Journal of Peptide Science, Vol. 3, 224–230 (1997)

Large-scale Production of Peptides Using the Solid-phase Continuous Flow Method. Preparative Synthesis of the Novel Tachykinin Antagonist MEN 10627

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Received 11 June 1996
Accepted 27 September 1996

Abstract: The large-scale solid-phase continuous flow synthesis of the bicyclic peptide MEN 10627, a new potent Neurokinin A receptor antagonist, is described using the Fmoc-polyamide method on both Macrosorb 125 and Macrosorb 250 resin. A new synthesizer designed in-house was realized by assembling Whitey valves and a Waters pump in order to allow small-scale (0.0001 mol; 1 × 10 cm Omnifit columns) synthetic studies which were strongly predictive of the conditions required for large-scale (0.01–0.10 mol; 3.6 or 4.9 × 46 cm Büchi columns) production, performed on the same apparatus. ©1997 European Peptide Society and John Wiley & Sons, Ltd.

J. Pep. Sci. 3: 224–230, 1997
(No. of Figures: 2. No. of Tables: 2. No. of Ref: 11.)

Keywords: large-scale peptide synthesis; SPPS; continuous flow; Macrosorb; Neurokinin A receptor antagonist

INTRODUCTION

Despite the great advancement in peptide chemistry, bulk quantities of peptides are not easily available in a standard organic chemistry laboratory because of the peculiar equipment and procedures necessary for their production. Specialized batchwise synthesizers are usually employed [1–4] for quantities from several grams to several hundred grams of pure peptide, required for pre-clinical and clinical programmes, and for production.

Those appliances are specially fashioned chemical reactors that cannot be straightforwardly scaled-up from the small-scale apparatus. Moreover, the reproducibility of the chemistry between small- and large-scale batch synthesis can be difficult to attain because of the different reaction conditions involved in the two processes.

The availability of a viable methodology to manufacture bulk quantities of peptides with the same practical work-up of a small-scale peptide synthesis would constitute an important asset for the industrial needs.

Previously, one of us reported [5] that the well-known continuous flow methodology [6] is a viable tool to manufacture important quantities of the 163–171 fragment of Interleukin 1β. More recently, a continuous flow methodology was applied [7] to the semi-large-scale synthesis of Leu^5-enkephalin (pen-

Abbreviations: Dap, diaminopropionic acid; DIPC, diisopropylcarbodiimide; DIPEA, diisopropylethyl-amine; PyBOP, benzotriazole 1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate.

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CCC 1075-2617/97/030234-07 $17.50

tapeptide) and human angiotensin I (decapeptide), resulting in an efficient process giving good yields of the final purified peptides.

In this paper we describe the large-scale synthesis of the novel bicyclic hexapeptide MEN 10627, a highly selective Neurokinin A receptor antagonist, endowed with remarkable potency and long duration of action *in vivo* [8–10], currently in the pre-clinical phase and aiming at clinical evaluation.

cyclo(−Met−A*sp*−*Trp*−*Phe*−*Da*p−*Leu*−)
MEN 10627

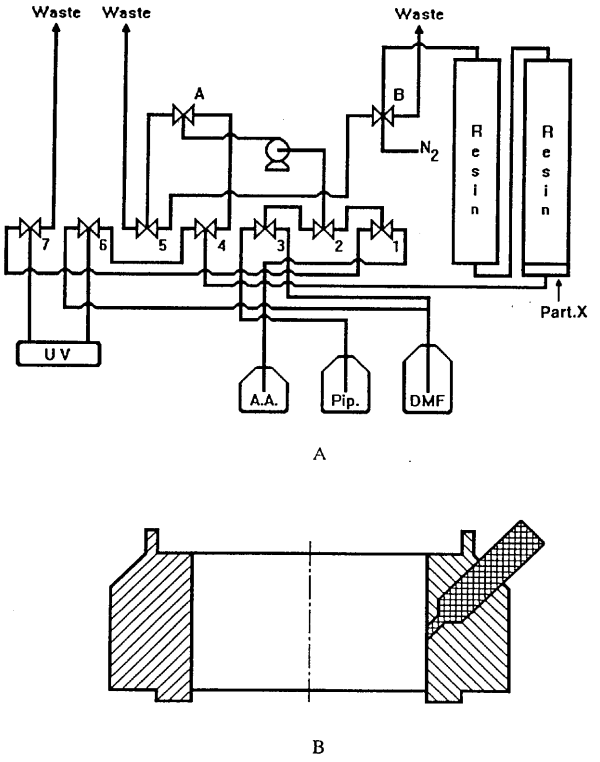
In order to provide sufficient compound for toxicological experiments, formulation and clinical studies, we developed a highly efficient synthetic process based on technology already described [5], but using a new synthesizer designed in-house and a well-defined methodology for the scale-up.

MATERIALS AND METHODS

General Procedures

The synthesis of MEN 10627 was performed with a new synthesizer designed in-house (Scheme 1), assembled from the materials already described [5]: a Waters 590 pump with a 1/4″ head, Whitey series 40 stainless steel valves, 1/8″ Teflon tubing and Büchi medium pressure chromatographic columns. The reactor is capable of working both in small scale (1 g of resin) and large scale (up to 300 g of resin), with comparable efficiency. The capacity of the whole process can be further improved (up to 600–700 g of resin) by substituting the 590 Waters pump (80 ml/min) with a more powerful 600 Waters pump (150 ml/min). In the preparative assembly the last column is connected to a short Teflon tube of the same size (Part X in Scheme 1), carrying an opening and a screw which allows sampling of the resin for analytical purposes (usually a Kaiser test), during the synthetic process. Undistilled, reagent grade DMF was used throughout the syntheses.

The versatility of the synthesizer enables one to experiment, at a well-defined linear velocity (L_v) of liquid flow, with both the chemical and the technological parameters of the process in inexpensive small-scale syntheses which become strongly predictive of the conditions required for bulk production. Thus, coupling methods, reaction times, pressure, viscosity of the fluids, formation of solids during the loading and recirculating steps, and



A

B

Scheme 1 (A) Large-scale synthesizer; (B) additional Teflon tube for removing samples (Part X).

reproducibility of the loading procedure when racemization is possible, are first set up and easily transferred to the bulk production.

By comparing small- and large-scale amidation reaction we found that if the final concentration of the acyl component (C_a) was the same in both cases, this ensured good reproducibility (efficiency and reaction times) between the small- and large-scale experiments. In this way, the difficulties due to the different weight of resin/dead volume ratio (R_w/V_d) in small ($R_w/V_d = 1/15$) and large- ($R_w/V_d = 1/3$) scale syntheses are overcome. Therefore, it became possible to determine the total amount of acylating reagent (n_t) to be added at the same step of the preparative synthesis by simply adding the quantity of the reagent (n_r), assigned to react with the resin, to the value of C_a times V_d, according to the equation:

$$n_t = n_r + C_a V_d$$

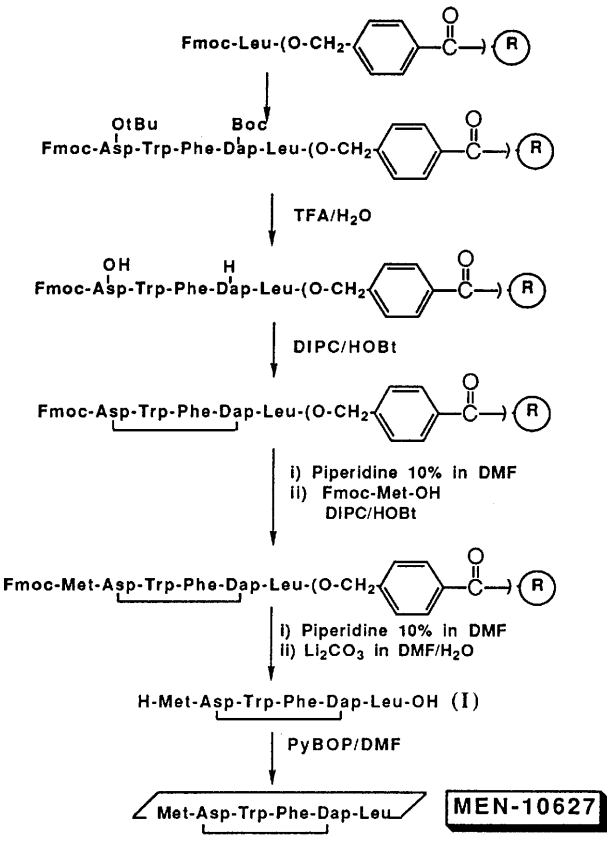
The quantity n_t, dissolved in the quantity of DMF scaled up from the small-scale experiment, was loaded into the preparative system and recirculated with the same L_v for the time previously found in the small scale. Since the concentration of the acylating mixture at the beginning of the amidation reaction is

superior in large scale than in small scale, owing to the different R_w/V_d, we found shorter reaction times in the large-scale synthesis than in the small scale (ca. 10% at $C_a = 0.1$ M). Nevertheless, the scaling-up procedure described above remains, in our experience, the most suitable correlation between small- and large-scale syntheses. Other basic parameters (e.g. starting concentration, excess of acylating agent compared with the theoretical loading of the resin) did not correlate well.

The esterification reaction of the first amino acid did not use the scaling-up procedure based on the C_a parameter because of the separate introduction into the column of $(FmocLeu)_2O$ and DMAP to minimize racemization, leading in general to difficulties in the reproducibility of the process.

The Synthetic Strategy

The synthesis of MEN 10627 was performed by using the Macrosorb SPR functionalized with *p*-hydroxymethylbenzoic acid, following the strategy shown in Scheme 2



Scheme 2 Synthetic pathway for MEN 10627.

RESULTS

Small-scale Synthesis

Five 1 × 10 cm Omnifit columns were each loaded with 1 g of Macrosorb SPR 125 resin (0.125 mmol/g), previously functionalized in batch by coupling with *p*-hydroxymethylbenzoic acid with the HOBt/DIPC method at a concentration of 0.268 M. The dead volume was adjusted with a piston to obtain $R_w/V_d = 1/15$.

The esterification step was optimized by accomplishing separate experiments on the five columns. The synthesizer was loaded with 5 ml solutions of $(FmocLeu)_2O$ in DMF in order to obtain concentrations (C_e) between 0.060 and 0.150 M with respect to V_d, followed by 0.1 equivalents of DMAP in 1 ml of DMF. A C_e of 0.114 M and a reaction time of 1 h proved optimal to obtain a Fmoc-Leu loading of 0.1 mmol/g (resin weight = 1.03 g).

The study of the amidation conditions was again performed separately on the five columns. The acylating agents dissolved in 5 ml of DMF were loaded in order to obtain a C_a between 0.030 and 0.080 M. A $C_a = 0.038$ M ensured complete acylation (Kaiser test) on the growing peptide in 1 h, using an L_v of 7.64 cm/min (flow = 6 ml/min).

The cleavage of the side-chain protective groups with 10 ml of $TFA/H_2O = 9/1$ had to be performed in batch, owing to the abnormal swelling of the resin induced by the deblocking mixture. The growing peptide-carrying resin was re-loaded into the columns to accomplish the first cyclization step which was studied by using HOBt/DIPC solutions at concentrations between 0.1 and 0.4 M. A 0.22 M solution was able to complete the intramolecular amidation in 20 h. After the last amidation step, set up as described above, the intermediate **I** was cleaved from the resin in batch with a 0.1N Li_2CO_3 solution (1 DMF/4 H_2O) obtaining, after precipitation at the isoelectric point, a crude product containing 52 mg of **I** (HPLC calculation), corresponding to a 65% yield (based on the first amino acid esterified on the resin). The HPLC purity was 85%. The ratio weight of peptide/weight of starting resin (P_w/R_w) was 0.052. Other basic reagents for the cleavage step, such as Na_2CO_3 or LiOH, afforded a less pure monocycle **I**. The two main impurities contained in the crude compound **I** were the corresponding sulphoxide-Met derivative and the *t*-butyl-Trp derivative (1.5%). The surprisingly low formation of the latter during the cleavage of the side-chain protective groups in TFA/H_2O (9/1) seems a

characteristic of the solid-phase synthetic methodology.

Large-scale Synthesis with Macrosorb 125

The large-scale preparation, performed on 300 g of Macrosorb 125 functionalized with *p*-hydroxymethylbenzoic acid, was set by using the reaction conditions selected as described above ($C_e = 0.114$ M, $C_a = 0.038$ M, $L_v = 7.64$, reaction time = 1 h, Fmoc-Leu loading = 0.092 mmol/g). Substantial reproducibility between small and large scales was observed for the amidation reactions, whereas the esterification step had a lower Fmoc-Leu-loading reproducibility. The cleavage of the t-Bu based protections, the first cyclization step and the last amidation (Fmoc = 0.089 mmol/g) were carried out as described for the small-scale reaction. The solid-phase preparation gave 323 g of the peptide–resin compound after Fmoc cleavage, MeOH and Et_2O washing and drying.

The peptide cleavage from the resin in 1.5 l of 0.1 N Li_2CO_3 solution (1 DMF/4 H_2O) gave 18.9 g of a yellow powder after concentration of the cleavage mixture and precipitation at the isoelectric point. This crude product contained 13.95 g of **I** (HPLC calculation). The ratio P_w/R_w was 0.046, confirming a good reproducibility between the small- and large-scale syntheses.

The Volumetric Capacity of Macrosorb

The Macrosorb SPR 125 resin has a poor volumetric capacity [11]. The suitability of other solid supports with higher functionalizations was then evaluated. Tentagel 220 mmol/g, Polyhipe 250 and 500 mmol/g and Macrosorb SPR 250 and 600 mmol/g were examined.

Tentagel resin, which proved in small-scale experiments as efficient as Macrosorb, was excluded because of its high commercial price. Polyhipe resin appeared poorly attractive due to its swelling characteristics: twice the column volume was needed with respect to the same mass of Macrosorb SPR, meaning that the same column volume would afford the same quantity of peptide using Macrosorb 250 or Polyhipe 500 or that Polyhipe 250 is equivalent to Macrosorb 125. Macrosorb SPR 600 was excluded because small-scale syntheses, with a loading of the first amino-acid between 0.350 and 0.400 mmol/g, afforded **I** of poor HPLC quality. A very low yield in the cleavage step was obtained, probably due to the unapproachability of the benzyl ester group caused by the hindrance of the large number of peptides chains.

Macrosorb 250 was extensively investigated in small-scale syntheses, proving as efficient as Macrosorb 125 after obvious operational adjustments. The most suitable esterification of the first amino acid was performed at $C_e = 0.143$ M for 3 h, obtaining an Fmoc-Leu loading of about 0.2 mmol/g without too large an increase in Leu racemization. The amidation reactions realized at $C_a = 0.053$ M and $L_v = 5.30$ cm/min resulted fully efficient without too large an increase in reaction times. The cleavage step of the t-Bu based protective groups was modified by introducing non-sulphurated scavengers (phenol and triisopropyl silane), which allowed yields similar to that described for Macrosorb 125.

Large-scale Synthesis with Macrosorb 250

The new high-capacity Macrosorb 250 resin was employed in large-scale preparations using 4.9 × 46 cm columns and the 600 Waters pump which allow one to work with at least half a kilogram of resin without too large an increase in the coupling-cycle time. The sequence of continuous flow and batch work-up was the same as that followed in the Macrosorb 125 large-scale experiment described above.

Starting from 490 g of *p*-hydroxymethylbenzoic acid functionalized resin (loaded into two columns) and using the C_e, C_a and L_v parameters found in small-scale experiments (Fmoc-Leu loading = 0.192 mmol/g), the monocyclic intermediate **I** was obtained by treating the final assembled resin (555 g) with 4.4 l of a 0.1 N Li_2CO_3 solution in DMF/H_2O = 1/4 for 20 h. The reaction mixture, after concentration (0.9 l) and precipitation at pH 5, gave 94 g of a yellow powder containing 47.57 g of **I** (P_w/R_w = 0.097). The differences between the two large-scale experiments performed on Macrosorb 125 and on Macrosorb 250, respectively, are listed in Table 1.

Final Cyclization to MEN 10627

The monocyclic intermediate **I** was cyclized with PyBOP in DMF using the method of infinite dilution. Some 47 g of the crude **I** in 1.4 l of DMF were added dropwise to 4.3 l of a solution of PyBOP (66 g) and DIPEA (43.4 ml) in DMF at −20 °C. The reaction mixture was stirred for 5 h. After evaporation of the solvent under vacuum the oily residue was treated with Et_2O (4 × 100 ml) and dissolved in 50 ml of acetone. On adding 500 ml of water, there was again

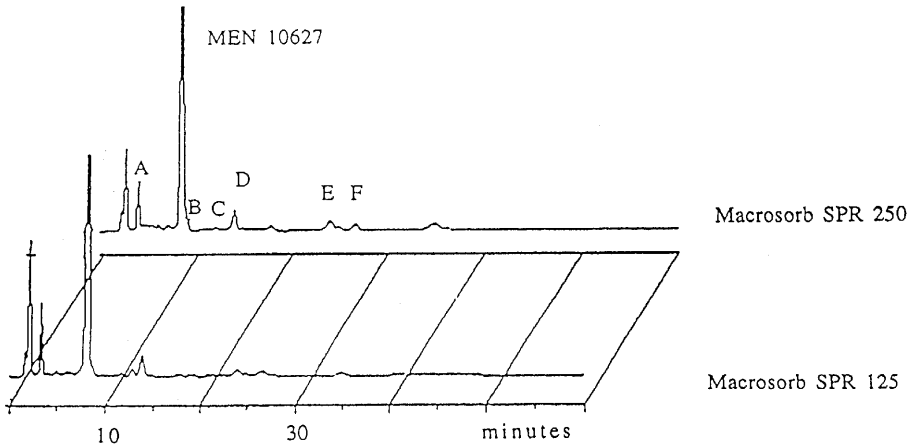
Table 1 Operating Parameters for the Large-scale Syntheses on Macrosorb 125 and Macrosorb 250

Resin	Macrosorb SPR 125	Macrosorb SPR 250
Capacity, theoretical (mmol/g)	0.125	0.250
Weight of resin (g)	300	490
Dead volume (ml)	1040	1680
Flow (ml)	80	100
L_v (cm/min)	7.86	5.30
Loading volume (ml)	350	950
C_e of $(FmocLeu)_2O$ (M)	0.114	0.143
C_a of Fmoc-AA-OH	0.038	0.053
% use of Fmoc-A-A-OH	41.1	51.3
FmocLeu-R (mmol/g)	0.092	0.192
$FmocMet(AA)_5$-R (mmol/g)	0.089	0.189
Reactive time (*T*) Dap (h)	1	3
Reaction time, Phe, Trp, Asp, Met (h)	1	1.5
Reaction time, first cyclization (h)	20	40
P_w/R_w	0.046	0.097

obtained a yellow oil which was repeatedly washed with water until a solid was obtained. The precipitate was filtered off, washed with water and dried to yield 40.1 g of a crude material containing 45.4% of MEN 10627 (yield = 78.34%).

Different crude MEN 10627, obtained from syntheses performed with Macrosorb 125 and 250, respectively, are compared in Figure 1. The major impurities were inorganic salts and by-products from the PyBOP, most of which were undetectable by HPLC at $\lambda = 220$ nm.

Some 10 g of crude cyclized material were purified by gel filtration through a 5 × 100 cm column packed with Sephadex LH-20. Elution with MeOH at 12 ml/min yielded 3.39 g of > 99% pure MEN 10627 (Figure 2).



Percentage of MEN 10627 and impurities in the crude product

	A	MEN 10627	B	C	D	E	F
Macrosorb SPR 250	2.14	87.6	1.09	-	2.95	2.13	1.62
Macrosorb SPR 125	3.84	86.4	0.64	1.10	3.42	1.92	1.23

A = Met-Sulphoxide; B = D-Leu MEN 10627; C = Di-Phe MEN 10627; D = Di-Leu MEN 10627; E = t-butylated MEN 10627; F = cyclic dimer of MEN 10627

Figure 1 Analytical HPLC of crude MEN 10627. Column, Lichrospher 100 RP18; eluent, 47% CH_3CN in 0.05 M HaH_2PO_4; flow rate, 1.0 ml/min; absorbance, 220 nm.

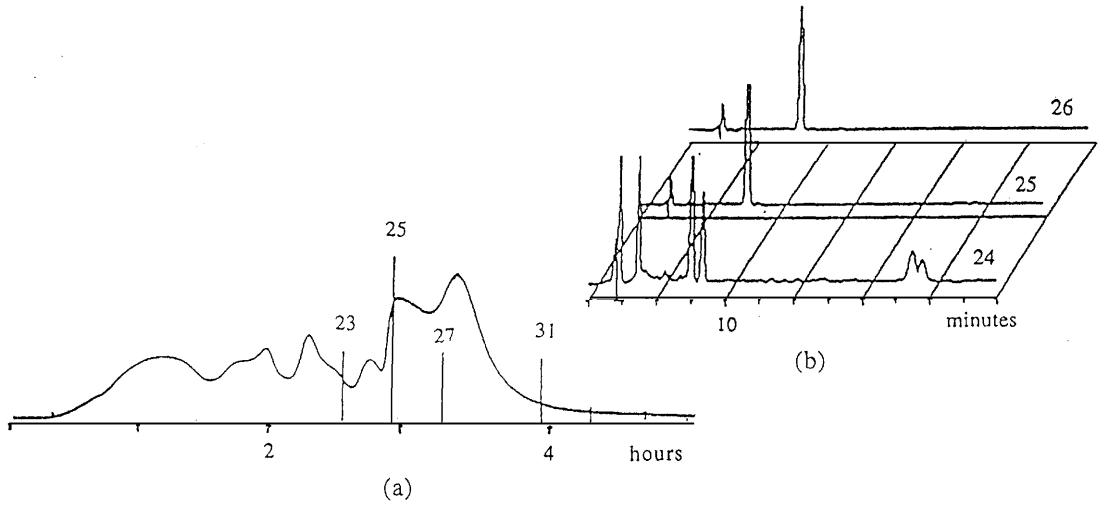


Figure 2 (a) Preparative Sephadex LH-20 chromatography of crude MEN 10627. Column, 1000 × 50 mm; eluent, MeOH; flow rate, 12 ml/min; absorbance, 230 nm; loading, 10 g of crude material containing 4.54 g of MEN 10627; pooled fractions, 25–31; yield, 4.05 g (> 99% HPLC), 89.2%. (b) HPLC of preparative Sephadex LH-20 fractions. Same conditions as Figure 1.

Analytical data of the purified product were in accordance with those already published [5].

DISCUSSION

The preparative method here described allowed the production of the quantities of MEN 10627 necessary for toxicological and clinical studies. This result was obtained in a standard organic chemistry laboratory without any industrial equipment. Both synthesis and purification could be performed by a single operator in one month. Two coupling reactions were usually run during a single day, notwithstanding that the synthesizer was entirely manually operated. The wastes did not exceed 20 l/day, also in the 500 g resin scale. The preparative method, at least for the amidation reactions, was directly scaled-up from small-scale pilot syntheses avoiding, in this way, tedious and expensive set-up for large scale. The concentration of the amino acids used during the acylation reaction was selected as a compromise between a relatively short reaction time and a relatively low concentration of reagent.

Owing to the difficulty in weighing the dry Fmoc-resin during the large-scale experiment, the synthesis yield based on the first amino acid esterified on the resin was impossible to calculate. P_w/R_w is a key parameter to evaluate the efficiency of the synthesis. The synthesis performed on 0.192 mmol/g Fmoc-Leu- esterified resin represented a great improvement in the method with respect to the 0.092 mmol/g resin, allowing a more suitable P_w/R_w, though, owing to the hindrance of the growing peptide, the linear velocity must be reduced and, as a consequence, the time of the synthesis must be proportionally increased.

The synthesis performed with the high-loading resin afforded 27.8 g of pharmaceutical grade pure MEN 10627.

Table 2 shows the percentage analysis of the costs resulting in a well-equilibrated sharing of the different items necessary for the synthesis. Under these conditions the recovery of the natural Fmoc-AA-OH was unnecessary: a standard recovery of 70% [7], in fact, reduced the total cost by only 6%.

In summary, the preparative method described in this paper is as practical as every other small-scale peptide synthesis. The high chemical efficiency obtained without an outlay on specialized equipment allows for further scale-up.

Table 2 Percentage Analysis of the Cost for the Synthesis of MEN 10627

Materials	% Cost
Resin	26.56
Linker	7.66
Fmoc-AA-OH	8.68
Fmoc-Dap(Boc)-OH	21.22
TFA	6.48
Solvents and reagent	24.10
Purification	5.30

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