Solid Phase Synthesis of Thymopoietin-(32–36) Using Adpoc Amino Acids and its Biological Activity+

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An exemplary peptide has been synthesized for the first time by an automated solid-phase instrumentation which utilized the new Adpoc (1-(1-adamantyl)-1-methylethoxycarbonyl-) group for protection of the α-amino groups of the amino acids. The pentapeptide, thymopoietin-(32–36) was synthesized, and shown to be pure by criteria including HPLC. Using a Cytoxan (cyclophosphamide) model system for suppression of a primary, hemolytic, humoral, antibody level, thymopoietin-(32–36) showed low activity, and reversed the suppressing effect of Cytoxan. This biological activity apparently is the result of cummulative effect.

Introduction

Kalbacher and Voelter [1] have designed a new protective group, identified as Adpoc, which is 1-(1-adamantyl)-1-methylethoxycarbonyl-, for the synthesis of peptides. This new protective group offers advantages over the commonly used protective group, *t*-butyloxycarbonyl-.

Regulations of the complex immune defense mechanisms by polypeptides, which are derived from the thymus gland, are being internationally studied as reviewed by Pahwa *et al.* [2]. G. Goldstein *et al.* [3] isolated two polypeptides from bovine thymus tissue, which have been designated thymopoietin I and II. The sequence of the 49 amino acids, constituting thymopoietin II, was elucidated [4]. Thymopoietin II can induce the expression of cortical thymocyte alloantigens, such as TL and Thy-1, on murine bone marrow precursor cells, which are negative for these differentiation antigens.

Schlesinger *et al.* [5] described a synthetic fragment, thymopoietin-(29–41). This fragment of 13 amino acids was considered to contain the "active site" of thymopoietin II, although this synthetic peptide had only 10% of the activity by weight or 3% of the activity based on molar concentration. This activity of the fragment was based upon induction, *in vitro*, of T-cell (Th-1) or B-cell (CR) differentiation.

In 1980, Verhaegen *et al.* [6] described a synthetic pentapeptide, Arg–Lys–Asp–Val–Tyr–OH, which is thymopoietin-(32–36), and its effects on peripheral E-rosette forming cells. Weksler *et al.* [7] found that the immune deficiencies in aging mice were reversed by thymopoietin-(32–36).

We have synthesized thymopoietin-(32–36) by using the new Adpoc-group [1] to block α-amino groups of four amino acids for incorporation by solid-phase synthesis. The pentapeptide was then tested in another model system representing immune mechanisms.

Materials and Methods

Synthesis

The pentapeptide was synthesized by the Merrifield solid-phase method using a Beckman Model 990 Automated Synthesizer. The Adpoc-group was used to protect the α-amino group of four of the amino acids during coupling. Aoc-Arg(Tos) was purchased from Peninsula Labs., San Carlos, CA. The Aoc-group was removed at the end of the synthesis during cleavage with hydrogen fluoride.

The four Adpoc amino acids were prepared from chloro-carbonyl fluoride and 2(1-adamantyl)-2-propanol (Kalbacher and Voelter [1]), and were pure by TLC in the three following systems.

+ *Dedicated to Prof. Dr. Dr. G. Weitzel on the occasion of his 65th birthday*

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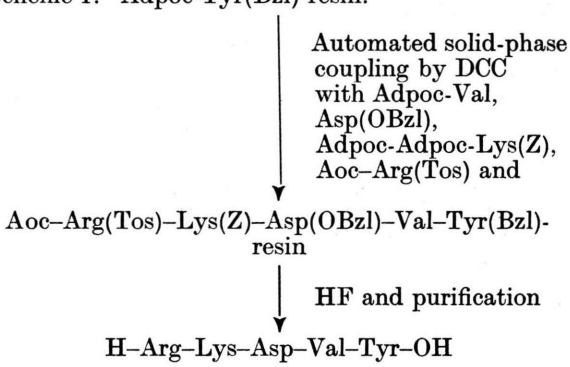
R_f^1, 1:1:1:1, BuOH:EtOAc:HOAc:H_2O
R_f^2, 5:5:1:3, AcOEt:Py:AcOH:H_2O
R_f^3, 2:2:1:1, BuOH:EtOAc:HOAc:H_2O

Adpoc-Tyr (Bzl)–OH	R_f^1 0.73;	R_f^2 0.9;	R_f^3 0.8
Adpoc-Val-OH	R_f^1 0.83;	R_f^2 0.87;	R_f^3 0.81
Adpoc-Asp (O–Bzl)–OH	R_f^1 0.85;	R_f^2 0.81;	R_f^3 0.74
Adpoc-Lys (Z)–OH	R_f^1 0.79;	R_f^2 0.88;	R_f^3 0.78

The NMR-data of the Adpoc amino acids were in agreement with the structures. As in Scheme 1, the C-terminal Adpoc-Tyr(Bzl) residue was linked to chloromethyl polystyrene resin (2 g, 1% cross-linked, 1.1 mM of Cl/1 g resin) through esterification of the cesium salt [8]. Amino acid analysis showed the substitution of 0.47 mM of Tyr per g of resin.

The synthesis was carried out using a program as described [9] for each synthetic cycle, except for the TFA-deprotection step (Scheme 1). For the removal of the Adpoc-groups, the protected peptide resin was allowed to react for 12 min in 10% TFA in CH_2Cl_2. Each residue was coupled twice with 2.5 equivalent of protected amino acid and DCC. On completion of the synthetic cycles, 3.04 g of protected peptide-resin were obtained.

Scheme 1. Adpoc-Tyr(Bzl)-resin.



HF cleavage

The peptide was cleaved from the polymer resin with concomitant removal of the side chain protecting groups using doubly-distilled, anhydrous HF (10 ml/g of resin) at 0 °C for 1 h in the presence of 1 ml anisole/1 g of peptide resin. The peptide/resin mixture was washed with ethyl acetate, followed by extraction with 10% acetic acid and water. The extracts were combined and lyophilized to give 518 mg of crude peptide.

Purification

About 500 mg of the pentapeptide was dissolved in 1 M acetic acid and the solution was centrifuged to remove a small amount of particulate material. The supernatant was applied to a Sephadex G-15 column (3.1 × 96 cm) and 1 M acetic acid was used for elution. UV absorption at 220 nm was determined for selection of the fractions. After lyophilization of the selected fractions, the partially purified product (238 mg) was applied to a CM-Sephadex ion exchange column. A linear gradient from 0.15 M ammonium acetate, pH 4.4, to 0.6 M ammonium acetate, pH 4.4, was used for elution. The optical densities of the fractions were determined. The desired fractions were collected, and yielded 136 mg of purified peptide. This material was dissolved in 1 M acetic acid, and the solution was applied to a Sephadex G-10 column, which was eluted with 1 M acetic acid to desalt the peptide.

Chemical characterization

The pentapeptide was analyzed for amino acid composition after acid hydrolysis (constant boiling HCl, 110 °C, 24 h); the results, expressed as moles of amino acid per mol of peptide, were: Asp, 1.05; Val, 0.98; Tyr, 0.96; Lys, 1.02; Arg, 0.98.

TLC provided evidence on the homogeneity of the synthesized peptide after the final purification, as well as having revealed progressive purification after each successive chromatographic step; R_f^1, 0.18; R_f^2, 0.55; R_f^4, 0.24 (R_f^4, 30:30:6:24, BuOH:Py:AcOH:H_2O).

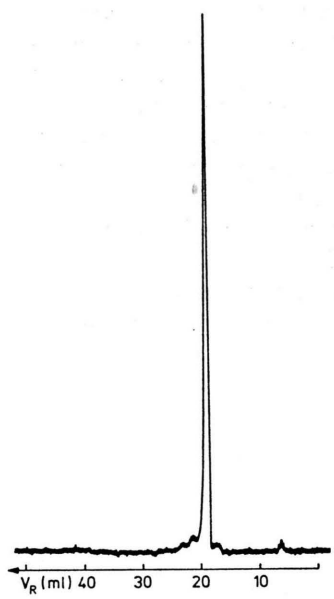


Fig. 1. Sample: Thymopoietin-(32–36); column: μ-Bondapak C_{18} (3.9 × 300 mm); elution: 25 min linear gradient from 0.1 M KH_2PO_4, pH 3.0 to 40%, CH_3CN/60% 0.1 M KH_2PO_4, pH 3.0; flow rate: 2 ml/min; wavelength: 210 nm; chart speed: 0.2 in/min; sensitivity: 2 A.

The HPLC-profile (Fig. 1) of the pentapeptide consisted of a single sharp peak, when chromatographed on a μ-Bondapak C_{18} column using a linear gradient from 0–40% acetonitrile in 0.1 M potassium dihydrogen phosphate pH 3.0.

Thymopoietin-(32–36) was tested for biological activity using a Cytoxan (cyclophosphamide) model for suppression of the primary, hemolytic, humoral, antibody. The peptide was subcutaneously injected at dosages from 6.25 to 200 μg/mouse for a total of seven times from day 1 through 7 after the injection of the antigen. Cytoxan alone at a level of 0.5 mg/mouse administered 24 h after the antigen, reduced the hemolytic level on day 6 to about 84% of the level of the saline control. Treatment with 50 μg/mouse/injection of pentapeptide restored the Cytoxan depressed hemolytic level ot about 98% of the saline control. A similar modest effect was also observed on days 7 and 8 after the antigen administration. This low activity of the pentapeptide was determined to be significant by computer analysis of the data, and is presumably the result of a cummulative effect. G. Goldstein *et al.* [10] reported the half-time of thymopoietin to be about 30 s, but that such a brief pulse could trigger long-ranging changes in responsive cells.

Discussion

The four Adpoc-amino acids, used for assembly of the peptide, were incorporated in the sequence by solid-phase reactions without difficulties. The reaction for the linkage of Adpoc–Tyr(Bzl) to the chloromethyl resin was conducted three times in order to obtain a product with a resonable ratio of Tyr/g resin. Since the Adpoc-group is thermolabile, the esterification was carried out at 40 °C instead of at 50 °C which is the temperature for the esterification of Boc-amino acids.

The TFA-concentration and the reaction time for the deprotection step were arbitrarily chosen. It is known that the removal of the α-protecting group in solid-phase synthesis requires a considerably longer reaction time than that in solution. It is believed that the Adpoc group could be removed under even milder acidic conditions than those described herein. The Adpoc-group appears to be an extremely attractive α-amino blocking group for solid-phase peptide synthesis, because its high acid lability ensures complete deprotection at low concentrations of acid. The use of lower concentrations of TFA in CH_2Cl_2 for deprotection minimizes difficulties in solid-phase synthesis, such as premature loss of protection of side chains and cleavage of the peptide-resin bond.

Since thymopoietin-(32–36) is a fragment of thymopoietin-(29–41), and since the latter peptide of 13 amino acids showed only 3% of the activity of thymopoietin II on a molar basis [5], it is hardly to be expected that the pentapeptide would show potent activity; another reason for this expectation is the projection of the biological activity of a fragment of only 5 amino acids with that of a parent peptide having 49 amino acids. Nevertheless, the low activity of the pentapeptide in this Cytoxan model system is of biological interest for the mechanisms of the immune system.

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[1] H. Kalbacher and W. Voelter, Angew. Chem. **90**, 998 (1978).
[2] R. Pahwa, S. Ikehara, S. Pahwa, and R. Good, Thymus **1**, 27 (1979).
[3] G. Goldstein, Nature (London) **247**, 11 (1974); R. S. Basch and G. Goldstein, Proc. Natl. Acad. Sci. USA **71**, 1474 (1974).
[4] D. H. Schlesinger and G. Goldstein, Cell **5**, 361 (1975).
[5] D. H. Schlesinger, G. Goldstein, M. P. Scheid, and E. A. Boyse, Cell **5**, 367 (1975).
[6] H. Verhaegen, W. DeCock, J. DeCree, and G. Goldstein, Thymus **1**, 195 (1980).
[7] M. Weksler, J. B. Innes, and G. Goldstein, J. Exp. Med. **148**, 996 (1978).
[8] B. F. Gisin, Helvetica Chimica Acta **56**, 1476 (1973).
[9] I. Yamaguchi, G. Rackur, J. J. Leban, V. Björkroth, S. Rosell, and K. Folkers, Acta Chem. Scand. **B 33**, 63 (1978).
[10] J. P. Tischio, J. E. Patrick, H. S. Weintraub, M. Chasin, and G. Goldstein, Int. J. Pept. Protein Res. **14(5)**, 479 (1979).