Solid-Phase Synthesis of Steroidogenesis-Activator Polypeptide under Continuous Flow Conditions[1)]

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4-(Hydroxymethyl)phenoxymethyl copoly(styrene–1% divinylbenzene) resin beads loosely packed in a glass column were satisfactorily employed as a solid support for synthesis of a triacontapeptide, steroidogenesis-activator polypeptide, under the Fmoc strategy. Peptide sythesis was carried out in a continuous DMF flow without generation of any considerable back pressure. Change of volume of the resin bed in the column was monitored in the course of peptide elongation. After cleavage from the resin, the product was purified on HPLC to give the desired triacontapeptide in an overall yield of 39% based on the C-terminal amino acid linked to the resin.

Solid-phase peptide synthesis[2)] can be performed in continuous flow mode.[3–5)] Many advantages of the continuous flow method of solid-phase peptide synthesis (CF-SPPS) over the traditional batchwise method are known,[3,6,7)] but the former method is still less popular. The limited application of CF-SPPS to peptide chemistry may be partly due to the recognition that soft resin supports commonly used in the batchwise method are troublesome for column operation required in CF-SPPS;[3,7)] the soft supports variably swollen in different solvents are often compressed in the column to disturb normal flow of the solvents and reagents and cause unexpected high back pressure. Indeed a high-pressure liquid delivery system was employed for CF-SPPS using copoly-(styrene–1% divinylbenzene) based supports and considerable back pressure was observed.[6)] Recently Krchňák, et al. showed that soft supports, benzhydryl or 4-methylbenzhydrylamine copoly (styrene-1% divinylbenzene) resin beads, are applicable to low pressure CF-SPPS under the Boc strategy, if sufficient space is left in the column packed with the resin.[8,9)]

In previous paper,[10)] the author showed that low pressure CF-SPPS of an eicosapeptide antibiotic, magainin 1,[11,12)] can be achieved on a soft support, 4-(hydroxymethyl)phenoxymethyl copoly(styrene–1% divinylbenzene) resin (Wang resin),[13)] under the Fmoc-protecting strategy.[14–16)] The satisfactory overall yield (31%) of the antibiotic showed utility of the CF-SPPS, where 2 hours' acylation was adopted using Fmoc-amino acid HOBt ester (3-fold moles per mole of the amine component). The investigation revealed that the soft resin beads swollen in DMF and packed in a glass column with sufficient space settled in the coulmn to form a gel bed, which is mechanically stable enough to allow easy permeation of liquids under low pressure conditions. However, during the arrangement of the eicosapeptide sequence, the volume of gel bed increased to about 1.5 fold of the original one, suggesting that preparation of peptides with longer sequences causes greater expansion of the support; more space may be required to be left in the reactor column for growing peptides and will result unfavourable dilution of the acylating reagents.

In order to obtain further information about the CF-SPPS, especially about the properties of swollen resin bed in the column, was tried preparation of a triacontapeptide, steroidogenesis-activator polypeptide (SAP) (Fig. 1).[17)] The peptide was chosen as the target compound because of its moderate molecular size containing various kinds of amino acid residues and of considerable recent interest in the peptide.

Peptide synthesis was performed according to a route illustrated in Fig. 2. Fmoc group was used for temporary protection of α-amino group and t-butyl ester or ether for side functional groups. A manual synthesizer similar to that designed by Dryland and Sheppard[7)] was used in the preparation. Schedule of peptide elongation (Table 1) was essentially similar to that shown in the literature,[7)] except for the acylating reagents. For removal of the Fmoc group, 20% piperidine in DMF[16)] was used. For acylation, 1 hour's circulation of a DMF–dioxane solution of preformed Fmoc-amino acid HOBt ester (3-fold moles per mole of the amine component) was carried out, the period for the coupling reaction being shortened compared to that adopted in the previous preparation in order to research conditions for rapid peptide synthesis. Single coupling procedure was employed and no monitor was carried out on the progress of the reaction. Throughout the synthesis a constant flow or circulation of the solvent or reagents were easily maintained without generation of any considerable back pressure. Table 2 shows heights of the resin bed in the column at some stages of the peptide elongation. The resin carrying a

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Ile-Val-Gln-Pro-Ile-Ile-Ser-Lys-Leu-Tyr-Gly-Ser-Gly-Gly-Pro-
 1                                  10
Pro-Pro-Thr-Gly-Glu-Glu-Asp-Thr-Asp-Glu-Lys-Lys-Asp-Glu-Leu
                  20                                  30
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Fig. 1. Amino acid sequence of steroidogenesis-activator polypeptide.

Table 1. Schedule of Coupling Cycle

Operation	Reagent	Mode of liquid delivery[a]	Time/min
Coupling	Fmoc-amino acid HOBt ester in DMF-dioxane[b]	Circulation	60
Washing	DMF	Flow[c]	10
Pause[d,e]	DMF	Stopped	10
Deblocking	20% piperidine in DMF	Flow	10
Washing	DMF	Flow	20

a) A flow rate of 2 ml min^{-1} was maintained by a pump. b) Added immediately after removal of dicyclohexylurea by filtration. c) The injector and the circulating line of the synthesizer were also rinsed with DMF. d) In a separate vessel, 1 M DCC in dioxane was added into a DMF solution of Fmoc-amino acid and HOBt to prepare the active ester used in the next coupling cycle. e) At the end of the pause, the height of resin bed was observed (Fig. 3).

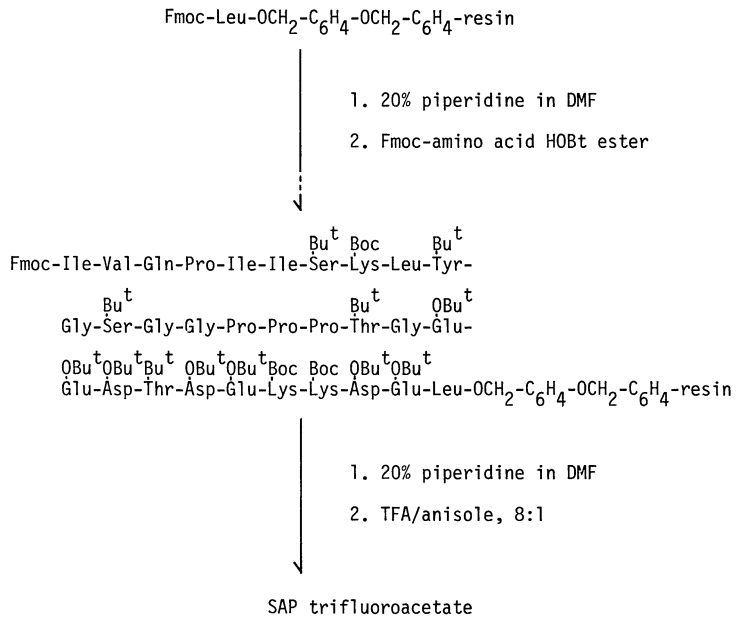

Fig. 2. Route of the synthesis of steroidogenesis-activator polypeptide.

certain peptide sequence swelled in a similar extent in DMF (washing), 20% piperidine in DMF (deblocking), and in a DMF–dioxane solution of Fmoc-amino acid HOBt ester (coupling). Presence or absence of the Fmoc group at the N-terminal of the sequence little affected the swelling volume. This stability of the bed volume must be quite favourable to maintenance of liquid delivery under low pressure conditions during the repeated operations.

It is noteworthy that the volumes of the peptide-resin changed not linearly in the course of peptide elongation (Fig. 3). The maximum volume (1.75 fold of the original volume) was observed when the resin contained the nonapeptide sequence, while the resin carrying the tridecapeptide sequence showed a less expanded volume (1.42 fold). The volume of resin containing the triacontapeptide sequence (1.67 fold) was still less than that of the support linked to the nonapeptide. The resin containing the eicosapeptide

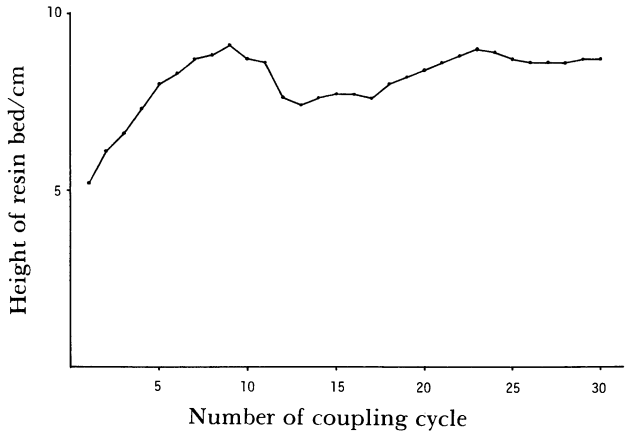

Fig. 3. Heights of Fmoc-peptide-resin bed settled in the reactor column. The height of Fmoc-Leu resin (0.372 mmol Leu/g, 0.600 g) packed in a 0.8×10 cm column is shown at coupling cycle No. 1. The heights were measured in DMF after 10 mins' pause of flow from the end of washing procedure.

Table 2. Heights of Resin Bed in the Reactor Column under Operation

Number of coupling cycle[a]	Height[b] (cm)/Operation			
	Coupling	Washing	Deblocking[c]	Washing[c]
9	8.2	8.3	8.3	8.0
14	7.4	7.5	7.7	7.6
18	7.9	8.0	7.7	8.0
21	8.7	8.7	8.8	8.8
27	8.6	8.7	—[d]	8.6

a) Coupling cycle No. 1 began at washing of Fmoc-Leu-resin (0.372 mmol Leu/g, 0.600 g) packed in a 0.8×10 cm column and ended at washing procedure after deblocking. b) Measured at the end of every operation without stopping liquid delivery. c) Fmoc group on the peptide-resin has been removed. d) Not observed.

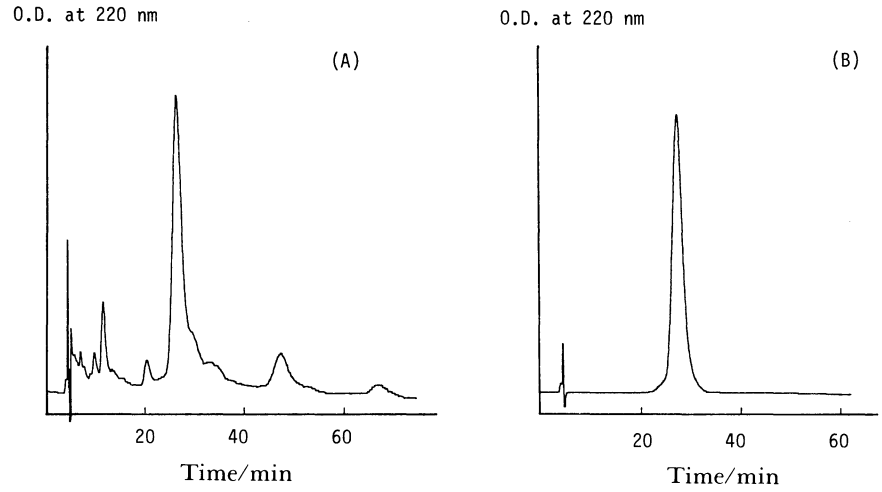

Fig. 4. HPLC profiles of synthetic Steroidogenesis-Activator Polypeptide. Crude peptide cleaved from the resin (A) and purified peptide (B). TSK-gel ODS 80TM column (0.46×15 cm); *i*-PrOH/0.1% TFA in water, 1:4; 0.5 ml min^{-1}.

sequence of SAP showed a more expanded volume (1.62 fold) than that containing the eicosapeptide sequence of magainin 1 (1.52 fold), but direct comparison of the volumes was impossible because of different loading of the C-terminal amino acids on the resin. This finding on the change of resin volume seems to contradict the fact that the swollen volume of copoly (styrene–1% divinylbenzene) resin beads increases in the course of peptide synthesis.[18] It is not likely that loss of peptides from the resin during synthesis caused the decrease of volume of resin observed, because the protected triacontapeptide-resin was obtained in a 103% yield based on the theoretical weight. A possible explanation for this finding is that the volume of peptide-resin reflects some conformational features of the growing peptides on the support in DMF. It seems difficult to forecast the volume of swollen support containing a certain peptide sequence without consideration of the folding of peptides on the support.

After completion of the arrangement of amino acids, the peptide was cleaved from the resin in TFA–anisole. The crude peptide (Fig. 4-A) was purified on a reversed-phase HPLC column to give the desired triacontapeptide in an overall yield of 39% based on the C-terminal leucine linked to the support. Homogeneity of the synthetic peptide was confirmed by means of analytical HPLC (Fig. 4-B), TLC, fast atom bombardment (FAB) mass spectroscopy, and amino acid analyses of the acid hydrolysates. The data showed an average yield of 96.9% per step was obtained in the CF-SPPS employing the 1 hour's single coupling procedure. The reason for the higher yield of SAP is not clear since SAP is more abundant in sterically hindered amide bonds than magainin 1, the latter compound being obtained with an average yield of 94.3% per step.

Present study showed that rapid and efficient CF-SPPS of a peptide with a considerably high molecular weight is possible on a copoly(styrene–1% divinylbenzene) based support employing the Fmoc strategy under low pressure conditions. Although further work is necessary to understand fully the properties of swollen peptide-resin, CF-SPPS using soft resin

supports commonly used in the batchwise method, which have superior loading capacities of amino acids and are less expensive compared to the rigid supports specially designed for CF-SPPS,[4,7] is a convenient technique in peptide synthesis under the Fmoc strategy.

Experimental

All reactions were carried out at room temperature if not mentioned otherwise. DMF (GR grade, Wako Junyaku Co.) was stored over Merck Molecular Sieve 4 A and was used without further purification. 4-(Hydroxymethyl)phenoxymethyl copoly(styrene–1% divinylbenzene) (0.67 mequiv OH/g) was purchased from Kokusan Kagaku Co. The manual peptide synthesizer consisted of a FMI PR-SY-ICSC ceramic pump, a Rheodyne 3 way, a 4 way, and a 6 position Teflon rotary valves, a Chemco Low Prep glass column (0.8×10 cm) with Teflon filter discs, and a glass injector barrel (2 ml), which were connected with Teflon tubes (0.1 cm in diameter) as shown in the literature.[7] The total volume within the circulating loop except for the column reactor was about 1 ml. Back-pressure was monitored using a FMI CD-100 LF pressure meter. For analytical HPLC, a TSK-gel ODS 80TM column (0.46×15 cm) was used with a solvent system of 2-propanol/0.1% TFA in water (1:4) at a flow rate of 0.5 ml min^{-1}. For preparative HPLC, a TSK-gel ODS 80TM column (0.8×50 cm) was employed with a stepwise gradient of 2-propanol/0.1% TFA in water (1:4.5 to 1:1.7) at a flow rate of 1.83 ml min^{-1}. Amino acid analyses were carried out on a JEOL 6AS automatic amino acid analyzer. Optical rotation was observed using a JASCO DIP-370 digital polarimeter. A JEOL JMS-SX 102 mass spectrometer was used for FAB mass spectroscopy.

Preparation of Fmoc-Leucyl Resin. To a suspension of 4-(hydroxymethyl)phenoxymethyl copoly(styrene–1% divinylbenzene) (1.012 g), Fmoc–Leu–OH (0.719 g), and 4-dimethylaminopyridine[19] (0.125 g) in DMF (10 ml), was added 1 M DCC (1 M=1 mol dm^{-3}) in dioxane (2.03 ml), and the mixture was stirred for 4.5 h. After washed with DMF and CH_2Cl_2, the dried resin was suspended in ice-cooled CH_2Cl_2 (12 ml) and pyridine (0.4 ml). Benzoyl chloride[13] (0.44 ml) was added to the suspension and the mixture was stirred for 15 min at ice-bath temperature. The resin was washed with CH_2Cl_2 and MeOH, and vacuum-dried over $CaCl_2$. The resin (25.33 mg) was treated with 20% piperidine in DMF for 20 min, washed with DMF, and suspended in TFA for 0.5 h. After filtration, the resin was washed with AcOH. The filtrate and the washing solution was combined and concentrated to give a residue, which was subjected to amino acid analysis to show 0.372 mmol leucine/g of the resin.

Preparation of Fmoc-Amino Acid HOBt Esters. To a solution of Fmoc-amino acid (0.67 mmol) and HOBt (0.091 g) in DMF (generally 1.5 ml. In case of Fmoc–Gln–OH, 2.5 ml), was added 1 M DCC in dioxane (0.67 ml) and the mixture was stirred for 40 min. The mixture was filtered and the precipitates were washed with DMF (0.6 ml). The combined filtrate was translated to the injector barrel of the synthesizer and pumped into the column. The vessels and the injector were rinsed with additional DMF (0.5 ml), which was also added to the column reactor.

Synthesis of SAP. The Fmoc-leucyl resin (0.600 g, containing 0.223 mmol Leu) was placed in the column reactor and swollen in DMF. The resin was subjected to the procedures for stepwise peptide elongation. After the final acylation, the pressure meter was equipped between the pump and the column. DMF was pumped into the column at a rate of 2 ml min^{-1} to find the back pressure less than 0.3 kg cm^{-2} in flowing or circulating mode.

The peptide-resin was translated on a filter, washed with DMF and MeOH, and vacuum-dried over P_2O_5 and NaOH. 1.624 g. A part of the peptide-resin (0.200 g) was suspended in 20% piperidine in DMF (20 ml) and the mixture was stirred for 20 min. After filtration, the peptide-resin was washed with DMF and MeOH, and vacuum-dried over $CaCl_2$ and NaOH. 0.189 g. The peptide-resin was suspended in TFA (8 ml) and anisole (1 ml), and the mixture was stirred for 1 h. The resin collected on a filter was washed with AcOH and the combined filtrate was concentrated to give an oil, which was solidified with trituration in ether. The solid collected with decantation was washed with additional ether, and vacuum-dried over $CaCl_2$ and NaOH. 0.122 g. The crude product (0.121 g) was purified on the preparative HPLC column (ten repeated runs). The fractions containing the desired peptide were combined and evaporated to give an oil, which was solidified by addition of ether. The solid collected by decantation was washed with additional ether and vacuum-dried over P_2O_5 and NaOH. Colorless solid, 42.82 mg. 39% based on the C-terminal Leu linked to the resin. Found: C, 46.71; H, 6.21; N, 11.40%. Calcd for $C_{141}H_{226}O_{51}N_{34}\cdot 7CF_3COOH$: C, 46.41; H, 5.81; N, 11.87%. Retention time on the analitical HPLC: 28 min. R_f 0.35 (Avicel-cellulose, *n*-BuOH/pyridine/AcOH/H_2O, 16:10:3:12, ninhydrin detection). *m/z*: 3213.38 $(M+H)^+$, 3236.28 $(M+Na)^+$. Amino acid ratio in acid hydrolysate (6 M HCl, 110 °C, 16 h) (Theoretical value in parentheses): Asp 3.06 (3), Thr 1.94 (2), Ser 1.95 (2), Glu 4.99 (5), Pro 3.80 (4), Gly 4.09 (4), Val 0.48 (1), Ile 1.73 (3), Leu 2.00 (2), Tyr 0.88 (1), Lys 3.17 (3). (6 M HCl, 110 °C, 48 h): Asp 3.01 (3), Thr 1.82 (2), Ser 1.72 (2), Glu 5.02 (5), Pro 3.84 (4), Gly 4.08 (4), Val 0.78 (1), Ile 2.49 (3), Leu 2.10 (2), Tyr 0.87 (1), Lys 3.04 (3). $[\alpha]_D^{23}$ −87.3° (*c* 0.3, 10% AcOH in water).

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1) Amino acids used are of L-configuration except glycine. Following abbreviations were used: Fmoc=9-fluorenylmethoxycarbonyl, Boc=*t*-butoxycarbonyl, Bu^t=*t*-butyl ether, OBu^t=*t*-butyl ester, DCC=dicyclohexylcarbodiimide, HOBt=1-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=*N*,*N*-dimethylformamide.

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