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TITLE: In Vitro Assessment of a Peptide Nucleic Acid (PNA) - Peptide
Conjugate Labeled with an Auger-Emitting Radionuclide for
Prostate Cell Killing

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13. ABSTRACT (Maximum 200 Words) The ultimate goal of the research is to develop new treatment reagents for metastatic prostate cancer. Unfortunately, treatment of metastatic prostate cancer is problematic as the cells can become resistant to the therapy being used. Therefore, this exploratory research was directed at developing new therapy reagents that will not be affected by changes within cells. The therapy reagents use a radioactive material, Auger electron emitting radionuclides, to help solve the problem. Auger emission is quite different from emission from other radioactive materials as they have short-range electron emissions that are very toxic to cells and are not affected by other biological factors. Importantly, the very short particle range can greatly decrease the toxicity outside of the cells that it is targeted to. The studies focused primarily on the synthesis of a peptide nucleic acid (PNA) that has an Auger-emitter (I-125) incorporated. By design the PNA will bind with mRNA and DNA associated with insulin-like growth factor receptor that is upregulated on PC-3 prostate cancer cells. The first objective was to obtain a thymine analog, an iodouracil-amino acid for incorporating into a PNA. This was accomplished in 8 synthetic steps. However, the tri-n-butylstannyluracil-amino acid required to couple with the PNA could not be prepared in the same manner. Therefore, a second synthetic route was undertaken which proved to be successful. The iodouracilamino acid derivative and tri-n-butylstannyluracil amino acid derivative were coupled with a free amine on the PNA to form new PNA molecules (iodouracil conjugate was used as standard for radioiodinated derivative). The stannyl-PNA was readily radioiodinated to give the desired [125I]iodo-PNA. To obtain selective targeting to PC-3 prostate cancer cells and cause internalization of the PNA, the peptide bombesin was coupled to the PNA. Because the PNA have very low solubility in water, a polyethyleneglycol (PEG) linker was used between the PNA and bombesin. Evaluation of the cell killing abilities of the radioiodinated PNA are underway.				
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I. Introduction:

These studies have been conducted as a preliminary step in the development of new therapeutic agents that are designed to improve the survival rates for prostate cancer patients with metastatic disease. Survival rates for prostate cancer drop from nearly 100% for cancers localized in the prostate to only 34% when metastases are present in other tissues (1). Unfortunately, treatment of metastatic prostate cancer is problematic as the cells can become resistant to the therapy being used. Therefore, new therapeutic agents that are not affected by changes in the cells are needed. In the studies, we used a specific type of radioactive material, an Auger electron-emitting radionuclide iodine-125 (^{125}I)(2). It is believed that Auger-emitting radionuclides targeted to nuclear material may hold the answer to the problem of cellular resistance. Auger emission is quite different from emissions from other radioactive materials as they have short-range electron emissions that are very toxic to cells when internalized, and are not affected by biological factors that cause resistance. Importantly, the very short particle range can greatly decrease the toxicity outside of the cells that it is targeted to. Our approach is to design a pharmaceutical that carries an Auger-emitting radionuclide and binds with the DNA in the cancer cell nucleus. Since Auger electrons have been shown to break DNA, such an agent could specifically kill the prostate cancer cell, or at a minimum cause damage to a gene (or gene products) that promote cancer growth.

In the exploratory studies, we have prepared a new type of radiopharmaceutical that is specifically designed to target prostate cancer cells, then be internalized and bind with messenger RNA in the cytoplasm and/or DNA in the nucleus. The radiopharmaceutical contains ^{125}I , on a targeting agent composed of a peptide nucleic acid (PNA)-peptide conjugate. Targeting to, and internalization in, prostate cancer cells is accomplished by the peptide portion of the conjugate. The peptide bombesin, can bind with cell surface gastrin-releasing peptide receptors and be internalized (3). Binding with mRNA and nuclear DNA specific to the insulin-like growth factor type 1 receptor (IGF-1R) is accomplished by an "antisense" PNA portion of the conjugate (4). When bound with IGF-1R mRNA or DNA, the radioiodine is physically located in close proximity to the phosphate backbone of the oligonucleotides, and decay of the radionuclide can cause extensive breakage of the backbone(s).

The following research report uses to statement of work to point out the progress made.

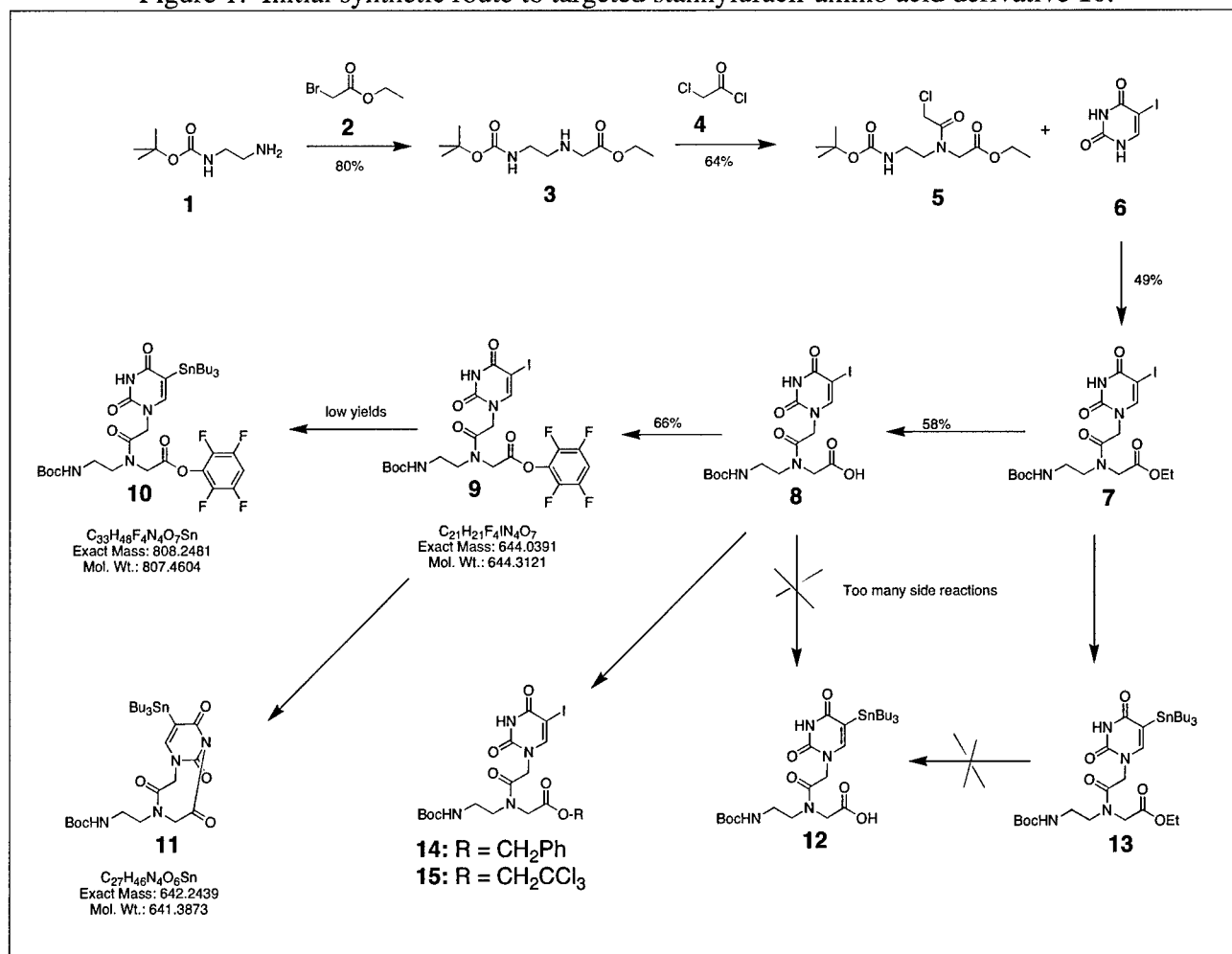
II. Research Conducted:

There were three objectives stated in the proposal. Those objectives, and the steps outlined to accomplish them in the statement of work, are listed in the following paragraphs. The progress and difficulties encountered in the research are provided after each objective.

- Objective 1.** Synthesize a stannyluracil (thymidine) analog that can be incorporated into a PNA and subsequently be radioiodinated (Months 1-3).
- Order chemicals (including PNA and bombesin) and synthesize glycine-ethylamine precursor (compound 5), months 1 & 2.
 - Prepare iodouracil adduct with glycine-ethylamine having N-tBoc and tetrafluorophenyl ester (compound 9), months 2 & 3.
 - Prepare stannyluracil derivative (compound 10), month 3.

The first objective was to prepare a novel reagent, a stannyluracil containing activated amino acid derivative **10**. The synthetic route proposed to prepare **10** is shown in Figure 1 (compounds **1-10**). This synthesis began with the preparation of the base-reactive intermediate **5** using the procedure previously published (5). The synthesis of the requisite iodouracil amino acid derivative **9** was achieved by coupling **5** with iodouracil **6**, followed by ethyl ester hydrolysis to form **8** and re-esterification. While this synthesis was successful, it took longer than projected due to difficulties with purification of some intermediates. Unfortunately, low yields of the stannyluracil amino acid derivative, **10**, were obtained when using the route shown in Figure 1. Attempts were made to prepare **10** from **9**, but the high temperature required in the reaction (i.e. refluxing toluene) caused **10** to cyclize, giving the unwanted **11** instead. Decreasing the time of reflux from 5 h to 2 h provided **10** in 29% yield, but this was not an optimal approach. Therefore, alternative routes to **10** were examined. One route explored was to prepare the stannyluracil derivative **12** from **8**. While a small quantity of **12** was formed under the reaction conditions, there were many other side reaction products formed so that route was not further explored. Another synthetic route explored was conversion of iodouracil **7** into its stannyluracil derivative **13**. Stannyluracil derivative **13** was readily prepared, but the stannyl group was not stable under the conditions for removing the ethyl ester. Other iodouracil derivatives, **14** and **15**, were prepared, and

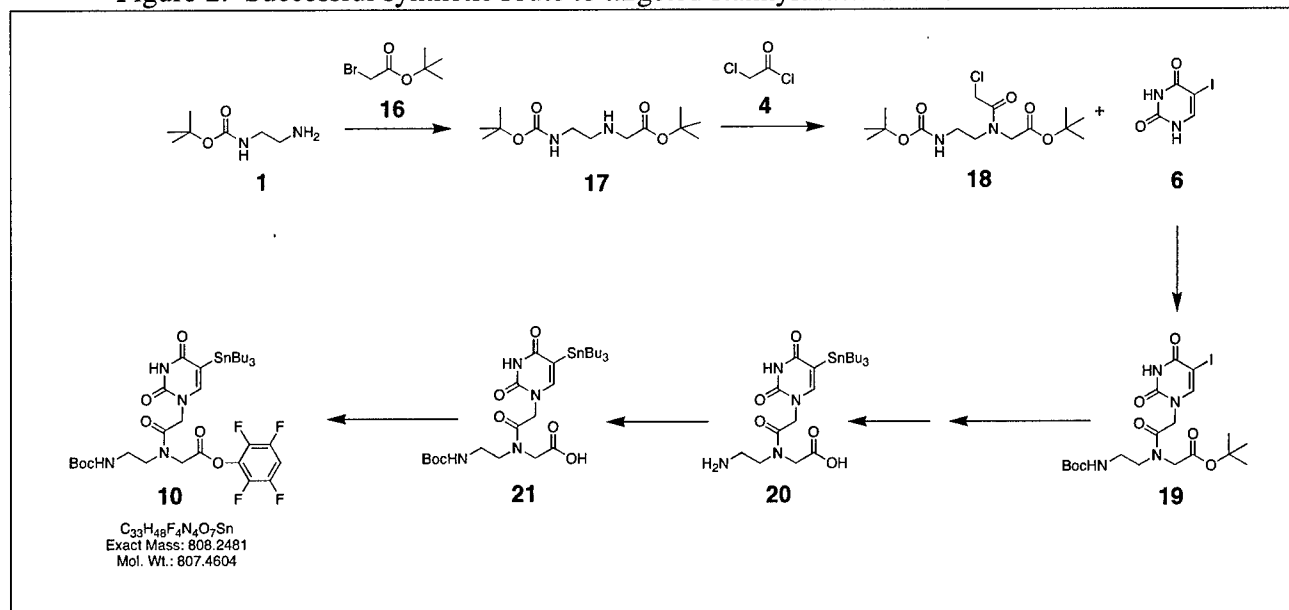
Figure 1: Initial synthetic route to targeted stannyluracil-amino acid derivative **10**.



these were converted to the corresponding stannyluracil derivatives, but again there were problems with removing the carboxylate protecting groups without also removing the stannyl group.

To circumvent the problem encountered with activated esters (i.e. TFP ester in **10**), and in removing the carboxylate protecting groups after preparation of the stannyl functionality, another synthetic route was devised for preparation of the stannyluracil derivative **10**. That synthesis is shown in Figure 2. Using reactions similar to those in Figure 1 to produce the ethyl esters **3** and **5**, the base-reactive **18** was prepared. Reaction of **18** with **6** provided iodouracil amino acid derivative **19**. The iodouracil derivative **19** was converted to the stannyluracil derivative **20** in two steps. The first step was preparation of the stannyl functionality and the second step was removal of the *tert*-butyl ester and *tert*-butoxycarbonyl (*t*Boc) groups in neat TFA. To prepare **10** from **20**, the free amine was reprotected as a N-*t*Boc group to give **21**, then the tetrafluorophenyl ester was prepared.

Figure 2: Successful synthetic route to targeted stannyluracil-amino acid derivative **10**.



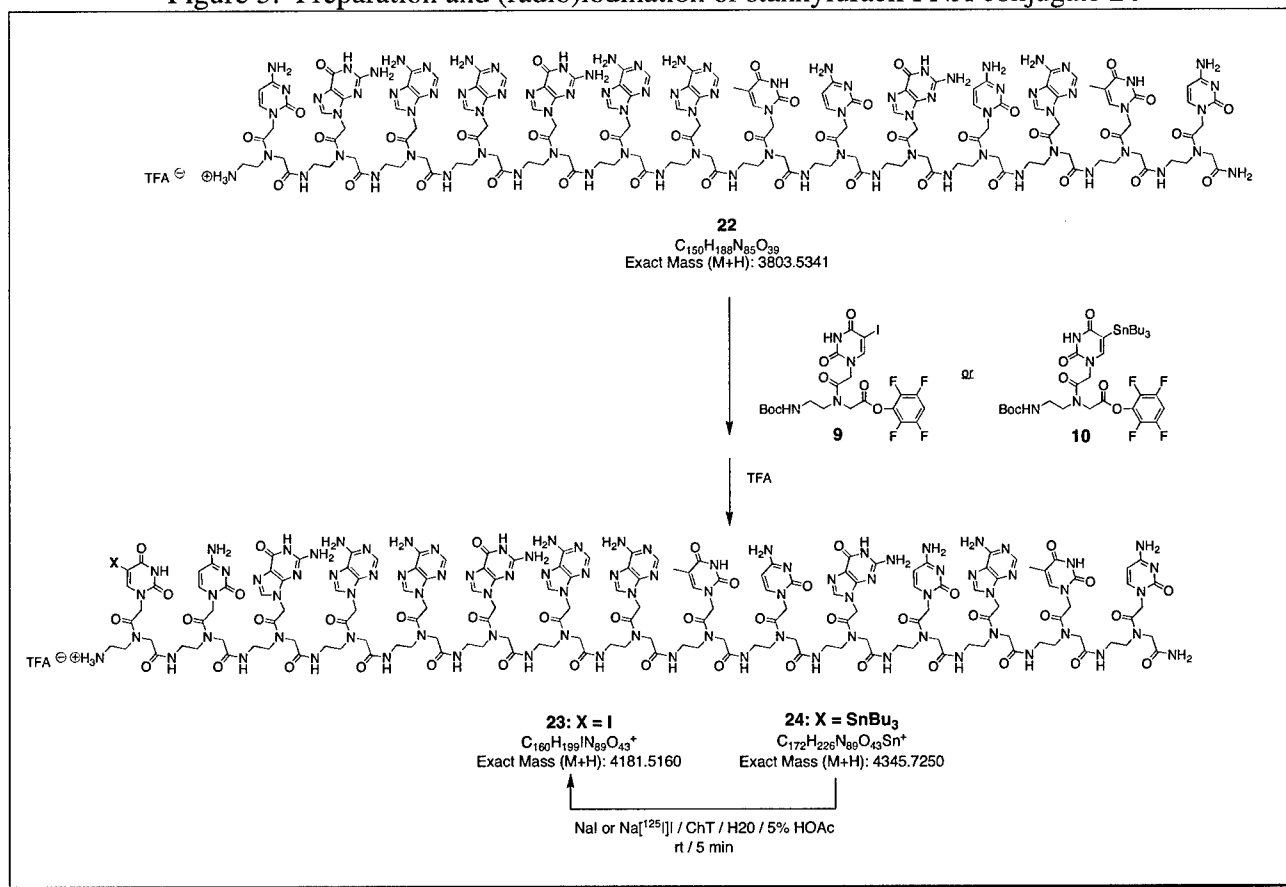
Objective 2. Prepare [125 I]PNA-bombesin adduct that incorporates a water solubilizing PEG linker (Months 4-8). All compounds will be fully characterized by NMR and mass spectral analysis. HPLC standards for radioiodinated compounds will be prepared using stable iodide.

- Conjugate stannyluracil derivative with PNA to prepare new PNA, which can be radioiodinated in high specific activity, month 4.
- Develop conditions for radioiodination, purification, and removal of the *t*Boc protecting group to yield [125 I]PNA **23**, month 5.
- Prepare PEG derivative (compound **29**) and conjugate with bombesin to obtain adduct, months 6 & 7.
- Conjugate radioiodinated PNA, [125 I]PNA with bombesin-PEG adduct (compound **31**) to prepare the [125 I]PNA-bombesin adduct (**32**), months 7 & 8.

A peptide nucleic acid (PNA), **22**, having a sequence for binding to IGF-1 mRNA ($\text{H}_2\text{N}-\text{CGAAGAATCGCATC}-\text{CO}_2\text{NH}_2$) was prepared in the Biotechnology Laboratory at the University of British Columbia (Dr. John Hobbs). This material is quite difficult to prepare and purify. We obtained two runs (5 mg and 6 mg) of this material. Our initial difficulty with the highly insoluble PNA was development of HPLC conditions to determine purity. Conditions were obtained which used a reversed-phase separation on a heated column using acetonitrile and water containing a small amount of trifluoroacetic acid. Mass spectral data and HPLC chromatograms indicated that the PNA synthesized was of sufficient purity (>95%) to work with.

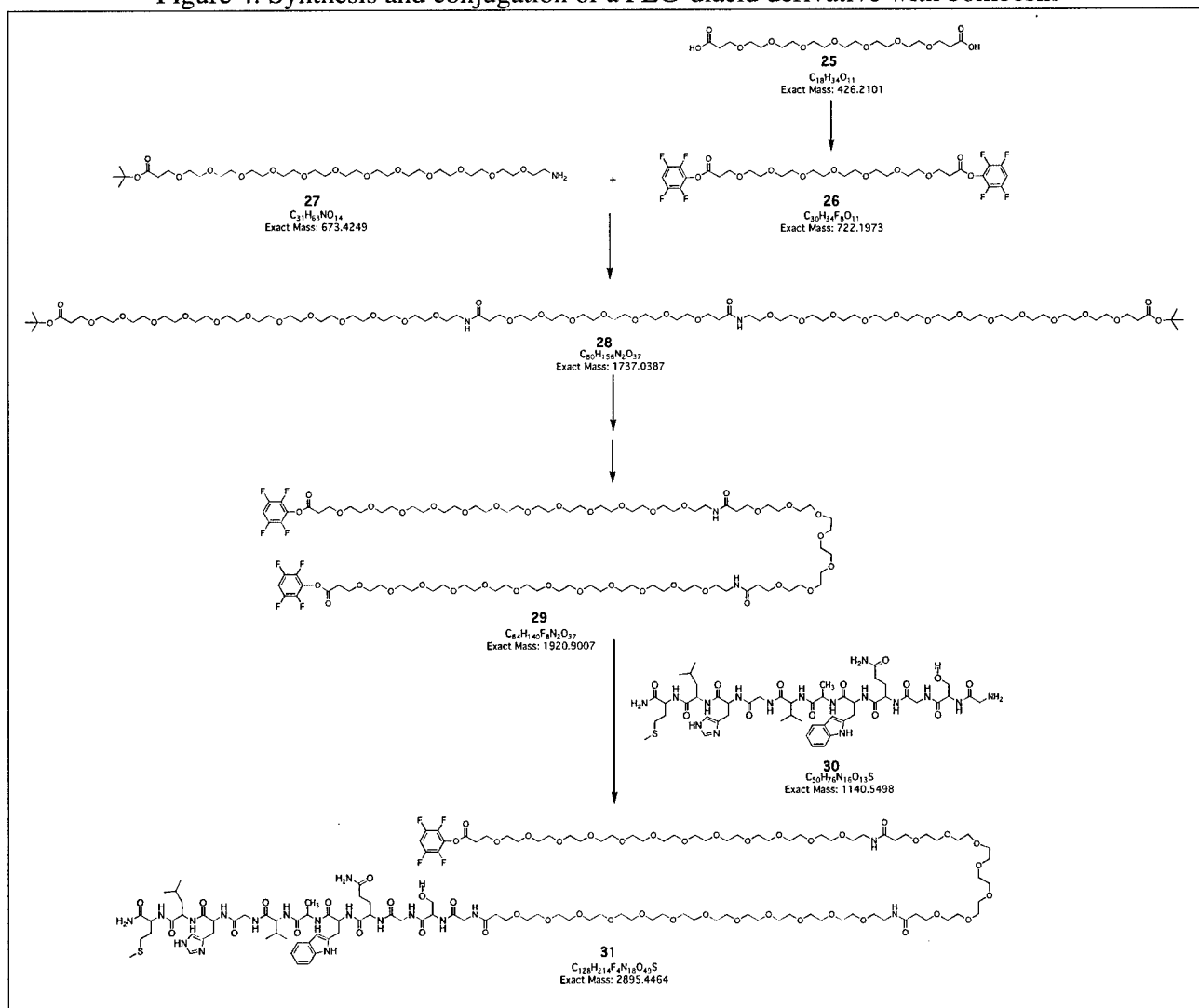
Reaction conditions were worked out to achieve coupling of the iodouracil derivative **9** with PNA **22** to prepare the new iodo-PNA **23**, as shown in Figure 3. Similar conditions were used to conjugate the stannyluracil derivative **10** with PNA **22** to prepare the stannyl-PNA **24**. Both new PNA were treated with trifluoroacetic acid to remove the tBoc protecting group. Deprotection of the amine was done to offset the very low solubility of the PNA in water. Higher solubility can be obtained when a free amine is present as that amine can be protonated with strong acid (e.g. TFA). Iodo-PNA **23** was prepared as a HPLC standard to be used when radioiodinating the stannyl-PNA **24**. In an initial reaction, non-radioactive sodium iodide was reacted with chloramine-T to give the iodinated PNA **23**. That material was identical to PNA **23** prepared from reaction of **9** with **22**. High specific activity radioiodination of **24** provided [^{125}I]**23** in 46% radiochemical yield.

Figure 3: Preparation and (radio)iodination of stannyluracil PNA conjugate **24**



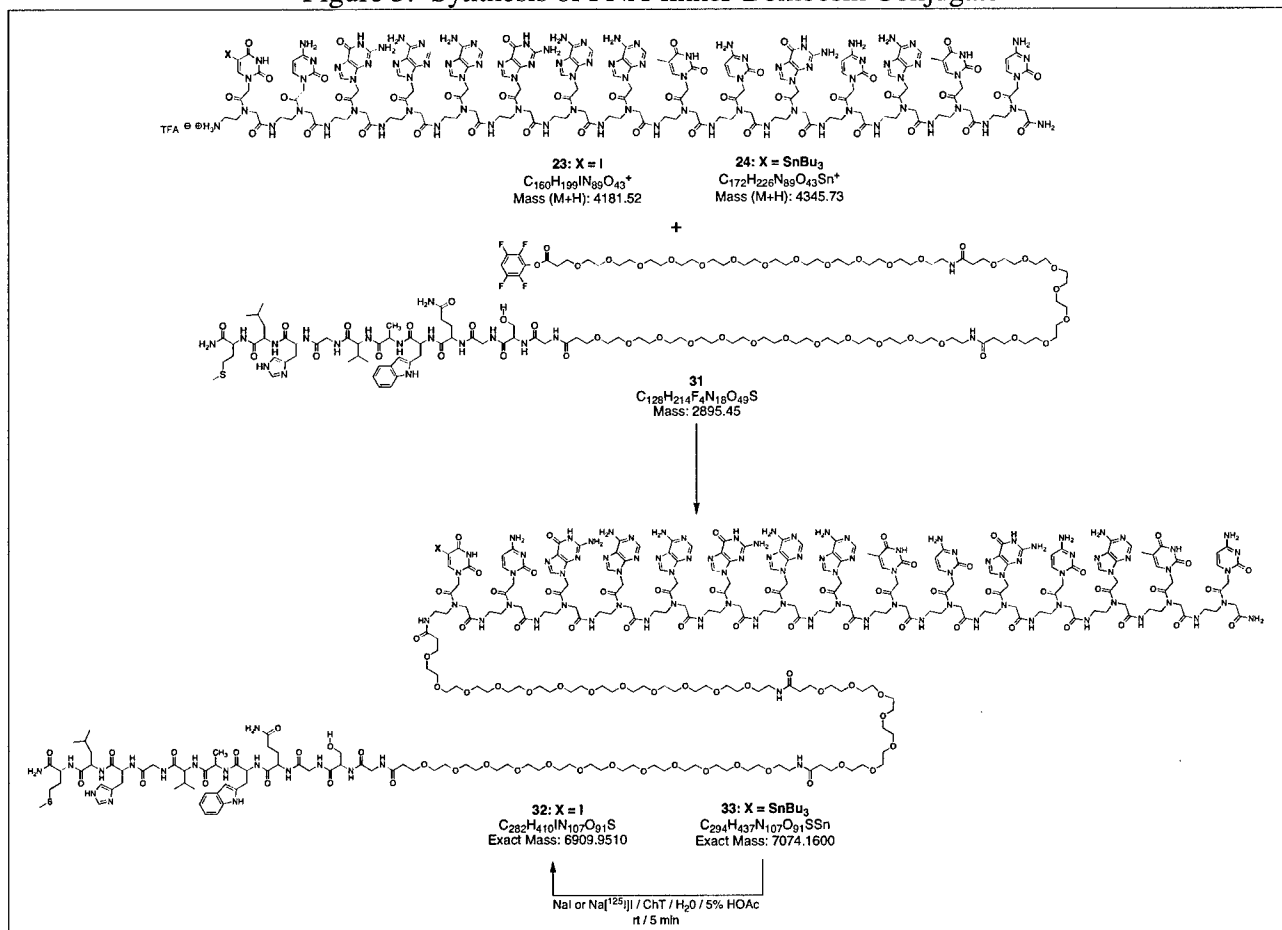
To offset the low solubility of the PNA in water, a new homobifunctional cross-linking polyethylene glycol (PEG) derivative, **29** was synthesized as shown in Figure 4. This molecule was prepared for use as a linker between the PNA and the cell targeting peptide bombesin. The bis-dPEG₈TM-acid **25** was obtained from Quanta BioDesign (Powell, Ohio) and the di-tetrafluorophenyl (TFP) activated ester **26** was prepared and purified. Reaction of **26** with two equivalents of an amino acid PEG derivative **27** (amino-dPEG₁₂TM-*t*-butyl ester; Quanta BioDesign) provided the new PEG derivative **28**. To make the PEG derivative **28** useful in our studies, the *t*-butyl esters were cleaved with TFA and tetrafluorophenyl (TFP) activated esters were prepared from the free acid to form bis-TFP ester PEG compound **29**. The next step in the synthesis was coupling the bis-TFP ester **29** with less than 1 equivalent of the peptide bombesin **30** to provide the adduct **31**. The bombesin derivative **30** was synthesized by Celtek Bioscience, LLC (Nashville, TN). This reaction was facile, but an excess of **29** in the reaction mixture with **31** made it difficult to purify.

Figure 4: Synthesis and conjugation of a PEG-diacid derivative with bombesin



The penultimate step in the preparation of radioiodinated PNA-bombesin conjugate was coupling of the bombesin-PEG-TFP ester **31** with the iodoPNA derivative **23** and (separately) with stannyl-PNA derivative **24**, as shown in Figure 5. Again, the iodoPNA-bombesin conjugate **32** was prepared as a HPLC standard for the radioiodinated PNA-bombesin conjugate [¹²⁵I]**32**. Based on the HPLC chromatogram, we have obtained the iodoPNA adduct **32**. To confirm the identity of **32**, we have isolated the peak from HPLC and have sent that material for mass spectral analysis. We have not yet received the mass spectral data. The stannylPNA **24** has also been coupled with the bombesin-PEG adduct, but we have not obtained the mass spectral data to confirm that coupling either. We anticipate obtaining the mass spectral data of these compounds within a week. The final radioiodination step has not been conducted. While the period of grant funding has expired, we plan to finish this project (we are very close) by conducting the radioiodination step and isolating the [¹²⁵I]**32** for testing in vitro cell survival.

Figure 5: Synthesis of PNA-linker-Bombesin Conjugate



- Objective 3.** Measure cell survival and quantify IGF-1R mRNA in PC-3 and LNCaP human prostate cancer cells treated with [125 I]PNA and [125 I]PNA-bombesin adduct (Months 9-12).
- Evaluate the cell survival and quantify IGF-1R mRNA for PC-3 and LNCaP cells treated with four concentrations of [125 I]PNA, months 9 & 10.
 - Evaluate the cell survival and quantify IGF-1R mRNA for PC-3 and LNCaP cells are treated with four concentrations of [125 I]PNA-bombesin, months 11 & 12.

The synthesis and radioiodination of reagents required for this objective took much longer than anticipated, so the cell survival studies have just begun. Initial experiments were designed to determine optimal conditions for transferring the PNA oligos into the 2 cell lines. For the first experiment, 1×10^5 cells of each line were suspended in electroporation media (phosphate buffered saline supplemented with 10 mM glucose), incubated at 4°C for 10 min, and subjected to electroporation using a Bio-Rad (Hercules, CA) Gene Pulser II system. The cells were shocked in 0.4 cm gap cuvettes using settings 0.25 kV and 960 μ F. Immediately following electroporation the cells were plated in 100 mm tissue culture dishes at 100 cells/plate. Cells that were not electroporated but otherwise identically treated were plated as well. Once the effect of electroporation on the cell lines plating efficiency has been quantified, we will repeat the electroporation using labeled and unlabeled PNA oligos. We should have that information shortly.

Assuming we do not have to modify electroporation conditions, we will then electroporate 1.2 and 12 μ Ci of I-125 labeled PNA oligo into each cell line. An equal mass of unlabeled oligo will be electroporated into a duplicate set of cells. Following electroporation a portion of the cells will be immediately plated to measure plating efficiency and toxicity. The remaining cells will be plated and maintained in mass culture at 37°C for 24 h to allow oligos to migrate (in cytoplasm and into nucleus) and hybridize to appropriate target site. After 24 h, these cells will be cryogenically frozen and stored at -80°C for 6 months to allow for a sufficient number of I-125 decays to effect survival. At this point, we don't know how much activity will be taken up, so we have arbitrarily chosen 6 months to examine. Future studies will make more careful measurements of activity and time experiments to examine effects of different numbers of disintegrations. We anticipate obtaining results from this experiment over the next 6 months.

III. Key Research Accomplishments:

- Synthesis of an amine protected and carboxylate activated iodouracil amino acid, **9**. This compound is important as a standard for preparation radioiodinated PNAs, but it has the potential to be used for more than that. The iodouracil base is sterically similar to a thymine base, but has different electronic properties. Therefore, it may be useful in preparing PNA that have tailored binding properties.
- Synthesis of the amine protected and carboxylate activated stannyluracil amino acid, **10**. As shown in this research, this base can be used to site-selectively introduce radioiodine into a PNA.
- Synthesis of iodo- and stannylPNA derivatives **23** and **24**. Using the synthetic methods developed with this funding, investigators can prepare these unique PNA, or similar ones, for other applications to cancer therapy.

4. Synthesis of a new PEG cross-linking reagent **29** that can be used for water solubilization and to provide a spacer between biomolecules being tested.
5. Synthesis of the bombesin-PEG adduct **31**. This adduct can be used to couple with molecules other than PNA to carry them across cell membranes using the GRP receptor system.
6. Synthesis of bombesin-iodoPNA adduct **32** and bombesin-stannylPNA adduct **33**. The stannyl derivative **33** can potentially be radioiodinated (or radiohalogenated) such that it can be used to break DNA or mRNA at specific locations in their sequence after binding. This reagent is of primary interest as a carrier of Auger electron emitter for therapy of metastatic cancer. Additional studies need to be conducted to prove its value.

IV. Reportable Outcomes:

1. This report
2. Abstract for preparation of radioiodinated PNA

V. Conclusions:

We have been successful in preparing a number of unique compounds (as listed above), and have begun cell survival testing of compounds of interest. We had several difficulties in the syntheses and those resulted in our progressing at a slower pace than anticipated. Our demonstration that a stannyluracil could be used to prepare high specific activity radioiodinated PNA is very important. This step was essential to the concept being investigated and in obtaining materials for testing. However this synthesis was accomplished late in the studies, resulting in our not obtaining testing of the radiolabeled reagents for cell killing at the time of this report. We are disappointed that we did not complete all of the studies outlined, particularly the in vitro testing. It is unfortunate that the manpower and time for the studies were limited. We believe that the studies are very important and plan to continue them to their completion. This should not take too much more effort as we have achieved most of what was planned.

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VII. Appendices

1. Abstract submitted to 12th International Symposium on Radiopharmaceutical Chemistry.

VIII. List of Personnel Receiving Pay for Research Effort

1. D. Scott Wilbur, Ph.D., Principal Investigator
2. Robert Vessella, Ph.D., Co-Investigator
3. Jeffrey Schwartz, Ph.D., Co-Investigator
4. Ming-Kuan Chyan, Ph.D., Research Scientist
5. Don Hamlin, B.S., Research Scientist
6. Robert Jordan, B.S., Research Scientist
7. Austin Odman, B.S., Research Scientist

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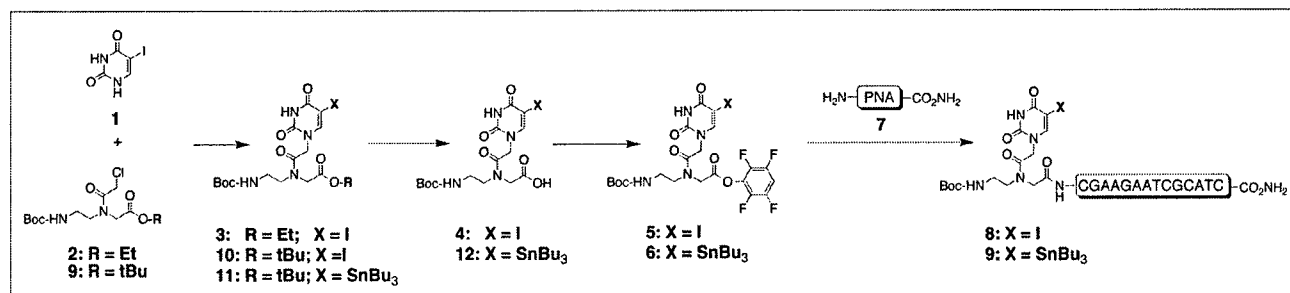
Synthesis and Radioiodination of a PNA Containing an [¹²⁵I]Iodouracil Moiety.

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Peptide nucleic acids (PNA) hold potential as agents for delivering radiation to specific gene sequences and mRNA. Previous investigators have shown that In-111-labeled PNA is effective at sequence-specific DNA cleavage [1]. We are interested in PNAs with Auger electron emitting radiohalogens directly incorporated into a base such that the radionuclide is held close to the DNA/RNA backbone. As a preliminary investigation, we have prepared a PNA that has an I-125 labeled uracil moiety in the place of a thymine moiety. The base sequence of the PNA was chosen as it has been shown to bind with the insulin-like growth factor type 1 receptor (IGF-1R) [2], which is upregulated in prostate cancer. The initial goal in the study was to produce uracil containing amino acid derivatives that could be incorporated into a PNA. The sequence of reactions is shown in the figure below. The first step was to prepare the base-reactive amino acid derivative **2**. That synthesis was carried out in four steps as described in the literature [3]. Reaction of **2** with commercially available iodouracil, **1**, was accomplished in 49% yield after purification. Hydrolysis of the ethyl ester in 1N NaOH at room temperature for 4 h gave the free acid **4** in 58% yield, and preparation of the TFP ester **5** was accomplished in 66% yield using tetrafluorophenyl trifluoroacetate (TFP-OTFA) and Et₃N in DMF at rt for 5 min. Unfortunately, low yields (e.g. <30%) of the desired stannyluracil **6** were prepared from **5** using the standard conditions, i.e. (nBu₃Sn)₂ / (Ph₃P)₄Pd in anhyd. toluene at reflux. An alternative pathway to **6** began with the preparation of **9** in a two-step synthesis. Reaction of **9** with **1** provided **10** in 44% yield. Conversion of **10** to the stannyluracil derivative **11** was readily accomplished using the standard stannylation conditions. Deprotection of **11** was accomplished in neat TFA at rt for 5 min, and reformation of the N-Boc to form **12** was accomplished with NaHCO₃/Boc₂O in acetone/H₂O for 3 h at rt. This was followed by preparation of the TFP ester using TFP-OTFA to give **6**. Once prepared, uracil amino acid derivatives **5** and **6** were reacted with PNA **7** to form PNA **8** (calc. 4181 Da, found 4185 Da; M+4H) and PNA **9** (calc. 4445 Da, found 4484 Da; M+K). Radioiodination of **9** was facile using Na[¹²⁵I]I and chloramine-T in H₂O/5% HOAc at rt for 5 min, providing a 46% (HPLC) isolated yield of [¹²⁵I]**8**.



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