Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 116–123
Published online 13 June 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.691

JOURNAL OF PEPTIDE SCIENCE

Convergent solid-phase synthesis of hirudin

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Received 2 February 2005; Revised 7 March 2005; Accepted 29 April 2005

Abstract: Hirudin variant 1 (HV1), a small protein consisting of 65 amino acids and three disulfide bonds, was synthesized by using Fmoc-based convergent methods on 2-chlorotrityl resin (CLTR). The linear sequence was assembled by the sequential condensation of 7 protected fragments, on the resin-bound 55–65 fragment. The conditions of fragment assembly were carefully studied to determine the most efficient synthetic protocol. Crude reduced [$Cys^{16,28}$(Acm)]-HV1 thus obtained was easily purified to homogeneity by RP-HPLC. Disulfide bridges were successfully formed by a two-step procedure, involving an oxidative folding step to form Cys^6–Cys^{14} and Cys^{22}–Cys^{39} linkages, followed by iodine oxidation to form the Cys^{16}–Cys^{28} bond. The correct disulfide bond alignment was established by peptide mapping using *Staphylococcus aureus V8* protease at pH 4.5. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hirudin; 2-chlorotrityl resin; convergent synthesis; protected peptide fragments; solid-phase fragment condensation; oxidative folding; solid-phase peptide synthesis

INTRODUCTION

Hirudin variant 1 (HV1, Figure 1) a small protein consisting of 65 amino acids, originally derived from the medicinal leech [1], is the most potent and specific inhibitor of thrombin known to date [2,3]. Owing to its strong anticoagulant properties, hirudin has been intensively investigated for research and therapeutic purposes [4–6]. Its therapeutic application makes its effective synthesis of great interest. Hirudin is produced in significant quantities through recombinant technology [7], and it is now in clinical use [8]. Although biological methods are still preferred for the large-scale synthesis of relatively complicated peptides and proteins, owing to the significant progress made, chemical synthesis methods have gained increasingly more attention by the pharmaceutical industry in recent years. The most important example is the production of the anti-HIV 36mer peptide T-20 (Fuzeon) in a multiton scale [9], by using the Fmoc-based convergent methodology [10,11].

Since the chemical synthesis of HV1 has not been reported, it was interesting to study the possibility to obtain the desulfated form of HV1 by applying convergent methods on CLTR, and in this paper we describe the results of our synthetic efforts.

Abbreviations: AcN, acetonitrile; AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; CLTR, 2-chlorotrityl resin; CPS, convergent protein synthesis; DCM, dichloromethane; DIC, *N,N*-diisopropylcarbodiimide; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; EDT, ethanedithiol; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; i-PrOH, isopropanol; MeOH, methanol; Mmt, 4-methoxytrityl; RP, reverse phase; RT, room temperature; SPFC, solid-phase fragment condensation; SPPS, solid-phase peptide synthesis; tBu, *tert*-butyl; TES, triethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane; Trt, triphenylmethyl, trityl.

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MATERIALS AND METHODS

2-Chlorotrityl chloride resin and Fmoc-amino acids were obtained from CBL-Patras. TLC-analyses: DC-Alufolien, Kieselgel 60, F_{254} (Merck). RP-HPLC: Waters 600E multisolvent delivery system, combined with Waters 991 photodiode array detector. Protected and free peptides were analyzed on a Lichrospher C-8 column (5 μm particle size; 4 × 125 mm) using linear gradients 50–100% B over 30 min (system A), 60–100% B in 30 min (system B), 70–100% B in 30 min (system C), 20–100% B in 30 min (system D) or 20–60% B in 40 min (system E), where A = 0.08% TFA in water and B = 0.08% TFA in AcN, at a flow rate of 1 ml/min, monitored at 214 and 265 nm. Semipreparative column: Lichrosorb RP-18 (7 μm particle size; 10 × 250 mm); ES-MS: Platform LC (Micromass), Cone Voltage 30 V.

General Procedure for Solid-phase Assembly of Protected Fragments

All syntheses were performed manually using plastic syringes equipped with porous polypropylene frits. Coupling reactions were performed in DMF, by *in situ* activation of a threefold molar excess of Fmoc-amino acid/DIC/HOBt (1 : 1.1 : 1.5) for 4 h. The completion of couplings was verified with the Kaiser test. Fmoc protective group was removed by a 2 × 15 min treatment with 25% piperidine in DMF. The completion of Fmoc-cleavage was checked with TLC after releasing the peptide from an aliquot of resin with the cleavage mixture AcOH/TFE/DCM (1 : 2 : 7). After each coupling/Fmoc-deprotection step, the resin was washed six times with DMF and four times with i-PrOH. Final peptide-resins were washed six times with DMF, two times with i-PrOH and once with

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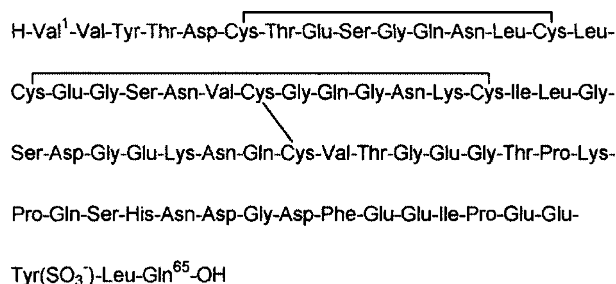

Figure 1 Hirudin variant 1.

n-hexane and dried. Protected fragments were prepared using a starting loading of 0.5 mmol/g.

Cleavage of Protected Fragments from Resin

Fully protected fragments were cleaved from CLTR by treatment with a 30:70 mixture of TFE-DCM (15 ml/g resin) for 2 h at RT. The resin was filtered off and washed twice with the cleavage mixture. The combined filtrates were concentrated on a rotary evaporator, and protected peptides were precipitated by the addition of cold diethyl ether/*n*-hexane mixture (1:1), collected by filtration and dried under vacuum. Table 1 summarizes the analytical data of the fragments used.

General Procedures for Solid-phase Fragment Condensation

Protocol A. Protected fragment (0.17 mmol) and HOBt (0.25 mmol) were dissolved in DMSO (1.8 ml). To this solution DIC (0.18 mmol) was added and after a brief shaking, the dried amino peptide-resin (400 mg, 0.056 mmol) was added. The resin suspension was left to stand at RT overnight, without shaking. After each condensation the peptide-resin was filtered and washed five times with DMSO, five times with DMF and four times with i-PrOH. The completion of the condensation reaction was checked with the Kaiser test and TLC.

Protocol B. Protected fragment (0.056 mmol) and HOBt (0.084 mmol) were dissolved in DMSO (0.6 ml). To this solution DIC (0.06 mmol) was added and after a brief shaking, the dried amino peptide-resin (400 mg, 0.056 mmol) was added. The peptide-resin suspension was left to stand at RT for 2 h, without shaking. After that, the peptide-resin was filtered and washed five times with DMSO, five times with DMF and four times with i-PrOH. The completion of the condensation reaction was checked with the Kaiser test. If the reaction was not complete, a new condensation was performed under the same conditions. This procedure was repeated until Kaiser test indicated complete condensation.

Table 1 Analytical data of the finally selected peptide fragments

Fragment	Yield (%)	HPLC purity (%)	t_R (min)	ES-MS[e]	
				Calc.	Found
2	88	96	23.8[a]	1101.22	1101.28 $(MH)^+$
3b	90	95	15.9[c]	1201.35	1201.10 $(MH)^+$
4	92	99	21.2[b]	886.95	886.95 $(MH)^+$
5b	96	95	18.5[b]	1173.43	1173.54 $(MH)^+$
7	89	96	16.0[c]	1197.26	1197.40 $(MH)^+$
8	85	99	26.5[d]	850.39	850.69 (MH)+
9	95	93	21.9[a]	880.85	880.62 $(MH)^+$
10	—	97	24.0[a]	587.25	587.33 $(MH_2)^{2+}$

[a] System A.
[b] System B.
[c] System C.
[d] System D, see *Materials and Methods*.
[e] After side-chain deprotection.

Cleavage from Resin-deprotection

Boc-HV1(1–65)-O-CLTR **12** (450 mg) was treated with the mixture AcOH/TFE/DCM (1:2:7) (4.5 ml) for 2 h at RT. The resin was filtered off and washed twice with the cleavage mixture. The filtrates were concentrated in a rotary evaporator and the protected peptide **13** was precipitated by addition of cold diethyl ether and collected by filtration. Yield: 204 mg (66%). A sample of the above product (20 mg) was totally deprotected with the mixture TFA/EDT/H_2O (90:5:5) for 4 h at RT. Crude peptide **14** was precipitated with diethyl ether after evaporation *in vacuo* of the deprotection mixture. The product was analyzed by RP-HPLC and purified by semipreparative RP-HPLC, using gradient elution (20% B to 60% B in 40 min). Pure peptide fractions were collected and lyophilized to afford **14** (3 mg, 26%).

Oxidation of Free Cys

The linear peptide **14** was dissolved in a buffer of Tris-HCl (pH = 8.5) in the presence of 200-μM β-mercaptoethanol at a concentration of 0.5 mg/ml and left at RT. The progress of the folding reaction was checked by RP-HPLC and ES-MS. Oxidation was completed after 5 h. The product **15** was collected, lyophilized and subsequently used without purification in the final oxidation step.

Enzymatic Digestion

The oxidized material from the previous step (0.5 mg) was dissolved in 50 mM acetate buffer, pH 4.5 (0.5 ml) and a solution of *Staphylococcus aureus V8* protease in the same buffer (5 μl) was added (protease to substrate ratio 1:10, by mass). After 20 min at RT, an aliquot of the digestion mixture was analyzed on an Agilent 1100 series LC-MS, using a Zorbax SB-C18 (3.5 μm particle size, 2.1 × 30 mm) column and a linear gradient of 10–50% B in 40 min at a 0.4 ml/min flow rate.

Oxidation of the Acm-protected Cys with Iodine

Iodine was dissolved in a AcOH/H_2O (4:1) mixture, at a concentration of 0.05 μmol/ml (*solution 1*). Peptide **15** was dissolved separately in the same mixture at a concentration of 0.5 mg/ml (*solution 2*). *Solution 2* was added dropwise to the stirred *solution 1* at RT. The completion of the

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reaction was checked with RP-HPLC and ES-MS. The reaction was completed in 30 min. Excess iodine was extracted with chloroform and the final product **16** was purified with RP-HPLC.

RESULTS AND DISCUSSION

Selection and Preparation of Protected Peptide Fragments

As previously described, in a convergent protein synthesis (CPS) the selection of protected fragments is crucial for its success [10,11]. Among other factors, the solubility and reactivity of fragments are the most important. It is, therefore, necessary to evaluate first the suitability of the fragments by performing preliminary small-scale syntheses. In our initial synthetic plan, hirudin sequence was divided into the relatively long (23 aa), resin-bound 43–65 fragment **1** and five additional protected fragments **2**–**6**, as shown in Figure 2. To avoid the possible racemization occurring during the condensation reactions, the C-components **2**–**6** were chosen to contain Gly as the *C*-terminal amino acid. All fragments were synthesized on the CLTR by the conventional SPPS method using Fmoc-amino acids. For side-chain protection the tBu (Glu, Thr, Tyr, Asp, Ser), Boc (Lys) and Trt (Gln, Asn, His) groups were used. Since hirudin is a three disulfide bridge (Cys^{6}–Cys^{14}, Cys^{16}–Cys^{28}, Cys^{22}–Cys^{39}) containing protein, we initially planed to investigate two general folding strategies, i.e. the one-step procedure where the three disulfides are simultaneously formed and the two-step approach where two disulfides are simultaneously formed and the third one in a subsequent step. In order to apply the first strategy, all six Cys-residues were protected with the acid-sensitive 4-methoxy trityl (Mmt) group [12]. Mmt group can easily be removed with dilute TFA solutions and thus offers clear advantage over the more acid resistant Trt groups for the synthesis of complicated Cys-rich peptides. In the two-step approach, we chose to protect $Cys^{6,14,22,39}$-residues with the Mmt group and $Cys^{16,28}$ -residues with the Acm group, in order to investigate the formation of the isolate 6–14 and 22–39 disulfides by an oxidative folding step and subsequently the formation of the 16–28 disulfide by iodine oxidation. The chain elongation

1 Fmoc-Glu(tBu)^{43}-Gly-Thr(tBu)-Pro-Lys(Boc)-Pro-Gln(Trt)-Ser(tBu)-His(Trt)-Asn(Trt)-Asp(tBu)-Gly-Asp(tBu)-Phe-Glu(tBu)-Glu(tBu)-Ile-Pro-Glu(tBu)-Glu(tBu)-Tyr(tBu)-Leu-Gln^{65}-O-CLTR

2 Fmoc-Glu(tBu)^{35}-Lys(Boc)-Asn(Trt)-Gln(Trt)-Cys(Mmt)-Val-Thr(tBu)-Gly^{42}-OH

3a Fmoc-Asn(Trt)^{26}-Lys(Boc)-Cys(Mmt)-Ile-Leu-Gly-Ser(tBu)-Asp(tBu)-Gly^{34}-OH

3b Fmoc-Asn(Trt)^{26}-Lys(Boc)-Cys(Acm)-Ile-Leu-Gly-Ser(tBu)-Asp(tBu)-Gly^{34}-OH

4 Fmoc-Ser(tBu)^{19}-Asn(Trt)-Val-Cys(Mmt)-Gly-Gln(Trt)-Gly^{25}-OH

5a Fmoc-Gln(Trt)^{11}-Asn(Trt)-Leu-Cys(Mmt)-Leu-Cys(Mmt)-Glu(tBu)-Gly^{18}-OH

5b Fmoc-Gln(Trt)^{11}-Asn(Trt)-Leu-Cys(Mmt)-Leu-Cys(Acm)-Glu(tBu)-Gly^{18}-OH

6 Fmoc-Val^{1}-Val-Tyr(tBu)-Thr(tBu)-Asp(tBu)-Cys(Mmt)-Thr(tBu)-Glu(tBu)-Ser(tBu)-Gly^{10}-OH

7 Fmoc-Val^{2}-Tyr(tBu)-Thr(tBu)-Asp(tBu)-Cys(Mmt)-Thr(tBu)-Glu(tBu)-Ser(tBu)-Gly^{10}-OH

8 Fmoc-Glu(tBu)^{43}-Gly-Thr(tBu)-Pro-Lys(Boc)-Pro^{48}-OH

9 Fmoc-Gln(Trt)^{49}-Ser(tBu)-His(Trt)-Asn(Trt)-Asp(tBu)-Gly^{54}-OH

10 Fmoc-Asp(tBu)^{55}-Phe-Glu(tBu)-Glu(tBu)-Ile-Pro-Glu(tBu)-Glu(tBu)-Tyr(tBu)-Leu-Gln^{65}-O-CLTR

Figure 2 Protected fragments synthesized in the present study. Underlined fragments were used in the final assembly.

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was performed using the DIC/HOBt activation method. Coupling reactions were followed with the ninhydrin test and TLC. The Fmoc-removal was performed by treatment with 25% piperidine in DMF. The quantitative removal of the Fmoc-group during the progress of the synthesis was checked with TLC. Cleavage of the protected peptides from the resin was performed by treatment of the peptide-resin esters with TFE/DCM (30 : 70) for 2 h at RT. Fragments **2**–**5** were, thus, obtained in >95% purity and were soluble as 0.1 M solutions in DMSO. However, fragments **1** and **6** could not be obtained in sufficient purity. In addition, **6** had a very limited solubility in DMSO. The purity and solubility of this fragment was greatly improved by omitting Val^1 from the *N*-terminus and thus, fragment **7** instead of **6** was used. Moreover, **1** was further divided into fragments **8**, **9** and **10**, which were easily obtained in high purity. The HPLC profiles of the finally selected crude fragments are shown in Figure 3. More analytical data are presented in Table 1. All fragments were used in the subsequent condensation reactions without further purification.

Fragment Assembly

Fragment condensations were studied in detail, in an attempt to find the most efficient synthetic protocol. At first, we carried out a small-scale synthesis in order to determine possible difficulties during fragment assembly. The condensations were performed by using

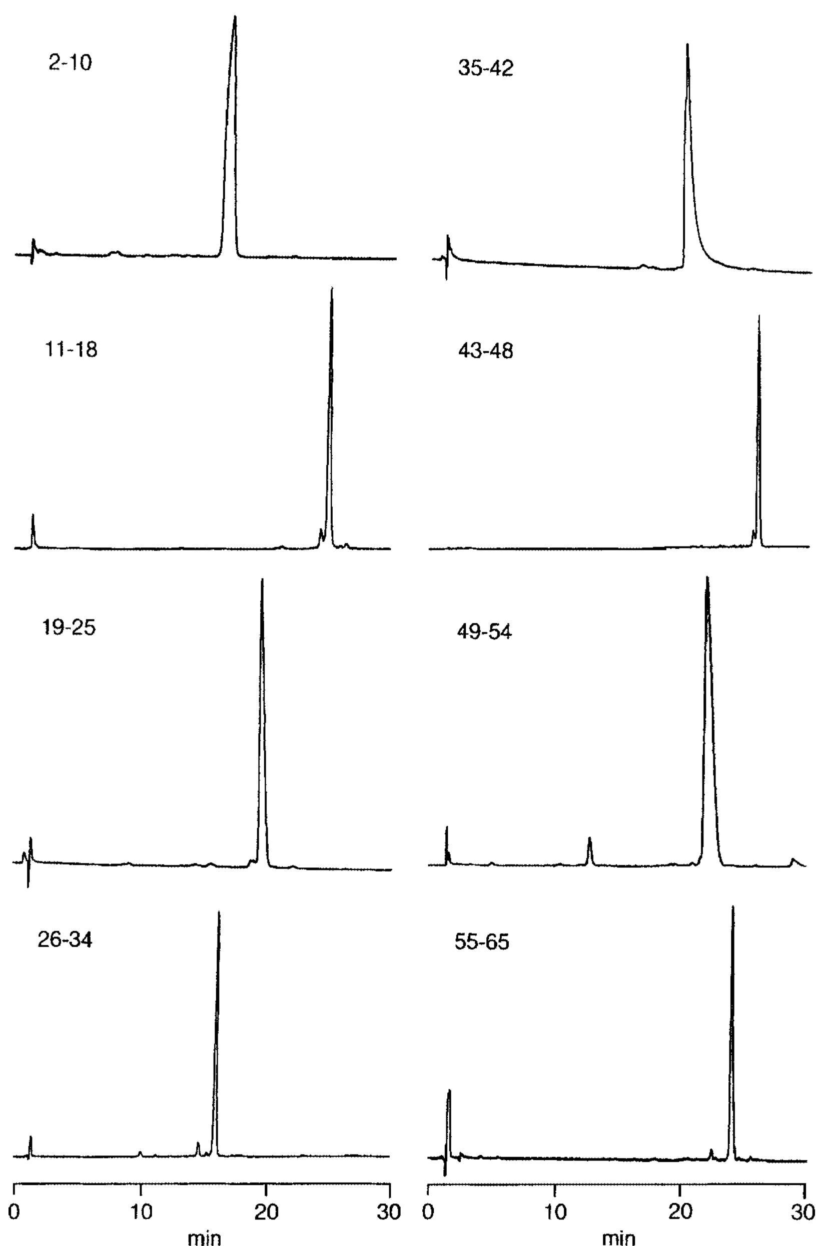

Figure 3 HPLC profiles of the protected fragments used in the final hirudin assembly.

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0.05–0.1 molar solutions in DMSO of a threefold molar excess of C-components over the resin-bound N-component **10**. As condensing agent, DIC/HOBt was applied. Coupling reactions were left overnight at RT. Samples from the obtained intermediate fragments, after every condensation, were cleaved from the resin, deprotected with TFA/EDT/H_2O (90 : 5 : 5) and analyzed with RP-HPLC and ES-MS. Until Glu^{43}(tBu), condensations proceeded very smoothly. However, a double coupling was necessary in the case of fragment 35–42 to drive reaction to completion. Even more difficult was the coupling of the 11–18 fragment **5a**. The condensation was incomplete after a double coupling under the same conditions. Fragment **5b** in which the Cys^{16}(Mmt) has been replaced by the Cys^{16}(Acm) exhibited better solubility and reactivity properties than **5a**. Therefore, in all subsequent synthetic attempts **5b** in combination with fragment **3b** was used, and thus the one-step folding procedure could not be further explored.

We also observed, from the RP-HPLC and ES-MS analyses, that after Glu^{43}(tBu), a portion of the excess C-components remaining after the coupling was almost impossible to be removed, even after extensive washings with DMSO and DMF. We assumed that these insoluble C-components might be responsible for the progressive accumulation of impurities, since they can be activated at a later condensation step. Thus, the crude product that was obtained from this trial assembly, after total deprotection with TFA/EDT/H_2O (90 : 5 : 5), was highly heterogeneous (data not shown).

Taking into consideration above results, we decided to adopt a modified fragment coupling protocol to improve the quality of the overall assembly. Thus, instead of using a threefold molar excess of C-components over the resin-bound N-component in one portion, we performed multiple condensations of each fragment using less than equimolar amounts at a time in the same DMSO volume, to prohibit the fragment-active esters precipitating during condensation and to facilitate the resin washings. Coupling reactions were checked after 2 h with Kaiser test and TLC and if they remained incomplete, a new condensation was performed. Condensations of fragments **9** and **8** were, thus, completed in 4 h and a total of two equivalents were required. Condensations of fragments **2, 3b, 4** and **7** were completed in 6 h and a total of three equivalents of each fragment were used. However, the condensation of **5b** could not be completed, independently of the excess used. Samples from the obtained intermediate peptides, after every condensation, were cleaved from the resin, deprotected with TFA/EDT/H_2O (90 : 5 : 5) and analyzed by RP-HPLC and ES-MS. We noticed from the RP-HPLC and

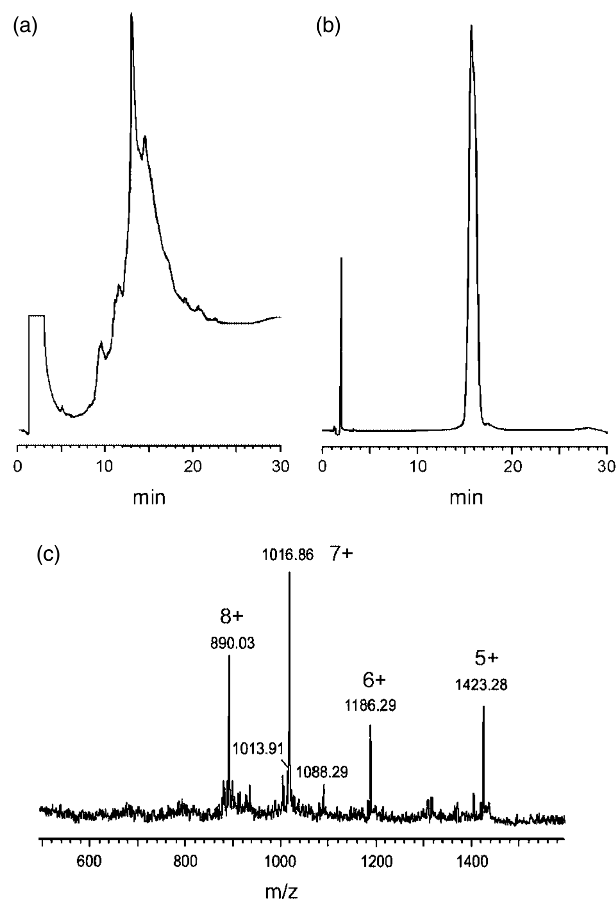

Figure 5 HPLC profiles of crude (a) and purified (b) linear $[Cys^{16,28}$(Acm)]-HV1. Conditions: system E (*see Materials and Methods*). (c) ES-MS of the purified product.

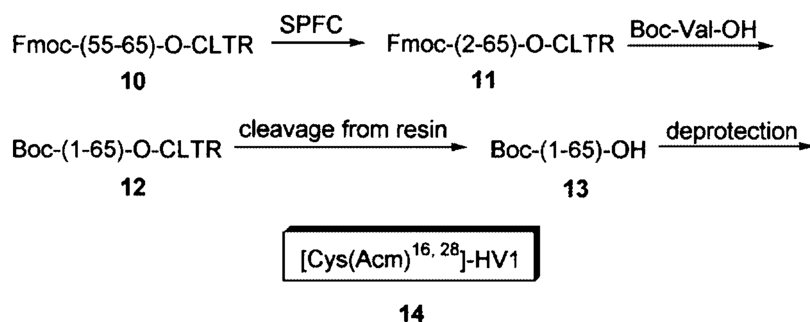

Figure 4 Convergent assembly of the linear hirudin sequence.

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ES-MS analysis that the quantity of the insoluble C-components was eliminated or greatly reduced and the purity of the obtained intermediates significantly improved. The Fmoc-HV1(2–65)-O-CLTR **11** obtained from this synthesis was coupled with Boc-Val-OH, using DIC/HOBt for its activation, to afford the final Boc-HV1(1–65)-O-CLTR **12** (Figure 4).

Cleavage from the Resin, Deprotection and Purification

Protected peptide **12** was cleaved from CLTR by treatment with AcOH/TFE/DCM (1:2:7) for 2 h at RT and precipitated with cold diethyl ether. Crude protected Boc-HV1(1–65)-OH **13** was isolated in 66% yield. The total deprotection of **13** (except of the 2 Acm groups) was studied by testing various TFA/scavengers mixtures, such as TFA/EDT (95:5), TFA/DCM/EDT (80:15:5), TFA/DCM/TES (80:15:5) and TFA/EDT/H_2O (90:5:5). Best results, in terms of purity, were obtained by using the last mentioned mixture for 4 h at RT. Crude deprotected [$Cys^{16,28}$(Acm)]-HV1 **14** was thus obtained in 40% purity, as determined by RP-HPLC analysis (Figure 5a). Peptide **14** was purified to homogeneity by semipreparative RP-HPLC and verified with ES-MS, giving an actual mass of 7111.6 Da, in excellent agreement with the calculated value of 7111.7 Da (Figures 5b,c).

Oxidative Folding

The purified [$Cys^{16,28}$(Acm)]-HV1 was subjected to a two-step oxidation procedure (Figure 6). At the first step, the peptide **14** was dissolved in a Tris-HCl buffer (pH = 8.5) in the presence of β-mercaptoethanol (200 μM) [13]. The folding reaction was essentially completed after 5 h at RT, as verified by RP-HPLC and ES-MS analysis (Figure 7). The measured mass of folded hirudin was consistent with the formation of two disulfide bonds (found: 7107.4 Da; calc. 7107.7 Da; i.e.

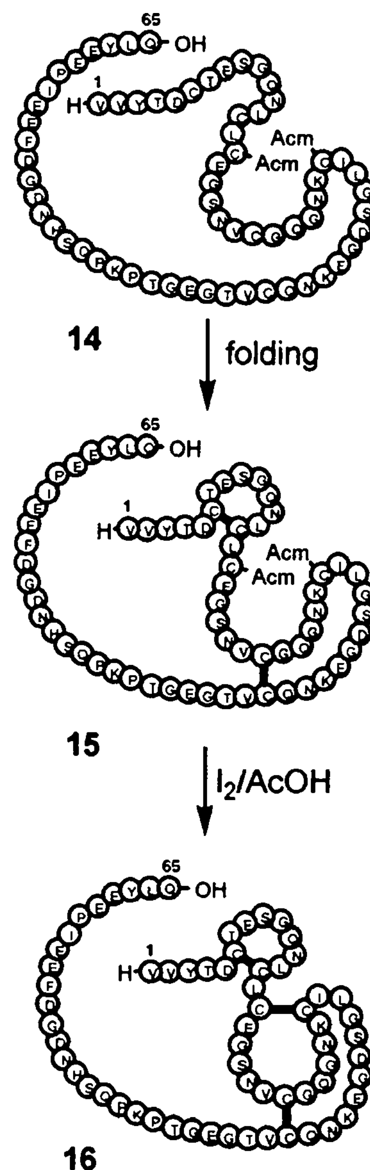

Figure 6 The two-step oxidation procedure.

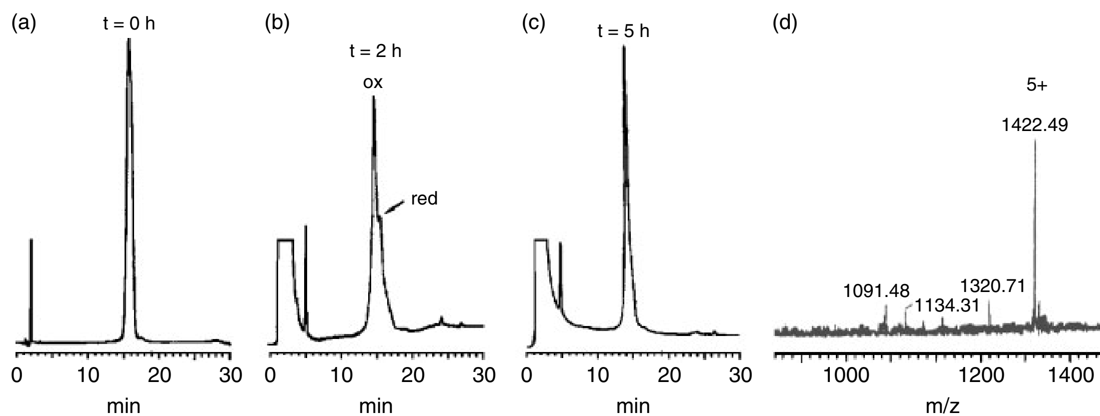

Figure 7 HPLC monitoring of oxidative folding of [$Cys^{16,28}$(Acm)]-HV1 (a-c). Conditions: system E. (d): ES-MS of the folded product.

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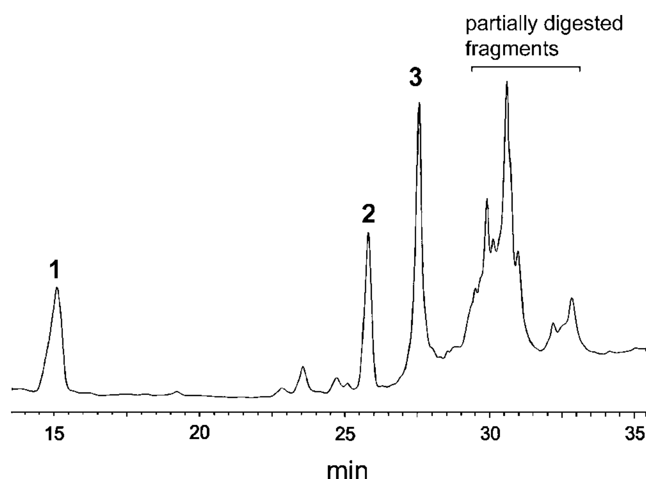
4.2 Da decrease, corresponding to the loss of 4 protons). Moreover, the disulfide bond alignment was determined by proteolysis using *Staphylococcus aureus V8* protease at pH 4.5 [14]. As shown in Figure 8, besides various partially digested fragments, three main fragments were produced corresponding to the sequence 62–65 (peak 1), the oxidized sequence 1–17 (peak 2) and the oxidized sequence 18–61 (peak 3), providing thus a clear-cut indication of the correct disulfide bond topology.

The final step was the simultaneous deprotection-oxidation of the two $Cys^{16,28}$(Acm)- residues. This step was performed by dissolving peptide **15** in a $AcOH/H_2O$ (4 : 1) mixture and adding it dropwise into a solution of iodine in $AcOH/H_2O$ (4 : 1). The formation of the disulfide bond was fast, completed in 30 min, as revealed by RP-HPLC analysis (Figure 9a). The pure fraction of the final product was collected, lyophilized and analyzed by RP-HPLC and ES-MS (Figures 9b,c). The actual mass of the fully oxidized hirudin was 6964.2 Da, in very good agreement with the calcd. value of 6963.5 Da. Table 2 summarizes the yields of the various hirudin synthesis steps and of the overall procedure.



Peak	Residues	ES-MS Calc.	ES-MS Found	Sequence
1	62-65	551.6	551.2	EYLQ
2	1-17	1946.2	1945.4	VVYTDCTESGQNLCLC(Acm)E
3	18-61	4645.9	4645.2	GSNVCGQGNKC(Acm)ILGSDGELNCV TGEGTPKPQSHNDGDFEEIPE

Figure 8 HPLC chromatogram of *Staphylococcus aureus V8* protease digested [$Cys^{16,28}$(Acm)]-HV1. In the inserted table, the assignment of the three main peptide fragments is shown.

CONCLUSIONS

Convergent methodology was successfully applied in the synthesis of hirudin. Difficulties concerning the limited solubility of certain activated fragments in the 1–42 peptide region, which caused precipitation problems during coupling, were overcome by using more dilute solutions of less than equimolar fragment quantities. The necessary repetitions of couplings were used to ensure complete condensations and keep fragment consumption to a minimum. Thus, the linear $Cys^{16,28}$(Acm)-sequence was obtained in good (∼40%) crude purity. The formation of the three disulfide bonds

Table 2 Summary yields of the various steps of HV1 synthesis

Step	Yield (%)
Fragment assembly	66
Total deprotection	98
Purification	26
Final purification after disulfide formation	72
Overall	**12**

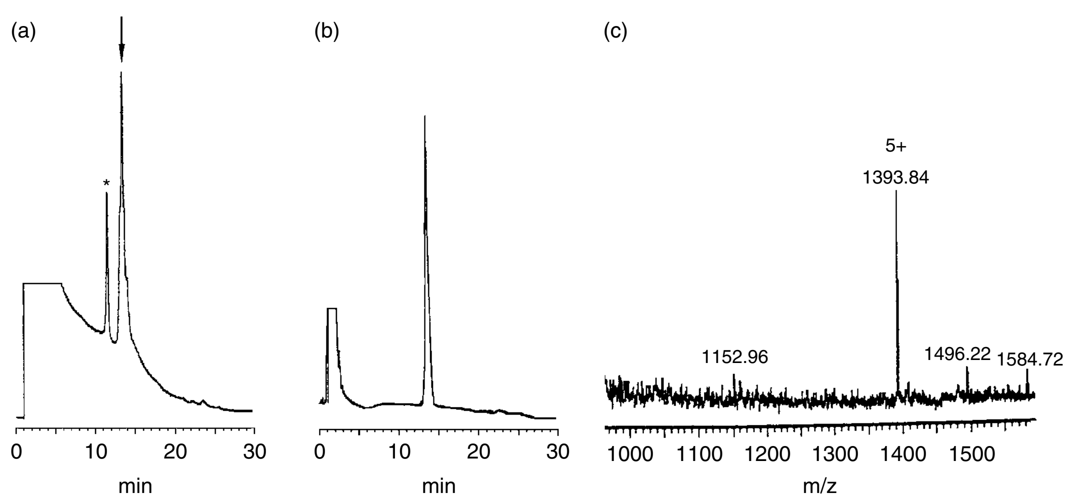


Figure 9 HPLC chromatograms of the crude (a) and purified (b) HV1, obtained after a 30-min iodine oxidation of [$Cys^{16,28}$(Acm)]-HV1. * = nonpeptide material. Conditions: system E. (c) ES-MS of purified HV1.

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was easily achieved by a two-step procedure, without the need for purification of the intermediate product. The results of this study indicate that complicated small proteins, like hirudin can efficiently be prepared in high purity by carefully optimized convergent methods.

Acknowledgements

The authors acknowledge CBL-Patras S.A. for financial support.

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