting Information Available: Purity data and spectroscopic data for complexes **1**−**4**, X-ray crystallographic data for complex **4**, and general information on instrumentation is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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