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Large-scale Production of Peptides Using the Solid Phase Continuous Flow Method. Part 2[1]: Preparative Synthesis of a 26-Mer Peptide Thrombin Inhibitor

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Abstract: A preparative method for the preparation of large peptides is described. An advantageous *theoretical weight of peptide/weight of starting resin* ratio (tP_w/R_w) of about 0.3 was successfully experimented. The esterification of the first amino acid was realized with a racemization of less than 1%. The study of the coupling conditions led to the use of a diluted acylating mixture that allowed a 56% consumption of the amino acid derivatives (percentage use of amino acids) introduced in the synthesis. The cost analysis of the synthesis showed that the recovery of the amino acid derivatives was not worthwhile. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

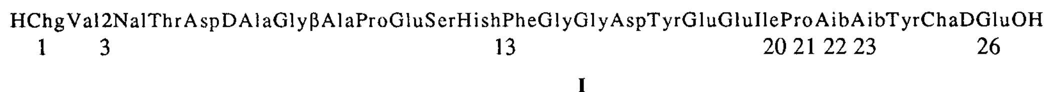
Keywords: large-scale peptide synthesis; continuous flow; macrosorb; hindered sequence; thrombin inhibitor

INTRODUCTION

Small peptides like the 163–171 fragment of Interleukin-1β [1], the Leu[5]-enkephalin (pentapeptide), the human angiotensin I (decapeptide) [2], and the double-cyclized MEN 10627 (hexapeptide) [3] have been successfully produced in preparative amounts by solid phase continuous flow methodology.

The synthesis of MEN 10627 allowed us to widely experiment with our large scale synthesizer and to establish the correct scaling-up methodology for the bulk production of peptides realized in a standard organic chemistry laboratory using low economic resources.

The potentiality of the described large scale continuous flow method based on Macrosorb resin and Fmoc chemistry for the preparation of peptides with a higher number of amino acid residues has to be established. The bifunctional thrombin inhibitor 26-mer peptide **I** [4], which is characterised by a high molecular weight and a peculiar chemical structure, represents a suitable test for this synthetic approach.



I

Abbreviations: DIPC, diisopropylcarbodiimide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, hydroxyazobenzotriazole; HOBt, hydroxybenzotriazole; PyBOP, benzotriazole-1-oxy-tris-phosphonium hexafluorophosphate; PyBroP, bromo-tris-pyrrolidino-phosphonium hexafluourophosphate; Ac_2O, acetic anhydride; Et_2O, diethyl ether; TIPS, triisopropylsilane; Cha, cyclohexylalanine; Chg, cyclohexylglycine; hPhe, homophenylalanine; 2Nal, 2-naphthylalanine.

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[1] cf. *J. Peptide Sci.* **3,** 224–230 (1997) for Part 1.

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In fact the large size of the peptide **I** can generate important increases in the weight and volume of the resin bed during the synthesis, leading to problematical percolation of the packed columns during the process.

I also appeared suitable to evaluate the chemical efficiency of the preparative method since its structure contains hindered aminoacids linked to one an other to form the strongly hindered sequence -Ile[20]ProAibAib[23]-.

Furthermore, this synthesis could potentially produce a process without economic viability using several very expensive non natural amino acids (DGlu, Cha, hPhe, 2-Nal).

MATERIALS AND METHODS

Peptide/Resin Ratio

In the case of a large peptide like **I** (M.W. = 2957 as medium average mass) an esterification of the first amino acid on the resin of 0.1 mmoles/g does not represent a poor volumetric characteristic of the solid support such as in the case of small peptides like MEN 10627 [3]. In fact, with this resin loading, a *theoretical weight of pepide/weight of starting resin* ratio (tP_w/R_w) of 0.295 could be obtained at the end of the synthesis of **I** against a value of 0.146 calculated for MEN 10627 (M.W. = 760) synthesized with the described 0.192 mmoles/g of first amino acid functionalization [3]. This 0.295 (tP_w/R_w) corresponds to 36.5% of resin weight increase during the synthesis, calculating also for the protective groups. From several experiments, the weight increase of the resin bed during the synthesis of **I** roughly corresponds, in DMF, to a volume increase of the same order of magnitude. A similar volume resin increase would be difficult to contain in a glass packed column because of radial expansion leading to a hindered percolation of liquids and, even, to the breakage of the column. However, we found that this volume increase can produce only longitudinal expansion. In fact, using short resin package of about 20 cm in height, through a reverse-flow washing applied at the end of each coupling cycle, a complete breakage of the packed resin bed is obtained. A new resin package, incorporating the volume expansion, is successively restored by normal-flow. In longer resin package of about 40 cm, which we previously employed [1,3], the breakage of the packed resin bed was more difficult. For this reason we chose 23 cm long Büchi columns packed, at the start of the synthesis, with a resin bed of 15–16 cm. A column of 3.6 cm of diameter carried 50 g of resin, whereas a column of 4.9 cm carried 100 g of resin. The dead volume of these packed columns was exactly regulated with suitable pistons that were adjusted after each coupling cycle during the synthesis. These packed columns were applied at our already described synthesiser [3] and used as usual.

The Synthetic Strategy

The synthetic strategy was based on the Fmoc/tBu-Boc chemistry using the Trt group for Histidine and *p*-hydroxybenzylphenoxyacetic acid as a linker between the resin and the peptide. The last amino acid (Chg) was protected with the Boc group.

Racemization of the First Amino Acid

To avoid the use of the commercially rare and very expensive Fmoc-D-GluOtBu, the esterification of the first amino acid was performed with Fmoc-D-Glu(-OtBu)OH. This reaction constitutes a crucial step in the pharmaceutical preparation of **I** since it must be performed preserving the chiral purity of the amino acid residue esterified on the hydroxybenzyl function. In fact in the case of large peptides the portion of racemization of the first amino acid residue produces a crude peptide containing also its epimeric analog. This epimeric mixture, due to the obvious small chromatographic differences between the components, can be difficult, or even impossible, to separate. For these reasons we considered unacceptable, in the preparation of **I**, a racemization of greater than 1%.

To ensure reproducibility of the esterification step, which was not attained with the flow method in the MEN 10627 synthesis [3] we used the batch esterification. In a preliminary experiment we esterified the Fmoc-D-Glu- residue (symmetrical anhydride 5/DMAP 1) on Macrosorb 125 (functionalization of 0.125 mmoles/g) at a concentration (C_e) of 0.070 M for 2 h, obtaining a 0.115 Fmoc-derivatization. Then we assembled the pentapeptide H-AibAibTyrChaDGlu-OH (**II**). This compound was cleaved from the resin with TFA 9/water 1 ($Y = 90\%$) and analyzed by HPLC (see Figure 1).

On the same analytical column was performed a semi-preparative purification of the peaks at 13.71 and 14.67 min (inset A and B of Figure 1), and the two isolated compounds were analyzed by FAB-MS (Matrix = glycerol 2/thioglycerol 1; keV = 10). We obtained for the two compounds the same quasi

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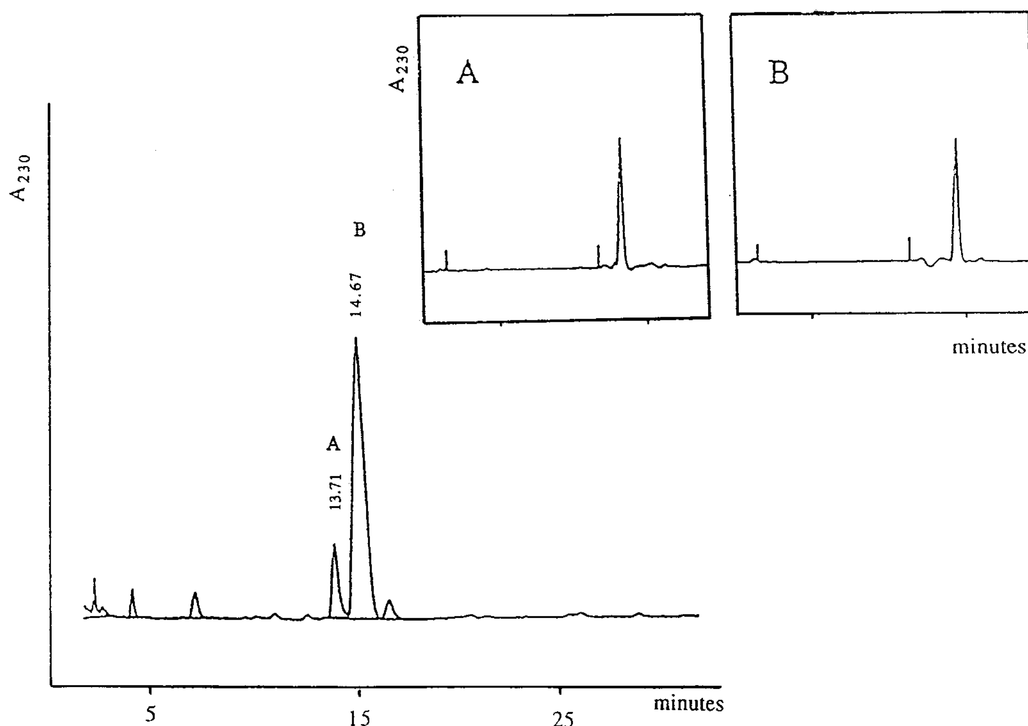

Figure 1 Analytical HPLC of the crude H-AibAib TyrCha DGlu-OH (**II**). Eluents = H_2O + 0.1% TFA; CH_3CN + 0.1% TFA. Isocratic conditions (10% of CH_3CN + 0.1% TFA) for 3 min, followed by a gradient from 10 to 60% of CH_3CN + 0.1%TFA for 60 min. Flow = 1 ml/min. Absorbance = 230 nm. A and B = purified fractions of semi-preparative chromatography under the same conditions.

molecular ion $(M+H)^+ = m/z$ 634 and the respective sodium cluster $(M+Na)^+ = m/z$ 656. These results demonstrated that the two peaks of Figure 1 were the epimeric analogs of the peptide **II**. The coelution with an authentic sample of H-AibAibTyr-ChaGlu-OH confirmed that the peak at 13.71 min in Figure 1 was the LGlu-epimer resulting from partial racemization (8.5%) during the esterification reaction.

The symmetrical anhydride and the acyl fluoride of Fmoc-D-Glu- esterified on the resin with 0.1 equivalents of DMAP (C_e = 0.05–0.150; DMF/Resin = 3/1) showed that the racemization was less than 1% if the reaction time was not superior to 20 min. The acyl fluoride derivative proved more efficient than the symmetrical anhydride both in minimising the racemization (0.3–0.7%) and in reaching, in the same time and at the same concentration, a higher level of esterification. Thus, esterification with Fmoc-D-GluF (C_e = 0.066 M; DMF/Resin = 3/1) on Macrosorb 125 for 20 min gave an Fmoc derivatization of 0.080 mmoles/g and a racemization of 0.35%.

The loading of 0.080 mmoles/g, though assuring a (tP_w/R_w) superior to that obtained in the synthesis of MEN 10627 with a first amino acid derivatization of 0.192 mmoles/g, resulted in a value that could be reasonably improved. The use of Macrosorb 250 functionalized with 0.250 mmoles/g of *p*-hydroxymethylphenoxyacetic acid allowed us to obtain, with a 0.066 M solution of Fmoc-D-GluF reacting for 20 min (DMF/Resin = 3/1), a Fmoc loading of 0.110 mmoles/g and a racemization of 0.35%. This value can be obtained, in the same time, with Macrosorb 125 only using a 20% more concentrated solution of the expensive (Fmoc-D-Glu-) acylating mixture. Of course a careful capping of the free OH groups with Ac_2O/DMAP was necessary after the esterification step.

Coupling Reaction Conditions

To obtain economically advantageous synthetic conditions, we tried to perform the amidation reactions during the stepwise synthesis in dilute conditions of the acyl component. This goal was most important for some, very expensive, non-natural

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amino acids present in the peptide (2Nal, hPhe, Cha).

Moreover it was necessary to study the coupling conditions of the bulky hindered sequence -Ile[20]-Aib[23]-. Therefore we preliminarly studied in small-scale (0.5 g of resin) and in batch conditions (to speed up a large number of experiments) the sequence -Glu[18]-Cha[25]-. Using the HOBt/DIPC acylation method in DMF, we found that a Fmoc-amino acid concentration after the amidation reaction (C_a) [3] of 0.030 M (21% less concentrated than the value used for MEN 10627 with a first amino acid functionalization of 0.092 mmoles/g) allowed complete acylation in 3 h for the residue Tyr[24] and Cha[25], but proved ineffective for the sequence -Ile[20]Pro[21]Aib[22]Aib[23]-.

This hindered sequence was carefully studied experimenting with many acylations methods in batch, analyzing the growing peptide by HPLC.

Aib[23] was coupled with PyBOP in 1 h ($C_a = 0.046$ M) and in 3 h ($C_a = 0032$ M) obtaining in both cases a 99% yield. The HOBt/DIPC method proved less efficient giving 85% yield in DMF and a 95% yield in DMA in 1 h at $C_a = 0.046$.

Table 1 shows the results for the coupling of Aib[22].

The Pro[21] coupling was performed with PyBOP, HATU and PyBrop at $C_a = 0.032$ in DMF obtaining a 97% yield in 3 h. The HOBt/DIPC method in DMF needed a higher concentration (0.138 M) to afford the same result. The fluoride derivative of Pro ($C_a = 0.037$ M) or in 3 h ($C_a = 0.032$ M) with PyBOP ($Y = 100\%$) in DMF afforded only a 70% yield in 1 h.

The fluorides derivatives, coupled in the presence of a tertiary amine in DMF, gave small quantities of double acylation.

The two Glu residues were acylated in the same conditions of Tyr[24]and Cha[25].

In the end the most suitable results for continuous flow, small scale pilot synthesis were found to be HOBt/DIPC for Glu[18,19], Tyr[24] and Cha[25] at $C_a = 0.030$ M, PyBOP for Aib[23], Pro[21] and Ile[20] at $C_a = 0.032$ M, and fluoride for Aib[22] at $C_a = 0.032$ M always using DMF as solvent.

Table 1 FmocAibX Coupling on 0.5g of H-Aib-TyrChaDGlu-® in 2 ml of DMF

Activation	C_a	Time (h)	Coupl. Y%
HOAt	0.046	3	21
PyBroP	0.046	3	10
PyBOP	0.046	3	48
HATU	0.032 or 0.046	3	57 or 96
Fluoride	0.032	3 or 16	98 or 99.5

Small Scale Synthesis

Two 1 × 10 cm Omnifit columns were each loaded with 1 g of *p*-hydroxymethylphenoxyacetyl-Macrosorb SPR 250 (0.0250 mmol/g), previously esterified in batch with 0.110 mmol/g of Fmoc-D-Glu-. The two columns were connected in series and the dead volume was adjusted with a piston to obtain a '*weight of resin/dead volume*' ratio (R_w/V_d) = 1/12.

The best amidation conditions selected in batch were applied to the small scale continuous flow synthesis (PyBOP at $C_a = 0.032$ M for Ile[20], Pro[21] and Aib[23], fluoride at $C_a = 0.032$ M for Aib[22] and HOBt/DIPC at $C_a = 0.030$ for all other amino acids). A linear velocity (L_V) of 4.2 cm/min was used throughout.

The second column of the series was opened at every acylation step to take a resin sample for the Kaiser test, which, after 3 h, resulted negative for all the detectable amino acid. The coupling of Aib, Pro and Ile residues were monitored by HPLC and gave, after 3 h, the same coupling yield as previously described.

Because of the partial acylation (97–99%) obtained with the sequence -Ile[20]-Aib[23], each amidation reaction was followed by an Ac_2O capping procedure to avoid the presence of deleted peptides in the final crude peptide mixture.

At the end of the assembling procedure, the resin was washed with MeOH and Et_2O and dried.

The resin from the first column (1.3 g) was then mixed with 5 ml of TFA98/TIPS2 for 3 h, the resin filtered off and the resulting solution evaporated to dryness. The resulting crude product was dissolved, under stirring, in water, adding drops of 0.05 M NH_4OH up to complete solution. The HPLC analysis showed the presence of 0.2367 g of **I**. Yield = 72.8% calculated on the first amino acid esterified on the resin.

RESULTS

Large Scale Synthesis

Large scale preparations were performed using the optimized parameters obtained in the small scale synthesis.

A standard experiment was realized with 325 g of resin mixed for 20 min in a 2 l pear-shaped flask

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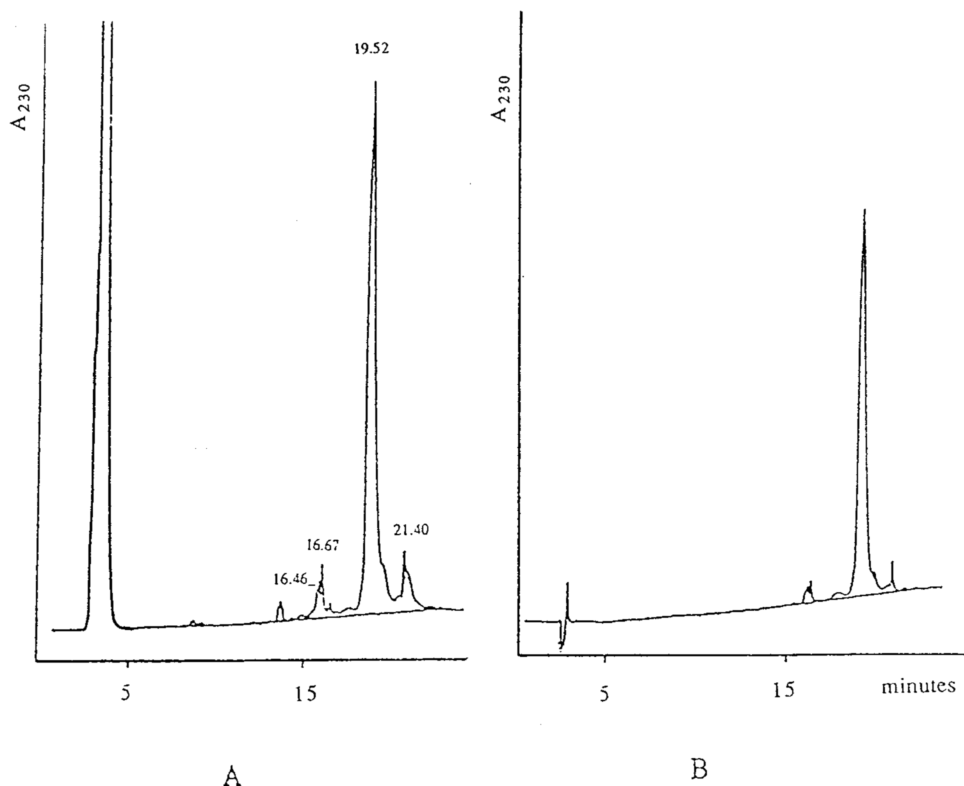

Figure 2 Eluents = H_2O + 0.1% TFA; CH_3CN + 0.1% TFA. Gradient = from 20 to 50% of CH_3CN + 0.1% TFA in 20′. Column = Superspher RP18 5μ. T = 45°C. Absorbance = 230 nm. A = Crude mixture after TFA treatment of the resin. B = Crude material after solution in NH_4OH 0.05 M and precipitation at the isoelectric pH.

with DMF (975 ml), Fmoc-D-GluF (C_e = 0.066 M) and DMAP (0.1 equivalents). A loading of 0.112 mmoles/g was obtained (racemization = 0.4%). This result shows that the batch esterification can be successfully scaled-up.

The resin was packed into three 4.9 × 23 cm Büchi columns (one of these carrying the described [3] teflon extension with an opening to remove samples of resin), the columns were connected in series onto the preparative synthesizer (the modified column being the last), and the dead volume of the apparatus adjusted to 940 ml with a suitable piston on the head of the columns.

The amidation reactions of the non-hindered amino acids were performed using C_a = 0.030 M. The hindered sequence was coupled at the established C_a of 0.032 M, following each amidation reaction with the described Ac_2O capping procedure. These acylations gave a percentage use of the amino acids employed in the synthesis of 54.7% for the hindered amino acids and of 56.3% for all other amino acids.

In this large scale experiment, in contrast to the previously presented preliminary results [5], we used a constant linear velocity (L_v) between small and large scale. At constant L_v we obtained identical chemical results to those presented in a preliminary communication [5],but the longitudinal expansion of the resin bed during the counter-current washing at the end of the acylation cycle (as described above) was more efficient.

After assembling **I** we washed the resin with MeOH and Et_2O obtaining 417.5 g of resin. The final peptide was cleaved from the resin in 3.3 l of TFA/TIPS 98/2 for 3 h. After TFA evaporation the crude product was suspended in 4 l of water and dissolved by adding 0.05 M NH_4OH under stirring until only a fine powder remained. Figure 2A shows the HPLC quality of this solution. The peak at 19.52 min, corresponding to the desired peptide, represented 80.5% of the integrated area. The total amount of **I** was calculated to be 77.49 g (Y = 72%).

The filtered solution of the crude reaction mixture was precipitated at its isoelectric pH obtaining 86.6

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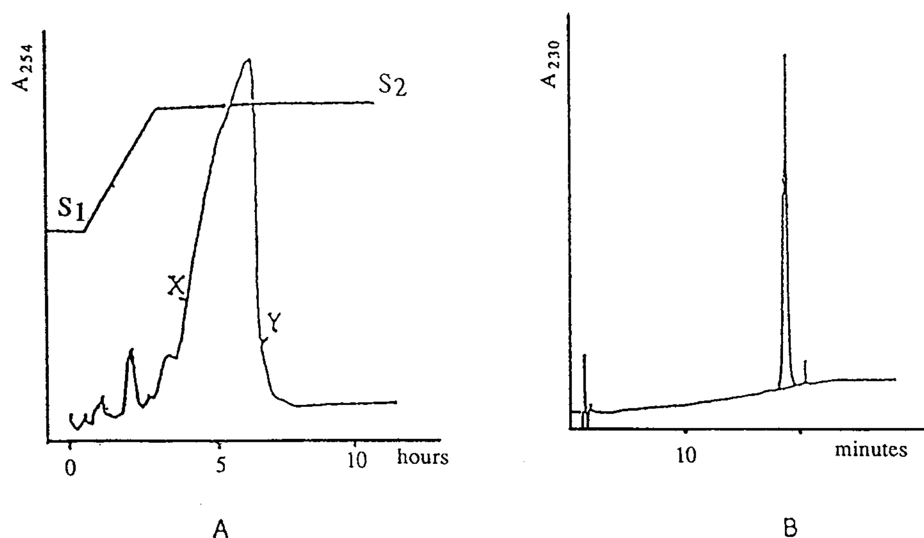

Figure 3 (A) Ion exchange chromatography of the crude material as in Figure 2B; Column = 4.9 × 23 cm packed with DEAE Sephadex A25; S_1 = NH4Cl 0.2 M, S_2 = NH4Cl 0.4 M; Flow = 20 ml/min. λ = 254 nm. (B) HPLC of fractions *X*–*Y*; Main peak = 97%; same conditions as Figure 2.

g of a white powder where the peptide **I** represented 87% of the amount (*Y* = 70%) (see Figure 2B).

The impurities at 16.46, 16.67 and 21.40 min in Figure 2A were identified as follows:

Impurity 16.46 min = Hirunorm V 1-22 [ES/MS = $(M + H)^+$ at *m*/*z* 2426];

Impurity 16.67 min = produced by crude mixture in TFA [ES/MS = (M + H) + at *m*/*z* 2510];

Impurity 21.40 min = Hirunorm V + 1 Aib residue [ES/MS = $(M + H)^+$ at *m*/*z* 3042];

Purification

The above described crude peptide mixture was purified by ion exchange (i.e.) chromatography. Figure 3 shows a preparative purification performed on 9 g of crude product, collecting fractions between X and Y in Figure 3A and obtaining, after precipitation at the isoelectric point, 6.5 g of a 97% pure peptide (*Y* = 58.1% calculated on the first amino acid esterified on the resin).

To reach a product of pharmaceutical quality, a final RP-HPLC purification of the 97% pure peptide was necessary. This purification was performed under isocratic conditions (83% of NH_4OAc 0.005 M/ 17% of CH_3CN) on a Lichcrospher RP-18, (12 μ) self-packed Merck column (5 × 18 cm). The peptide sample (0.4 g) was dissolved in the aqueous component of the chromatographic run by adding drops of 0.1 M NH_4OH (the final pH was never superior to 7.4). The purification afforded 0.316 g of a 99.9% pure peptide (*Y* = 45.8% calculated on the first amino acid esterified on the resin).

The ES/MS spectrum of the purified product (obtained with a VG Quattro Triple Quadrupole Fisons spectrometer equipped with an electrospray source) showed a quasi molecular ion $(M + H)^+$ at *m*/*z*

Table 2 Technical Data and Results of the Small and Large Scale Synthesis of Hirunorm V

	Small scale	Large scale
Resin	Macrosorb SPR 250	Macrosorb SPR 250
Resin weight	1 g	325 g
Flow	3 ml/min	78 ml/min
L_v	4.2 cm/min	4.2 cm/min
Fmoc-D-Glu-functionalization	0.110 mmoles/g	0.112 mmoles/g
C_e (conc. of esterification)	0.066 M	0.066 M
C_a (end acylation conc.)	0.030	0.030
% use of AA		56%
Amidation time	3 h	3 h
Yield of **I** in deblock. Mixture	72.8%	72%
Grams and yield after i.e. chr.		62.5 g *Y* = 58.1%
Grams and yield after HPLC		49.3 g *Y* = 45.8%

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Table 3 Percentage analysis of the cost for the synthesis of Hirunorm V

Materials	Cost (%)
Resin	16.50
Linker	2.69
Fmoc AA OH [1,3,13,26]	12.32
Fmoc AA OH (all others)	16.38
Solvents and reagents	23.76
TFA and TIPS	22.79
Purification	5.56

2958, the sodium cluster $(M + Na)^{+}$ at m/z 2980 and the double charge ion $(M + 2H)^{2+}$ 1479.

The biological activity was as previously described [4].

DISCUSSION

The large scale preparation of **I** demonstrates the complete small-large scale reproducibility of the method both for chemical and technical parameters (see Table 2).

Due to the esterification in batch, in the synthesis of **I** it was also possible to compare the yield based on the first amino acid. The ratio *weight of peptide/weight of starting resin* P_w/R_w, previously [3] used to assess the reproducibility and the efficiency of the synthesis, becomes redundant. Nevertheless we used the ratio *theoretical weight of peptide/weight of starting resin* (tP_w/R_w) as a key parameter to organize the conditions for the synthesis. The loading of 0.112 mmoles/g (straightforwardly scaled-up from small scale) for a 26-mer peptide is a reasonable compromise between the necessity to obtain a (tP_w/R_w) as high as possible and the possibility to percolate a continuous-flow column. This result indicates that the loading of the resin must be based upon the molecular weight of the peptide to be synthetized (the higher the molecular weight, the lower the loading).

Selecting the concentration and reaction time parameters we were able to use up to 56% of the acylating reagent introduced in the synthesis. This result makes the amino acid recovery an unnecessary step; in fact, a standard recovery of 70% [2] leads to a reduction of the total cost of the synthesis of only 8.6%. Table 3 shows a well equilibrated sharing of the different items of the synthesis and a low cost of the amino acid derivatives on the entire cost of the synthesis (28.7%). This relative low cost of the AA makes the long discussion [6,7] about the cost of the Fmoc AA in large scale synthesis obsolete. If their cost is of the same order of magnitude as the solvents and others reagents used in the synthesis, it is evident that they can't fundamentally affect the final price of the peptide. On the other hand, the well equilibrated sharing of the cost among the different items shows that the economics of the process depends upon the general efficiency of the synthesis and not on the improvement of a single parameter.

The marked difference between our preparation and the semi-large scale preparation of human Angiotensin I [2] is essentially due to the different cost of the resin and to the different use of amino acid derivatives employed at a concentration four times superior (0.25 M) to that described in this paper.

Our preparative method can be compared with other successful preparative-scale syntheses of large peptides performed in batch via a stepwise [8] or a fragment-condensation [9] approach. It is easy to verify that the present continuous-flow methodology is, at least, simpler and less time-consuming than the above described methods.

The particular chemical efficiency of our process leads us to believe that this method could be further studied for kilogram scale production, particularly important for compounds like antithrombotic agents used in therapy in large quantities. It is easy to foresee that in similar very large industrial production the total cost will be close to the cost of materials.

Moreover the low cost of the employed Fmoc-amino acids the simplicity of the scaling-up approach and the low economic resources necessary for the Macro-Lab let to think that a good number of peptide molecules used today in terapy could be synthesized with this method to obtain more advantageous industrial production methods.

GRF, Somatostatin and even LH-RH agonists could be reasonable candidates to apply the described preparative method.**

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