Tetrahedron Letters,Vol.28,No.38,pp 4469-4472,1987 0040-4039/87 $3.00 + .00
Printed in Great Britain Pergamon Journals Ltd.

CONTINUOUS-FLOW SOLID-PHASE PEPTIDE SYNTHESIS

Viktor Krchňák*, Josef Vágner
Léčiva-Pharmaceuticals, 143 10 Praha 4,
Martin Flegel
Institute of Pharmacology, Czechoslovak Academy of Sciences
128 00 Praha 2,
and Otakar Mach
Institute of Molecular Genetics, Czechoslovak Academy of Sciences,
166 37 Praha 6, Czechoslovakia

Summary: We describe a simple manually operated synthesizer for solid-phase peptide synthesis (SPPS). The synthesis is performed on standard polystyrene-based resin in a flow reactor under low-pressure conditions. The usefulness of the present configuration of SPPS is exemplified on the synthesis of two octapeptide and one decapeptide amides.

Solid-phase peptide synthesis, developed and introduced by R.B.Merrifield[1,2], can by carried out either discontinuously, batchwise or in a continuous-flow (CF) manner. It is generally accepted that CF SPPS under low-pressure conditions requires specially designed, mechanically rigid carriers to prevent compression of the resin and to facilitate a reasonable flow rate through the reaction column[3]; otherwise a high-pressure flow system is necessary for passing solvents through the resin-packed column[4]. Below, we describe a low-pressure CF SPPS configuration that utilizes standard copoly(styrene-divinylbenzene) resin.

A scheme of the synthesizer for CF SPPS has been delineated in Fig. 1. Reservoirs R for solvents and reactants are connected via distribution valve V1 with the flow reactor F and, via four-port valve V2, with waste. Solvents and reactants are passed through the flow reactor by means of 20-50 kPa overpressure of nitrogen gas applied into reservoirs R, the flow rate being controlled by this overpressure. The reservoir for activated amino acid A is connected via the four-port valve V2, a peristaltic pump P and distribution valve V1 with the flow reactor F. Switching over valve V2 enables recycling of the activated amino acid by means of the peristaltic pump P. All connections are made with teflon tubing; the only exception is the silicon rubber tubing used in the peristaltic pump, which can withstand the N,N-dimethylformamide (DMF) used to dissolve activated amino acid.

Reportedly, neither standard polystyrene-based resin nor poly(dimethyl-acrylamide) resin can be used in low pressure CF SPPS[3,4]. However, we found that copoly(styrene-divinylbenzene) resin can be advantageously used for low-pressure CF SPPS providing the resin is given sufficient space in the flow reactor (approximately equal to the volume of the freely swollen resin) and

constant moderate overpressure (20-50 kPa) is applied to effect solvent flow (rather than maintaining constant flow by a pumping system). When the available space in the flow reactor was smaller than the swollen-resin volume, the flow rate decreased, reaching practically zero value when the column volume was cca 60% that of the swollen resin (V.K., J.V., unpublished results). The solvent flows through the interstitial channels among the resin beads and when the beads are compressed (e.g. owing to inadequate column volume) the solvent cannot pass. The moderate overpressure we use precludes such compressing of resin beads as occurs in pumped systems (cf.[3]). To make a solvent flow through the internal channels of the beads the pressure has to be several orders higher in view of the small size of these channels. Because the swelling volume of peptidyl-resin changes in different solvents, and also in the course of the synthesis, we devised a simple flow reactor with an adjustable inner volume. The reactor consists of a polypropylene syringe (20-ml volume) equipped with a moving teflon piston (Fig. 2) and can be charged with up to 2 g of resin.

Fig. 1. Scheme of the synthesizer

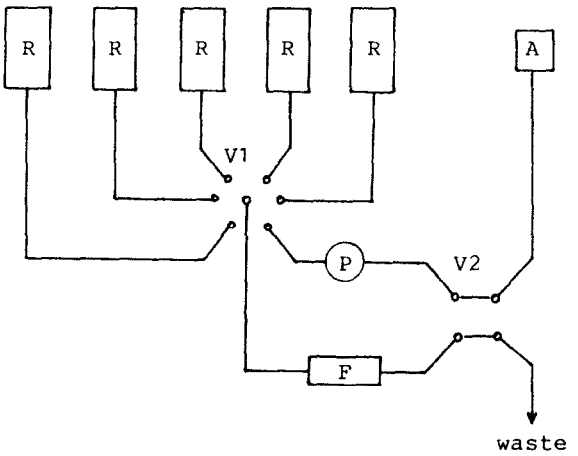
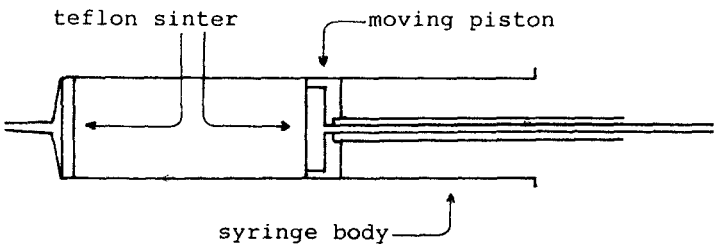


Fig. 2. Flow reactor with adjustable volume



The synthesis was accomplished using the most common t-butyloxycarbonyl (Boc)-benzyl (Bzl) protection strategy[2] on 1.5 g of p-methylbenzhydrylamine copoly(styrene-1% divinylbenzene) resin (0.4 meq/g; Chemical Dynamic Corporation, New Jersey). Temporary protecting groups were removed by trifluoroacetic acid (TFA), condensation was performed with a four molar excess of preformed N-hydroxybenzotriazole (HOBt) esters in DMF. The condensation was monitored by 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, the compound recommended by Sheppard for the preparation of self-indicative active esters[5]. We used this triazine, in contrast to Sheppard[5], only in an indicative amount. After dissappearence of the yellow colour we checked the sample of peptidyl-resin for the presence of free amino groups by the ninhydrin test[6], which was negative in all cases thus showing the applicability of this triazine in an indicative amount to the standard polystyrene-based resin (the resin itself, obviously, must be white or pale yellow). One typical synthetic cycle consisted of the following steps: (1) deprotection, 40% TFA in DCM (v/v), 30 min (3 min flow, 27 min respite); (2) washing, DCM, to slightly acidic effluent (checked by wetted pH paper); (3) neutralization, 5% N-ethyl-N,N-diisopropylamine in chloroform (v/v), 3 min; (4) washing, DMF, to neutral effluent; (5) condensation: take protected amino acid, 4 equiv in DCM (or DCM:DMF, 4:1, if neccessary), add HOBt, 4 equiv in DMF, add DCC, 4 equiv in DCM, 30 min, 20 °C, filter, rinse with DCM, evaporate DCM under reduced pressure ($t<25$ °C), dilute with DMF, filter, rinse with DMF, adjust with DMF to 0.2 M solution, add indicative amount of triazine, place in reservoir A, recycle till yellow colour has disapeared (flow rate 2 ml/min), withdraw a small sample of peptidyl-resin, test with ninhydrin[6], recycle if neccessary; (6) washing, DCM, 5 min. The flow rate during the washing steps was cca 20-30 ml/min, overpressure 20-50 kPa. Typical duration of one reaction cycle was cca 1-1.5 h. The synthesis ended with the last Boc group being removed (step (1)) and the peptidyl-resin being washed with DCM and methanol and dried in a stream of nitrogen. Crude peptides were obtained after "low-high" hydrogen fluoride treatment as described by Tam at al[7].

We used this method to synthesize the octapeptide amide Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr (P1), the so-called T-peptide[8] coded for by the env gene of the human immunodeficiency virus (HIV)[9], and the octapeptide amide Asp-Asn-Asp-Thr-Thr-Ser-Tyr-Thr (P2), which corresponds to T-peptide in other isolates of HIV[10-12]. We also synthesized the decapeptide amide Leu-Glu-Asn-Glu-Trp-Leu-Ser-Arg-Leu-Phe (P3) coded for by the pol gene of bovine leukaemia virus[13]. The yield of the peptidyl-resins was nearly quantitative. The amino acid analyses, retention times in high performance liquid chromatography (HPLC), and purity of the crude peptides are summarized in Table 1.

The present work showed, that low-presure CF SPPS was compatible with use of standard polystyrene-based resin providing the resin was given sufficient space in the flow reactor for full or nearly full swelling. The above configuration of the SPPS method combines the advantages of the continuous-flow methodology

(fast and efficient washings, low consumption of solvents, high condensation-reaction rates) with the use of a readily available standard polystyrene resin and the well-documented Boc-Bzl protection strategy and gives high yields of crude peptides of a high degree of purity. The application of the method has recently been extended to multiple synthesis of peptides (manuscript in preparation).

Table 1. Analytical data on individual peptides

Pep-	Amino acid analyses										Rt*	purity$
tide	Ala	Arg	Asp	Glu	Leu	Phe	Ser	Thr	Trp	Tyr	(min)	(%)
P1	1.00	-	1.08	-	-	-	1.04	3.93	-	0.94	16.8	91.3
P2	-	-	3.15	-	-	-	1.07	2.80	-	0.97	13.3	90.0
P3	-	0.98	1.02	2.09	2.97	1.02	0.92	-	0.93	-	15.0#	96.3

*Retention times in HPLC under isocratic conditions, the mobile phase being 15% methanol in water and containing 0.1% TFA, column 25x0.4 cm, reversed stationary phase Separon SGX C18, flow rate 50 ml/h; $the area of the peak of the product to the area of all peaks meassured at 220 nm; #60% MeOH.

References

1.Merrifield, R.B., J.Am.Chem.Soc. 85, 2149 (1963).
2.Barany, G., Merrifield, R.B., in The Peptides, Analysis, Synthesis, Biology, (Gross, E., Meienhofer, J., eds.), vol.2, p. 1, Academic Press, New York (1980).
3.Dryland, A., Sheppard, R.C., J.Chem.Soc., Perkin Trans. I 125 (1986), and references cited therein.
4.Lukas, T.J., Prystowsky, M.B., Erickson, B.W., Proc.Natl.Acad.Sci. USA 78, 2791 (1981).
5.Atherton, E., Cameron, L., Meldal, M., Sheppard, R.C., J.Chem.Soc., Chem.Commun. 1763 (1986).
6.Kaiser, E., Colescott, R.L., Bossinger, C.D., Cook, P.I., Anal.Biochem. 34, 595 (1970).
7.Tam, J.P., Heath, W.F., Merrifield, R.B., J.Am.Chem.Soc. 105, 6442 (1983).
8.Pert, C.B., Hill, J.M., Ruff, M.R., Berman, R.M., Robey, W.G., Arthur, L.O., Ruscetti, F.W., Farrar, W.L., Proc.Natl.Acad.Sci. USA 83, 9254 (1986).
9.Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stempien, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A., Dina, D., Luciw, P.A., Science 227, 484 (1985).
10.Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., Alizon, M., Cell 40, 9 (1985).
11.Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., Wong-Staal, F., Nature 313, 277 (1985).
12.Muesing, M.A., Smith, D.H., Cabradilla, C.D., Benton, C.V., Lasky, L.A., Capon, D.J., Nature 313, 450 (1985).
13.Sagata N., Yasunaga T., Tsuzuku-Kawamura J., Ohishi K., Ogawa Y., Ikawa Y., Proc.Natl.Acad.Sci. USA 82, 7879 (1985).

(Received in UK 13 July 1987)