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Synthesis of Tritium Labeled (±) 15-Deoxyspergualin Trihydrochloride

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Summary

Tritium labeled (±) 15-deoxyspergualin, (±)-7-[(aminoiminomethyl) amino]-N-[2-[[4-[(3-aminopropyl)amino]butyl]amino]-1-hydroxy-2-oxoethyl][4,5-$^3H_2$]heptanamide trihydrochloride was prepared from the acid catalyzed condensation of 7-guanidino[4,5-$^3H_2$]heptanamide hydrochloride and glyoxyloylspermidine dihydrochloride in a total yield of 14.5%. The radiochemical purity was determined to be 91.1% and 95.4% on two different HPLC systems.

Keywords: Tritium, (±) 15-deoxyspergualin, DSG, immunosuppressive.

Introduction

(±) 15-Deoxyspergualin, DSG, **1a**, is a synthetic derivative of spergualin, a fermentation product isolated from Bacillus laterosporus.[1] (±) 15-Deoxyspergualin has exhibited good antitumor and immunosuppressive activity in preclinical animal models[1,2] and is currently in Phase II clinical trials for these indications. The mechanism of action of DSG has yet to be elucidated but appears to be distinct from other immunosuppressive agents (e.g. cyclosporin A, FK506).[3] Recently the putative intracellular binding protein has been identified as the cognate form of the heat shock protein 70 (hsc 70).[4] In order to further characterize the mechanism of action of DSG, and its interaction with its target binding protein, tritium labeled DSG was prepared as described herein.

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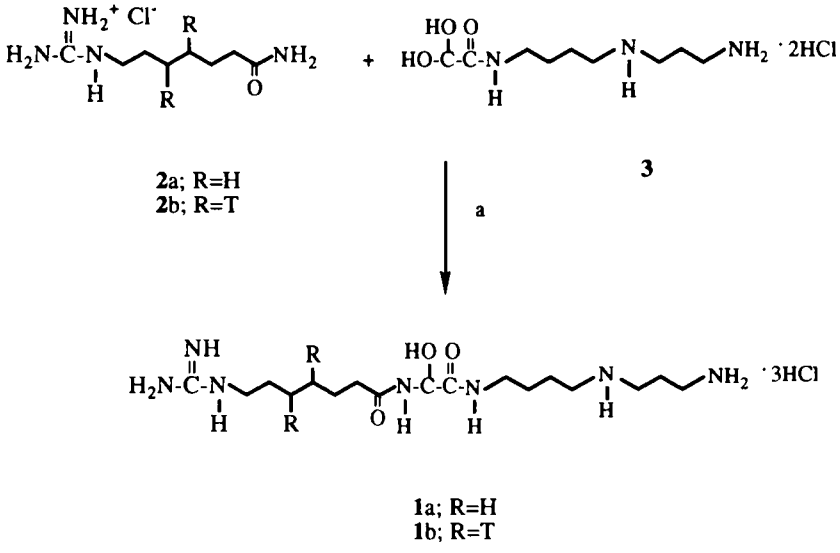
Discussion

The synthesis of radiolabeled DSG was based on the original nonradioactive synthesis of DSG reported by Umeda.[5] Accordingly, 7-guanidino[4,5-$^3H_2$]heptanamide hydrochloride, **2b**, and glyoxyloylspermidine dihydrochloride, 11-dihydroxy-10-oxo-4,9-diazaundecane-1-amine dihydrochloride, **3**, were condensed in the presence of glutaric acid and water to yield **1b** in a 14.5% yield (Scheme 1).

Scheme 1. Synthesis of Tritiated (±) 15-Deoxyspergualin, **1b**.

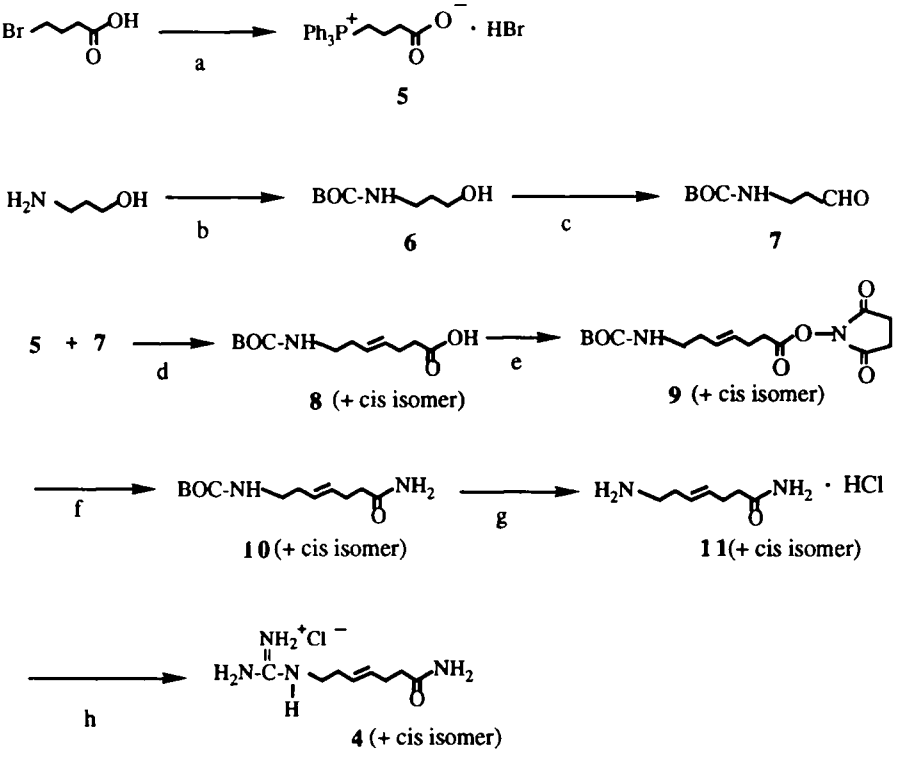


Reagents: a, glutaric acid, H_2O, 55-60°C, 8h.

Tritiated 7-guanidinoheptanamide hydrochloride **2b** was prepared by the catalytic reduction of a cis/trans mixture of 7-guanidino-[4,5]heptenamide hydrochloride, **4**, with tritium gas. The synthesis of 7-guanidino[4,5]heptenamide hydrochloride, **4**, from 4-bromobutyric acid is shown in Scheme 2.

Since (±) 15-Deoxyspergualin has limited stability in aqueous solution to both heat and base as a result of the fragile nature of the carbinolamine functionality at the C-11 position,[6] aqueous solutions of **1b** were lyophilized to minimize decomposition.

Scheme 2. Synthesis of cis/trans 7-guanidino[4,5]heptenamide hydrochloride, **4**.



Reagents: a, PPh_3, xylene, RT; b, BOC-ON, 50% aq. 1,4-dioxane, RT; c, PCC, CH_2Cl_2, RT; d, $LiN(TMS)_2$, THF, RT; e, 1-hydroxybenzotriazole hydrate, 1,3-dicyclohexylcarbodiimide, N-hydroxysuccinimide, THF, RT; f, 2.0 M NH_3/CH_3OH, RT; g, 3 M HCl/EtOAc, RT; h, 3,5-dimethylpyrazole-1-carboxamidine nitrate, K_2CO_3, EtOH, CH_3OH, RT, 10 d.

The identity and purity of the final radioactive product was established by co-elution of the radiolabeled substance with authentic unlabeled DSG on two different HPLC systems (Methods 1&2). The radiochemical purity was determined to be 91.1% and 95.4% (HPLC methods 1&2 respectively). In each system the major radioactive impurity was ^{3}H-7-guanidinoheptanamide.

Additional proof of structure comes from two chemical modification studies which were conducted on ^{3}H-DSG, namely conversion of ^{3}H-DSG to its 11-methoxy analog and hydrolysis of ^{3}H-DSG to ^{3}H-7-guanidinoheptanamide. DSG is known to be easily derivatized to the corresponding 11-methoxy analog by reacting it with methanolic HCl at RT overnight.[5] DSG can also be hydrolyzed to 7-guanidinoheptanamide by treatment with dilute NaOH at RT

overnight.[5] The reaction products from both of these chemical modification studies can be evaluated by HPLC (Method 1). In both studies, the radioactive and non-radioactive DSG behaved in a similar manner.

Experimental

7-Guanidino[4,5-$^3H_2$]heptanamide hydrochloride, **2b**, (1.3 Ci, 70 Ci/mol) was prepared from a cis/trans mixture of 7-guanidino-[4,5]heptenamide hydrochloride, **4**, by a method developed by Amersham International plc using tritium gas. Sephadex-CM C-25 and Sephadex LH-20 resins were purchased from Pharmacia LKB. Brij-35 was obtained from Sigma Chemical Co. All other reagents were ACS grade or the highest quality material commercially available. (±) 15-Deoxyspergualin was obtained from Nippon Kagaku Co., Ltd. Distilled deionized water was used throughout this study. Radioactivity was measured by a Beckmann LS 9000 liquid scintillator. Radiochemical purity was determined by HPLC.

Analytical Methods

During the purification of the radiolabeled DSG via column chromatography by either Sephadex-CM C-25 or LH-20 resins, the effluent was monitored by ninhydrin or the Sagakuchi reaction. 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

Samples were resuspended in 0.2 M perchloric acid, filtered through a 0.45 micron filter and aliquots were then used for determination of DSG content by ion-pair reversed-phase HPLC separation and post column derivatization with o-phthalaldehyde (OPA) essentially as described by Seiler and Knodgen.[7] Briefly, samples were analyzed by a Beckman System Gold HPLC using a Beckman Ultrasphere ODS 5 um column (4.6 mm x 25 cm) at a flow rate of 1 ml/min. Samples (200 ul) were loaded onto a column equilibrated with 70% Buffer A (0.2 M sodium acetate and 0.01 M sodium octanesulfonate, pH 4.5) and 30% Buffer B (62.5% of Buffer A plus 37.5 % of a 1:1 mixture of acetonitrile and methanol). After 10 minutes of 70% A/30% B a linear gradient starting at 70% A/30% B and ending at 100% B was run over 40 min. The system is then maintained at 100% B for 10 min and returned to 70% A/30% B over

a 10 minute period. Post-column derivatization was carried out by using a Beckman 106 pump at a flow rate of 0.5 ml/min with an OPA solution composed of 50g/L of Boric acid, 44 g/L of KOH, 400 mg/L of OPA, 3 mL/L of Brij-35 and 3 mL/L of 2-mercaptoethanol. The post column buffer was mixed 1:2 with the column effluent and then analyzed via a Beckman 171 fluorescent detector (excitation 345 nm, emission 455 nm) and a Beckman radioactive detector. In this system, the desired ^{3}H-product, **1b**, elutes at approximately 51.5 min, whereas the ^{3}H-precursor, **2b**, elutes at approximately 33 min. Known samples of DSG obtained from Nippon Kayaku Co., Ltd., were used to determine a standard curve from which the mass of DSG could be determined.

Method 2

Samples were analyzed on a Zorbax RX-C18 (4.6 x 250 mm) column at a flow rate of 1 mL/min. Radioactivity was monitored with a Radiomatic A280 radioactive detector. Samples (2 ul) were loaded onto a column equilibrated with 85% Buffer A (0.010 M NaH_2PO_4, 0.005 M sodium octanesulfonate, pH 3.0, 35% CH_3OH) and 15% Buffer B (0.010 M NaH_2PO_4, 0.005 M sodium octanesulfonate, pH 3.0, 80% CH_3OH). Immediately upon injection a linear gradient starting at 85% A/15% B and ending at 70% A/30% B was run over 20 min. At 20 min, a linear gradient starting at 70% A/30% B and ending at 25% A/75% B was run over 10 min. At 30 min, a linear gradient starting at 25% A/75% B and ending at 100% B was run over 5 minutes. The system is then returned to 85% A/15% B over a 10 minute period. In this system the desired ^{3}H-product, **1b**, elutes at approximately 32 min, whereas, the ^{3}H-precursor, **2b**, elutes at approximately 7 min.

SYNTHESIS

(3-Carboxypropyl)triphenylphosphonium bromide, **5**[8]

To a solution of 4-bromobutyric acid (50.21 g, 0.30 mol) in 400 mL of xylene (freshly distilled from CaH_2) was added triphenylphosphine (78.86 g, 0.30 mol) and the reaction allowed to stir at RT under N_2 until all of the triphenylphosphine was in solution (30 min). The solution was refluxed for 7 d. The solution was cooled and the white precipitate collected by filtration. The crystalline material was rinsed with 300 mL of cold hexane to yield **5** (104.24 g, 81%). ^{1}H- NMR (CD_3OD) (ppm) 1.8 (m, 2H), 2.5 (tr, 2H), 3.4 (m, 2H). 13C-NMR (CD_3OD) (ppm) 6.92, 6.96, 9.54, 10.23, 21.94, 22.17, 106.83, 107.98, 118.19, 118.36, 122.48, 122.62, 123.99, 163.28. DCI-M.S. m/e 348 $(M+H)^+$. Anal. calc'd for $C_{22}H_{21}O_2P{\cdot}HBr$: C, 61.55; H, 5.17. Found: C, 61.64; H, 5.14.

1,1-Dimethylethyl-(3-hydroxypropyl) carbamate, 6[9]

To a solution of 3-amino-1-propanol (28.17 g, 0.38 mol) in 400 mL of 50% aqueous 1,4-dioxane was added BOC-ON (101.60 g, 0.41 mol) and the solution was allowed to stir at RT for 1 h. The solution was diluted with 200 mL of H_2O and extracted with EtOAc (4 X 200 mL). The organic layer was washed with H_2O (2 x 100 mL), cold dilute HCl (100 mL), brine (100 mL) and dried over Na_2SO_4. The organic layer was removed on a rotary evaporator and the crude product purified via flash chromatography (silica gel, 60% CH_2Cl_2/hexane, followed by 100% CH_2Cl_2, the product was eluted off the column with 5% CH_3OH/CH_2Cl_2). The solvent was removed on a rotary evaporator to yield **6** (38.5 g, 58%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) (ppm) 1.44 (s, 9H,), 1.66 (quintet, 2H,), 2.96 (s, 1H,), 3.29 (m, 2H,), 3.66 (m, 2H,), 4.77 (br s, 1H,). ^{13}C NMR ($CDCl_3$) (ppm) 28.57, 33.03, 37.24, 59.60, 79.94, 157.82. DCI-M.S. m/e 175 $(M+H)^+$. Anal. calc'd for $C_8H_{17}NO_3$: C, 54.84; H, 9.78; N, 7.99. Found: C, 54.51; H, 9.56; N, 7.72.

1,1-Dimethylethyl (3-oxopropyl) carbamate, 7[9]

To a solution of **6** (21.39 g, 0.12 mol) in 600 mL of anhydrous CH_2Cl_2 was added pyridinium chlorochromate (52.63 g, 0.24 mol) in a single portion. The reaction was allowed to stir at RT for 2 h and then diluted with an equal volume of hexane and purified via flash chromatography (silica gel). The column was initially eluted with 1 L of CH_2Cl_2 and the compound eluted off the column with a gradient of 25% to 50% EtOAc/hexane to yield **7** (8.12 g, 39%) as a colorless oil. ^{13}C NMR ($CDCl_3$) (ppm) 28.25, 33.68, 44.19, 79.08, 155.78, 201.15. DCI-M.S. m/e 174 $(M+H)^+$.

7-[[(1,1-Dimethylethyl)carbonyl]amino]-4-heptenoic acid, 8

To **5** (16.34 g, 0.05 mol) in an oven dried 1 L flask purged with Ar, was added 300 mL of anhydrous THF. The slurry was allowed to stir for 5 min and then 122 mL (0.122 mmol) of lithium bis(trimethylsilyl)amide (1.0 M in THF) was added over 10 min. After stirring for 40 min, a solution of **7** (8.12 g, 0.05 mol) in 50 mL of anhydrous THF was added and the solution allowed to stir for an additional 60 min at RT. The solution was diluted with 100 mL of H_2O, the organic solvent removed on a rotary evaporator and the aqueous phase extracted with Et_2O (75 mL). The pH of the aqueous phase was adjusted to 2 with 1.0 N HCl. The aqueous phase was extracted with Et_2O (2 x 75 ml) and the organic layer extracted with brine and dried (Na_2SO_4). The solvent was removed on a rotary evaporator to yield **8** as a yellow viscous oil. The crude material was used as is in the next step without purification. ^{13}C NMR (CD_3COCD_3) (ppm) 23.33, 28.67, 33.80, 34.21, 40.87, 78.72, 128.3, 130.59, 156.82, 175.21. DCI-M.S. m/e 244 $(M+H)^+$.

1,1-dimethylethyl [7[(2,5-dioxo-1-pyrrolidinyl)oxy]-7-oxo-3-heptenyl] carbamate, **9**

To a solution of **8** (2.50 g, 0.010 mol) in 125 mL of THF was added 1-hydroxybenzotriazole hydrate (0.040 g, 0.030 mol), 1,3-dicyclohexylcarbodiimide (2.98 g, 0.014 mol), and N-hydroxy-succinimide (1.88 g, 0.0163 mol). The reaction was allowed to stir at RT overnight under Ar. The solvents were removed on a rotary evaporator and the crude product dissolved in 50 mL of EtOAc and purified via flash chromatography (silica gel, EtOAc) to yield 1.95 g (56%) of **9** as a viscous amber oil which solidified on standing at -20°C to a waxy solid. ^{1}H NMR ($CDCl_3$) (ppm)1.4 (s, 9H), 2.2 (m, 2H), 2.4 (m, 2H), 2.7 (m, 2H), 2.8 (s, 4H), 3.1 (q, 2H), 4.6 (br s, 1H), 5.4 (m, 2H). DCI-M.S. m/e 341 $(M+H)^+$. Anal. calc'd for $C_{16}H_{24}N_2O_6$: C, 56.46; H, 7.10; N, 8.23. Found: C, 56.81; H, 7.34; N, 8.23.

1,1-dimethylethyl (7-amino-7-oxo-3-heptenyl) carbamate, **10**

To a solution of **9** (1.95 g, 0.0057 mol) in 50 mL of CH_2Cl_2 was added 10 mL (0.020 mol) of a 2.0 M solution of NH_3 in CH_3OH (Aldrich) and the solution allowed to stir at RT for 25 h. The reaction mixture filtered and the solid rinsed with 20 mL of CH_2Cl_2. The filtrate was concentrated on a rotary evaporator to afford a yellow oil which solidified on standing at -20°C to yield 1.8 g of crude **10**. The crude product was purified via flash chromatography (silica gel, EtOAc) to yield 0.85 g (62%) as a solid. DCI-M.S. m/e 243 $(M+H)^+$. m.p. 82-84°C.

7-amino-4-heptenamide, **11**

To a solution of **10** (0.33g, 0.0013 mol) in 5 mL of EtOAc was added a freshly prepared solution of 3 M HCl in EtOAc (10 mL, 0.030 mol). The solution was allowed to stir for 30 min at RT. The solvent was removed on a rotary evaporator and the residue dried under vacuum at RT to yield crude **11**, which was used as is in the next step without purification.

7-guanidino-4-heptenamide hydrochloride, **4**

To a solution of crude **11** in absolute EtOH (8 mL) was added K_2CO_3 (0.21 g, 0.0015 mol) and the suspension allowed to stir for 5 min. To this was added 3,5-dimethylpyrazole-1-carboxamidine nitrate (0.25 g, 0.0013 mol) in 5 mL of CH_3OH. The reaction was allowed to stir at RT for 10 d and monitored by NMR. The solvent removed on a rotary evaporator and 20 ml of 1 M HCl in CH_3OH was added and the solution concentrated on a rotary evaporator. The residue was dissolved in 10 mL of H_2O and applied to a (1 x 50 cm) column of Trisacryl resin. The column was eluted with H_2O (50 ml) and then with 0.25 M NaCl (225 mL) 0.5 M NaCl (225 mL), 0.75 M

NaCl (225 mL) and 1.0 M NaCl. Fractions that were positive for the Sagakuchi reaction were then concentrated on a rotary evaporator at 50°C. The residue was suspended in CH_3OH, sonicated and filtered. The filtrate was then applied to a (4 x 60 cm) column of LH-20. The column was eluted with CH_3OH, and the fractions that were positive for the Sagakuchi test were combined and the solvent removed on a rotary evaporator to yield **4**, (0.16 g, 55%) as a white solid. ^{13}C NMR (CD_3OD)(ppm) 24.00, 27.57, 35.55, 42.11, 48.16, 127.41, 132.68, 158.66, 178.32. High Resolution M.S. m/e 185.1402 $(M+H)^+$.

(±)-7-[(aminoiminomethyl)amino]-N-[2-[[4-[(3-aminopropyl) amino]butyl]amino]-1-hydroxy-2-oxoethyl][4,5-$^3H_2$] heptanamide trihydrochloride, ^{3}H-DSG, **1b**

Into a 5 ml round bottom flask containing 1.3 Ci of ^{3}H-7-guanidino[4,5-$^3H_2$]heptane hydrochloride, **2b**, was added 7-guanidinoheptane hydrochloride[5], **2a**, (0.077 g, 0.00035 mol), glyoxyloylspermidine dihydrochloride[10], **3**, (0.075 g, 0.00026 mol), glutaric acid (0.034 g, 0.00026 mol) and 40 microliters of H_2O. The flask was sealed with a rubber septum and allow to stir at 55-60°C for 8h. The reaction mixture was diluted with 1.5 mL of H_2O and the solution applied to a 1 x 20 cm column of Sephadex CM C-25. The column was then eluted with a 100 mL aliquots of 0.1M, 0.2M, 0.3M, 0.4M, and 0.5M NaCl. The flowrate through the column was 3-4 mL/min. The 9 mL aliquots of the column eluent were collected via a fraction collector and each fraction was analyzed via both the Sagakuchi and Ninhydrin spray reagents. Fractions analyzing positive for both tests (corresponding to 6 fractions eluting with 0.4M NaCl effluent) were then collected and analyzed via HPLC. Those fractions (5 out of 6) considered essentially the same via HPLC were then lyophilized. The residue was then suspended in 10 mL of CH_3OH and filtered through a 0.45 micron filter. The filtrate was then applied to a 2.5 x 25 cm column of LH-20 in CH_3OH. The column was eluted with CH_3OH at a flow rate of 3-4 ml/min. The column effluent was collected 10 mL fractions via a fraction collector. Fractions which were positive for both the Sagakuchi and Ninhydrin spray reagents were then combined and the solvent removed on a rotary evaporator (water bath, 30°C). The residue was then dissolved in 5 mL of H_2O and the solution lyophilized overnight to yield **1b** (0.0248 g, 188 mCi, 14.5%). The specific activity of **1b** was determined to be 3.78 Ci/mmol. The radiochemical purity of **1b** was determined to be 91.1% via HPLC method 1 and 95.4% via HPLC method 2. ^{3}H-7-guanidinoheptanamide was found to be the major impurity in each analysis.

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