SOLUTION SYNTHESIS OF ^{14}C-LABELED THYMOPOIETIN$_{32-36}$ USING CATALYTIC TRANSFER HYDROGENATION AS THE FINAL DEPROTECTION STEP

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Summary

A ^{14}C-labeled pentapeptide, [^{14}C]Arg-Lys-Asp-Val-Tyr (6), corresponding to the active fragment 32-36 of thymopoietin II has been synthesized by a solution method with a 44 percent radiochemical yield. ^{14}C-labeled Z-Arg(HCl)-OH was coupled to the protected tetrapeptide Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole to give the protected pentapeptide 5 with a specific activity of 340 mCi/mmol. Catalytic transfer hydrogenation was used to remove the protecting groups and the free peptide was purified by gel filtration and high performance liquid chromatography.

Key Words: synthesis, [^{14}C]-TP-5, Transfer Hydrogenation

Thymopoietin II is a 49 amino acid polypeptide that was isolated from bovine thymus by G. Goldstein.[1] Thymopoietin was shown to induce early T cell differentiation and this effect was quite selective in that thymopoietin inhibited early B cell differentiation.[2] The pentapeptide Arg-Lys-Asp-Val-Tyr (TP-5), which corresponds to the fragment 32-36 of thymopoietin II, has shown the same selective T cell differentiating capacity as thymopoietin[3] and is being studied as an immunoregulatory compound. In vitro stability studies of the pentapeptide in human plasma indicated that most of the pentapeptide has a half life of approximately 30 seconds.[4] In this study tritium labeled pentapeptide (TP-5) was prepared from Arg-Lys-Asp-Val-3,5-dibromo-Tyr by catalytic reduction with tritium gas. The success of this method depends upon the stability of both the starting compound and the product toward reduction conditions, the quantitative reduction of the bromide and the successful purification of the tritiated peptide. Error in the calculation of the specific activity of the labeled peptide can occur because of contamination of the unlabeled peptide. Some decomposition has been observed on tritiated TP-5 with the label in the aromatic

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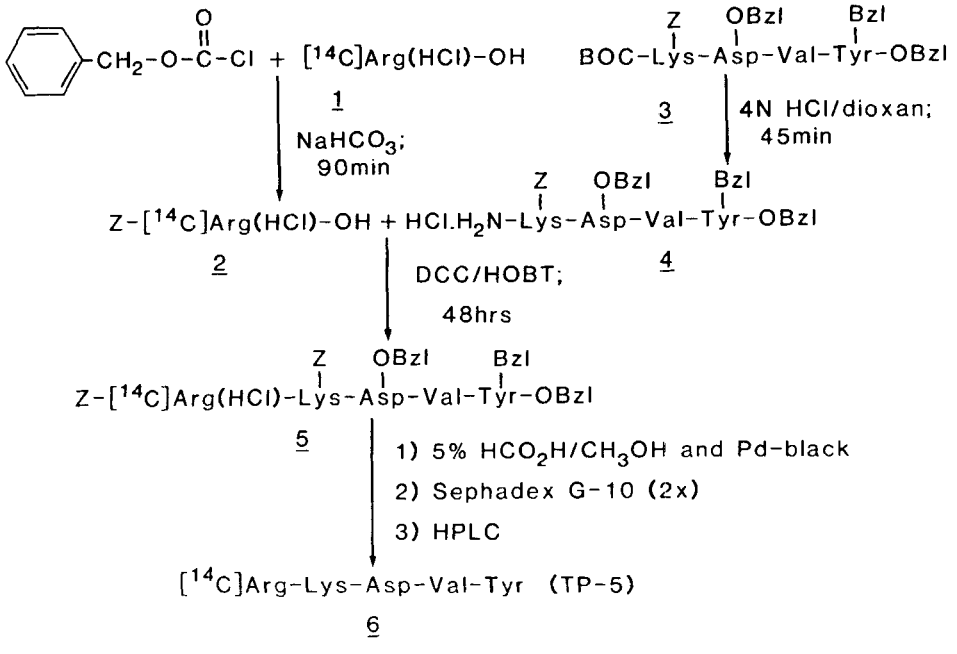
ring of tyrosine on prolonged standing.

For binding studies we have synthesized a different radio labeled pentapeptide which does not show the stability problem exhibited by (3,5-$^{3}H_2$-Tyr^{5})-TP-5. The synthetic design allows for the synthesis of material with a desired specific activity in which unreacted materials and reagents can be readily removed.

In this synthesis, we used ^{14}C-isotope instead of tritium because of its higher radioactive counting efficiency (scheme 1). In addition, we labeled arginine rather than tyrosine since arginine, as the amino terminal residue, could be added last thereby avoiding radioactive intermediates in earlier steps of the synthesis.

SCHEME I



The ^{14}C-labeled Z-Arg(HCl)-OH (2) was prepared from uniformly labeled Arg(HCl)-OH (1) and benzylchloroformate. The resulting Z-[^{14}C]Arg(HCl)-OH (2) was coupled to the protected tetrapeptide, Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl·HCl (4), which was synthesized via classical solution techniques, in the presence of 1-hydroxybenzotriazole by dicyclohexylcarbodiimide in DMF. The pentapeptide was totally deprotected by catalytic transfer hydrogenation with 5 percent formic acid/CH_3OH and Pd-black. Catalytic transfer hydrogenation has been used increasingly in solution[5] as well as in solid phase peptide synthesis[6], because of the rapidity and selectivity of the reaction in addition to its simplicity and mildness. Usually no strong acid or base, which may result in side reactions, is required; the reaction can be carried out at ambient temperature and atmospheric pressure. The catalytic transfer hydrogenation technique is, therefore, ideally suited for the preparation of a radioactive compound. In addition to formic acid, other agents such as 1,4-cyclohexadiene, cyclohexene, hydrazine, sodium formate and ammonium formate have been used as hydrogen donors in the catalytic transfer hydrogenation.[7]

The deprotected peptide was purified by gel filtration on a Sephadex G-10 column equilibrated with 0.1M CH_3COOH and H_2O followed by high performance liquid chromatography on a strong cation exchange column.

Results and Discussion

Our synthetic strategy was to use arginine with minimum protection in the last coupling step and to obtain the final product in a single deprotection step. Due to the complex nature of the highly basic guanidino moiety, the incorporation of an arginine residue into synthetic peptides has posed more problems than have been encountered with most other protein-derived amino acids. Z-Arg(HCl)-OH has been used successfully in the synthesis of arginine containing peptides by classical techniques and, therefore, was our choice in this synthesis.

The remaining amino acids have benzyl and benzyloxycarbonyl protecting groups. ^{14}C-labeled Z-Arg(HCl)-OH (2) was prepared from [^{14}C]Arg(HCl)-OH (1) and benzylchloroformate in a sodium bicarbonate solution using the method of Zervas et al.[8] with slight modification in the work-up procedure. The reaction was completed within one and one half hours, as shown by thin layer chromatography on a silica Gel GF plate (n-butanol/acetic acid/H_2O, 3:1:1), where R_f of Arg(HCl)-OH was 0.225 and Z-Arg(HCl)-OH was 0.700. Excess of benzylchloroformate was removed by washing the reaction mixture with ether several times. To insure that the guanidino group of arginine remained fully protected as HCl salt, the sample was dissolved in 0.1 N HCl and lyophilized. The coupling of Z-[^{14}C]Arg(HCl)-OH (2) to Lys(Z)-Asp-(OBzl)-Val-Tyr(Bzl)-OBzl·HCl (4) was accomplished by using the DCC/HOBT coupling method[9]. The ^{14}C-labeled Z-Arg(HCl)-OH (2) was preformed in situ with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole prior to the addition of the tetrapeptide and diisopropylethylamine to the reaction mixture. The coupling was monitored by thin layer chromatography which showed that most of the Z-[^{14}C]Arg(HCl)-OH (2) reacted after 48 hours. The two radioactive positive spots which corresponded to the Z-Arg(HCl)-OH and protected TP-5 were collected from the thin layer chromatography plate and counted. The results indicated that TP-5 accounted for 95 percent of the total counts. Excess Z-[^{14}C]Arg(HCl)-OH was removed by washing the crude protected pentapeptide thoroughly with water. The removal of the protecting group was achieved by catalytic transfer hydrogenation in 5 percent HCOOH/CH_3OH (v/v) with Pd-black as the catalyst. The reaction was completed in 1 hour. The crude product was applied to a Sephadex G-10 column equilibrated with 0.1 M acetic acid. The pentapeptide was eluted with the same solvent (void volume of 4.2 ml as determined with blue dextran 2000) between 4.5-9.0 ml which contained the highest counts. The peptide was placed back into the Sephadex G-10 column and eluted with H_2O under similar conditions.

Finally, the peptide was purified by HPLC on a Partisil-10 SCX column using 35 percent acetonitrile in 0.02 M ammonium phosphate buffer pH 5.1 as mobile phase (Figure 1). The peptide was eluted 9 minutes after sample injection and the desired peak was collected and analyzed by thin layer chromatography which showed only a single ninhydrin and chlorine/KI-starch positive spot. The peptide co-eluted with the unlabeled TP-5 when they were injected together to the Partisil-10 SCX column; no breakdown of the sample was observed even when the injection was repeated again a week later.

The combination of using a synthetic scheme with guanidino group of arginine protected as the HCl salt and catalytic transfer hydrogenation as the final deprotection step followed by purification with HPLC has enabled us to prepare a very pure ^{14}C-labeled TP-5 with good stability.

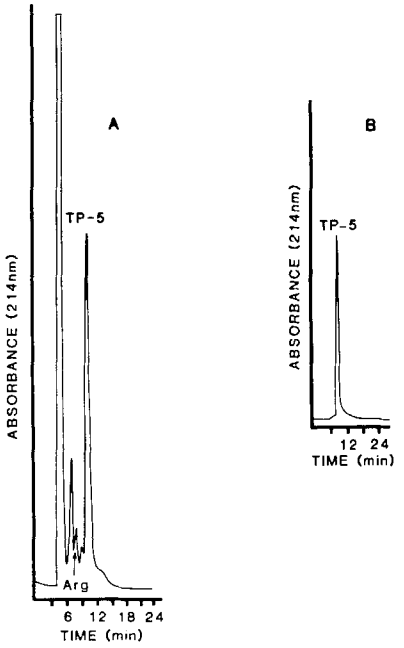


Figure 1. HPLC PURIFICATION OF CRUDE [^{14}C]Arg-Lys-Asp-Val-Tyr ON PARTISIL-10 SCX COLUMN (25cm x 0.46cm), 35% ACETONITRIL IN 0.02M AMMONIUM PHOSPHATE, pH 5.1 AS MOBILE PHASE.
A. CRUDE SAMPLE B. PURIFIED SAMPLE, REINJECTED

The catalytic transfer hydrogenation has proved to be a useful technique in the synthesis of radiolabeled compound because of its convenience and completeness of reaction.

Experimental

Materials and Methods

L-[U-^{14}C]arginine (340 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Illinois. High pressure liquid chromatography was performed using a Laboratory Data Control system equipped with a Constametric III solvent delivery system, a Rheodyne injector 7126 and a model 1203 UV detector with a single wavelength of 214 nm, on a Whatman Partisil-10 SCX column using 35 percent acetonitrile in 0.02 M ammonium phosphate buffer, pH 5.1 as mobile phase.

Synthesis of [^{14}C-Arg]-TP-5

Z-[^{14}C]Arg(HCl)-OH (2)

Sodium bicarbonate (23 mg 270 μmoles) was added slowly with stirring to [^{14}C]Arg(HCl)-OH (6.33 mg; 29 μmoles; 10 mCi) in 2.5 ml of H_2O followed by benzylchloroformate (5.61 mg; 33 μmoles). The reaction was monitored by thin layer chromatography. After 1 hour, additional benzylchloroformate (2.8 mg; 16 μmoles) was added to the mixture and the reaction continued for another one half hour. The reaction mixture was washed with ether in order to remove the excess of carbobenzoxychloride. The aqueous phase was lyophilized to give a white solid which had a R_f value that corresponded to the R_f value of Z-Arg(HCl)-OH (0.70; n-Butanol/HOAc/H_2O, 3:1:1; Silica Gel GF, 250 microns). The material was dissolved in 5 ml of 0.1 N HCl, lyophilized and used without further purification.

H-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl·HCl (4)

BOC-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl (3; 30 mg; 28.6 μmoles) was treated with 8 ml of 4 N HCl/dioxane for 45 minutes. The solvent was removed and the residue was triturated with ether to give a white solid material. The product was filtered, washed with ether (3 x 15 ml) and dried in a dessicator under vacuum over KOH pellets. The compound showed only a single ninhydrin and UV positive spot on Silica Gel GF plate (R_f 0.28; $CHCl_3/CH_3OH$, 90:10).

Z-[^{14}C]Arg(HCl)-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl (5)

1-Hydroxybenzotriazole (4.5 mg; 28.6 μmoles) and dicyclohexylcarbodiimide (6 mg; 28.6 μmoles) were added to Z-[^{14}C]Arg(HCl)-OH (2) in 1 ml of DMF with stirring. After one half hour, H-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl·HCl (4) and diisopropylethylamine (3.72 mg; 28.6 μmoles) were added to the mixture. The coupling was carried out for 48 hours and monitored by thin layer chromatography. Two radioactive spots corresponding to Z-Arg(HCl)-OH (R_f 0.22 Silica Gel F_{60}, 200 microns; n-Butanol/HOAc/H_2O, 4:1:5) and TP-5 (R_f 0.55) were observed and were subsequently collected and counted. The fraction that corresponded to TP-5 accounted for 95 percent of the total counts. The solvent was removed under reduced pressure at ambient temperature. The residue was triturated with water (5 ml) and the solid was filtered and washed with water (3 x 2 ml). The crude product was dried and used without further purification.

[^{14}C]Arg-Lys-Asp-Val-Tyr (6)

Z-[^{14}C]Arg(HCl)-Lys(Z)-Asp(OBzl)-Val-Tyr(Bzl)-OBzl (5) was hydrogenated with 5 ml of 5 percent HCO_2H/CH_3OH (v/v) and 25 mg of Pd-black for 18 hours. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was applied to a Sephadex G-10 column (30 cm x 0.8 cm) equilibrated with 0.1 M acetic acid. The flow rate was 9 ml/hr and fractions of 0.5 ml were

collected. The desired fractions (9-18) were pooled and lyophilized. The residue was divided into two parts and placed back into a Sephadex G-10 column equilibrated with H_2O under similar conditions. The desired fractions (9-15) were collected, lyophilized and purified on a Partisil-10 SCX column (25 cm x 0.46 cm) equilibrated with 35 percent acetonitrile in 0.02 M ammonium phosphate buffer, pH 5.1. The flow rate was 1.5 ml/min and absorbance was read at 214 nm. The peak, with a retention time that corresponded to TP-5, was collected and counted to give ^{14}C-labeled TP-5 with a specific activity of 340 mCi/mmol. The peptide was then re-injected into the Partisil column together with unlabeled TP-5 and only a single peak was obtained. The peptide showed the same R_f value as the unlabeled TP-5 on Silica Gel GF plate (R_f was 0.21 in n-Butanol/HOAc/H_2O, 4:1:5, upper phase and 0.52 in n-Butanol/HOAc/H_2O/Pyridine, 15:3:12:10) using ninhydrin and KI/starch-Chlorine as spray reagent. The radiochemical yield of the peptide was 44 percent based on [^{14}C]Arg(HCl)-OH.

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