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PEPTIDE SYNTHESIS. PART 11. A SYSTEM FOR CONTINUOUS FLOW SOLID PHASE PEPTIDE SYNTHESIS USING FLUORENYLMETHOXYCARBONYL-AMINO ACID PENTAFLUOROPHENYL ESTERS

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Instrumentation and operating procedures are described for the continuous flow solid phase synthesis of peptides using fluorenylmethoxycarbonyl-amino acid pentafluorophenyl esters. The technique is illustrated by synthesis of the tetradecapeptide (1) corresponding to a part sequence of human cytomegalovirus and the pentadecapeptide sequence (2) from an interferon-induced RNA transcript. The value of spectrometric monitoring of deprotection reactions in solid phase synthesis is demonstrated.

In Part 8 of this series,[1] we described a new technique and equipment for solid phase peptide synthesis under continuous flow conditions. A key feature in this technique was use of a novel physically rigid support consisting of polydimethylacrylamide gel polymerised within the pores of macroporous kieselguhr particles. This provided a functionalised support freely permeated and solvated by polar aprotic solvents suitable for peptide (and oligonucleotide[2]) synthesis set in a rigid framework stable to pumped liquid flow in packed columns.

In this system, peptide bond formation used Fmoc-amino-acids exclusively, usually activated as symmetrical anhydrides. Exceptions were asparagine, glutamine, and lysine, for which activated p-nitrophenyl esters were preferred. Although chemically very efficient, the use of anhydrides may be considered disadvantageous in that manual preparation is required for each derivative immediately before use. This limited operation to a semi-automatic mode in which only one complete cycle of amino-acid addition could be carried out without operator intervention. Simple p-nitrophenyl esters, on the other hand, could be prepared beforehand and stored, but were relatively unreactive and sometimes required lengthy coupling reaction periods.

In Part 10 we gave details of our more recent work using Fmoc-amino-acid pentafluorophenyl esters.[3] These were much more reactive than the corresponding p-nitrophenyl derivatives, yet were usually crystalline and stable to storage. Examples were given of their use in a conventional solid phase technique using a shaken reactor vessel, as well as in a pumped flow

system. Times for complete reaction were usually acceptably short and could be further reduced by use of 1-hydroxybenzotriazole catalysis.

Use of these esters has permitted a further step towards automatic operation. We now describe instrumentation and reaction protocols suitable for unattended sequential addition of several amino-acid residues using continuous flow synthesis. Of course, Fmoc-amino acid pentafluorophenyl esters may also be used in more conventional agitated reaction vessel equipment.

It should be emphasised that entirely unattended operation of solid phase peptide synthesis is not to be commended in every case. It is now well established that reaction rates may vary substantially as synthesis proceeds, and that these variations are a function of amino-acid sequence as well as of individual amino-acid structure.[4,5] It is for this reason that we have also devoted substantial effort to the development of reaction monitoring techniques. In Part 8 we discussed the value of continuous spectroscopic measurement of fluorenyl derivatives in the flowing stream during both acylation and deprotection reactions.[1] For acylation using symmetrical anhydrides when (apart from some amino-acid side chains) the only major uv absorbing species present are the Fmoc-amino-acid anhydride and free acid, useful quantitative information about amino-acid uptake can frequently be obtained as the reaction is proceding. When reliable spectroscopic data is not easily available, we would routinely make extensive use of simple colour tests (ninhydrin[6] and trinitrobenzenesulphonic acid[7]) on withdrawn resin samples to verify completion of acylation reactions (absence of free amino groups). With the system described here, the flowing stream passes at all times through a variable wavelength, uv monitoring system, and the analogue trace obtained provides a continuous check on correct machine (and operator) function. During the acylation phase the additional ultra-violet absorption of pentafluorophenyl derivatives and especially of catalyst hydroxybenzotriazole when used renders difficult quantitative interpretation of spectroscopic data based on uptake of Fmoc-amino-acids from solution onto the solid phase. Thus automatic synthesis using these esters or any other technique not providing feedback information requires experience and exercise of considerable caution by the operator if colour tests are dispensed with. Acylation times should be preset generously.

On the other hand, quantitative monitoring of the deprotection reaction[1] is still easily feasible and was used in the illustrative syntheses of sequences 1 and 2 described below. In normal operation, consistent areas of successive deprotection peaks provide welcome reassurance that all is proceding smoothly and efficiently. Machine failure or operator error is often easily detected. In one synthesis not described here, complete omission of an amino-acid due to

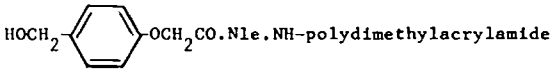
machine malfunction was detected at an early stage. At a more subtle level, its value in detecting slowing of the synthesis reactions due to internal aggregation effects within the resin matrix is shown in the second example below.

H-Thr-Pro-Glu-Val-Asn-Pro-Ile-Asp-Ala-Glu-Gly-Leu-Ser-Gly-OH

(1)

H-Cys(Acm)-Tyr-Ala-Thr-His-Lys-Tyr-Leu-Asp-Ser-Glu-Glu-Asp-Glu-Glu-OH

(2)



(3)

Continuous flow solid phase synthesis can be mechanised very simply. The basic requirement is for a pump, reaction column, and a single valve allowing flow (Fig 1a) and recirculation (fig 1b) modes. For automatic operation, additional valving and controlling mechanism is required for reagent and amino-acid selection. For use with pre-prepared crystalline Fmoc-amino-acid pentafluorophenyl or other esters, a system for dissolving the dry samples and introducing the solutions into the machine is also required. It is convenient if means for flow reversal through the column are also incorporated.

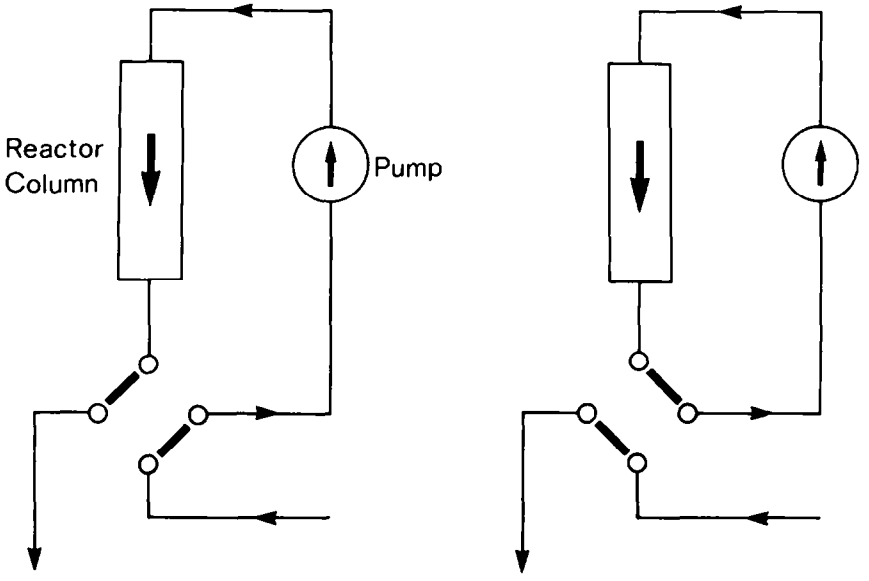


Fig. 1. (a) Flow and (b) recirculate modes.

The synthesiser presently in operation in our laboratory has the flow system depicted in Figure 2. The key valve 1 switches between flow and recirculate functions. Valve 4 controls the direction of flow through the reactor column, and valves 7a and 7b solvent/reagent selection. The Fmoc-polyamide solid phase synthesis is very economical in reagents and only two reservoirs are usually required, one for the single solvent (dimethylformamide, Dmf) employed throughout, and one for the deprotection reagent, 20% piperidine in Dmf. Valve 8 controls nitrogen pressurisation of the reservoirs (which is mantained only during an initial flush and pump priming period), or directs nitrogen to the sample dissolving system for agitation purposes. Flow from the pump is directed by valve 2a either to the reactor column or to the sampling system through valve 2b.

Valve 3 similarly selects the effluent from the column or sampler device. The remaining valves 5 and 6 govern the flow to and from the sample chambers, choosing between Dmf and nitrogen (for agitation) during sample dissolution, and sample and Dmf during transfer to reactor and rinsing on. Valve pairs 2a,b and 7a,b can be ganged with single pneumatic operators and spring returns, requiring only eight control lines in all. Additional control lines are required to operate the amino-acid rotary selector valve RV, the pump, spectrophotometer, and chart recorder. The sample chambers selected in turn

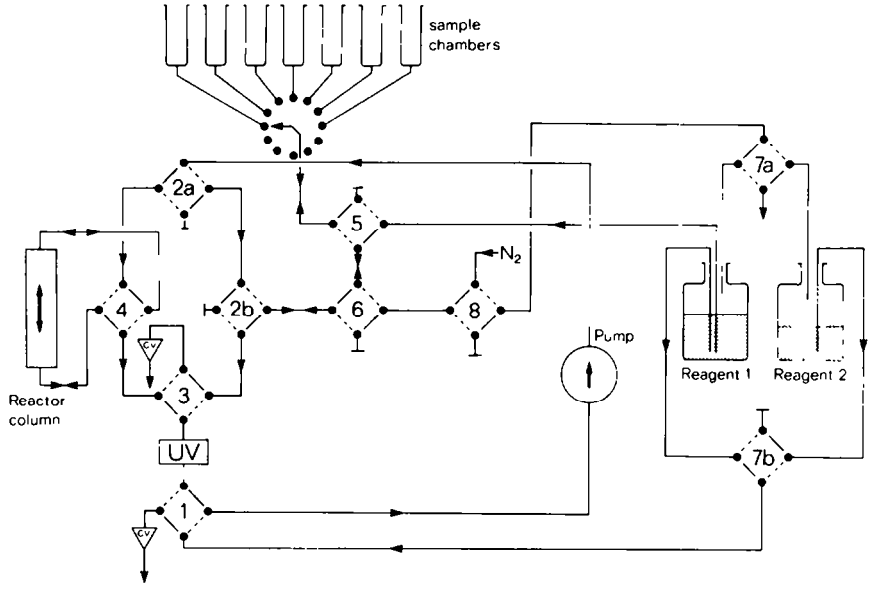


Fig. 2. Fluidics system in the automatic synthesiser. All the valves are shown in their OFF or resting state.

by the rotary valve are simple Luer fitting 5ml syringe barrels equipped with porous glass or ptfe filters. In our instrument, provision has also been made for automatic control of pump speed, but no variation in flow rate is employed in the procedures described. Two simple ball check valves reduce the possibility of syphonage back into the reagent bottles. Additional more positive valving could be advantageous here.

With this valving arrangement the various liquid control functions listed in Table 1 are easily implemented with the valve configurations shown. The manual and automatic control systems are essentially as described for the earlier instrument. Simple toggle switches and indicator lights allow direct manual operation of individual valves. A controlling microcomputer interfaced to relays in parallel with these manual switches provides a higher level of manual control through the keyboard, as well as programmed sequential operation.

	V1	V2	V3	V4	V5	V6	V7	V8	PUMP	RV
Shut down	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	
Recirculate	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	ON	
Flow Dmf	ON	OFF	OFF	OFF	OFF	OFF	OFF	OFF	ON	
Reverse flow Dmf	ON	OFF	OFF	ON	OFF	OFF	OFF	OFF	ON	
Flow piperidine	ON	OFF	OFF	OFF	OFF	OFF	ON	OFF	ON	
Select next amino-acid	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	ON*	STEP
Fill sample chamber	ON	ON	OFF	OFF	ON	OFF	OFF	OFF	ON	
Mix	OFF	OFF	OFF	OFF	ON	ON	OFF	OFF	ON*	
Empty sample chamber	OFF	OFF	ON	OFF	ON	OFF	OFF	OFF	ON	
Rinse sample in	OFF	OFF	ON	OFF	OFF	OFF	OFF	OFF	ON	

Table 1. Valve and pump configurations for the various liquid control functions. * Optional, but see below.

In these last modes, valves are operated in combination through software defined functions generally corresponding to those listed in Table 1. Software facilities for manually extending or curtailing preset reaction times and for data collection from the ultra-violet spectrophotometer and its interpretation are also provided.

Operation of the synthesiser depicted in Fig 2 and the general protocol for flow synthesis using Fmoc-amino acid pentafluorophenyl esters is best illustrated with reference to Table 2. This is a facsimile of the automatic mode function list in the controlling Basic programme. Each data statement**

** This programming technique was adopted largely because the Basic interpreter associated with the controlling microcomputer (Hewlett Packard HP85) did not have string array facilities. It has the advantage of ease of readability within the program and ease of modification.

is read in turn by the computer, the function and any additional numerical parameters interpreted, and the appropriate commands passed to the synthesiser. A permanent record of each operation is also printed with its step number and the time. The parameters following each function and separated from it by commas are of several types. After the commands FLOW or RECIRCULATE, the constant 1 selects a corresponding pump speed, presently 3ml/min. After other liquid or gas transfer functions (FILL, MIX, EMPTY, WASH) relating to the sampling system, the numerical value determines the time duration (in min) for each operation. Exceptionally for FLOW and RECIRCULATE, these times are set in separate TIME statements.* Other parameters control reagent selection and data collection. REAGENT,1 is Dmf (this is the default and need not be specified except to cancel selection of REAGENT,2 (20% piperidine in Dmf). MONITOR,90,4,312 sets the spectrometer to a wavelength of 312nm, zeros the baseline and collects 90 optical density readings at 4 second intervals.

```
100 DATA #01 FLOW,1
105 DATA #02 TIME,2
110 DATA #03 SAMPLE
115 DATA #04 FILL,1
120 DATA #05 MIX,1
125 DATA #06 FILL,.13
130 DATA #07 EMPTY,1.3
140 DATA #09 WASH,.5
150 DATA #11 RECIRCULATE,1
155 DATA #12 TIME,30
156 DATA #XX PAUSE
160 DATA #13 TIME,30
165 DATA #14 FLOW,1
170 DATA #15 TIME,6.5
175 DATA #16 RECIRCULATE,1
180 DATA #17 TIME,.5
185 DATA #18 FLOW,1
190 DATA #19 TIME,5
195 DATA #20 FILL,.5
200 DATA #21 MIX,.2
205 DATA #22 REAGENT,2
210 DATA #23 FLOW,1
215 DATA #24 TIME,.5
220 DATA #25 MONITOR,90,4,312
225 DATA #26 TIME,9
230 DATA #27 REAGENT,1
235 DATA #28 FLOW,1
240 DATA #29 TIME,10
241 DATA #XX PAUSE
245 DATA #30 TIME,10
250 DATA #31 CYCLE
255 DATA #32 HALT
260 DATA #33 END
```

Table 2. Standard controlling protocol for sequential addition of Fmoc-amino-acid pentafluorophenyl esters and deprotection.

Thus the reaction protocol of Table 2 stipulates an initial pre-wash with Dmf for 2min, selection of the next sample chamber containing the solid pentafluorophenyl ester (and hydroxybenzotriazole catalyst if used), filling

* This programming inconsistency has purely historical origins.

it with Dmf for 1 min and then agitation with nitrogen for 1 min. A second brief FILL operation displaces nitrogen from the sample chamber lines, and the sample is then pumped onto the reaction column (EMPTY) and rinsed on briefly with Dmf (WASH). The first recirculation period of 30 min is followed by a PAUSE command. This stops further reading of the programme data statements, and sounds an alarm calling the operator to remove a sample of resin for qualitative ninhydrin or other colour tests. This and the second PAUSE command (line 241; at this point a resin sample is often removed for quantitative amino-acid analysis) are removed for unattended overnight operation. An additional PAUSE is sometimes inserted as line 201. This prevents automatic (and irrevocable) progression to the deprotection cycle without positive action by the operator. Operation of the synthesiser is resumed after the PAUSE (at line 156) has been cancelled from the keyboard, and acylation allowed to continue for a second 30 min recirculation period. Generally, ninhydrin tests indicate complete reaction after the first 30 min period. The excess pentafluophenyl ester and liberated pentafluorophenol are washed from the column in two stages by FLOW (Dmf) for a total of 11.5 min. The brief intermediate RECIRCULATE (lines 175/180) is used to rinse the flow/recirculate valve V1, and the final FILL and MIX operations (lines 195/200) flush and empty the lines to the sample chamber. The acylation step is now complete.

In the absence of an inserted PAUSE command or manual intervention, deprotection follows immediately. Reagent 2 is selected and flowed through the column. This continues while the monitor command is issued to the spectrometer. The optical density readings are immediately displayed on the screen and, when data collection has ceased, plotted, integrated, and printed (see, for example, ref 1). The flow of deprotecting reagent (20% piperidine) is terminated after a total of about 10 minutes. This is unnecessarily long for almost all deprotection reactions. Completion of deprotection can be verified by visual inspection or computer interpretation of the deprotection data (see below), and some experienced operators reduce deprotection times to 2 min or less with the option of manual or automatic repetition. Under these circumstances, the deprotection product (presumably piperidine-dibenzofulvene adduct, though we have not verified complete conversion of the initially liberated dibenzofulvene on this time scale) continues to emerge from the column during the following Dmf wash (lines 230-240). As indicated above, the optional PAUSE at line 241 enables a resin sample to be removed for analysis. The wash is then continued (total 20min) before the CYCLE command restores the reading of DATA statements to line 100 and synthesis continues with the next programmed amino-acid residue.

Operation is illustrated below for the straightforward tetradecapeptide (1), a part sequence of human cytomegalovirus. In this synthesis, pentafluorophenyl ester derivatives were used throughout except for O-t-butyl-serine and threonine residues, and the single asparagine. As we have already discussed, crystallisation of the first two very soluble pentafluorophenyl esters is difficult, and in this early synthesis preformed anhydrides were introduced manually. Esters of 3-hydroxy-4-oxodihydrobenzotriazine now provide[8] appropriate alternative activated derivatives for these two Fmoc-amino-acids (see below). For the asparagine residue, the p-nitrophenyl ester was used as no sample of the pentafluorophenyl derivative was available in the laboratory at the time.

The starting resin (3) functionalised with the acid labile linkage agent and internal reference norleucine residue was prepared on a large (10g) scale. With approximately four fold excesses and equivalent amounts of hydroxybenzotriazole, both acylation reactions using the pentafluorophenyl esters of Fmoc-norleucine and 4-hydroxymethylphenoxyacetic acid were complete when colour tests were taken after 45 min using the flow rate (3ml/min) customary for smaller scale syntheses. Intermediate deprotection of the Fmoc-norleucyl resin was carried out with 20% piperidine in dimethylformamide in the usual manner. The C-terminal glycine residue was transesterified to part (ca 2.5g) of this resin using the Fmoc-amino-acid pentafluorophenyl ester (five fold excess) in the presence of dimethylaminopyridine catalyst. This reaction was repeated and the resin then acetylated as precautionary measures, though later amino-acid analysis showed that incorporation of glycine was approximately 90% after just one transesterification reaction.

Addition of the following serine residue utilised the preformed symmetric anhydride as discussed above. The following 11 amino-acid residues preceding the final threonine were added automatically without intervention. Four fold excesses of each pentafluorophenyl ester together with hydroxybenzotriazole were used and 60 min was allowed for each acylation reaction. Quantitative spectroscopic data were collected for the release of piperidine-dibenzofulvene adduct into the flowing stream in every deprotection step. The scope and limitations of this monitoring procedure has been discussed in detail previously.[1] The results (Experimental part) were very satisfactory and were consistent with a smooth assembly. In our experience, more reliable data is obtained from flow systems during fully automatic operation than with manual intervention. Probably this is attributable to greater constancy of flow rate when the pump is left undisturbed throughout the synthesis. The final residue (O-t-butyl-threonine) was added as its symmetrical anhydride. The first

attempted coupling was immediately terminated and the system washed out when the uv monitor showed a much smaller peak than anticipated for introduction of the anhydride into the flowing stream. This was due to a weighing error. A repeat acylation proceeded uneventfully.

The peptide was detached from the resin and the side chain protecting groups cleaved with 95% aqueous trifluoroacetic acid in the usual manner. The hplc profile of the crude product is shown in Fig. 3. The two peaks eluting at the start of the gradient did not contain significant peptide material as determined by amino-acid analysis.

They were easily removed by preparative hplc, giving purified product with the elution profile of Fig. 4. Analytical and other details of the assembly are given in the Experimental part.

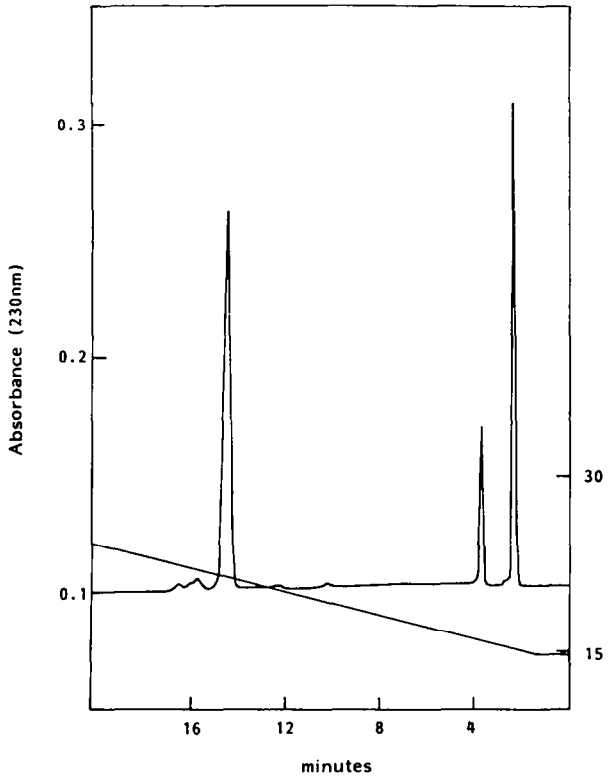


Fig 3. Hplc profile of total crude tetradecapeptide (1). Conditions: Aquapore 300 reversed phase column; reservoir A contained 0.1% aq trifluoroacetic acid and, B 90% acetonitrile, 10% A; linear gradient 15-30% B over 30 min at 1.5ml/min. Absorbance measurement at 230nm.

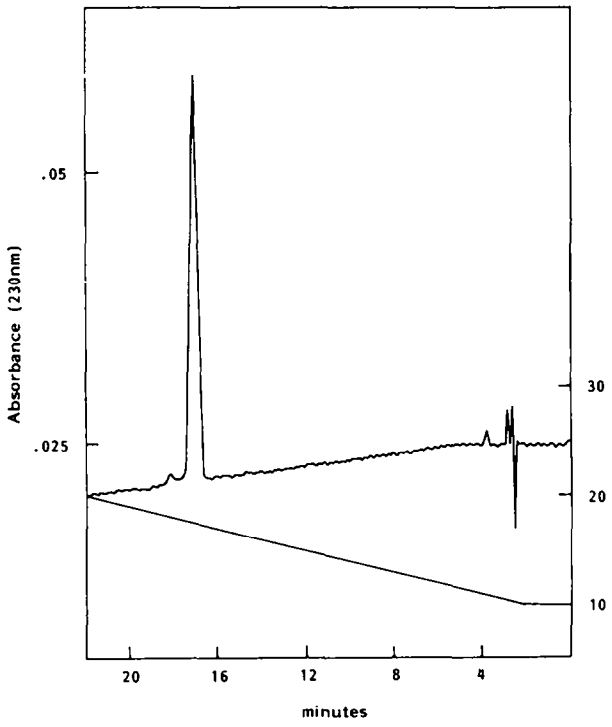


Fig 4. Hplc profile of purified tetradecapeptide (1): Conditions as for fig 3 except that the gradient was 10-25% B over 30 min.

The synthesis technique is further illustrated by the pentadecapeptide (2), derived from an interferon-induced RNA sequence. This is predictably a distinctly more difficult sequence than that of the foregoing tetradecapeptide. All but two of the constituent amino-acids contain reactive side chains, including those of histidine and cysteine. In addition the particularly labile aspartylserine sequence is present. In the protected form, there is a substantial build up of apolar side chains from the carboxy terminus backwards, with no intervening glycine or proline residues. Such a situation has previously been thought to contribute to aggregation effects within the resin matrix with consequent slowing of the synthesis reactions.[9] The assembly was carried out much as in the foregoing, though between times there had been some useful software refinement. Spot optical density readings were made automatically after sample entry and washout, as a check for correct machine operation. The areas of deprotection peaks were automatically compared with a running average of those of previous residues, and their shapes were examined for evidence of trailing. By this time the value of Fmoc-amino-acid 3-hydroxy-4-oxodihydrobenzotriazine esters in solid phase synthesis had been recognised,[8] and these derivatives were now utilised for

O-t-butyl-serine and threonine, permitting fully automatic assembly. The S-acetamidomethyl group was used for side chain protection of the N-terminal cysteine residue. The pentafluorophenyl ester of this derivative has proved to be a superior reagent to the symmetrical anhydride, which is markedly insoluble and commonly precipitates along with dicyclohexylurea during its preparation.

The carboxy terminal residue (t-butyl glutamate) was again added using the pentafluorophenyl ester in the presence of dimethylaminopyridine. The usual precautions[1] were taken to avoid prior contact of the optically active pentafluorophenyl ester and the basic catalyst, minimising the risk of racemisation.[10] Transesterification was later shown to be 85% complete after a single coupling, but the reaction was repeated and the resin acetylated as before. Chain extension was then carried out automatically. Spectrometric monitoring of the deprotection peaks gave unexceptional results for the first 11 residues, but at this stage a sudden fall in relative peak area occurred from 32.6 (histidine) to 23.3 (threonine). The controlling microprocessor flagged a warning for this fall to 71% of the previous average and automatically transferred to a safe close-down routine. It had also noted in the printed record that the previous histidine deprotection peak was trailing, though its area was normal. Inspection of the threonine peak shape (Fig 5a; cf, for example, the typical profile for the second aspartyl residue, Fig 5b) showed the characteristic severe trailing effects which we have previously recognised[1,11] and associated with slowing of deprotection* (and subsequent acylation reactions) due to onset of internal peptide-resin aggregation phenomena.[5] With trailing deprotection peaks,** the reduced area is usually attributable not to incomplete deprotection (or incompletion of the previous acylation reaction) but to delayed elution of part of the deprotection product in a different concentration of piperidine when its extinction coefficient is less.[1] This point is established in the present case by the absence of significant amounts of failure peptides in the final product. All deprotection profiles prior to the histidine residue were similar to Fig 5b, and all subsequent to alanine were similar to Fig 5a. These last were all recognised as anomalous by the computer, though since a new running average of deprotection areas was established at the restart, the synthesis then continued automatically from alanine to the end of the sequence.

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* Or slower physical release of the deprotection product from the resin column into free solution.

** Note that in the present experiments, deprotection data was collected for 6min whereas the total deprotection time was about 10 min.

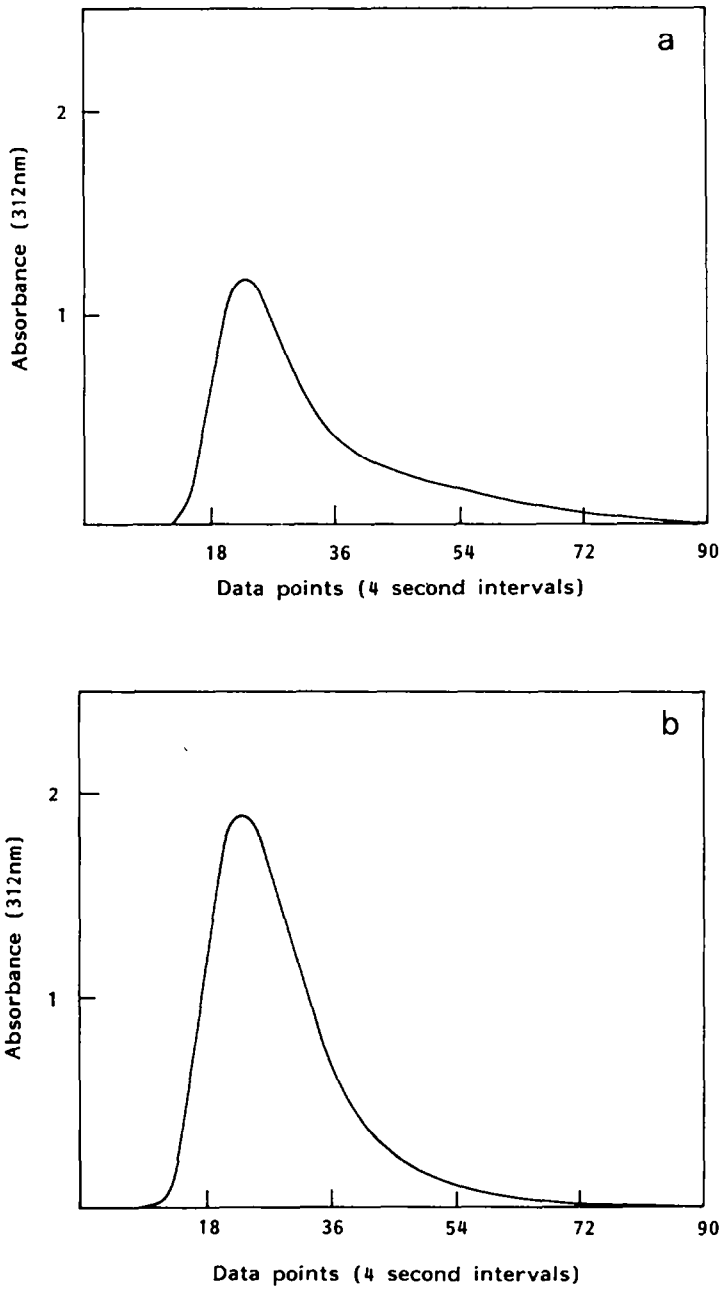

Fig 5. Deprotection profiles for (a) O-t-butyl threonine, and (b) O-t-butyl aspartate residues (see text).

The acylation and deprotection times (60 and 10min respectively) preset for this synthesis proved adequate despite the reduced reaction rates in the final stages and a very satisfactory result was obtained. Detatchment of the peptide from the resin and cleavage of all acid-labile protecting groups was achieved with aq trifluoroacetic acid or trifluoroacetic acid-phenol mixture; the latter has previously[7] been found particularly effective for peptides containing S-acetamidomethyl-cysteine. It yielded the hplc profile depicted in Fig 6 for the total crude cleavage product. The early eluting peak did not contain peptide material. In this small scale preliminary experiment the isolated yield was only 60%, probably due to losses incurred in removing the phenol. On a larger scale using aqueous trifluoroacetic acid for cleavage, the isolated crude yield was 78%. Analytical data and other details are collected in the Experimental section.

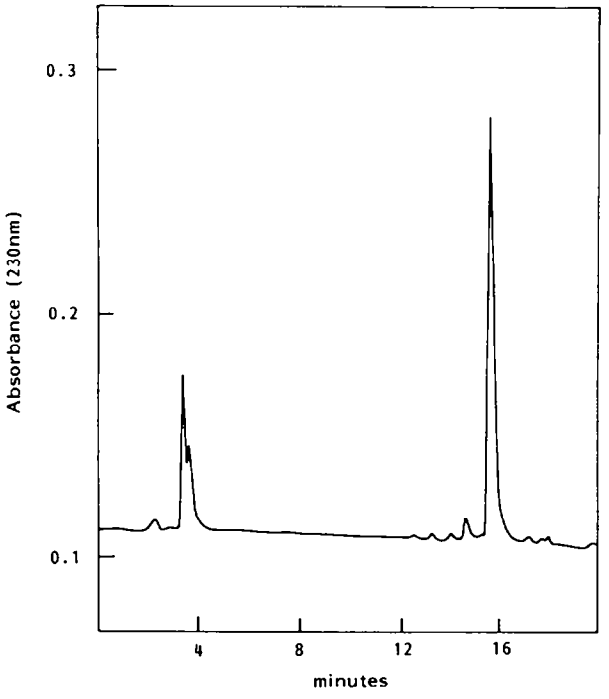

Fig. 6. Hplc profile of total crude pentadecapeptide (2): Conditions as fig. 3 except that the gradient was 5-35% B over 30 min.

Experimental

General procedures for solvent and reagent purification and for the conduct of continuous flow solid phase synthesis have been given previously.[1] Pentafluorophenyl esters of Fmoc-amino-acids were prepared as already described (ref 3 and references there cited). The automatic instrument used

in the present work utilised the same valve types and control mechanisms as the semi-automatic synthesiser already described.[1] A variable speed LDC/Milton Roy miniPump VS was used, set in these experiments to a pumping speed of 3ml/min. The automatic sample changer consisted of two six-way Altex rotary valves attached to a common motor drive but offset to each other by 30° of rotation. The 12 sample chambers were 5ml Luer syringe barrels into which porous glass frits had been fused at the Luer end, or in which porous ptfe discs were retained by stainless steel spring rings.

Functionalisation of Methoxycarbonyl-polydimethylacrylamide Support.

Copoly(dimethlacrylamide-bisacryloylethylenediamine-acryloylsarcosine methyl ester) polymerised in the presence of macroporous kieselguhr particles[1] (10g, 0.108 meq sarcosine/g) was placed in a round bottom flask, covered with ethylenediamine, and shaken gently (0.5min, 10min rest and repeat) overnight. The ethylenediamine was removed and the resin washed with Dmf by swirling and decantation. The resin was transferred to a glass column equipped with adjustable solvent resistant ptfe end pieces and washed further with Dmf until the effluent gave a negative reaction with ninhydrin. A solution of Fmoc-L-norleucine pentafluorophenyl ester (2.23g, 4.3mmole) and hydroxybenzotriazole (0.655g, 4.5mmole) dissolved in Dmf (10ml) was loaded onto the column manually and recirculated for 45min. Ninhydrin and trinitrobenzenesulphonic acid colour tests were then both negative. The following machine operations were then carried out for the times indicated: FLOW (15min); RECIRC (0.5min); FLOW (7min); REAGENT 2 (20% piperidine/Dmf); FLOW (13min); REAGENT 1 (Dmf); FLOW (40min). The whole procedure was then repeated using 4-hydroxymethylphenoxyacetic acid pentafluorophenyl ester[3] (1.61g, 4.6mmole) and hydroxybenzotriazole (0.657g, 4.5 mmole). The functionalised resin was removed from the column, washed in a funnel with Dmf and then ether, dried in vacuo over silica gel, and stored at 4°.

Solid Phase Synthesis of H-Thr-Pro-Glu-Val-Asn-Pro-Ile-Asp-Ala-Glu-Gly-Leu-Ser-Gly-OH (1).

(i) Esterification of the first residue. Fmoc-glycine pentafluorophenyl ester (0.58g, 1.25mmole) was dissolved in Dmf (2ml) and added manually to the foregoing resin (ca 2.3g) contained in a glass column. The solution was rinsed on with Dmf (0.5ml), and followed by 4-dimethylaminopyridine (30.5mg, 0.25mmole) dissolved in Dmf (0.5ml), and then Dmf (0.5ml). The mixture was recirculated through the column for 30min and then washed out automatically (steps 14-19, Table 2). After removal of a resin sample for analysis, the whole procedure was immediately repeated, and then again using acetic anhydride (48μl) in place of Fmoc-glycine pentafluorophenyl ester. A sample of this resin and the foregoing were deprotected on a sintered glass funnel

with 20% piperidine/Dmf, washed (Dmf and ether), dried, and hydrolysed with redistilled constant boiling hydrochloric acid in vacuo for 18h at 110° [Found: (30min sample) Gly, 1.00; Nle, 1.11; (60min sample) Gly, 1.00; Nle 0.93].

(ii) Manual addition of Fmoc-(O-t-butyl)-L-serine anhydride. The symmetrical anhydride was prepared[1] from Fmoc-(O-t-butyl)-L-serine (766mg, 2mmole) dissolved in dichloromethane (2ml) and dicyclohexylcarbodiimide (196mg, 0.95mmole) dissolved in dichloromethane (1ml) during 10min at room temp. The mixture was filtered from precipitated dicyclohexylurea, evaporated, and the oily residue dissolved in Dmf (2ml) and applied to the resin column immediately. After recirculation for 60min colour tests were negative and the rest of the synthesis cycle (from step 14) carried out automatically.

(iii) Automatic chain extension using Fmoc-amino-acid activated esters. The following solid Fmoc-amino-acid derivatives (all of the L-configuration except glycine) were placed in successive sample chambers: leucine pentafluorophenyl ester (515mg, 1mmole); glycine pentafluorophenyl ester (465mg, 1mmole); t-butyl glutamate pentafluorophenyl ester (592mg, 1mmole); alanine pentafluorophenyl ester (482mg, 1mmole); t-butyl aspartate pentafluorophenyl ester (573mg, 1mmole); isoleucine pentafluorophenyl ester (516mg, 1mmole); proline pentafluorophenyl ester (509mg, 1mmole); asparagine p-nitrophenyl ester (476mg, 1mmole); valine pentafluorophenyl ester (506mg, 1mmole); t-butyl glutamate pentafluorophenyl ester (598mg, 1mmole); proline pentafluorophenyl ester (511mg, 1mmole). Each chamber also contained 1-hydroxybenzotriazole (ca 150mg, 1mmole). The synthesiser was started using the program given in Table 2 [with both PAUSE functions (lines 156 and 241) deleted] and run without interruption. The release of dibenzofulvene-piperidine adduct was followed spectrometrically for each residue.[1] The following peak areas were obtained by direct summation and are uncorrected for non-linearity: Ser, 29.4; leu, 29.1; gly, 30.4; Glu, 30.4; Ala, 31.0; Asp, 30.3; Ile, 30.9; Pro, 27.4; Asn, 29.6; Val, 28.6; Glu, 30.0; Pro, 28.2. All peak shapes were normal.

(iv) Manual addition of Fmoc-O-t-butyl-threonine anhydride. The anhydride preparation was commenced as for the similar O-t-butyl serine derivative above except that only 1mmole of Fmoc-L-threonine was used in error. This was detected by the low optical density reading obtained at the start of the acylation cycle. The column was immediately washed out with Dmf and a new preparation of the anhydride [from Fmoc-O-t-butyl-L-threonine (794mg, 2mmole) and dicyclohexylcarbodiimide (196mg, 1mmole) in dichloromethane as usual] introduced manually. After recirculation for 60 min, the subsequent steps (from line 165, Table 2) were performed automatically.

(v) Isolation and characterisation of the tetradecapeptide. The resin was removed from the column, washed with Dmf and ether, and dried in vacuo over silica gel (Found: Asp, 2.00; Thr, 0.96; Ser, 0.91; Glu, 2.08; Pro, 2.13; Gly, 2.10; Ala, 1.07; Val, 0.97; Ile, 0.95; Leu, 1.00; Nle, 1.06). A sample (999mg) was treated with 95% aq trifluoroacetic acid for 60min at room temp. The filtrate and washings were evaporated and the residue re-evaporated with ether (3 times) [Found: Asp, 2.00; Thr, 0.96; Ser, 0.93; Glu, 2.12; Pro, 2.15; Gly, 2.08; Ala, 1.09; Val, 0.94; Ile, 0.93; Leu, 1.00 (45μmoles, 78%)]. The residual resin had Leu, 1.00; Nle, 41.8 (97% cleavage). Hplc was carried out on an analytical Aquapore RP300 column. Reservoir A contained 0.1% aq trifluoroacetic acid; reservoir B 90% acetonitrile, 10 % A. A linear gradient of 15-30% B was developed over 30min at a flow rate of 1.5ml/min. The elution profile measured at 230nm is shown in Fig. 3. The major peak eluting at 14.3min was collected fom several similar runs on a semi-preparative column using a gradient of 10-25%B over 30min (Found: Asp, 2.01; Thr, 0.96; Ser, 0.89; Glu, 2.04; Pro, 1.98; Gly, 2.01; Ala, 1.03; Val, 1.01; Ile, 0.95; Leu, 1.00), hplc profile Fig. 4. The early eluting peaks were also collected; neither contained significant peptide material (2% Gly in the peak eluting at 2.2min, 0.1% Gly in that eluting at 3.6min).

Solid Phase Synthesis of H-Cys(Acm)-Tyr-Ala-Thr-His-Lys-Tyr-Leu-Asp-Ser-Glu-Glu-Asp-Glu-Glu-OH (2).

(i) Esterification of the first residue. Fmoc-t-butyl-L-glutamate pentafluorophenyl ester (745mg, 1.25mmole) dissolved in Dmf (2ml) was manually introduced into the column containing the same functionalised resin as above (ca 2.3g), and washed on with Dmf (0.5ml), a solution of 4-dimethylamino-pyridine (30.2mg, 0.25mmole) in Dmf (0.5ml), and then Dmf (0.5ml) as before. After 30min recirculation, the column was washed, a resin sample removed for analysis, and the esterification repeated. The resin was finally acetylated as already described, and a sample deprotected [found: (30min sample) Glu, 1.00; Nle 1.18; (60min sample) Glu, 1.00; Nle 0.94].

(ii) Automatic chain extension using Fmoc-L-amino-acid activated esters. 1.00mmole of each of the following pentafluorophenyl esters (3-hydroxy-4-oxo-dihydrobenzotriazinyl esters for serine and threonine) were placed in successive sample chambers: t-butyl glutamate, t-butyl aspartate, t-butyl glutamate, t-butyl glutamate, O-t-butyl-serine, t-butyl aspartate, leucine, O-t-butyl-tyrosine, ε-Boc-lysine, im-Boc-histidine, O-t-butylthreonine, alanine, O-t-butyl-tyrosine, S-acetamidomethyl-cysteine. The synthesiser was started using the program given in Table 2 with both PAUSE functions removed and the following modifications or additions: line 153, TIME,20; line 155, TIME,20; line 156, TEST,312,1.4,2.6 (measure optical density at 312nm; error

if less than 1.4 or greater than 2.6); line 157, PLATEAU,312 (measure slope at 312nm); line 160, TIME,20; line 191, TEST,312,-.1,.1 (measure optical density at 312nm; error if less than -0.1 or greater than 0.1). Additional changes within the Basic program examined the shape and area of each deprotection peak (see text). The fllowing peak areas were obtained: Glu, 33.9; Asp, 33.0; Glu, 34.95; Glu, 35.6; Ser, 33.65; Asp, 34.8; Leu, 33.3; Tyr, 31.3; Lys, 33.1; His, 32.6; Thr,23.3 (error close down initiated: REAGENT,1; FLOW,1; TIME,10; HALT; END); after restart, Ala, 26.0; Tyr, 23.0; Cys, 27.9.

(iii) Characterisation and isolation of the pentadecapeptide derivative. The resin was removed from the column, washed on a funnel with Dmf and ether, and dried in vacuo over silica gel (2.72g) (Found: Asp, 2.09; Thr, 0.87; Ser, 0.90; Glu, 4.30; Ala, 0.87; Cys, n.d.; Leu, 1.00; Nle, 1.08; Tyr, 1.65; His, 0.91; Lys, 0.97). A resin sample (20.9mg) was treated with a solution of trifluoroacetic acid (7.5ml) containing phenol (0.38g) for 60 min at room temp. The combined filtrate and washings were evaporated in vacuo, evaporated twice with ether, and the residue dissolved in water and washed three times with ether. The combined ether extracts were washed with water and the combined aqueous phases evaporated (Found: Asp, 2.16; Thr, 0.93; Ser, 0.88; Glu, 4.33; Ala, 0.93; Leu, 1.00; Tyr, 1.83; His, 0.93; Lys, 1.01). The cleavage yield was 69% (Found; Leu, 1.00; Nle, 3.48 for residual resin), and the overall crude isolated yield 60%. On a larger scale (894mg) using 95% aq trifluoroacetic acid for 60min at room temp, the cleavage yield was 77% and the crude isolated yield 78%. Preparative hplc gave purified peptide (72% of the material applied to the column) (Found: Asp, 1.98; Thr, 0.90; Ser, 0.86; Glu, 4.17; Ala, 0.95; Leu, 1.00; Tyr, 1.95; His, 0.95; Lys, 0.95).

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