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SPECIALIA

A new solid-phase synthesis of Thymopoietin II by a mild procedure

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Summary. The solid-phase synthesis of a nonatetracontapeptide corresponding to the entire amino acid sequence of Thymopoietin II is described. Use of the recently developed, base-labile, fluorenylmethyloxycarbonyl-amino acids in combination with *tert*-butyl based side chain protecting groups and p-alkoxybenzyl ester peptide to resin linkage enabled the synthesis to be carried out under much milder reaction conditions than previously.

It is now evident that various factors are involved in the many activities of the thymus and of its cells[1]. Among these factors, Thymopoietin I and II, 2 closely related polypeptide hormones (formerly known as 'Thymin I and II') isolated from bovine thymus in 1974 by Goldstein[2], induce the selective differentiation of thymocytes from precursor

cells. The primary structure of Thymopoietin II (TP II) was proposed to be[3]

H-Ser-Gln-Phe-Leu-Glu-Asp-Pro-Ser-Val-
Leu-Thr-Lys-Glu-Lys-Leu-Lys-Ser-Glu-
Leu-Val-Ala-Asn-Asn-Val-Thr-Leu-Pro-Ala-
Gly-Glu-Gln-Arg-Lys-Asp-Val-Tyr-
Val-Gln-Leu-Tyr-Leu-Gln-Thr-Leu-
Thr-Ala-Val-Lys-Arg-OH.

The chemical synthesis by solution methods of a nonatetracontapeptide corresponding to the entire amino acid sequence of TP II has been described[4], and solid phase synthesis of TP II has also been reported[5]. The strategies of synthesis employed in both of these approaches required, for the final cleavage and deprotecting step, strongly acidic conditions (methanesulfonic acid at 18 °C[4] or anhydrous HF at 0 °C[4,5]), which are known to cause serious decomposition. The damaging effect of these destructive treatments often leads to the formation of undesirable side products which are difficult to eliminate[6], and may interfere with in vitro and in vivo studies on structure-activity relationships.

Recent studies in 2 laboratories[7,8] have led to the application of a base-labile amino protecting group, 9-fluorenylmethyloxycarbonyl (Fmoc), in solid-phase peptide synthesis. The use of N^{α}-Fmoc group, in combination with *tert*-butyl based side chain protecting groups and p-benzyloxybenzyl ester peptide to resin anchorage[9] which are both cleavable by mild acid, provides an alternative strategy for a solid-phase synthesis under very mild reaction conditions. I now describe an improved synthesis of TP II in which treatment with acidic reagents is minimised in order to reduce any potential side reaction during the synthesis.

Material and methods. The solid support was the p-benzyloxybenzyl alcohol resin, prepared as described by Wang[9]. The α-amino protecting group was Fmoc in all but the first (Arg_{49}) and the last step (Ala_1), where 2-biphenylisopropyloxycarbonyl (Bpoc) and *tert*-butyloxycarbonyl (Boc) were used respectively. Fmoc-amino acids were prepared by the procedures of Carpino and Han[10] and of Chang et al.[11]. The following groups were employed for side-chain protection: Arg and Lys, Boc; Asp and Glu, *tert*-butyl ester; Ser, Thr and Tyr, *tert*-butyl ether. Assembly of the TP II sequence was initiated by the coupling of Bpoc-Arg(Boc)-OH to the resin with the aid of N,N'-dicyclohexylcarbodiimide, in the presence of 4-dimethylaminopyridine. After washing, the Bpoc-Arg(Boc)-resin was dried to constant weight (2.157 g) in vacuo at 35 °C. A substitution of 0.154 mmoles/g was estimated by weight gain and by the method of Gisin[12]. The substituted resin (1.30 g, 0.20 mmoles) was transferred to the glass reaction vessel of a manual apparatus and acetylated with a mixture of pyridine and acetic anhydride (1:1 v/v, in the presence of a catalytic amount of 4-dimethylaminopyridine[9]). After filtration and washing with DMF, CH_2Cl_2, $(CH_3)_2CHOH$ and CH_2Cl_2, the protected amino acid resin was treated with 0.5% trifluoroacetic acid in CH_2Cl_2 to remove the Bpoc group, and submitted to the program shown in table 1 for stepwise synthesis from the carboxyl terminus.

Fmoc-glutamine and Fmoc-asparagine were coupled to the resin as p-nitrophenyl esters (2 mmoles, 10 equiv.) in DMF in step 12 and 16. Immediately preformed symmetrycal anhydrides of Fmoc amino acids were used in all other couplings. Completeness of coupling reactions was monitored both by the ninhydrin color test of Kaiser[13] and the fluorometric method[14]. The progress of the synthesis was checked 7 times (after coupling of Thr_{43}, Tyr_{36}, Gly_{29}, Asn_{22}, Leu_{15}, Ser_8 and Ser_1) by total acid hydrolysis (12 M HCl per propionic acid, 1:1 v/v, 2 h, 135 °C)[15] with subsequent amino acid analysis (table 2).

Table 1. Synthetic program for Thymopoietin II

Operation	Reagent or solvent[a]	Mixing time (min)	Applications
1	50% piperidine in DMF[b]	1	1
2	50% piperidine in DMF	10	1
3	DMF	1	3
4	CH_2Cl_2	1	3
5	$(CH_3)_2CHOH$	1	3
6	CH_2Cl_2	1	3
7-9	repeat operation 1-3		
10	DMF	1	3
11	CH_2Cl_2	1	3
12	Fmoc-amino acid anhydride[c] (4-fold excess)	180	1
13	CH_2Cl_2	1	3
14	$(CH_3)_2CHOH$	1	3
15-16	repeat operation 11-12		
17	CH_2Cl_2	1	3
18	DMF	1	3
19	CH_2Cl_2	1	3
20	Coupling monitoring (if necessary, repeat operation 12-13)		
21	DMF	1	3

[a] Wash volumes were 20 ml. [b] Percentages express v/v ratios. [c] Fmoc-asparagine and Fmoc-glutamine were introduced as p-nitrophenyl esters (10-fold excess); mixing time: 360 min.

Table 2. Check of progress of synthesis by amino acid analysis

Amino acid	Sequence spanned by peptide-resin[a] 43-49	36-49	29-49	22-49	15-49	8-49	1-49
Lys	1.07 (1)	1.11 (1)	2.21 (2)	2.05 (2)	2.99 (3)	5.07 (5)	4.94 (5)
Arg	0.98 (1)	0.96 (1)	1.96 (2)	1.95 (2)	1.93 (2)	1.91 (2)	1.96 (2)
Asx	(0)	(0)	1.01 (1)	3.10 (3)	3.12 (3)	3.27 (3)	4.01 (4)
Thr[b]	1.71 (2)	1.88 (2)	1.81 (2)	2.91 (3)	2.93 (3)	3.79 (4)	3.80 (4)
Ser[b]	(0)	(0)	(0)	(0)	0.79 (1)	1.64 (2)	2.51 (3)
Glx	(0)	2.13 (2)	4.14 (4)	4.10 (4)	5.03 (5)	5.98 (6)	7.95 (8)
Pro	(0)	(0)	(0)	1.08 (1)	1.03 (1)	1.04 (1)	2.09 (2)
Gly	(0)	(0)	1.00 (1)	1.01 (1)	1.08 (1)	0.99 (1)	1.13 (1)
Ala	1.12 (1)	1.21 (1)	1.08 (1)	2.15 (2)	3.17 (3)	3.04 (3)	3.05 (3)
Val	1.05 (1)	2.02 (2)	3.07 (3)	4.00 (4)	5.05 (5)	5.99 (6)	6.10 (6)
Leu	1.00 (1)	3.08 (3)	3.11 (3)	3.96 (4)	5.98 (6)	7.03 (7)	7.94 (8)
Tyr	(0)	1.89 (2)	1.87 (2)	1.92 (2)	1.85 (2)	1.94 (2)	1.83 (2)
Phe	(0)	(0)	(0)	(0)	(0)	(0)	1.12 (1)

[a] The theoretical number of residues is indicated between brackets. [b] Uncorrected for loss in hydrolysis.

At the end of the synthesis, after the last operation (number 21), the protected nonatetracontapeptide resin was extensively washed with CH_2Cl_2 and dried to constant weight (1.74 g) in vacuo over P_2O_5 (35 °C). To a suspension of the peptide resin in CH_2Cl_2 (10 ml), TFA (25 ml) in CH_2Cl_2 (15 ml) was added at 25 °C with vigorous stirring to cleave all the protecting groups and the peptide to resin bond. After 50 min at room temperature, the resin particles were removed by filtration, and the solvent evaporated under reduced pressure (bath temperature: 30 °C). The obtained residue was triturated with diethyl ether to give 211 mg of crude product as a white powder (19% overall yield).

Results and discussion. The crude peptide was purified by gel filtration on sephadex G-50 followed by Sephadex LH-20. Further purification was accomplished with CM-cellulose by gradient elution using pH 6.5 ammonium acetate buffer (0.001–0.3 M)[4]. The chromatographic purifications were monitored by absorbance at 205 nm due to the peptide bond. After desalting on Bio Gel P-2 of symmetrical main peak fractions in 1 N acetic acid, lyophilization gave a colorless residue of synthetic peptide, 155 mg (14% overall yield).

Thin-layer chromatography (Silica gel, Merck 60 F-254) in n-butanol-ethyl acetate-acetic acid-water (1:1:1:1) gave a single ninhydrin-positive, Sakaguchi-positive spot, R_f 0.59 (Fujino et al.[4]: 0.60). Paper electrophoresis on Whatman 3MM at pH 1.9 (formic acid-acetic acid buffer), 600 volt, 60 min, gave a single ninhydrin-positive spot, R_{Lys} 0.62 (Fujino et al.[4]: 0.59). $[\alpha]_D^{25}$ was found to be −72.4° (c = 0.3, 5% aqueous AcOH), which is in good agreement with the value (−75°) reported for the nonatetracontapeptide synthesized by conventional solution methods[4]. Serine was found to be the only amino terminal amino acid by the dansyl technique[15,16]. Arginine was shown to be the C-terminal amino acid by carboxypeptidases A and B digestion[17]. The results of amino acid analysis of acid and enzyme hydrolysates of a sample of purified peptide are reported in table 3.

From the reported data of physicochemical analyses applied to the synthetic nonatetracontapeptide corresponding to the sequence proposed for TP II, it appears that the product obtained possesses an acceptable degree of homogeneity which enables it to be used for biological studies.

To conclude, the present results suggest that the base-labile N^{α} temporary protecting group Fmoc may be a useful and favourable alternative to the acid-labile groups widely employed up to now during the solid phase synthesis of medium-sized natural peptides.

Table 3. Amino acid analyses of synthetic Thymopoietin II

Amino acid	Theoretical	Acid hydrolysate[a,c]	Enzyme digest[b,c]
Lys	5	5.11	5.24
Arg	2	1.96	1.92
Asp	2	4.20	2.02
Asn	2		
Thr	4	3.88[d]	13.12
Ser	3	2.69[d]	
Gln	4	8.17	
Glu	4		3.87
Pro	2	2.05	1.89
Gly	1	1.02	1.11
Ala	3	3.12	3.07
Val	6	5.94	6.20
Leu	8	8.15	8.14
Tyr	2	1.89	2.07
Phe	1	1.04	0.95

[a] Hydrolysis was carried out with 6 N HCl for 24 h at 110 °C in sealed evacuated tubes. [b] Digestion with acid protease for 24 h at 37 °C, followed by 24 h digestion with trypsin and chymotrypsin, followed by 48 h digestion with leucine amino-peptidase. [c] Average of 3 determinations. [d] Uncorrected for loss in hydrolysis.

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Localization of sucrase activity in the cocoon of *Bombyx mori*

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Summary. Enzymes capable of hydrolyzing sucrose, trehalose, lactose and cellobiose were detected in the cocoon of *Bombyx mori*. Sucrase activity was found to be highest and was present only in the outermost layer of the cocoon.

The occurrence of enzymes capable of hydrolyzing disaccharides has been reported in different insect species[1]. Most of these enzymes involved in digestion and metabolism of carbohydrates are found in salivary glands, gut and haemolymph[1,2]. The presence of sucrase activity in liquid silk has been shown in *Bombyx mori*[3] and in *Philosamia ricini*[4]. This