Peptide Synthesis. Part 8. A System for Solid-phase Synthesis Under Low Pressure Continuous Flow Conditions

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Details are given of a novel technique for solid-phase peptide synthesis under continuous flow conditions. The resin support is a poly(dimethylacrylamide) gel polymerised within the pores of rigid macroporous kieselguhr particles. Manual and semiautomatic (microprocessor controlled) instrumentation is described for the use of this resin in synthesis. The method is illustrated by preparation of a pentadecapeptide sequence related to the repeating unit of adenovirus tail fibre. The value of continuous spectrometric monitoring of solid-phase synthesis is discussed.

Previous papers in this series[1-7] and elsewhere[8-19] have described the development and application of the 'Fmoc-polyamide' method of solid-phase peptide synthesis. This technique differs substantially from conventional solid-phase systems which have generally been patterned closely on the original Merrifield proposals.[20] In the Fmoc-polyamide method the hitherto almost universally employed polystyrene support resin has been replaced by a polar, poly(dimethylacrylamide) gel,[7] and t-butoxycarbonyl (Boc) protected amino acids replaced by fluorenylmethoxycarbonyl (Fmoc) derivatives.[6] The first of these changes provides a resin–peptide system which is freely permeated and solvated by the dipolar, aprotic solvent dimethylformamide, minimising aggregation phenomena within the resin matrix and at the same time providing a particularly favourable reaction medium for both acylation and deprotection reactions.† The second change enables replacement of the customary treatments with strong acid used for repetitive cleavage of α-amino protecting groups by milder base treatments. Typically, contact with 20% piperidine in an anhydrous organic solvent is sufficient to cleave completely Fmoc-peptide-resins within a few minutes. Equally importantly, use of Fmoc-derivatives also permits restructuring of the overall protecting group strategy with replacement of benzyl groups by more labile t-butyl or *p*-alkoxybenzyl derivatives. This leads to substantial further mildening of reaction conditions. Another significant departure from the standard Merrifield procedure has been adoption of pre-formed symmetrical anhydrides‡ as acylating species,[7] in place of *in situ* activation by dicyclohexylcarbodi-imide. All these changes have, we believe, provided the basis for a highly efficient solid-phase system conducted under mild and chemically optimised reaction conditions.

Merrifield-style synthesis is usually carried out in a shaken, stirred, or nitrogen-agitated reaction vessel containing the insoluble polymer-supported peptide. Reagents are added sequentially to this vessel and removed by filtration and thorough washing. The process is thus essentially a discontinuous, batchwise one. On the other hand, continuous flow techniques offer clear advantages. Removal of excess reagents by continuous solvent flow through a packed column bed is inherently more efficient, economical, and rapid than batchwise washing. This is particularly true for gelatinous solids with high liquid retention.§ Resin columns may be connected in series, providing a simple and economical method for simultaneous multiple analogue synthesis. Flow systems are particularly easily and simply mechanised. Continuous monitoring of the soluble reactant concentration by spectrometric or other means is possible, in principle providing opportunity for true automation with feedback control.

This paper describes the development and application of a practical continuous flow solid-phase peptide synthesis system based on established 'Fmoc-polyamide' chemistry.

Soft, gelatinous polymer supports may not be generally suitable for use in pumped flow systems. Marked changes in bed volume may occur with change of solvent and as the various chemical reactions take place. This is particularly noticeable when the peptide resin becomes protonated, as in the cleavage of Boc groups. These effects are minimised in the Fmoc-polyamide system which operates in a single solvent throughout and without acidic reagents. Nevertheless, our early experiences using poly(dimethylacrylamide) resin packed in small glass columns showed that such resin beds may be physically unstable. On occasions, a previously stable flowing column would suddenly begin to compress and shrink in size, ultimately to a volume less than that of the dry unswollen resin. At the same time there was an associated steep rise in pressure. These observations explained why early investigators of continuous flow systems employed rigid surface functionalised silica[21] or coated glass supports,[22] and why more recent studies using polystyrene gel have required high-pressure stainless-steel technology and h.p.l.c.-type pumping systems.[23-25] None of these investigations has, as far as we are aware, provided a fully practical system. We attribute this compression of the gel support to closure of the interstitial channels. Solvent flow through a column of loosely packed swollen gelatinous beads probably occurs largely through these channels, solvent and reactant penetration of the internal resin matrix being diffusion controlled. Deformation of the soft swollen beads or blockage by small particles (especially at the top of the column) leads to restriction of solvent flow, development of increasing pressure, and a progressive piston effect squeezing solvent from the expanded matrix.

High-pressure flow systems are technically inconvenient and may be unrealiable. More importantly, any collapse of the internal open matrix character of the resin support and consequent reduction in internal volume must reduce reactant accessibility and increase steric crowding of the growing peptide chains. A deleterious effect on the efficiency of synthesis seems inevitable.

One solution to this problem is to enclose the gel polymer in

† Addition of dimethylformamide to polystyrene resins has now been found beneficial in some instances (S. B. H. Kent and R. B. Merrifield, in 'Peptides 1980,' Proceedings of the 16th European Peptide Symposium, Helsinger, Denmark 1980, Seriptor, Copenhagen 1981, p. 321).
‡ These reagents are also useful in polystyrene-based synthesis.

§ Poly(dimethylacrylamide) gel has a swollen bed volume of *ca.* 20 ml g^{-1} in dimethylformamide (DMF). Washing with an equal volume of solvent and filtration would require repetition 9 times before the soluble reactant concentration was reduced to *ca.* 0.1% of its original value.

a rigid framework, constructed so as to mantain channels for liquid flow and yet permit rapid diffusion of reactants into and out of the gel matrix. If this framework is itself particulate, the critical factors are particle and pore size. By controlling reactant accessibility and the rate of diffusion into and out of the gel, these parameters effectively determine reaction and wash times in synthesis. Of several possibilities considered, macroporous inorganic particles prepared by sintering the powdered mineral in the presence of an organic binder were selected as offering the best promise.[26] They may be prepared from a number of inorganic substances (kieselguhr, alumina, titania, hydroxyapatite, *etc*). They have interconnecting pores several thousand Å in diameter, larger than, for example, currently available controlled pore glass. For the present work, kieselguhr was selected because of its generally low adsorptive properties, the fabricated particles used having a size range of 350—500 μm.

Preparation of a typical poly(dimethylacrylamide)–Kieselguhr composite resin has been described previously.[16] The monomer mixture consisting of dimethylacrylamide, ethylene bisacrylamide (crosslinking agent), and acryloylsarcosine methyl ester (functionalising agent) together with initiator ammonium persulphate was soaked into the highly absorbent kieselguhr and polymerisation allowed to proceed.[16] This resin, with sarcosine content of 0.1 mequiv. g^{-1}, is now available commerically under the name Pepsyn K.* The bare kieselguhr element is also available.† The composite poly(dimethylacrylamide)–kieselguhr support forms a free flowing powder which packs easily into glass columns. Negligible back pressure is generated by solvent flow at normal rates (*ca.* 100 ml h^{-1} cm^{-2}). There is usually no significant change in bed volume as the chemical reagents and reactions vary.‡ Diffusion of reagents and reactants into and out of the physically supported gel matrix is evidently very rapid (see below), and the support appears to behave chemically indistinguishably from unsupported resin.[13]

Only simple equipment is required for use of the composite support in solid-phase peptide synthesis. A typical manual system is depicted in Figure 1. Essentially, provision is required for flowing solvent and reactants through the resin bed to waste, and for recirculation. This can be accomplished with flow/recirculate (V1) and solvent/reagent selection (V2) valves as indicated. Addition of activated Fmoc-amino-acids may take place directly to the top of the column or, if V2 is a multiway rotary valve as shown, through a syringe barrel connected to V2. Since under normal circumstances negligible pressure is generated in the system, thin flexible poly(tetrafluoroethylene) (PTFE) tubing may be used for connections and a glass column fitted with a sintered support filter and Quickfit joint for containing the resin. The pump is usually a simple reciprocating type with stainless steel or sapphire piston and check values. Only PTFE and glass surfaces contact the solvent or reagent elsewhere. Details of the pump and valves used in our laboratory are given in the Experimental section.§

Simple manually controlled equipment of this type has been in regular use in this laboratory. Most of our work however, has used more advanced semi-automatic or automatic microprocessor-controlled equipment. All the chemical processes for

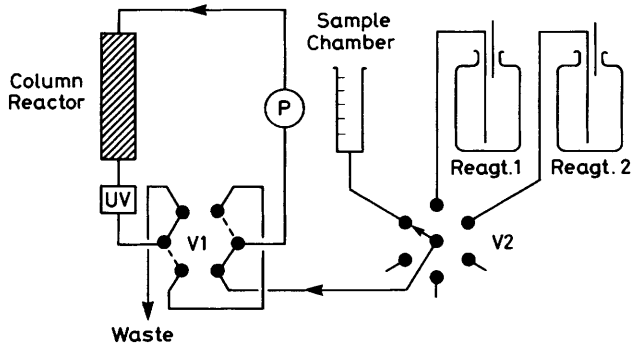


Figure 1. Manually operated equipment for continuous-flow solid-phase synthesis

deprotection (Fmoc group cleavage), washing, and acylation (activated Fmoc-amino acid addition) are easily mechanised using flow techniques. Construction and operation of a typical semiautomatic instrument capable of unattended single residue addition is described below. Fully automatic operation (sequential addition of several amino-acid residues) requires special consideration of the amino acid activation step. It is possible to design and construct systems for the automatic conversion of protected amino-acids into their symmetrical anhydrides,¶ but the mechanical problems to be overcome in a fully reliable manner are formidable. We chose instead a chemical solution—to develop the use of alternative activated species of acylating efficiency equal to symmetrical anhydrides but which could be pre-prepared, stored, and delivered sequentially to the flow system in a simple, fully automatic manner. Fmoc-amino acid pentafluorophenyl esters[28] have proved particularly suitable.[8] Design and operation of a fully automatic microprocessor controlled flow synthesizer using this chemistry will be described in a forthcoming publication.‖

The liquid flow system of a semiautomatic (single residue addition) flow synthesizer utilising symmetrical anhydrides or activated esters is outlined in Figure 2. Essentially it consists of a pumped reagent selection, flow, and recirculation system as before, with additional facilities for reversing the direction of flow through the column, activated sample introduction through a sample loop, nitrogen pressurisation of the selected reagent bottle, and continuous spectrometric monitoring of reactant concentration. A single valve type (see Experimental section) is used throughout. Individual valves or ganged pairs (V2a,b; V4a,b) are operated through pneumatic actuators and return springs. Each actuator has been modified by addition of an opto-electronic sensing mechanism (equivalent to a simple microswitch contact) enabling its status to be determined electronically. Gas flow to the actuator is controlled by a simple low voltage solenoid.

In Figure 2, all the valves are shown in their off or resting state. This is the fail-safe situation with V1 (flow/recirculate) in recirculate mode; V2 (loop in/out), loop out; V3 (reverse flow), flow down column; V4 (reagent and nitrogen selection), reagent 1; and V5 (nitrogen on/off), off. A non-return check valve (CV) reduces the possibility of flow back of reagent into the (incorrect) reservoir. The drain outlet is raised above the maximum liquid level in the reservoirs preventing siphoning to waste. The control system is outlined in Figure 3. The chosen

* From Cambridge Research Biochemicals Ltd., Button End Industrial Estate, Harston, Cambridge CB2 5NX.

† From Sterling Organics Ltd., Dudley, Cramlington, Northumberland NE23 7QG.

‡ Remarkably this is not always the case when Pepsyn K is used for oligodeoxyribonucleotide synthesis[27] when quite large changes in bed volume have been observed. It is possible that these effects may have been due in part to use of resin samples with substantial poly(dimethylacrylamide) gel *on the surface* of the kieselguhr particles.

§ A complete manual system (the PEPSYNthesiser) based on the foregoing is available from Cambridge Research Biochemicals Ltd.

¶ As in the recently introduced Applied Biosystems Model 430A Synthesiser. This instrument operates in the normal batchwise, not continuous flow, mode.

‖ A commercial instrument is under development by LKB Biochrom, Trinity Science Park, Cambridge.

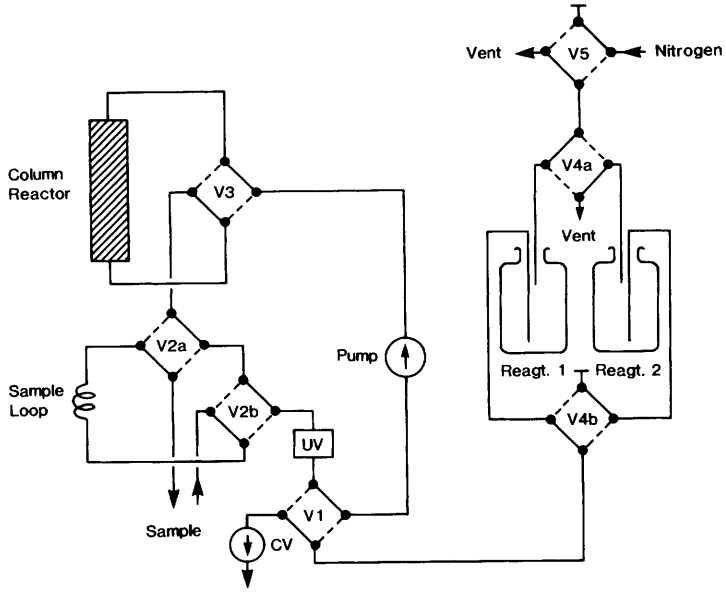


Figure 2. Semi-automatic (single residue addition) equipment for continuous-flow synthesis

microcomputer (Hewlett Packard HP85) provides in a single unit all the facilities depicted in the upper half of the figure, *viz.* microprocessor and keyboard, display screen, printer, and program/data storage cassette. Plug-in Hewlett Packard interface units provide serial (RS232) communication with a digital single beam u.v. monitor (LKB Ultrospec), and a versatile parallel (IEEE488) interface. The latter transmits control signals to relay assemblies which, in turn, operate the nitrogen solenoids. It also receives status signals from the valve actuators through a contact sensing assembly.

Three levels of control are provided. Simple toggle switches with indicator lights actuate the solenoids directly and provide manual control of the individual valves and pump, even in the absence of the computer. A higher level of manual control is available through the computer keyboard, in this case through a series of software defined keys corresponding to control words FLOW, RECIRCULATE, *etc.* (see Experimental). Each initiates a series of valve and/or pump operations appropriate to the named function. Alternate sets of software-defined functions facilitate data collection by the spectrometer, data processing *etc.* In the third level of control, the same or similar control words are executed automatically in a stored sequence and at preselected time intervals. In this mode, provision is also made for manual intervention through HOLD, RESUME, and ADVANCE functions, as well as reversion to fully manual control.

Completely manual operation of the flow systems outlined in Figures 1 and 2 is straightforward. A typical sequence of operations for addition of a single amino acid residue (acylation and deprotection) is given in Table 1. The Fmoc-amino-acid anhydride[4] prepared immediately beforehand is dissolved in the minimum amount of DMF and loaded onto the top of the resin column, either directly or by way of the sample chamber (a syringe barrel equipped with a glass or PTFE sintered filter) of Figure 1 or sample loop (Figure 2). In the first two cases the sample is run onto the column using the pump, rinsed on with a little additional DMF, and set in recirculating mode. In the third case the sample is rinsed into the loop with DMF and the sample loop introduced into the already recirculating stream through operation of V2. The loop is removed as soon as the sample has been washed out (either timed or as indicated by the u.v. monitor, see below) to reduce the dead volume and

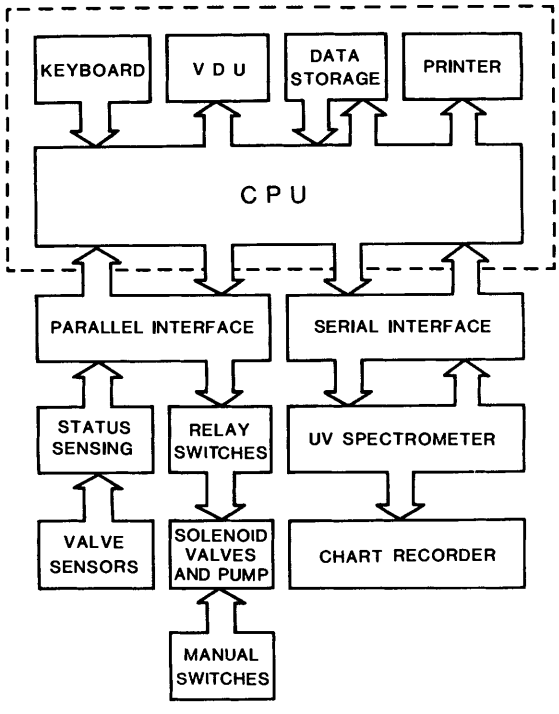


Figure 3. Microprocessor-based control system for continuous flow synthesizer

minimise eventual dilution of the recirculating sample. The following operations are as indicated in Table 1. Withdrawal of samples of resin for qualitative colour tests (with ninhydrin[29] and trinitrobenzenesulphonic acid[30]) at step 3 and for colour tests and later amino acid analysis at step 10 is optional but is strongly commended. The second acylation period (step 4) may be curtailed or extended depending on the result obtained at step 3, and further tests can be carried out as necessary. Very exceptionally, the acylation step may need to be repeated.

The various operations in Table 1 may be followed visually if the flowing stream is passed through a narrow path length u.v.

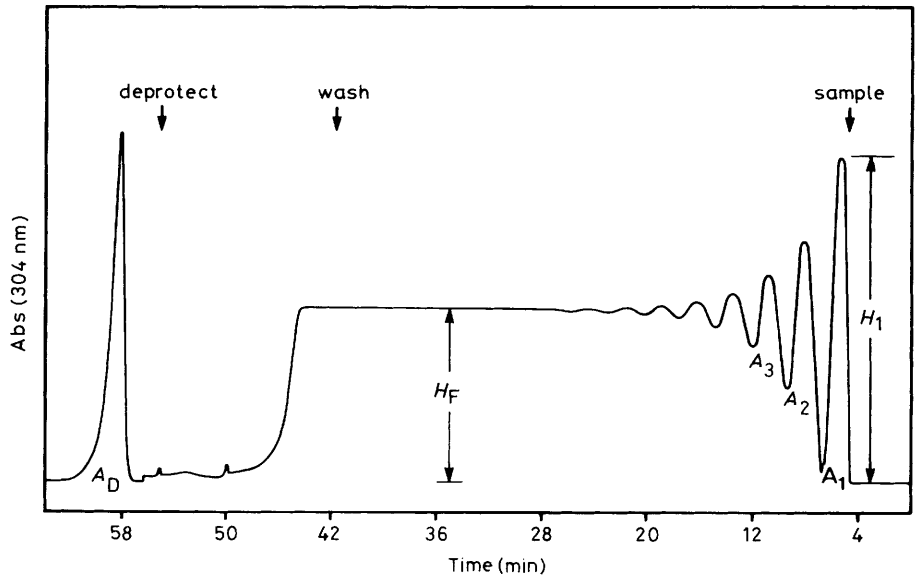


Figure 4. Typical spectrometric record for one cycle of Fmoc-amino acid addition and deprotection

Table 1. Procedure for flow synthesis under manual control

Acylation:	1. Apply preformed Fmoc-amino acid anhydride to resin column	
	2. Recirculate	20 min
	3. Pause, remove resin sample	
	4. Recirculate	20 min
Wash:	5. Flow DMF	6.5 min
	6. Recirculate (rinse flow/ recirculate valve V1)*	0.5 min
	7. Flow DMF	5 min
Deprotect:	8. Flow 20% piperidine	10 min
Wash:	9. Flow DMF	8 min
	10. Pause, remove resin sample	
	11. Flow DMF	12 min

* The particular sample introduction system in use should also be rinsed at this time.

cell. Qualitative information is easily obtained and is extremely informative. A typical spectrometric record of a complete cycle of amino acid addition and deprotection is shown in Figure 4.* Introduction of the activated Fmoc-amino acid and recirculation through the resin bed produces the characteristic oscillating pattern at the beginning of the trace. Diffusion processes, largely in the resin bed, result in broadening and lowering of successive peaks, ultimately to a uniform concentration throughout the recirculating system. Reaction of the activated Fmoc derivative with the peptide resin reduces the area of successive peaks (see later). Qualitatively, completion of the acylation reaction is indicated by plateauing of the u.v. trace after diffusion processes have removed the oscillating character. The wash phase is shown to be complete by return to steady baseline optical density. Rinsing of the flow/recirculate valve (step 6 in Table 1) and of the sampling system usually results in small peaks appearing in this region. Deprotection liberates the piperidine adduct of dibenzofulvene into solution with generation of a characteristic peak in the trace. The area of this peak should be a measure of overall efficiency of amino acid addition and deprotection, especially in comparison with preceding residues (see later). The rapidity with which this peak rises and falls is an indication not only of the rate of the deprotection reaction (in free solution Fmoc-valine has a half life of *ca.* 6 s [6]), but also of the speed of diffusion of reagent into and the product out of the supported resin. The same steepness of the falling trace is shown in the rapid washout following acylation, strong indications that enclosure of the gel resin within the pores of the inorganic support has not hindered diffusion processes markedly.

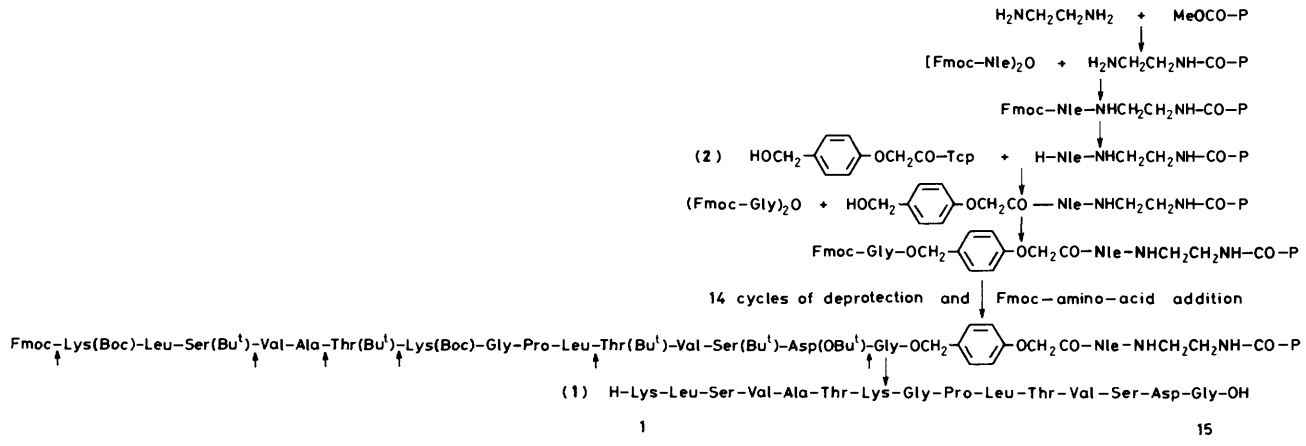
Operation of the semi-automatic synthesizer outlined in Figure 3 is illustrated in Table 2. This is a facsimile of the computer printout recording one complete cycle of synthesis under program control. Each operation is recorded along with the current time and program step number. The pre-activated Fmoc-amino acid is introduced into the sample loop during the initial wash period (steps 1—3). Before this is introduced into the flowing stream, a series of commands set up the u.v. monitor to collect quantitative data during the acylation step. In this case 350 optical density measurements are to be made at 2 s intervals at 304 nm. The base line is reset to zero and data collection commences immediately. The sample loop is then switched into the recirculating stream and removed after a predetermined time when the sample has left the loop. The time interval specified in step 7 has been fine tuned to ensure that time out for this operation does not coincide with the exact moment of command or data transfer from the spectrometer which is proceeding simultaneously with other timing and control functions. When data collection is complete it is automatically stored in a cassette data file and then processed as described later. Count down of the main timer is suspended during data storage operations when the computer cannot be interrupted to perform other control functions. Subsequent operations may, therefore, be delayed a variable amount depending on access time to the mass storage medium. The pause command (step 11) at the end of the first acylation period is again optional as are those at steps 22 and 32. It serves to sound a warning tone for resin sample removal if desired and provides flexibility in adjusting reaction times in the light of colour tests or spectrometric monitoring. The rest of the steps

* This may be compared with a published (C.-D. Chang, A. M. Felix, M. H. Jimenez, and J. H. Meienhofer, *Int. J. Peptide and Prot. Res.*, 1980, **15**, 485) spectrometric record of a discontinuous synthesis using a centrifugal reactor.

are largely self explanatory. Step 15 rinses the flow/recirculate valve and steps 18—20 the sample loop. The pause at step 22 gives the operator final decision as to whether to proceed (irrevocably) to the deprotection cycle. The deprotection time has been substantially shortened over that given in Table 1, in line with results of spectrometric monitoring of this reaction, but see below.

A typical synthesis using the semi-automatic flow synthesizer is illustrated in the Scheme. The target peptide (**1**) is a 15 residue anhydride. Reaction was allowed to proceed for 30 min. All further acylation and deprotection reactions followed the general procedure given in the Table. Samples of deprotected peptide-resin were removed for quantitative amino acid analysis at the points indicated (arrows) in the Scheme. The results are collected in Table 3.

In this synthesis, the amount of symmetrical anhydride used at each acylation step was deliberately kept to a minimum (*ca.* 2 equiv.)‡ so that the value of both colour tests and continuous



Scheme. Assembly of adenovirus pentadecapeptide sequence

sequence related to the repeating unit[15] of adenovirus fibre.* Kieselguhr-supported poly(dimethylacrylamide) gel[16] functionalised with methoxycarbonyl groups was converted into the amine form by overnight reaction with excess ethylenediamine. The briefly washed resin was then placed in the column of the synthesizer and washed further with dimethylformamide until the effluent gave a negative colour with ninhydrin. While this was in progress, the internal reference amino-acid derivative, Fmoc-norleucine, was converted into its symmetrical anhydride (*ca.* 2 equiv. relative to the expected amine content of the resin) by reaction with a slight deficiency of dicyclohexylcarbodiimide in dichloromethane solution in the usual way. The anhydride, isolated by filtration and evaporation, was introduced in dimethylformamide solution into the sample loop and the synthetic cycle outlined in Table 2 commenced. Completion of this and all subsequent amine acylation reactions was checked by colour tests using both ninhydrin and trinitrobenzenesulphonic acid reagents on resin samples withdrawn at the end of the first acylation period (25 min) and at further intervals if necessary. Quantitative spectrometric data was also collected for both acylation and deprotection reactions (see below). Following deprotection of the reference norleucine residue, the reversible active ester linkage agent (**2**) was coupled in the presence of hydroxybenzotriazole catalyst using steps 1—22 of the standard procedure (Table 2). The special procedure† for esterification of the first amino-acid residue (Fmoc-glycine) to the resin-bound linkage agent was carried out manually by adding 4-dimethylaminopyridine catalyst to the top of the column followed, after rinsing, by the preformed symmetric spectrometric monitoring could be assessed. Because of this, some acylations were appreciably slower than usual and gave weakly positive colour reactions at the time of the first tests (25 min). These acylations were prolonged and a second test carried out after 50 min. In three acylation reactions of sterically hindered residues [Thr(OBu^t)-11, Thr(OBu^t)-6, and Val-4], marginal or doubtful colour tests for residual amine groups were obtained after 50 min, and these reactions were repeated. Only in the last of these did later amino acid analysis give any indication of further amino acid incorporation, and this was not substantiated by further analysis. A generally satisfactory assembly was achieved (see Table 3). There was little loss of peptide from the resin as shown by the near constancy of the internal reference norleucine residue.

Most of the completed peptide–resin was retained for further experimentation and only a small part cleaved at this pentadecapeptide stage. The total crude product obtained by treatment of this resin with 95% aqueous trifluoroacetic acid was characterised by amino acid analysis (Table 3) and h.p.l.c. in 4 systems (*e.g.* Figure 5; the other systems gave very similar profiles). The cleavage yield determined by analysis of residual resin was 97% and the actual yield of crude pentadecapeptide recovered was 82%.

During the foregoing assembly of the adenovirus-related pentadecapeptide both acylation and deprotection reactions were monitored by optical density measurements of the flowing reactant stream. The results obtained are illustrative of the problems encountered and progress towards continuous real time monitoring of solid-phase synthesis.

Successful solid-phase peptide synthesis demands very high yields at each acylation and deprotection step, ideally >99% per cycle. To be useful, data must be collected, processed, and evaluated before synthesis is allowed to proceed irrevocably to

* This sequence was suggested to us by Dr. N. M. Green of the National Institute for Medical Research, Mill Hill, London, and was required for model studies on the β-sheet structure proposed[31] for the virus fibre.

† Dimethylaminopyridine is a powerful racemising agent for optically active Fmoc (and other urethane protected) α-amino acid anhydrides.[18] The special procedure adopted for the esterification step ensures that the two reactants are mixed in the presence of the hydroxy component and not beforehand.

‡ In general a larger excess of anhydride (*ca.* 4-fold) is to be preferred. This should be dissolved in the minimum amount of dimethylformamide to keep the initial concentration as high as possible.

Table 2.

```
PROGRAM OPSY11
EXPERIMENT: ALEU6
08:00 # 01 REAGENT DMF
08:00 # 02 FLOW
08:05 # 03 TIME    5.00 MIN
08:05 # 04 MONITOR
           DATA POINTS, 350
           TIME INTERVAL, 2 SEC
           WAVELENGTH, 304mM
           BASELINE,   0.000
08:06 # 05 RECIRCULATE
08:06 # 06 LOOP IN
08:06 # 07 TIME    1.74 MIN
08:08 # 08 LOOP OUT
08:08 # 09 TIME   25.00 MIN
08:17   DATA COLLECTION CEASED
08:19   STORED IN FILE ALEU6
08:33 # 11 PAUSE
08:33      RESUMED
08:33 # 12 TIME   10.00 MIN
08:43 # 13 FLOW
08:43 # 14 TIME    6.50 MIN
08:50 # 15 RECIRCULATE
08:50 # 16 TIME    0.50 MIN
08:50 # 17 FLOW
08:50 # 18 LOOP IN
08:50 # 19 TIME    5.00 MIN
08:55 # 20 LOOP OUT
08:56 # 22 PAUSE
08:57      RESUMED
08:58 # 23 REAGENT 20%PIPERIDINE
08:58 # 24 FLOW
08:58 # 25 TIME    1.00 MIN
08:59 # 26 MONITOR
           DATA POINTS, 90
           TIME INTERVAL, 4 SEC
           WAVELENGTH, 304nM
           BASELINE,   0.000
08:59 # 27 TIME    0.85 MIN
09:00 # 28 REAGENT DMF
09:00 # 29 FLOW
09:00 # 30 TIME    8.00 MIN
09:05   DATA COLLECTION CEASED
09:07   STORED IN FILE DLEU6
09:08 # 32 PAUSE
09:08      RESUMED
09:06 # 33 FLOW
09:08 # 34 TIME   12.00 MIN
09:20 # 35 HALTED AND RESET
09:20   CYCLE COMPLETE
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the next stage. Spectrometric monitoring of column effluent produces data related to soluble reactant concentration within minutes, and it can be processed rapidly. However, many factors combine to reduce the precision attainable below that strictly required.

Experimental conditions must be optimised for synthesis rather than analysis. Using only an (exactly) two-fold excess of symmetrical anhydride in the acylating step [equation (1)], complete reaction would result in a fall in solution Fmoc concentration of 25% below the initial value. An unacceptable 95% coupling yield results in a theoretical fall in Fmoc concentration of 23.75%. With the larger initial excesses commonly used in solid-phase chemistry the situation worsens rapidly. The fall in Fmoc concentration anticipated for complete acylation is also a function of the resin content of the column, resin functionality, and efficiency and manipulative losses in the anhydride-forming step. To maximise reaction rate

Table 3. Amino acid analysis for intermediate and final peptide resins [cols. (1)—(6)], total crude pentadecapeptide [col. (7)], and h.p.l.c.-purified peptide [col. (8)]. For column (1), the analysis is related to Gly = 1.00; for all other columns Asp = 1.00. Numbering is from the amino terminus.

Residues	(1) 15	(2) 11—15	(3) 7—15	(4) 6—15	(5) 4—15	(6) 1—15	(7) 1—15	(8) 1—15
Nle	1.13	1.15	1.15	1.16	1.17	1.18		
Gly	1.00	1.03	1.97	1.93	1.95	1.95	1.93	1.95
Asp		1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ser		0.87	0.86	0.86	0.87	1.72	1.68	1.74
Val		0.96	0.95	0.96	1.83	1.91	1.83	1.98
Thr		0.87	0.88	1.81	1.82	1.81	1.81	1.88
Leu			0.94	0.94	0.95	1.86	1.82	1.86
Pro			0.92	0.89	0.89	0.91	0.93	0.92
Lys			0.89	0.89	0.91	1.83	1.77	1.88
Ala					0.92	0.96	0.95	1.02

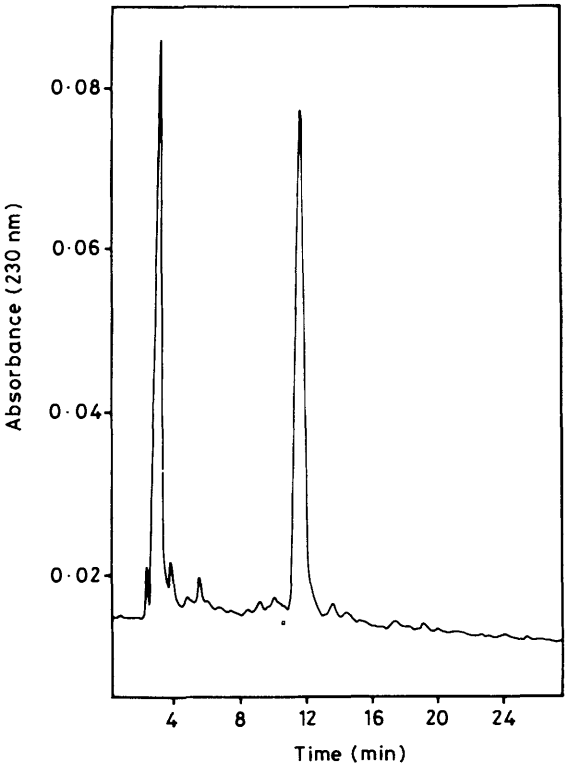


Figure 5. H.p.l.c. of total crude synthetic pentadecapeptide (**1**). For conditions see Experimental section

$$2(\text{Fmoc.NHCHRCO})_2\text{O}\ (\text{solution}) + \text{H}_2\text{N-Polymer}\ (\text{solid}) \longrightarrow \begin{cases} \text{Fmoc.NHCHRCO.NH-Polymer}\ (\text{solid}) \\ + \\ \text{Fmoc.NHCHRCO.OH}\ (\text{solution}) \\ + \\ (\text{Fmoc.NHCHRCO})_2\text{O}\ (\text{solution}) \end{cases}$$

Equation (1)

and minimise the effect of dilution in the recirculating system, the initial anhydride concentration is maintained as high as possible. Commonly 0.5 mmol is dissolved in only 2 ml of

dimethylformamide. There may, therefore, be considerable variability in introducing this small volume into the synthesizer. Even disregarding errors introduced by the spectrometer, pumping system, *etc.*, it is clear that the accuracy attainable at this stage in development is unlikely to indicate more than that the synthesis has proceeded (or is proceeding) normally *towards* completion. The prospects for determining quantitatively and in real time reaction efficiencies meaningful in solid-phase synthesis terms, *i.e.* when the difference between 95, 97, or 99% yield may be highly significant, are not good.

Assuming a linear relationship between Fmoc concentration and optical density, the typical spectrometric record illustrated in Figure 4 can, in principle, be analysed in a number of ways. Most simply, the final Fmoc concentration is given by the plateau height (H_f) and total recirculating Fmoc derivative by the product of this with the total system volume. If this last parameter is known accurately the amount of Fmoc derivative taken up onto the resin can easily be calculated from the known amount of anhydride introduced. The main errors in this procedure are likely to be manipulative losses in anhydride formation and introduction, and baseline drift in the spectrometer. The height of the first peak (H_1) may be a direct measure of initial Fmoc concentration if the small volume in which the sample is introduced is sufficient to flush the spectrometer flow cell and connecting tubing completely. The total Fmoc derivative introduced is then the product of this height with the sample volume. Uncertainty about the latter will reduce accuracy in this approach. The area (A_1) of the first peak should be a true measure of the total Fmoc derivative introduced. This peak is always well separated from that following and direct summation is easy. In extension of this, the areas of succeeding peaks (A_2, A_3, *etc.*) should follow the progress of the acylation for successive passes of reactant through the resin bed, though these overlap substantially and will be much more difficult to determine. All these area measurements are critically dependent on constant flow rate from the pumping system. The area of the deprotection peak (A_D) should be a simple measure of the overall efficiency of synthesis at each cycle, reflecting both the acylation and deprotection steps and any loss of peptide from the resin. It can be related, in principle, to that of the previous residues only providing no resin sample is removed from the system for analytical purposes.

A number of technical problems needed to be solved at the outset. The initial Fmoc concentrations (*ca.* 0.5M in dimethylformamide) are too high for spectrometric measurement at $\lambda_{max.}$ (266 nm), the optical density rising above 2.5 even using the thinnest (0.1 mm) flow cells available. With this optical path length and 0.5 mmol of Fmoc-amino acid anhydride (each molecule of which contains two Fmoc groups), measurement can only be made at 304 nm or above. Use of such narrow cells is, however, technically troublesome. Substantial back pressure is generated using dimethylformamide at a flow rate of *ca.* 3 ml min^{-1}, and in recirculating mode ingress of air is encouraged during the suction part of the pumping cycle. The spectrometric results described below for the adenovirus tail fibre synthesis were obtained using a 0.1 mm cell, but measurements at 1.0 mm path length and 312 nm are now proving equally satisfactory and are likely to be generally adopted in the future. Both wavelengths lie on the falling edge of the absorption curve where optical density is changing sharply with wavelength. This is likely to be a further significant source of inaccuracy and care has to be exercised in the initial calibration and in maintaining a stable wavelength setting of the spectrometer.

It was early apparent that the relationship between Fmoc concentration and optical density in dimethylformamide solution was not linear. In part this departure from Beer's Law may be due to the high concentrations involved, but fluorescence phenomena are also important. In dimethylformamide, the fluorenylmethyl chromophore has a strong emission band at 312 nm,* and indeed Fmoc derivatives have been advocated as fluorescent labels.[32] With some simple optical systems it is possible to obtain negative peaks during the monitoring process. The curvature of the concentration/optical density relationship is easily corrected by constructing a calibration curve. This linearisation process forms part of the data processing software of the semi-automatic synthesizer.

Of the various possibilities mentioned above for analysing the acylation reaction spectrometric data, we have thus far concentrated on area analysis of successive peaks in the reaction profiles. This provides interpretable data early in the acylation period and has thus far given the most indicative results. Figures 6a—c show microprocessor-generated graphical presentations of the data collected at the first Fmoc-amino acid addition step in the synthesis, incorporation of the internal reference norleucine residue. The raw data is plotted in Figure 6a. The first peak represents activated Fmoc-amino acid passing through the spectrometer cell direct from the sample loop, subsequent peaks after one, two, and three passages through the resin bed. A simple integration procedure in which peak areas are defined by verticals dropped from successive valleys to the base line applied to this data gives progressively *increasing* peak area, a consequence of the non-linear relationship between Fmoc concentration and optical density. Linearisation of this data by interpolation and replotting gives Figure 6b. The same integration procedure now gives areas which have also been plotted in the figure and which are consistent with a rapid and complete reaction. Peak areas corresponding to completion of the second and third passes through the column are equal, indicating cessation of anhydride uptake by the resin. This particular result is deceptively clearcut with near ideal plateauing of successive areas. Within the accuracy of the measurements and integration procedure, a steady Fmoc concentration of 77% of the starting value has been reached after two passes through the column. Often, however, this simple method of area evaluation gives much less satisfactory results. Substantial overlap of peaks occurs after just a few passes through the column, and the peaks are asymmetric. Definition of peak area by dropping verticals from the valleys is commonly inadequate under these circumstances, and successive peaks may show a continuing slight downward or more commonly upward trend. These effects are illustrated by the next two residues for which data was collected, Asp-14 (Figure 7a) and Ser-13 (Figure 8a), the second and third residues in order of addition.† An alternative curve-fitting procedure[33] for peak summation developed for us by Dr. R. Diamond ‡ overcomes this problem. In Figure 6c, 7b, and 8b, the same data has been evaluated by curve fitting for all peaks except the first. This does not overlap, is particularly steep, and experience has shown is best integrated by direct summation. The area plots now indicate progress towards complete or nearly complete acylation after three passes through the column (contact time at 50% maximum concentration or above is *ca.* 3 min), in agreement with the results of the first colour tests (complete reaction after 25 min).

These results are encouraging and provide welcome reassur-

* We are grateful to Dr. D. A. East for these fluorescence measurements and for advice.

† Useful data cannot be obtained for the two intermediate steps (addition of the reversible linkage agent and esterification of the first residue to this) since the first does not involve the Fmoc chromophore and in the second absorption is dominated by dimethylaminopyridine catalyst. Likewise, acylation reactions involving other chromophores (nitrophenol, hydroxybenzotriazole) in the presence of the Fmoc group cannot be followed directly by the general procedure.

‡ We are grateful to Dr. Diamond for developing the necessary mathematical procedures and for preparing the Basic program.

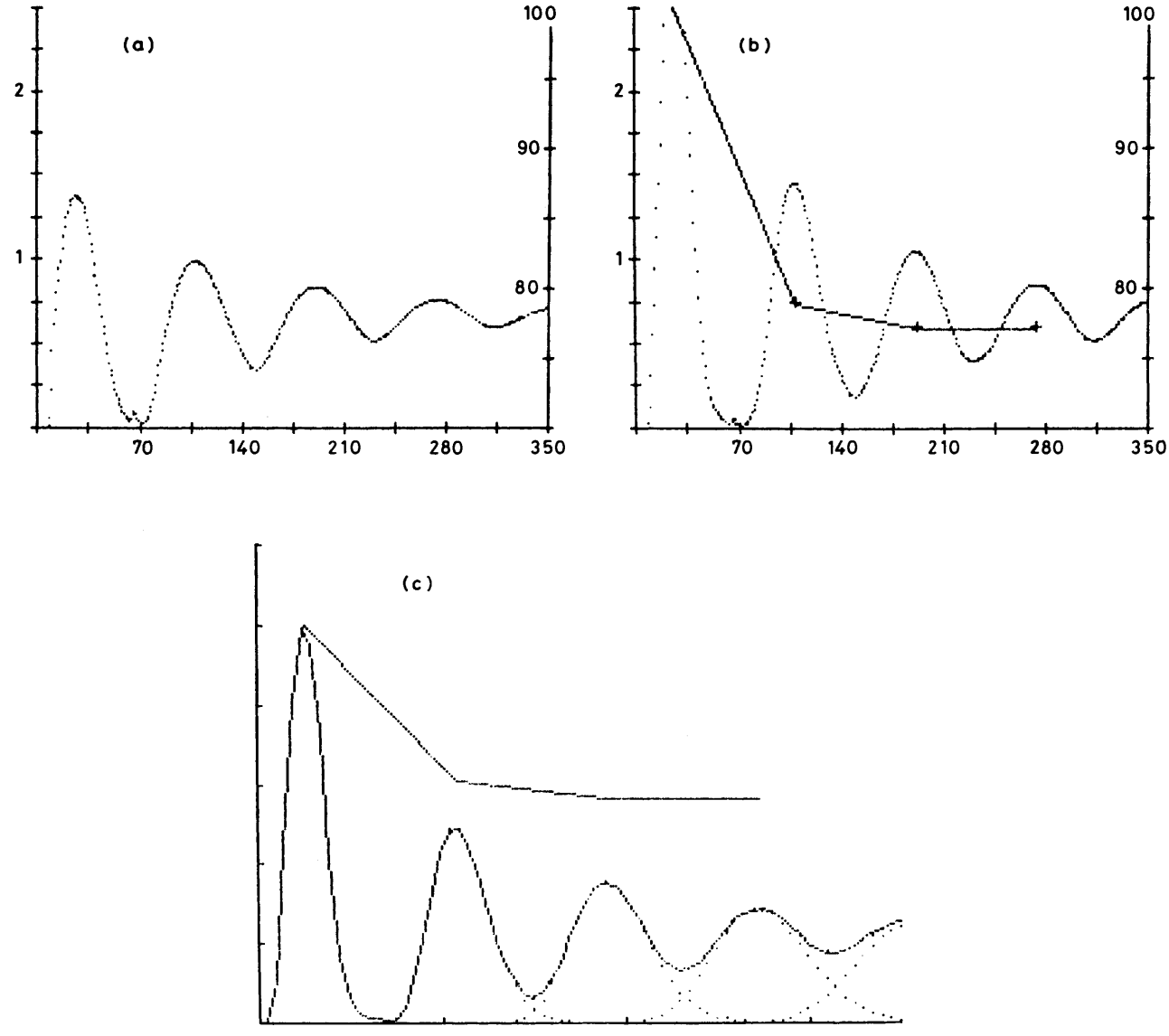


Figure 6. Computer monitoring of the acylation reaction. Acylation of amino-resin by Fmoc-norleucine anhydride. (a) Raw data: 350 measurements of optical density have been made at 2 s intervals; x-axis, measurement number; y-axis, optical density. (b) The same data corrected for non-linearity in the optical density–concentration relationship. An arbitrary scaling factor is included in the correction algorithm. Areas of individual peaks have been evaluated by summation from valley to valley and are plotted referred to peak 1 = 100%. (c) Area evaluation by fitting and summation of individual peak curves to the corrected reaction profile [33]

ance that the synthesis is proceeding normally. The results are obtained at a time when remedial action is possible should this prove necessary. However the sensitivity is at best marginally adequate. The one step in the synthesis where subsequent amino acid analysis indicated incomplete acylation (addition of the twelfth residue, Val-4) gave the reaction profile of Figure 9. This does indeed show a continuing downward trend in the incorporation profile after three passes through the column, though the difference from the earlier figures is small. Much experience has now indicated that the curve fitting and summation procedure slightly underestimates the area of the first fitted peak (the second peak in the figure), in agreement with the profile observed.

As expected from the discussion above, the absolute values for amino acid uptake show wide variations, and under the experimental conditions of the synthesis are not significant. Our more recent results have shown clearly that a major disturbing factor is inconstancy of flow rate, caused by frequent (two or three times per cycle) stopping and restarting the pump. This interruption of the flow is necessary only for removal of resin samples for colour tests and amino acid analysis, not for introduction of activated amino acid derivatives. As progress towards full automation continues these interruptions should no longer be necessary.

The spectrometric data for the successive deprotection steps are of chemical as well as analytical interest. In principle, the areas of successive peaks could be a simple measure of the Fmoc content of the resin prior to deprotection, and successive areas may be compared to follow the progress of amino acid incorporation throughout the synthesis. The same caveats regarding inaccuracies in measurement apply (particularly fluctuation in pumping rate); additionally the regular removal of resin for chemical analysis will lead to a progressive decline in peak areas. The results obtained for the early residues were in

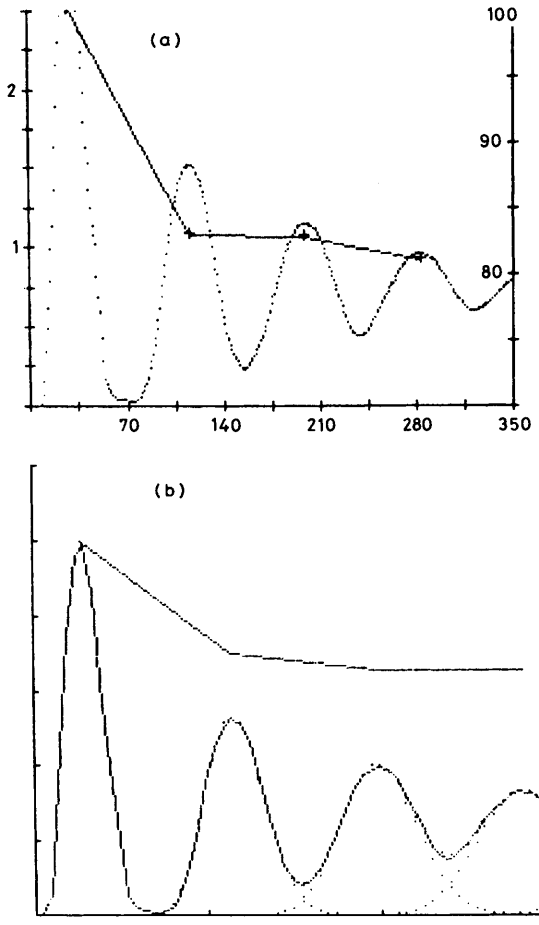


Figure 7. Acylation of glycyl-resin by β-t-butyl Fmoc-aspartic acid anhydride. Area evaluation by (a) direct summation of the linearised data; (b) curve fitting

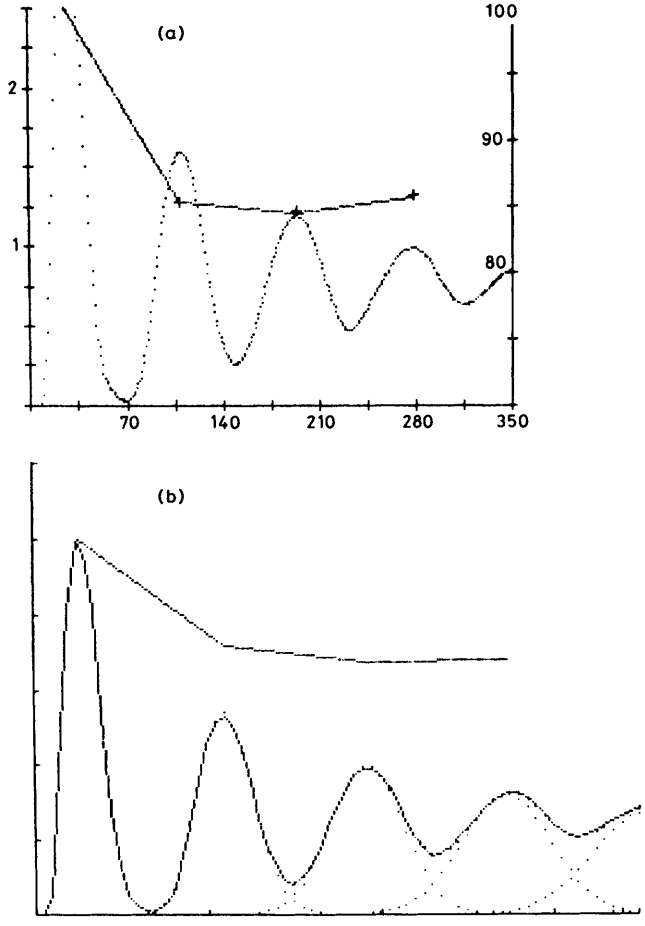


Figure 8. Acylation of aspartyl-resin by *O*-t-butyl Fmoc-serine anhydride. Area evaluation as in Fig. 7

reasonable agreement with this although again there are wide variations. However, we have observed previously that on occasions the deprotection peaks may undergo a rather sudden change in shape (*e.g.* see ref. 11). Such a change was also encountered in the present work. The transition is illustrated by the three successive deprotection profiles for Ala-5 (the 11th residue added) (a typical result; all the previous residues were similar), Val-4 (a slow and evidently hindered acylation reaction), and Ser-3 (Figures 10a—c). The following profile was similar to the last of these. The effect is characterised by a much lower and broader deprotection peak corresponding to slower release of chromophore into solution. Deprotection reactions were repeated or the times increased for all the following steps. It is not possible to say whether this apparent slowness is due to reduced rate of cleavage of the Fmoc-protected peptide resin or to restricted diffusion of the fluorene derivative out of the resin matrix. In either event, the observations suggest a change of structure within the matrix, probably a consequence of increased association between the protected peptide chains. Similar effects may have been encountered at similar chain lengths in polystyrene-based solid phase peptide synthesis.[34] From time to time in earlier polyamide solid-phase synthesis using beaded gel resins (*e.g.* ref. 17) visible changes in the structure of the resin have been observed. These are characterised by the reduced volume and more granular nature of the swollen gel, and generally slower reaction rates, and are consistent with effectively increased crosslinking through peptide

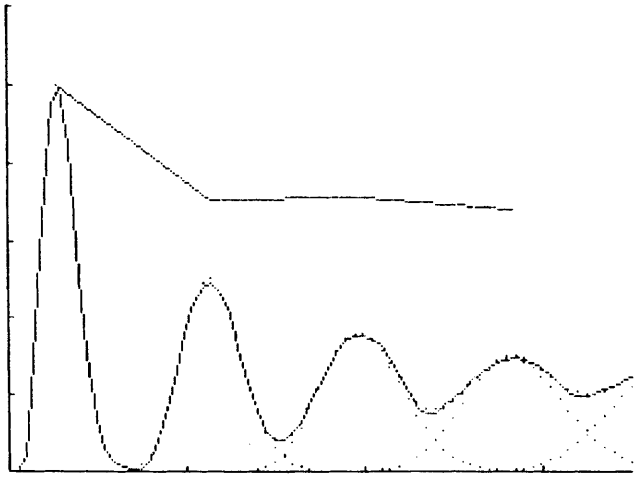
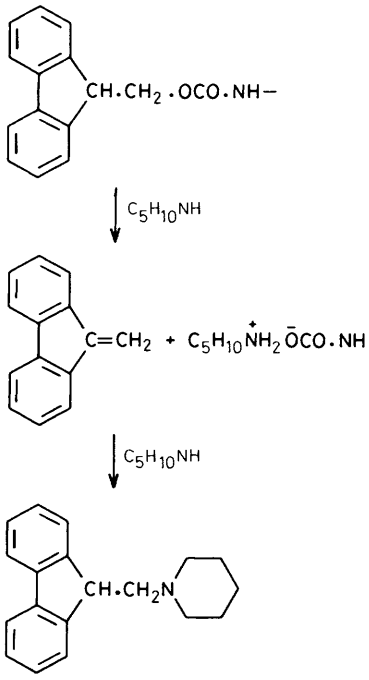


Figure 9. Acylation of alanyl-resin by Fmoc-valine anhydride. Area evaluation by curve fitting

association. In the present case, the succeeding acylation and deprotection reactions were brought to completion without great difficulty, but the areas of the deprotection peaks were appreciably lower, around 85% of those expected. We attribute this change in peak area not to incompletion of the Fmoc cleavage reaction (though this may be slower), but to liberation

of the absorbing species into solution and its measurement under different conditions. The Fmoc-cleavage reaction is usually so fast that it is occurring at the leading edge of the piperidine reagent as it passes down the column. The peak is effectively eluted on a gradient of piperidine in dimethylformamide, produced not only by mixing at the interface but also by neutralisation of the initially formed carbamate [equation (2)] and uptake of piperidine by reaction with



Equation (2)

dibenzofulvene. Thus when entry of the absorbing species into the cuvette is delayed (as in Figure 10c), the optical density is measured in a different solvent mixture. We have confirmed that the extinction of dibenzofulvene-piperdine adduct in dimethylformamide-piperidine mixture is strongly dependent on the piperdine concentration (Experimental section), and in the appropriate direction. Thus equal peak areas for successive deprotection reactions are to be anticipated only under strictly equivalent experimental circumstances.

Spectrometric monitoring of continuous-flow solid-phase synthesis is attractive and in a qualitative or semi-quantitative sense provides useful and reassuring information. There are evidently many pitfalls in attempting strictly quantitative interpretation of the data. At the present stage of development, the technique is useful in indicating normal and satisfactory progress of both acylation and deprotection reactions, without establishing their completion. It can also be used to verify the effectiveness of the first wash operation and to check generally on correct operation of the synthesizer. Much experience has shown how valuable this information feedback is.

Experimental

The following procedures for solvent and reagent purification were rigorously followed. Dimethylformamide: freshly fractionally distilled under reduced pressure through a 12 in Fenske column packed with steel gauze coils. Acetic acid: distilled from chromium trioxide. 2-Methylbutan-2-ol: fractionally distilled from anhydrous potassium carbonate. Dichloromethane: distilled from phosphorus pentaoxide less than 48 h before use. Piperidine: distilled from potassium hydroxide. Di-isopropylethylamine: distilled from ninhydrin and then from potassium hydroxide. Ethylene diamine: distilled from potassium hydroxide. Dicyclohexylcarbodi-imide: distilled under reduced pressure using a nitrogen bleed. 1-Hydroxybenzotriazole: recrystallised from water.

General procedures for solid-phase synthesis using beaded poly(dimethylacrylamide) supports[7] and fluorenylmethoxycarbonylamino acids[6] have been given previously. Fmoc-amino acids were commercial samples (Cambridge Research Biochemicals Ltd., Button End Industrial Estate, Harston, Cambridge) or were previously prepared in the laboratory (Fmoc.Nle.OH, Fmoc.Lys(Boc).ONp). All were checked for purity and identity by melting point, optical rotation, and t.l.c. (chloroform, methanol, acetic acid, 85:10:5 v/v).

Resin samples for amino acid analysis were taken after removal of the terminal Fmoc group and were hydrolysed *in vacuo* using redistilled 6M-hydrochloric acid at 120 °C for 18 h.

Equipment for Continuous Flow Solid-phase Synthesis.—(a) Manual instrument. This was assembled from Altex rotary valves types 243291 (three way) and 243288 (six way) (Anachem Ltd., 20 Charles Street, Luton, Beds LU2 0EB), and a Dosapro Milton Roy Minipump 'A' (Usine Dosapro Milton Roy, 27360 Pont-Saint-Pierre, France). The flow rate was 3.3 ml min^{-1}. Connections utilised 1/16 in (within the recirculating loop) or 1/8 in o.d. PTFE tubing and Altex fittings. The column was of all glass construction, 15 mm i.d. with a sintered glass support and Altex connector at the lower end and a B14 Quickfit taper joint at the upper. A mating B14 cone was also fitted with a sintered filter and Altex connector. A column length of 44 mm between the upper and lower filters in the assembled column accommodates about 2.3 g of poly(dimethylacrylamide)–kieselghur resin.

(b) Semi-automatic instrument. This was assembled similarly using 4 way 0.031 in bore Altex slider valves type 243265 (Anachem), each fitted with one pneumatic actuator type 243270 and one return spring type 243271. Valves V2 and V4 (Figure 2) were assembled with two slider valves per actuator unit. All return spring units were modified by attachment of a small projecting blade at the rear which interrupted the beam of an optoelectronic sensor when the valve was actuated. An adjustable nitrogen supply of *ca.* 90 lb in^{-2} controlled by 24 V DC solenoid air valves type 243629 (Anachem) was provided for the pneumatic acutators. A second nitrogen supply of *ca.* 3 lb in^{-2} was provided for the reagent bottles. The pump and column assembly were as in (a) above. A non-return valve (Lee Products Ltd., The Vale, Chalfont St Peter, Gerrards Cross, Bucks SL9 9SU, type TCKA 6201070A) was included in the drain outlet (Figure 3). The sample loop was constructed of 1/8 in o.d. PTFE tubing and had a volume of 3.5 ml. It was filled by suction from a 5 ml glass syringe with the sample contained in a 5 ml syringe barrel fitted with a glass or PTFE sintered filter.

The micro-controller consisted of a Hewlett Packard HP85 microcomputer fitted with an additional 16K memory unit (minimum memory requirement is presently 32K), I/O Rom, and serial (RS232) and parallel (HPIB) interfaces. The serial interface connects directly with an LKB Ultrospec 4050 u.v. monitor (LKB Biochrom, Science Park, Cambridge). The parallel interface connects with a Microlink IEEE 488 Interface Unit (Biodata Ltd., 6 Lower Ormond St., Manchester MI 5QF) containing two 4 way heavy duty relay modules type HDR4, an 8-way level sensor module type CC8, and bus control and power supply modules. A regulated 24 V 3 A supply for the solenoid valves is also provided in the Microlink Unit. In a separate unit, a series of toggle switches are connected in

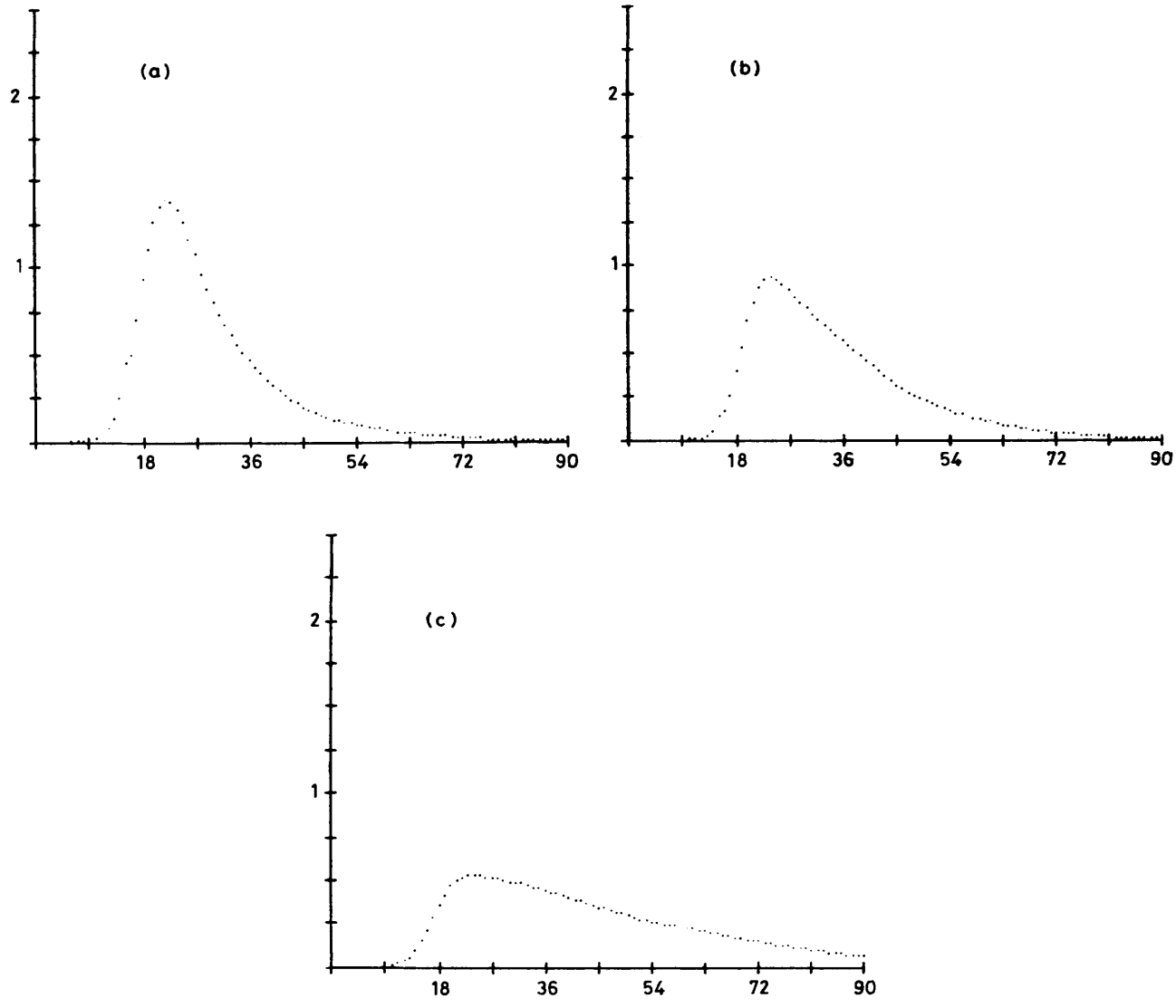


Figure 10. Release of dibenzofulvene or its piperidine adduct into solution during deprotection of (a) Fmoc-Ala-, (b) Fmoc-Val, and (c) Fmoc-Ser(Bu')-peptide-resins

parallel with the relay contacts, enabling direct manual control of the solenoid valves and of the pump.

Control Functions for Semi-Automatic Synthesizer.—The following sequences of control valve and pump operations were defined and initiated either by keyboard commands (manual operation) or during execution of the control program (automatic mode). REAGENT: kbd entry '1', V4 OFF; kbd entry '2', V4 ON. FLOW: V1 OFF, V5 ON, delay 3 sec (purge lines), V1 ON, Pump ON, V5 OFF. RECIRCULATE: V1 OFF, Pump ON. REVERSE: V3 toggles OFF/ON or ON/OFF. LOOP I/O: V2 toggles OFF/ON or ON/OFF. TIME: variable time delay before execution of next command (automatic mode) or warning tone (manual mode). PAUSE: (automatic mode only) PUMP OFF, cancelled by CONT(inue) key. MONITOR: displays alternate set of functions for control and data output from the u.v. spectrometer, data storage and recall, processing, printout, and plotting.

Linearisation of Optical Density Data for Fmoc-derivatives in Dimethylformamide Solution.—The optical densities of standard solutions (2.0—0.1M) of Fmoc-isoleucine in freshly redistilled DMF at 304 nm using a 0.1 mm silica flow cell were measured: [O.D., (concn.)], 2.27 (1.19), 2.04 (0.81) 1.83 (0.62), 1.485 (0.40), 1.33 (0.31), 1.09 (0.20), 0.95 (0.15), 0.75 (0.10)*, 0.57 (0.05), and 0.345 (0.02). [* A better fit to a smooth curve is obtained with these co-ordinates replaced by 0.75 (0.093)]. A similar calibration curve was determined for the deprotection product (piperidine-dibenzofulvene adduct in 20% piperidine/DMF), but use of this was discontinued when it was found that measured optical densities were strongly dependent on piperidine concentration. Solutions of Fmoc-cyclohexylamine [(a) 0.507 and (b) 0.502 g)] in 20% piperidine/DMF (10 ml) were kept for 20 min and then diluted 2, 4, and 8 fold with DMF and with 20% piperidine/DMF respectively. The optical densities were measured at 312 nm (a) 1.35, 1.16, 0.97, 0.77; (b) 1.34, 0.84, 0.52, 0.31.

Physically Supported Copoly(dimethylacrylamide-bisacryloylethylenediamine-acryloylsarcosine Methyl Ester) (with E. Atherton and A. E. Rosevear).—*N,N*-Dimethylacrylamide (33.3 g, 336 mmol) and then acryloylsarcosine methyl ester (2.83 g, 18

mmol) were added to a solution of bisacryloylethylenediamine (3.90 g, 23.2 mmol) in water–dimethylformamide (3.12:2 v/v; 137 ml). 10% Aqueous ammonium persulphate (25 ml) was added and the mixture poured immediately onto the kieselguhr support (335—500; μm 100 g) and stirred thoroughly with a glass rod. The polymerisation mixture was put into a desiccator and evacuated with a water pump vacuum for 2 min when vigorous degassing occurred. It was left *in vacuo* for 15 min after which the vacuum was broken by the introduction of nitrogen and the mixture left for 2.5 h. After being washed thoroughly with water on a sintered glass filter, the kieselguhr-polymer was passed through a 700 μm sieve, which released excess of surface polymer, and was then washed on a sintered funnel by back-flowing with water. The support was then washed by decantation (× 4) before being filtered off, washed with acetone and then ether, and dried under high vacuum over P_2O_5; yield 125.5 g (Found: Sar, 0.108 m equiv. g^{-1}).

Preparation of Fmoc-amino Acid Symmetrical Anhydrides.—The Fmoc-amino acid (1 mmol) was dissolved in the minimum amount of dichloromethane (less than 5 ml; a few drops of DMF were added to complete solution if necessary) in a 50 ml round-bottomed flask containing a small magnetic stirrer bead. A solution of dicyclohexylcarbodi-imide (98 mg, 0.475 mmol) in dichloromethane (1 ml) was then added and the mixture stirred for 10 min. Precipitated dicyclohexylurea was filtered off and the filtrate evaporated under reduced pressure. The residue was dissolved in DMF (2 ml), transferred with a Pasteur pipette to the sampler syringe barrel, and drawn into the sample loop. The flask was rinsed with further DMF (1 ml) which was likewise transferred into the sample loop. Anhydride preparation was timed so that the acylation reaction could commence immediately.

Some Fmoc-amino acid anhydrides (*e.g.* norleucine, glycine, alanine) were precipitated from the dichloromethane solution during preparation. When this occured the suspension in dichloromethane was evaporated without prior filtration, the residue swirled with DMF, the whole transferred to the sampler syringe barrel, filtered into the sample loop, and rinsed in as usual.

Functionalisation of Methoxycarbonyl-polymer Support.—The following preliminary operations may be conveniently carried out on a large scale to provide resin for several syntheses. The foregoing kieselguhr-polymer (2.5 g, *ca.* 0.27 mequiv. sarcosine) was shaken gently in a sealed round bottomed flask with an excess of ethylenediamine (sufficient to cover the resin) overnight.* The ethylenediamine was removed and the resin washed with dimethylformamide by swirling and decantation. Most of the resin was transferred to the flow synthesizer glass column described above and washed with DMF until the effluent gave no colouration with ninhydrin (*ca.* 30 min). The symmetrical anhydride (0.475 mmol) of Fmoc-norleucine was prepared as described above. The synthesizer was then put into automatic mode and the program given in Table 2 commenced. During the prewash (Table 2, step #1), the anhydride was introduced manually into the sample loop and the following steps allowed to proceed sequentially. Progress of the acylation reaction was followed photometrically (Figures 6a—c, see text). Negative ninhydrin[29] and trinitrobenzenesulphonic acid[30] tests were obtained after *ca.* 25 min. The total acylation time was 40 min. The wash and deprotection cycles continued as in Table 2.

Addition of the reversible linkage agent followed immediately. A solution of *p*-hydroxymethylphenoxyacetic acid 2,4,5-trichlorophenyl ester (0.184 g, 0.5 mmol) and 1-hydroxybenzotriazole (0.076 g, 0.5 mmol) in DMF (2 ml) was introduced into the sample loop as before and rinsed in with further DMF (1 ml). The same sequence of synthesizer operations was used for the acylation reaction but the program was terminated at step #22 (Table 2). The spectrometric record was dominated by absorption due to 1-hydroxybenzotriazole and the data was not processed. Negative responses to ninhydrin and trinitrobenzenesulphonic acid was obtained at the first test after *ca.* 25 min.

Esterification of Fmoc-glycine to the Functionalised Resin and Cleavage of the Fmoc Group.—Fmoc-glycine (0.743 g, 2.5 mmol) was converted into its symmetrical anhydride with dicyclohexylcarbodi-imide (0.246 g, 1.19 mmol) in dichloromethane (10 ml) for 10 min as usual. The symmetrical anhydride was precipitated and the whole reaction mixture was evaporated under reduced pressure. A solution of 4-dimethylaminopyridine (30.8 mg, 0.25 mmol) in DMF (0.5 ml) was added directly to the top of the resin column and rinsed on with more DMF (0.5 ml). The foregoing mixture of Fmoc-glycine anhydride and dicyclohexylurea was swirled with DMF (3 ml), filtered directly onto the top of the resin bed, and rinsed on with DMF (1 ml). The synthesizer was set in recirculation mode (manual control). After 30 min the reaction was terminated and the column washed with DMF for 30 min. The spectrometric record of the acylation reaction was dominated by absorption due to 4-dimethylaminopyridine and the data was not processed. No colour tests are available for this step. The synthesizer was set in automatic mode and the program commenced at step #23 (Table 2). Deprotection proceeded normally. A resin sample was removed for amino acid analysis at step #32 (Table 3).

Solid-phase Assembly of H-Lys-Leu-Ser-Val-Ala-Thr-Lys-Gly-Pro-Leu-Thr-Val-Ser-Asp-Gly-OH.—The acylation and deprotection procedures of Table 2 were used to add the following 14 amino acid residues to the foregoing glycyl resin. With the exception of lysine (see below), 1.0 mmol of Fmoc-amino acid was activated with a slight deficiency of dicyclohexylcarbodi-imide (0.475 mmol) as described above, timed to coincide closely with completion of the previous cycle. The following side chain protected Fmoc-amino acids were used: Fmoc.Asp(OBu^t).OH, Fmoc.Ser(Bu^t).OH, Fmoc.Thr-(Bu^t).OH. Both lysine residues were introduced using the activated ester derivative, Fmoc.Lys(Boc).ONp (0.5 mmol) in the presence of 1-hydroxybenzotriazole (0.5 mmol), dissolved together in DMF (2 ml). Acylation reaction times (min) were as follows: Asp-2, 39; Ser-3, 40; Val-4, 40; Thr-5, 71 + 41 (see text); Leu-6, 40; Pro-7, 63; Gly-8, 67; Lys-9, 65; Thr-10, 115 + 60; Ala-11, 73; Val-12, 111 + 41; Ser-13, 51; Leu-14, 74; Lys-15, 99. Spectrometric monitoring of the deprotection reaction indicated a marked slowing at Val-12 (Figure 10) and the deprotection reaction was repeated. Deprotection times (min) were increased for the following residues: Ser-13, 2.85 + 2.85; Leu-14, 3.85; Lys-15, 3.85.

At the completion of the synthesis, the resin was washed on a sintered glass funnel successively with DMF, 2-methylbutan-2-ol, acetic acid, 2-methylbutan-2-ol, DMF, and ether, and dried *in vacuo*. Yield, 2.24 g, amino acid analysis, Table 3. A sample (26.3 mg) was cleaved with aqueous trifluoroacetic acid (95%, 3 × 5 ml, 20 min each) and the extracts evaporated under reduced pressure, finally at 0.1 mmHg. The residual resin was thoroughly washed with 2-methylbutan-2-ol, DMF, 10% diisopropylethylamine/DMF, DMF, and ether, Found: *Nle 1.00*; Gly 0.04, corresponding to 97% cleavage. The crude peptide was examined by h.p.l.c. on Aquapore RP300 and μ-Bondapak C_{18} columns. System 1, reservoir A contained 0.1% aqueous tri-

* Our current practice is to make use of the flow synthesis microcontroller to actuate a conventional solid phase wrist action shaker on 0.5 min, off 10 min for about 100 cycles.

fluoroacetic acid, reservoir B, 90% acetonitrile, 10% A; system 2, reservoir A contained 0.01M-ammonium acetate, pH 4.5, reservoir B, 90% acetonitrile, 10% A. Flow rate 1.5 ml min^{-1}; effluent monitoring at 230 nm. System 1, linear gradient 15—30% B over 30 min, Aquapore RP300, t = 11.9 min (Figure 5); μ-Bondapak C_{18}, t = 14.9 min. System 2 linear gradient 15—30% B over 30 min, Aquapore RP300, t = 8.8 min; μ-Bondapak C_{18}, t = 14.3 min. Amino acid analysis of h.p.l.c. purified sample, Table 3.

Acknowledgements

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