Tetrahedron Vol. 45, No. 24, pp. 7759 to 7764, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00
Pergamon Press plc

FULLY AUTOMATIC SIMULTANEOUS MULTIPLE PEPTIDE SYNTHESIS IN MICROMOLAR SCALE - RAPID SYNTHESIS OF SERIES OF PEPTIDES FOR SCREENING IN BIOLOGICAL ASSAYS

GERD SCHNORRENBERG* and HELMUT GERHARDT#

*** Department of Medicinal Chemistry, Boehringer Ingelheim KG, D-6507 Ingelheim, FRG**
 # Zinsser Analytic GmbH, Eschborner Landstrasse 136, D-6000 Frankfurt 94, FRG

(*Received in Germany* 19 *June* 1989)

Abstract - A new method of simultaneous multiple peptide syntheses on polystyrene support in the wells of microtiterplates is illustrated by the syntheses of 31 overlapping segments of endothelin. All reagent and solvent handling is automatically performed by a freely programmable robotic sample processor.

Structure activity studies on peptides and proteins are of great importance for biochemistry, pharmacology and immunology. Overlapping segments are useful tools to disclose domains which are responsible for the biological activity whereas substitution analogues of active segments can help to detect essential amino acids. For these evaluations there is need for series of different peptides. Quick progress often is hampered by time consuming peptide synthesis. Therefore there is growing interest in techniques enhancing the speed of peptide synthesis.

In this respect improvements were achieved by the introduction of the simultaneous multiple peptide synthesis[1,2]. The impact of this new technique on the evaluation of structure activity data has been demonstrated at the example of a 12 residue peptide of fibrinogen alpha chain[3]. The major drawback of this method is that all technical operations of peptide synthesis have to be done by hand.

We now want to report on a novel fully automatic simultaneous synthesis of up to 48 different peptides which was applied to the syntheses of 31 overlapping segments of endothelin[4]. Due to the small scale only minimum quantities of reagents are required.

Solid phase peptide synthesis is performed in the wells of a 96-well microtiter plate which were loaded by about 12 mg of resin yielding 5 umol of peptide[5], a quantity usually sufficient for analysis (HPLC, FAB-MS and microsequencing) and biological assay (radio receptor assay, enzyme assay or radio immunological assay).

All liquid handling in the microtiterplate is performed by a robotic sample processor (Tecan RSP 5052) which has been adapted to peptide synthesis. A special software has been developed for controlling peptide synthesis allowing free choice of sequences and chain length.

RESULTS AND DISCUSSION

Syntheses of peptides are performed applying Fmoc strategy[6,7] on a wide variety of commercially available polystyrene resins. For peptide acids the Wang resin (4-benzyloxy-benzylester-type linkage)[8] or the more labile 4-benzyloxy-2-methoxy-benzyl-ester linkage[9] turned out to be well suited. For peptide amides the Barany-linker[10] proved to be superior.

Coupling is achieved by N,N'-diisopropylcarbodiimide (DIC)/ 1-hydroxybenzotriazole (HOBt)[12] in single or double coupling steps. A tenfold excess of reagents is used for every coupling step. Despite of this high excess of reagents syntheses on the robotic sample processor are extremely economical. For the total syntheses of the 31 overlapping endothelin segments only quantities between 0.3 g and 1 g of Fmoc amino acids were necessary. Optionally completion of all couplings can be checked by ninhydrin or TNBSA[13] test.

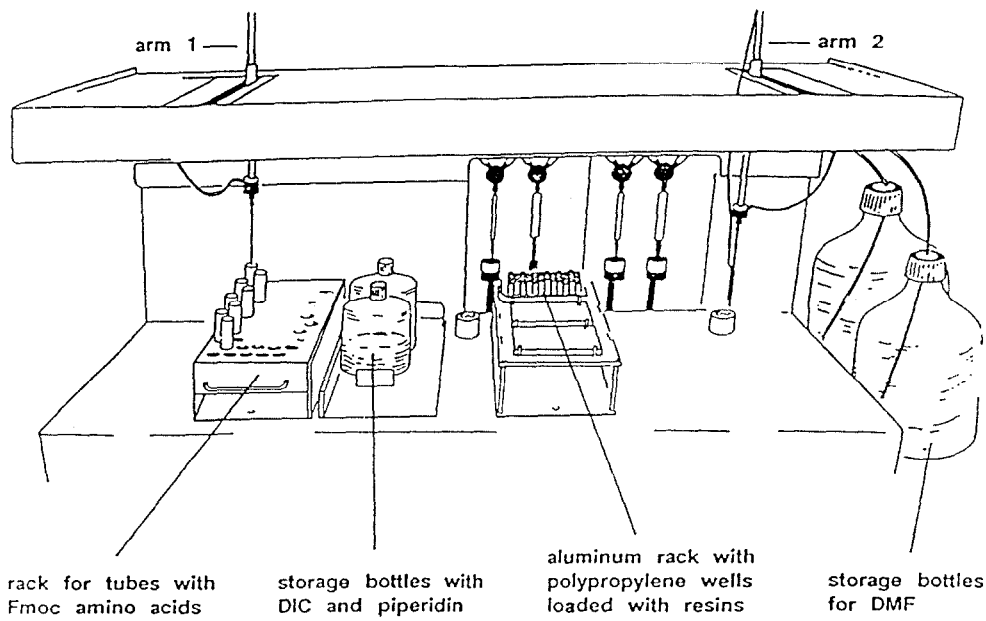


Figure 1.

Figure 1 shows the robotic sample processor for automatic reagent and solvent handling. The sample processor has two pipetting arms one being responsible for dosage of all reagents. All washing steps are performed by the other one.

Reagent handling of arm one:

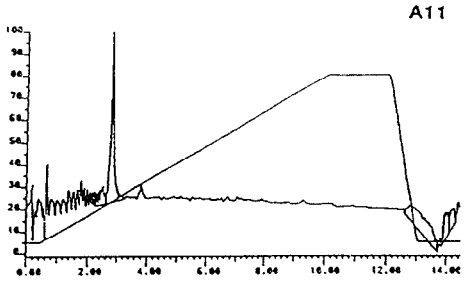
Up to 80 different solutions of Fmoc amino acids in DMF containing one equivalent of 1-hydroxybenzotriazole can be stored in septa sealed tubes in the rack on the left hand side. Aliquots are transferred into the resin loaded wells. Exact dosage is secured by a motor driven syringe. Coupling is initiated by pipetting a DMF solution of DIC (0.75 molar) from a septum sealed storage bottle into each well. Fmoc deprotection is achieved by addition of a 50% piperidine/DMF solution into each well. After each of these operations the tip of arm one is intensively washed with DMF in a wash station to avoid cross contamination.

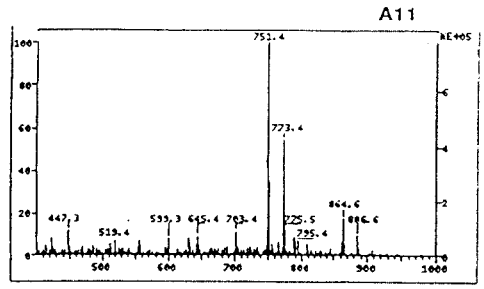
Solution handling of arm two:

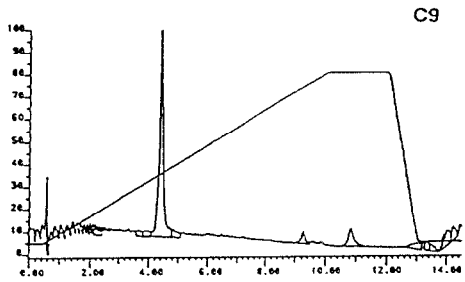
Sucking off all solubles from the wells and all washing steps are carried out by arm two connected to a motor driven syringe transferring all solutions to the waste. To avoid loss of resin the tip is protected by a narrow stainless steel net. Resin sticking to the net is rinsed back to the wells by dosage of DMF through an attached cannula thus initiating the next washing step (see figure 1). Cross contamination is prevented by driving the tip to a wash station before proceeding to the next well. After each reaction step the resins in the wells are ten times intensively washed with DMF. A typical protocol of a synthetic cycle for 5 umol resin bound Fmoc amino acid or Fmoc peptide is given in table 1.

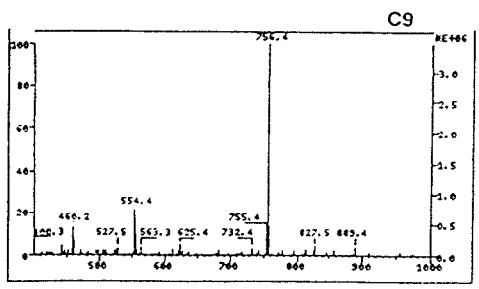
Table 1 Protocol of a synthetic cycle:

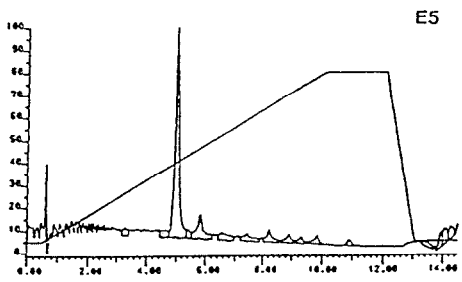
step	arm 1	arm 2	operation
1		add 300 ul DMF	wash resin
2		removal of supernatant	
3	add 300 ul 50 % piperidine/DMF		deprotect
4		removal of supernatant	
5	add 300 ul 50% piperidine/DMF, 15 min		deprotect
6		removal of supernatant	
7		add 300 ul DMF	wash resin
8		removal of supernatant	
9-26	repeat steps 7 and 8 nine times		
27	add Fmoc amino acid (50 umol)/HOBt (50 umol) in 200 ul DMF		coupling
28	add DIC (75 umol) in 100 ul DMF, 40 min		
29		removal of supernatant	
30		add 300 ul DMF	wash resin
31		removal of supernatant	
31-32	repeat steps 27 and 28		coupling
33		removal of supernatant	
34-53	repeat steps 30 and 31 ten times		wash resin











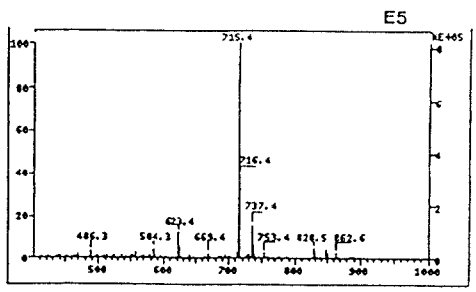


Figure 2

HPLC profiles of some crude peptides, sequences

A11: H-Ala-Ser-Ser-Leu-Met-Asp-Lys-OH
(endothelin 3-9 [Ala3])

C9: H-Lys-Glu-Ala-Val-Tyr-Phe-OH
(endothelin 9-14 [Ala11])

E5: H-Phe-Ala-His-Leu-Asp-Ile-OH
(endothelin 14-19 [Ala15])

Figure 3

Positive ion FAB mass spectra of peptides (sequences given in figure 2)

A11: 751.4
(M-H+, nominal mass being 750.4)
773.4 (M-Na+)

C9: 756.4
(M-H+, nominal mass being 755.4)

E5: 715.4
(M-H+, nominal mass being 714.2)

were deposited on a copper target in a solution of glacial acetic acid/3-nitrobenzyl-alcohol.

Peptide Syntheses

12 mg of 4-benzyloxy-2-methoxy-benzylester resin loaded with Fmoc protected amino acid (loading between 0.4 and 0.7 mmol/g resin) were placed into 31 polypropylene wells of a microtiterplate each. Assembly of the peptides on the resins was achieved by applying the synthesis protocol outlined above. To synthesize 31 endothelin segments 170 double couplings were performed. The total amount of amino acid derivatives is given below.

Table 3

No. of double couplings of	[mg]	[mmol]
30 Fmoc-Ala-OH	933 mg	3.0
22 Fmoc-Ser(tBu)-OH	845 mg	2.2
19 Fmoc-Leu-OH	671 mg	1.9
11 Fmoc-Met-OH	408 mg	1.1
17 Fmoc-Asp(tBu)-OH	700 mg	1.7
11 Fmoc-Lys(Boc)-OH	516 mg	1.1
11 Fmoc-Glu(tBu)-OH	469 mg	1.1
11 Fmoc-Val-OH	373 mg	1.1
11 Fmoc-Tyr(tBu)-OH	506 mg	1.1
11 Fmoc-Phe-OH	426 mg	1.1
10 Fmoc-His(Trt)-OH	620 mg	1.0
6 Fmoc-Ile-OH	212 mg	0.6

Cleavage from the resin was performed manually using equipment for the liquid handling of microtiterplates. The resins were 5 times washed with dichlormethane, dried in a desiccator under reduced pressure and treated twice with 300 ul of trifluoroacetic acid / 5% anisol for ten min. The solution was filtered and the resin washed three times with each 300 ul trifluoroacetic acid / 5% anisol. After evaporation in vacuo the residues were treated with diethylether to precipitate the peptides. Crude peptides were again washed with diethylether dried and lyophilized, yield 3-5 mg.

ACKNOWLEDGEMENTS

The authors want to thank A. de Haas for the development of the software. The excellent technical assistance of W. Knapp, M. Pudlo and S. Klein is gratefully acknowledged. We wish to thank H.-J. Foerster and E. Ecker for FAB-MA analyses.

REFERENCES

1. R.A. Houghten, Proc.Natl.Acad.Sci. U.S.A. 82, 5131 (1985).
2. R. Frank and R. Doering, Tetrahedron, 44, 6031 (1988).
3. Z.M. Ruggerie, R.A. Houghton, S. Russell, and T.S. Zimmermann, Proc.Natl.Acad.Sci. U.S.A. 83, 5708 (1986).
4. M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M.-M. Kobayashi, Y. Mitsui, Y.Yazaki, K. Goto, and T. Masaki, Nature 332, 411 (1988).
5. G. Schnorrenberg, and W. Knapp, German patent application P 3823576 (1988).
6. J. Meienhofer, M. Waki, E.P. Heimer, T.J. Lambros, R.C. Makofske, and C.-D. Chang, Int.J.Pept.Protein Res. 13, 35 (1979).
7. E.Atherton, E.J. Gait, R.C. Sheppard, and B.J. Williams, Bioorg.Chem. 8, 351 (1979).
8. S.-S. Wang, J.Am.Chem.Soc. 95, 1328 (1973).
9. M. Mergler, R. Tanner, J. Gosteli, and P. Grogg, Tetrahedron Lett. 29, 4005 (1988).
10. F. Albericio, and G. Barany, Int.J.Pept. Protein Res. 30, 206 (1987).
11. W. Koenig, and R. Geiger, Chem.Ber. 103, 788 (1970).
12 D. Sarantakis, Biochem.Biophys.Res.Commun. 73, 336 (1976).
13 D.M. Benjamin, J.J. McCormack, and D.W. Gump, Anal.Chem. 45, 1531 (1973).

Table 2 **Endothelin segments synthesized**

endothelin	C S C S S L M D K E C V Y F C H L D I I W
entry	
A1	A S A S S L
A3	A S A S S L M
A5	S A S S L M
A7	S A S S L M D
A9	A S S L M D
A11	A S S L M D K
B2	S S L M D K
B4	S S L M D K E
B6	S L M D K E
B8	S L M D K E A
B10	L M D K E A
B12	L M D K E A V
C1	M D K E A V
C3	M D K E A V Y
C5	D K E A V Y
C7	D K E A V Y F
C9	K E A V Y F
C11	K E A V Y F A
D2	E A V Y F A
D4	E A V Y F A H
D6	A V Y F A H
D8	A V Y F A H L
D10	V Y F A H L
D12	V Y F A H L D
E1	Y F A H L D
E3	Y F A H L D I
E5	F A H L D I
E7	F A H L D I I
E9	A H L D I I
E11	A H L D I I W
F1	H L D I I W

Synthetic example

Overlapping hexa- and heptapeptide segments of endothelin (all cysteines replaced by alanine) have been synthesized (table 2). 31 peptides have been assembled on the 4-benzyloxy-2-methoxy-benzylester resins loaded with the Fmoc amino acids (12 mg per well corresponding to about 5 umol resin bound Fmoc amino acid) using double coupling cycles with DIC as indicated above. Splitting from the resins and side chain deprotection have been achieved by treatment with trifluoroacetic acid / 5% anisol two times for 10 minutes. Removal of solvent under reduced pressure, precipitation and washing with diethylether and lyophilization yielded crude peptides in quantities from 3 up to 5 mg. Some HPLC analyses and FAB-MS are shown in figures 2 and 3 giving evidence for the purity of the crude products.

EXPERIMENTAL

All reagents, solvents, Fmoc protected amino acids and resin-bound Fmoc amino acids were purchased from various commercial suppliers. Side chain protection for Ser,Tyr,Asp and Glu were tBu, for Lys tBoc, for His trityl (Trt). HPLC analyses were carried out on a reversed phase silica gel column (Dynamax C18 3 u 50 x 21.4 mm) with gradients of acetonitrile/water acidified with 0.2% trifluoroacetic acid, solvent A being water/acetonitrile 95/5, solvent B water/acetonitrile 20/80. Positive ion FAB spectra were recorded on a Finnigan MAT 90 equipped with an Ion Tech 11 FN saddle field gun delivering 8-9 keV xenon atoms. Samples