Protection of Tyrosine in Solid-Phase Peptide Synthesis[1]

DONALD YAMASHIRO AND CHOH HAO LI*

The Hormone Research Laboratory, University of California, San Francisco, California 94122

Received August 24, 1972

N^{α}-*tert*-Butyloxycarbonyl-*O*-(*o*-bromobenzyloxycarbonyl)tyrosine has been synthesized and employed in the solid-phase peptide synthesis of the octapeptide Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe, which occurs in the human growth hormone sequence. The new protecting group for tyrosine was found to be stable and gave no significant side product upon its removal in hydrogen fluoride.

In solid-phase peptide synthesis[2] benzyl protection of the phenolic hydroxyl group in tyrosine has generally been employed. This protection is known to be unsatisfactory since it is not only unstable under the acidic conditions required for the removal of N^{α}-Boc protection[3-5] but also yields a side product,[4,6] 3-benzyltyrosine,[4] when it is removed in hydrogen fluoride.[6] Modification of the tyrosine residue in HF has also been observed in solid-phase synthesis of tyrosine-containing peptides.[7]

We recently proposed use of the very stable Z(*o*-Br)[8] group for the protection of the side chain of lysine.[3] We have now successfully applied it for similar protection of tyrosine in the solid-phase synthesis of the octapeptide Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe (**1**) corresponding to amino acid residues 138–145 in the human growth hormone molecule.[9,10] Evidence is presented that this protection is stable and gives no significant side product upon its removal in HF.

Quantitative data on the stabilities of protecting groups commonly used in solid-phase synthesis have been obtained on acetylated amides of amino acids with protected side-chain functions.[3] A comparable test was carried out with N^{α}-acetyl-*O*-Z(*o*-Br)tyrosinamide by treatment with 50% TFA in CH_2Cl_2 for 24 hr. As judged by tlc only 1% of the protection was lost as compared to standard benzyl protection where at least 50% is lost. The new protecting group was completely removed in HF in 10 min at 0° and only a single product was detected. Since the new protecting group forms a phenolic ester with tyrosine, the possibility of instability under basic conditions exists. Therefore, the acetylated amide was treated with 10% diisopropylethylamine in DMF for 24 hr; only about 5% of the protection was lost.

For use of the new protecting group in peptide synthesis N^{α}-Boc-O-Z(*o*-Br)Tyr was synthesized and isolated as its dicyclohexylamine salt. The compound was treated in HF and amino acid analysis showed a virtually quantitative yield (98%) of tyrosine. Octapeptide **1**, which has already been synthesized by solid-phase procedures,[5] was resynthesized with the following side-chain protecting groups: Z(*o*-Br) for Lys[3] and Tyr; Bzl for Ser and Thr.

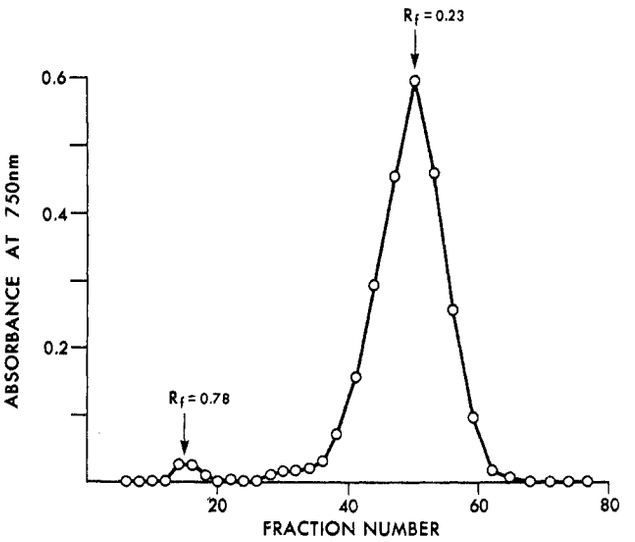

Figure 1.—Partition chromatography of octapeptide **1** on Sephadex G-25; absorbance by Folin–Lowry analysis.

The finished peptide was cleaved from the polymer and deprotected in HF[6,11] and purified as described previously.[5] The partition chromatography on Sephadex G-25 is shown in Figure 1. The R_f value of 0.23 is in close agreement with the value of 0.22 previously reported[5] for chromatography of **1** under these conditions. In the previous synthesis of **1**, either with Bzl protection of tyrosine or Bzl(*m*-Br) protection, significant amounts of side products were observed travelling faster than **1** in the partition chromatography.[12] In the present synthesis only a minute trace of peptide side product (R_f 0.78) could be detected. When peptide **1** from the partition chromatography was then subjected to chromatography on CM-cellulose[13] only one peak was obtained (Figure 2). The overall yield of highly purified octapeptide **1** was about 86% based on the starting Boc-Phe polymer, higher than the yields previously attained.

Experimental Section

Melting points were determined on a Fisher-Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of

(1) This work was supported in part by the American Cancer Society, the Allen Foundation, and the Geffen Foundation.

(2) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).

(3) D. Yamashiro, R. L. Noble, and C. H. Li, presented at the 3rd American Peptide Symposium, Boston, Mass., June 1972.

(4) B. W. Erickson and R. B. Merrifield, presented at the 3rd American Peptide Symposium, Boston, Mass., June 1972.

(5) D. Yamashiro and C. H. Li, *Int. J. Peptide Protein Res.*, **4**, 181 (1972).

(6) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jap.*, **40**, 2164 (1967).

(7) R. Shapira, F. C-H. Chou, S. McKneally, E. Urban, and R. F. Kibler, *Science*, **173**, 736 (1971).

(8) Symbols and abbreviations are in accordance with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1971). Other abbreviations: TFA, trifluoroacetic acid; DIA, diisopropylethylamine; CM-cellulose, carboxymethylcellulose; tlc, thin layer chromatography.

(9) C. H. Li, J. S. Dixon, and W.-K. Liu, *Arch. Biochem. Biophys.*, **133**, 70 (1969).

(10) C. H. Li and J. S. Dixon, *ibid.*, **146**, 233 (1971).

(11) J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.*, **89**, 181 (1967).

(12) Recently, similar results were obtained when octapeptide **1** was synthesized with Bzl (2,6-Cl_2) protection of tyrosine (D. Yamashiro, unpublished observations).

(13) E. A. Peterson and H. A. Sober, *J. Amer. Chem. Soc.*, **78**, 751 (1956).

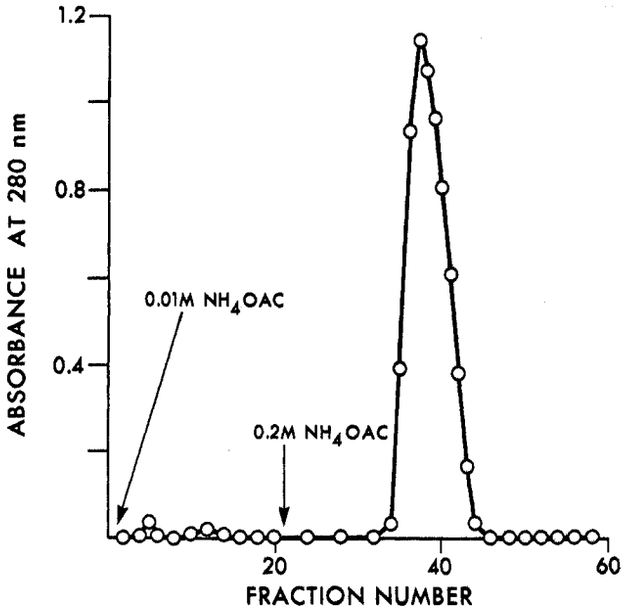

Figure 2.—CM-Cellulose chromatography of octapeptide 1.

California, Berkeley. Thin layer chromatography was run on silica gel in the following solvents: 1-butanol–acetic acid–water, 4:1:1 (BAW); 1-butanol–pyridine–acetic acid–water, 30:20:6:24 (BPAW). Thin layer data cited refer to single spot chromatograms unless otherwise noted.

***O*-(*o*-Bromobenzyloxycarbonyl)tyrosine.**—A solution of tyrosine (13.9 g, 77 mmol) in 78 ml of 2 *N* NaOH was mixed with a solution of $CuSO_4 \cdot 5H_2O$ (9.4 g) in 39 ml of water, heated to 60°, and then cooled to 24°. The pH was adjusted to about 5 with glacial HOAc; the solid was collected on a filter and washed with water and acetone. This solid was suspended in 1100 ml of 70% aqueous DMF, and *p*-nitrophenyl *o*-bromobenzyl carbonate[14] (41.5 g, 118 mmol) and $NaHCO_3$ (14.2 g) were added. The mixture was stirred for 20 hr and the product was collected on a filter and washed with 75% aqueous DMF, water, and acetone, yield 21.4 g of blue solid. This solid was stirred in 1 *N* HCl (300 ml) for 1 hr, filtered, and washed with 1 *N* HCl (250 ml), water, and acetone, yield 15.9 g (50%) of colorless solid. For analysis a sample was recrystallized from 50% HOAc: mp 203–208° dec; tlc (BAW) R_f 0.57 (ninhydrin and chlorine color developments); $[\alpha]^{24}D$ −4° (*c* 2, 80% HOAc).

Anal. Calcd for $C_{17}H_{16}NO_5Br \cdot H_2O$ (412.24): C, 49.53; H, 4.40; N, 3.40. Found: C, 49.49; H, 4.02; N, 3.82.

Dicyclohexylamine Salt of N^{α}-Boc-*O*-(*o*-bromobenzyloxycarbonyl)tyrosine.—The dimethyl sulfoxide method[15] was adapted for the preparation of the Boc derivative. Thus, *O*-Z(*o*-Br)-tyrosine (4.12 g, 10 mmol) was dissolved in dimethyl sulfoxide (50 ml) with 3.4 ml (20 mmol) of DIA, and Boc azide (2.8 ml, 20 mmol) was added. After 2 hr of stirring, an additional 1.7 ml of DIA was added. A solution was obtained from the initially gelatinous mixture and this was allowed to stand overnight. The following work-up was performed at 4°. Water (100 ml) was added to the solution, which was then washed with 100 ml of ether. The ether layer was back-extracted with 60 ml of 7% NaCl. The combined aqueous phases were mixed with 24 ml of saturated NaCl and extracted successively with 250-, 100-, and 100-ml portions of ethyl acetate. The combined ethyl acetate extracts were mixed with 150 ml of 12% NaCl and acidified to pH below 3 with 3 *N* HCl (5 ml). The ethyl acetate layer was washed with three 100-ml portions of 17% NaCl and then dried over anhydrous $MgSO_4$ at room temperature. Removal of drying agent and solvent gave 3.9 g of oil which was dissolved in ether (*ca.* 35 ml), cooled to 0°, mixed with dicyclohexylamine (1.6 ml), and diluted at 0° with petroleum ether (bp 30–60°) (30 ml). Crystallization at 4° gave 5.09 g (75% yield), mp 137–140°, $[\alpha]^{24}D$ +29.3° (*c* 2.04, absolute EtOH).

Anal. Calcd for $C_{34}H_{47}N_2O_7Br$ (675.67): C, 60.44; H, 7.01; N, 4.15. Found: C, 60.52; H, 7.08; N, 4.34.

For determination of optical purity a sample (407 mg) was treated in HF (15 ml) in the presence of anisole (1.5 ml) for 30 min at 0° and worked up as previously described.[5] Quantitative amino acid analysis for tyrosine gave a 98% yield. Determination of optical rotation gave $[\alpha]^{24}D$ −10.2° (*c* 2.13, 1 *N* HCl) based on the amount of tyrosine obtainable from the starting sample. The starting tyrosine gave $[\alpha]^{24}D$ −10.3° (*c* 2.07, 1 *N* HCl).

N^{α}-Acetyl-*O*-(*o*-bromobenzyloxycarbonyl)tyrosinamide.—This compound was prepared by procedures previously described,[3] mp 202–204°, tlc (BAW) R_f 0.80.

Anal. Calcd for $C_{19}H_{19}N_2O_5Br$ (435.28): C, 52.43; H, 4.40. Found: C, 52.36; H, 4.54.

Solid-Phase Peptide Synthesis Procedures.—For the esterification step[16] Boc-phenylalanine (1.33 g, 5.0 mmol) in 6.5 ml of methanol was mixed with 7.8 ml of 0.61 *N* tetramethylammonium hydroxide in methanol and evaporated *in vacuo*. The oil was reevaporated from dioxane and then methanol and dried *in vacuo* over P_2O_5 for 5 hr. The salt was treated with 2.52 g of chloromethylated polymer (0.57 mmol Cl/g) in DMF (25 ml) for 17 hr at 24°. The resin was filtered off and washed with DMF, DMF–H_2O (1:1), water, glacial HOAc, water, and EtOH, yield 3.03 g. A sample of the resin deprotected and neutralized gave an amine content[17] of 0.30 mmol/g. An aliquot (1.00 g) of Boc-Phe polymer was placed in a Beckman Model 990 peptide synthesizer and treated at 24° by the same schedule for synthesis as described previously.[5] Side-chain protecting groups were Bzl for Ser and Thr and Z(*o*-Br) for Lys and Tyr. Yield of protected peptide polymer was 1.54 g.

Phe-Lys-Gln-Thr-Tyr-Ser-Lys-Phe (1).—A portion (655 mg) of protected peptide resin was treated in HF and then submitted to gel filtration on Sephadex G-10 as described previously,[5] yield 157 mg. An aliquot (82 mg) of this material was submitted to partition chromatography[18] on a 2.21 × 59.6 cm Sephadex G-25 column in the solvent system 1-butanol–pyridine–0.1 *N* aqueous NH_4OH containing 0.1% HOAc (4:1:5) with collection of 5.9-ml fractions as shown in Figure 1. Isolation of material in fractions 38–60 gave 73 mg. This material was chromatographed on CM-cellulose[5] with collection of 10.2-ml fractions as shown in Figure 2. Isolation of material in fractions 35–43 gave 70 mg of octapeptide 1 (86% yield based on starting resin and assuming ϵ 1340 for tyrosine at 276 nm in 1 *N* HOAc): tlc (BPAW) R_f 0.30, identical with that of an authentic sample[5] of 1 (ninhydrin and Pauly reagents); $[\alpha]^{24}D$ −27° (*c* 0.5, 1 *N* HOAc) [lit.[5] $[\alpha]^{24}D$ −28° (*c* 0.5, 1 *N* HOAc)].

Paper electrophoresis in collidine acetate buffer (pH 6.9, 400 V, 4 hr) showed a single spot (ninhydrin and Pauly reagents) with R_f 0.55 relative to lysine, identical with that of an authentic sample[5] of 1. Amino acid analyses[19] of an acid hydrolysate and a leucine aminopeptidase digest (pH 8, 24 hr, 37°) gave $Lys_{1.9}$-$Thr_{1.0}Ser_{0.9}Glu_{1.0}Tyr_{1.0}Phe_{2.0}$ and $Lys_{1.9}(Thr + Gln + Ser)_{3.0}$-$Tyr_{1.0}Phe_{2.0}$, respectively.

Registry No.—**1**, 37440-42-9; *O*-(*o*-bromobenzyloxycarbonyl)tyrosine, 37440-25-8; tyrosine, 60-18-4; *p*-nitrophenyl *o*-bromobenzyl carbonate, 37440-43-0; dicyclohexylamine salt of N^{α}-Boc-*O*-(*o*-bromobenzyloxycarbonyl)tyrosine, 37440-44-1.

Acknowledgment.—We thank Mr. Richard L. Noble, Mr. W. F. Hain, and Mr. Kenway Hoey for their skilled technica lassistance.

(14) D. Yamashiro and C. H. Li, *J. Amer. Chem. Soc.*, in press.

(15) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1969, p 29.

(16) A. Loffet, *Int. J. Protein Res.*, **3**, 297 (1971).

(17) B. F. Gisin, *Anal. Chim. Acta*, **58**, 248 (1972).

(18) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).

(19) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).