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Award Number: W81XWH-05-1-0556

TITLE: Diagnostic and Therapeutic Radiopharmaceutical Agents for Selective Discrimination of Prostate Cancer

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REPORT DATE: October 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-10-2006	Annual	1 Oct 2005 – 30 Sep 2006

4. TITLE AND SUBTITLE

Diagnostic and Therapeutic Radiopharmaceutical Agents for Selective Discrimination of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0556

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Paul Benny

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Washington State University
Pullman, WA 99164

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The project investigated the development of ligand linked Flutamide analogs for complexing a M(CO)3 (Re,99mTc) organometallic species to target prostate cancer for imaging and therapy. The project has been successful in developing new synthetic strategies for this application. A general method was developed for preparing a universal flutamide analog that can be applied to number of ligands to yield a flutamide targeted species capable of delivery of M(CO)3 to the cancer cell. The chemical stability (heat, pH) was investigated and the application of this general coupling methodology was applied to several tridentate ligands(i.e., cysteine, histidine). The resulting flutamide linked ligand system were complexed with the M(CO)3 to yield the desired metal complex. The 99mTc complexes were prepared in excellent yields(>95%) at (10-4,10-5 M) ligand concentration at biologically relevant pH (7.4) and compared to the corresponding rhenium analogs through radioHPLC techniques.

15. SUBJECT TERMS
Imaging and therapy of Prostate cancer

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction

The focus of the research as highlighted in the proposal is the development of new diagnostic agents for identifying and probing prostate cancer. Flutamide, a non steroidal antagonist of the androgen receptor, is a current medical treatment of prostate cancer.[1] The proposed work involves the synthesis of novel modified flutamide derivatives that incorporates radionuclides (^{99m}Tc, ^{188}Re) into the framework of the system as unique organometallic species, $M(CO)_3^+$. These radionuclides have an important contribution to the molecule by providing a mechanism to directly image and therapeutically treat prostate cancer at the primary and potentially secondary sites. The potential outcome of this work would be the development of radioactive incorporated flutamide compounds that can be used to actively monitor existing treatment protocols and to provide an enhanced therapeutic value in conjunction with associated emissions. The compounds may also have potential use is evaluating hormone refractory syndrome as cancer cells become drug resistant or mutations occur.2

Body

The proposed scope of work for year one has been primarily achieved. The objectives listed in the scope of work initiate the synthesis of flutamide modified compounds and the preliminary characterization of complex experimentation with Re and Tc-99m as listed in the year one objectives illustrate in bold below.

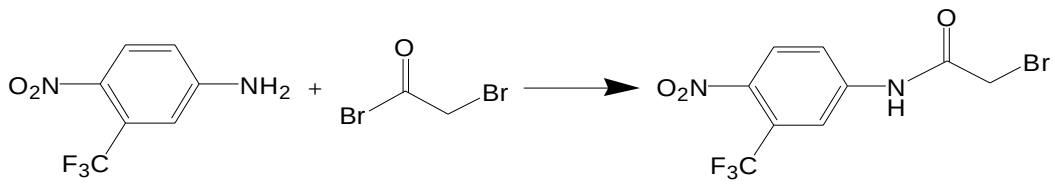
Year One Objectives

- **Synthesize and characterize flutamide linked L-amino acid ligands**

The laboratory progress has succeeded in the development of a synthetic pathway for the formation of a general multi-functional precursor of flutamide to link with ligands. (Figure 1) Although the synthesis was originally considered to be straightforward, product decomposition and relative low synthetic yields initially hindered results. Initial work focused on the development of a generic flutamide species that had the potential to serve as a general alkylation species for a number of ligands. The proposed species is formed by the reaction of 3-trifluoromethane 4-nitro aniline with bromo acetyl bromide to yield an amide linkage between the aryl amine and the acetyl bromide to yield 2-Bromo-N-(4-nitro-3-trifluoromethyl-phenyl)-acetamide (**1**). Compound **1** is suggested due to the reactivity of alkyl bromides with variety of nucliphiles (i.e., N, S, O, P), which would the number of potential systems explored. The initial synthesis of this compound **1** yielded a mixture of results. The reaction was initially prepared in a fashion to analogous aryl amide formation by reacting the above reagents in a 1:1 fashion in an ice bath over an hour. The starting materials were found to have only partially reacted to form the desired amide (20-30%). Albeit lower than desired yields, purification of the compound **1** from the 3-trifluoromethane 4-nitro aniline starting material proved more tedious than expected. After many attempts at purification via recrystallization and column methods, a small amount of pure compound **1** was obtained. However, the isolated yields of pure **1** were much lower (~10%) than the original reaction yields due to the coelution of the 3-trifluoromethane 4-nitro aniline starting material with the product. Modifications in the synthesis were required to achieve the desired yield and purity of compound. The yield of **1** was observed to improve with the elongation of mixing time (40-50%). However, the dilemma of purification of the product from unreacted starting material still existed. The increased addition of bromoacetyl bromide to 1.5 equivalents to 1 equivalent of the 3-trifluoromethane 4-nitro aniline starting material combined with the elongation of reaction time proved to be significant in increasing the yields (80-90%) and the isolated purity by minimizing the unreacted starting material. Using this

modified method, multi-gram synthesis of compound **1** have been carried with excellent yield and purity.

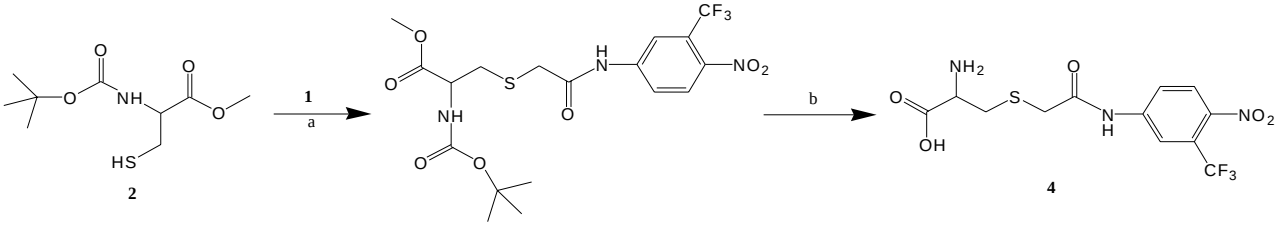
Figure 1. Synthesis of the generic linkable Flutamide derivative, 2-Bromo-N-(4-nitro-3-trifluoromethyl-phenyl)-acetamide (**1**)



1

With a generic linkable flutamide species available, the first series of ligands investigated for linkage with compound **1** were cysteine and histidine. Both of these amino acids serve as excellent tripodal ligands for $M(CO)_3$ M=Tc, Re that are known for their high labeling efficiency.[4,5] The initial attempt for cysteine linked flutamide compounds involved the alkylation of 2-tert-Butoxycarbonylamino-3-mercapto-propionic acid methyl ester (**2**) with **1** at the thiol.(Figure 2) The isolation was followed by removal of the boc and the methyl ester protecting groups via stepwise reaction a) 1:3 LiOH/ Methanol b) 1:2 Trifluoroacetic acid (TFA)/Methylene chloride). Although the initial formation of **3** (85%) proved to be very successful, the proposed standard deprotection methods results in the cleavage of the amide bond. The analogous aniline derivative was also prepared and deprotected according to the preceding method. Unlike the flutamide derivative, the aniline derivative did not result in amide cleavage in high basic conditions for methyl ester cleavage. The strong electron withdrawing groups of nitro and trifluoromethane may be the reason for cleavage compared to the aniline derivative.

Figure 2. Reaction pathway for a Flutamide link Cysteine compound



2 **3** **4**

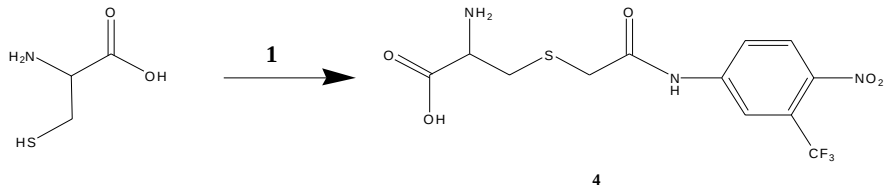
a) NEt_3, 40 °C, 2 hrs, acetonitrile b) Step 1: 1:3 LiOH/ MEOH Step 2: 1:2 TFA/$MeCl_2$

An alternative approach was developed to directly react cysteine with **1** without the use of protecting groups thus avoiding potential cleavage of the amide bond. This approach utilizes the reactivity of the thiol group over the amine in an aqueous sample. (Figure 3) In the literature reports alkylation of a thiol can be achieved in a strongly

alkaline media (1M NaOH, pH=12). From initial experiments, the strong basic solution initiated cleavage of the amide bond in **1**. To avoid cleavage, the reaction was carried out at a pH between 8 and 9 and stirred at room temperature overnight. This method did yield some of the desired product (20%), however, the majority of the material was the poorly soluble unreacted starting material, which proved very challenging to purify **4** from the starting material **1**.

The reaction was carried out at a higher temperature at 50 °C to facilitate product formation. However excessive heating led to the isolation the 3-trifluoromethane 4-nitro aniline, the amide cleavage product/starting material. It was apparent the insolubility of **1** in water was hindering product formation at room temperature and excessive heating at basic pH increase the cleavage of the amide bond in **1** so the addition of a miscible organic solvent, dimethyl formamide, was selected to add to the reaction. Although initially a good idea to improve solubility, it did not yield the desired product. Other miscible organic solvents were also investigated yielding similar poor results as before. Surprisingly, the addition of an immiscible organic solvent, methylene chloride, proved to be very successful to yield the desired product in excellent yields (>95%). The alkylation reaction of the thiol in the biphasic system was so successful at ph=8 that desired product was formed at room temperature within an hour and the product analytically precipitates from the reaction mixture, where the starting materials remain in solution. This new biphasic approach has been applied to other moieties to provide a facile synthesis for linking the cysteine ligand with other alkyl halides coupled with other targeting small molecules and peptides. More importantly, racemization of the alpha carbon in the sample won't occur as readily at the higher pH's minimizing any sterochemical effects. A presentation on this topic was offered at an American Chemical Society in April 2006 (See appendix for abstract) and a manuscript is in preparation.

Figure 3. Direct synthesis of Cysteine linked flutamide compounds

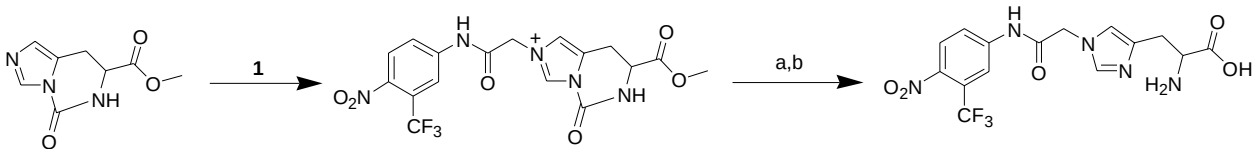


4

Flutamide linked Histidine ligands were also attempted in parallel with the cysteine analogs. (Figure 4) The initial synthetic route proved to be partially successful. Alkylation to the imidazole ring was achieved in refluxing acetonitrile, indicating thermal

stability in a neutral organic solvent. The first step in the deprotection was also successful by the ring opening carbamate formation reaction with 9-Fluorenylmethanol appeared to be successful. However, after the second step involving the cleavage of the carbamate and the methyl ester with LiOH: MeOH 1:3 the resulting the amide cleavage product/starting material, 3-trifluoromethane 4-nitro aniline, was observed. The development of an alternative starting material with an acid cleavable group, such as tert butyl alcohol, is in current developmental stages for the histidine system.

Figure 4. Synthetic route for Histidine linked flutamide derivatives



a) FMOC, ACN b) DIPEA, LiOH/MeOH

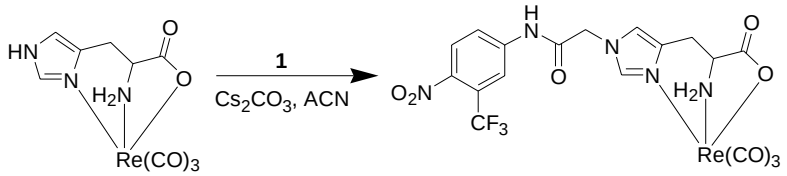
- **Prepare and characterize the metal complexes with the flutamide linked amino acid ligands**

Preliminary results in forming the desired metal complexes are positive. A general procedure involving the complexation of rhenium analogs has been developed. The method was applied with compound **4** successfully. The procedure involves the dissolution of $[NET_4]_2[fac\text{-}ReBr_3(CO)_3]$ in 25 mL of water followed by the addition of the ligand to the solution. The pH is carefully adjusted to 6.5 with 0.1 M $NaHCO_3$. Excessively high pH's are strongly avoided as it have been found to decrease yields due to metal cluster formation of the rhenium starting material and cleavage of the amide bond. The product precipitates from solution and is collected dried. Purification is currently achieved by Prep HPLC or column chromatography. The yields of this reaction with **4** are limited by the purification step as other methods of purification are being currently investigated. A small amount of pure $Re(CO)_3$**4** was utilized as a HPLC reference for the ^{99m}Tc studies.

Even though the preparation of the free histidine linked flutamide ligand **7** is currently unsuccessful, an alternative approach for the production of the rhenium complex was utilized.[4] The desired product was formed by alkylation of the $^{\varepsilon}N$ on the imidazole ring of $Re(CO)_3$histidine[6] complex when reacted with **1** in the presence of Cs_2CO_3 in a acetonitrile solution at room temperature overnight. The reaction yielded the desired complex $Re(CO)_3$**7** in moderate yields 50-60%. The product was isolated as the major species when stirred at room temperature. However, heating the sample even at 40 °C with the base caused multiple products to be observed in the HPLC trace.

The product was purified by column chromatograph. This method may also provide the free ligand for the ^{99m}Tc studies by selective removing the Re metal from the complex by acidic oxidation. This approach is currently under investigation to determine the stability of the ligand during Re deprotection.

Figure 5. The synthesis of the $Re(CO)_3$**7** from $Re(CO)_3$His with compound **1**



- **Optimize radiosynthesis conditons for Tc-99m and Re-188**

Efficient radiochemical methods were investigated to overall best application for product formation. A general procedure was developed that produced excellent radiochemical yields. The method involves the reaction of $^{99m}Tc(OH_2)_3(CO)_3^+$ (~20 uCi /mL)with the flutamide linked ligands in a biologically relevant pH 7.4 for 30 minutes at 95 °C. The radiochemical products were characterized by HPLC and compared with the retention times of the rhenium analogs. The cysteine linked flutamide analog **4** was examined according to the procedure outlined above. Ligand **4** was tested at 10^{-4}, 10^{-5},10^{-6} M ligand concentration to an constant ^{99m}Tc concentration. The 10^{-4} and 10^{-5} M ligand concentrations exhibited excellent labeling yields (>95%) as a single product matching the rhenium retention times. The 10^{-6} M concentration of **4** partially labeled (60%) after 30 minutes of heating. Under this general method developed with the cysteine analogs, the amide linkage appears to remain intact while the ^{99m}Tc complex is formed.

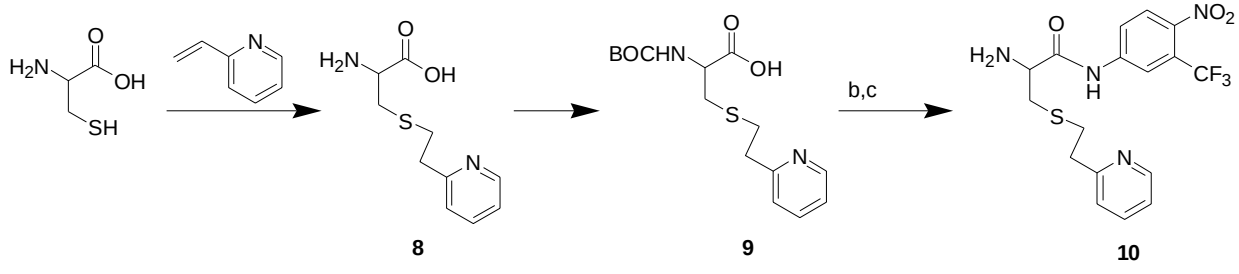
- **Conduct preliminary stability studies**

Several studies investigating the stability of the ligand and metal (Re, ^{99m}Tc) ligand complex have been highlight in the preceding sections. Both on the chemical and radiological species appear to have a reasonable degree of stability. The main challennge in the study is the synthetic pathways, where prolonged exposure to heat, sensitivity to basic pH's, and the combined effect can lead to degradation of the flutamide analogs. The studies illustrate the metal complex is robust, while the most sensitive portion of the ligand system is the amide bond that is particularly prone to cleavage. By selecting neutral or acidic conditions, the degradation of the amide bond is minimized yielding the desired complex.

- **Synthesize and characterize flutamide linked with linear tridentate ligands**

Due to the initial challenges with the synthesis and coupling with compound **1**, efforts were primarily focused to understand the chemistry of the tripodal ligand systems. A linear ligand system was developed based on the cysteine system. Cysteine was modified by alkylating a pyridyl group on the thiol. (Figure 6) This linear system (N,S,N) would provide a charged species compared the tripodal systems perhaps enhancing the interactions and solubility of the compound. Compound **8** is prepared according to the literature.[7] The amine was protected with an acid labile tert-butoxy carbonyl (BOC) by reacting **8** with BOC anhydride in methanol to give compound **9** in good yields. Coupling of the 3-trifluoromethane 4-nitro aniline with **8** was more difficult that previously anticipated. Several coupling methods and coupling agents were investigated for forming the amide bond in compound **9**. The coupling agent dicyclohexylcarbodiimide (DCC) yield a very low yield of **9** (<5%), where the majority of the isolated product was the activated **8** DCC adduct. Attempts to react further with the starting material were unsuccessful. It was believed that the steric bulk of the BOC protecting group and the pyridyl linkage may have been sterically blocking or inhibiting the amide formation in combination with the **8** DCC adduct. The same reaction conditions were utilized with the surrogate analog, aniline, to yield the analogous desired product similar to **9** (see addition data). This suggested that the reaction is chemically possible, but would require stronger reaction conditions for amide coupling. A stronger coupling agent O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) was utilized to yield compound **9** in better yields (40%). Current work in this area involves the improvement of yields of the coupling step, deprotection of the BOC group, and the addition of a glycine residue on the C terminus to increase the linker distance from the complexing portion of the ligand and the improve bio recognition. Studies with $M(CO)_3$ M= Re, ^{99m}Tc will be conducted as soon the deprotected ligand is obtained with the general procedure developed for the tripodal ligands with rhenium and technetium-99m.

Figure 6. Synthetic route for linear S-Pyridyl Cisteine linked flutamide derivatives.



Key Research Accomplishments

*Development of basis chemistry of the synthetic flutamide derivatives

*Development of coupling mechanisms for flutamide linked analogs

*Developed a new alkylation technique for thiol for polar and nonpolar species utilizing a biphasic system

*Development of an excellent radiochemical labeling system (>95%) of achieved with $^{99m}Tc(CO)_3$ that maintains the stability of the amide bond in flutamide.

Reportable Outcomes

Presentations

1) Glenn Fugate American Chemical Society San Francisco 9/2006

2) Brienne Bottenus American Chemical Society Atlanta 3/2006

Degrees

Brienne Bottenus BS 5/2006 Washington State University

Manuscripts

1) S-functionalized cysteine ligands for complexation with M(CO)3+ M=Re, 99mTc for diagnostic applications. Bottenus, Brienne N.; Fugate, Glenn A.; Benny, Paul. (in progress)

Conclusions

The results presented here illustrate potential viability of the compounds for prostate cancer. The first years research has establish several important milestones to measure the success of the project. Several key synthetic hurdles were overcome to demonstrate the chemistry of derivitization of flutamide analogs as a real possibility. Several new synthetic strategies were developed for the project. A general method for preparing a derivitized flutamide analog that can be coupled with a ligand for complexing $M(CO)_3$ was developed for wide application to a variety of ligand types. A new method involving the synthesis of flutamide linked cysteine analogs using a biphasic approach to improve yield and purification of the desired product.. This new simple method has other potential applications beyond the current work by providing an easy method for cysteine to be attached other targeting agents for prostate cancer, such as peptides and other small molecules. In the past year, a number stability of issues were investigated and it was found that the proposed compounds are stable in biological pH and the radiochemical/ metal complexes can be form in good yields. The development of radiochemical labeling methods that gave the desired compound in high yield (>95%) in a biologically friendly media was critical to the potential application in humans. This insures the products have potential application for the next stage of research investigating the cellular interaction of the compounds with the androgen receptors. A solid foundation understanding the basis chemistry of the compounds was imperative in the first year as was explored. This approach will should greatly assist in the next step in research application to cancer cells that may require structural adjustments in the compound and refining the targeting methodology to improve target specificity towards prostate cancer cells. The development of the synthetic pathways developed during the first year can be utilized and adjusted to facilitate the synthesis of additional compounds for imaging and treating prostate cancer.

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Appendices

Abstracts of posters

1) In situ formation of tridentate ligands in aqueous media from 2, 4-pentanedione to form imine complexes around [M(CO)3(OH2)3]+ (M=Re, 99mTc) centers. Fugate, Glenn A.; Bottenus, Brienne N.; Benny, Paul. Department of Chemistry, Washington State University, Pullman, WA, USA. Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, United States, Sept. 10-14, 2006 (2006), INOR-184. Publisher: American Chemical Society, Washington, D. C CODEN: 69IHRD Conference; Meeting Abstract; Computer Optical Disk written in English. AN 2006:860578 CAPLUS

Abstract

Exploration of new methods for radiolabeling that enhance biol. affinity and sepn. has led this investigation to prep. a multidentate compds. by reacting 2 ligands on a metal center. A family of 2, 4-pentanedione (acac) complexes with the [M(CO)3(OH2)3]+ (M=Re, 99mTc) species has been synthesized on the macroscopic and radiotracer level. Several "2 + 1" type donor systems complexes of the form [M(CO)3(acac)L] were made where M = Re or Tc-99m and L is a simple amine ligand such as pyridine. Tridentate ligands were synthesized using the metal center as a template. The reaction of acac with a compd. contg. two amine groups such as 2-(2-aminoethyl)pyridine forms a tridentate ligand via the formation of an imine. These two step, one pot type of reactions may be a useful means of attaching chem. sensitive target seeking compds. to radioisotopes centers of interest.

2) S-functionalized cysteine ligands for complexation with M(CO)3+ M=Re, 99mTc for diagnostic applications. Bottenus, Brienne N.; Fugate, Glenn A.; Benny, Paul. Department of Chemistry, Washington State University, Pullman, WA, USA. Abstracts of Papers, 231st ACS National Meeting, Atlanta, GA, United States, March 26-30, 2006 (2006), INOR-298. Publisher: American Chemical Society, Washington, D. C CODEN: 69HYEC Conference; Meeting Abstract; Computer Optical Disk written in English. AN 2006:247626 CAPLUS

Abstract

This work focuses on the functionalization of cysteine by conversion of the free thiol to a thioether moiety to improve the complexation of fac-M(CO)3+ (M=Re, 99mTc) and to serve as a synthetic handle for coupling biomols. Several model amino acid and peptide analogs were coupled with cysteine. The cysteine linked peptides were examd. with rhenium and structurally characterized by std. chem. analyses to det. the specific coordination at the N, O, S donors of the S-modified cysteine section of the compd. Radioactive 99mtechnetium complexes were prepd. and compared to the retention times of the analogous rhenium compds. on HPLC showing >90% labeling efficiency.

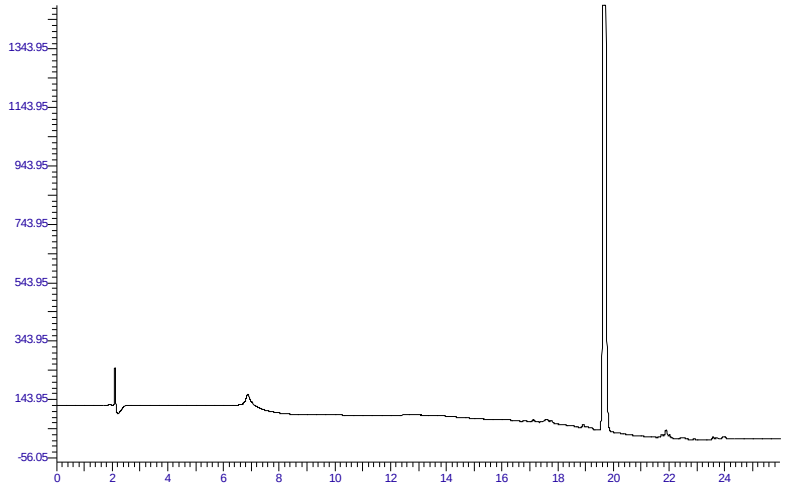
List of Personnel supported by this funding

Personnel	Position
Paul Benny	Professor, director of research
Glenn Fugate	Post Doc (10/1/06-8/1/06)
Brienne Bottenus	Undergraduate researcher (10/1/06-6/1/06)
Elieen Morley	Undergraduate researcher (06/1/06-current)

Supporting Data

2-bromo-N-3-trifluoromethyl-4-nitro phenylacetamide, 1. 3-trifluoromethyl-4-nitro aniline, (1.2605g, 6.1mmol) was dissolved in 40.0mL CH_2Cl. 1.29mL (9.2mmol) of $(C_2H_5)_3N$ was added. Reaction mixture was chilled in ice bath for 30 minutes. 802µl (9.2mmol) of bromoacetyl bromide where dissolved in 10.0mL of CH_2Cl and added dropwise to the reaction mixture. Reaction turned from yellow solution to amber color solution. Reaction was followed by HPLC. After two hours a single peak was seen at 19.67min. Solution was washed 3X (50.0mL each) with 0.1M NaOH and H_2O. The reaction mixture was evaporated and dried. The compound was purified by a silica gel column (5% Ethyl Acetate, 95% Hexane) RF=0.1 and followed by TLC (25%Ethyl acetate, 75% Hexane). 1.6126 g of product was collected (81%yield).^{1}H NMR (Varian Mercury Vx 300MHz, CD_3CN, 25ºC) δ= 3.98 (s, 2H; CH_2), 7.94-8.03 (m, 2H, CH aromatic), 8.16 (d, 1H, CH aromatic). ^{13}C NMR (Varian Mercury Vx 300MHz, CD_3CN, 25ºC) δ= 29.145 (CH_2), 76.825-77.670 (ACN), 118.575 (CF_3), 118.575-127.340 (5C aromatic) 141.257(1C aromatic), 164.259(CO).

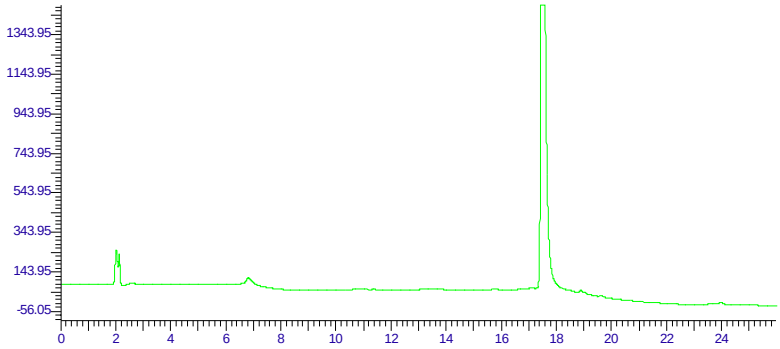
Figure 7. HPLC trace of the formation reaction of 2-bromo-N-3-trifluoromethyl-4-nitro phenylacetamide, **1.**



S-N-3-trifluoromethyl-4-nitro phenylacetamide-cysteine, 4. Compound **1** (253.2g, 0.77mol) was dissolved in 5.00mL CH_2Cl. 283.1mg (2.3mol) of L(+)-cysteine and 10.0mL of H_2O was added. Reaction was adjusted to pH 8 by using 1.0M $NaHCO_3$. Reaction was left stirring at RT overnight . White precipitate was filtered, dried in vacuo, and weighed, where180.2mg of product was collected (65%yield). HPLC confirms a single peak at 17.53 min characteristic of the product and the absence of any starting material. Increased yields (85-95%) can be isolated by prep HPLC of the entire aqueous portion of the reaction. ^{1}H NMR (Varian Mercury Vx 300MHz, D_2O/D_2OCl, 25ºC) δ= 1.90 (m, 1H, NH), 2.90-3.12 (m, 3H), 3.36 (d, 2H, NH_2), 4.09

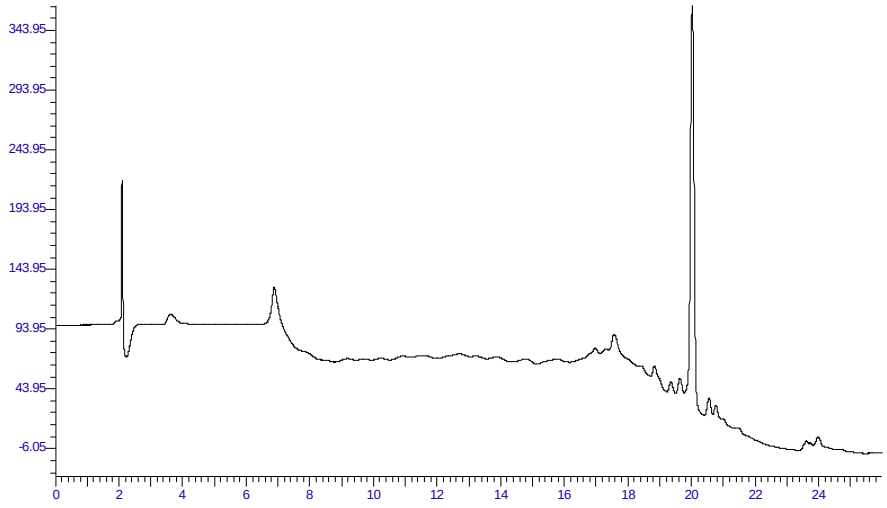
(q, 1H, CH), 7.61-7.66 (d of t, 1H, CH aromatic) 7.80-7.84 (d of d, 2H, CH aromatic). ^{13}C NMR (Varian Mercury Vx 300MHz, D_2O/D_2OCl, 25ºC) δ= 35.651-36.023 (CH_2), 51.228-51.847 (CH), 118.775 (CF_3), 123.053-127.722 (4C aromatic), 141.717-142-109 (2C aromatic), 169.645-170.312 (1CO), 173.819 (1CO)

Figure 8. The formation of S-N-3-trifluoromethyl-4-nitro phenylacetamide-cysteine, **4**, from a biphasic system with compound **1**.



Re(CO)$_3$4. $ReBr_3(CO)_3$ (112.3mg, 0.146mmol) and CysFlute (51.5mg, 0.146mmol) where dissolved in 15.0mL of H_2O. The pH of reaction was carefully adjusted to 6.5 using 1M $NaHCO_3$ and at stirring at room temperature for 3 days. A yellow precipitate belonging to our product was seen. The precipitate was filtrated and dried. The compound was further purified by prep HPLC or a silica column (95%MeCl2/ 5% MeOH). 43 % yield

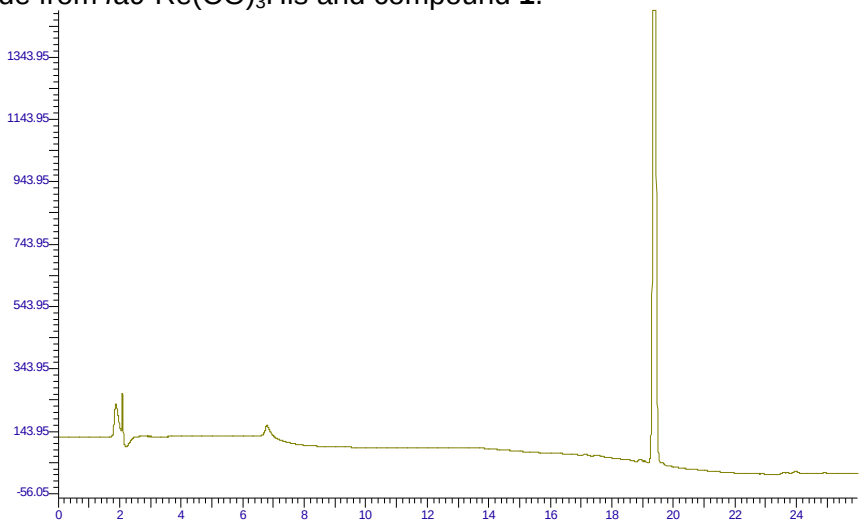
Figure 9. HPLC trace of the formation of Re(CO)$_3$**4** from *fac*-$ReBr_3(CO)_3$ and compound **4**.



Re(CO)$_3$His-^{e}N-N-3-trifluoromethyl-4-nitro phenylacetamide. Re(CO)$_3$His (52.9mg, 0.12mmol) was dissolved in acetonitrile (5.0 mL) followed by the addion of compound **1** (45.4mg, 0.14mmol) and Cs_2CO_3 (44.7mg, 0.14mmol) to the reaction. The reaction was stirred at room temperature and followed by HPLC. After 2.5 hours the reaction was observed to have gone to completion. The majority of the product was present in the solution. The solution was

filtered to remove the salt precipitate and dried. The solid was further purified by column chromatography. 59.2mg of product was collected (69%yield) HPLC RT 19.32

Figure 10. HPLC trace of the formation of $Re(CO)_3$His-^{e}N-N-3-trifluoromethyl-4-nitro phenylacetamide from *fac*-$Re(CO)_3$His and compound **1**.



Boc-cys-S-ethylpyridine, 9. To a solution of cys-ethyl-pyr (.3060g, 1.352mmol) in methanol (100mL) were added at 40°C, triethylamine (.418mL, 1.487mmol) and di-tert-butyl-dicarbonate (.6270mL, 4.462mmol). The mixture was then stirred at room temperature for 24h. After removal of the solvent, the product was purified by silica gel column chromatography using a solvent system of 5% methanol in methylene chloride, then 10% methanol in methylene chloride after 1L of 5% solution was used. (99.82%yield) HNMR (CD_3OD) pyr (dd, 1H, 8.518), pyr (td, 1H, 7.936), pyr (d, 1H, 7.526), pyr (t, 1H, 7.421), -CH_2-pyr (t, 2H, 3.126), -CH_2-CH_2-pyr (t, 2H, 2.940), -CH_2-S- (d, 2H, 3.019), -CH-COOH (t, 1H, 4.315), $(CH_3)_3$-C-O- (s, 9H, 1.432) CNMR ($CDCl_3$) 173.421, 156.021, 155.678, 144.874, 142.721, 127.358, 124.799, 80.181, 53.642, 34.640, 34.131, 31.477, 28.465

Boc-cys- aniline-S-ethylpyr. To a solution of boc-cysethylpyr (.0516g, 0.1583mmol) in methylene chloride (25mL) were added N,N'-Dicyclohexylcarbodiimide (.0877g, .4250mmol) and aniline (.0357mL, .3559mmol) . The mixture was stirred at room temperature for 48h. After removal of the solvent, the product was purified by silica gel column chromatography using a solvent system of 35% ethyl acetate in hexanes. (83.48%yield) HNMR (CD_3OD) pyr (ddd, 1H, 8.428), pyr (td, 1H, 7.721), pyr (td, 1H, 7.305), pyr (td, 1H, 7.245), -CH_2-pyr (m, 2H, 3.448), -CH_2-CH_2-pyr (t, 2H, 2.948), -CH_2-S- (dd, 2H, 3.023), -CH-COOH (t, 1H, 4.379), $(CH_3)_3$)-C-O- (s, 9H, 1.446), anal (dd, 1H, 7.547), anal (t, 1H, 7.279), anal (tt, 1H, 7.103)

NH_2-cys- aniline-S-ethylpyr. To a solution of boc-cysethylpyr+aniline (.053g, .1321mmol) in methylene chloride (4mL) was added trifluoroacetic acid (1mL). The mixture was stirred at room temperature for 1h. (61.64%yield) HNMR (D_2O) pyr (dd, 1H, 8.405), pyr (td, 1H, 8.247), pyr (td, 1H, 7.297), pyr (td, 1H, 7.256), -CH_2-pyr (t, 2H, 3.210), -CH_2-CH_2-pyr (t, 2H, 2.931), -CH_2-S- (d,

2H, 3.094), -CH-CO- (t, 1H, 4.226), anal (dd, 1H, 7.686), anal (td, 1H, 7.288), anal (td, 1H, 7.143)

Boc-cys- 5-Amino-2-nitrobenzotrifluoride -S-ethylpyr, 10. To a solution of Boc-cys-S-ethylpyridine **8** (0.036g, 0.1104mmol) in dry DMF (20mL) were added 5-Amino-2-nitrobenzotrifluoride (0.0228g, 0.1106mmol) and O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (0.0419g, 0.1105mmol). To the mixture, a solution of N-ethyldiisopropylamine (0.0400mL, .4171mmol) in dry DMF (5mL) was added dropwise over 2min. The mixture was then stirred at room temperature under nitrogen with mol. sieves for 24h. After removal of the solvent, the product was purified by preparative HPLC. HNMR (CD_3OD) pyr (dd, 1H, 8.767), pyr (t, 1H, 7.934), pyr (dd, 1H, 7.729), pyr (m, 1H, 7.518), -CH_2-pyr (t, 2H, 3.404), -CH_2-CH_2-pyr (t, 2H, 3.103), -CH_2-S- (d, 2H, 3.234), -CH-CO- (t, 1H, 4.271), $(CH_3)_3$-C-O- (s, 9H, 1.210), nbenz (tt, 1H, 8.529), nbenz (d, 1H, 8.038), nbenz (dd, 1H, 7.876)