AUTOMATIC MONITORING OF SOLID PHASE SYNTHESIS OF A DECAPEPTIDE

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1. Introduction

A method for monitoring of solid phase peptide synthesis has been described [1, 2]. The method is based upon a potentiometric titration of the whole batch of resin bound peptide suspended in a mixture of methylene chloride and glacial acetic acid 1/1 (v/v). Perchloric acid dissolved in glacial acetic acid was used as titrant. A description is given below of an automation of this method in connection with the previously described automatic system for peptide synthesis [3–5]. The combined equipment is tested by the synthesis of the amino acid sequence of antamanid, HPhe–Pro–Pro–Phe–Phe–Val–Pro–Pro–Ala–Phe–resin.

2. Experimental

2.1. *Titration*

The titrations were carried out with 0.04 N $HClO_4$/HOAc as described in [6]*. After 7 to 14 days of operation it was necessary to regenerate the electrodes; the glass electrode by immersion for 48 hr in phosphate buffer, pH 6.5, and the calomel electrode by renewal of both salt solutions. During the synthetic procedure the hole for filling of the outer saltbridge of the calomel electrode was blocked.

The initial potential was approx. –290 mV, and the preset endpoint –482 mV. The delay shut off time was 5 min, the proportional band 0.05 and the chart paper speed 1 cm/min.

The reactor used was of the type previously described [2]. In the present experiment, however, a reactor with exchangeable filters of sintered glass and teflon was used.

2.2. *Pipetting of* $(C_2H_5)_3N$

Due to instability of the $(C_2H_5)_3N/CH_2Cl_2$ mixture, separate pipetting of the two components was carried out (fig. 1). When the code for $(C_2H_5)_3N$ is read, 0.3 ato. N_2 is applied on the $(C_2H_5)_3$ N container by energizing the three-way valve D_1. The liquid is transferred to vessel A through a teflon tubing. When the detector C_2 is sensing the liquid [7], D_1 is de-energized and the pressure vented off. Because of communicating vessels $(C_2H_5)_3N$ flows back into the container to a level in A, determined by the position of the tip of the teflon tubing. When the tubing is empty, which is ascertained by the liquid detector C_1, the control unit signals to the reader to advance the punched tape. The code for emptying of A is now read and N_2-pressure is applied by energizing the three-way valve D_2. Simultaneously the liquid valve of the synthesizer's metering flask is energized and the liquid flows on to the reactor. After the liquid detector has sensed end of flow, the control unit commands punched tape advance. The accuracy with which $(C_2H_5)_3N$ is measured off is ± 40 µl independent of the total volume. In order to let the control unit of the synthesizer be able to control the metering of $(C_2H_5)_3N$, a sequential circuit was developed for the control unit. The circuit secures that alarm will be activated by mal-

*FEBS Letters 19 (1972) 346: In the explanation for the coding: T: Glacial acetic acid should be: T: abs. ethanol, U: glacial acetic acid.

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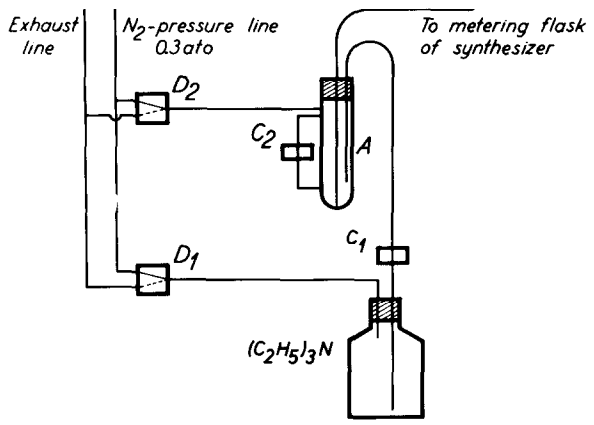

Fig. 1. Diagram of $(C_2H_5)_3N$ pipetting system.

functions of the detectors C_1 and C_2 and also by an empty container.

2.3. *Automation*

All operations were coded on punched tape and automatically carried out by the combined synthesizer–titration equipment (fig. 2). The titration equipment consisted of Radiometer titrator TTT2b, autoburette ABU13 (25 ml), stripprinter PRS1 and recorder Servograph REC51b. The standard delay shut off times of the titrator were increased by a factor of 10 by modifying the shut off timer. A relay control was installed for the stand-by function allowing the titration equipment to be operated by the synthesizer's control unit.

An interface was constructed in order to let the titration equipment be connected to the synthesizer. The interface allowed the following functions to be carried out: 1) initiation of the titration equipment, 2) stirring and chart paper advance in a preselected period (1 min), 3) operations mentioned under 2 plus pen down for recording of the initial potential in a preselected period (1 min), 4) operations mentioned under 3 plus start of addition of the titrant, and 5) shut off involving advance of punched tape, stand-by, pen up and chart paper stop.

2.4. *Cycles performed*

The following cycles were performed for titration, cleavage of the Boc-Group , and coupling (table 1). The same titration cycle was carried out before and after cleavage. The numbers in columns I, II and III indicate how many times each operation was performed. The reactor was drained after each operation.

2.5. *Reagents and solvents*

Boc-L-amino acids were prepared according to [13] or purchased from Reanal. DCC purum was obtained from Merck. Methylene chloride from Merck was pro analysi grade and not further purified, ethanol content max. 0.3%. HOAc was May and Baker pronalys grade. C_2H_5OH was obtained from the Danish Distillers Ltd., Pharmacopoeia Nordica grade. Triethylamine puriss was obtained from Fluka. The resin used was Bio-Rad, Bio-Beads S-X2, 200–400 mesh. Before the chloromethylation the resin was suspended in methylene chloride for removal of smaller particles. 1-^{14}C-acetic acid was obtained from NEN Chemicals.

2.6. *Synthetic procedure*

The following resins were used: A) 1.036 g Boc–Pro–resin with a substitution degree of 0.972 meq Boc–Pro/g Boc–Pro–resin. B) 5.00 g Boc–Pro–resin with a substitution degree of 0.681 meq Boc–Pro/g Boc–Pro–resin. In the preparation of resins A and B chloromethylation and esterification, respectively, were carried out according to [8, 9]. C) 2.231 g Boc–Phe–resin with a substitution degree of 0.456 meq/g Boc–Phe–resin. The chloromethylation was carried out at room temp in 1, 2-dichloroethane by addition of chloromethyl–methyl–ether and $SnCl_4$ [10]. The esterification was carried out according to [11] in order to avoid formation of quarternary ammonium groups. The substitution degree was determined by perchloric acid titration. The Boc-amino acids and DCC were added in 3-fold excess based on the resin-bound amino acid.

2.7. *Amino acid analysis*

Hydrolysis for 96 hr at 110° of the resin-bound peptides was performed in a 1:1 mixture of 6 N hydrochloric acid/glacial acetic acid in evacuated, sealed ampoules. The hydrolysis medium was removed by evaporation at reduced pressure and by low temperature [12].

2.8. *Tracer experiments*

Counting of the radioactive samples was carried out using a Packard Tri-Carb 2420 liquid scintillation spectrometer. The resin samples were combusted in a Packard Tri-Carb 305 sample oxidizer.

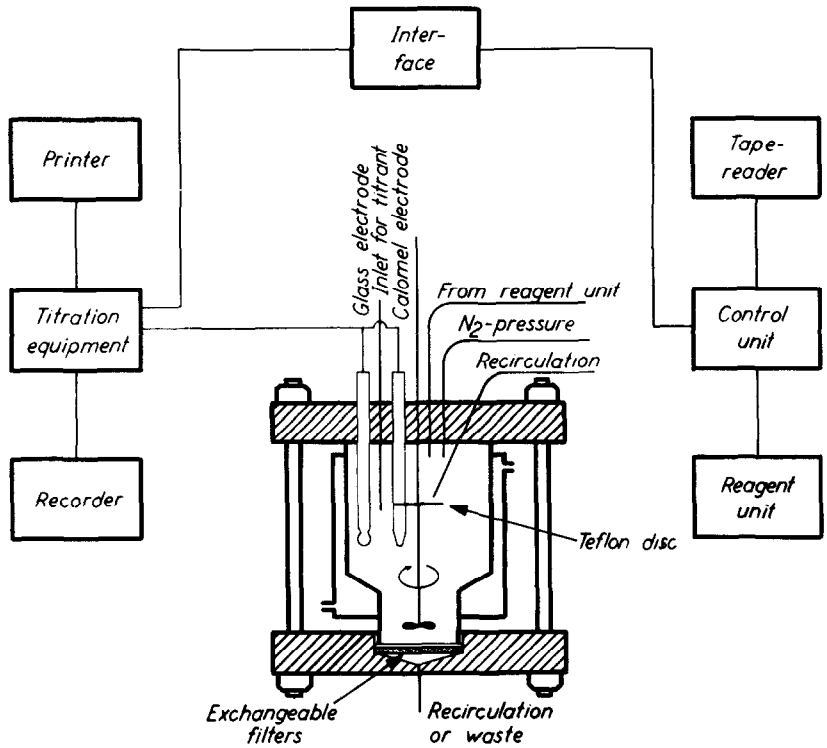

Fig. 2. Diagram of the combined synthesizer–titration equipment. Hatching indicates teflon.

3. Results

3.1. *Formation of proline diketopiperazine, cyclo-(Pro–Pro–)*

In an experiment in which the synthesis was commenced by coupling of Boc–Pro to Pro-resin, resin A, the following titration values of the total batch were found: Boc–Pro–resin 0.180 meq, Pro–resin 1.187 meq, Boc–Pro–Pro–resin 0.183 meq, Pro–Pro–resin 0.535 meq, and after repeated treatment with HCl/HOAc, 0.459 meq. Thus the coupling should be near 100%. The titration value after cleavage revealed, however, a pronounced decrease in the amount of detectable amino groups.

The synthesis was repeated in a larger scale using resin B and followed by amino acid analysis only. The combined CH_2Cl_2 and EtOH filtrates were collected after the treatment with $(C_2H_5)_3N/CH_2Cl_2$ and evaporated to dryness under reduced pressure. The solid residue was analyzed by mass spectrometry and pro-

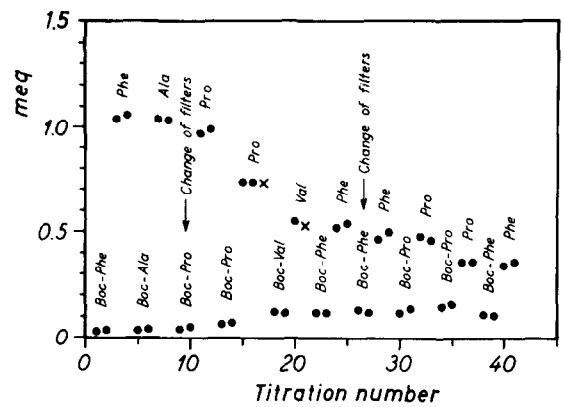

Fig. 3. Titration values determined during the synthesis of the sequence of antamanid: HPhe–Pro–Pro–Phe–Phe–Val–Pro–Pro–Ala–Phe–resin. x indicates that the procedure for cleavage of the Boc-group was repeated before the titration.

Table 1
Cycles performed.

I	II	III	Solvents and reagents	Volume (ml)	Time (min)
1	0	1	CH_2Cl_2	35	2.5
1	0	1	$(C_2H_5)_3N/CH_2Cl_2$	4.2 + 35	10
6	0	5	CH_2Cl_2	35	2.5
3	3	0	EtOH	35	2.5
3	3	0	HOAc	35	2.5
1	0	0	HOAc + CH_2Cl_2	35 + 35	10–30*
0	1	0	N HCl/HOAc	35	5
0	1	0	N HCl/HOAc	35	30
0	3	0	HOAc	35	2.5
0	0	1	Boc-Amino acid/CH_2Cl_2	35	30**
0	0	1	DCC/CH_2Cl_2	35	120
3	0	3	CH_2Cl_2	35	2.5
0	3	4	EtOH	35	2.5
0	2	2	CH_2Cl_2	35	2.5

I: Titration cycle. II: Boc-group cleavage cycle. III: Coupling cycle.
* Titration.
** No reactor drain.

nounced peaks at masses 194, 166 and 70 were found, corresponding to the molecular ion of proline diketopiperazine, loss of CO from the molecular ion, and the amine fragment of proline, respectively.

The amino acid analysis of the Boc–Pro–Pro–resin showed 1.01 mM proline/g Boc–Pro–Pro–resin and, after cleavage of the Boc group, treatment with $(C_2H_5)_3N/CH_2Cl_2$ and washings with CH_2Cl_2, EtOH, and HOAc, 0.42 mM proline/g Pro–Pro–resin was found.

3.2. *Titration experiments*

In fig. 3 the titration values determined during the synthesis of the amino acid sequence of antamanid on resin C are shown. Correction has been carried out for the sample removed at the heptapeptide stage. The titration values of the Boc protected N-terminals show an increase during the synthesis. The values after cleavage of the Boc-group show a pronounced decrease in the number of titrable groups at the tetra, penta, and nona peptide stage. A minor decrease was obser-

Table 2
Amino acid content of the synthesized products.

		Theoretical	Titration	Amino acid analysis		
Heptapeptide	Phe	3	3.00	1) 3.00		
	Ala	1	1.68	1.56		
	Pro	2	2.68	2.83		
	Val	1	0.70	0.68		
Decapeptide	Phe	4	4.00	1) 4.00	2) 4.00	3) 4.00
	Ala	1	1.97	1.74	1.88	1.92
	Pro	4	4.23	4.62	4.65	4.83
	Val	1	0.82	0.76	0.77	0.82

1) Resin-bound product. 2) Cleaved crude product. 3) Ether precipitated product.

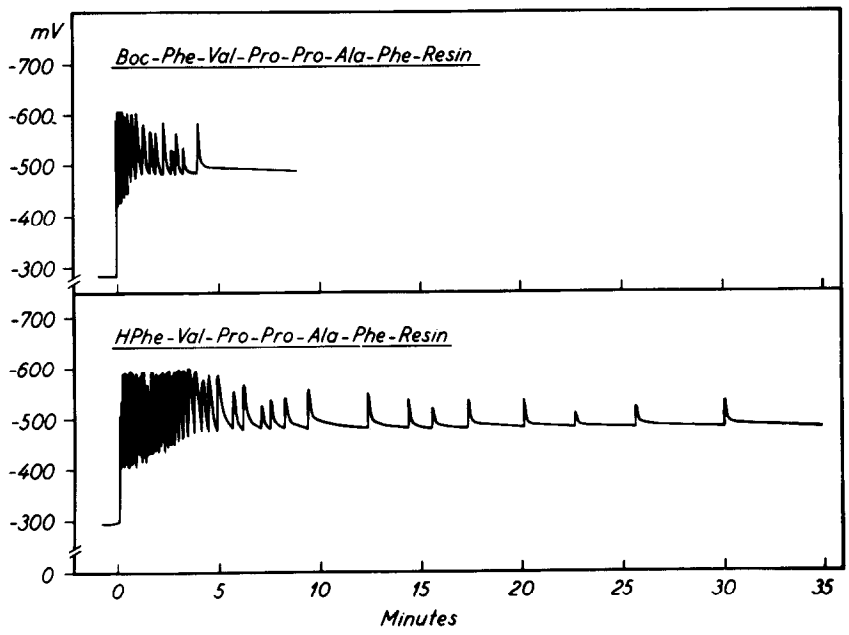

Fig. 4. Recording of variations of the potential during the titration.

ved after the Pro to Ala and the Phe to Phe coupling, which, however, may have been due to a slight loss of material caused by the change of filters. As indicated in fig. 3 extra treatment with HCl/HOAc was performed twice, but did not result in an increase in the titration value.

The average accuracy of the means of the double determinations calculated from SEM = $\sqrt{\Sigma(\Delta)^2/4n}$ was found to be ± 0.007 meq.

In table 2 the results of the amino acid analyses are compared with the amino acid composition calculated from the titration values after 7 steps, after the complete synthesis and after cleavage of the peptide from the resin. By the calculation of the amount of the single residues, the titration value obtained before cleavage of the Boc-group has been subtracted from the value obtained after cleavage. A reasonably good agreement was found between the values obtained by the two methods.

In fig. 4 the potential is recorded during the titration after coupling of Boc–Phe to Val and after cleavage of the Boc-group. A stable initial potential was recorded as well as the changes of the potential due to the additions of the titrant. At the end of the titration the potential slowly approached the preset endpoint.

3.3. *Tracer experiments*

The experiments were performed in all glass equipment; for the resin the reactor described by Kusch [14] was used. The solvents were in both experiments applied in 2 ml amounts and stirring was performed during the washings.

0.2 g Teflon was placed in ^{14}C-HOAc (10 μCi/ml) for 22 hr. The sample was thereafter subjected to washings with CH_2Cl_2 for 4 X 2.5 min, $(C_2H_5)_3N$/CH_2Cl_2 for 1 X 30 min, CH_2Cl_2 for 5 X 2.5 min, and finally with CH_2Cl_2 for 1 X 120 min. 4.3 μMole of HOAc could be demonstrated to be present in the final volume of CH_2Cl_2.

0.2 g of resin C was treated for 30 min with N HCl/^{14}C-HOAc (10 μCi/ml HOAc). The normal cycle for cleavage of the Boc-group was then performed, followed by the coupling cycle until the coupling step where washing for 120 min with CH_2Cl_2 was carried out. 0.1 μMole of HOAc was washed out during the final washing.

0.7 μMole HOAc was found to remain in the whole resin batch as determined after combustion of 50–80 mg samples. In the coupling step thus a total of 0.8 μmole was present.

No ^{14}C-HOAc could be detected to leak out from the outer saltbridge of the calomel electrode into the reactor.

4. Discussion

The experiments with C-terminal proline have shown that low yields must be expected by the coupling of another proline on account of the diketopiperazine formation. This certainly is part of the explanation for the low yield found in solid phase synthesis of angiotensin-converting enzyme inhibitors [15]. Diketopiperazine formation by coupling of Leu to C-terminal Gly or Val has been described [16].

The overall agreement in our experiments between the amino acid composition of the synthesized products as determined by the two methods, table 2, shows that the pronounced decreases in the titration values cannot be due to a loss of material, as in the experiments with C-terminal proline. Intrachain diketopiperazine formation has been described for peptides in solution [17]. A reaction of this type, however, would not have influenced the titration value, unless a simultaneous blocking of the liberated amino groups had taken place. Thus the titration values most likely show the exact amount of amino acid incorporated at the various coupling stages. Further experiments must, however, be performed in order to explain the differences in table 2 between the values obtained by the two methods, as these differences must be considered to be real. The differences may be due to a loss of material by changing of the filters or, to a minor degree, due to a slight loss of material throughout the synthesis [18, 19].

Cleavage of the Boc-group was repeated in the positions shown in fig. 3 without resulting in an increase of the titration value. As some increase should be expected if only partial cleavage had occurred, it strongly indicated that the decrease in the number of titratable amino groups during the synthesis was due to an irreversible blocking under the procedure applied.

Thin-layer chromatography of the ether precipitated product with 1-butanol/pyridine/acetic acid/water, 30:20:6:24 as eluent showed 5 spots. Two of these which had the highest R_f values were the most pronounced. For visualizing, spraying with tert. butyl hypochlorite, followed by *o*-tolidine/potassium iodide was used [20].

A mass spectrometric investigation confirmed the presence of the four peptides expected according to the titration values [21]. After cleavage, the crude product was deuteroacetylated and permethylated [22, 23]. The following N-terminal acylated sequences were found: CH_3CO–Pro–MeAla–MePhe, CH_3CO–Pro–Pro–MeAla–MePhe, CH_3CO–Pro–MePhe–MePhe–MeVal–Pro–Pro–MeAla– and CD_3CO–MePhe–Pro–Pro–MePhe–MePhe–MeVal–Pro–.

The fact that a deuteroacetylated N-terminal amino group could only be demonstrated at the decapeptide stage, whereas all the shorter peptides were acetylated, shows that the blocking is due to acetylation. In earlier experiments [6] it was shown that the titration procedure in itself did not result in a blocking of the amino groups as constant titration values could be demonstrated by repeated titration of an alanine resin. The acetylation cannot have taken place during the cleavage of the Boc-group as otherwise a higher content of proline and valine should have been found by the amino acid analysis. The acetylation therefore must have been due to a factor involved in the coupling cycle. Thus the most likely explanation seems to be that the acetylation is caused by the simultaneous presence of DCC and residual acetic acid in an amount large enough to cause an acetylation of approx. 1/4 mmole. The reason that the acetylation especially took place during the Pro to Pro and the Val to Pro couplings in the present experiment may have been due to a slow coupling rate of the two amino acid derivatives involved, allowing a larger degree of acetylation to take place. It seems unlikely that the presence of EtOH in the CH_2Cl_2 should have caused a blocking of free amino groups. Esterification of the Boc-amino acid, however, must be expected to occur to a certain extent, thus reducing the excess of the derivative.

The acetylation in the present experiment can be explained by the demonstrated presence of acetic acid in the resin as well as in the teflon parts of the reactor system.

As seen in fig. 3 a slight increase in the titration values of the Boc protected N-terminals occurred. This increase could be due to amino groups not easily accessible for the Boc-derivatives or DCC and there-

fore a potential source for failure sequences, which also, but only to a slight degree, have been demonstrated by mass spectrometry [21].

The potentiometric recording turned out to be a reliable control of proper function of the titration system. The slow approach of the potential to the preset value at the end of the titration may be due to an impeded reaction with not easily accessible amino groups, or to a slow diffusion of the titrant into the resin.

Further experiments must be carried out in a reactor system made from materials which will not absorb any of the components present in the synthetic procedure. Furthermore a titration procedure without the use of HOAc should be considered. In connection with this possibly another strong acid, e.g. trifluoromethanesulfonic acid may be used.

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