Tryptic Condensation Combined with Peptide Segment Synthesis–Condensation Strategy for the Efficient Synthesis of Human Growth Hormone Releasing Factor (1–29) Amide

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Tryptic final condensation strategy combined with peptide segment synthesis–condensation strategy was applied to the efficient synthesis of the active fragment of human growth hormone releasing factor [hGRF(1–29)-NH_2].

A new strategic achievement is required for the efficient chemical synthesis of valuable biologically active peptides in high yield and purity with a few processes. Kaiser has proposed the peptide segment synthesis–condensation approach for the efficient synthesis of long polypeptides.[1] This approach involves short protected segments being synthesized by the stepwise solid-phase-synthesis (SPS) on *p*-nitrobenzophenone oxime resin.[2] The protected oligopeptides (from penta- to deca-peptide in length) are cleaved from the resin and satisfactorily purified by reprecipitation. The time-consuming preparation and purification of protected peptide intermediates can be thus replaced by the speedy SPS on an oxime resin. The intermediates are further condensed for the synthesis of longer polypeptides. However, the condensation between long segments often proceeds slowly, with considerable racemization.

As a variation of Kaiser's strategy, we attempted to employ trypsin for 'the final condensation step'. The protease-catalysed reaction[3] is well accepted as racemization frec. Trypsin has a very narrow specificity, namely it acts only on Arg and Lys, but once they are protected at side chains, trypsin does not recognize them as substrates. Therefore, the intermediate segment ending with Arg or Lys can be condensed with any other peptide segment containing protected Arg and/or Lys by the reversed reaction catalysed by trypsin with no secondary hydrolysis. Very recently, we have discovered a mixed solvent, hexafluoroisopropyl alcohol (HFIP)–dimethylformamide (DMF) (1 : 1, v/v) containing 4% water, as a good medium for the tryptic condensation of a model tetrapeptide[4] and 13-peptide, α-melanocyte stimulating hormone.[5] We further attempted to expand the utility of the tryptic condensation strategy for longer, more complicated and more valuable

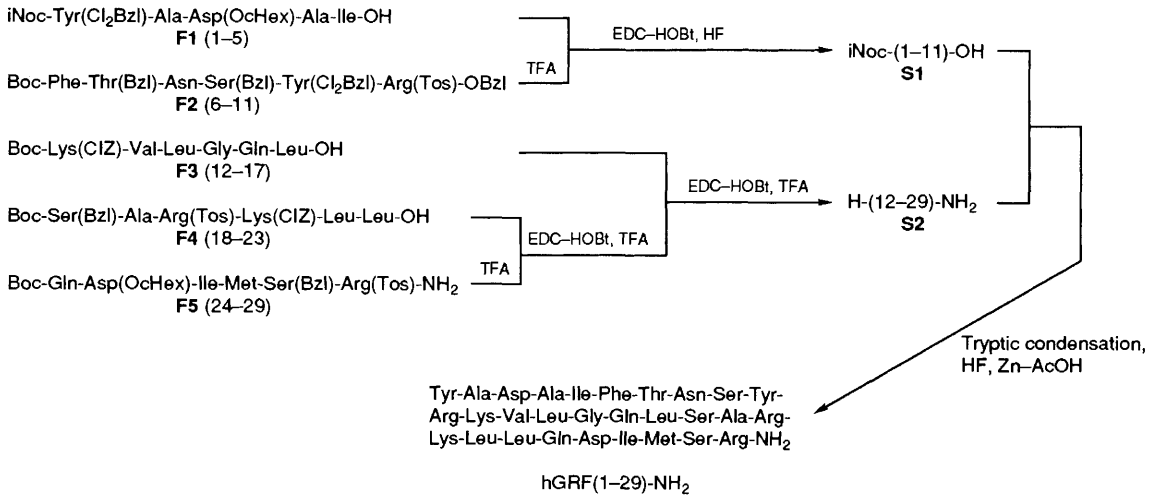

Fig. 1 Synthetic scheme of hGRF(1–29)-NH_2 by the combined strategy of segment synthesis–condensation and tryptic condensation. The protected fragments (**F1–F5**) were synthesized using the oxime resin. *Abbreviations used*: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; cHex, cyclohexyl; Cl_2Bzl, 2,6-dichlorobenzyl; ClZ, 2-chlorobenzyloxycarbonyl; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBt, 1-hydroxybenzotriazole; iNoc, isonicotinyloxycarbonyl; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl.

hormonal peptides such as the human growth hormone releasing factor (1–29) NH_2 (hGRF(1–29)-NH_2).[6] This report describes the synthesis of hGRF(1–29)-NH_2[7] by the combined strategy of segment synthesis–condensation and tryptic condensation (Fig. 1).

For the synthesis of hGRF(1–29)-NH_2, Arg11 was chosen as a stitching point by trypsin with the *C*-terminal segment headed by Lys(ClZ)12. Other Arg and Lys residues were kept protected in the segment to prevent secondary hydrolysis. The sequence of the hormone peptide was divided into five protected peptide fragments (**F1–F5**) which were easily synthesized by the stepwise solid-phase method on the oxime resin and cleaved as being protected at side chains.[8] They were obtained in high yield (81–87%) and purified satisfactorily by reprecipitation [the purity >95% by reversed phase high performance liquid chromatography (RP-HPLC)]. These fragments were appropriately combined in solution by EDC–HOBt method[9] to give two long protected segments. They were subsequently treated with either anhydrous HF or TFA to give **S1** or **S2**. Both segments were conveniently purified by passing through the columns of Sephadex G-25 (1 mol dm^{-3} AcOH) and Sephadex LH-60 (DMF), respectively.

These two components were condensed with the aid of trypsin (TPCK treated, Sigma) in 4% H_2O–HFIP–DMF (18 ml) under the following condition; [**S1**] = 1 mmol dm^{-3} (30 mg), [**S2**] = 5 mmol dm^{-3} (280 mg), pH 7, [trypsin] = 50 μmol dm^{-3}. The progress of the tryptic condensation was monitored by RP-HPLC [Vydac C4 column (0.46 × 25 cm) with a linear gradient of 18–100% propan-2-ol–0.1% TFA for 50 min]. The conversion rate of this step was estimated as 90% by the decrease of **S1** (Fig. 2). It should be noted that any byproduct was not observed during the condensation. Trypsin was specific to free Arg as expected even in the crucial organic condition and effected no secondary hydrolysis.

After incubation for 2 days, the reaction mixture was subjected to gel filtration (Sephadex LH-60, DMF) and the product was treated with anhydrous HF and Zn–AcOH [deprotection of iNoc group[10]], consecutively, to give very pure (80% by RP-HPLC) hGRF(1–29)-NH_2. Further purification was carried out by RP-HPLC [YMC C18 column (1.0 × 25 cm) with a linear gradient of 28–55% acetonitrile–0.1% TFA for 30 min]; yield 20 mg (33%). Total yield from the short protected fragments was 20%. The desired product was identical with the authentic hGRF(1–29)-NH_2 on RP-HPLC. In addition, HPLC peptide mapping of the tryptic hydrolysates of the product confirmed the amino acid sequence by five

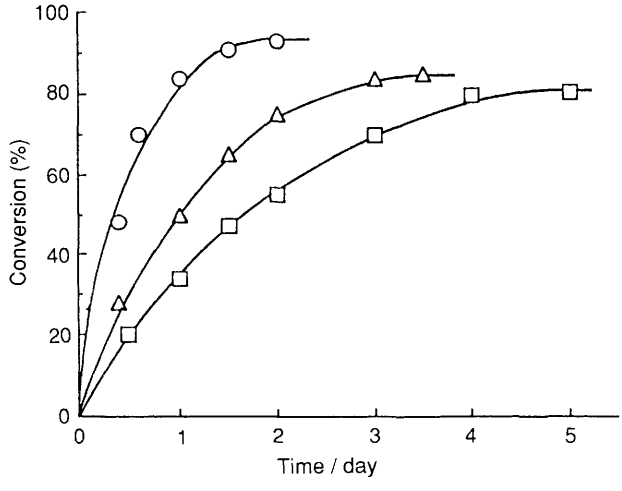

Fig. 2 Progress curves of the tryptic condensation. The ratios of **S2** to **S1** are 5.0 (○), 2.0 (△) and 1.0 (□), respectively. When the ratio decreased from 5.0 to 2.5 and 1.0, the reaction rates slowed but high yields were still obtained. This fact indicates that the aminolysis of the acyl enzyme intermediate would be the rate-determining step responsible for high molecular weight of **S2**.

major digested fragments. Amino acid analysis of the synthetic hGRF(1–29)-NH_2 was also in good agreement with the theoretical values.

In conclusion, the low water content mixed solvent, 4% H_2O–HFIP–DMF, could dissolve the protected 18-peptide segment **S2** and keep trypsin active for the condensation with **S1** to give partially protected 29-peptide amide. Thus, the synthesis of a long hormonal peptide by tryptic final condensation strategy was achieved with the aid of the new solvent system, which is applicable to the poorly soluble protected peptide segments.† As demonstrated above, the combination of the tryptic condensation with the segment synthesis–condensation approach could be a useful strategy for the synthesis of valuable peptide hormones on a large scale.

† Though Klibanov reported the use of *tert*-pentyl alcohol for the protease mediated condensation,[3e] this solvent could neither dissolve the long peptide segments with partial protection nor allow trypsin to mediate the condensation.

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8 The SPS on the oxime resin was carried out manually according to the literatures.[1,2] Each protected fragments (**F1**–**F5**) was purified by reprecipitation with MeOH to give satisfactory purity on RP-HPLC and identified by fast atom bombardment mass spectrometry.

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