Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 493-499

SOLUTION SYNTHESIS OF [ASN^{76}]-HUMAN PARATHYROID HORMONE(1-84)

Terutoshi Kimura, Michihiro Takai, Kumiko Yoshizawa, and Shumpei Sakakibara

Peptide Institute, Protein Research Foundation
Minoh, Osaka 562, JAPAN

Received May 23, 1983

Human parathyroid hormone, hPTH(1-84), was synthesized by the conventional solution procedure applying the maximal-protection approach. All protecting groups were removed simultaneously by the HF method. The product was purified by CM-cellulose column chromatography, gel-filtration on Sephadex G-50 and in the final stage, by reversed phase HPLC. The structure of the final product was confirmed not only by HPLC analysis but also by peptide mapping of tryptic digests on HPLC. The present product showed 350(249-480) IU/mg on *in vitro* rat renal adenylate cyclase assay.

The amino acid sequence of human parathyroid hormone, hPTH, was originally determined by Keutmann *et al.*(1) in 1978 as a single-chain polypeptide with 84 amino acid residues, and the total synthesis was successfully done for the first time by our research group in 1981 (2). Immediately after our synthesis, the structure was revised by Hendy *et al.* (3) to have Asn instead of Asp at position 76 in Keutmann's structure, based on deduction from sequence analysis of cDNA cloned for human preproPTH. This communication reports the solution synthesis of [Asn^{76}]-hPTH(1-84) and the characterization of the product.

MATERIALS AND METHODS

Materials. DPCC-treated trypsin(EC 3.4.21.4.) was purchased from Sigma Chemicals Co., and amino peptidase-M(EC 3.4.11.2.) was purchased from Pierce Chemical Co. Boc-amino acids and other reagents for peptide synthesis were obtained from Peptide Institute, Inc., Osaka, Japan.

Abbreviations: WSCI, water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOBT, 1-hydroxybenzotriazole; Pac, phenacyl ester; DMF, N,N-dimethylformamide; DPCC, diphenyl carbamyl chloride.

0006-291X/83 $1.50

Peptide Synthesis. [Asn^{76}]-hPTH was synthesized by the conventional solution procedure as shown in Fig. 1. Condensation reaction of each segment was carried out in DMF or N-methylpyrrolidone by the WSCI/HOBT method after removal of the terminal Boc- or Pac group. The fully protected product was deprotected by the HF method using an HF reaction apparatus, Protein Research Foundation Type I. The crude product was purified on a column of CM-cellulose, Sephadex G-50 and then by reversed-phase HPLC.

Reversed-Phase HPLC. HPLC was performed on a Hitachi Liquid Chromatograph Model 638 equipped with a column of Nucleosil 5C$_{18}$ (150 x 4 mm). All runs were performed at ambient temperature at a flow rate of 1.0 ml/min. Other conditions are given in each figure legend.

HPLC Mapping of Tryptic Digests. A solution of Peptide (40 µg) in 40 µl of water were treated with a water-solution of DPCC treated trypsin(2.5 µg/5 µl) at 37°C; pH of the mixture was 6. After 30 min, 12 µl of the whole mixture was applied to the reversed-phase HPLC system.

Biological activity. Biological potency of hPTH was measured by in vitro assay of the rat-kidney adenylate cyclase activity following the procedure developed by Marcus and Aurbach (4) using WHO bovine PTH(1-84) as the standard.

RESULTS AND DISCUSSION

The principle of the present synthesis is based on the maximum protection strategy using stable protective groups at the side chains (5). The whole molecule was assembled with 13 segments by the route shown in Fig. 1. Boc-amino acids developed for Merrifield's solid phase procedure were used for stepwise synthesis of each segment in solution, which was started from the C-terminal amino acid Pac ester by the WSCI/HOBT method or by the active ester method. Each segment was also coupled by the WSCI/HOBT method after removal of the terminal Boc or Pac group; the latter group was removed by warming the peptide with Zn powder in acetic acid. No particular difficulty was encountered in the segment condensation reactions in solution when DMF or N-methyl-pyrrolidone was used as the solvent.

The fully protected 84-peptide thus obtained was treated with HF at 0°C for 1 hr in the presence of anisole, methionine, dimethylsulfide and ethanedithiol as scavengers. The crude

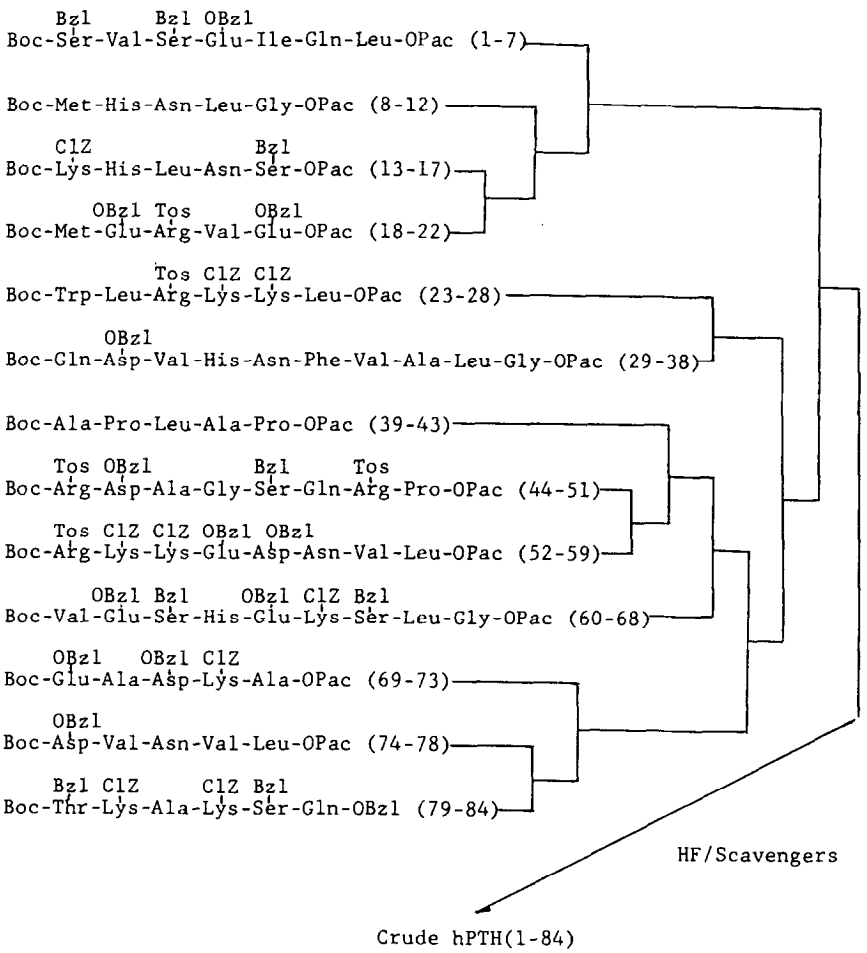

Fig. 1. Coupling route for the synthesis of [Asn^{76}]-hPTH(1-84).

product was fractionated on a CM-cellulose column using ammonium acetate solution with linear gradient concentrations from 0.05 M at pH 5 to 0.4 M at pH 6, and then by gel-filtration on Sephadex G-50 using 1 M acetic acid as the solvent. The main peak of the Sephadex chromatogram was developed on a gradient HPLC system (Fig. 2); the main peak here was collected from several different runs and rechromatographed on an isocratic HPLC system; the elution profile is shown in Fig. 3. The homogeneity of each fraction of the broad peak was checked with a similar HPLC system under analytical conditions; fractions showing a single peak on

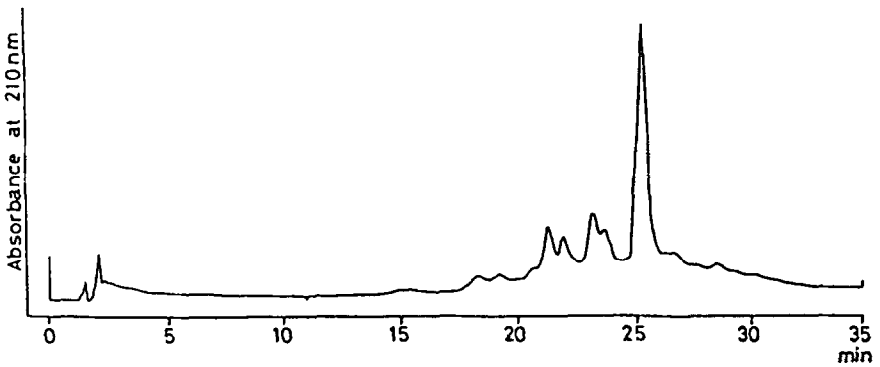

Fig. 2. HPLC profile of crude product obtained after gel-filtration on Sephadex G-50.
Eluant: 0.1 M NaCl(pH 2.4) containing MeCN, which was gradiently increased from 27.5% to 40%.

analytical HPLC were collected and lyophilized to obtain a final product.

Amino acid analysis of an acid hydrolyzate and an Ap-M digest revealed that the product contained all component amino acids in the expected ratios (Table 1). When the final product was treated with a dilute aqueous hydrogen peroxide solution, the reaction mixture showed four peaks on HPLC including that of the intact molecule (Fig. 4). The formation of three additional

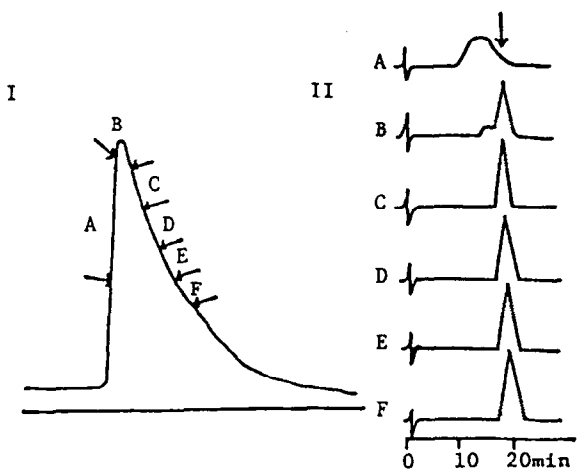

Fig. 3. Separation of final product, h-PTH(1-84), on HPLC.
I. Isocratic HPLC profile of collected materials.
II. Purity check of each fraction in I under analytical conditions; fractions C,D,E and F were pooled for lyophilization as the purified material. Eluant: 31% MeCN in 0.1 M NaCl(pH 2.4).

Table 1. Amino acid analyses of synthetic [Asn^{76}]-hPTH.

Amino Acid	Expected	6 N-HCl	Ap-M
Lys	9	9.36	8.92
His	4	3.60	3.60
NH_3	9	10.08	
Arg	5	5.15	
Asp	5		3.76
Asp+Asn	10	10.00	
Thr	1	0.99	
Thr+Gln	5		3.85
Ser	7	5.74	
Ser+Asn	12		11.40
Glu+Gln	11	10.32	
Cit+Glu	12		10.80
Pro	3	2.74	3.24
Gly	4	4.00	4.00
Ala	7	7.07	6.86
Val	8	7.76	7.52
Met	2	1.20	1.12
Ile	1	0.77	0.90
Leu	10	9.90	9.20
Phe	1	1.01	1.04
Trp	1	0.53	0.52

peaks may be explained by the partial or complete oxidation of the Met residues to the sulfoxides since the hPTH molecule contains two Met residues at positions 8 and 18. This observation proved that the purified material was free of contamination from oxidized peptides.

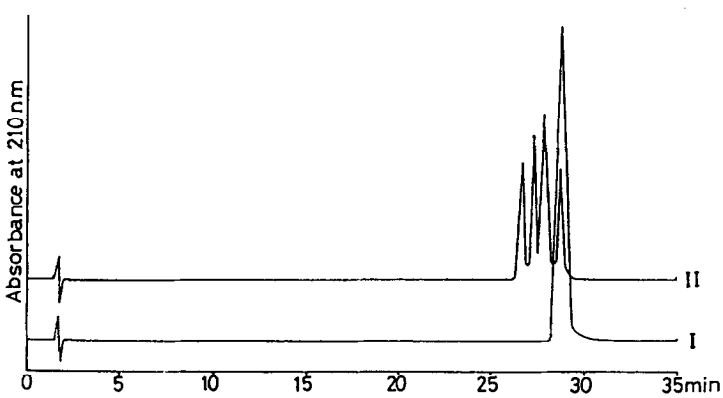

Fig. 4. HPLC profile of purified synthetic hPTH(1-84) and its hydrogen peroxide-treated products.
I. hPTH(1-84) II. Oxidized products. Eluant: 0.1 M NaCl (pH 2.4) containing MeCN, which was gradiently increased from 10% to 50%.

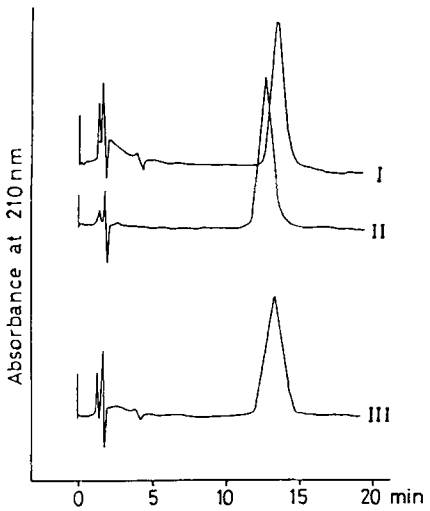

Fig. 5. Separation of Asn- and Asp-hPTH(1-84) on HPLC. I. [Asp^{76}]-hPTH II. [Asn^{76}]-hPTH III. Mixture of Asp- and Asn-PTH. Eluant: 31% MeCN in 0.1 M NaCl (pH 2.0).

Our HPLC system showed enough resolution power for analyzing analogs of hPTH(23-84) containing Asp or Asn at position 76. However, the system failed to separate [Asn^{76}]- and [Asp^{76}]-hPTH(1-84) molecules into two peaks when they were injected onto the column as a mixture (see Fig. 5). Thus, the homogeneity had to be checked by other means. HPLC mapping of the trypsin digest was compared with those of some other synthetic peptides as references (Fig. 6). All peaks in the chromatograms were assigned as shown in the figure. In the chromatogram of the present product, all the expected fragments were observed and no unwanted peaks appeared. The present product might have been contaminated by D-Glu^{22}-containing peptide since the last coupling reaction was performed between segments(1-22) and (23-84), but no D-Glu^{22}-containing fragment(21-25) was detected by tryptic mapping within the range of resolution. Thus, We concluded that our present product was reasonably homogeneous. The product showed 350(249-480) IU/mg in the rat-kidney adenylate cyclase assay. The specific optical rotation value was observed to be: $[\alpha]_D^{20}$ -89.2°(c 0.2, 1% AcOH).

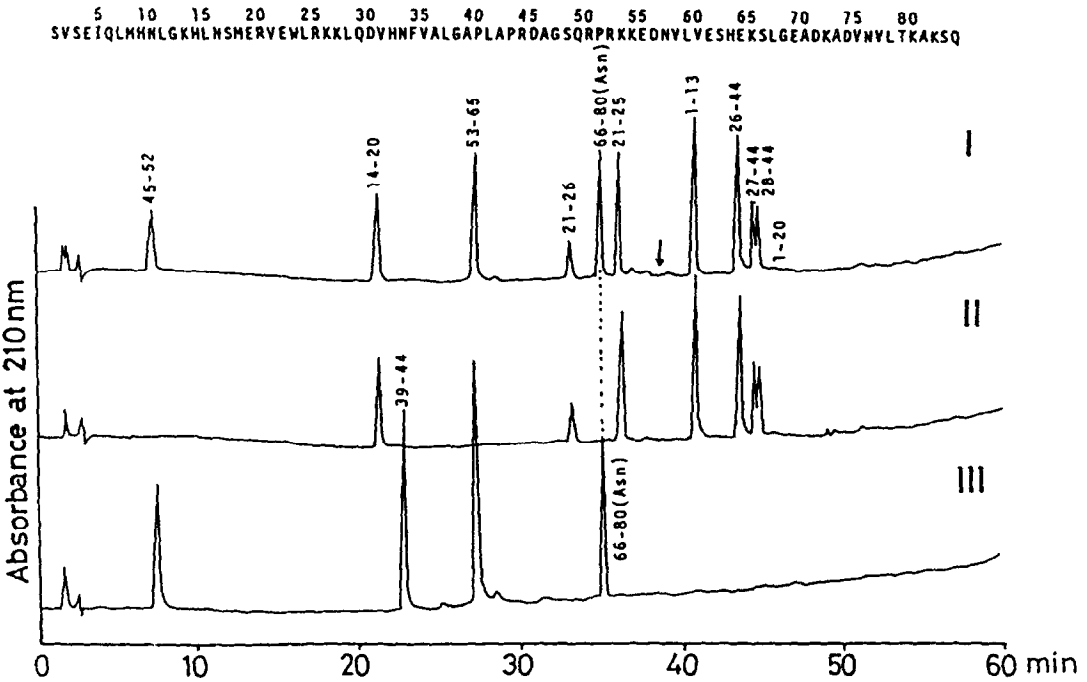

Fig. 6. HPLC mapping of a tryptic digest of [Asn^{76}]-hPTH in comparison with those of some other synthetic peptides. I. Asn-hPTH(1-84). II. hPTH(1-44) III. Asn-hPTH(39-84) Arrow indicates the position of [D-Glu^{22}]-containing fragment(21-25). Eluant: 10 mM phosphate buffer(pH 2.6) containing 50 mM Na_2SO_4 and MeCN, which was increased gradiently from 2% to 50%.

ACKNOWLEDGEMENT

We wish to thank Toyo Jozo Co., Ltd. for conducting the adenylate cyclase assay.

REFERENCES

1. Keutmann, H.T., Sauer, M.M., Hendy, G.H., O'Biordan, J.L.H., and Potts,Jr., J.T. (1978) Biochemistry 17, 5723-5729.
2. Kimura, T., Morikawa, T., Takai, M., and Sakakibara, S. (1981) J. Chem. Soc. Chem. Commun. 340-341.
3. Hendy, G.H., Kronenberg, H.M., Potts, Jr., J.T., and Rich, A. (1981) Proc. Natl. Acad. Sci. USA 78, 7365-7369.
4. Marcus, R. and Aurbach, G.D.(1969) Endocrinology 85, 801-810.
5. Kimura, T., Takai, M., Masui, Y., Morikawa, T., and Sakakibara, S. (1981) Biopolymers 20, 1823-1832.