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Synthesis of Carbon-14 Labeled (±) 15-Deoxyspergualin Trihydrochloride

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Summary

C-14 labeled (±) 15-Deoxyspergualin trihydrochloride, 1-amino-19-(^{14}C-guanidine)-11-hydroxy-4,9,12-triazanonadecane-10,13-dione trihydrochloride was prepared in a 3% overall yield from Carbon-14 labeled S-Methyl isothiourea hemisulfate, [imino-^{14}C]. The radiochemical purity of the sample was 97.0% and the specific activity was 41.3 microcuries/mg.

Keywords: Carbon-14, 15-Deoxyspergualin Trihydrochloride, immunosuppressive.

Introduction

(±) 15-Deoxyspergualin trihydrochloride, DSG, **1a**, 1-amino-19-guanidine-11-hydroxy-4,9,12-triazanonadecane-10,13-dione trihydrochloride, is a synthetic derivative of spergualin, a fermentation product isolated from *Bacillus laterosporus*.[1] The potent immunosuppressive activity of DSG has been known since the mid-1980's.[2] In humans, DSG has proven effective for the prevention and treatment of solid organ rejection.[3] In 1994, the Nippon Kayaku Co. Ltd., (Toyko, Japan) received marketing approval in Japan for the use of DSG in the treatment of acute renal graft rejection episodes. In the United States, Bristol-Myers Squibb is conducting clinical trials with DSG in rheumatoid arthritis, islet cell and renal transplantation.

Although the mechanism of action of this agent has yet to be fully elucidated, it is clearly distinct from other immunosuppressive agents (e.g. cyclosporin A, FK 506).[4] Recent studies by Nadler et al,

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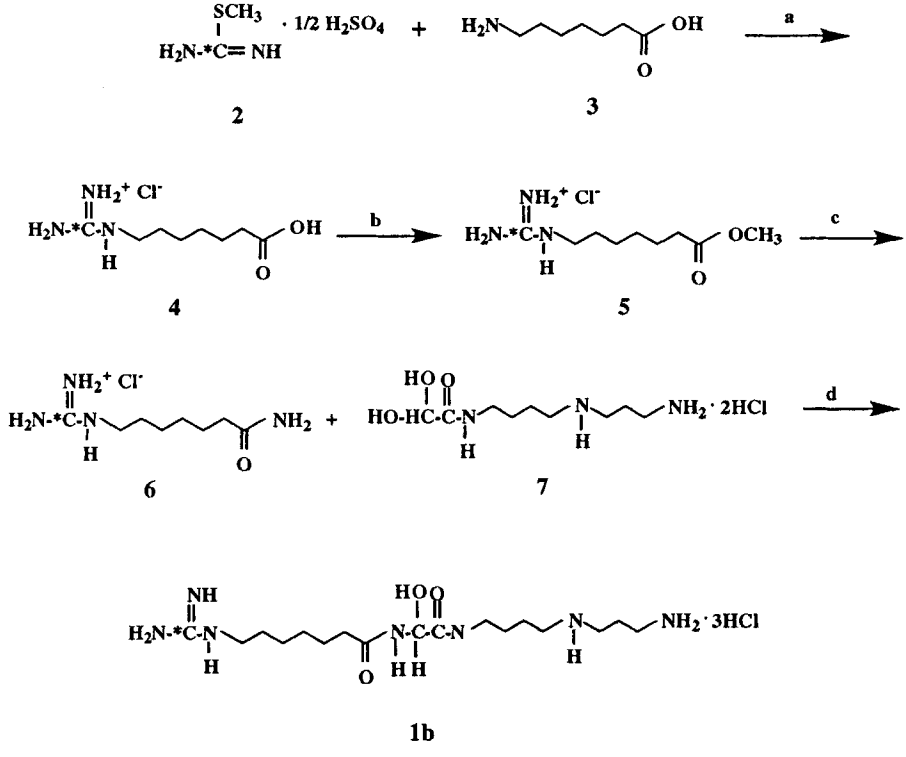
demonstrate that DSG binds to the constitutive heat shock protein 70 (Hsc 70) which is involved in the binding and intracellular transport of antigenic peptides.[5]

We previously reported the synthesis of H-3 labeled DSG.[6] In this paper we report the synthesis of Carbon-14 labeled (±)-15-deoxyspergualin trihydrochloride, **1b**, for use in studies designed to further elucidate the mechanism of action of this novel immunosuppressive agent.

Scheme 1. Synthesis of Carbon-14 labeled (±)-15-Deoxyspergualin Trihydrochloride.



* Denotes Position of Radiolabel

Reagents: a, 3N NaOH, 55°C, 8h; b, CH_3OH, Dowex 50W (H^+ form); c, NH_4OH; d, glutaric acid, H_2O, 60°C, 6h.

Discussion

The synthesis of radiolabeled DSG was based on the original nonradioactive synthesis of DSG reported by Umeda.[7] In our synthesis, Carbon-14 labeled S-Methyl isothiourea hemisulfate,

[imino-^{14}C], **2**, was reacted with 7-aminoheptanoic acid, **3**, to yield Carbon-14 labeled 7-(^{14}C-guanidino)heptanoic acid, which was then converted to its hydrochloride salt, **4**. The crude C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride was purified by crystallization and subsequently converted to the corresponding amide, **6**. Condensation of crude Carbon-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride, with glyoxyloylspermidine dihydrochloride, 11-dihydroxy-10-oxo-4,9-diazaundecane-1-amine dihydrochloride, **7**, in the presence of glutaric acid and water yielded the desired product (Scheme 1). Purification of the crude Carbon-14 labeled DSG, **1b**, was subsequently achieved on ion exchange chromatography followed by desalting on a Sephadex LH-20 column.

Conversion of 7-guanidinoheptanoic acid hydrochloride, **4**, to 7-guanidinoheptanamide hydrochloride, **6**, can be effected in two steps via the initial formation on the corresponding methyl ester, **5**, followed by ammonolysis. In nonradioactive experiments, we routinely obtained an 80-85% yield of 7-guanidinoheptanamide hydrochloride, **6**, with the remaining 15-20% being the corresponding acid, **4**. It is not necessary to purify the radiolabeled 7-guanidinoheptanamide hydrochloride, **6**, since the corresponding radiolabeled acid does not interfere with the final condensation reaction and it can be easily removed during ion exchange chromatography.

The final step in the purification procedure involves removing excess NaCl from the C-14 labeled DSG via LH-20 chromatography. In this chromatography, although the desired product elutes before the NaCl, the volume of effluent separating the two materials is small. Therefore it is important to collect the column effluent in small aliquots (appr 8 mL) to insure complete separation of the unwanted salt from the desired product. Confirmation that the desired separation has been achieved can also be obtained by testing each fraction for chloride ion via ethanolic silver nitrate.

The identity and purity of the final radioactive product was established by co-elution of the radiolabeled substance with authentic unlabeled DSG by HPLC (Method 2). The radiochemical purity of the final product was 97.0% (with 2.1% of the radioactivity being Carbon-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride and 0.9% being Carbon-14 labeled 7-(^{14}C-guanidino)heptanoic acid). The specific activity of the final product, **1b**, was 41.3 uCi/mg.

Experimental

Carbon-14 labeled S-Methyl isothiourea hemisulfate, [imino-^{14}C] , **2**, was obtained from the ViTrax Company (Placentia, CA). Sephadex-CM C-25 and Sephadex LH-20 resins were purchased from Pharmacia LKB. Brij-35 solution (30% w/v) was obtained from the

Sigma Chemical Co (St. Louis, MO). DSG, **1a**, was obtained from Nippon Kagaku Co., Ltd. Glyoxyloylspermidine dihydrochloride, **7**, was obtained from the hydrolysis of DSG.[7] In this procedure, DSG, was refluxed in 1N acetic acid for 4 h, concentrated in vacuo, and then diluted with H_2O. The hydrolysis reaction was then purified via ion chromatography on Sephadex CM-25 (Na^+ form) followed by desalting on Sephadex LH-20 as described herein for the purification of C-14 labeled DSG, **1b**. All other reagents were ACS grade or the highest quality material commercially available. Distilled deionized (DI) water was used throughout this study. Radiochemical purity and specific activity were determined by HPLC.

Analytical Methods

During the column chromatography of radiolabeled DSG, the column effluent was monitored by both the ninhydrin and the Sagakuchi spray reagents. The Sagakuchi reaction, which indicates the presence of the guanidino group, involves first spraying the sample on a TLC plate with a 0.1% solution of 8-hydroxyquinoline in acetone, drying the plate and then lightly spraying it with a solution of 0.2 mL of bromine in 100 mL of 0.5 N NaOH.

HPLC Analysis

Method 1

Reversed-phase HPLC was performed using an Zorbax Cyano column, 4.6 x 250 mm, Rainin pumps, Rainin model UV-1 spectrophotometer for UV analysis and a *IN/US B*-RAM radioactive flowthrough detector for radioactivity measurements. The HPLC conditions were set using unlabelled reference samples with a mobile phase of 50% CH_3OH and 50% 0.01 M NaH_2PO_4 (pH 5.6), uv 205 nm. The flowrate of the mobile phase was 1 mL/min. In this system C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride, **4**, has a R_t of approximately 3.6 min, and C-14 labeled 7-(^{14}C-guanidino)-heptanamide hydrochloride, **6**, has a R_t of approximately 8.5 min.

Method 2

Ion-pair reversed-phase HPLC was performed using an Zorbax Rx C-18 column, 4.6 x 250 mm, and a Rainin model FL-1 fluorescence detector which was connected in line between the uv detector and radioactivity detector. In this system, those compounds containing primary amines could be visualized by post-column derivatization with o-phthalaldehyde (OPA) essentially as described by Seiler and Knodgen.[8] In this configuration, the HPLC effluent was mixed immediately upon exiting the uv detector with the OPA derivatization cocktail. (A 1L solution of the postcolumn derivatization cocktail is prepared by dissolving 25 g of Boric acid, 10 g/L of NaOH, and 1.5 mL/L of Brij-35 in approximately 950 mL of DI H_2O. The

solution is adjusted to pH 9.7, filtered, and to this solution is added 1.5 mL/L of 2-mercaptoethanol, and 0.2 g of OPA dissolved in 5 mL of CH_3OH. The solution is then brought up to a total volume of 1L with DI H_2O). This postcolumn buffer was mixed 1:2 with the column effluent (0.5 mL cocktail to 1.0 mL HPLC effluent) and then pumped through a 10 feet reaction coil made of PEEK tubing (0.062" I.D.) submerged in a water bath maintained at 40°C. The effluent was then analyzed via fluorescent detector (excitation 340 nm, emission 418 nm) and subsequently via the radioactive flowthrough detector.

Samples (3 ul) were loaded onto a Zorbax Rx C-18 column equilibrated with 85% Buffer A (35% CH_3OH, 0.010 M NaH_2PO_4, 0.005 M sodium octanesulfonate, pH 3.0) and 15% Buffer B (80% CH_3OH, 0.010 M NaH_2PO_4, 0.005 M sodium octanesulfonate, pH 3.0). The flowrate of the mobile phase was 1.0 mL/min. At 3 min post-injection, a linear gradient starting at 85% A/15% B and ending at 25% A/75% B was run over 7 min and then this composition maintained for 5 min. At 15 min post-injection, the column is then returned to 85% A/15% B over a 2 minute period. In this system the C-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride, **6**, has a R_t of approximately 8.7 min, while C-14 labeled 7-(^{14}C-guanidino)-heptanoic acid hydrochloride, **4**, has a R_t of approximately 11.4 min and C-14 labeled DSG, **1b**, has a R_t of approximately 15.9 min.

SYNTHESIS

C-14 labeled 7-(^{14}C-Guanidino)heptanoic Acid Hydrochloride, **4**.

A 100 mCi sample of Carbon-14 labeled S-Methyl isothiourea hemisulfate, [imino-^{14}C] (56 mCi/mmol), **2**, dissolved in 7 mL of 95% EtOH was concentrated to dryness in a 15 mL RB flask. To this was then added 0.247 mg (1.78 mmol) of nonradioactive S-Methyl isothiourea hemisulfate. The solids were then suspended in CH_3CN and concentrated to dryness.

To this vessel was then added 0.403 g (2.77 mmol, 0.78 eq) of 7-aminoheptanoic acid, **3**, and 2.1 mL of 3 N NaOH. The solution was allowed to stir at 55°C for 8 h. The resulting solid was filtered and rinsed with cold water and then cold acetone to yield 300 mg of crude carbon-14 labeled 7-(^{14}C-guanidino)heptanoic acid. (*It is important not to dilute the reaction mixture prior to filtration since this will result in poor recovery of the desired material*). The crude C-14 labeled 7-(^{14}C-guanidino)heptanoic acid was then crystallized by treating the solid with 2.2 mL of 2 N HCl, briefly heating the suspension and then allowing the solution to cool. The product was then collected by filtration and rinsed with dilute HCl and then acetone to yield 240 mg (21.6 mCi) of C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride, **4**, as fine white needles. The radiochemical purity of the crystallized C-14 labeled

7-(^{14}C-guanidino)heptanoic acid hydrochloride, **4**, was >99% (HPLC Method 1).

C-14 labeled 7-(^{14}C-Guanidino)heptanamide Hydrochloride, **6**.

The C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride, **4**, was suspended in 10 mL of anhydrous MeOH and to this was added 0.5 g of Dowex 50W resin (H^+ form). The solution was refluxed for 4 h, after which time the solution was filtered and the solvent removed on a rotary evaporator under high vacuum to yield the corresponding methyl ester, **5**, as a clear syrup. To this syrup was added 15 mL of conc. NH_4OH and the mixture allowed to stir in a closed container for 14 h. After 14 h, the mixture was concentrated to a white solid, **6**, (133 mg, 12 mCi), and used in the next reaction without purification. The radiochemical purity of the crude C-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride, **6**, was 80%, with the remaining 20% of the radioactivity being C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride, **4** (HPLC Method 1).

C-14 labeled 15-(±)-Deoxyspergualin Trihydrochloride, **1b**

To the crude C-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride, **6**, was added 0.29 g (0.00099 mol) of glyoxyloyl-spermidine, **7**, 0.132 g (0.001 mol) of glutaric acid and 160 uL of H_2O. The resulting viscous syrup was allowed to stir at 60°C for 6 h in a closed vessel. After 6 h, the vessel was placed in the freezer (-20°C) for 14 h. After 14 h, 20 mL of water was added to the reaction mixture and the solution applied to a Sephadex CM-25 column (Na^+ form, 3 x 22 cm). Purification of the reaction mixture was achieved by eluting the column sequentially with 300 mL of 0.1 M, 0.2 M, 0.3 M, 0.4 M, and 0.5 M NaCl. The flowrate through the column was 4-5 mL/min. The effluent was collected in 15 mL fractions via a fraction collector. Fractions containing radioactivity were then analyzed via the Sagakuchi and Ninhydrin spray reagents as well as HPLC (Method 2). C-14 labeled 7-(^{14}C-guanidino)-heptanoic acid hydrochloride, **4**, (2.4 mCi) eluted off the column with 0.1M NaCl, while unreacted C-14 labeled 7-(^{14}C-guanidino)-heptanamide hydrochloride, **6**, (4.0 mCi) eluted off the column with 0.2M NaCl. The desired product, C-14 labeled DSG, **1b**, eluted off the column with the last 100 mL of 0.4 M NaCl. Those fractions containing C-14 labeled DSG were then combined and lyophilized overnight. The C-14 labeled DSG was then extracted from NaCl with 50 mL of MeOH. The mixture filtered and the filtrate was concentrated to 10 mL on a rotary evaporator at R.T. The solution was then filtered through a 0.4 micron filter and further desalted by passage through a 3 x 30 cm column of LH-20 resin. The column was eluted with MeOH, at a flowrate of 3-4 mL/min. The eluent was collected in 8 mL aliquots on a fraction collector and the desired fractions were collected, and concentrated to a clear syrup on a

rotary evaporator at R.T. The C-14 labeled DSG was then dissolved in 7 mLs of H_2O and lyophilized to yield 3.0 mCi of Carbon-14 labeled DSG, **1b**, as a white solid. The radiochemical purity of the sample was 97.0% (HPLC Method 2). The radiolabeled impurities in the sample were C-14 labeled 7-(^{14}C-guanidino)heptanamide hydrochloride (2.1%) and C-14 labeled 7-(^{14}C-guanidino)heptanoic acid hydrochloride (0.9%). The specific activity of the final product, **1b**, was determined to be 41.3 uCi/mg.

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