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Synthesis of a Tritium Labeled Rat Enterostatin, Val-[3,4-^{3}H-Pro]-Gly-[3,4-^{3}H-Pro]-Arg-OH

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Summary

A tritium labeled rat Enterostatin, Val-[3,4-^{3}H-Pro]-Gly-[3,4-^{3}H-Pro]-Arg-OH, was prepared by the catalytic tritiation of the didehydroproline analog of rat Enterostatin, Val-[3,4-dehydroPro]-Gly-[3,4-dehydroPro]-Arg-OH, over Pd/C in methanol. The product had a specific activity of 59.2 Ci/mmol and a radiochemical purity of greater than 99%.

Keywords: Enterostatin, 3,4-dehydroproline, 3,4-^{3}H-proline.

Introduction

Enterostatin is a pentapeptide that is cleaved from the N-terminus of the pancreatic enzyme procolipase in the small intestine.[1] In rat, one molecular form of Enterostatin has the following amino acid sequence, Val-Pro-Gly-Pro-Arg, **1a**, which was determined by cloning of procolipase cDNA.[2] This pentapeptide has been reported to have a pronounced effect on food uptake in rats, especially those foods that are high in fat.[1] It has been postulated that this effect could be attributed to an interaction between the peptide and receptors in the brain.

The objective of this study was to prepare high specific activity rat Enterostatin for use in localizing specific receptor populations through autoradiography and brain-region specific receptor binding studies. Catalytic tritiation of small peptides containing dehydroamino acids (e.g. dehydroleucine, dehydroproline, etc.) is a widely used method for the synthesis of tritium labeled peptides. In our study, we prepared high specific activity rat Enterostatin via the tritiation of the didehydroproline analog of Enterostatin, **2**, over Pd/C in methanol (Scheme 1).

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Scheme 1. Synthesis of tritium labeled rat Enterostatin, **1b**.

Val-[3,4-dehydroPro]-Gly-[3,4-dehydroPro]-Arg-OH

2

T_2
Pd/C, CH_3OH

Val-(3,4-^{3}H-Pro)-Gly-(3,4-^{3}H-Pro)-Arg-OH

1 b

Results and Discussion

The synthesis of non-radiolabeled rat Enterostatin, **1a**, the didehydroproline analog, Val-[3,4-dehydroPro]-Gly-[3,4-dehydroPro]-Arg-OH, **2**, and each of its monodehydroproline analogs, Val-[Pro]-Gly-[3,4-dehydroPro]-Arg-OH, **3**, and Val-[3,4-dehydroPro]-Gly-[Pro]-Arg-OH, **4**, was accomplished via solid phase methods using Fmoc L-amino acids and diisopropylcarbodiiimide (DIC) / hydroxybenzotriazole (HOBt) activiation (**Scheme 2**). The crude peptides were subsequently purified via reversed phase HPLC.

During our initial hydrogenation studies, we considered the possibility that an incomplete reduction of **2** to **1a** might occur. We therefore developed an HPLC method capable of resolving Enterostatin, **1a**, the didehydroproline analog, **2**, and each of its monodehydroproline analogs, **3**, and **4**.

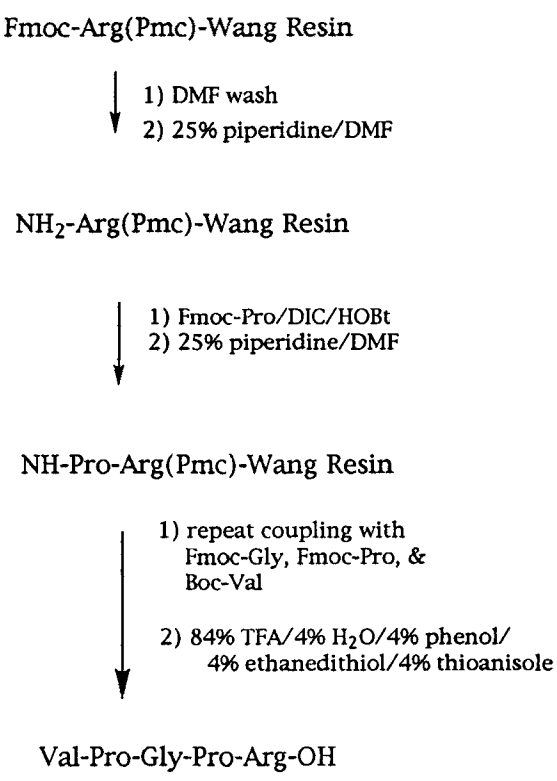
The hydrogenation of **2** to **1a** over Pd/C in methanol proved to be an efficient transformation and using these conditions we found that the tritiation of **2** to **1b** also proceeded very well. HPLC analysis of the radiolabeled material showed that the didehydroanalog had been cleanly reduced to the desired radiolabeled product **1b** (Figure 1). The radiochemical purity of **1b** was found to be greater than 99% and the specific activity of the sample was found to be 59.2 Ci/mmol.

Despite the successful preparation of high specific activity tritium labeled rat Enterostatin, our preliminary biological and autoradiographic studies with this radiolabeled compound have proven inconclusive.

Experimental

Fmoc-Arginine(Pmc)-Wang resin was purchased from Advanced Chemtech, Louisville, KY. All of the amino acids used in this work were

Scheme 2. Solid phase synthesis of Val-Pro-Gly-Pro-Arg, **1a**.



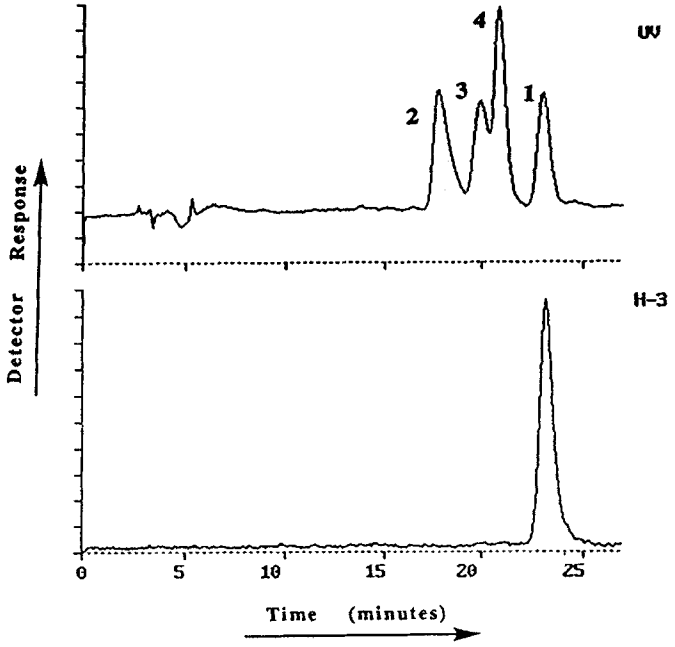
1a

L-amino acids. Boc-valine and Fmoc derivatives of proline, 3,4-dehydroproline and glycine were purchased from Bachem California, Torrance, CA. All of the other solvents or reagents were ACS grade or the highest quality material commercially available. Solid phase peptide synthesis was carried out on an Advanced Chemtech model 200 synthesizer. All experimental conditions were optimized using non-radioactive materials. The identity of the final product **1b** was confirmed by co-elution via HPLC of the radiolabeled substance with authentic unlabeled compound. The specific activity of **1b** was determined by UV and radioactivity measurements.

HPLC Method

HPLC was performed using a Waters microBondapak C18 column, (3.9 x 300 mm), Rainin CPX pumps, a Rainin UV-1 detector for UV analysis (220 nm) and a *IN/US B*-RAM radioactive flowthrough detector for radioactivity measurements. The flowrate of the column was 1 mL/min. The HPLC conditions were optimizing using unlabeled reference samples.

Figure 1. HPLC analysis of H-3 labeled Enterostatin spiked with a standard mixture of Enterostatin, **1a**, the didehydroproline analog, **2**, and each of its monodehydroproline analogs, **3** and **4**.



In this analysis, the samples were loaded onto the column equilibrated with 5% Buffer A (0.1% TFA/ CH_3CN) and 95% Buffer B (0.1% TFA). Immediately following the injection of the sample a linear gradient starting at 5% Buffer A and 95% Buffer B and ending at 8% Buffer A and 92 % Buffer B was run over 25 minutes. The system maintained at 8% Buffer A and 92 % Buffer B for 5 min and then returned to 5% Buffer A and 95 % Buffer B over a 5 min period. In this system the didehydroproline analog, Val-[3,4-dehydroPro]-Gly-[3,4-dehydroPro]-Arg-OH, **2**, has a R_t of approximately 17.4 min, the monodehydroproline analogs, Val-[3,4-dehydroPro]-Gly-Pro-Arg-OH, **3**, and Val-Pro-Gly-[3,4-dehydroPro]-Arg-OH, **4**, have R_t of approximately 19.5 and 20.4 min. respectively, and the desired product, Val-[3,4-^{3}H-Pro]-Gly-[3,4-^{3}H-Pro]-Arg-OH, **1b**, has a R_t of approximately 22.7 minutes.

Synthesis

Val-Pro-Gly-Pro-Arg, **1a**.

Into a 100 mL reaction vessel on an Advanced Chemtech 200 peptide synthesizer was placed 2.22 g (1 mmol) of Fmoc-Arg(Pmc)-Wang Resin. The resin was washed with 80 mL of DMF (2x), and then

subjected to two 10 min treatments with 25% piperidine/DMF to remove the Fmoc group. The resin was then washed with 80 mL of DMF (2x), 80 mL of CH_2Cl_2 (2x), and finally with 80 mL of DMF (2x) to remove residual piperidine. To the free amine-resin was added 1.35 g (4 mmol) of Fmoc-proline, 4 mmol of diisopropylcarbodiimide (2 mL of a 2M solution in CH_2Cl_2), and 0.54 g (4 mmol) of hydroxybenzotriazole, HOBt, in 12 mL of 20% CH_2Cl_2/DMF. The resin slurry was mixed gently for 3 h, filtered, washed with 80 mL of DMF, 80 mL of CH_2Cl_2, and 80 mL of DMF. This complete coupling procedure was then repeated with identical stoichiometry using Fmoc-glycine, Fmoc-proline and finally Boc-valine. The protected peptide-resin was washed with 80 mL of DMF (3x), 80 mL of CH_2Cl_2 (5x), and then dried under vacuum. The dried resin was transferred to a 125 mL erlenmeyer flask containing 75 mLs of a cleavage mixture composed of 84% TFA/ 4% H_2O/ 4% ethanedithiol/ 4% thioanisole/ and 4% phenol. After 1 h, the reaction mixture was filtered through a coarse sintered glass funnel, and the filtrate transferred to a 100 mL round bottom flask and concentrated to a brown syrup on a rotary evaporator. The syrup was then triturated with 25 mL of cold diethyl ether, whereupon, the peptide precipitated as a white solid. The solid was filtered and rinsed with 25 ml of cold diethyl ether (2x). IS-M.S, m/e 525 $(M+H)^+$.

Val-[3,4-dehydroPro]-Gly-[3,4-dehydroPro]-Arg-OH, **2**.

Compound 2 was prepared using similar procedures as described for compound **1a** except Fmoc-(3,4-dehydroproline) was used in place of Fmoc-proline. IS-M.S., m/e 521 $(M+H)^+$.

Val-[3,4-dehydroPro]-Gly-Pro-Arg-OH, **3**.

Compound **3** was prepared using similar procedures as described for compound **1a** except Fmoc-(3,4-dehydroproline) was used in place of Fmoc-proline in amino acid number 2. IS-M.S., m/e 523 $(M+H)^+$.

Val-Pro-Gly-[3,4-dehydroPro]-Arg-OH, **4**.

Compound **4** was prepared using similar procedures as described for compound **1a** except Fmoc-(3,4-dehydroproline) was used in place of Fmoc-proline in amino acid number 4. IS-M.S., m/e 523 $(M+H)^+$.

Hydrogenation of **2**.

To 10 mg of Pd/C in a 250 mL Parr hydrogenation flask was added 10 mg (0.02 mmol) of **2** dissolved in 5 mL of anhydrous CH_3OH. The flask was evacuated and then pressurized with 10 psi of H_2 gas, and shaken for 30 minutes on Parr Hydrogenator. After 30 minutes a sample was removed from the flask, the catalyst filtered. HPLC analysis of the reaction mixture showed complete conversion to the desired

product, **1a**, with no evidence of partial reduction to Val-[3,4-dehydroPro]-Gly-Pro-Arg-OH, **3**, or Val-Pro-Gly-[3,4-dehydroPro]-Arg-OH, **4**.

Tritiation of **2**.

The tritiation of **2** was conducted at Amersham Life Science, Buckinghamshire, England using conditions as described above for the hydrogenation of **2**. The radiochemical purity of Val-[3,4-^{3}H-Pro]-Gly-[3,4-^{3}H-Pro]-Arg-OH, **1b**, as obtained from the tritiation reaction, was found to be greater than 99%. No subsequent HPLC purification was performed.

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