NORTHERN ILLINOIS UNIVERSITY

The Synthesis and Biological Evaluation of Fluorescein-Tagged
Carborane for Boron Neutron Capture Therapy

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1. Abstract

Boron Neutron Capture Therapy (BNCT) is a bimodal cancer treatment that involves killing cancer cells through a nuclear reaction of two nontoxic species, boron-10 (^{10}B) and thermal neutrons. There are currently only two FDA-approved drugs for this promising area of cancer therapy, so present research focuses on the synthesis of novel compounds with high boron content as well as methods for their delivery into the tumor cell. A fluorescent tag can allow the observation of the delivery vehicle's uptake during biodistribution studies. Accordingly, fluorescein was conjugated with iodinated 1-methyl-*o*-carborane and characterized using Fourier-transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR), elemental analysis, and mass spectrometry (MS). The biological potential of the fluorescein-tagged 1-methyl-*o*-carborane conjugate was then evaluated using flow cytometry, cytotoxicity, and biological distribution studies with squamous cell carcinoma (SCC-VII) and pancreatic cancer (MIA PaCa-2) cell lines using phase-contrast, fluorescent, and confocal microscopy.

2. Introduction

Boron Neutron Capture Therapy (BNCT) is a binary method of cancer treatment in which a drug containing boron-10 (^{10}B) is selectively transported into tumor cells and irradiated with thermal neutrons.[1,2] The larger nuclear cross-section of ^{10}B nuclei allow the capture of a thermal neutron to form an excited ^{11}B nucleus, which undergoes a fission reaction that results in α–particles (^{4}He^{2+}), a ^{7}Li^{3+} ion, and weak gamma radiation (**Figure 1**).[2] The highly charged particles damage the surrounding tissue, but dissipate their kinetic energy over 5-9 µm, or approximately one cell diameter, therefore avoiding damage to the surrounding healthy tissue.[3]
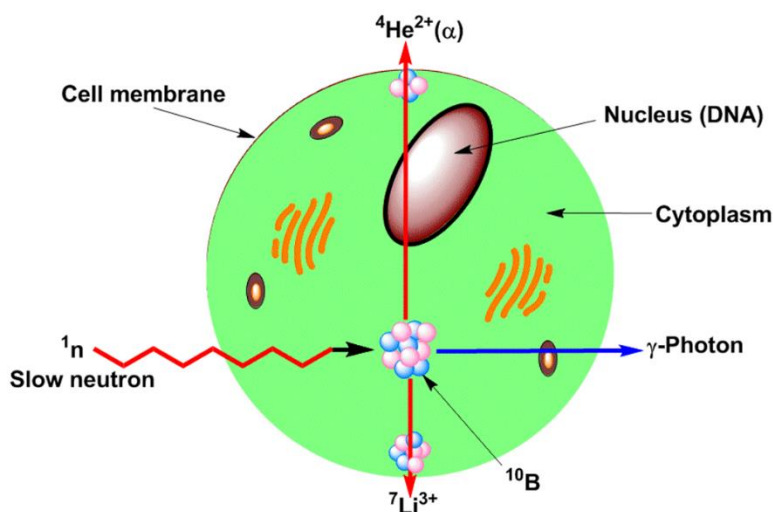

Figure 1: BNCT reaction mechanism.[1]

The lethality of these particles is limited to only boron-containing cells, and therefore the most important requirement of BNCT is the amount and selective accumulation of ^{10}B in the cancer cells with only small amounts entering the surrounding healthy tissue. To meet these requirements, researchers have examined both small- and macro-molecule-based boron delivery agents.[3] Unfortunately, to date there are only two clinically used drugs: sodium mercaptododecaborate (BSH) and boronophenylalanine (BPA), as shown in **Figure 2**.[4] The boron content of BPA is low

and the thiol group in BSH can form a disulfide linkage, which damages DNA, and both exhibit low tumor-to-brain and tumor-to-blood ^{10}B ratios.[1,3]
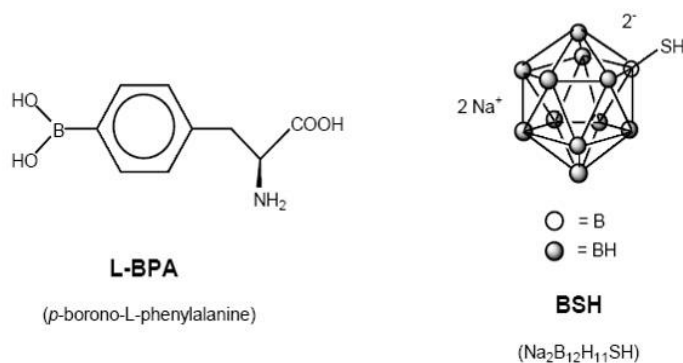
Figure 2: Molecular structure of boronophenylalanine (BPA) and sodium mercaptododecaborate (BSH).[3]

Developing boron delivery agents that address these issues is the focus of current BNCT research. One such method involves the conjugation of a carborane cage with high boron content with biocompatible organic compounds that are regularly used in cellular processes, such as purines, carbohydrates, or imidazoles.[5] Fluorescein (**Figure 3**), the biomolecule studied here, has been applied in a variety of medical applications, such as in the diagnosis of corneal abrasions, ulcers, and infections.[6] Fluorescein angiography (**Figure 3**) is a procedure used for diagnosing retinal vascular tissue disorders and intraocular tumors.[7]
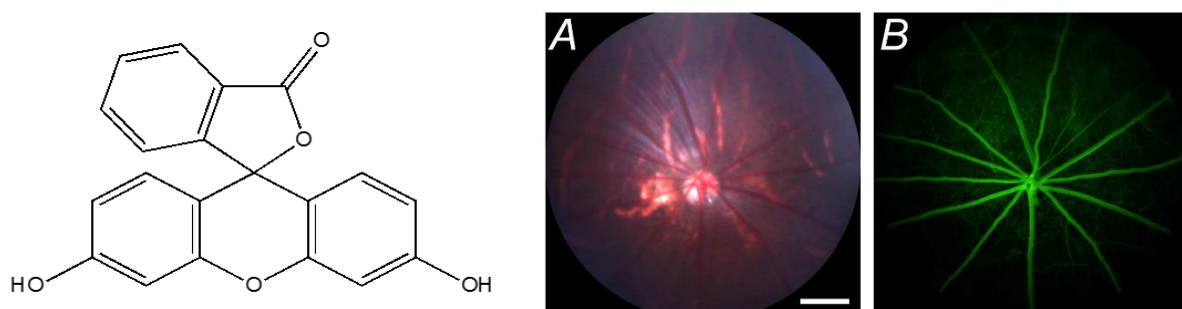

Figure 3: Left -The molecular structure of fluorescein. Right - Fluorescein angiography in rat retina before (a) and after (b) dye administration.[7]

Recently, fluorescein has increasingly been used as tumor marker for brain cancer, which makes it an attractive candidate for BNCT. Here, fluorescein was conjugated with 1-methyl-o-carborane to allow better observation of cellular uptake. 1-methyl-*o*-carborane was first iodinated using 1,4-diiodobutane. The resulting 1-methyl-2-iodobutyl-*o*-carborane was then conjugated with fluorescein through a Williamson ether synthesis reaction.

3. Materials and Methods

The reactions described in this study were generally performed under argon conditions using Schlenk lines. Various laboratory equipment was also required throughout these synthesis procedures and included round bottom flasks (100 mL and 250 mL), magnetic stir bars, hotplates, condenser, syringes, funnel and filtration paper, separatory funnel, TLC paper, capillary tubes, ultraviolet lamp, silica-gel column, rotary evaporator, and distillation apparatus. The required chemicals such as 1-methyl-*o*-carborane, *n*-butyllithium, diethyl ether, ethyl acetate, hexane, 1,4-diiodobutane, fluorescein, sodium hydroxide, and dimethylformamide were used without further purification. Biological evaluation was performed Dr. Sherine Elsawa and Jason Misurelli of the Northern Illinois University Department of Biological Sciences, as well as Dr. Masao Takagaki and Dr. Kazuko Uno of Kyoto University in Japan.

4. Synthesis and Analytical Data of Compounds

Me

1. *n*-Butyllithium
2. 1,4-Diiodobutane

Toluene/Diethyl Ether

Me

1-Methyl-o-carborane

● = C

○ = B-H
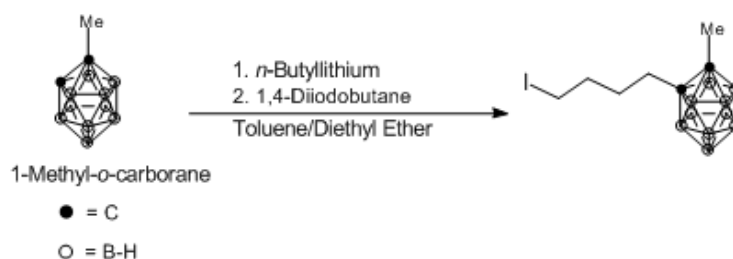
Scheme 1: Synthetic route used to prepare 1-methyl-2-iodobutyl-*o*-carborane.

1-methyl-*o*-carborane (2.018 g, 13.99 mmol) was added to an oven-dried 250 mL three-necked round-bottom flask and dissolved in a 100 mL 2:1 mixture of diethyl ether and toluene. This solution was then cooled to -78 °C using dry-ice before *n*-butyllithium (17.33 mL, 27.73 mmol) was added dropwise *via* syringe. The temperature of the mixture was maintained at -78°C for 30 minutes before being allowed to return to room temperature. After stirring overnight, the reaction mixture was cooled to 0 °C using ice before 1,4-diiodobutane (3.7 mL, 27.75 mmol) was added to the flask *via* syringe and the mixture was stirred for 30 minutes and allowed to warm to room temperature. The reaction was stirred overnight and refluxed for 8 hours the following day. After reflux, an ice bath was used to cool the reaction to 0 °C before the mixture was diluted with deionized water. Extraction was performed with diethyl ether (3 x 50 mL) and deionized water. All the extracts from aqueous layer were combined with organic layer, and the solvents were removed leaving a yellow, sticky residue. The product was purified using thin-layer chromatography (SiO$_2$, developed with a 6:1 mixture of hexane and ethyl acetate) and silica-gel column chromatography before being taken for NMR characterization (^{1}H, ^{11}B, and ^{13}C), as shown in **Figures 4, 5,** and **6**.
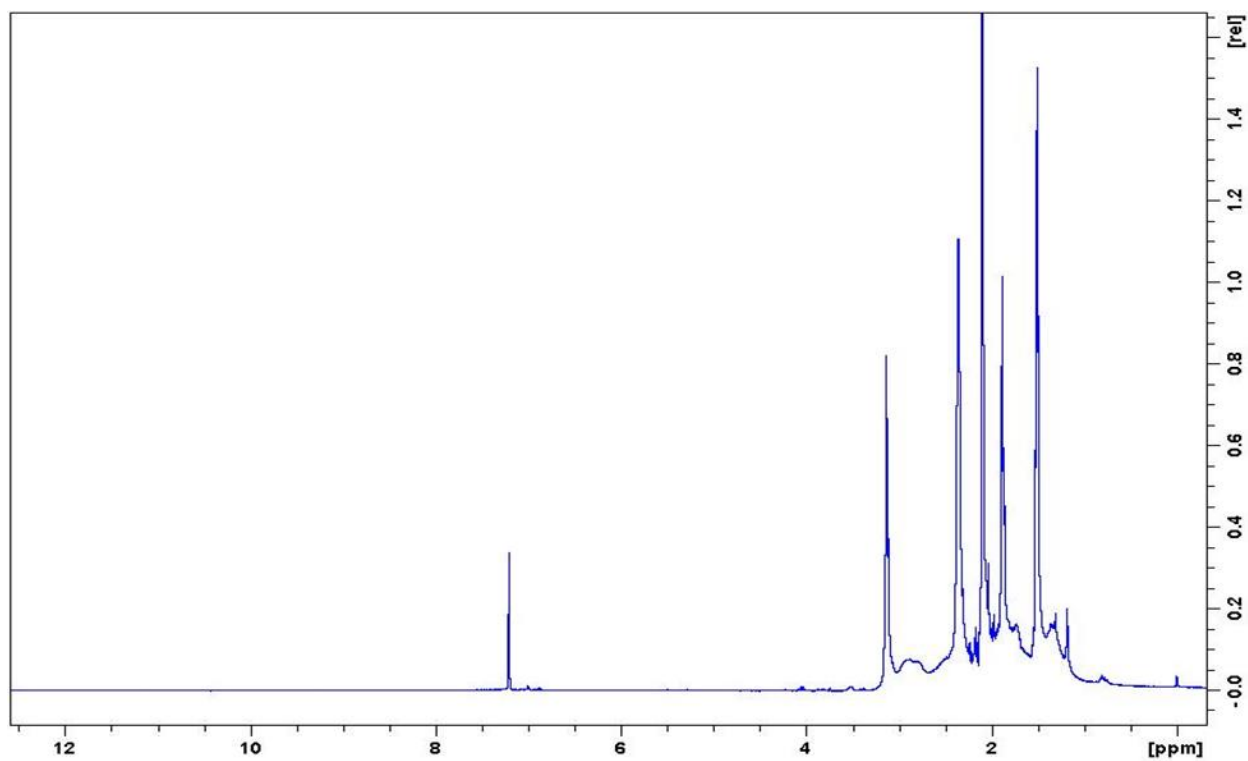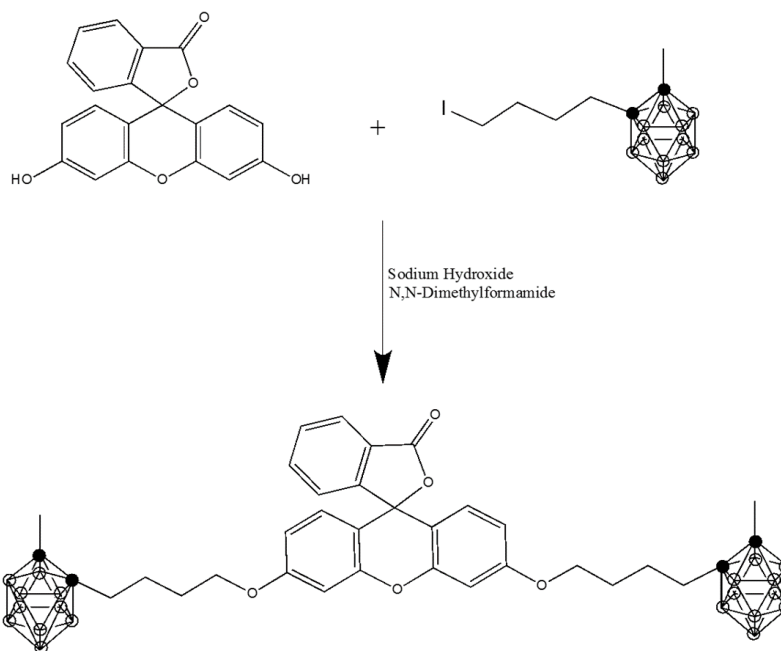
Figure 4: ^{1}H-NMR Spectrum (300 MHz) of 1-methyl-2-iodobutyl-*o*-carborane.
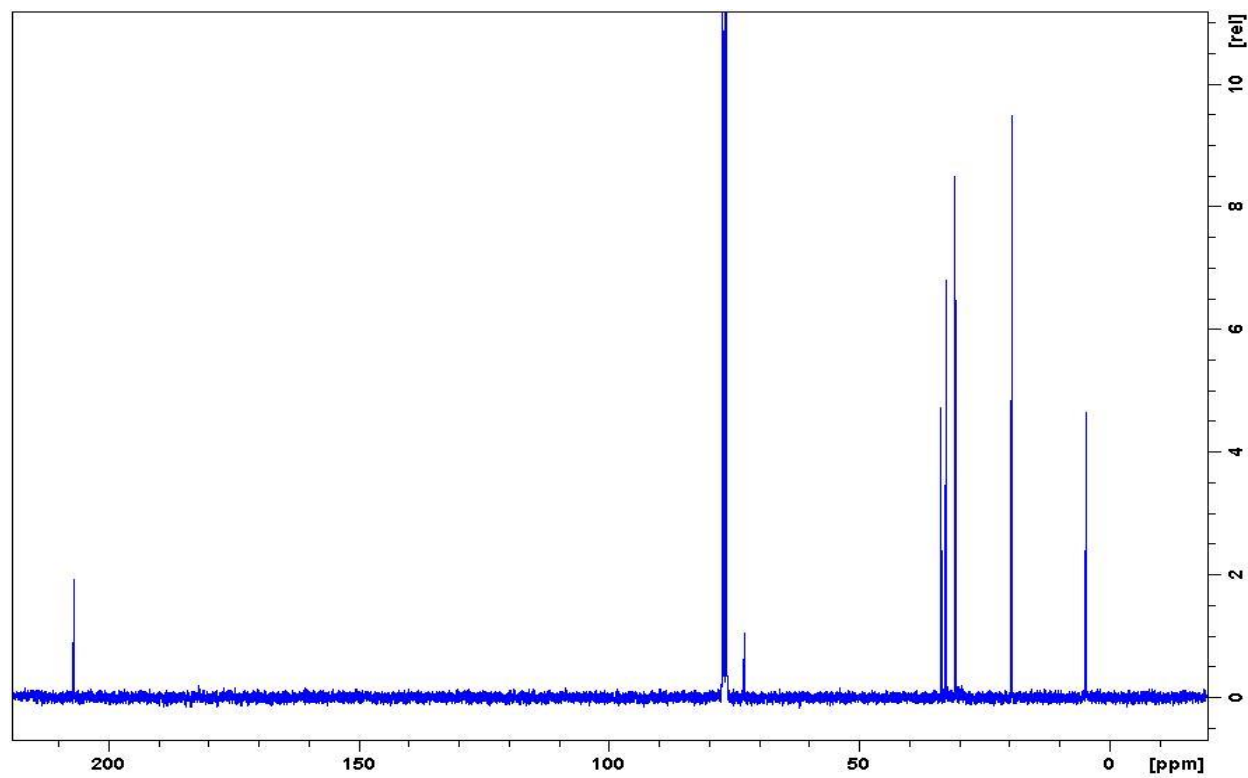
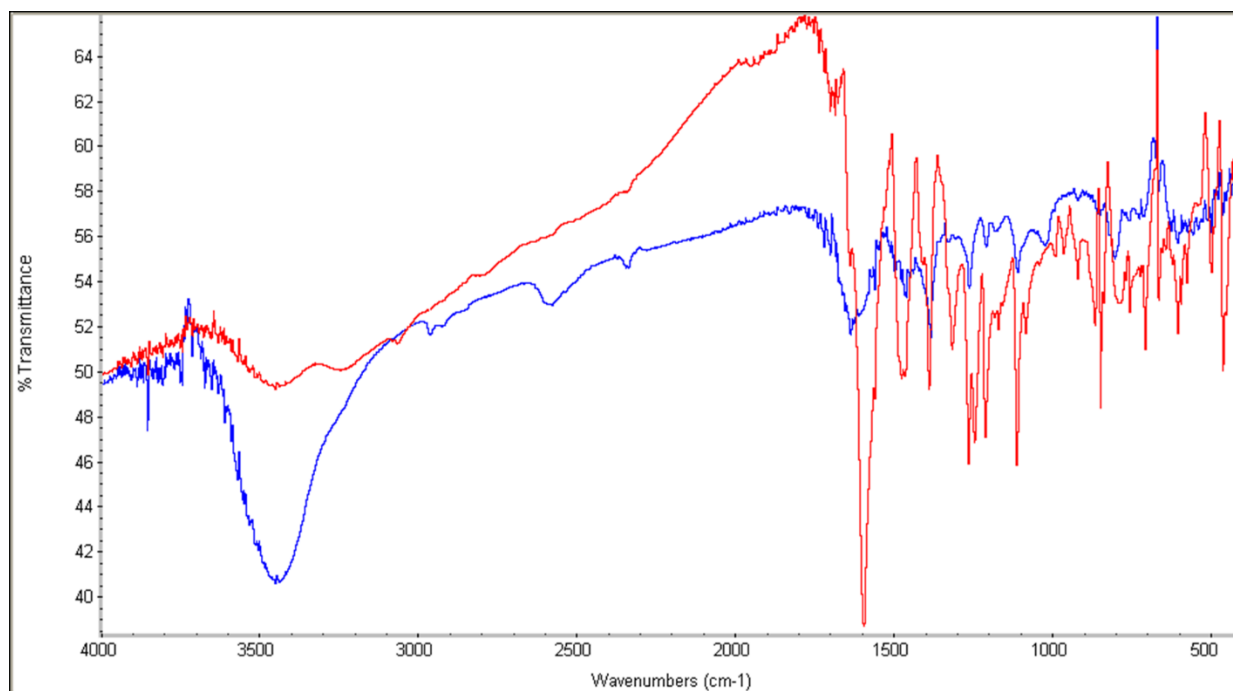**Figure 5**: ^{13}C-NMR Spectrum (300 MHz) of 1-methyl-2-iodobutyl-*o*-carborane.
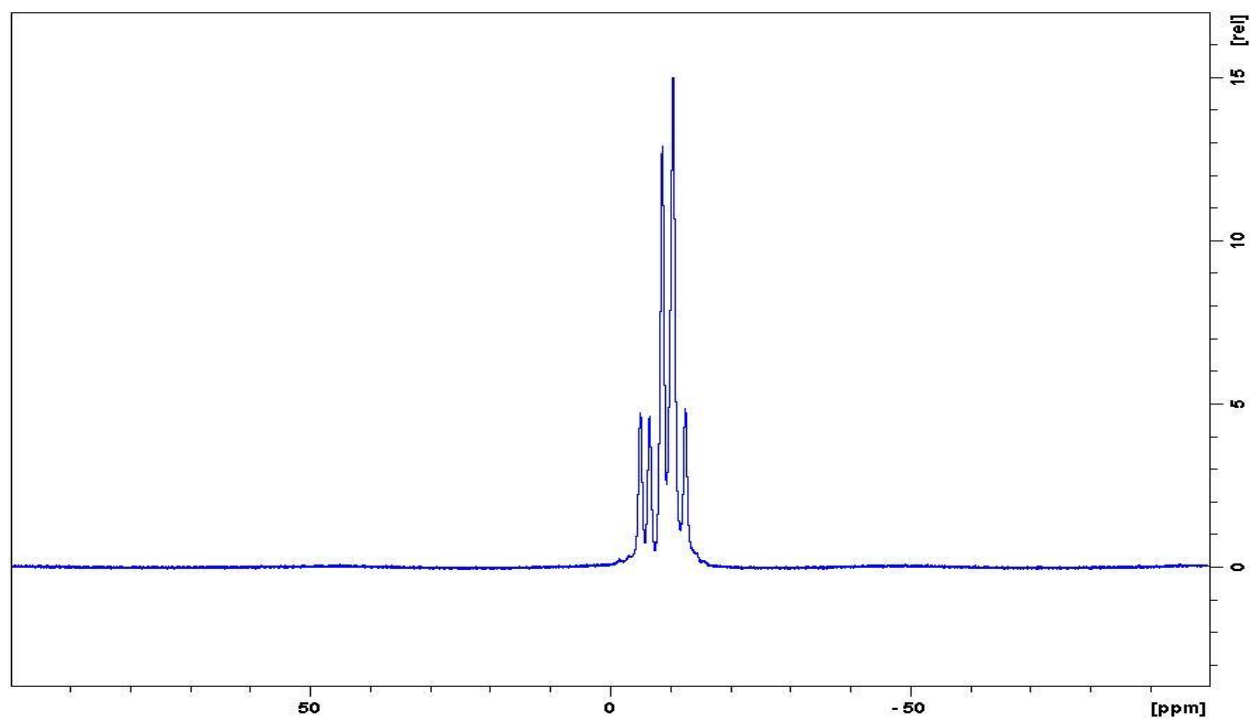
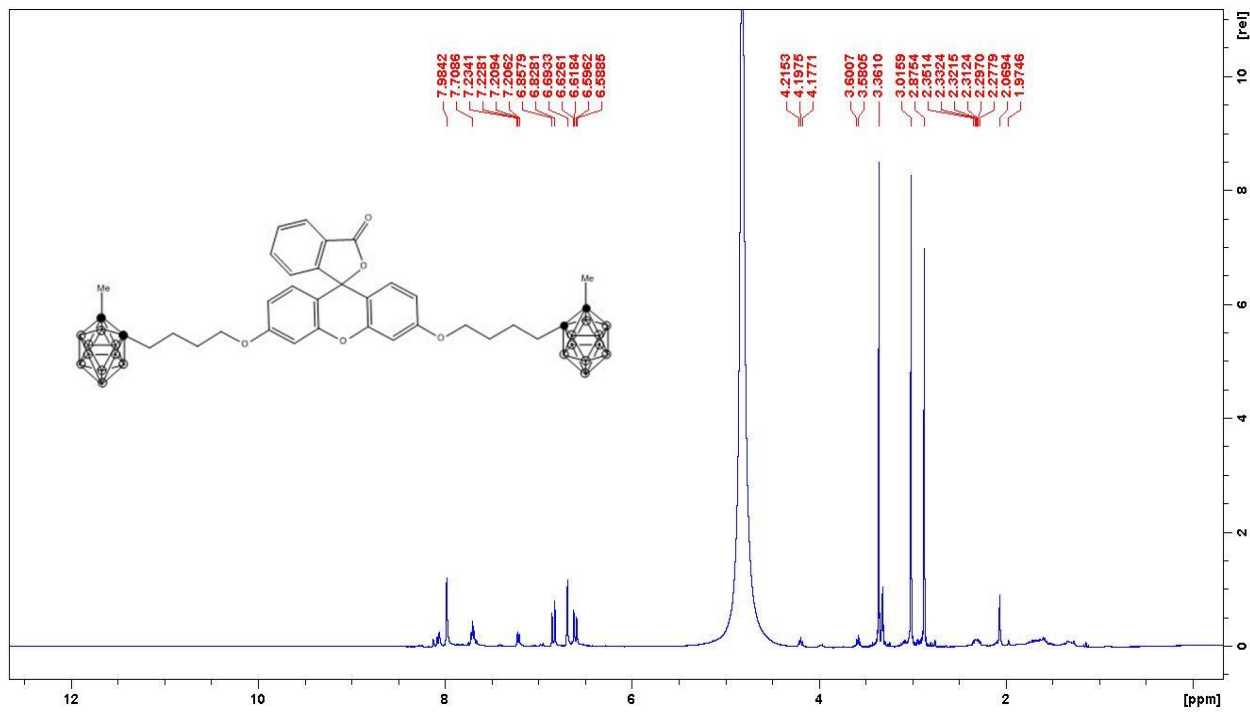**Figure 6**: ^{11}B-NMR Spectrum (300 MHz) of 1-methyl-2-iodobutyl-*o*-carborane.

Scheme 2: The synthesis of fluorescein-tagged 1-methyl-*o*-carborane.

Sodium hydroxide (8.0 mg, 0.20mmol) was brought together with 50 mL of DMF in a 250 mL round-bottom flask. The resulting mixture was sonicated for 30 minutes before fluorescein (33.0 mg, 0.0993 mmol) and previously synthesized 1-methyl-2-iodobutyl-*o*-carborane (119 mg, 0.234 mmol) were added and the reaction was refluxed for 24 hours. Following reflux, the crude mixture was purified by TLC (SiO$_2$, developed with DMF) and silica gel column chromatography (SiO$_2$, eluted with mixed solvents of hexane and DMF). Solvent was removed through distillation and the dark orange purified product (81.3% yield) was then taken for characterization using IR (**Figure 7**), NMR (**Figures 8**, **9**, and **10**), mass spectrometry (**Figure 11**), and elemental analysis (**Table 1**).
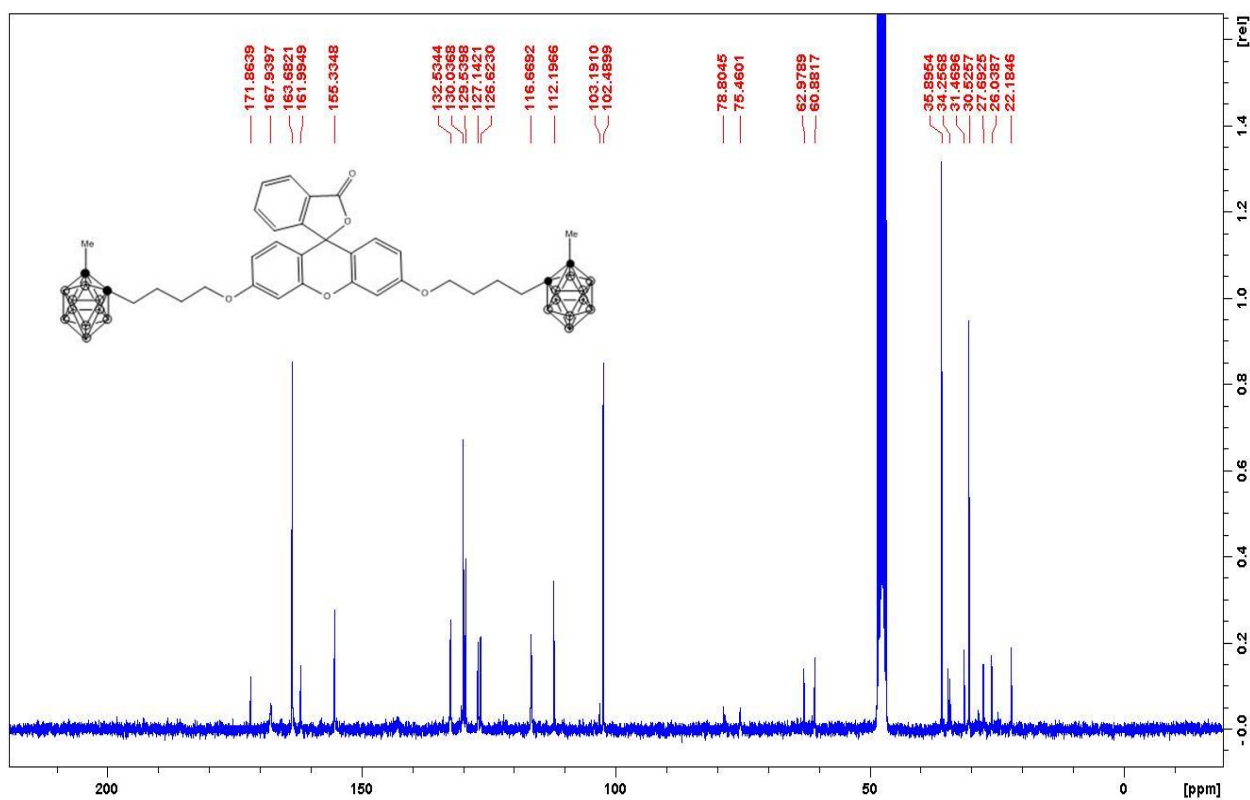

Figure 7: FT-IR spectra of fluorescein (red) compared to fluorescein-tagged 1-methyl-*o*-carborane (blue).
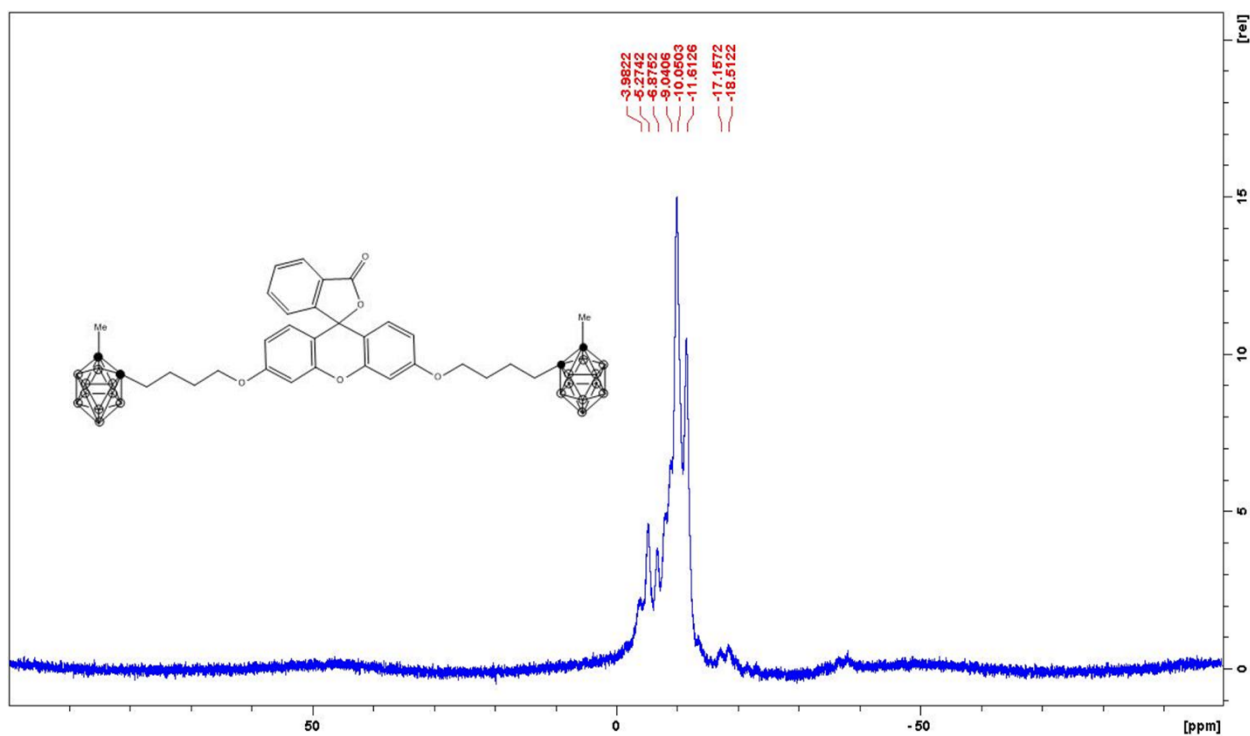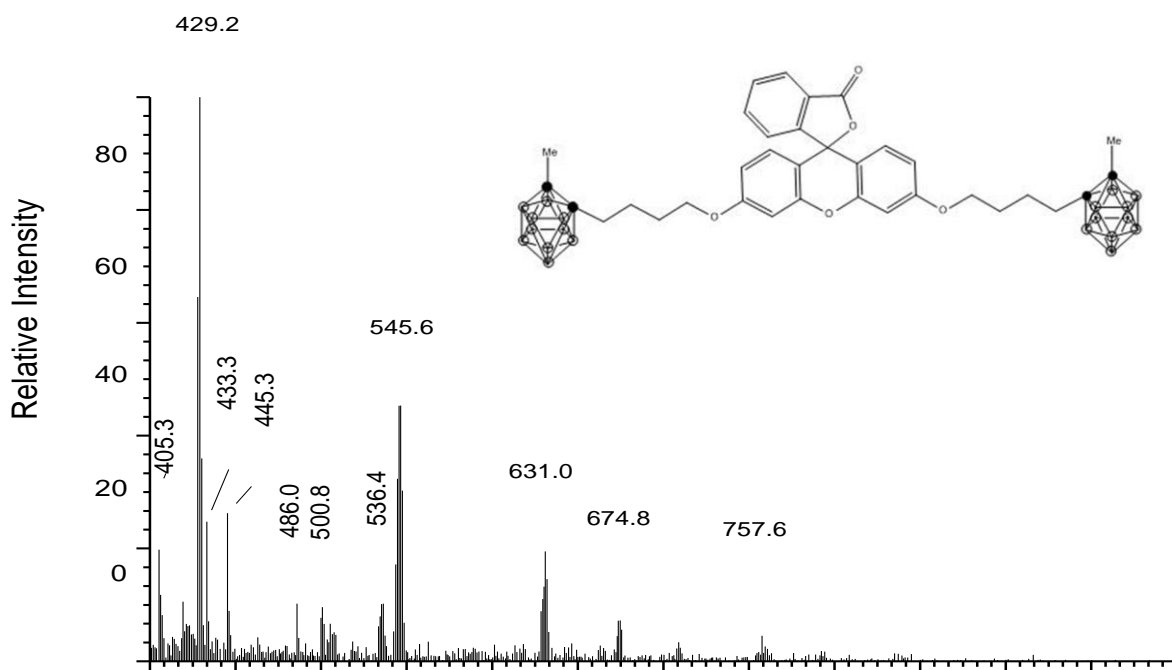
Figure 8: ^{1}H-NMR Spectrum (300 MHz) of fluorescein-tagged 1-methyl-*o*-carborane.

Figure 9: ^{13}C-NMR Spectrum (300 MHz) of fluorescein-tagged 1-methyl-*o*-carborane.

**Figure 10**: ^{11}B-NMR Spectrum (300 MHz) of fluorescein-tagged 1-methyl-*o*-carborane.

Figure 11: Mass spectrometry of Fluorescein-Tagged 1-methyl-o-carborane. Cald: 756.5. Found: 757.6 (M$^+$ + 1, 100%).

|	Fluorescein		Fluorescein-Tagged Carborane	
	Expected	**Observed**	**Expected**	**Observed**
% H	3.64	3.60	6.92	5.86
% C	72.29	69.19	53.95	52.20
% N	0.00	1.96	0.00	1.89

Table 1: Elemental analysis showing the expected and observed percentages of hydrogen, carbon and nitrogen in both starting fluorescein and fluorescein-tagged 1-methyl-o-carborane.

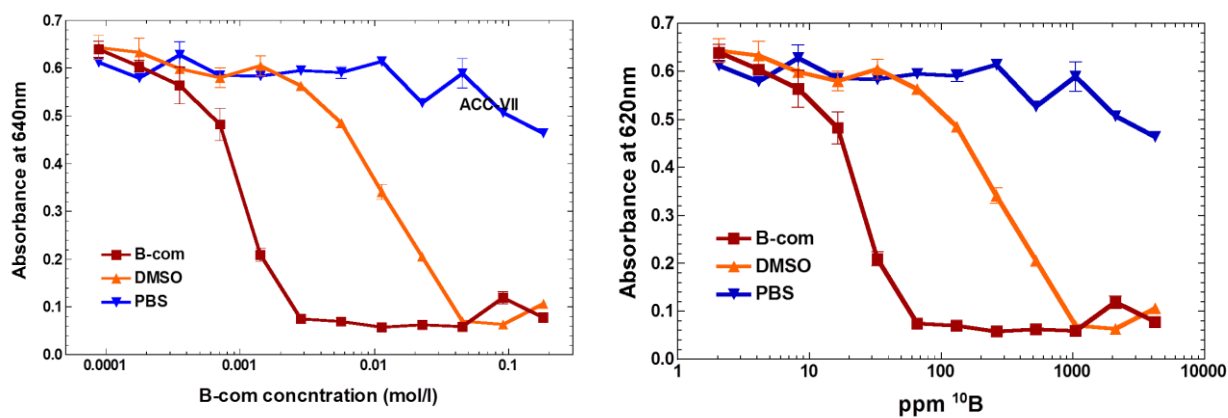### 5. Biological Evaluation of Fluorescein-Tagged 1-methyl-*o*-carborane
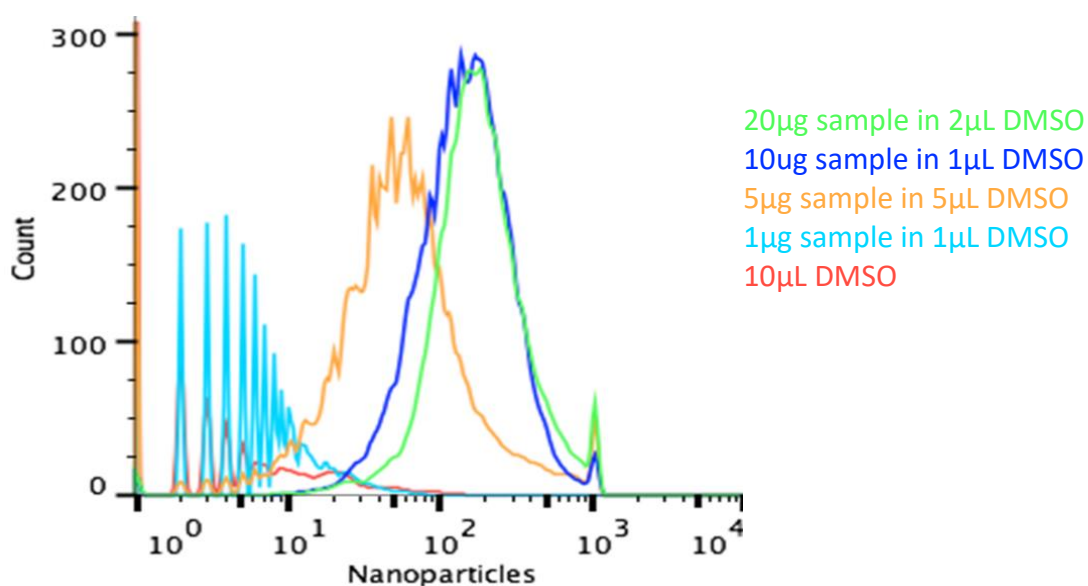
Figure 13: Flow cytometric analysis of samples containing fluorescein-tagged 1-methyl-*o*-carborane in DMSO with increasing concentration.
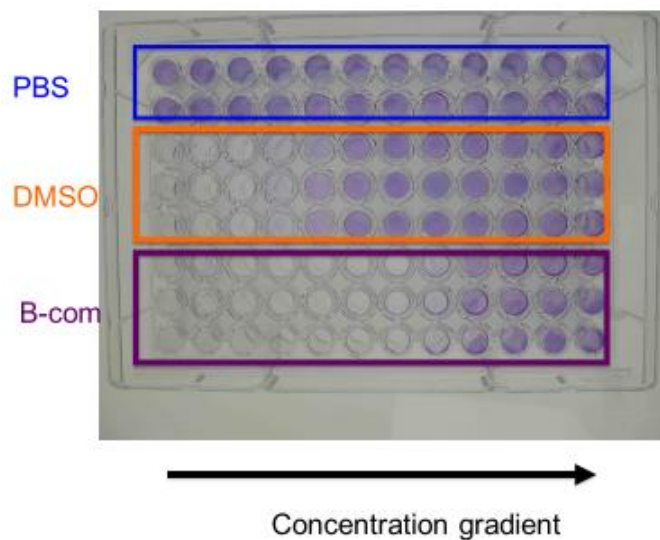
**Figure 14**: Cytotoxicity assay performed with 1-methyl-o-carborane (B-com), dimethylsulfoxide (DMSO) and phosphate buffered saline (PBS).


Figure 15: Plots of absorbance versus sample molarity (left) and ^{10}B accumulation in parts-per-billion (right) showing the cytotoxicity of 1-methyl-o-carborane. The observed IC_{50} value is 0.001 M or 21ppm ^{10}B, revealing moderate toxicity.
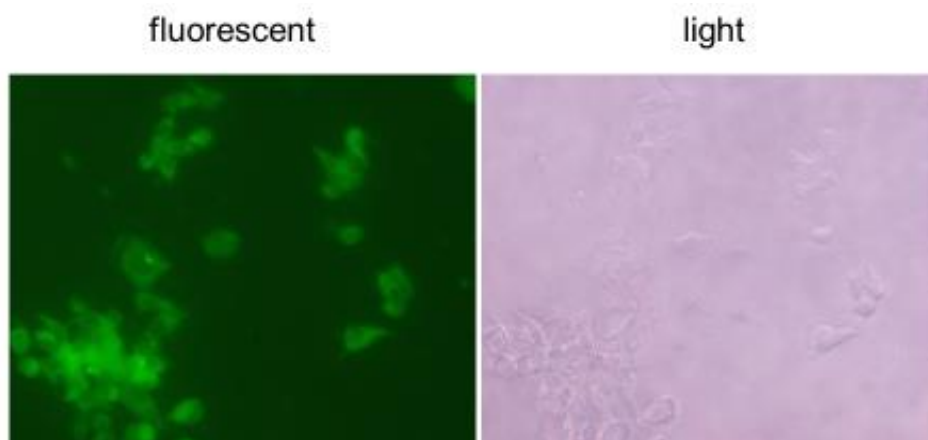
Figure 16: Fluorescent microscopic images of squamous cell carcinoma (SCC-VII) after the addition of fluorescein-tagged 1-methyl-*o*-carborane and an incubation period of 2 days.
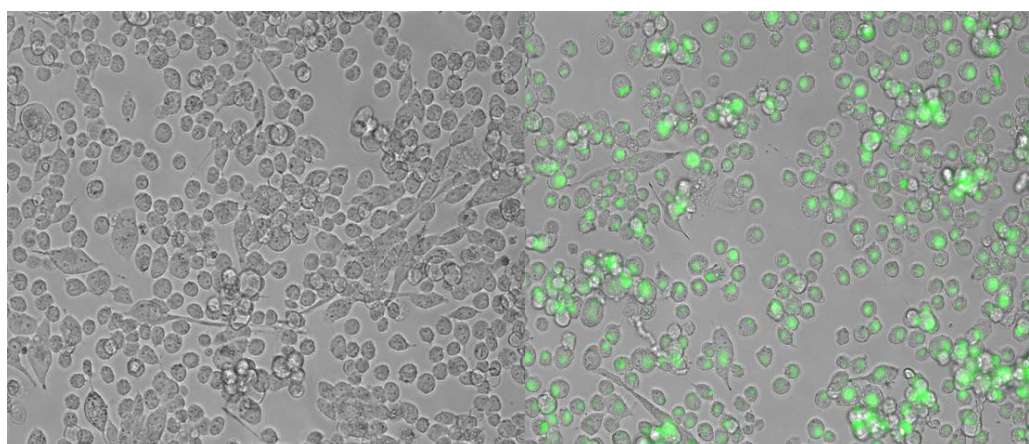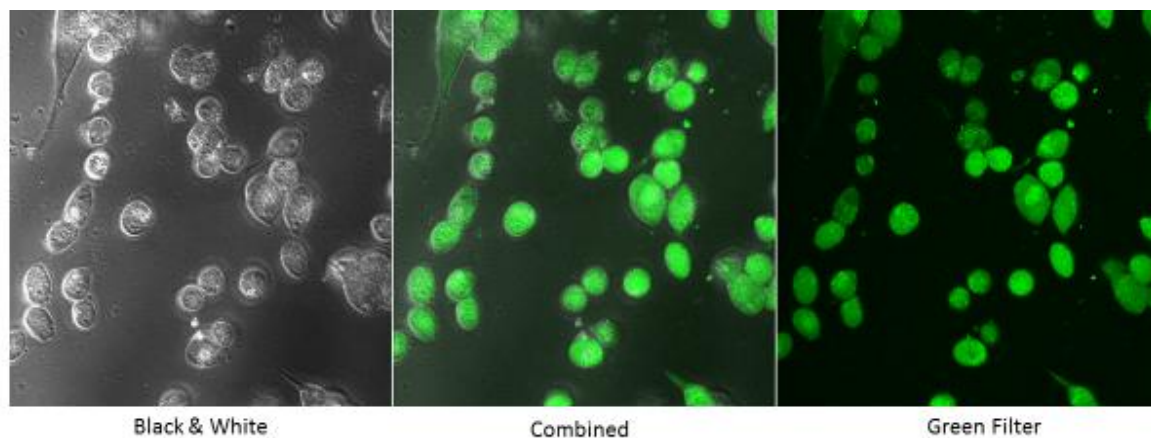

Figure 17: Phase-contrast microscopic images of pancreatic cancer cells (MIA PaCa-2) 24 hours after the introduction of 2μL DMSO as a control (left), and a 20μg sample in 2μL DMSO (right).
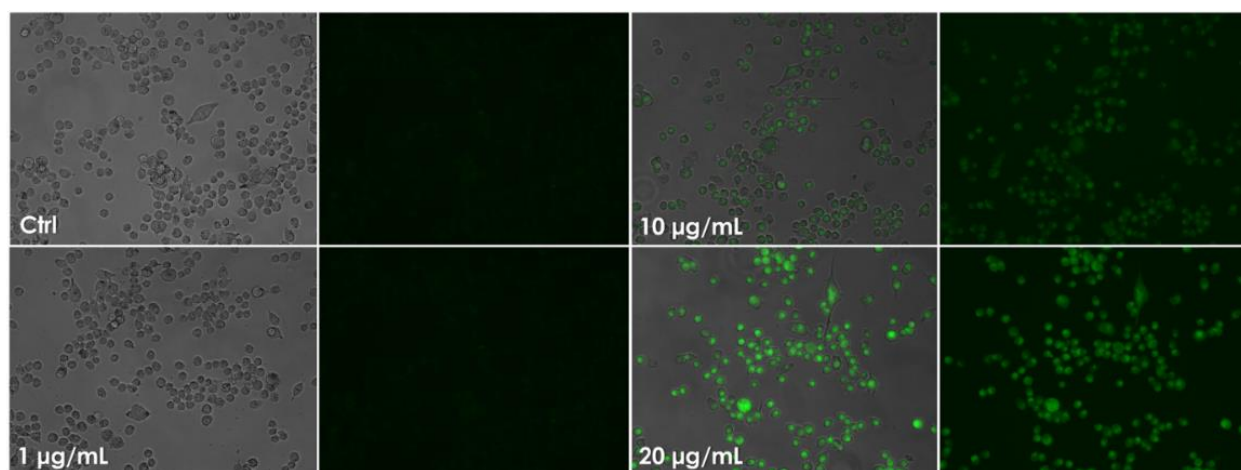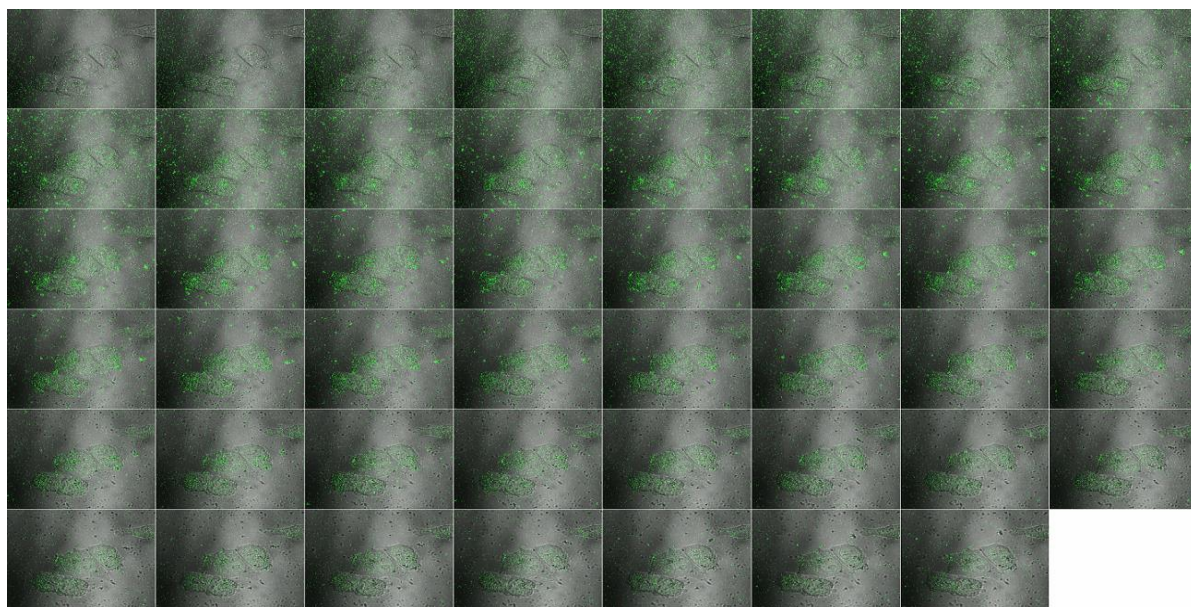

Figure 18: Fluorescent microscopic images of pancreatic cancer cells (MIA PaCa-2) 24 hours after the introduction of samples with varying concentrations of 1-methyl-*o*-carborane in DMSO.

Figure 19: Fluorescent microscopic images of pancreatic cancer cells (MIA PaCa-2) containing 1-methyl-*o*-carborane. Black & white shows cells only, Green filter shows compound only, and Combined shows both cells and compound.

Figure 20: Confocal microscopic images of pancreatic cancer cells (MIA PaCa-2) treated with a solution of 1-methyl-*o*-carborane in DMSO.

6. Discussions

All characterization methods supported the successful synthesis of the fluorescein-tagged 1-methyl-*o*-carborane. Chemical shifts in NMR spectra for the starting fluorescein were present, as well as peaks characteristic of the appended carborane cage. Additionally, a distinct peak was observed near 2600 cm^{-1} in the IR spectra of the product, which would correspond with the B-H

bonds of the carborane cage moieties. The peak of the parent ion was found at 757.6 during mass spectrometry of the product, which would correspond with the $M^+ + 1$ peak.

With successful synthesis of the product confirmed, further biological analysis was performed. The positive correlation between nanoparticles and sample concentration in flow cytometry confirm cellular uptake in pancreatic cancer cells. Uptake in this cell line is further reflected in phase-contrast and fluorescent microscopic images, showing even distribution throughout the cytoplasm. Confocal microscopy, which consists of cross-sections of pancreatic cancer cells treated with fluorescein-tagged 1-methyl-*o*-carborane in DMSO, also shows cellular uptake as the middle cross-sections would not exhibit fluorescence if the compound was not present.

Accumulation in a second cell line is shown in the fluorescent microscopic images of squamous cell carcinoma after treatment with the compound. The cytotoxicity assay allowed the determination of the product's IC_{50} value, which is the concentration that can be administered before 50 percent cell death is observed. The IC_{50} value of 1-methyl-*o*-carborane was calculated as 0.001 M or 21ppm ^{10}B, reflecting moderate toxicity relative to that of relative to BSH (28 mM) and BPA (7.9 mM).[5] This is most likely due to the increased boron content of fluorescein-tagged 1-methyl-*o*-carborane compared to BSH and BPA.

7. Conclusions

Fluorescein was conjugated with 1-methyl-*o*-carborane using a Williamson ether synthesis reaction, and the identity of the product was confirmed through characterization using NMR, IR, mass spectrometry, and elemental analysis. During biological evaluation studies done by the NIU Department of Biological Sciences as well as Kyoto University in Japan, cellular uptake was

confirmed in squamous cell carcinoma and pancreatic cancer cell lines with moderate cytotoxicity. Further work will involve the decapitation of the carborane cage moieties, which has been shown to improve cytotoxicity while also resolving water-solubility.[7] Nonetheless, the current results are promising and BNCT evaluation through animal studies at Kyoto University are scheduled to begin in Spring 2017.

8. References

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