Final Report

for the Project Period of:

June 1, 2017 through June 30, 2020

Project Title:

Automated Isolation of Astatine-211 and Chemistry Evaluation

Award #: DE-SC0018013

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I. Summary of Research Project

This research project was undertaken to further radiochemistry involved in astatine-211 (^{211}At)-labeled cancer therapy agents, which tremendous potential for treating cancer, particularly blood-borne and metastatic cancers. To achieve that potential, the chemistry of ^{211}At needs to be better understood, more efficient methods for isolation of ^{211}At from irradiated targets needs to be developed, new highly efficient methods for stably labeling cancer targeting agents needs to be developed, and clinical trials need to be conducted. Development of an automated system for isolation of ^{211}At from bismuth targets is critically important in achieving widespread use of ^{211}At. This is particularly true when expanding clinical trials involving ^{211}At-labeled agents to clinical sites that lack the radiochemistry personnel needed for ^{211}At isolation. Although methods for labeling large slowly metabolized cancer targeting agents, such as antibodies, have been developed, there are presently no good methods for labeling small molecules with ^{211}At. Thus, development of new methods for stably labeling small molecules with ^{211}At is also critical to the development of new ^{211}At-labeled agents for cancer treatment.

The research in this investigation was a collaborative effort between the research groups of the PIs, Dr. D. Scott Wilbur in the Department of Radiation Oncology at the University of Washington (UW), Matthew O'Hara at the Pacific Northwest National Laboratory (PNNL), Dr. Stosh Kozimor at the Los Alamos National Laboratory, and Dr. Silvia Jurisson at the University of Missouri. In this report only those studies conducted at UW are reported. Separate final reports will be provided by PIs at LANL and PNNL. The overall objectives of the research effort were to: (1) improve the availability of the α-emitting radionuclide astatine-211 (^{211}At) through development of a simple and automatable method for isolation of ^{211}At from irradiated bismuth targets, (2) investigate chemistry that would help understand ^{211}At species present under various conditions, and (3) evaluate new chemical labeling methods that can be used for preparation and evaluation of ^{211}At-labeled small molecules, and (4) stimulate Postdoctoral Fellow and student interest in radiochemistry, and train them to become, the next generation of the radiochemists.

A part of the investigation was focused on developing column methods for separation of ^{211}At from large quantities of bismuth salts so that an automated system for rapid and efficient isolation of ^{211}At from irradiated bismuth targets could be obtained. There were difficulties with automation of steps in the wet chemistry method that involved extraction of ^{211}At from 8M HCl into diisopropyl ether (DIPE) and back-extraction into 4N NaOH from DIPE. Those difficulties appeared to be due to mixing difficulties and miscibility of two phases. Prior DOE funded studies at PNNL (Matt O'Hara, P.I.) developed a novel flow-through mixer and separation module which successfully addressed the problems using a semi-automated setup. In this investigation, there was (1) a continued effort to optimize the PNNL L/L wet chemistry separation method, (2) evaluation of resins, including a commercial resin (LANL/UW) for the separation, and evaluation of a tellurium solid support system and its (semi)-automation to simplify and shorten the time to isolate ^{211}At from the irradiated bismuth targets. Another major portion of the studies involved using the isolated ^{211}At in the development of new radiolabeling moieties for use in protein conjugates and small molecule labeling. Those studies included looking at dithiol coupling with ^{211}At to potentially provide a chelate method for labeling, evaluation of the use of iodonium salts as a method for nucleophilic substitution of ^{211}At onto boron cage molecules, evaluation of monocarbon carborane labeling with ^{211}At and labeling using moieties that contain two *closo*-decaborate(2-) groups. The results of those studies are provided herein.

This final report outlines the experiments conducted and results obtained at UW from June 1, 2017 thru June 30, 2020 (*2-year project with 1-year no-cost extension*). The report divides the information on the experiments and results obtained into the 4 specific objectives of the research efforts. Three of the specific objectives are research related and the fourth is training related. The objectives are listed below:

Specific Objective 1: *Compare L/L extraction and column separation methods for automation of the wet chemistry isolation of ^{211}At from irradiated bismuth targets.*

Specific Objective 2: *Evaluate ^{211}At species produced and its reactivity under various conditions*

Specific Objective 3: *Evaluation of new methods for labeling small molecules with ^{211}At*

Specific Objective 4: *Training of students in radiochemical methods involved in production, automation of ^{211}At isolation, radiochromatographic characterization and radiolabeling with ^{211}At.*

Note that some of the tasks within the original individual specific objectives changed during the research period as experiments indicated that either the results were difficult to interpret or research results indicated that there were other more promising approaches that might be taken.

Summary of Major Findings from Research/Training Activities at UW:

- A flow-through chamber was developed at PNNL for efficiently removing bismuth metal and ^{211}At from irradiated targets. That was published in *Applied Radiation and Isotopes* (2017) 122: 202-210.
- Automation of the "wet chemistry" isolation approach was successfully demonstrated. That was published: M.J. O'Hara et al. "Development of an autonomous solvent extraction system to isolate astatine-211 from dissolved cyclotron bombarded bismuth targets." *Scientific Reports* (2019) 9:20318.
- Additional optimization runs of the wet chemistry semi-automated system demonstrated that the results were reproducible. Those results have not yet been published.
- Isolation of ^{211}At from PEG columns were not efficient at releasing ^{211}At, so this approach was not further pursued. Other approaches were deemed more likely to provide the separation sought.
- An efficient method for isolating ^{211}At from bismuth targets that used a packed tellurium metal column was developed. Results were published this work: Y. Li et al. "Investigation of tellurium-packed column for isolation of astatine-211 from irradiated bismuth targets and demonstration of a semi-automated system", *Scientific Reports* (2019) 9:16960.
- Optimization of the semi-automated system using a tellurium column is required before the method can be finalized and used routinely. This work is on-going in our laboratory.
- The column matrix investigated by Dr. Kozimor et al. at LANL gave promising results, but non-optimized isolation yields were not as good as the tellurium column results. The results were published: D. Woen et al. A solid-state support for separating astatine-211 from bismuth. *Inorganic Chemistry* (2020) 59, 6137-6146.
- Difficulties in identifying species in the evaluation of ^{211}At species via chemical methods resulted in our looking for a more conclusive method for identification. We plan to evaluate chemical methods with new spectral analyses approaches in future studies.
- Our iodonium salt nucleophilic labelings were successful but did not provide high enough labeling yields to be useful in ^{211}At labelings.
- Chelation of ^{211}At using di- and tri-thiol compounds was studied, but the results with sulfhydryl moieties indicated that reduction was occurring rather than chelation. Prior chelation attempts using amine and carboxylate containing ligands resulted in forming unstable attachment of the ^{211}At.
- Two monocarbon carborane derivatives were converted to their phenyl iodonium salts for labeling studies. The iodonium salt that had a carboxylate directly attached to the boron cage carbon was found to be not very stable, whereas the iodonium salt that had a phenyl ring attached to the carbon atom was stable.
- Nucleophilic reaction of the iodonium salts with [^{211}At]NaAt resulted in production of several radiolabeled species.
- Direct electrophilic ^{211}At-labeling of a monocarbon carborane at elevated temperatures provided high yield of one of the products observed in the nucleophilic reaction.
- Reaction of the monocarbon carborane phenyliodonium salt with dilute NaOH provided the *p*-hydroxy-monocarbon carborane in good yield.
- Direct ^{211}At labeling of the p-OH-monocarbon carborane was efficient at room temperature, but animal studies (through a collaboration) indicated that it was not stable in vivo.
- New reagents in which the ^{211}At atom is bound in a higher oxidation state provided high labeling yields. These types of compounds will be investigated further as labeling reagents for small molecules.

II. Research Results

A large amount of data was generated in the experiments conducted under this research effort. To assist the reader in finding the information that is of interest, the results have been compiled into tables where possible and they are organized under the specific objectives that to be achieved in the proposed research.

A. *Specific Objective 1:* *Compare L/L extraction and column separation methods for automation of the wet chemistry isolation of ^{211}At from irradiated bismuth targets.*

This specific objective is very important in our continuing effort to obtain an automated system for isolating ^{211}At from bismuth targets for use in clinical trials. Automation (or semi-automation) of the ^{211}At separations can allow a less qualified person than needed to manually conduct the isolation process and will decrease the radiation exposure to the personnel conducting the separations. Isolation of ^{211}At from bismuth targets can be fairly efficiently conducted by dry distillation (ref). That is the method that was used in our laboratory for over a decade, but inconsistencies in recovered yields, particularly when scaling up to clinical level ^{211}At production runs, and radiation safety issues, led us to develop a "wet chemistry approach (ref). It should be noted that an automated system for ^{211}At recovery from ^{209}Bi targets using the dry distillation method has been reported (1). While their automated system appears to provide good recovery of ^{211}At from bismuth metal targets, the fact that we use much larger quantities of bismuth in our targets would require major alteration of their system. Further, their automation approach requires mechanical removal of bismuth from the aluminum target backing, which was deemed a radiation hazard by our Radiation Safety personnel when proposed by us.

In prior studies we found that a "wet chemistry" ^{211}At isolation approach provided very consistent and high recovery yields (2). In collaboration with Matt O'Hara at the Pacific Northwest National Laboratory, we investigated development of modules that could be used for automation of the wet chemistry approach and made significant progress towards that end. In this research effort we chose to continue to develop an automated ^{211}At isolation method that uses the wet chemistry isolation approach. Although not part of the funding under this grant, Matt O'Hara's (PNNL) effort continued to optimize the L/L separation system that he designed and built as there were some funds remaining from the previous related grant. He also conducted some studies at UW in collaboration with a company (Global FIA; Graham Marshall, President; https://www.globalfia.com/index.php) to refine the automation system used. Additionally, Dr. Stosh Kozimor (LANL) used his extensive knowledge of solid support separations to evaluate ether-containing resins for separation of ^{211}At from bismuth salts. In our laboratory, Dr. Yawen Li (UW) evaluated the use of a tellurium metal column to separate and isolate ^{211}At from bismuth dissolved in 10M HNO_3. Information on those studies is provided below and also in more detail in quarterly reports submitted separately by Matt O'Hara and Stosh Kozimor.

SO1a: Design and optimize L/L extraction module (at PNNL – continuation of previous funding)

This research was a continuation of studies conducted previously by Matt O'Hara (PNNL) in collaboration with UW personnel. Matt made trips to UW to evaluate changes in his automation protocol. While each step was programmed to run on the computer, no attempts were made previously to the system to run automatically all the way through the isolation process. Matt modified the software so that can be tested. He carried out three ^{211}At isolation runs in our laboratory. ICP-MS samples were saved and were analyzed. Anion exchange radio-HPLC and radio-iTLC QC analysis were performed on the runs, as well as QC tests.

SO1b: Evaluate PEG columns with different resin cores (at UW)

In our previous research effort, several polyethylene glycol (PEG) resins were prepared and evaluated as an alternative to diisopropyl ether (DIPE) in isolation of ^{211}At from irradiated bismuth targets. A large number of studies were conducted with high column capture (e.g. 90%), but only moderate recovery of ^{211}At (e.g. 40-60%). In the proposed studies, we requested that a commercially-available full automated isolation system be purchased so that we could further optimize the isolation from the PEG-resin columns, and compare that with other solid-support column recoveries. However, that part of the proposed studies was not funded. Therefore, this specific objective was not investigated.

SO1c: Evaluate new ether containing columns for ^{211}At isolation (at LANL)

In this effort, the UW collaborated with Stosh Kozimor at the Los Alamos National Laboratory. It was decided that was best to ship irradiated bismuth targets to LANL, so they could dissolve the targets and have the solutions (10M HNO_3, 8M HCl, DIPE, 4M NaOH) in hand to evaluate adsorption and release from the resins that they tested. Prior to sending irradiated targets to LANL, we had to obtain a certified shipping container that could be used for the target shipments. Stosh Kozimor obtained a certified shipping container in which a large (B52) lead pig could be placed (see Figure 1). He also was able to get fabricated a foam insert that would hold the lead pig in place during shipment. Pictures of the shipping container, foam insert and the lead pig are provided below. In the last picture, an aluminum backed target is set inside the pig, but in practice the target will be sealed in two plastic bags (Ziploc) and that enclosure will be placed inside a plastic bottle that fits into the pig. Inside the plastic bottle will be charcoal pellets to adsorb any volatile ^{211}At.

Figure 1: Pictures of a certified shipping system to be used for shipments of irradiated bismuth targets from UW to LANL. The heavy B52 pig was not required for shielding, but it will be used as it was provided at no cost to the studies.

Both LANL and the University of Washington Radiation Safety approved this shipping container, and a number of shipments were made from UW to LANL. It should be noted that some shipments did not make it to LANL in the 1-day shipment period. In those cases, LANL had very little to work with. Due to this difficulty, some trips were made from UW to LANL to conduct experiments. Postdoctoral Fellows from Dr. Kozimor's group, Drs. David Woen and Laura Lilley visited the UW laboratory and observed the "wet chemistry" ^{211}At isolation process, as well as ^{211}At MAb labeling. Dr. Woen came back to Seattle to conduct K_d measurements for his new resins in our laboratory. The results of his studies were provided in his separate quarterly progress reports.

SO1d: Test and compare separation methods developed at PNNL, LANL and UW (at UW).

This task was modified since we did not have funds to optimize the PEG columns. One of the goals of our continued studies was to eliminate the distillation of 10 M HNO_3 in the isolation process, as that could decrease the overall isolation time and retain the ^{211}At species in a low oxidation state. To achieve this, we chose to evaluate another method for ^{211}At isolation, that of using a tellurium column to separate the ^{211}At from the 4-5 grams of bismuth target material. Use of a tellurium column separation was previous described in the literature (3) and in a monogram on Radiochemistry of Astatine (4). The procedure uses hydroxylamine hydrochloride to destroy nitrate making it possible to circumvent the distillation process.

We previously conducted preliminary studies to use Te column method for separating ^{211}At from irradiated Bi target. In those preliminary studies, we evaluated the ^{211}At yields obtained from a Te column and determined whether the $SnCl_2$ used in the literature was required. The literature method involved destroying nitrate ions using hydroxylamine hydrochloride then reducing astatine to astatide using $SnCl_2$ prior to loading onto the column. After washing off Bi, Sn and other impurities including excess hydroxylamine with 8 M HCl and water, ^{211}At was eluted in about 1.5 mL of 2 N NaOH to provide a recovery yield of 75%. The radio-HPLC analysis indicated that 100% of the product was in the astatide form. This is very important as we had some difficulty obtaining only astatide from the L/L isolation method. While it is possible to convert the other species after evaporation of a basic solution, it was encouraging that the astatide form could be obtained directly from the Te column. An assessment of the percentage of astatide obtained when using DIPE extractions (i.e. L/L separation), a proprietary column (PNNL) or the Te column is provided in Table 1. Note that ^{211}At from the Te column has the highest percent astatide without evaporation to dryness. Studies where ^{211}At was isolated using the Te column with and without $SnCl_2$ demonstrated that its' use was not necessary to obtain pure astatide. We believe this is true as hydroxylamine hydrochloride may be a reductant and the Te column may possibly also keep ^{211}At in the reduced form.

Radio-HPLC analyses were performed to assess the effect of evaporating ^{211}At solutions to dryness on the radiochemical purity. It was encouraging to find out that evaporating the ^{211}At solutions to dryness did not cause ^{211}At to stick to the vial or decrease % astatide available for reactions. With these encouraging results with ^{211}At from a Te column, we decided to evaluate semi-automation the Te column isolation process (did not have equipment to fully automate the process). For the automation setup, a Hamilton dual syringe pump was used, along with three Hamilton MVP valves. The valves and pumps are run by a laptop computer. The automated isolation procedure used is described below.

Tellurium column ^{211}At Isolation Procedure: The irradiated Bi target was manually put into the Teflon dissolution chamber (see ref (5)), then FEP tubing for flowing 10 M HNO_3 through the dissolution chamber were connected. Using the 10-mL syringe installed on the Hamilton syringe pump, 15 mL of 10 M HNO_3 was pumped through the dissolution chamber to dissolve the target. The HNO_3 solution coming from the dissolved target was collected into a 250-mL round bottom flask. Air was pushed through the dissolution chamber to ensure all the HNO_3 was transferred into the round bottom flask. Deionized water was used to rinse the HNO_3 from the syringe, and that solution was transferred into a waste vial used for non-radioactive waste. Using the same 10-mL syringe, at a flow rate of 2.4 mL/min, 80 mL of 34% $NH_2OH{\cdot}HCl$ was added into the dissolved target to destroy the nitrate, followed by 22 mL of 8 M HCl to make the solution of dissolved target 1.5 M HCl, resulting a total volume of about 117 mL. Using a 25-mL syringe on the Hamilton syringe pump, 21.5 mL of the dissolved target solution was aspirated into a 25-mL loop and loaded onto the Te-packed column at a flow rate of 5.2 mL/min. This step was repeated 6 times to load all the radioactivity onto the column. The effluent from the column collected Bi and other impurities into a radioactive waste vial. The Te-packed column was then rinsed with 20 mL of 1.5 M HCl, followed by 20 mL of D.I. water. Finally, the column was eluted using 5 mL of 2 M NaOH. The NaOH solutions were collected in 1 mL fractions manually and about 95% of the ^{211}At was recovered in the 4th and 5th fractions. The transfer of all the liquids were controlled by a custom software program running on a laptop outside the fume hood. The overall process took ~ 1 hour and 50 min. A schematic of the automation setup is shown in Figure 2 and a picture of the setup is shown in Figure 3. The results from the first run of the automated system are provided in Table 2.

Table 1. Radiochemical purity of ^{211}At solutions determined using an anion exchange radio-HPLC method.

Date (mm/dd/yy)	Separation method	^{211}At solution	Peak 1[a] (%area)	Peak 2[a] (%area)
01/24/17	DIPE extraction-manual	pH ~7	3.6	96.3
02/2/17	Proprietary Column-semi-automated	pH ~7	2.2	97.5
		pH ~7, ^{211}At solution was evaporated to dryness and redissolved in PBS buffer	0	99.9
02/2/17	DIPE extraction-automated	pH>13	0	100
		pH ~7	2.4	97.5
		pH>13, ^{211}At solution was evaporated to dryness and re-dissolved in water	0	100
		pH ~7, ^{211}At solution neutralized after evaporation	4.7	95.0
03/07/17	DIPE extraction-manual	pH>13	7	93
		pH ~7	25.1	74.9
	Te column-manual	pH>13	0	100
03/14/17	DIPE extraction-manual	pH>13	62.6	33.6
		pH ~7	0	75[b]

[a]Peak 1 corresponds to the astatine species that elutes shortly after the void peak and peak 2 corresponds to the radiopeak of astatide. [b]An unknown radio-peak that accounts for 24% of the radioactivity was observed near the end of the run, 18 min after the peak of astatide.

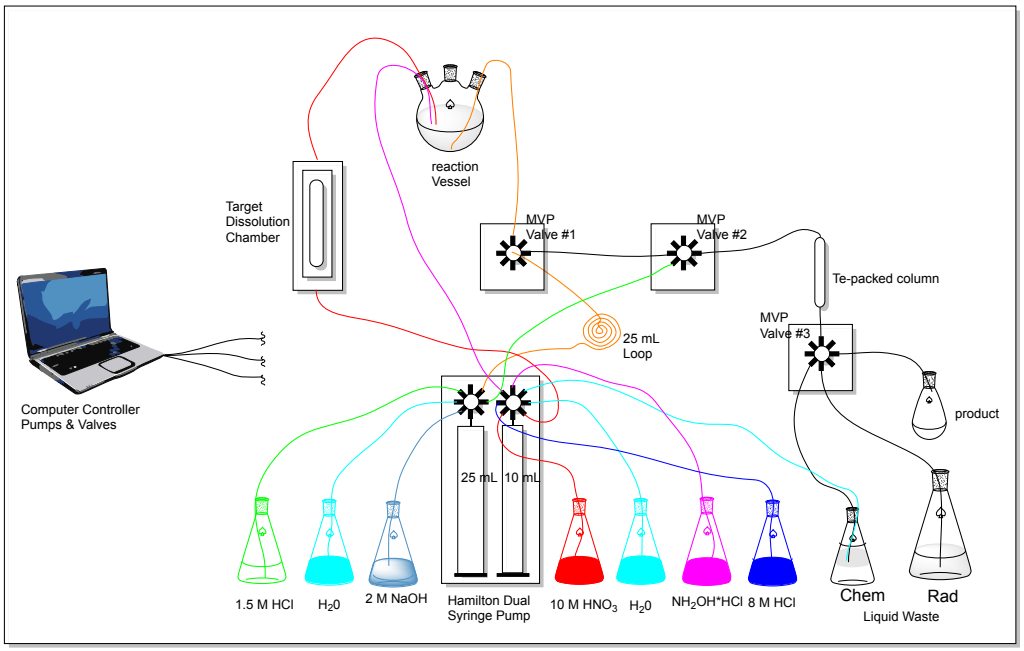

Figure 2: Schematic representation of the computer-controlled automation setup using syringe pumps to move the liquids for loading the Te column, rinsing and eluting ^{211}At from the Te column. Note that in subsequent isolations, gaseous effluents from the reaction vessel will be passed through a calcium carbonate and/or alumina column to trap nitric acid and NOx fumes.

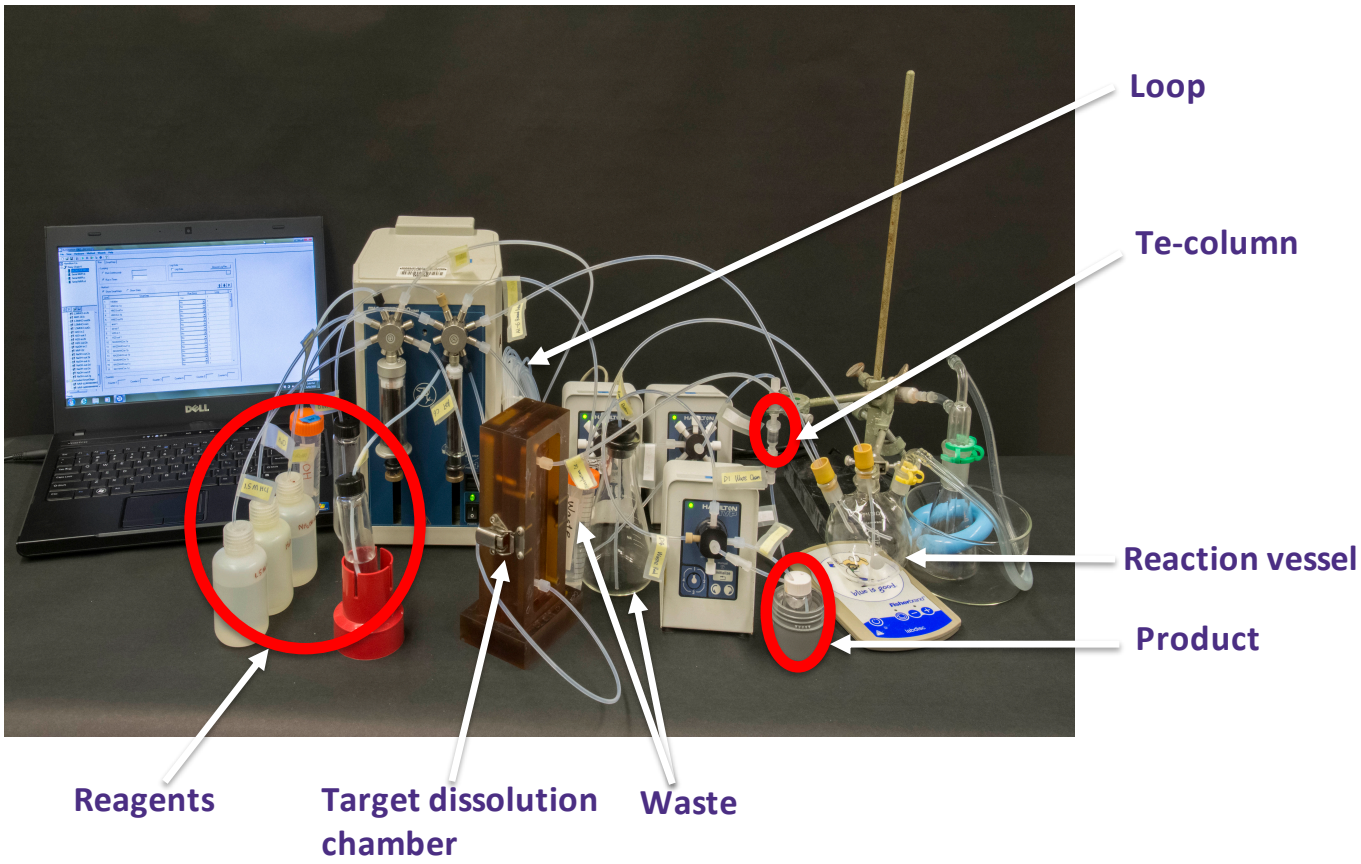

Figure 3. Automated system using Te-packed columns for isolation of ^{211}At. The computer is used outside of the glovebox and the rest of the system is housed in a negative pressure glovebox when being used.

Table 2: Results obtained from 1st automated run of Te column

18.6 mCi	on target	8:14		attenuation corrected:			24.18	mCi			
Product (1 mL fractions)											
F1	3.3	uCi									
F2	12.8	uCi									
F3	9.8	uCi									
F4	12.3	mCi									
F5	8.7	mCi									
					delta T (min)						
Total of F4+5:	21	mCi	10:03		109		decay correct to 8:14 am:			25.00	mCi
At-211 half life	7.214 h	432.84	min				**%yield=**	**103.41**			
At-211 decay constant		0.001601	min-1								
				delta T(min)		decay correct to 8:14 AM			**%**		
radio-waste:~45 mL	34	uCi	10:10 AM	116		40.94			**0.17**		
total vol is estimated to be 157 mL	118.6	uCi				142.81			**0.59**		
Column:	210	uCi	10:13 AM	119		254.09			**1.05**		
Target waste/clean up	241	uCi	10:32 AM	138		300.60			**1.24**		
target after clean up	344	uCi	10:31 AM	137		428.39			**1.77**		

Note that the total recovery was estimated to be 103% decay corrected yield, but that there was ~4% in the waste from washes of the target. One might estimate that the overall recovery was 96% after decay correctiion. The >100% estimated recovery likely comes from the fact that we use an attenuation factor of 1.33 to estimate the total ^{211}At produced (2), and that factor is not an exact number. What is important though is that 21 mCi (~87% non-decay corrected) of Na[^{211}At]At was obtained from the column in 2 mL of NaOH solution after 109 min (1.8 h). This was by far the highest recovered yield of ^{211}At obtained to that date. Prior to that experiment we generally obtained ~14 mCi ^{211}At to work with after a 3-hour work-up time. As we want to control the basicity of the ^{211}At that is used or shipped out, a portion of the isolated ^{211}At was neutralized, then made basic.

After isolation of the ^{211}At from the Te columns, we conducted QC tests on them. Those tests showed the ^{211}At was only in the astatide form. This was consistent for all of the Te column studies conducted, including the automated isolation. We thought it was also important to evaluate labeling of a monoclonal antibody with the astatine from the Te columns. In one study, two labelings were done using our BC8-B10 control. One of the labelings used the oxidant chloramine-T and the other did not use an oxidant. A 95% labeling yield was obtained when ChT was used, and a 91% labeling yield was obtained in the reaction without ChT. A BC8-B10 radiolabeling was conducted with the ^{211}At obtained using the automated Te column isolation procedure. A 77% radiolabeling yield was obtained in that labeling. It must be noted that the 91% and 95% labeling yields are higher than we normally get for BC8-B10, but the 77% yield is in the range that other ^{211}At labelings of BC8-B10. Both the isolated Na[^{211}At]At and the proteins labeled by the different methods were evaluated by ICP-MS to determine if the approaches have different amounts of bismuth and other trace metals. As expected, using 2 N NaOH to elute the Te column resulted in having some Te in the solution. However, after purification of the radiolabeled BC8-B10, the concentrations of bismuth, tellurium and other metals are extremely low (within release criteria). These results suggest that there is no need to further purify the ^{211}At from Te if it is being used for protein labeling.

A second automated ^{211}At production run was conducted following the same protocol described in the previous quarterly report, and the results obtained reproduced those in the 1st automated run. The isolation yield after decay correction was 88% (actual yield was 80%). Note that although the decay corrected percent yield was calculated to be 99.6% based on the estimated activity in the irradiated target (as shown in Table 3), there was about 1.8 mCi (10.6%) activity in the target dissolution chamber, 116 µCi (0.7%) left on the column and 120 µCi (0.7%) in the waste. Therefore, we adjusted the percent yield (decay corrected) to 88%.

Table 3: Results obtained from the 2nd automated run of Te column

19.6 mCi	on target	7:17	am	Attenuation corrected:			25.48	mCi			
Product (1 mL fractions)											
F1	23.6	uCi									
F2	33	uCi									
F3	8.99	mCi									
F4	10.5	mCi									
F5	0.95	mCi									
F6	82.2	uCi									
					delta T (min)						
Total of F3+4+5:	20.44	mCi	9:32		135		decay correct to 7:17 am:			25.37	mCi
%yield (before decay correction)=	**80.22**	%									
At-211 half life	7.214 h	432.84	min				%yield (decay corrected)=			99.58	%
At-211 decay constant		0.001601	min-1								
				delta T(min)		decay correct to 7:17 AM			%		
radio-waste:~45 mL	27	uCi	11:35 AM	258		40.81			**0.16**		
total vol is estimated to be 200 mL	120	uCi				181.39			0.71		
Column:	116.5	uCi	11:41 AM	264		177.80			**0.70**		
Target waste/clean up	1.1	mCi	11:25 AM	248		1.64			**6.42**		
target after clean up	716	uCi	11:30 AM	253		1073.66			**4.21**		

The radiochemical purity of the product from the second run was analyzed using anion exchange radio-HPLC and radio-iTLC. Radio-HPLC analyses were performed for the ^{211}At solutions before and after neutralization on a Hewlett-Packard model 1050 HPLC (Hewlett-Packard Company, Palo Alto, CA, USA) with a Beckman Model 170 Radioisotope Detector (Beckman-Coulter, Brea, CA, USA). A Dionex IonPac AS-20 anion exchange column with a Dionex AG-20 guard column (Dionex, Sunnyvale, CA, USA) was eluted with a 50 mM NaOH at 1 mL/min in isocratic mode. As shown in Figure 4, essentially 100% of the ^{211}At was in the form of astatide.

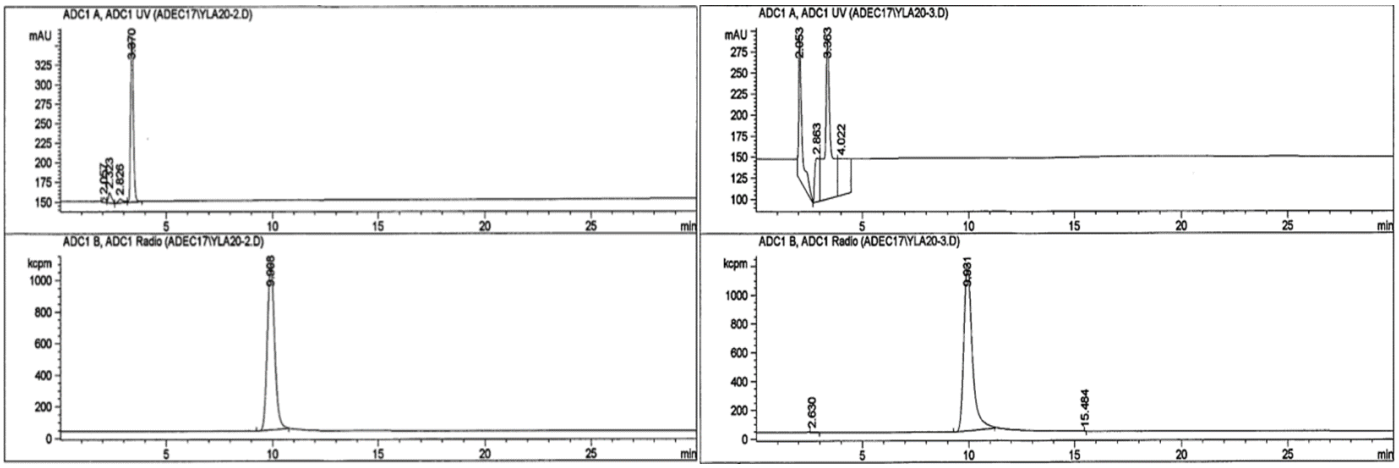

Figure 4. Anion exchange radio-HPLC chromatograms of ^{211}At solution before (left) and after pH neutralization (right).

Radio-iTLC analyses were performed using Agilent iTLC-SG strips (7 mm*90 mm) and n-butanol saturated with 3 N NH_4OH (pH 11). The iTLC strips were scanned at 0.2 mm/s using a LabLogic ScanRAM scanner equipped with a 1-mm NaI probe and the Laura software. The results indicated that before pH neutralization, 100% of the activity moved to the solvent front. However, for the ^{211}At solution of neutral pH, 78.3% (integrated) yield of the activity stayed at the origin as shown in Figure 5. It seems likely that At+ or AtO+ are formed under neutral conditions, and that species have reacted with the iTLC-SG at the origin.

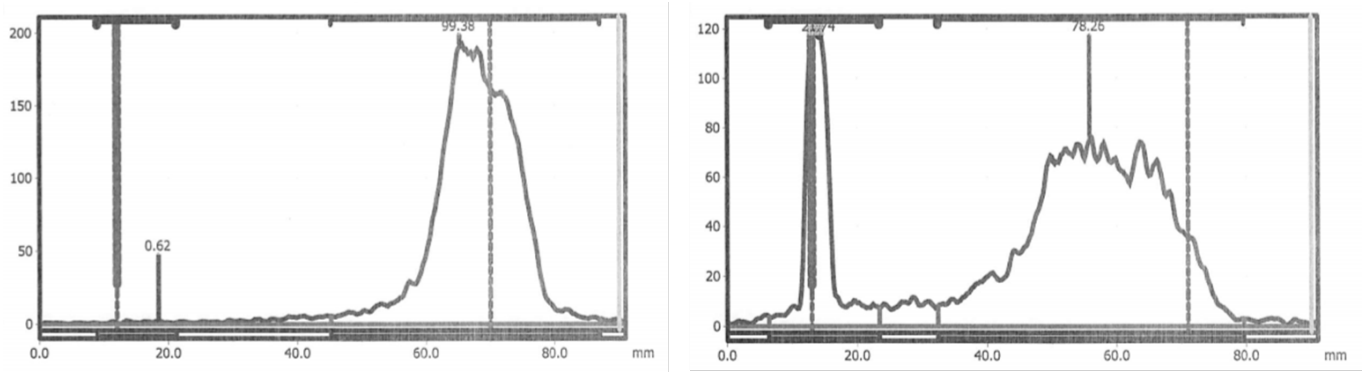

Figure 5. Radio-iTLC chromatograms of ^{211}At solution before (left) and after pH neutralization (right).

The proximity of BC Cancer Research Centre (BCCRC) (Vancouver, Canada) to Seattle, WA, made it feasible to have a professional courier (HealthEx Norwest) drive ^{211}At across the Canadian border and deliver it to BCCRC in 3-4 hours. A trial shipment containing about 4 mCi of ^{211}At isolated using the automated Te column method was sent to Jason Crawford, a Postdoctoral Fellow at BCCRC. The activity was ready for shipment before 11:30 am at UW Radiation Safety. However, the activity failed to go through the Canadian border because the courier missed one of the required documents.

A 3rd automated ^{211}At production run using the Te-column method was conducted and the isolation yields obtained in the 1st and 2nd automated runs was reproduced. The isolation yield after decay correction was 96.7% (actual yield was 92.3%). Note that although the decay corrected percent yield was calculated to be 109.1% based on the estimated activity in the irradiated target (as shown in Table 4), there was about 1.6%, 1.1% and 0.6% activity left in the target dissolution chamber, on the column and in the waste, respectively. Therefore, we adjusted the percent yield (decay corrected) to 96.7%. Radio-HPLC indicates that the ^{211}At was only present as astatide. However, the radiolabeling yield of B10-conjugated antibody using the isolated ^{211}At was very poor. The radiolabeling yield was 20-26% under the normal conditions (no oxidant). The yield increased to 40.6%, 53.9% and 88%, respectively, when 85 μg, 156 μg and 780 μg of chloramine-T was added to the reaction mixtures. We believe residual reductive $NH_2OH{\cdot}HCl$ in the product might have resulted in the poor yields.

Table 4. Results of the 3rd automated run of Te-column

20.3 mCi	on target	8:13	am	Attenuation corrected:			26.39	mCi			
Product (1 mL fractions)											
F1+F2	11.5	uCi									
F3	2.6	uCi									
F4	14.8	mCi									
F5	8.87	mCi									
F6	711	uCi									
F7	127	uCi									
					delta T (min)						
Total of F4+5+6:	24.381	mCi	9:57		104		decay correct to 7:17 am:			28.80	mCi
%yield (before decay correction)=	92.39	%									
At-211 half life	7.214 h	432.84	min				%yield (decay corrected)=			109.13	%
At-211 decay constant		0.001601	min-1								
				delta T(min)		decay correct to 8:13 AM			%		
radio-waste:~40 mL	23.9	uCi	12:45 PM	272		36.95			0.14		
total vol is estimated to be 160 mL	95.6	uCi				147.78			0.56		
Column:	201	uCi	12:09 PM	236		293.31			1.11		
Target waste/clean up	192.7	uCi	12:04 AM	231		278.96			1.06		
target after clean up	105.5	uCi	12:07 PM	234		153.46			0.58		
						%yield (based on loss)=			96.69		

A shipment of about 4 mCi of the product isolated using the automated Te-column method was made to the BCCRC. The activity was ready at UW Radiation Safety for the local courier HealthEx to picked up before 11:30 am. All the documents required to go through the country border were provided and the activity was delivered to BCCRC successfully.

In an attempt to improve the protein radiolabeling yield, the volume of water wash was increased from 20 mL to 40 mL to remove residual $NH_2OH{\cdot}HCl$ from the Te column and tubing tubing. In addition, the volume of $NH_2OH{\cdot}HCl$ was reduced to 65 mL. Unfortunately, a fraction of the collected 2 N NaOH containing ^{211}At was spilled inside the glovebox by accident and we could not measure the total amount of radioactivity eluted from the column. However, the fact that only small amounts of ^{211}At was found on the target, Te column and waste after the run suggests most of the ^{211}At was eluted by 2 N NaOH. The remaining ^{211}At was used for labeling B10-conjugated OKT10 antibody, but the radiolabeling yield was only 6.3% without chloramine-T and 19.0% with 120 µg of chloramine-T.

It is important to know the minimal amount of $NH_2OH{\cdot}HCl$ required to destroy all the nitrate ions in the dissolved Bi target solution. Therefore, we had a work study undergraduate student, Taylor Morscheck perform titration studies using 35% $NH_2OH{\cdot}HCl$. The completion of the $NH_2OH{\cdot}HCl$ and nitric acid reaction was indicated by the cessation of visible bubbling. Nitrate/nitrite test paper (EMD Millipore™) was used to verify the nitrate ion was below it's detection limit (10 ppm). To mimic the automated isolation process, 4.25 g of Bi metal was dissolved using 15 mL of 10 M HNO_3. The resultant solution was split into three 5-mL fractions and 35% $NH_2OH{\cdot}HCl$ was added dropwise at a flow rate about 2 mL/min using a 25-mL burette. On average, 17.4 ± 0.5 mL was used for each fraction, suggesting about 52.2 mL is needed for a full target. Taylor also monitored the temperature of the exothermic reaction and found the highest temperature of the reaction mixture was 95°C during the addition of $NH_2OH{\cdot}HCl$.

In some of the studies, radiolabeling yields of < 20% were obtained for B10-conjugated MAb using ^{211}At isolated from the Te-columns. However the yields could be increased by the addition of the oxidant chloramine-T. We believe that the reducing agent $NH_2OH{\cdot}HCl$ used to destroy nitrate ions might have contaminated the final product and hindered the electrophilic astatination reaction. Studies were conducted to determine whether using reduced amounts of $NH_2OH{\cdot}HCl$ could improve the radiolabeling yield. To mimic the conditions used in automated ^{211}At isolation runs, 3 mL of 10 M nitric acid was used to dissolve 1.0 g of Bi beads and the resultant solution was spiked with 0.5 mL nitric acid solution containing ^{211}At and Bi. Various amounts of 35% $NH_2OH{\cdot}HCl$ was added dropwise, followed by 3-4 mL of 8 M HCl to reconstitute the solution to 1.5 M HCl. Based on Taylor Morscheck's titration studies, to completely destroy the nitrate ions in the nitric acid used to dissolve an irradiated Bi target, about 52.2 mL of 35% $NH_2OH{\cdot}HCl$ is needed. For small-scale isolation runs that have a volume of 3.5 mL, 12.5 mL is the minimal amount of 35% $NH_2OH{\cdot}HCl$ required to destroy all the nitrate ions. Four small-scale ^{211}At isolation runs were conducted using 14.5 mL, 12.5 mL, 11.0 mL and 10.5 mL of $NH_2OH{\cdot}HCl$, respectively. Hydrochloric acid (1.5 M) and water were used to wash the column, and the ^{211}At was eluted using 2 mL of 2 M NaOH. More than 90% of the radioactivity was collected in the first 1 mL of NaOH.

As shown in Table 5, reducing the amount of $NH_2OH{\cdot}HCl$ from 14.5 mL to 10.5 mL did not significantly affect the isolation yield, suggesting reasonable isolation yields could be achieved without completely destroying the nitrate ions. The radiolabeling yields for B10-conjugated MAbs were still low (12-23.4%) without using an oxidant, in spite of the reduction in the amount of $NH_2OH{\cdot}HCl$. As reported in the previous quarterly report, when excess $NH_2OH{\cdot}HCl$ was used for ^{211}At isolation, radiolabeling yields of 40.6%, 53.9% and 88% were obtained when 85 µg, 156 µg and 780 µg of chloramine-T was added, respectively. Here we demonstrated that by reducing the amount of $NH_2OH{\cdot}HCl$, a radiolabeling yield of 82.9% could be obtained by adding just 70 µg of chloramine-T, the same amount regularly used for MAb radioiodination (Table 5). Subsequent small-scale ^{211}At isolation runs were conducted to determine the minimal amount of $NH_2OH{\cdot}HCl$ required to obtain good ^{211}At isolation yields (>80%) and automated runs using full targets will conducted to validate the optimized Te-column isolation method.

Table 5. Isolation and MAb radiolabeling yields of Te-column isolation runs using various amounts of $NH_2OH{\cdot}HCl$

Run#	35% $NH_2OH{\cdot}HCl$ (mL)	Isolation Yield %[a]	B10-conjugated MAb Labeling Yield%
1	14.5	79.6(83.3)	10.4
2	12.5	83.3(86.6)	17
3	11.0	81.5(84.8)	12
4	10.5	77.3(80.5)	23.4 (82.9)[b]
5	11.0	78.3(82.6)	26%(58%)[b]
6	9.0	82.2(85.5)	21%

[a]Decay corrected isolation yields are in parentheses. [b]30-70 µg chloramine-T was added (10% of the total volume of the reaction mixture).

Two ^{211}At isolation experiments were performed using the Te-packed columns to evaluate whether B10-conjugated MAb radiolabeling experiments could be improved by decreasing the amount of $NH_2OH{\cdot}HCl$ used to destroy nitrate ions. The first experiment was performed to repeat conditions previous runs. In the second experiment, the amount of $NH_2OH{\cdot}HCl$ was further reduced to 9 mL, which is about 64% of the $NH_2OH{\cdot}HCl$ needed for the reaction to complete/bubbling to stop. As shown in Table 5, the isolation yields and radiolabeling yields were about the same as before. The radiolabeling yield was increased more than 2 times in presence of chloramine-T, as expected.

An evaluation of another approach to dissolution of the bismuth target without using strong nitric acid was conducted as an alternative to destroying the nitrate ions. An undergraduate research student, Marina Llewellyn, tried a number of mixed solutions of H_2O_2 and HCl for dissolving Bi metal. It is known that concentrated HNO_3 dissolves Bi metal rapidly at room temperature, whereas concentrated HCl slowly dissolves Bi metal in presence of oxygen and hot H_2SO_4 only slightly attacks Bi. The DIPE extraction method and the Te-packed column method requires a distillation step and a $NH_2OH{\cdot}HCl$ reduction step, respectively, to get rid of HNO_3. Being able to directly dissolve Bi targets in HCl could simplify the isolation process and reduce isolation time. Therefore, we wanted to evaluate whether the addition of an oxidant could improve the solubility of Bi in HCl. Hydrogen peroxide is an attractive oxidant for this purpose because it only generates oxygen and water. Marina evaluated 5% H_2O_2 in 6 M HCl, 10% H_2O_2 in 6.7 M HCl and 20% H_2O_2 in 4 M HCl for dissolving Bi metal at room temperature. Unfortunately, none of the mixtures could dissolve Bi metal at a reaction rate comparable to concentrated HNO_3. Some bubbling (colorless) was observed in all of those experiments, which might be mostly O_2 generated from decomposition of H_2O_2. When NaOH was slowly added to the solution, white precipitate was observed, suggesting a small amount of Bi was dissolved.

The previous results suggested that ^{211}At isolated using the Te column requires an oxidizing agent to provide high astatination yields for B10-conjugated antibodies. We tried to mitigate this problem by reducing the amount of $NH_2OH{\cdot}HCl$ used to reduce the nitrate in the ^{211}At and Bi mixture, as we found it is unnecessary to eliminate all the nitrate to achieve high isolation yields. We previously determined the minimal volume of 35% $NH_2OH{\cdot}HCl$ required to eliminate the nitrate in the Bi/HNO_3 solution obtained by dissolving a full target is about 52 mL (V_{min}). In order to determine the optimal volume of $NH_2OH{\cdot}HCl$ that provides high isolation and radiolabeling yields, our effort progressed to performing a number of small-scale isolations using different amounts of $NH_2OH{\cdot}HCl$. The conditions and results of six ^{211}At isolation experiments conducted are summarized in Table 6.

Table 6. Isolated yields of ^{211}At and antibody labeling yields using the Te column with various amounts of $NH_2OH{\cdot}HCl$

Run#	35% $NH_2OH{\cdot}HCl$ (%V_{min})	Isolation Yield %[a]	B10-conjugated MAb Labeling Yield%
1	52	80.1(84.7)	82.5
2	46	67.2(69.5)	72.9
3	46	67.4(70.9)	7.5
4	100	75.9(79.6)	23.7
5	70	67.5(70.7)	38.5
6	52	71.7(74.9)	34.0

[a]Decay corrected isolation yields are in parentheses.

Runs 1-2 provided high antibody radiolabeling yields that are comparable to those obtained with ^{211}At isolated using the "wet chemistry" method. For the isolation of ^{211}At, we followed the similar protocol described before. Briefly, we dissolved 1 gram of Bi in 3.5 mL of 10 M HNO_3 and the resultant solution was spiked with 0.5 mL concentrated HNO_3 containing dissolved Bi target (irradiated). After the addition of $NH_2OH{\cdot}HCl$, 8 M HCl was added to make the solution approximately 1.5 M HCl. Then the ^{211}At/Bi mixture was loaded onto the Te column, followed by 20 mL of 1.5 M HCl and 20 mL of H_2O. The purified ^{211}At was eluted using 1 M NaOH. About 85% and 70% decay corrected isolation yield was obtained when 52% and 46% of V_{min} was used, respectively.

Encouraged by the results obtained, the same conditions (Runs 3 and 6) were repeated. However, the radiolabeling yields were 59-90% lower than what was obtained previously. There were some differences in how the ^{211}At/Bi mixture was obtained. An irradiated Bi target was dissolved in concentrated HNO_3 (15.8 M) by being placed Bi face down in a plastic container. The solution containing the dissolved target was split into four 3.7 mL fractions, diluted 1.58 times and used for 4 parallel ^{211}At isolation experiments (Run 3-6). By visually examining the target body after the residual ^{211}At was decayed, we observed a significant amount of Bi metal left on the target body, suggesting there is less Bi in the ^{211}At/Bi HNO_3 solution than there usual.

Run 4 was conducted as a control group. $NH_2OH{\cdot}HCl$ was added until the cessation of bubbling. A 79.6% decay-corrected isolation yield was obtained, and the isolation yields of run 3 and run 5-6 were about 5-10% lower. Run 5 was conducted using 70% of V_{min} to compare with the conditions where 52% or 46% of V_{min} was used. It appeared that a slight better antibody radiolabeling yield could be obtained, but the experiment needed to be repeated a few more times.

Based on the previous studies, using about 52% of the minimal volume of 35% $NH_2OH{\cdot}HCl$ required to destroy all the nitrate (V_{min}) prior to loading onto the Te column provides high ^{211}At isolation yields as well as high B10-conjugated MAb labeling yields. Two ^{211}At isolation experiments were conducted using 45% and 40% of V_{min}. The results are summarized in Table 7. Using 45% and 40% of V_{min} provided good MAb labeling yields, however, the ^{211}At isolation yields are about 5% lower than those obtained using 52% of V_{min}.

Table 7. Percent yields of ^{211}At isolation and MAb labeling conducted using various amounts of 35% $NH_2OH{\cdot}HCl$

Run#	35% $NH_2OH{\cdot}HCl$ (%V_{min})	Isolation Yield %[a]	B10-conjugated MAb Labeling Yield%
1	45	78.4(80.5)	76.6
2	40	77.4(79.8)	70.0

[a]Decay corrected isolation yields are in parentheses.

B. *Specific Objective 2:* *Evaluate ^{211}At species produced and its reactivity under various conditions*

This task was not conducted as planned as there were difficulties in identifying the radiopeaks on radio-HPLC. It was felt that these studies would be better conducted where mass spectral data could be obtained to better confer the product structure. Those studies are planned. However, as a first step we thought it important to ascertain whether the conversion of astatide to the reactive At+ or AtO+ required air for the oxidation process. Therefore, some simple labeling experiments with and without oxygen or other oxidant (i.e. chloramine-T) present were conducted. The experiments were conducted with B10 conjugated MAb in sodium phosphate solution at pH 6.8. The reactions were conducted in air-tight microwave sealed tubes where the reactions could be conducted under Ar atmosphere. All reagents were sparged with Ar for 1-hour before use. The MAb-B10 conjugate used was stored under Ar for 3 days prior to use. When used, chloramine-T was added immediately after the ^{211}At solution. Radiolabeling results are shown Figure 6 as differences in the peaks obtained by size-exclusion radiochromatography.

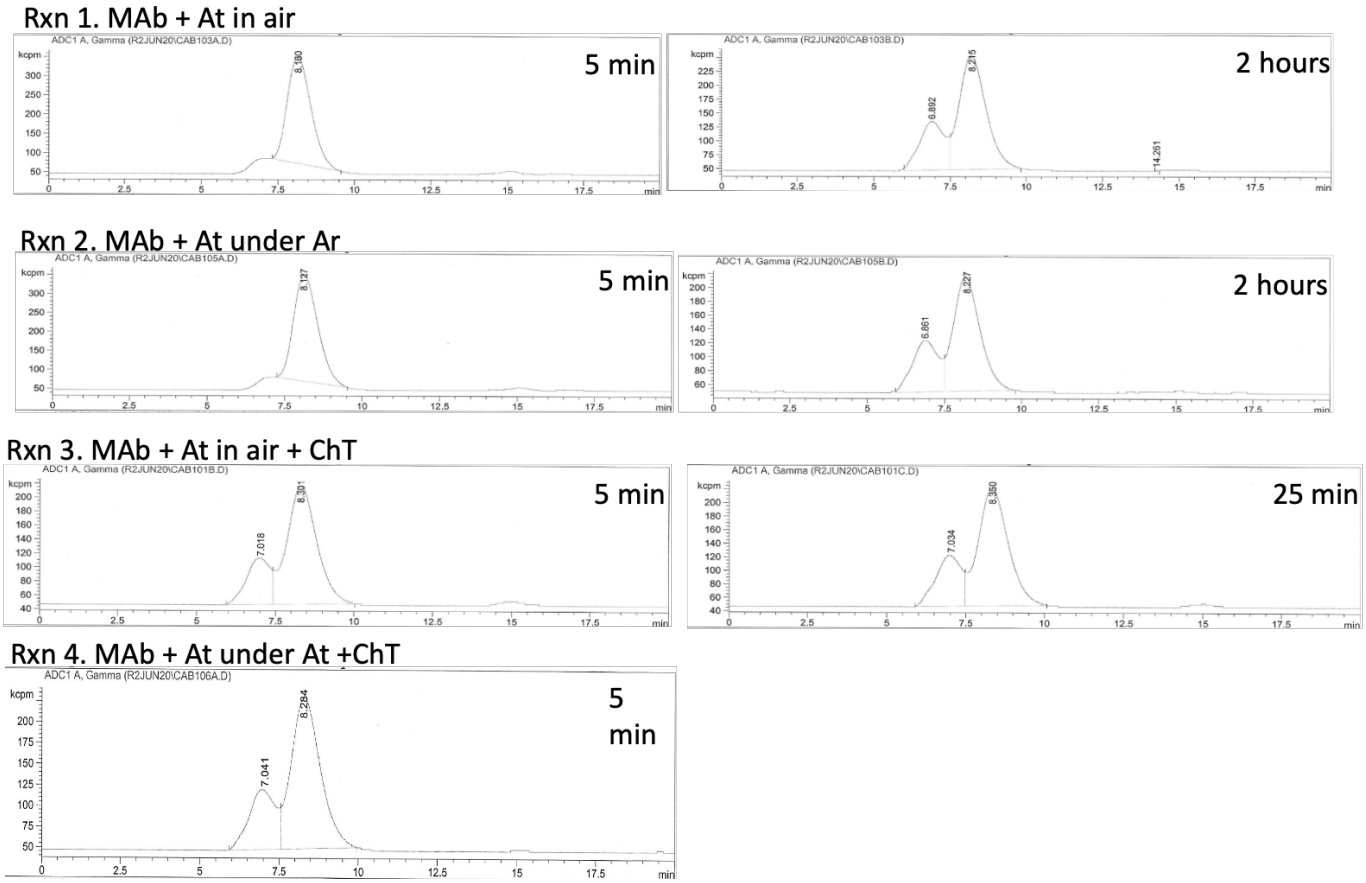

Figure 6: Size exclusion radiochromatograms of At-211 labeling reactions in the presence and absence of air (under Ar) and with/without the oxidant chloramine-T.

The results suggest that oxygen is not the agent that oxidized At- to At+ (or AtO+). The radiolabeling yields appear equivalent with/without air, and the subsequent formation of a higher molecular weight MAb species (probably MAb-B10-MAb) occurs over time irrespective. Addition of chloramine-T causes dimer formation more rapidly. These results need to be confirmed by additional testing. Another reaction that was run included DTPA to chelate trace metals and determine if metals were responsible for the oxidation process. The results of that experiment suggest that there is an effect on oxidation, but it needs to be conducted again.

C *Specific Objective 3: Evaluation of new methods for labeling small molecules with ^{211}At*

Our research group has been investigating ^{211}At-labeled molecules for over 30 years, but neither we (nor anyone else) have been able to obtain a suitable chemical structure or conjugation moiety that can be used for ^{211}At-labeling of small molecule cancer-targeting agents. While we have demonstrated that ^{211}At-labeled antibody can eradicate disseminated B-cell lymphoma (6), our inability to prepare ^{211}At-labeled biotin derivatives for pretargeting has kept us from employing this important approach to cancer targeting. We have prepared many ^{211}At-labeled biotin derivatives (7-10), but have not been able to obtain derivatives that are both stable in vivo and provide cancer targeting similar to the radiometal derivatives of biotin-DOTA derivatives (11-14).

Our goal in the studies was to find an alternative pendant group for labeling small molecules and peptides with ^{211}At, with more favorable in vivo pharmacokinetic properties. It was of interest to determine if phenyl iodonium salts would facilitate astatination reactions of borane cage molecules. There was an interest in evaluating thiol-containing molecules as reagents to chelate ^{211}At. The dithiol-containing molecule lipoic acid and some trithiols (from Dr. Jurisson's lab) were investigated as chelates for ^{211}At, but attempts to get that reaction to work failed. We now believe that the thiols are too reductive, resulting in disulfide and astatide formation. Several attempts indicated that this approach would not provide the chelated astatine (+3 or +5 state) that we wanted (under the conditions tried). Therefore, our focus was turned to developing new labeling reagents that used boron cages. We have prepared a large number of protein-reactive derivatives of the different types of boron cage molecules (9, 15-22).

There have been a large number of examples where the boron cage moieties have been incorporated into biomolecules for studies in Boron Neutron Capture Therapy (23-27). It should be noted that these *boron cage aromatic compounds are similar in size to a phenyl group* (~25% larger), so they can be incorporated into biological molecules readily. Importantly, it was also shown that the aromatic boron cage moieties underwent electrophilic halogenation reactions similar to halogenation of phenyl groups (28-30). Since, in general, boron-halogen bonds have a higher bonding energy than the corresponding carbon-halogen bonds, it seemed possible that such moieties could be more stable to in vivo deastatination. Our prior studies had shown that monoanionic monocarbon carboranes could be astatinated, but very slowly and in low yields. Therefore, we wanted to evaluate reagents that would facilitate astatination of monocarbon carboranes. One method that was chosen to study was using an iodonium intermediate in a nucleophilic displacement reaction using astatide.

SO3a: ^{211}At-labeling of monocarbon carboranes using iodonium salts

Two monocarbon carboranes (**2** & **6**) along with their iodonium salts (**3** & **7**) were targeted for synthesis. Each of the monocarbon carboranes contained a carboxylate group that could be used to attach the moiety to disease-targeting agents. Syntheses of two monocarbon carboranes (**2** & **6**) and their iodonium salts (**3** & **7**) were conducted as described in a literature reports (31-33). The syntheses are shown in Figure 7.

Some steps of the monocarbon carborane syntheses are not shown for simplicity, but are outlined here (31). The first step in the synthesis of the monocarbon carborane **2** is reaction of glycolic acid monohydrate, **1**, with decaborane ($B_{10}H_{14}$) under basic conditions to form the borane adduct, *arachno*-6-CB_9H_{13}-6-CO_2H anion. The second step in the synthesis of **2** involves removal of two protons with base and ring closure to form the *closo*-2-CB_9H_9-CO_2H anion. The more stable *closo*-1-CB_9H_9-CO_2H anion is formed from the *closo*-2-CB_9H_9-CO_2H using thermal rearrangement in refluxing acetonitrile. The same reaction conditions were used to prepare the 1-(*para*-carboxyphenyl-*closo*-CB_9H_9 anion, **6**. Iodination of carborane **2** was previously accomplished using N-iodosuccinimide (NIS) in acetonitrile at ice bath temperature (32), but was not needed here as the next step involves preparation of an iodonium salt directly from a phenyl iodonium diacetate derivative. Preparation of the B-iodonium salts **3** and **7** followed the literature procedure for making the corresponding *closo*-$B_{12}H_{11}$-1-iodophenyl derivative (33).

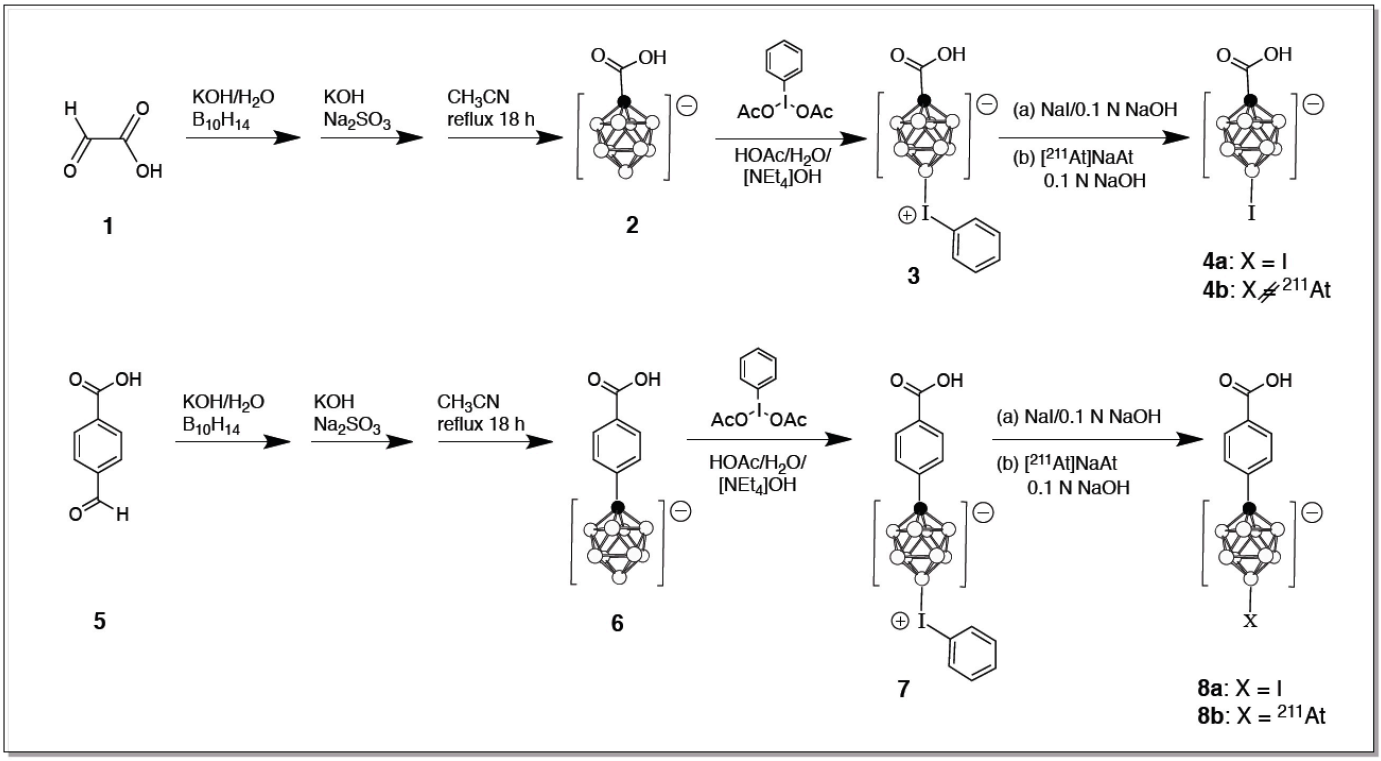

Figure 7: Schemes showing the syntheses of phenyl iodonium salts of monocarbon carboranes, iodination and astatination reactions. (Hydrogen atoms are not shown on boron cage for simplicity; thus, white circles represent BH atoms and the dark circle represents a carbon atom)

Reaction of sodium iodide with the iodonium salt **3** provided the iodinated derivative **4a**, but the reaction of [^{211}At]NaAt to form **4b** gave unexpected results (not sure of product). The RP-HPLC retention time for **4b** was quite different from the iodinated derivative (12.9 min vs. 14.9 min), making it questionable whether that compound was obtained. The experimental procedures for these reactions are not repeated here as they have been reported in the literature. However, synthetic methods for the remaining synthetic steps are provided below.

Initial studies with monocarbon carboranes **2** and **3** demonstrated that they were not very stable, and the questionable results obtained after astatination, led us in subsequent studies to concentrate on the monocarbon carborane substituted with a *p*-benzoate moiety, **6**. Therefore, we prepared the 10-phenyliodonium salt of The iodonium salt **7** was obtained in high purity, as shown in Figure 7. Reaction of the phenyl iodonium salt of C-1-*para*-benzoate monocarbon derivative **7** with NaI appeared to provide the iodinated derivative **8a**, but that needed to be confirmed with an iodo standard. As an alternative to the previously reported iodination using NIS, an iodination reaction using the conditions that we have found to be efficient in iodination was conducted with 1-(*para*-carboxyphenyl-*closo*-CB_9H_9 anion, **6** to obtain an iodinated standard, **8a**. To show the conversion to iodinated product **8a**, only half of the quantity of iodide was used, so the HPLC shows both starting material, **6** (t_R = 10.9 min) and iodinated product **8a** (t_R = 12.1 min), as shown in Figure 8.

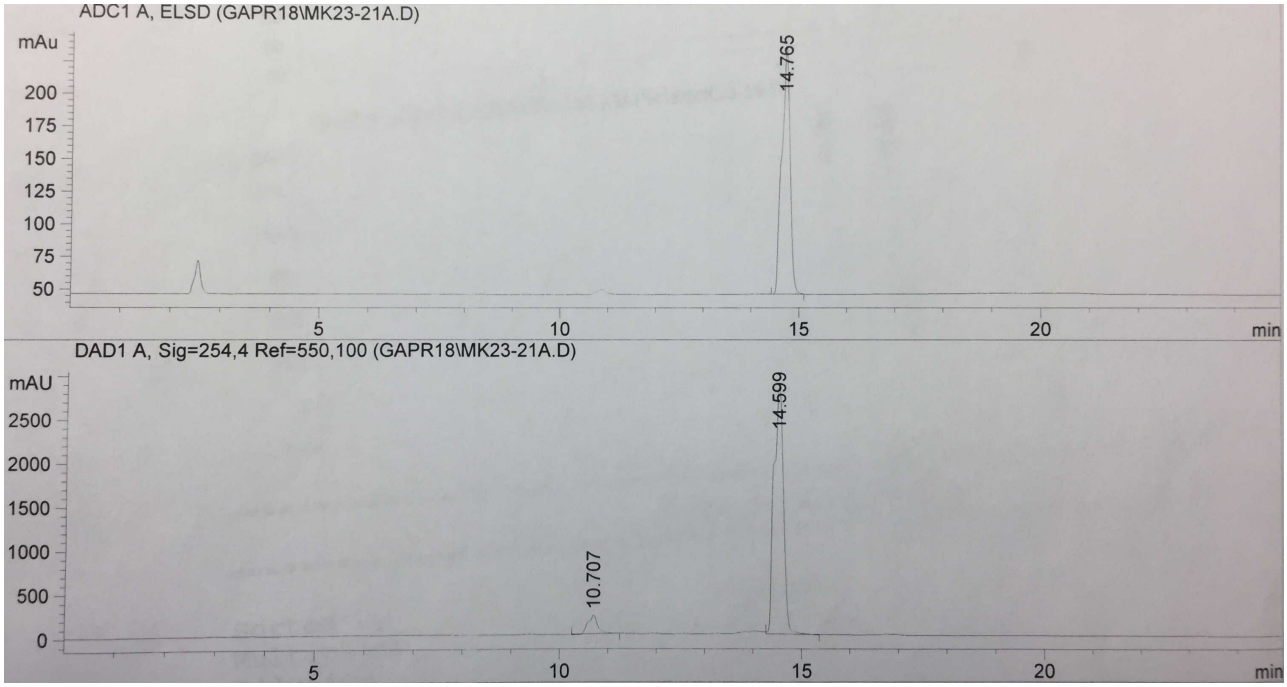

Figure 7: Reversed-phase HPLC chromatograms of the phenyliodonium salt of 1-(*p*-benzoate)-CB_9H_9, **7**. Bottom chromatogram is UV (254) detection and top chromatogram is detection by ELSD. Difference in retention times comes from time to flow from first detector (UV) to the second detector (ELSD).

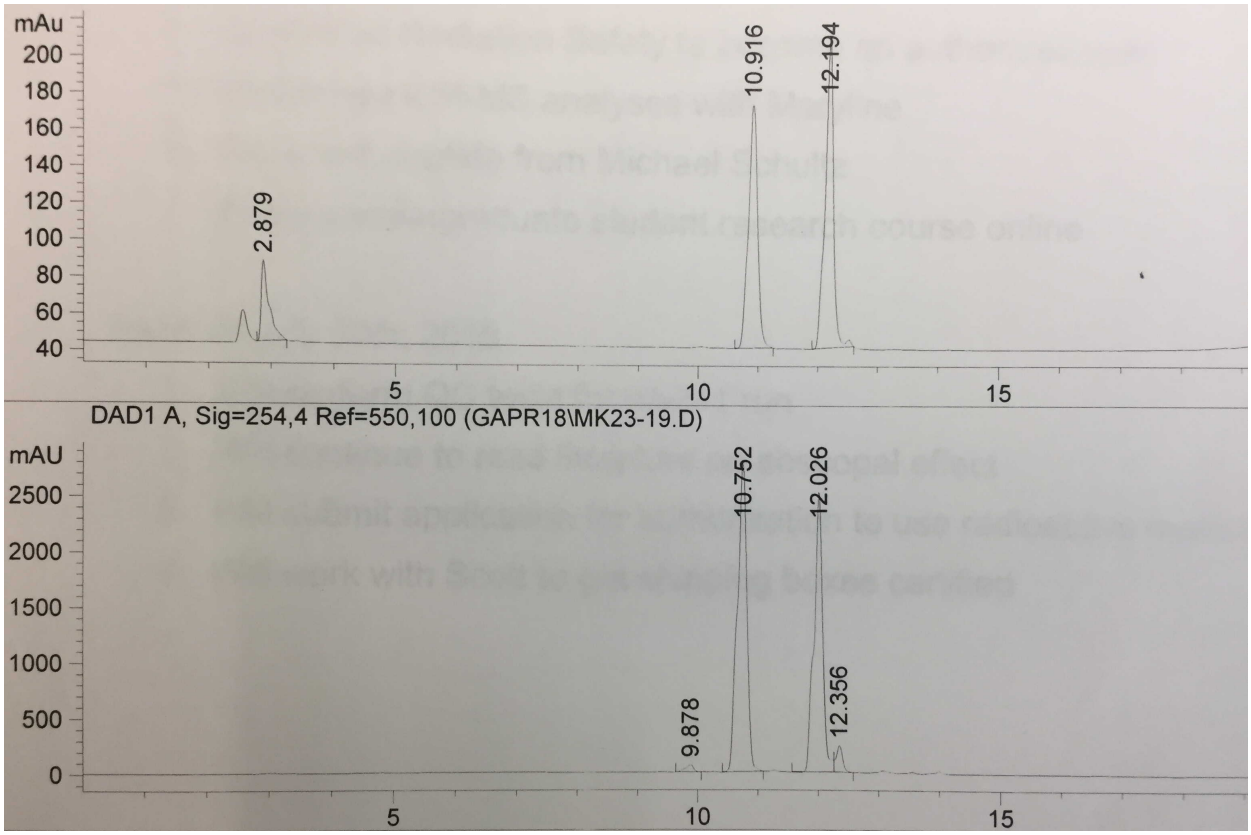

Figure 8: Reversed-phase HPLC chromatograms of reaction product from electrophilic iodination (50% equivalence of iodide) of 1-(*p*-benzoate)-CB_9H_9, **6**, to yield 1-(*p*-benzoate)-CB_9H_8-10-I, **8a**. Bottom chromatogram is UV (254) detection and top chromatogram is detection by ELSD. Difference in retention times comes from time to flow from first detector (UV) to the second detector (ELSD).

The iodonium salt **7** was reacted with [^{211}At]NaAt in 0.1 M NaOH/CH_3CN at 80°C for 10 min to determine if we could prepare the astatinated product **8b** using this approach. The product mixture obtained is shown in Figure 9. Since the corresponding (electrophilic) iodinated compound **8a** had a retention time of 12.2 min, it

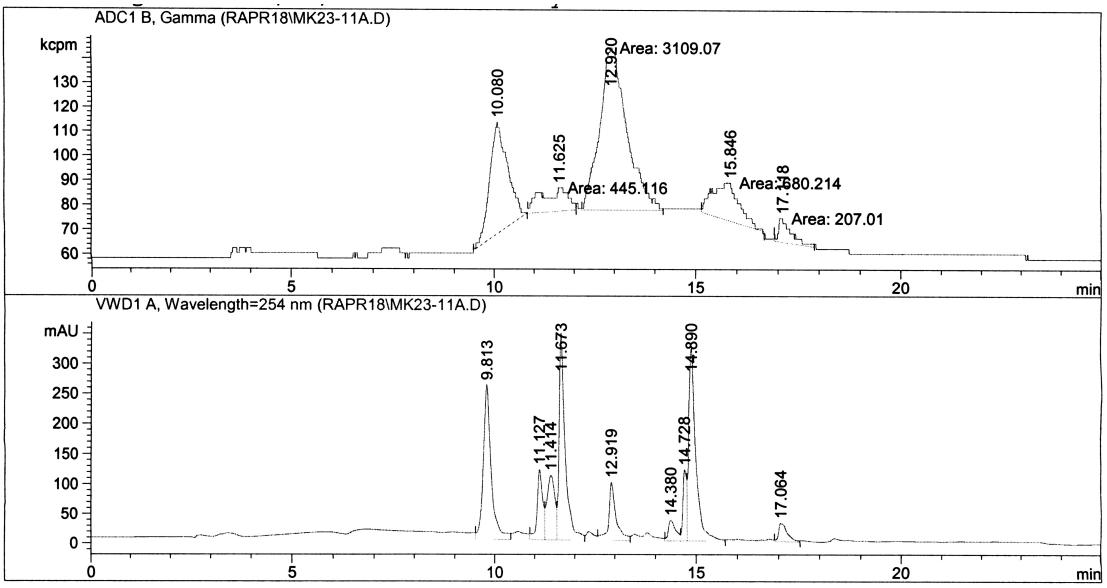

Figure 9: Product mixture from reaction of UV (bottom) and radio(top) chromatograms obtained from the reaction of iodonium salt **7** with [^{211}At]NaAt

seems likely that the reaction provided **8b** in 53% yield based on the radio-HPLC results (t_R = 12.9 min). However, the astatination reaction conditions also resulted in other ^{211}At-labeled species and converted the starting iodonium salt (t_R = 14.9 min) into other derivatives. Therefore, studies were conducted to determine the nature of the impurities found in the astatination reaction of iodonium salt **7**. We were concerned that some of the non-radioactive UV absorbing peaks seen in Figure 9 came from decomposition of the iodonium salt (t_R = 14.9 min), so studies were conducted to determine if decomposition of the iodonium salt **7** occurred under the reaction conditions without adding the halide. Reactions were carried out in the Biotage microwave at 40°C, 50°C and 80°C for up to 3 hours. All temperatures appeared to result in some decomposition of the iodonium salt, but the decomposition at 40°C reaction temperature was slow, whereas higher temperature reactions showed major decomposition of the iodonium salt, **7** (Figure 10). From the HPLC chromatograms, it is evident that the decomposition of the starting iodonium salt is rapid at 80°C and that the new peaks formed in the UV chromatogram from the astatination reaction are due to decomposition or side reactions to form new carborane species. This data suggests that when using the iodonium salt of the monocarbon carborane **7**, the temperature of reaction should not exceed 40°C. Subsequent astatination reactions will be conducted at room temperature and 40°C, in different solvents with varying quantities of base or reductant.

HPLC chromatograms for products obtained when the iodonium salt **7** was subjected to different temperatures are shown in Figure 10. It can be noted that there are pairs of peaks in the chromatograms. We originally thought that the duplicate peaks might be from a channel in the reversed-phase HPLC column, or more likely they were from regioisomeric products. Interestingly, in the original RP-HPLC chromatogram of pure **7**, there is a leading-edge shoulder apparent on the peak. We thought that there might be a slow rearrangement to another regioisomeric product (e.g. 6 substituted) that occurs, and the leading-edge peak becomes more prominent with time. We noted that at lower temperatures the iodonium salt **7** was primarily converted to another species, so we sought to find out what that species is. Mass spectral analysis of peaks collected from 9.4-9.8 min retention time indicated that the product was a hydroxyl monocarbon carborane derivative. Additionally, an electrophilic N-chlorosuccinimide/sodium iodide reaction with phenyliodonium salt of monocarbon carborane **7** was conducted to evaluate whether a different iodinated reaction product would be obtained from the iodonium salt.

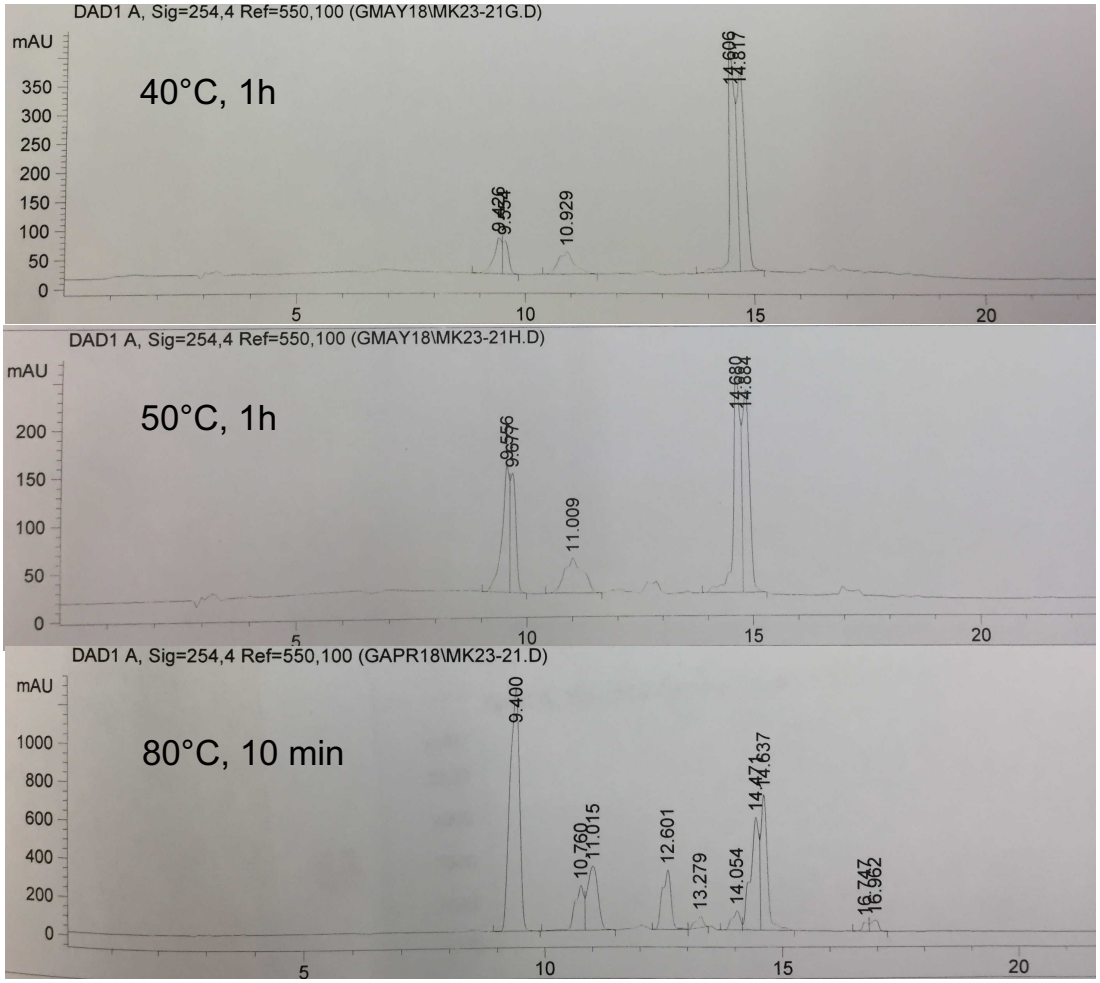

Figure 10: Product mixtures from stability tests on iodonium salt **7** in mixture of NaOH/H_2O/CH_3CN conducted at 40°C, 50°C and 80°C. All chromatograms used UV detection.

Iodonium salt **7** provided a 53% radiolabeling yield when reacted with [^{211}At]NaAt at 80 ºC for 10 min. However, the UV chromatogram suggested iodonium salt **7** rapidly decomposes at 80 ºC (Figure 10). We evaluated astatination reactions at room temperature and 50 ºC. As shown in Figure 11, the labeling yield was 7.9% at room temperature but increased to 41.9% at 50 ºC. Increasing the reaction time from 30 min to 60 min at 50 ºC further improved the radiolabeling yield to 55.7%, which is similar to the radiolabeling yield obtained at 80 ºC. In all these reactions, the retention time of the product peak, around 12.7 min, is consistent with that of the [^{125}I]**8a** standard obtained from electrophilic reaction (Figure 12). Although lowering the reaction temperature might slow down the decomposition of the iodonium salt **7**, it did not significantly improve the ^{211}At radiolabeling yield. It should be noted that two radiopeaks around 6.8 min and 14.4 min increased as the reaction time extended to 1 h, which is likely due to the decomposition of the iodonium salt.

SO3b: ^{211}At-labeling of monocarbon carboranes directly

Although astatination of *closo*-decaborate(2-) provided high radiolabeling yields (>80%) at room temperature without use of oxidant, it was expected monocarbon carborane derivatives might require higher reaction temperature or use of an oxidant to achieve such high radiolabeling yields. Preliminary labeling studies showed the addition of chloramine-T did not provide the desired product but generated a radiopeak around 3.6 min. While not confirmed, this peak was believed to be that of astatate ion.

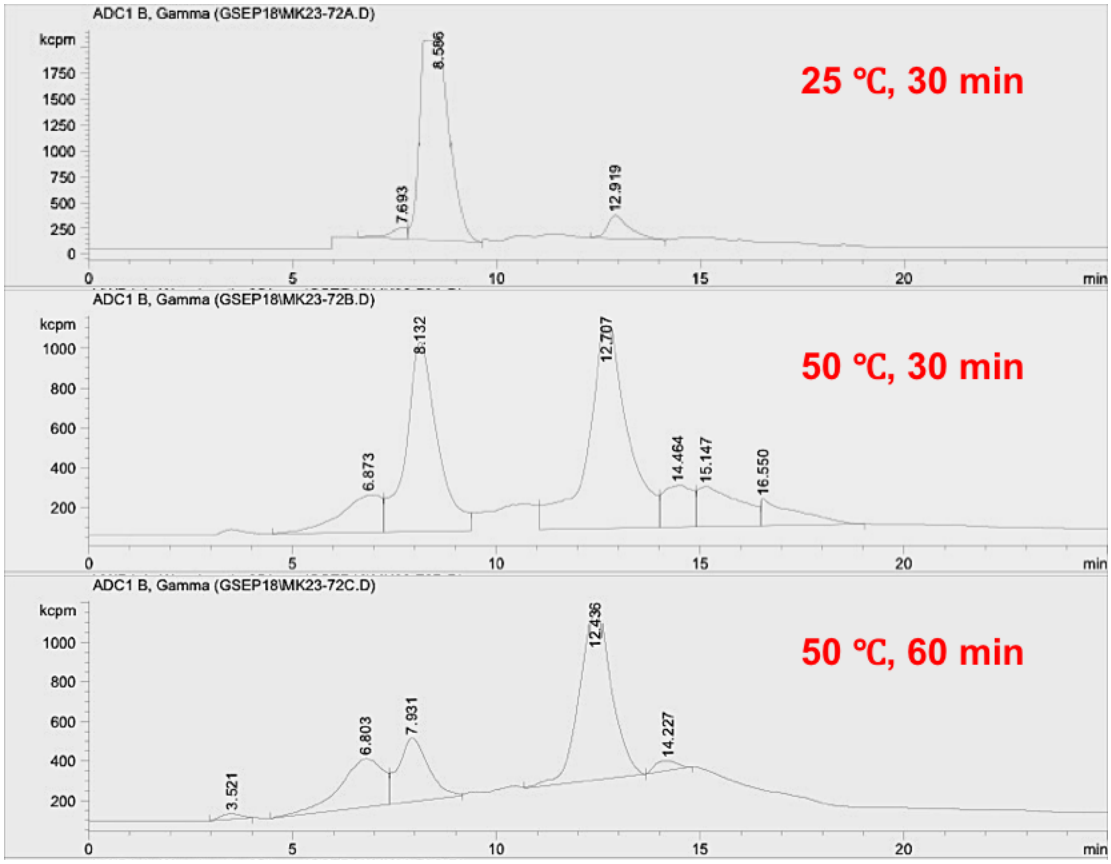

Figure 11. Radio-chromatograms of product mixtures obtained from the reactions of iodonium salt **7** with [^{211}At]NaAt. Peak at ~ 8 min is astatide.

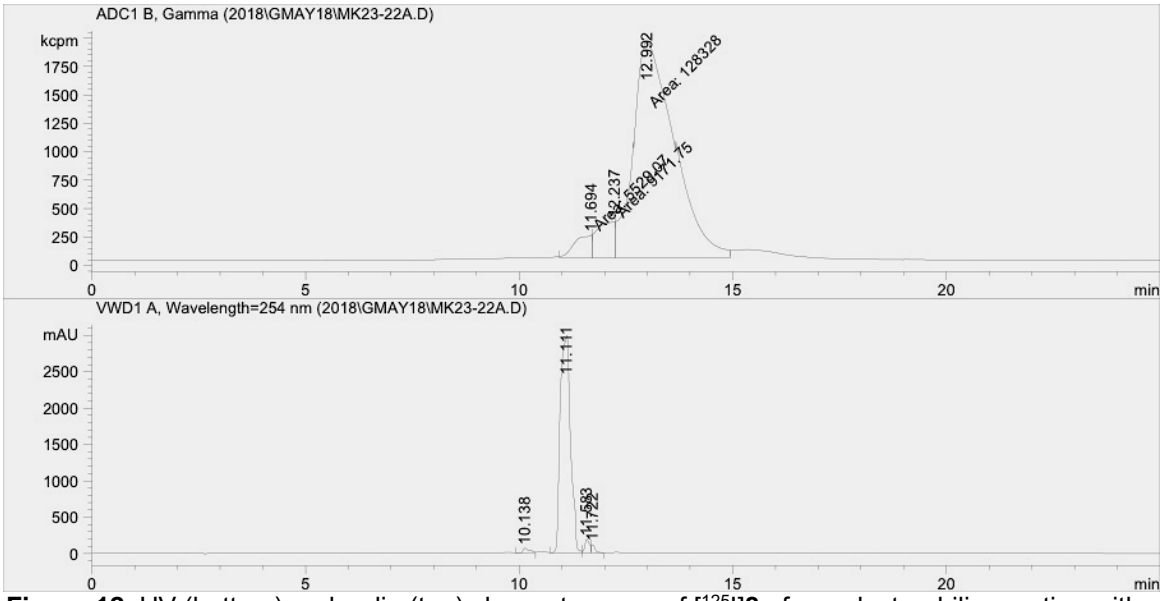

Figure 12. UV (bottom) and radio (top) chromatograms of [^{125}I]**8a** from electrophilic reaction with ^{211}At.

From the earlier studies it appeared that the 10-hydroxyl derivative **9** might be readily prepared as depicted in Figure 13. A small (50 mg) preparative reaction was conducted at 80°C for 30 min, and a 77% isolated yield of **9** was obtained. This new monocarbon carborane was of interest as it is less lipophilic and should be more activated to radiohalogenation than the monocarbon carborane **6**. Indeed, electrophilic iodination

of 10-hydroxyl-monocarbon carborane **9**, provided the iodinated product (Figure 15). As done previously, only 50% of the equivalent of NaI was used so that one could see both the starting material, **9**, and the product, **10a**.

Radioiodination and astatination reactions with compound 7 were conducted.

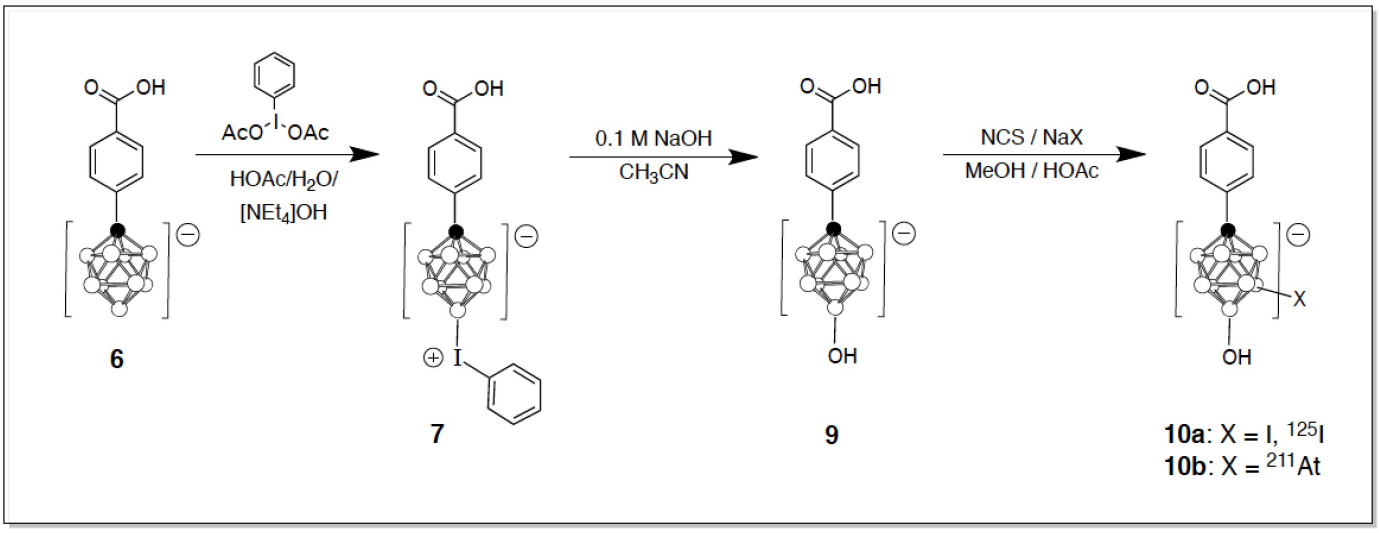

Figure 13: Schemes showing the syntheses of 10-hydroxyl-1-*p*-benzoate-monocarbon carborane and its possible iodination and astatination reaction products. (Hydrogen atoms are not shown on boron cage for simplicity; thus, white circles represent BH or B atoms and dark circle represents a carbon atom).

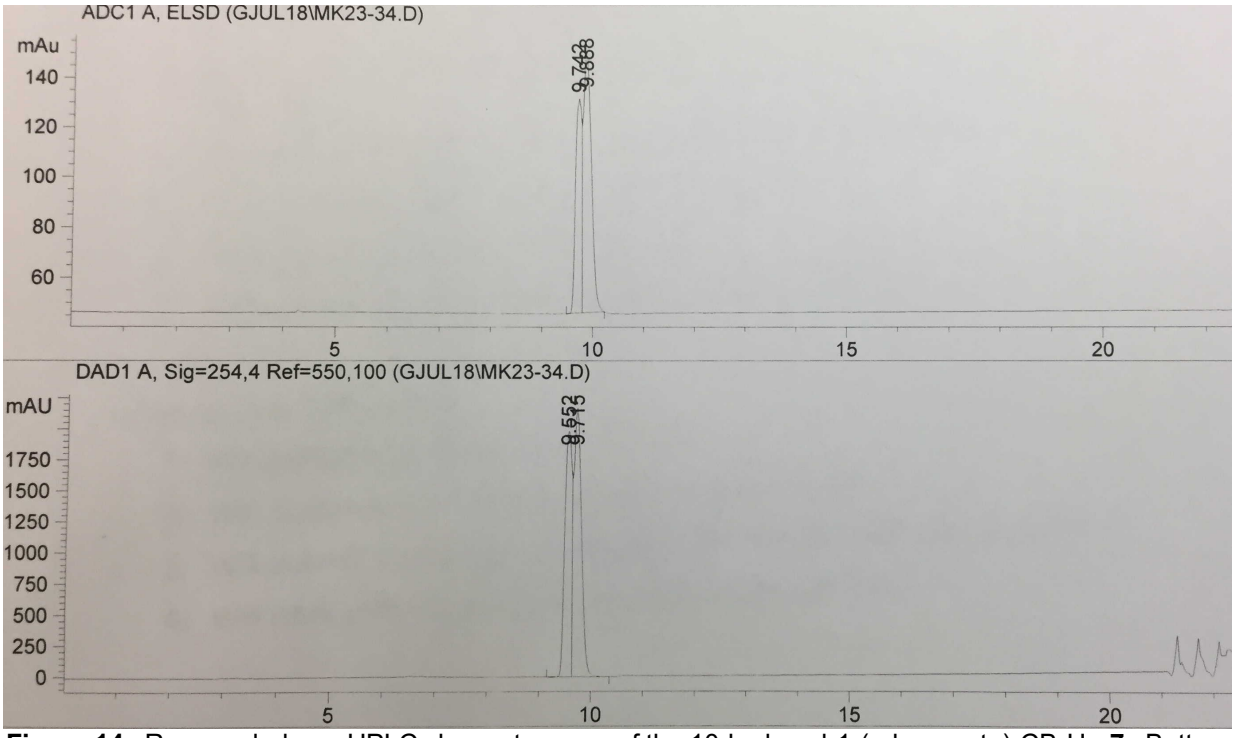

Figure 14: Reversed-phase HPLC chromatograms of the 10-hydroxyl-1-(*p*-benzoate)-CB_9H_9, **7**. Bottom chromatogram is UV (254) detection and top chromatogram is detection by ELSD. Difference in retention times comes from time to flow from first detector (UV) to the second detector (ELSD).

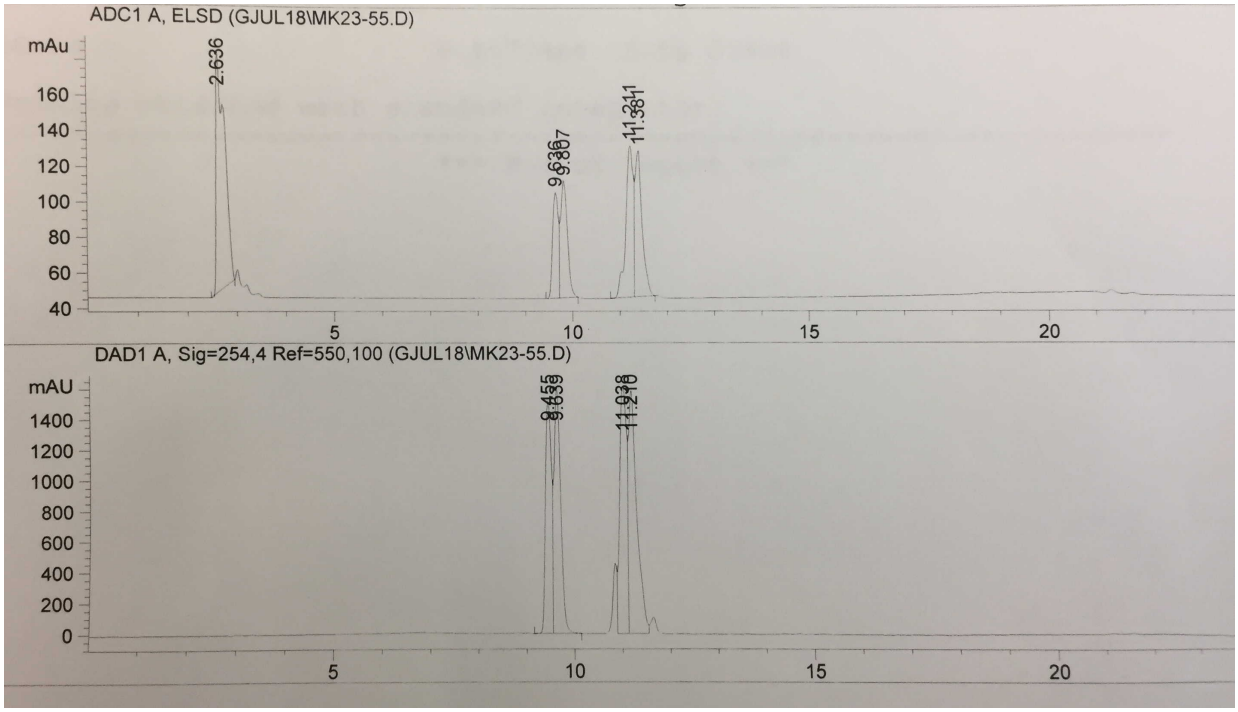

Figure 15: Reversed-phase HPLC chromatograms of reaction product from electrophilic iodination (50% equivalence of iodide) of 10-hydroxyl-1-(*p*-benzoate)-CB_9H_9, **9**, to yield 10-hydroxyl-1-(*p*-benzoate)- CB_9H_8-6-I, **8a**. Bottom chromatogram is UV (254) detection and top chromatogram is detection by ELSD.

In addition to radioiodination, astatination reactions of **9** were evaluated. Interestingly, astatination of **9** at 80 ºC for 30 min resulted in a radiolabeling yield > 78% (Figure 17D). Electrophilic radioiodination using [^{125}I]NaI and NCS at room temperature provided a radiolabeling yield >98%, as shown in Figure 16 (integration yield). The retention time of [^{125}I]**10a** is around 11.7 min, which is consistent with the iodinated **10a** standard (Figure 13). However, the astatination reaction at room temperature in NH_4Ac buffer (pH 5.5) provided a modest yield of 35.4% with a shoulder peak around 11 min (Figure 17A). The radiopeak at 8.2 min is believed to be unreacted astatine. Two other radiopeaks were observed around 3.4 min and 13.4 min, respectively. Interestingly, astatination of **9** at 80 ºC for 30 min resulted in a radiolabeling yield > 78% (Figure 17D).

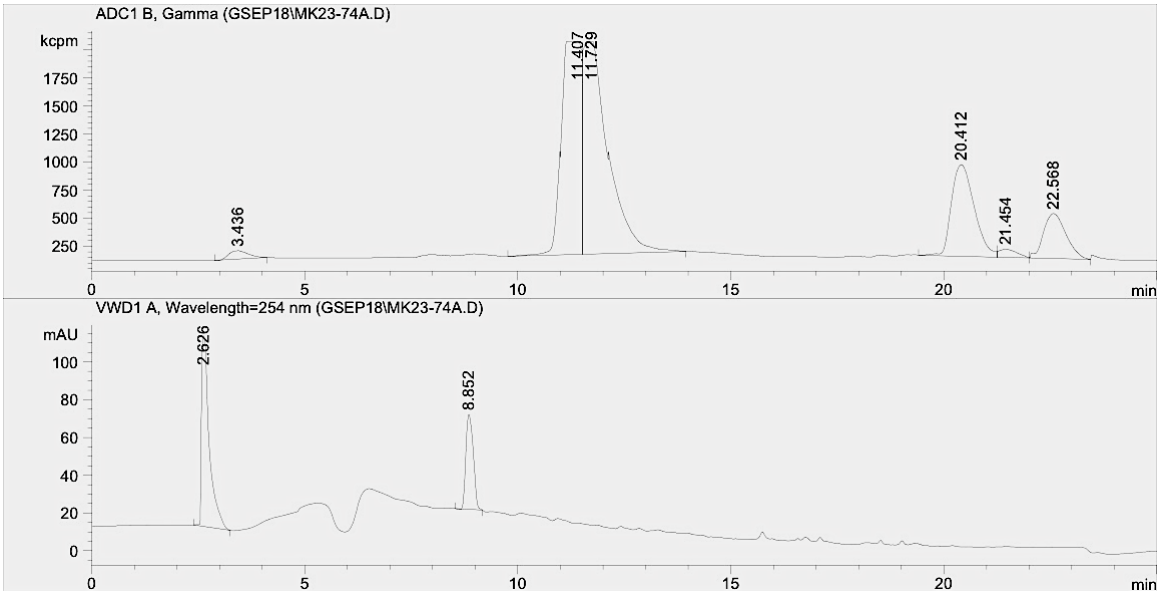

Figure 16. Radio (top) and UV (bottom) chromatograms of reaction mixture obtained from the reaction of 10-hydroxyl-monocarbon carborane **9** with [^{125}I]NaI.

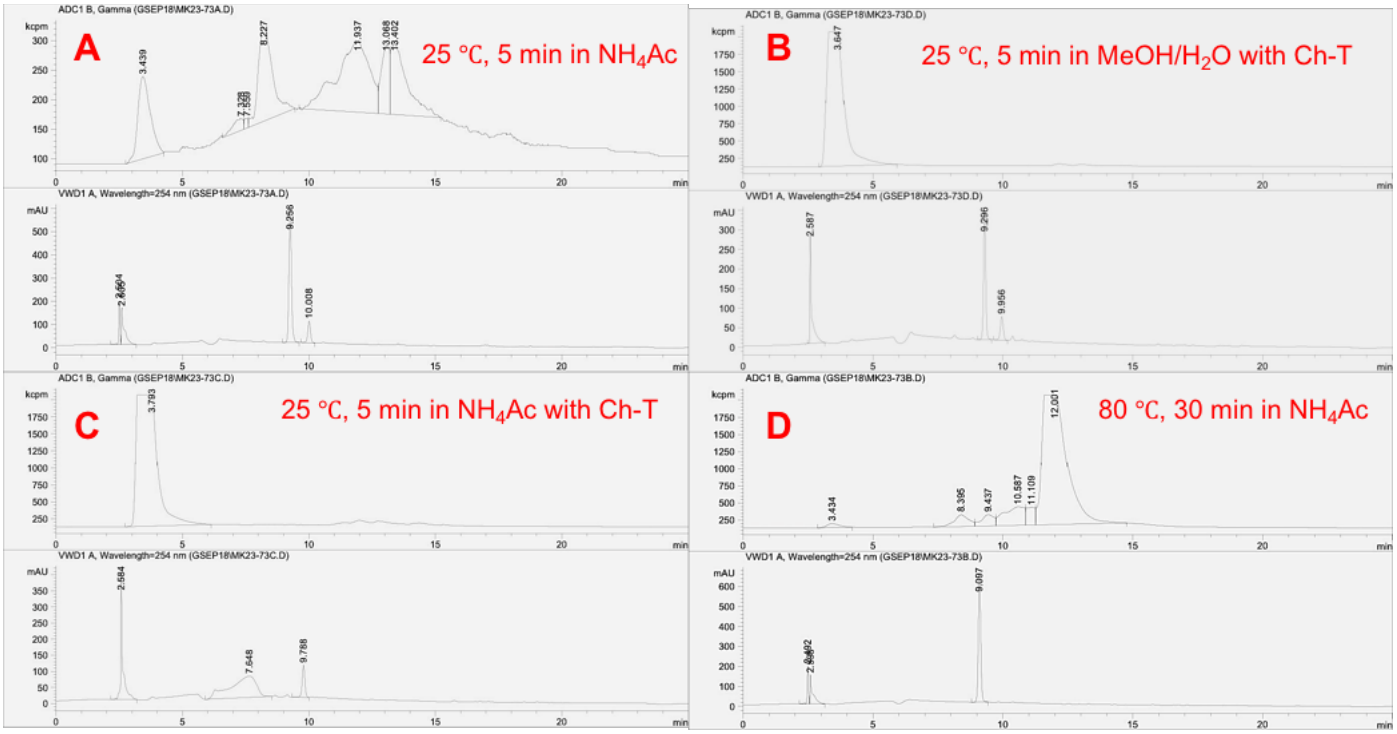

Figure 17. Radio (top) and UV (bottom) chromatograms of reaction mixtures obtained from the reactions of 10-hydroxyl-monocarbon carborane **9** with [^{211}At]NaAt. (A) 0.1 mg of **9** was reacted with ^{211}At in NH_4Ac buffer (pH 5.5) at room temperature for 5 min. (B) 0.1 mg of **9** was reacted with ^{211}At in MeOH/H_2O with 20 µg of Ch-T at room temperature for 5 min. (C) 0.1 mg of **9** was reacted with ^{211}At in NH_4Ac buffer (pH 5.5) with 20 µg of Ch-T at room temperature for 5 min. (D) The reaction mixture (A) was heated at 80 °C by microwave for 30 min.

Additional ^{211}At labeling experiments were conducted without oxidant (no chloramine-T) to evaluate radiolabeling yields. Since the 10-hydroxyl monocarbon **9** provided the best yields previously (Fig. 17, panel D), an ^{211}At labeling experiment was conducted under the same reaction conditions (MeOH, NH_4OAc, pH 5.5., 80°C, 30 min) but with 0.2 mg **9**. The reaction provided nearly quantitative yield of the desired ^{211}At-labeled compound **10b**, as shown in Figure 18. Radiolabeling reactions of the non-hydroxylated monocarbon

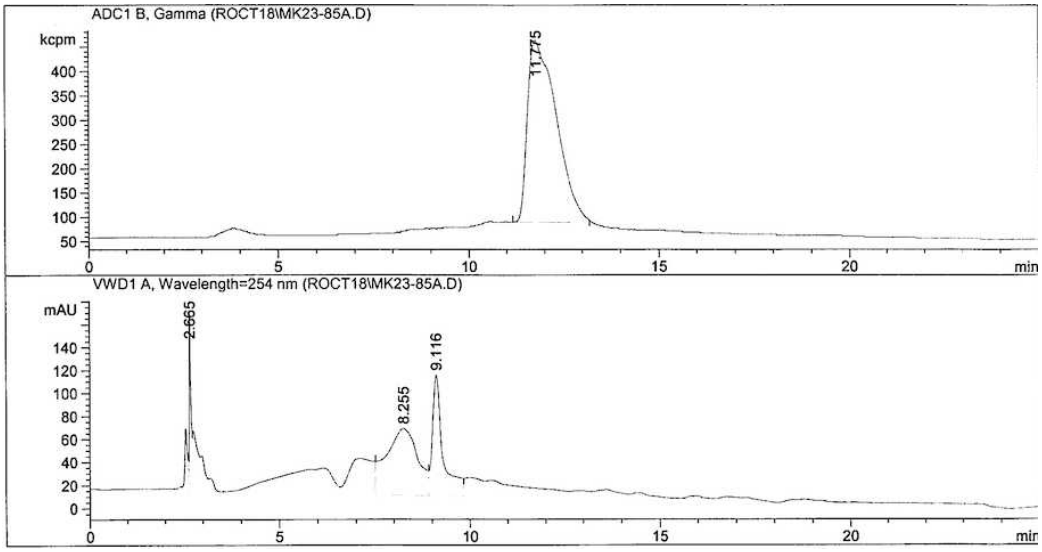

Figure 18 Radio (top) and UV (bottom) chromatograms of reaction mixture obtained from the reactions of 10-hydroxyl-monocarbon carborane **9 (0.2 mg)** with [^{211}At]NaAt when reacted in NH_4Ac buffer (pH 5.5) at 80°C in a microwave for 30 min.

carborane, **6**, were conducted using the same reaction conditions, except the temperature was gradually increased in increments of 10°C from an initial 40°C. Results from each increment of temperature were evaluated by radio-HPLC, then the next temperature increase was made, followed by radio-HPLC analysis again. Figure 19 shows the reaction results by radio-HPLC after 30 min at 40°C, 30 min at 50°C, 30 min at 60°C, and finally 30 min at 70°C. A significant amount (> 85%) of the desired product (t_R = 12.5 min) was obtained. Interestingly, after the next incremental increase in temperature + 80°C for 30 min, a substantial amount of an unknown radiolabeled material is seen by radio-HPLC, as shown in Figure 20.

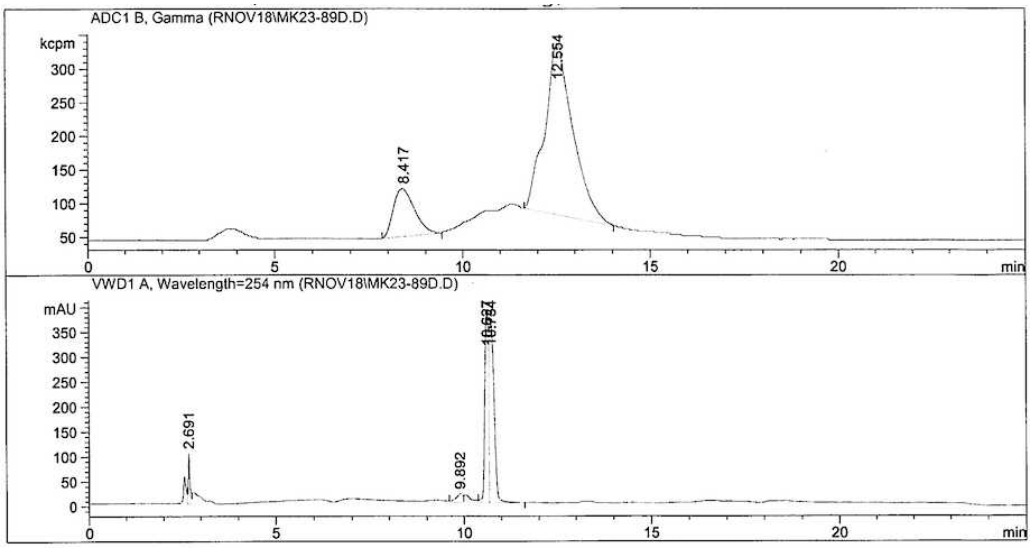

Figure 19 Radio (top) and UV (bottom) chromatograms of reaction mixture obtained from the reaction of benzoyl monocarbon carborane **6 (0.2 mg)** with [^{211}At]NaAt when reacted in NH_4Ac buffer (pH 5.5) in a microwave at 40°C, 30 min; 50°C, 30 min; 60°C, 30 min; and finally 70°C for 30 min.

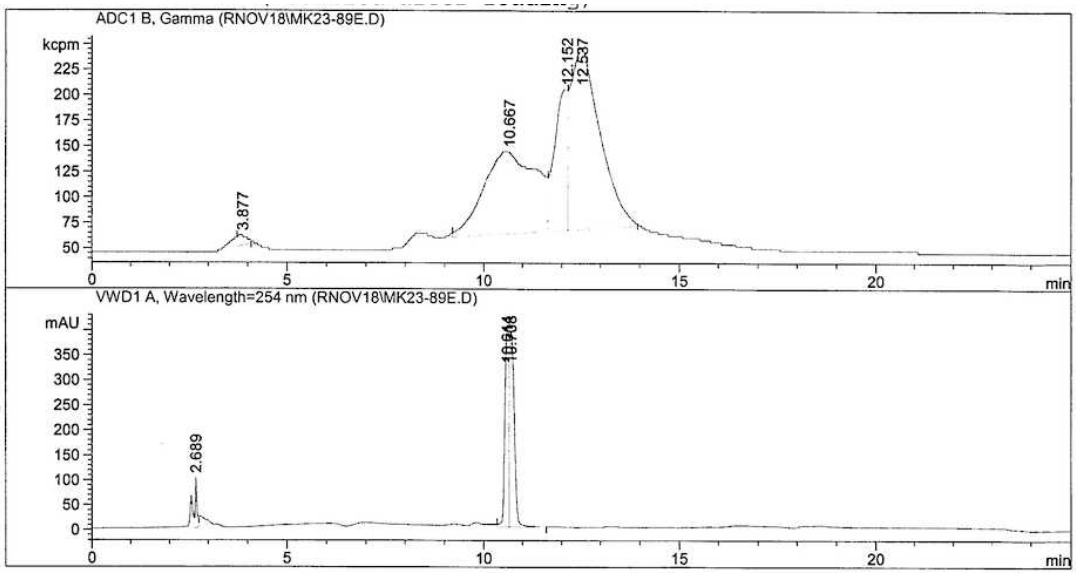

Figure 20 Radio (top) and UV (bottom) chromatograms of reaction mixture obtained from the reaction of benzoyl monocarbon carborane **6 (0.2 mg)** with [^{211}At]NaAt when reacted in NH_4Ac buffer (pH 5.5) in a microwave at 40°C, 30 min; 50°C, 30 min; 60°C, 30 min; 70°C, 30 min; and finally 80°C for 30 min.

Since we were uncertain what caused the increase in other reaction products at the higher temperatures, additional labeling studies were conducted at room temperature and 40°C and evaluated at 10 min and 60 min after reaction initiation. At room temperature very little of the product was obtained, even at 60 min

reaction time (<20%). Similarly, at 40°C only 33% yield was obtained at 60 min reaction time. In the reactions studied, there were radio-HPLC peaks at ~3.8 min and ~8.5 min. We thought those peaks may be due to non-reacted ^{211}At species, so a reaction was conducted at 40°C without having a monocarbon carborane derivative present. Radio-HPLC evaluations were done at 10 min and 60 min. Figure 21 shows the radio-HPLC at 10 min reaction time. The 60-minute reaction time product mixture is essentially the same. It can be noted that there is a very small amount of the a monocarbon carborane derivative present (t_R = 10.7 min). We believe that compound may be from the injection port of the HPLC.

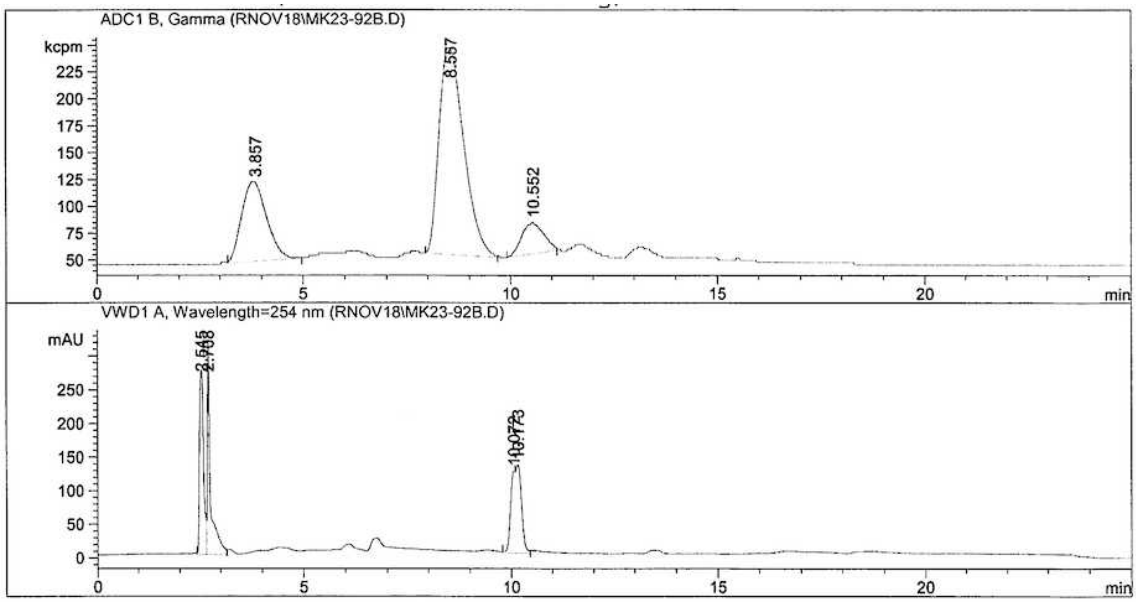

Figure 21 Radio (top) and UV (bottom) chromatograms of reaction mixture obtained from the reaction with [^{211}At]NaAt when reacted in NH_4Ac buffer (pH 5.5) in a microwave at 40°C for 10 min.

Additional ^{125}I- and ^{211}At-labeling reactions of Bz-CB_9H_9, **6**, and Bz-CB_9H_8-OH, 9 were conducted to evaluate the labeling conditions identified in the previous studies at a larger scale and demonstrate reproducibility of the labeling yields obtained earlier. We obtained [^{125}I]**8a** and [^{125}I]**10a** in high yields (69-77%) via electrophilic reactions using *N*-chlorosuccinimide (NCS). The reactions were conducted at room temperature for 5 min, and the products were purified using radio-HPLC. The collected product radiopeaks were evaporated to dryness and re-dissolved in a small volume of PBS. Figure 22 shows the radiochemical purity of the collected products [^{125}I]**8a** and [^{125}I]**10a**. The free radioiodine peak (11.3%) in Figure 22, panel A suggests [^{125}I]**8a** might have partially deiodinated during the evaporation process. The radiopeak at 11.9 min (19.8%) is likely to be an isomer of the primary product at 12.6 min, which could not be separated under the current HPLC conditions. But it is also possible that a fraction of the primary product has converted to this isomer during evaporation. The isolated [^{125}I]**10a** had higher radiochemical purity (98.8%) as shown in Figure 22, panel B. It is expected that ^{125}I labeling of Bz-CB_9H_8-OH, **9** would result in two or more isomers as indicated by the shoulder peak around 12 min.

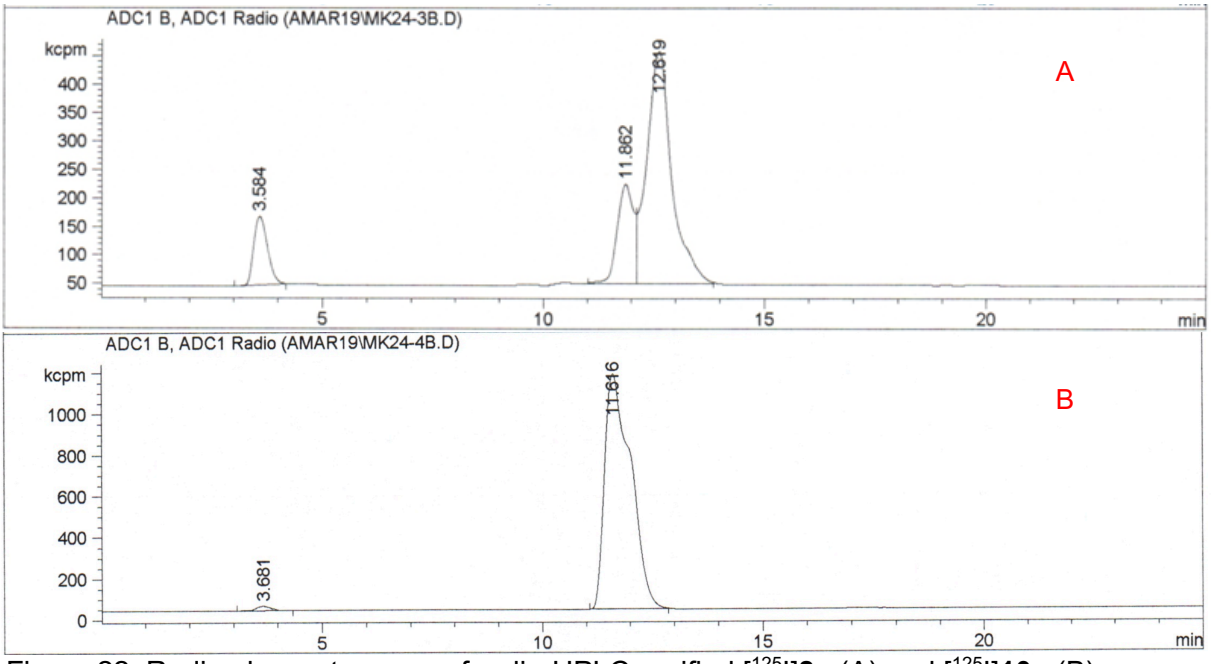

Figure 22. Radio-chromatograms of radio-HPLC purified [^{125}I]**8a** (A) and [^{125}I]**10a** (B).

The ^{211}At labeling reactions were conducted in NH_4Ac buffer (pH 5.5) in the absence of additional oxidant. Bz-CB_9H_9, **6**, and Bz-CB_9H_8-OH, **9** were mixed with about 3 mCi of ^{211}At at 60 °C for 30 min. Similarly, the products were purified using radio-HPLC and the collected product peaks were evaporated to dryness, then re-dissolved in PBS. A significant amount of unknown radiolabeled material (10.4-11.3 min) was observed in the reaction mixture of **6**, which is consistent what we observed before for the astatination of **6**. It is encouraging to see that the desired products, isomers of [^{211}At]**8b** could be isolated from the unknown radiolabeled material using the current HPLC conditions. As shown in Figure 23, panel A, the [^{211}At]**8b** isomers were eluted around 12.2-13.4 min, with about 8% of free astatine. The astatination of **9** provided a good yield of ~73%, which is similar but slightly lower than the radiolabeling yields obtained before at 80 °C. Figure 23, panel B shows the radio-HPLC purified [^{211}At]**10b** has a radiochemical purity of 90%.

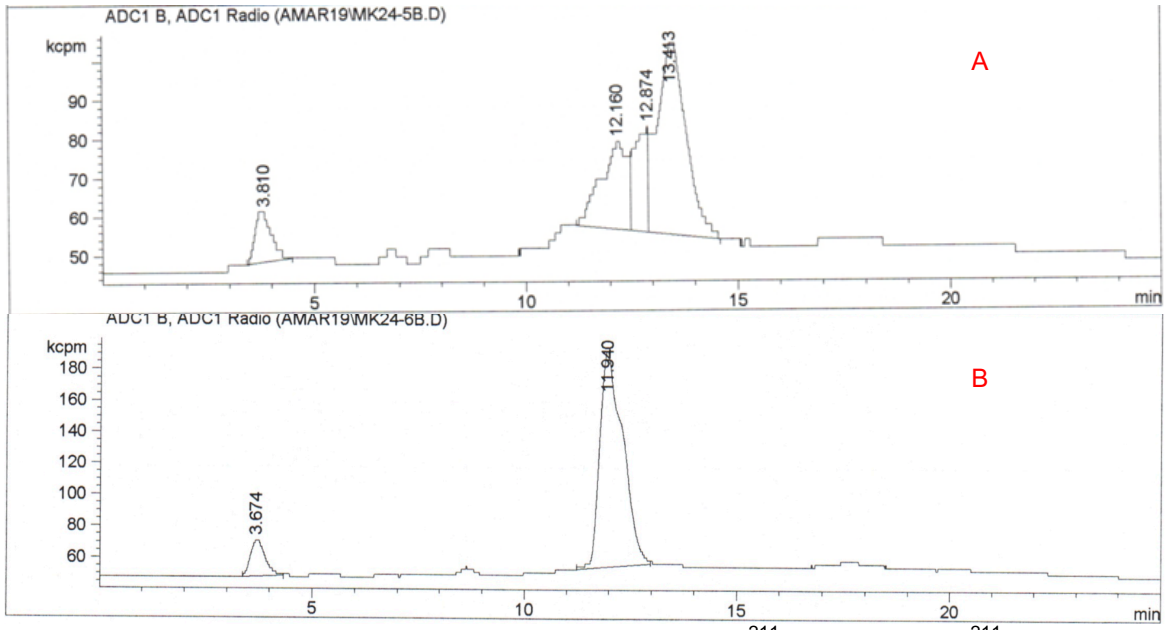

Figure 23. Radio-chromatograms of radio-HPLC purified [^{211}At]**8b** (A) and [^{211}At]**10b** (B).

Preparation of [^{211}At]**10b** was efficient so this compound was of interest for in vivo use. However, there was a question as to whether the hydroxylated monocarbon carborane would provide the high in in vivo stability of the ^{211}At. As part of our NIH-funded collaboration with Dr. Damian Green at the Fred Hutch involving ongoing studies to pretarget ^{211}At to treat multiple myeloma, a simple biodistribution in mice was conducted. That study indicated that the introduction of a hydroxyl functional group on the monocarbon carborane estabilized the ^{211}At towards in vivo deastatination.

SO3c: ^{211}At-labeling of molecules with bis-closo-decaborate(2-) moieties

The disappointing in vivo results of [^{211}At]**10b** led to investigation of an alternate small molecule ^{211}At-labeling moiety. We had previously observed that coinjected Fab´-B10 conjugates radiolabeled with radioiodine and ^{211}At had high concentrations in the kidney of mice, but when a cleavable linker was used the radioiodinated Fab´-B10 conjugate was cleared from the kidney, but not the ^{211}At-labeled Fab´-B10 (20). We believe the difference in retention in the cell may be due to oxidation of the B10-^{211}At atom by cellular oxidases, whereas the oxidases did not oxidize the B10-iodine atom. Once oxidized the B10-At atom may react with materials in the cell causing its' retention. Our hypothesis is that pre-oxidation of the At atom can eliminate the retention in cells. Further, it may be that oxidation in the blood system of aryl-bonded ^{211}At-labeled compounds has caused the difficulties we have had with developing ^{211}At-labeled small molecules for cancer pretargeting protocols. Thus, we began investigating a bis-B10 moiety as depicted in Figure 24. In the astatinated compound, **21**, the At atom is in the formal +3 state (or +5 state if AtO+ is the reactant) and is bonded to 2 boron cages.

A biotin-sarcosine adduct was prepared for this example as it could be used directly in our NIH-funded collaborative studies with Dr. Green. In initial studies the bis-B10 reagent was labeled under neutral conditions to get the mono-^{211}At product **20**, then chloramine-T (ChT) was used to oxidize that such that the second B10 moiety can react with it to potentially provide the ^{211}At-bridged B10s, as depicted in compound **21**. It should be noted that when labeled in the manner shown in Figure 24, the resultant ^{211}At label is oxidized to the +3 state.

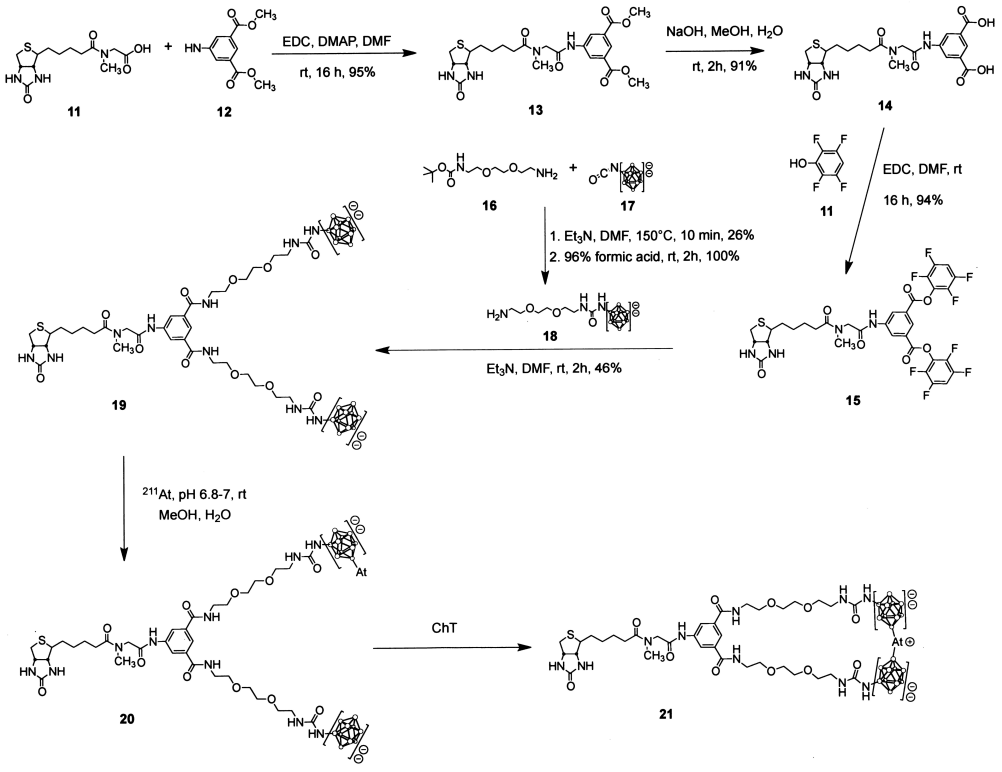

Figure 24. Synthesis and potential ^{211}At-labeling of biotin-sarcosine-bis-B10 reagent.

Astatine-211 labeling reactions were conducted for biotin-sarcosine-bis-B10, the compound **19** shown in Figure 24. Compound **19** was reacted with [^{211}At]NaAt in 5% HOAc in MeOH/H_2O at room temperature for 5 min without an oxidant (i.e. no chloramine-T). The reaction was quenched with 10 μL of 1 mg/mL $Na_2S_2O_5$. The reaction provided one ^{211}At labeled product (95%) as suggested by the radio-HPLC peak at ~10.1 min (Figure 25, panel A). No ChT was used in this reaction, so the product might be **20** in Figure 24. However, it is also possible that the product is astatine-bridged bis-B10 **21** as the two B10 groups of **19** might rapidly form intramolecular bonds with astatine. Astatine-211 labeling reactions with ChT were also conducted for **19**, and a representative radio-HPLC chromatogram is shown in Figure 25, panel C. The reaction with chloramine-T provided multiple products that were eluted from 9-11 min. After sitting at room temperature for 2 h, a significant amount of free ^{211}At was observed (29%) (Figure 25, panel D). It is likely that the biotin moiety in **19** was oxidized by chloramine-T, so it is not clear whether astatine-bridged bis-B10 **21** was produced.

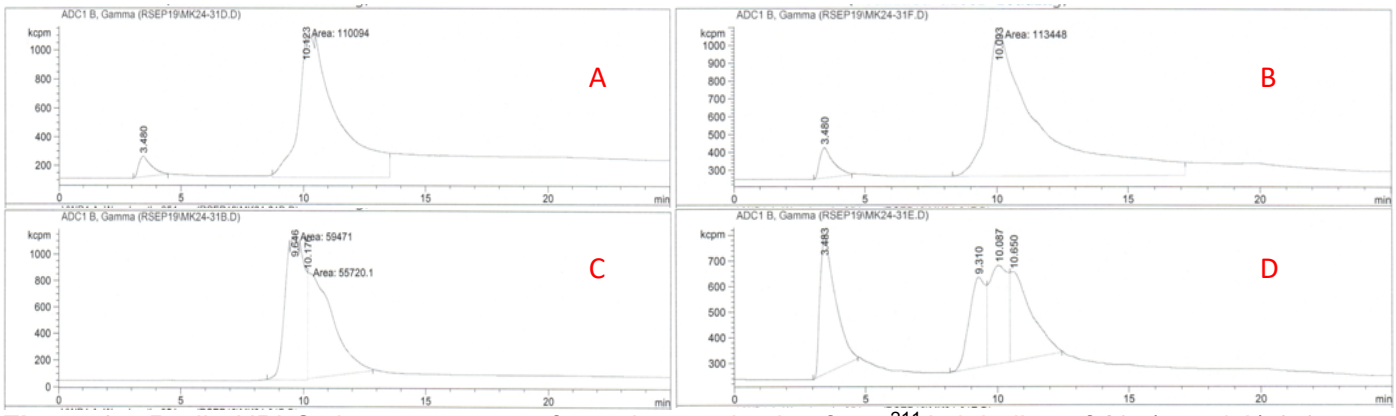

Figure 25. Radio-HPLC chromatograms of reaction production from ^{211}At labeling of **19**. (panel A) 0.1 mg of **19** was reacted with ^{211}At in 5% HOAc in MeOH/H_2O without chloramine-T at room temperature for 5 min. (panel B) the reaction mixture (A) sat at room temperature for about 1 h. (panel C) 0.1 mg of **19** was reacted with ^{211}At in 5% HOAc in MeOH/H_2O with 10 μg of chloramine-T at room temperature for 3 min. (Panel D) the reaction mixture (C) sat at room temperature for about 2 h.

Reaction of ^{211}At (pH 6-6.5) with the biotin-sarcosine-bis-B10 reagent in 5% HOAC in MeOH/H_2O at room temperature produced a single product without added oxidant (i.e. chloramine-T). Radio-HPLC analyses suggested the retention time of the astatinated product and labeling yield did not change significantly between 5 min and 1 h reaction time (Figure 26). However, two radiolabeled products were observed on the radio-HPLC chromatogram. As shown in the top panel in Figure 26, the two products obtained after 5 min eluted at ~9.0 min and 10.4 min, respectively. In addition, the ratio of those two radio-peaks changed over time. As shown in the middle and bottom panels in Figure 26, the radio-peak eluted around 9.0 min decreased from 38.7% to 4.5% after 100 min, and completely disappeared after 130 min. The radiochromatogram obtained after reacting at room temperature for approximately 100 min also has a significant amount of free ^{211}At, which eluted at 3.5 min. It is possible that the radio-peak at 9.0 min corresponds to the radiolabeled product, compound **20** (Figure 24) and the radio-peak at 10.4 min corresponds to the same product obtained from the astatonium salt, compound **21** (Figure 24). Although no oxidant was added to the reaction mixture, **20** might be able to slowly convert to **21** in the presence of air.

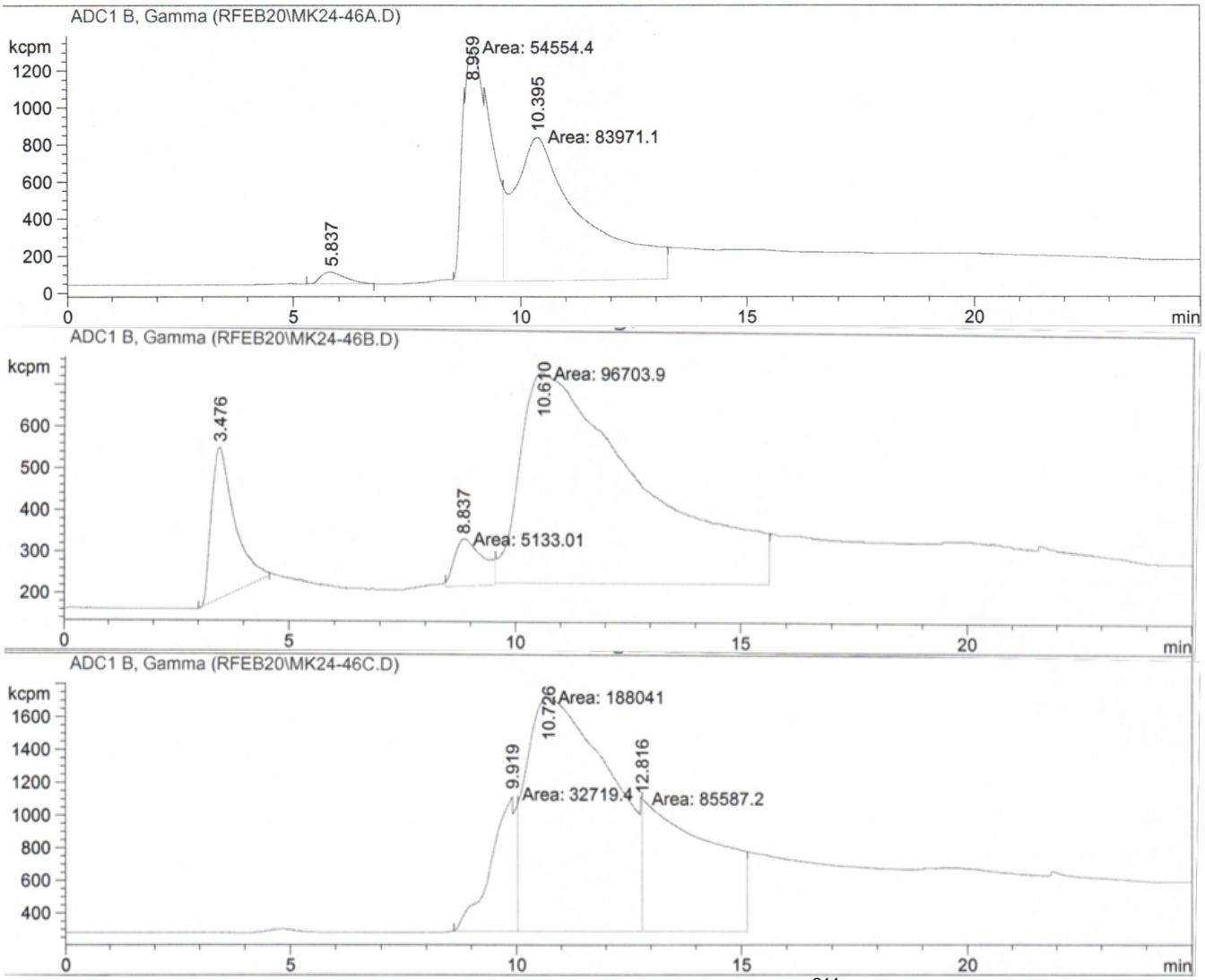

Figure 26. Radio-HPLC chromatograms of reaction production from ^{211}At labeling of **19**: 0.1 mg of **19** was reacted with ^{211}At in 5% HOAc in MeOH/H_2O at room temperature for approximately 5 min (top),100 min (middle) and 130 min (bottom).

The radio-peak starting at 10.4 min was isolated. The isolated radiolabeling yield was approximately 60% after reacting ~130 min at room temperature. The collected HPLC eluent was evaporated to dryness using the Biotage evaporator. No ^{211}At activity was volatilized during evaporation. The product was re-dissolved in 400 μL PBS and analyzed by radio-HPLC to check identity (retention time) and radiochemical purity. The radiochromatogram in Figure 27 showed the collected product had high radiochemical purity. Further studies are planned with this compound.

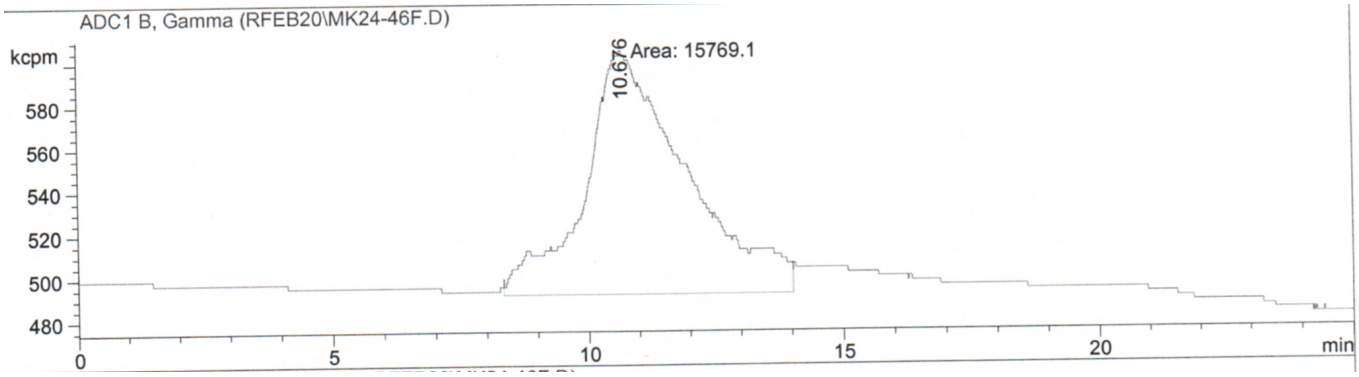

Figure 27. At-211 labeled compound **19** after HPLC purification. The purified was evaporated to dryness, re-dissolved in PBS, then analyzed by radio-HPLC.

The ^{211}At labeling experiments conducted with the biotin-sarcosine-bis-B10 reagent suggested that only one primary labeled product ($R_T \approx 10.6$ min) was observed on radio-HPLC when chloramine-T was absent in the reaction mixture (Figure 25, panels A and B). However, when chloramine-T was added to the reaction mixture, two additional labeled species were observed on the radio-HPLC chromatograms. It was unclear whether those additional species were due to the oxidation of the biotin moiety by chloramine-T. Therefore, we prepared a new bis-B10 reagent where the biotin-sarcosine groups were replaced with N_α-Acetyl-L-Lys. The synthetic steps followed to obtain Ac-Lys-bis-B10 is shown in Figure 28.

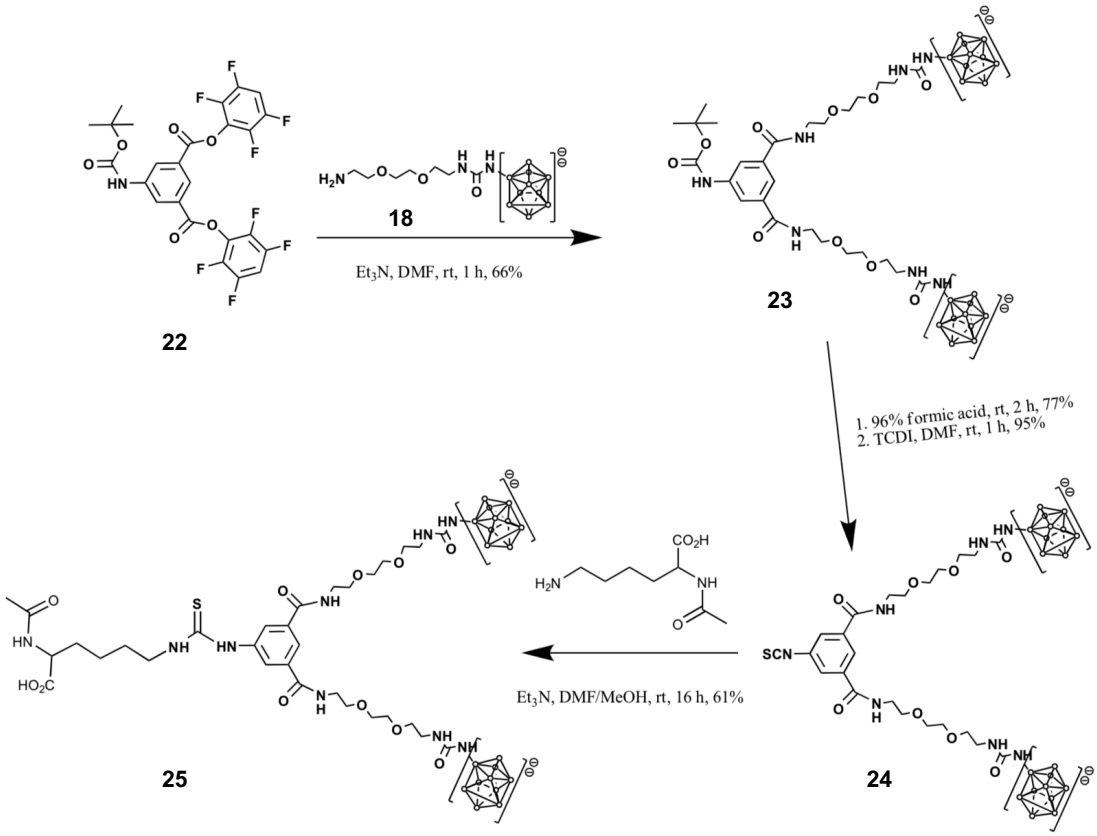

Figure 28. Synthesis of Ac-Lys-bis-B10 reagent

Radiolabeling experiments with compound **25** were conducted using ^{211}At at room temperature in 5% HOAc in MeOH/H_2O solutions in the presence or absence of chloramine-T. As shown in the radio-HPLC chromatograms in Figure 29, panel A, in the absence of chloramine-T, the reaction provided a radiolabeling yield of 100% after 5 min, based on HPLC analysis of the crude product. However, about 26% of free ^{211}At

was observed after 2 h (Figure 29, panel B). Interestingly, the retention time of the product changed from 9.6 min to 10.4 min (a 0.8-min difference in retention time), after 2 h. It is known that ^{211}At can react with mono-B10 very rapidly (in 1 min) without using added oxidant. It is possible that the reaction of the second B10 group with the B10-bound ^{211}At can also occur without added oxidant but at a much slower rate. More labeling studies are required to test this hypothesis. The ^{211}At labeling reaction that used chloramine-T provided radiolabeling yields of 50% and 79% after 5 min and 2 h, respectively. The radio-peaks that correspond to the ^{211}At labeled product eluted around 10.6-10.9 min (Figure 29, panels C and D).

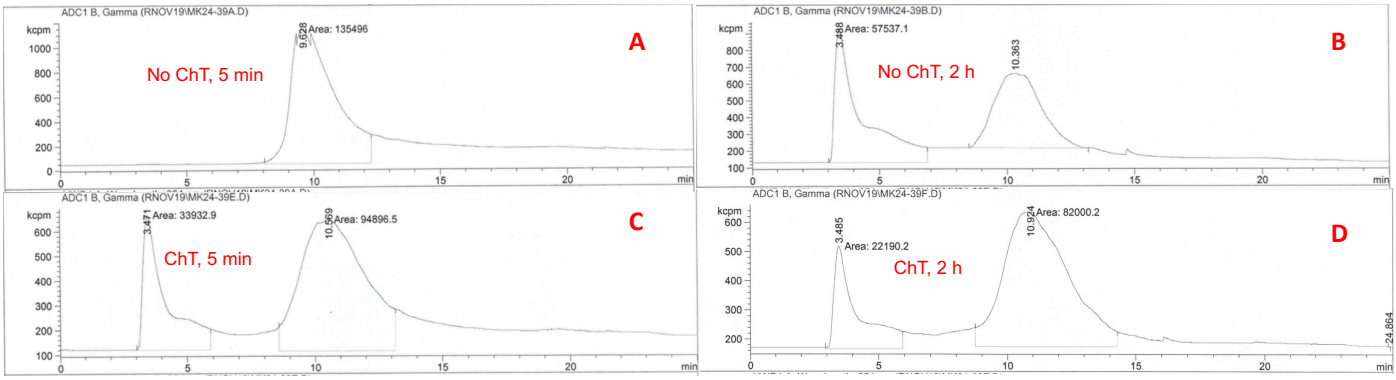

Figure 29. Radio-HPLC chromatograms of reaction production from ^{211}At labeling of **25**. (Panel A) 0.1 mg of **25** was reacted with ^{211}At in 5% HOAc in MeOH/H_2O without chloramine-T at room temperature for 5 min. (Panel B) the reaction mixture (from A) sat at room temperature for 2 h. (Panel C) 0.1 mg of **25** was reacted with ^{211}At in 5% HOAc in MeOH/H_2O with 10 μg of chloramine-T at room temperature for 5 min. (Panel D) the reaction mixture (from C) sat at room temperature for 2 h.

Synthetic Procedures:

Synthetic and radiolabeling procedures that were used to prepare compounds employed in this investigation and some procedures used to test stability of radiolabeled compounds are provided below. The compound numbers are shown in bold.

[Net_4]*closo-1*-CB_9H_8-*1*-CO_2H-*10*-I-Ph, **3**.

A solution containing [Net_4] *closo-1*-CB_9H_9-*1*-CO_2H, **2** (50 mg, 0.17 mmol) and 70% HOAc/H_2O (3 mL) was treated with Ph-I-$(Oac)_2$ (54.8 mg, 0.17 mmol) at 0 °C for 4 hours. The volatiles were evaporated by a rotary evaporator under vacuum. The crude residue was dissolved in methanol/water (1/1) and purified via Biotage C18 FLASH 25+M column; The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 80% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 20 min). Yield 49.5 mg (58.5%). HPLC t_R = 12.7 min. HRMS (ES^-) $C_8H_{13}B_9IO_2$ $(M-H)^-$ Calcd: 367.0798, Found: 367.0811

[Net_4]*closo-1*-CB_9H_8-*1*-CO_2H-*10*-I, **4a**. (iodide reaction on iodonium salt)

A reaction mixture of [Net_4] *closo*-1-CB_9H_8-1-CO_2H-10-I-Ph, **3** (5 mg, 10.1 μmol), NaI (15 μmol, 22.6 μL, 0.667 mol/L aqueous solution), 0.1 N NaOH (10.1 μL, 0.1 N aqueous solution) and acetonitrile (0.5 mL) was stirred and heated by microwave at 80°C for 10 min. HPLC t_R = 9.6 min.

[Net_4]*closo-1*-CB_9H_8-*1*-CO_2H-*10*-I, **4a** *(preparation of an iodo standard by electrophilic reaction)*

N-Chlorosuccinimide (0.5 eq, 114 μL, 8.51 μmol, 10 mg/mL, 0.0749 mol/L in MeOH) was added to a solution of [Net_4] *closo-1*-CB9H9-*1*-CO_2H, **2** (1.0 eq, 5.0 mg, 17.0 μmol), NaI (0.5 eq, 12.8 μL, 8.51 μmol, 100 mg/mL, 0.667 mol/L in H_2O) and 5% HOAc/MeOH (0.4 mL) at room temperature. After the reaction mixture was stirred at room temperature for five minutes, sodium metabisulfite (0.5 eq, 162 μL, 8.51 μmol, 10 mg/mL, 0.0526 mol/L in H_2O) was added to quench the reaction. HPLC t_R = 9.7 min.

[Net_4]*closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-I-Ph, **7**.
A solution containing *closo-1*-CB_9H_9-*1*-(4-Ph-CO_2H), **8** (55 mg, 0.149 mmol) and 70% HOAc/H_2O (3 mL) was treated with Ph-I-$(Oac)_2$ (47.9 mg, 0.149 mmol) at 0 °C for 2 hours. The volatiles were evaporated by a rotary evaporator under vacuum. The crude residue was dissolved in methanol/water (1/1) and purified via Biotage C18 FLASH 25+M column; The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 80% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 20 min). Yield 45 mg (52.8%). HPLC t_R = 14.9 min. LRMS (ES^-) $C_{14}H_{17}B_9IO_2$ $(M-H)^-$ Calcd: 443, Found: 443

Stability Testing of closo-1-CB_9H_8-*1*-CO_2H-*10*-I-Ph, **7**.

(a) Test with NaOH: A reaction mixture of [Net_4] *closo-1*-CB_9H_8-*1*-CO_2H-*10*-I-Ph (1 mg, 2.0 µmol), 0.1 N NaOH (20 µL, 0.1 N aqueous solution), H_2O (150 µL) and acetonitrile (0.6 mL) was stirred and heated by microwave at 80 °C for 10 min. (needs HPLC)

(b) Test without NaOH: A reaction mixture of [Net_4] *closo-1*-CB_9H_8-*1*-CO_2H-*10*-I-Ph (1 mg, 2.0 µmol), H_2O (150 µL) and acetonitrile (0.6 mL) was stirred and heated by microwave at 80 °C for 10 min.

(c) Test under radiohalogenation conditions: A reaction mixture of [Net_4]*closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-I-Ph, **7** (1 mg, 1.75 µmol), 0.1 N NaOH (20 µL, 0.1 N aqueous solution), H_2O (150 µL) and acetonitrile (0.6 mL) was stirred and heated by microwave at 40, 50, 80 °C with or without NaOH. This compound was only stable at 40 °C. Mass spec. showed the major decomposed compound was CB_9H_8(OH)-Ph-CO_2H.

[Net_4]*closo-1*-CB_9H_8-*1*-(4-Ph-CO_2H)-*10*-I, nucleophilic reaction to make **8a.**
A reaction mixture of [Net_4] *closo*-1-CB_9H_8-1-(4-Ph-CO_2H)-10-I-Ph (5 mg, 8.7 µmol), NaI (13 µmol, 19.6 µL, 0.667 mol/L aqueous solution), 0.1 N NaOH (8.7 µL, 0.1 N aqueous solution) and acetonitrile (0.5 mL) was stirred and heated by microwave at 80°C for 10 min. HPLC t_R = 12.3 min.

[Net_4]*closo-1*-CB_9H_8-*1*-(4-Ph-CO_2H)-*10*-I, **8a** *(preparation of iodo standard by electrophilic reaction)*
N-Chlorosuccinimide (0.5 eq, 90 µL, 6.76 µmol, 10 mg/mL, 0.0749 mol/L in MeOH) was added to a solution of [Net_4]*closo-1*-CB_9H_9-*1*-(4-Ph-CO_2H) (1.0 eq, 5.0 mg, 13.5 µmol), NaI (0.5 eq, 10.1 µL, 6.76 µmol, 100 mg/mL, 0.667 mol/L in H_2O) and 5% HOAc/MeOH (0.4 mL) at room temperature. After the reaction mixture was stirred at room temperature for five minutes, sodium metabisulfite (0.5 eq, 129 µL, 6.76 µmol, 10 mg/mL, 0.0526 mol/L in H_2O) was added. HPLC t_R = 12.2 min.

closo-1-$CB_9H_8{}^{125}I$-*1*-(*4*-Ph-CO_2H)-10-I-Ph, alternative approach to making [^{125}I]**8a**.
A reaction mixture of [Net_4]*closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-I-Ph (0.3 mg), $Na^{125}I$ (1 µL), 0.1 N NaOH (8.7 µL, 0.1 N aqueous solution) and acetonitrile (0.5 mL) was stirred and heated by microwave at 80°C for 10 min. Radio-HPLC showed 7.9% yield. HPLC t_R = 13.0 min

closo-1-CB_9H_8-*1*-(4-Ph-CO_2H)-*10*-I, **8a** (preparation of ^{125}I standard by electrophilic reaction)
N-Chlorosuccinimide (10 µL, 1 mg/mL, 0.00749 mol/L in MeOH) was added to a solution of [Net_4] *closo-1*-CB_9H_9-*1*-(*4*-Ph-CO_2H) (**CRV1-30**, 0.1 mg), $Na^{125}I$ (7 µL, 1.3 mCi) and 5% HOAc/MeOH (0.1 mL) at room temperature. After the reaction mixture was at room temperature for five minutes, sodium metabisulfite (10 µL, 1 mg/mL, 0.00526 mol/L in H_2O) was added. The HPLC collection of the product peaks was evaporated to dryness by a Biotage evaporator, then dissolved in PBS for quality control.
HPLC (RadioHPLC, Boron-20, Alltech Altima column): t_R = 11.9 & 12.6 min.

[Net_4]*closo-1*-CB_9H_8-*1*-(4-Ph-CO_2H)-*10*-^{211}At, [^{211}At]**8b**.(iodonium salt labeling)
A reaction mixture containing [Net_4] *closo*-1-CB_9H_8-1-(4-Ph-CO_2H)-10-I-Ph (0.3 mg), ^{211}At (150 µL, pH 7, 140 µCi), 0.1 N NaOH (20 µL) and acetonitrile (0.6 mL) was stirred and heated by microwave at 80°C for 10 min. HPLC t_R = 12.9 min. Yield 53% (by radioHPLC)

closo-1-CB_9H_8-*1*-(*4*-Ph-CO_2H)-10-^{211}At, [^{211}At]**8b.** (^{211}At labeling at lower temperatures)

(a) Reaction at room temperature: A reaction mixture containing [Net_4] *closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-10-I-Ph (0.3 mg), ^{211}At (100 µL, pH 6.5, 1.6 mCi), 0.1 N NaOH (20 µL) and CH_3CN (700 µL) was stirred at room temperature for 30 min. HPLC t_R = 12.9 min. Yield 7.9% (by radio-HPLC).

(b) Reaction at 50 °C for 30 min: The reaction mixture *(a)* was heated by microwave at 50 °C for 30 min. HPLC t_R = 12.7 min. Yield 41.9% (by radio-HPLC).

(c) Reaction at 50 °C for 60 min: The reaction mixture *(b)* was heated by another 30 min. HPLC t_R = 12.4 min. Yield 55.7% (by radio-HPLC).

Closo-1-CB_9H_8-*1*-(4-Ph-CO_2H)-*10*-^{211}At, [^{211}At]**8b**. (direct labeling)

A mixture of *closo-1*-CB_9H_9-*1*-(*4*-Ph-CO_2H) (0.1 mg), ^{211}At (200 µL, pH 6-6.5, 3.0 mCi), MeOH (100 µL), NH_4Ac pH 5.5 (200 µL), was stirred and heated by microwave at 60 °C, 30 min. The HPLC collection of the product peaks was evaporated to dryness by a Biotage evaporator, then dissolved in PBS for quality control. HPLC (RadioHPLC, Boron-20, Alltech Altima column): t_R = 12.5 min.

closo-1-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-OH, **9**.

A reaction mixture of [Net_4] closo-1-CB_9H_8-1-(4-Ph-CO_2H)-10-I-Ph (50 mg, 0.087 mmol), H_2O (200 µL) and acetonitrile (1.0 mL) was stirred and heated by microwave at 80°C for 30 min. The crude product was purified via Biotage (C18 FLASH 25+M column; The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 80% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 20 min). Yield 26 mg (77%). HPLC t_R = 9.6 min. LRMS (ES^-) $C_8H_{14}B_9O_3$ $(M)^-$ Calcd: 257; Found: 257

closo-1-CB_9H_7I-*1*-(*4*-Ph-CO_2H)-*10*-OH, **10a**.

N-Chlorosuccinimide (0.5 eq, 87 µL, 6.48 µmol, 10 mg/mL, 0.0749 mol/L in MeOH) was added to a solution of [Net_4] *closo-1*-CB9H8OH-*1*-(4-Ph-CO_2H) (1.0 eq, 5.0 mg, 13 µmol), NaI (0.5 eq, 9.7 µL, 6.48 µmol, 100 mg/mL, 0.667 mol/L in H_2O) and 5% HOAc/MeOH (0.4 mL) at room temperature. After the reaction mixture was stirred at room temperature for five minutes, sodium metabisulfite (0.5 eq, 123 µL, 6.48 µmol, 10 mg/mL, 0.0526 mol/L in H_2O) was added. HPLC t_R = 10.9 min. LRMS (ES^-) $C_8H_{13}B_9IO_3$ $(M)^-$ Calcd: 383.07; Found: 383.07

closo-1-$CB_9H_7{}^{125}I$-*1*-(*4*-Ph-CO_2H)-*10*-OH, [^{125}I]**10a.**

N-Chlorosuccinimide (10 µL, 1 mg/mL, 0.0749 mol/L in MeOH) was added to a solution of [Net_4] *closo-1*-CB9H8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), $Na^{125}I$ (2 µL, 796 µCi) and 5% HOAc-MeOH/H_2O (0.1 mL) at room temperature. After the reaction mixture was at room temperature for five minutes, sodium metabisulfite (10 µL, 1 mg/mL, 0.00526 mol/L in H_2O) was added. HPLC t_R = 11.5 min.

N-Chlorosuccinimide (10 µL, 1 mg/mL, 0.00749 mol/L in MeOH) was added to a solution of [Net_4] *closo-1*-CB9H8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), $Na^{125}I$ (7 µL, 1.3 mCi) and 5% HOAc/MeOH (0.1 mL) at room temperature. After the reaction mixture was at room temperature for five minutes, sodium metabisulfite (10 µL, 1 mg/mL, 0.00526 mol/L in H_2O) was added. The HPLC collection of the product peaks was evaporated to dryness, then re-dissolved in PBS for quality control.

Closo-1-$CB_9H_7{}^{211}$At-*1*-(*4*-Ph-CO_2H)-*10*-OH, **10b.**

(a) A mixture of *closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), ^{211}At (100 µL, pH 6.5, 1.4 mCi), MeOH (150 µL) and NH_4Ac pH 5.5 (500 µL) was stirred at room temperature for 5 min. HPLC t_R = 11.9 min. Yield 35.4% (by radio-HPLC).

(b) The reaction mixture *(a)* was heated at 80 °C by microwave for 30 min. HPLC t_R = 12.0 min. Yield 78.1% (by radio-HPLC).

(c) A mixture of *closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), ^{211}At (100 µL, pH 6.5, 1.2 mCi), MeOH (100 µL), NH_4Ac pH 5.5 (100 µL) and chloramine-T (20 µL, 1 mg/mL in H_2O) was stirred at room temperature for 5 min. No labeling.

(d) A mixture of *closo-1*-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), ^{211}At (50 µL, pH 6.5, 0.5 mCi), 5% HOAc-MeOH/H_2O (100 µL) and chloramine-T (15 µL, 1 mg/mL in H_2O), was stirred at room temperature for 5 min. No labeling.

(e) A mixture of closo-1-CB_9H_8-*1*-(*4*-Ph-CO_2H)-*10*-OH (0.1 mg), ^{211}At (200 µL, pH 6-6.5, 3.0 mCi), MeOH (100 µL), NH_4Ac pH 5.5 (200 µL) was heated 60 °C by microwave for 30 min. The HPLC collection of the product peaks was evaporated to dryness by a Biotage evaporator, then re-dissolved in PBS for quality control. HPLC t_R = 11.9 min

3,5-CO_2Me-Ph-*Sar*-Biotin, **13**.
A solution of Biotin-Sar-OH (**11**, 1.0 g, 3.17 mmol), *3,5*-CO_2Me-Ph-NH_2 (**12**, 0.603 g, 2.88 mmol), EDC (0.608 g, 3.17 mmol), DMAP (0.035 g, 0.288 mmol) and anhydrous DMF (15 mL) was stirred at room temperature for 16 h. H_2O (90 mL) and 1 N HCl (5 mL) was added to the reaction solution and stirred for additional 30 min. The precipitate was filtered, washed with water (500 mL), dried under vacuum for 16 h to give the product as white solid. HPLC t_R = 15.3 min. Yield 1.39 g (95%). LRMS (ES^+) $C_{23}H_{31}N_4O_7S$ $(M+H)^+$, Calcd: 507.19, Found: 507.19

3,5-CO_2H-Ph-*Sar*-Biotin, **14**.
A solution of *3,5*-CO_2Me-Ph-*Sar*-Biotin (**13**, 1.0 g, 1.974 mmol), NaOH (0.197 g, 4.94 mmol) and 50% MeOH/H_2O (20 mL) was stirred at room temperature for 2 h. H_2O (90 mL) and 1 N HCl (6 mL) was added to the reaction solution and stirred for additional 30 min. The precipitate was filtered, washed with water (500 mL), dried under vacuum for 16 h to give the product as white solid. HPLC t_R = 12.0 min. Yield 0.86 g (91%). LRMS (ES^+) $C_{21}H_{27}N_4O_7S$ $(M+H)^+$ Calcd: 479.16, Found: 479.16

3,5-CO_2TFP-Ph-*Sar*-Biotin, **15**.
A solution of *3,5*-CO_2H-Ph-*Sar*-Biotin (**14**, 0.406 g, 0.848 mmol), EDC (0.358 g, 1.867 mmol), TFP-OH (0.310 g, 1.867 mmol) and anhydrous DMF (10 mL) was stirred at room temperature for 16 h. 90 mL of H_2O was added to the reaction solution and stirred for additional 30 min. The precipitate was filtered, washed with water (500 mL), dried under vacuum to give the product as white solid. HPLC t_R = 16.7 min. Yield 0.62 g (94%). HRMS (ES^+) $C_{33}H_{27}F_8N_4O_7S$ $(M+H)^+$ Calcd: 775.1473, Found: 775.1465

$[Et_3NH]_2$ $B_{10}H_9$-dioxa-NH_2, **18**.
Step 1: A solution of $[Et_3NH]_2$ $B_{10}H_9$-N=C=O (**17**, 0.215 g, 0.591 mmol), BOC-dioxa-NH_2 (**16**, 0.147 g, 0.591 mmol), Net_3 (0.165 mL, 1.183 mmol), DMF (15 mL) was stirred and heated by microwave at 150 °C for 10 min. The crude solution was mixed with H_2O (2 mL) and purified via Biotage on an C18 FLASH 25+M column. The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 80% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 20 min). The crude product also can be purified by silica gel column (1.5 cm x 25 cm) eluted with gradient solution from 100% EtOAc to 50% MeOH/EtOAc. HPLC t_R = 9.8 min. Yield 0.094 g (26%). HRMS (ES^-) $C_{12}H_{34}B_{10}N_3O_5$ $(M+H)^-$ calcd: 410.3434, found: 410.3427

Step 2: A solution of $[Et_3NH]_2$ $B_{10}H_9$-dioxa-NH-BOC (94 mg, 0.154 mmol) and 96% formic acid (2 mL) was stirred at room temperature for 2 h. The reaction solution was evaporated under vacuum to dryness. HPLC t_R = 4.1 min. Yield 79 mg (100%). HRMS (ES^-) $C_7H_{26}B_{10}N_3O_3$ $(M+H)^-$ Calcd: 310.2910, Found: 310.2912

3,5-$B_{10}H_9$-dioxa-Ph-*Sar*-Biotin, **19**.
A solution of *3,5*-CO_2TFP-Ph-*Sar*-Biotin (50 mg, 0.065 mmol), $B_{10}H_9$-dioxa-NH_2 (79 mg, 0.155 mmol), Net_3 (45 µL, 0.323 mmol) and anhydrous DMF (2 mL) was stirred at room temperature for 2 h. The solution was then triturated with EtOAc. The crude residue was dissolved in 50% MeOH/water and purified via Biotage on a C18 FLASH 25+M column; The gradient mixture was composed of MeOH and 0.05 M triethylammonium acetate. Starting with 80% 0.05 M triethylammonium acetate, increased to 100% MeOH over the next 15 min). HPLC t_R = 7.6 min. Yield 44 mg (46%). LRMS (ES^-) $C_{35}H_{72}B_{20}N_{10}NaO_{11}S$ $(M+Na)^-$ Calcd: 1082.7, Found: 1082.7

C. *Specific Objective 4:* *Training of students in radiochemical methods involved in production, automation of ^{211}At isolation, radiochromatographic characterization and radiolabeling with ^{211}At.*

The research was set up so that Postdoctoral Fellows and students could learn radiochemistry techniques from being involved in the research effort and by visiting and working at the collaborating institutions. The Postdocs and students were trained in all the radiochemical methods involved in target preparation, irradiation, radionuclide isolation/purification, quality assurance methods and shipments of radionuclides. Dr. Yawen Li who began the studies as a Postdoctoral Fellow was promoted to Assistant Professor at the University of Washington on January 1st, 2018. A second Postdoctoral Fellow, Dr. Maryline Kerlin, joined our group in April, 2018. When she left UW she moved to the Lawrence Livermore National Laboratory. During the research period, a Postdoctoral Fellow at LANL, Dr. David Woens came to UW twice to conduct studies on properties of column matrices for separation of ^{211}At from bismuth targets. An undergraduate student Taylor Morscheck was involved in the research effort to determine the minimal amount of NH_2OH•HCl required to eliminate nitrate ions in the dissolved bismuth target solution. Another undergraduate student, Marina Llewellyn, was trained in radiochemical methods and was involved in the research effort.

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