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Synthesis and Radiolabeling of a Biotin-CHX-B Chelate for Bi-213

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SUMMARY

The synthesis and radiolabeling of a biotin derivative which contains a chelating group (CHX-B) for binding the α-emitting radionuclide, ^{213}Bi, is described. The biotin-CHX-B derivative was readily prepared in high yield through a convergent synthesis that coupled N-methylglycylbiotinamide with anilino-CHX-B. Bismuth-213 labeling of the biotin-CHX-B molecule was facile at room temperature, resulting in >95 % radiochemical yield within 10 min reaction time.

Key words: Bismuth-213, Biotin Derivative, Pretargeting, Radioimmunotherapy.

INTRODUCTION

Radioimmunotherapy utilizes monoclonal antibodies (mAbs) to selectively deliver radionuclides to tumors for therapy. However, the use of radiolabeled mAbs is problematic in that the radionuclide decays in blood and non-target tissues during the process of tumor localization and blood clearance. To circumvent this problem, an alternate method of radioimmunotherapy which separates the delivery of the radionuclide from the pharmacokinetics of mAbs, termed "pretargeting", is being investigated (1, 2). Pretargeting has several potential advantages over using conventional radiolabeled antibodies for therapy of cancer (3). Most of the studies involving the pretargeting approach utilize reagents consisting of combinations of monoclonal antibodies (mAbs), biotin binding proteins (streptavidin or avidin), and radiolabeled biotin derivatives (4). The very strong binding affinity of biotin with the proteins avidin (5) and streptavidin (6) have made compounds containing biotin attractive for *in vitro* bioassays (7, 8) and *in vivo* medical applications. The high binding affinity of biotin is particularly attractive for delivering

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therapeutic radionuclides attached to biotin in pretargeting as this results in nearly covalent attachment of the radionuclide with the antibody/streptavidin localized on tumors.

The most common radioisotopes used for radioimmunotherapy are ^{131}I and ^{90}Y, both of which emit β-particles with energetics and half-lives favorable for therapy (9). Isotopes emitting α-particles offer several advantages over β-emitters (9-13): a) their high linear energy transfer (100 keV/μm) render them very cytotoxic; b) the path length of an α-particle with an energy of 5 to 8 MeV is on order of 40-80 μm, greatly reducing the nonspecific irradiation of normal tissue around the targeted tumor; c) the DNA damage caused by α-particles is not easily repaired by the cell; and d) the cytotoxicity of α-particles is not affected by oxygen, making them much more effective at killing cells under hypoxic conditions. The most promising α-emitters for clinical use at this time are astatine-211, bismuth-212 and bismuth-213 (14). Unfortunately, these radionuclides have short half-lives making their application to radioimmunotherapy difficult. However, if the pretargeting approach is taken, the very short half-lives of bismuth-212 ($t_{1/2}$ = 60.6 min) and bismuth-213 ($t_{1/2}$ = 45.6 min) do not limit their application. Indeed, considering the rapid tumor penetration and blood clearance of biotin derivatives in pretargeting, biotin conjugates which contain chelated α-emitting radionuclides of bismuth appear to be particularly suited for radioimmunotherapy.

There have been a number of studies involving chelating agents for labeling mAbs with bismuth nuclides (15-19). The chemical constraints on the chelator to be employed are stringent. Complexes must be thermodynamically stable and kinetically inert so that the radioisotope is irreversibly bound to protein for at least five half lives to avoid renal toxicity caused by kidney deposition of released radiobismuth. Brechbiel and co-workers have shown that bismuth chelates of the macrocyclic ligand DOTA are inert both *in vitro* (17) and *in vivo* (16, 18). However, attempts to label DOTA linked to mAbs with bismuth iodide according to established procedures were unsuccessful due to slow complex formation kinetics (20). To obviate this difficulty, Brechbiel et al. (19) have prepared a C-functionalized derivative of trans-cyclohexyldiethylenetriaminepentaacetic acid (CyDTPA) which is inert *in vivo* and useful for preparation of bismuth-labeled mAbs. In this study, we have evaluated the suitability of one of the diastereomers of the CyDTPA, signified by CHX-B, as a chelator for a preparing stable Bi-213 labeled biotin derivative.

EXPERIMENTAL

General: Chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Biotin was obtained from Sigma Chemical Co. (St. Louis, MO).

The nitrophenyl-CHX-B, **1** (as diastereomeric pair) was prepared as previously reported (15). 2,3,5,6-Tetrafluorophenyl trifluoroacetate (TFP-OTFA) was prepared as previously reported (21). Ultrapure anhydrous sodium acetate and Ultrapure glacial acetic acid were obtained from Alfa Aesar (Ward Hill, MA). Ultrex Hydrochloric Acid was obtained from J. T. Baker (VWR). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). The Bi absorber membrane (25 mm disposable syringe filter apparatus) was obtained from 3M (St. Paul, MN) as an experimental 3M Empore™ absorber membrane loaded with a proprietary active particle. Water was double distilled using Quartz glassware. Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. NAP-10 (Sephadex G-25) columns were obtained from Pharmacia Biotech AB (Uppsala, Sweden). ^{1}H NMR were obtained on either a Bruker AC-200 (200 MHz) or Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as ppm using tetramethylsilane as internal standard (δ = 0.0 ppm). Mass spectral data were obtained on a VG 70SEQ mass spectrometer with 11250J data system.

Radioactive Materials: Reactions involving radioactive materials were conducted in 1.7 mL microcentrifuge tubes. Standard radiation safety procedures, including double gloves, were used. Radiation monitoring followed approved procedures using gamma and alpha detection probes. Actinium-225 was obtained from Pacific Northwest National Laboratory (PNNL), Richland, WA. Measurement of Bi-213 was accomplished on a Capintec CRC-15R dose calibrator using the I-131 channel (measured value x 4). Accurate quantification of the Bi-213 was not possible as the dose calibrator was not calibrated with a Bi-213 standard.

Chromatography: HPLC separations of non-radioactive compounds (Scheme 1) were obtained on Hewlett-Packard quaternary 1050 gradient pumping system with a variable wavelength UV detector (254 nm) and a Varex ELSD MKIII evaporative light-scattering detector. Analysis of the HPLC data were conducted on a Hewlett-Packard HPLC ChemStation software. All reactions were monitored by HPLC. Reverse-phase HPLC chromatography was carried out using an Alltech Altima C-18 column (5 μm, 250 X 4.5 mm) using a gradient solvent system at a flow rate of 1 mL/min. Solvent A in the gradient was 0.05 M Ammonium acetate pH 5.5. Solvent B was methanol. Starting from 100% A, the gradient was increased to 100% B over the next 25 min. Retention times under these conditions were: t_R: **1** = 10.7 min; **2** = 8.2 min; **3** = 5.1 min; **4** = 11.4 min; **5** = 12.8 min.

HPLC separations of the Bi-213 labeled biotin derivative, **6**, was conducted using a gradient system which consisted of two Beckman model 110B pumps, a Beckman 420 controller, model 153 UV detector (254 nm), and a model 170 radioisotope detector. Chromatograms were obtained on an Altima C18 column (250 x 4 mm, Altech), eluting with a gradient starting at 20% MeOH/ 80% (0.05M

ammonium acetate pH 5.5). The gradient was increased to 100% MeOH over 20 min., then was held at 100 % MeOH for 5 min. Retention times under these conditions were: t_R: **6** = 13.1, 13.7 min (diastereomers).

N-[2-Amino-3-(*p*-aminobenzyl)propyl]-trans-cyclohexane-1,2-diamine-N,N',N',N",N"-pentaacetic acid (2): Nitrophenyl CHX-B, **1** (150 mg, 0.258 mmol) was dissolved in 9 mL of $NaHCO_3$ solution at pH 8.5. 10% Pd/C (30 mg) suspended in water (15 mL) was added to the reaction flask and the flask was placed on a Parr Hydrogenation Apparatus. The reaction solution was stirred under H_2 atmosphere until H_2 uptake ceased (3 h). It was then purged of H_2, filtered and the filtrate was lyophilized to leave an off-white solid, **2** (140 mg, 98%). ^{1}H NMR (D_2O, pH 11.0) δ 7.08 (m, 2H), 6.82 (m, 2H), 3.90-2.40 (m, 17H), 2.06 (d, J = 13.0, 1H), 1.9 (br d, 1H), 1.63 (br m, 2H) and 1.4-0.95 (m, 4H).

N-Methylglycylbiotinamide (3): This compound was prepared as previously described (23).

N-[2-Amino-3-(p-N-methylglycylbiotinamide-benzyl)propyl]-trans-cyclohexane-1,2-diamine-N,N',N',N",N"-pentaacetic acid (5): To a 51 mg (0.163 mmol) quantity of **3** dissolved in 10 mL of DMF under argon atmosphere was added 46 mg (0.18 mmol) of 2,3,5,6-tetrafluorophenyl trifluoroacetate, followed by 0.025 mL (0.18 mmol) of Et_3N. The reaction mixture was stirred at rt for 20 min. To the solution containing **4** was added 90 mg (0.163 mmol) of **2** in 15 mL of DMF. That mixture was stirred at rt for an additional 1 h, and the solvent was removed under vacuum. The residue was triturated with ether and the solid was filtered. The isolated solid was dried under vacuum to yield 0.13 g (94%) of **5** as an oil. ^{1}H NMR (D_2O, pH 11.0) δ 7.5 (m, 2H), 7.3 (m, 2H), 4.6 (m, 1H), 4.4 (m, 1H), 3.90-2.80 (m, 29H), 2.6-2.3 (m, 2H), 1.8-1.2 (m, 14H). HRMS: mass calcd for $C_{38}H_{55}N_7O_{13}S$ $(M + H)^+$, 850.3657; found, 850.3641.

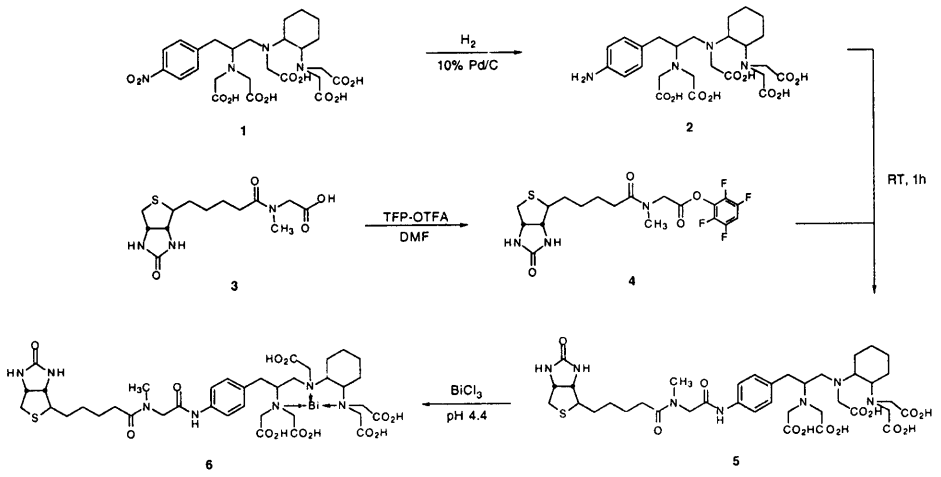
Separation of Bi-213 from Ac-225: The Ac-225 was dissolved in 4 mL of ultrapure 0.5 M HCl. A 25 mm anion exchange disc was pre-treated with 0.5 M HCl by passing 4 mL of acid through the membrane via syringe. This step insures that the anion resin is in the chloride form. The Ac-225 and its daughters (including Bi-213) are drawn up through the anion exchange disc into the syringe. The syringe was then removed from the absorber disc apparatus, and the Ac-225 in the syringe was returned to the vial. Useful quantities of Bi-213 can be separated from the Ac-225 after a 2 h decay period. The Bi-213 absorbed on the membrane, is contaminated with minor traces of Ac-225/Fr-221. To remove the Ac-225/Fr-221, a new syringe was attached to the Bi-213 "loaded" absorber disc and 4 mL of 0.005M HCl was drawn through it and discarded. Following this, the Bi-213 was released from the anion exchange disc by eluting with 2-4 mL ultrapure 0.05M NaOAc (pH 4.4).

Radiolabeling of Biotin-CHX-B conjugate: A fraction of Bi-213 collected from the Bi absorber disc contained 360 μCi of activity in 0.4 mL of 0.05 M NaOAc, pH 4.4. To 100 μL of this solution (45 μCi) was added 10 μL of a 10 mg/mL solution of biotin-CHX-B, **5** chelate. The reaction was allowed to progress for 10 min at rt before injecting on the HPLC.

RESULTS AND DISCUSSION

At the onset of these studies there were several factors that had to be considered in preparing a biotin derivative to carry Bi-213 in pretargeting protocols. Those factors included: 1] using a chelate that binds Bi-213 tightly such that it is not released *in vivo*; 2] using a chelate that binds Bi-213 very rapidly to minimize loss of the 46 min half-lived Bi-213 to decay; and 3] using a biotin derivative that is stabilized to the action of the serum enzyme, biotinidase (22, 23). From previous studies it was learned that the cyclohexyl-DTPA derivatives, CHX, labeled very rapidly with bismuth, forming a complex that was inert *in vivo*. Other studies have shown that the cleavage of biotin from biotinamide derivatives could be blocked by using N-methylbiotinamide moieties (23, 24). Thus, in this investigation, a CHX chelate moiety was combined with N-methylglycylbiotin to prepare the biotin derivative of choice.

The synthesis of the biotin-CHX-B derivative is shown in Scheme 1. The C-functionalized *p*-nitrobenzylcyclohexyl-DTPA, **1**, was obtained as previously described (15). Reduction of the nitro group in **1** to form the amine derivative **2** was accomplished by hydrogenation (10 % Pd/C) at pH 8.5 in 98 % yield. The TFP ester of biotin-N-methylglycine, **4** was prepared from **3** *in situ* as previously



Scheme 1. Synthesis and bismuth-labeling of biotin-CHX-B.

described (23). The amino derivative, **2**, was then conjugated to biotin derivative, **4** to prepare, **5** in 94 % yield. Biotin derivative **5** was >95% pure by HPLC analysis. A NMR spectrum for biotin derivative **5** was obtained in D_2O at pH 11. The NMR (Figure 1) is complicated by the presence of the diastereomeric pair of compounds, but the area under peaks integrates correctly for the number of protons. The identity of the compound was confirmed by high resolution mass spectrometry.

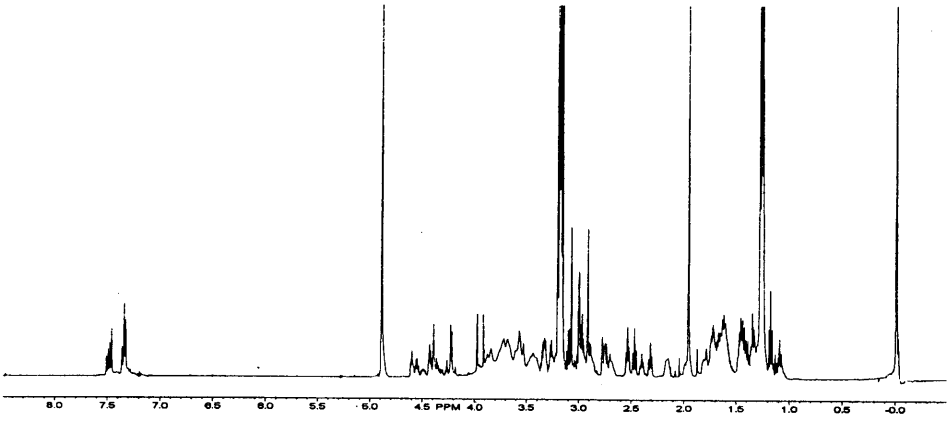


Figure 1: ^1H NMR (500 MHz) of Biotin-CHX-B, **5**, in D_2O (pH 11).

Bismuth-213 is an α-emitting radioisotope that has properties which makes it suitable for radioimmunotherapy when the method of tumor pretargeting is used (14). It can be readily obtained from a generator system which employs Ac-225 as the parent. The decay scheme for the Ac-225 generator for Bi-213 and its daughter nuclides are shown in Figure 2. The generator system, developed

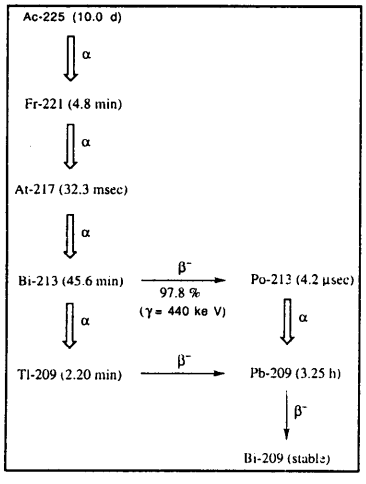


Figure 2: Decay Scheme for Generation of Bi-213 and Its Daughter Nuclides.

at Pacific Northwest National Laboratory, is composed of an proprietary "experimental" Bi absorbing membrane in a disposable syringe filter apparatus. With the appropriate elutions and washings, complete separation of the Bi-213 from Ac-225 and Fr-221 was obtained. Using that separation system, recoveries of >90% were obtained and the recovered bismuth acetate was used directly in the labeling step.

Bi-213 labeling of the biotin-CHX-B derivative, **5**, was accomplished at room temperature in >95% radiochemical yield. In the radiolabeling experiments, Bi-213 was separated from Ac-225, then **5** was mixed with the eluant and after 10 min reaction time, the bismuth labeled biotin derivative **6** was isolated from the HPLC. A typical HPLC chromatogram is shown in Figure 3. Re-injection of the HPLC isolated **6** after 2 h indicated that it was stable over that period of time. Mixing **6** with streptavidin in solution, followed by size exclusion separation on a NAP-10 column demonstrated that over 97% of the bismuth labeled biotin derivative bound with streptavidin.

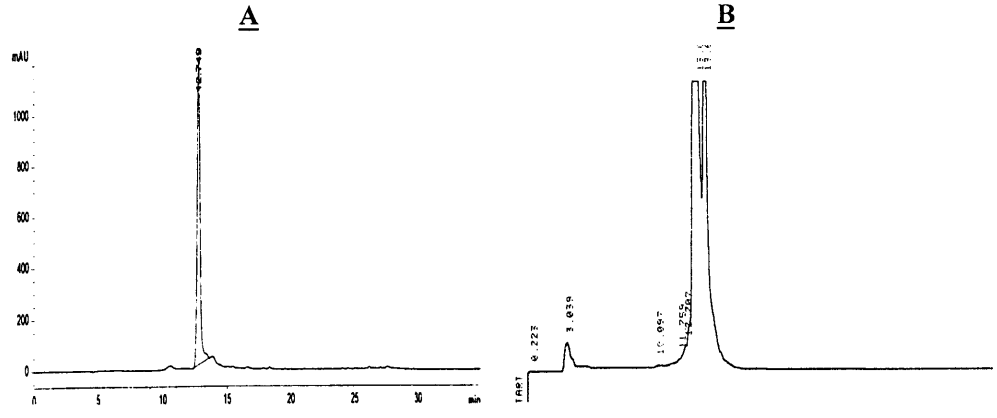


Figure 3: (Panel A) HPLC chromatogram of HPLC purified biotin-CHX-B, **5**. (Panel B) Radiochromatogram of reaction mixture containing Bi-213-labeled biotin-CHX-B, **6**.

In conclusion, a method for the synthesis and ^{213}Bi labeling of biotin-N-methylglycine-CHX-B conjugate has been described.

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