Peptide Synthesis. Part 13.[1] Feedback Control in Solid Phase Synthesis. Use of Fluorenylmethoxycarbonyl Amino Acid 3,4-Dihydro-4-oxo-1,2,3-benzotriazin-3-yl Esters in a Fully Automated System

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A spectrometric technique is described for monitoring the course of acylation reactions by the title esters in solid phase peptide synthesis. The procedure has allowed construction of a fully automated peptide synthesizing system in which acylation and deprotection steps may be checked automatically for completion before proceeding to the next amino acid residue.

Recent years have seen a dramatic growth in the application of synthetic peptides in biological research, especially in immunology. Antisera raised against synthetic peptides have become important tools in protein research, and some investigations have required synthesis of complete sets of overlapping peptides spanning an entire protein sequence. Of the method of synthesis presently available, probably only the solid phase technique[2] can satisfy the present demand economically.

The success of solid phase peptide synthesis has been due in part to its ease of mechanisation. Many of the manipulative operations (reagent metering, transfer, and washing of the solid support) can be machine-aided. Punched tape and more recently microcomputer-controlled instrumentation capable of carrying out these processes automatically have been constructed. In principle these machines are able to continue peptide synthesis through addition of many amino acid residues without human intervention or presence, though under these circumstances, no analytical control can be exercised within the time scale of the synthesis. This practice has been further encouraged by the development of techniques[3] for multiple, simultaneous solid phase syntheses in which individual analytical control is likewise currently impossible.

It is implicit in the operation of all current peptide synthesis instruments in automatic mode that the coupling and deprotection reactions are carried out under predetermined, preprogrammed conditions. This approach is, in our view, not completely satisfactory. It is well established[4,5] that the rates of peptide bond formation may vary substantially as chain elongation proceeds, and that these variations are not easily predictable. They are not related exclusively to particular amino acid residues, nor to short peptide sequences. The rate of formation of any given peptide bond may thus be influenced by the *total* structure of the peptide already synthesized, as well as by the dispersing medium, nature of the solid support, acylating reagent employed *etc.* Bearing in mind the vast number of possible amino acid sequences even within the limitations of the protein amino acids,† it seems unlikely that useful controlling computer programs can be devised which will automatically adjust reaction parameters to accommodate anticipated sequence-related problems.

Much can be done in a chemical sense to minimise the importance of some rate-slowing effects. Internal aggregation of peptide chains within the flexible resin matrix may be less likely in dispersing media which, as well as being kinetically suitable, are good solvating agents for the protected peptide chain. Support effects may be reduced if this is similarly well solvated. These factors were very much in mind in our development of the Boc-[6] and Fmoc-polyamide[7] techniques of synthesis, which utilise polar, polydimethylacrylamide resins which may be regarded as 'transparent' within the good solvent dimethylformamide. Nevertheless, the ultimate, ideal situation requires that the rate or progress towards completion of each individual chemical reaction be actually measured as the reaction is proceeding. Only in this way can rate-distorting effects be detected with the prospect of reliable automatic adjustment of reaction conditions.

A significant first step towards continuous reaction monitoring was the development of a practical flow technique for solid phase synthesis.[8–10] Earlier techniques had largely employed agitated resin suspensions, and under these conditions, continuous determination of reagent concentration in solution or of the functional composition of the solid phase was excessively difficult. Conversion to a pumped flow system required development[8] of a new, physically supported gel resin which could be packed into columns and through which reagents could be pumped at low pressures and without resin compression. With a stationary packed bed resin support and a reagent stream passing continuously through one or more sensor flow cells, measurement in both phases became easily feasible.

In earlier papers in this series[9,12] and elsewhere,[11] we have described procedures for following acylation reactions (by Fmoc-amino acid anhydrides) and deprotection of the resin-bound Fmoc-peptides by spectroscopic measurements on the flowing reagent stream. These measurements could be carried out automatically by the controlling microprocessor and provided useful real time information about the progress of the synthesis. We now report an entirely new approach in which residual amino groups on the resin support are continuously monitored as the acylation reaction proceeds.[13,14] The technique is totally non-invasive and has permitted development of truly automated instrumentation in which reaction parameters are under complete feedback control.

In Part 12 we gave details of the use of 3,4-dihydro-4-oxobenzotriazin-3-yl (Dhbt) esters (**1**) of Fmoc-amino acids in solid phase synthesis.[1] These activated derivatives were excellent acylating agents for resin-bound amino groups, coupling rapidly in high yields and without racemisation. Most importantly, we observed that during the acylation reaction, a transient deep yellow colour ($\lambda_{max.}$ *ca.* 440 nm) appeared on the resin support, fading as the reaction progressed. In the absence of added base, the solution remained colourless throughout. This observation, which we ascribed to ionization and ion-pair formation of the liberated 3,4-dihydro-3-hydroxy-4-oxobenzotriazine (**2**) with resin-bound amino groups, provided opportunity for visual[1,13] and now spectrometric monitoring of

† There are, for example, 400 possible dipeptide, 8 000 tripeptide, and 64 000 000 linear hexapeptide combinations of the 20 genetically coded amino acids.

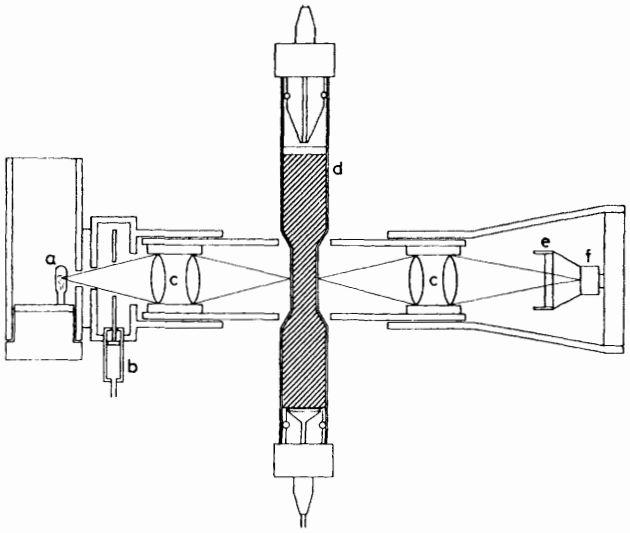


Figure 1. The solid phase photometer. (a) Lamp; (b) pneumatically operated shutter; (c) focussing lenses; (d) column reactor; (e) optical filter; (f) photo detector/pre-amplifier assembly

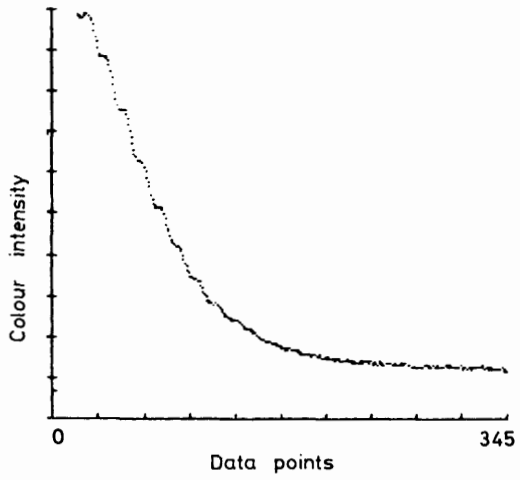


Figure 2. Plot of resin colour intensity versus time for a model coupling of Fmoc-Val-ODhbt with isoleucyl resin. 345 Readings were collected at 12 s intervals

the acylation reaction.[14] Deprotection continues to be assessed usefully by following release of the protecting group into solution.[9] Recently we have indicated a new means for attaching the first protected amino acid to the resin support which may enable this step also to be monitored automatically.[15]

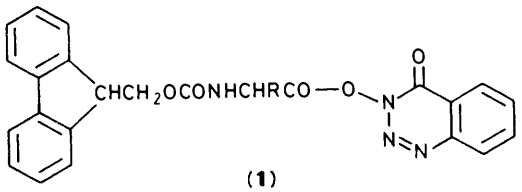
After some initial experimentation, the photometric device illustrated in Figure 1 was constructed to measure the intensity of the resin colour. This is a second prototype system* assembled in Cambridge from readily available components. A similar system has since been constructed by one of us (M. M.) in the University of Copenhagen, and this paper also includes some observations relating to the use of this instrument. The Kieselguhr-supported resin[8,9] used in our continuous flow procedure is only translucent† and with the limited sensitivity detector available required a narrow resin bed and a concentrated light source. The resin is contained in a standard chromatographic column (for details see the Experimental section) which had been narrowed and flattened. A resin bed of uniform thickness about 4 mm has proved appropriate. Light from a low voltage quartz-halogen lamp is focussed onto the surface of the resin, and the diffused, larger light patch on the far side of the resin bed focussed again onto the semiconductor photodetector. A narrow band pass filter with maximum transmission at 440 nm is placed immediately in front of the photodetector. The photodetector and integral preamplifier employed in this prototype were originally designed for use in an LKB amino acid analyser.‡ The detector has maximum sensitivity at much longer wavelength than that employed here, and incident long wavelength stray light was initially a problem. This is eliminated by placing the 440 nm filter immediately in front of the detector with a light-tight seal. In early experiments it was noted that continued exposure of the resin–benzotriazine complex to the high intensity light source resulted in some photodegradation, with slight permanent yellowing of the resin and an associated steady fall in photodetector output. This was eliminated completely by introducing a mechanical shutter mechanism between the lamp source and the focussing lens which is opened automatically immediately prior to each reading. Both electromagnetic solenoid and pneumatic actuators have been employed satisfactorily, though the latter is to be preferred giving a more controlled, less jarring opening and closing action. Use of the shutter enables exposure of the resin bed to be reduced twenty-fold or more. Alternatively, in the Danish instrument photodegradation was eliminated by inserting a second narrow bandpass filter between the first condenser lens and the column reactor. A gallium arsenide photodiode detector with enhanced sensitivity at shorter wavelengths was also employed.

Depending on the resin used, the output from the photodetector swings up to about 100 mV when the resin passes from its white to yellow state and *vice versa*. It is connected directly to a commercial 10 or 12 bit analogue to digital converter incorporating variable gain and offset controls. The digitised output is sampled by the controlling microcomputer at regular preset intervals and processed and displayed directly.

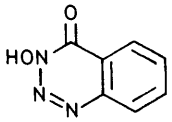
Figure 2 shows the plotted output for a very early model coupling reaction, that of Fmoc-valine Dhbt ester to isoleucyl resin. The controlling system was set to collect 345 readings at 12 s intervals, giving a total data collection period of 69 min. Plateauing of the colour density indicates that this very hindered acylation was complete after about 40 min into this period. The fluctuations in the descending trace of Figure 2 correspond in position and diminishing relative amplitude to the typical recirculation pattern observed simultaneously for the liquid phase using a flow cell.[9] They are thus due to end absorption by the acylating species (**1**) and unionised hydroxy component (**2**) in solution. The normal unsupported polydimethylacrylamide gel swells about tenfold in dimethylformamide, so that after allowing for the inert Kieselguhr element, probably considerably more than half of the column volume is occupied by recirculating liquid. These fluctuations are easily removed or reduced by averaging the data over a time corresponding approximately to the recirculation period.

Only very simple software is required to utilise the photometric data to control the acylation step in solid phase synthesis. Data presentation has been improved (*e.g.* Figure 3) to show more clearly the input stage when the Dhbt ester is first

* A more refined device is under development by Pharmacia LKB Biochrom as an extension to the Biolynx peptide synthesizing system.
† Studies have also been carried out on more transparent supports, notably on polydimethylacrylamide contained in controlled pore glass[16] and on surface functionalised glass,[17] and we expect to report on these in due course.
‡ We are grateful to Pharmacia LKB for generously making these components available to us.



(1)



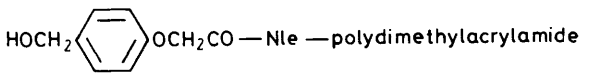
(2)

H—Val—Gln—Ala—Ala—Ile—Asp—Tyr—Ile—Asn—Gly—OH

(3)

H—Asp—Asp—Glu—Val—Asp—Val—Asp—Gly—Thr—
—Val—Glu—Glu—Asp—Leu—Gly—Lys—Ser—Tyr—Gly—OH

(4)



(5)

introduced into the column (rapid white to yellow resin transition), as well as the slower acylation reaction (yellow to white transition). The former may be sensed by the controlling microprocessor to verify previous deprotection (see below) and correct sample introduction.

During acylation, data averaging commenced after sufficient readings had been collected, and the rolling average is plotted on a vertical scale of 0 to 1 000.* It falls smoothly with little contribution from the recirculating liquid. To detect the plateau point, the differences between successive data readings are evaluated and are also displayed on the screen at the much enhanced sensitivity of -5 to $+5$ units full scale. We have adopted the arbitrary criterion that five successive difference points should lie between the $+1$ and -1 delimiters shown before automatic termination of the reaction period can occur. Additionally, the colour intensity plot should have fallen below the 400 ordinate but not below zero. This prevents premature termination if the density readings are over- or under-range (when the differences between successive digitised values become zero), and helps to ensure that extremely slow reactions do not cause false termination by nearly plateauing at an early stage. There is, in our view, no great merit in striving to achieve maximum speed in the conduct of solid phase peptide synthesis, and our current controlling program therefore allows an additional arbitrarily fixed 10 min safety period after the above termination criteria have been met. If automatic termination does not occur within the present data collection period, acylation continues for a maximum of 999 min before proceeding automatically to the next step.† This allows ample time for operator intervention if desired. It is of course a simple matter to program the controller to carry out alternative automatic actions include safe shut down or alarm signals if preferred. With only little extra equipment, the instrument could even summon human help by making an appropriate telephone call!

Operation of the prototype system in actual synthesis has been extremely encouraging. There has been some variability in baseline position from resin sample to resin sample, and also as the synthesis proceeds. Initially changes in ambient light levels was one cause of this. Repositioning of the optical filter as discussed above and shielding of the central part of the reactor column has effectively abolished sensitivity to room lighting. The upper and lower parts of the resin bed remain exposed for visual checking of reaction progress. It is essential to maintain an undisturbed, uniformly packed resin bed as far as possible; only the top of the bed should be disturbed for the removal of resin samples for amino acid analysis or confirmatory colour tests thought necessary, and the downwards direction of liquid flow should not be reversed. The baseline position is also altered if the recirculating solution becomes yellow. This is usually due to incomplete washing of all traces of piperidine from the system after the deprotection step. Redesign of the upper column end piece as shown in Figure 1 so that liquid no longer becomes trapped around the sealing O-ring has solved this problem in our instrument.‡ The liquid level is set initially below the orifice of this end piece and is stable under low pressure conditions. The lower column fitting appears to be relatively unimportant. Probably a more sophisticated electronic system with automatic adjustment of gain and/or offset levels would be advantageous in compensating for baseline shift effects.

We have already described[13] synthesis of our standard test case, the difficult acyl carrier protein decapeptide sequence residues 65—75 (**3**), using this system. Slowing of coupling reactions due to simple steric hindrance at isoleucine residues§ was easily detected, as was the extreme hindrance due to the previously recognised internal aggregation effect which occurs at the last (valine to glutamine) coupling. We now describe a second exploratory synthesis which also shows anomalous rate behaviour, that of the more substantial nineteen residue sequence (**4**) related to part of the calcium-binding protein endoplasmin. A later but similar synthesis of the same sequence using purely visual monitoring has already been discussed in Part 12 of this series.[1] It completely confirmed the results reported here.

Kieselguhr-supported polydimethylacrylamide resin previously functionalised with an internal reference norleucine residue and an acid-labile linkage agent[9] as in (**5**) was transesterified with Fmoc-glycine pentafluorophenyl ester in the presence of 4-dimethylaminopyridine catalyst. Incorporation of glycine onto the resin was 83% after a single 45 min reaction period. This compares with 96% incorporation in a similar, later experiment when the transesterification step was repeated.[1] Double acylation for short reaction periods rather than a single,

* Resolution in the figures is limited by the visual display unit to 192 (vertical) × 256 points.

† More sophisticated software has also been written which allows continuous data collection until termination. The fixed length data array is compacted when full by discarding alternate data points and continuing data collection at double the previous time interval. Data presentation then becomes similar for both fast and slow reactions with differing time scaling along the horizontal axis.

‡ In the Danish instrument, the pump has also been identified as a source of contamination when its operation is paused and the back pressure reduced. It has proved advantageous to halt pump operation deliberately several times during wash periods to ensure rinsing of the valves and pump interior. In the Cambridge instrument, pump operation is normally maintained continuously and this effect has not been observed.

§ The caption to Figure 3 in ref. 13 is incorrect. The coupling data shown is that for Fmoc-isoleucine Dhbt ester with asparaginylglycine resin, as indicated in the text.

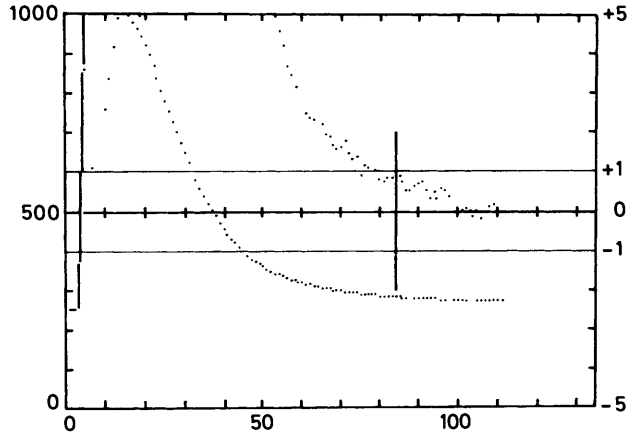


Figure 3. Solid phase monitoring of the coupling of Fmoc-Glu(OBu^t)-ODhbt with glutamyl resin (see text). The vertical line marks the computer-determined end point for the acylation reaction

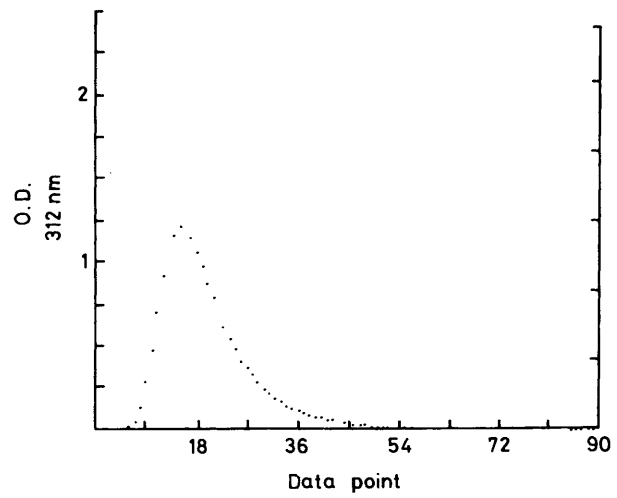


Figure 4. Release of fluorene derivative into solution during deprotection of the Fmoc-nonapeptide resin formed in Figure 3. Absorbance was measured at 312 nm

prolonged reaction is generally to be preferred for dimethylaminopyridine-catalysed esterifications, especially for amino acids other than glycine where racemisation is a potential problem.* Residual hydroxy groups were blocked by acetylation, and the resin then placed in the reaction column illustrated in Figure 1. The synthesis was continued in the automatic, continuous flow synthesizer already described[11] using Fmoc-amino acid Dhbt ester exclusively, initially in two-fold excess. Photometric data (135 readings at 20 s intervals) were collected for the solid phase during the acylation reaction; deprotection was monitored by following release of the protecting group into solution as usual.

The assembly proceeded uneventfully to the ninth residue in the sequence. In every case, complete reaction was indicated within the data collection period at times ranging from 17 to 27 min, and as discussed above, autotermination occurred 10 min later.† The data for the last of this first group of coupling reactions (Glu-9) is shown in Figure 3 and is typical. The deprotection curve obtained by monitoring at 312 nm the release of fluorene derivatives into solution is shown in Figure 4, and is also typical. A very different result was obtained for the next residue in the sequence (Val-10, Figure 5). The reaction had slowed dramatically and was incomplete when data collection ceased after 45 min. Similar behaviour was encountered previously (*cf.* Figure 4 in ref. 13) in the acyl carrier protein sequence at a stage where there was strong previous evidence for very hindered reaction, presumably due to internal aggregation effects. Reaction continued until next morning when the resin was washed and the acylation immediately repeated. The photometric data (Figure 6) then showed that the acylation was already complete; no significant yellow colour was produced on addition of the Dhbt ester and both the density and difference traces showed only residual effects due to incomplete compensation for the recirculating solution. These are exaggerated in the very early stages when concentration fluctuations are very large, and prevent more immediate

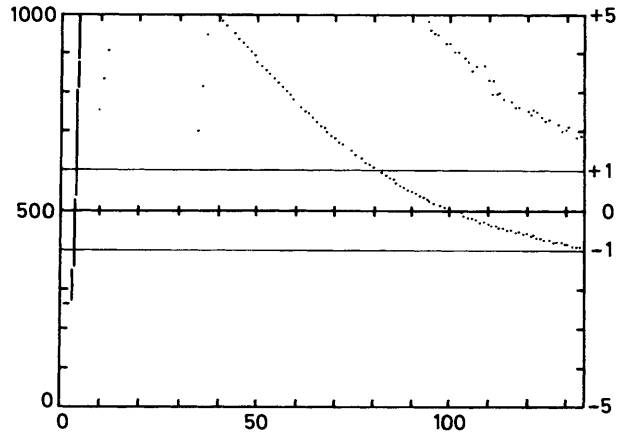


Figure 5. Solid phase monitoring of the coupling of Fmoc-Val-ODhbt with glutamyl-glutamyl resin (see text)

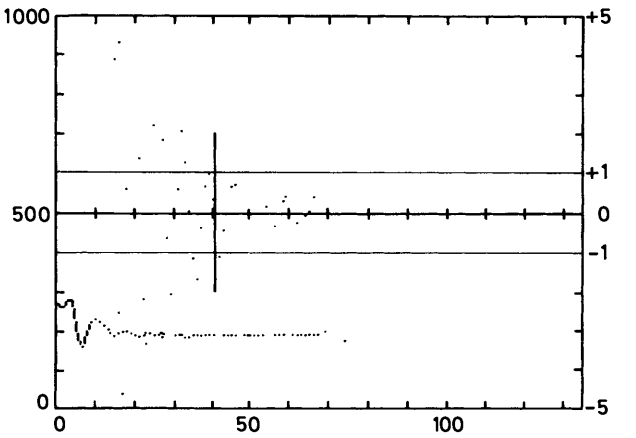


Figure 6. Repetition of the acylation reaction shown in Figure 5

* Fmoc-amino acid 2,3-dihydro-4-hydroxy-3-oxo-2,5-diphenylthiophene dioxide esters may now provide a generally superior procedure for linking the first amino acid in solid phase synthesis.[15] Dimethylaminopyridine catalyst is not required in this technique.

* These experiments were carried out on Kieselguhr-supported resin originally prepared in the laboratory.[8,9] In our experience, the less dense resin now commercially available (Macrosorb SPR, Pepsyn K, Ultrasyn) reacts about twice as fast.

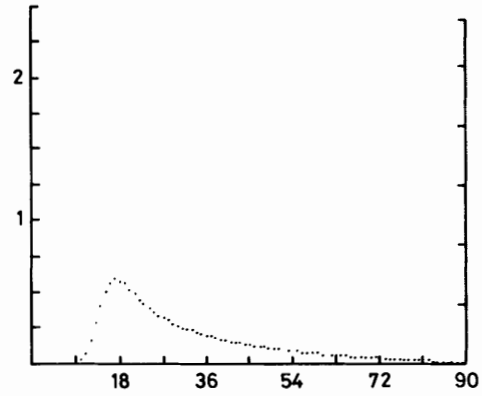


Figure 7. Hindered release of fluorene derivative in the deprotection of Fmoc-decapeptide resin (*cf.* Figure 4)

autotermination. The deprotection curve for this residue is shown in Figure 7. It has the anomalous shape and low apparent area characteristic of very highly hindered, aggregated situations.[9,11]

At this stage the molar excess of Fmoc-amino acid Dhbt ester used was increased from two- to four-fold. Addition of Thr-10 was similarly slow with a photometric record similar to that of Figure 5. Autotermination did not occur within the data collection period, but in the light of the previous experience, the reaction was terminated manually after a much shorter reaction time (3 h). Attempted repeat acylation again showed that reaction was already complete, the data obtained being almost identical to that shown in Figure 6. Very remarkably, the following amino acid residues reacted normally. Addition of Gly-12 was indicated as complete at 17.8 min, and Asp-13 at 23.4 min. Sterically hindered Val-14 was slow and just failed to terminate within the 45 min data collection period.* It was terminated manually after about 100 min. The remaining residues (including Val-14) were all signalled as complete within the time range 28—35 min.

The relative times indicated photometrically for complete reaction compare well with those obtained by visual observation.[1] As expected, the photodetector is much more sensitive than the eye and consistently indicated complete reaction substantially later. In fact, the visual and photometric procedures are probably not comparable. The former provides a rather subjective estimate of the time when the resin returns close to its original (remembered) shade; the latter measures the time when the photometer output stabilises. This is an important difference and is overwhelmingly in favour of the photometric system, since slight colouration of the solution or even slight permanent colouration of the column changes only the plateau level, not the plateau time. Our belief from the present data and that previously reported is that the system described here is probably also markedly more sensitive than the common ninhydrin and trinitrobenzene sulphonic acid visual colour tests in detecting the end point of coupling reactions.

The synthesis of the 19 residue sequence (**4**) was completed somewhat differently from that already described,[1] but without difficulty. The total crude product obtained from the peptide resin by treatment with aqueous trifluoroacetic acid gave the h.p.l.c. profile of Figure 8. Analytical details are collected in the Experimental section. Peaks A and B did not contain peptide

* Current practice is to collect data for 85 min (255 readings at 20 s intervals) or to collect data continuously using the data compacting program referred to earlier.

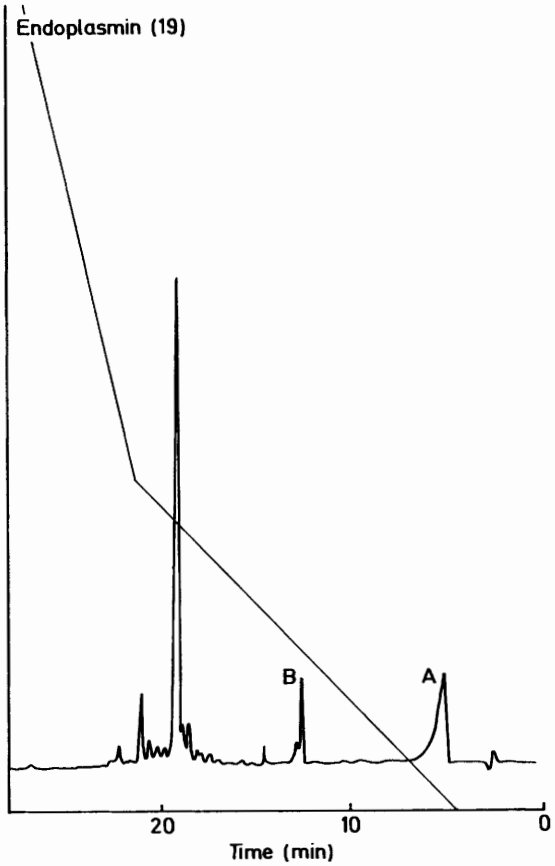


Figure 8. H.p.l.c. profile of the total crude reaction product in the synthesis of nonadecapeptide (**4**)

material. For preparative purposes, the crude mixture was purified by anion-exchange chromatography. The major peak was collected and then gave the h.p.l.c. profile of Figure 9.

We believe that the reaction monitoring technique described will prove valuable for the analytical control and automation of solid phase peptide synthesis. No additional reagents, resin removal, or other interference with the synthetic procedure is required. Measurements take place largely on the solid phase itself rather than in solution. Unexpectedly slow reactions are easily detected, and the data are obtained, displayed to the operator and computer interpreted as the reactions are proceeding and at a time when remedial action can be taken automatically or manually if required. Excessively long reaction periods may be avoided and the overall conduct of synthesis speeded. The procedure appears to be general for addition of most protein amino acids. Histidine behaves curiously with a large upward shift of baseline for the addition of Fmoc-His(Boc)-ODhbt but not for subsequent residues. This appears to be associated with deep colouration of the Dhbt ester itself in dimethylformamide solution; other amino acid Dhbt esters usually give essentially colourless solutions. We hope to report further experience covering the full range of common amino acids in due course.

Finally it is worth noting that the measurement principle may also be applicable to deprotection steps. The initial white to yellow transition as the acylamino acid Dhbt ester (**1**) is introduced is a measure of the initial concentration of free amine on the resin support. The activated ester itself is not a suitable reagent for determining this quantitatively, as the yellow colour is developed only after some degree of acylation has already

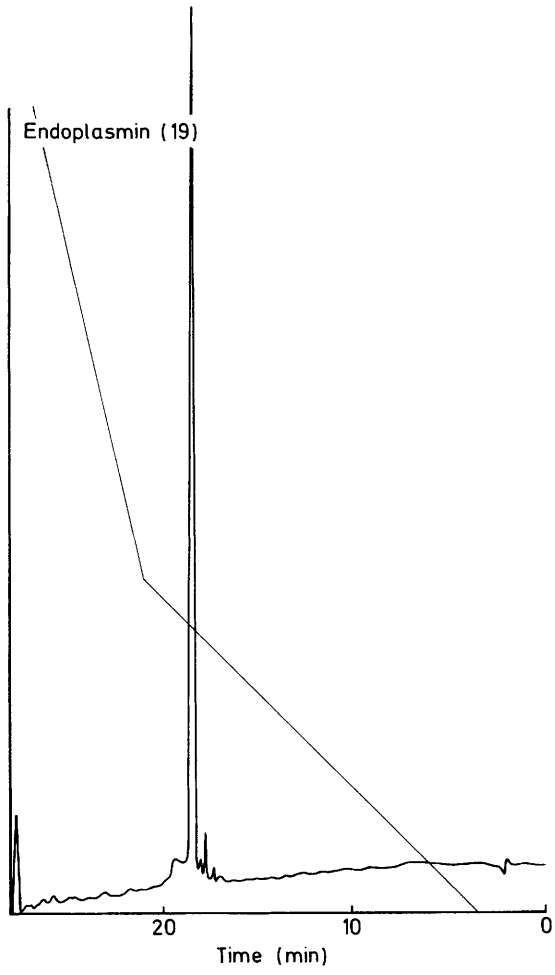


Figure 9. H.p.l.c. profile of (**4**) after purification

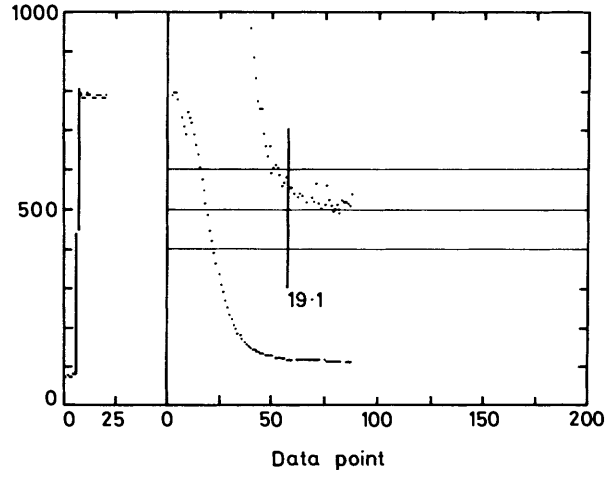


Figure 10. Solid phase monitoring of deprotection and acylation reactions. The initial colour was developed by flowing the hydroxybenzotriazine (**2**) through the resin followed directly by Fmoc-Pro-ODhbt

occurred, *i.e.*, after (**2**) is liberated. We have examined briefly an alternative procedure in which a solution of the parent alcohol (**2**) flows through the resin bed prior to addition of the ester (**1**). The data obtained now has the form shown in Figure 10. The sharp rise in level shown in the left hand box should be a quantitative measure of free amine content of deprotected resin; the curve on the right is the following normal acylation profile. The height of the left hand transition may be compared with that for previous amino acid residues. This is a different type of application where quantitative measurement is necessary and there will be problems of linearity and accuracy. Presently, completion of deprotection is usefully followed by the release of Fmoc groups into solution.[9]

Experimental

General procedures for the purification of solvents and reagents,[9] the preparation of Fmoc-amino acid 2,3-dihydro-4-oxobenzotriazin-3-yl esters,[1] and the conduct of solid phase synthesis using these esters,[1] have been given previously. The automatic continuous flow synthesizer used in the present work was adapted from that previously described [12] by addition of the photometric system below.

Solid Phase Photometric System for Monitoring Acylation Reactions.—The general layout is illustrated in Figure 1. The casing is of turned aluminium in three parts comprising lamp housing and shutter assembly, optics and column holder, and photodetector and filter assembly. The miniature 12 V 20 W quartz halogen lamp is mounted off centre in a cylindrical holder adjustable for height and rotation. The electrical leads connecting the two pin lamp socket are firmly tied back to a terminal block mounted on the outside of the holder assembly. A separate 24 V muffin fan directs a cooling air stream through and around the cylindrical housing. The lamp is mounted as close as practicable to a sliding shutter assembly actuated by an internal miniature pneumatic cylinder (Clippard Minimatic type SM-3-3). This is connected through external regulator (45 psi) and needle valve to an electrically switchable nitrogen supply. The complete lamp and shutter assembly is a sliding fit onto a central column housing turned to size to accommodate focussing lenses (eyepieces from Dixon's 8 × 30 Prinz binoculars, *ca.* 1965), at each end. The central part is cut away at the front to accommodate the column reactor which is gripped firmly by two Terry clips; a detachable, half-cylindrical plate covers the central part of the column when in use. The photodetector assembly consists of a circular printed circuit board with a centrally mounted phototransitor/operational amplifier integrated circuit (Radiospares, type RS 308-067) and passive components. The circuit board is adjustable in the plane orthogonal to the light beam and the entire detector assembly slides on the column holder along the axis of the light beam. The 440 nm narrow band pass filter (LKB part no. 41440022) is mounted in a light tight holder fitting directly over the photodetector. The output from the photodetector is connected to a 0.2/2 V dual range digital voltmeter and to analogue input (Biodata Microlink AN1) and 12 bit A/D converter (Biodata Microlink A12D) units. The controlling Hewlett Packard HP85 computer is connected through an IEEE 488 interface.

Solid Phase Synthesis of H-Asp-Asp-Glu-Val-Asp-Val-Asp-Gly-Thr-Val-Glu-Glu-Asp-Leu-Gly-Lys-Ser-Tyr-Gly-OH (**4**).—4-Hydroxymethylphenoxyacetyl-norleucyl-polydimethylacrylamide resin was prepared as already described.[1] Fmoc-glycine pentafluorophenyl ester (649 mg, 1.4 mmol) was transesterified to the resin (2.5 g, *ca.* 0.28 mol equiv.) suspended in dimethylformamide (DMF, 5 ml) in the presence of 4-dimethylaminopyridine (34.2 mg, 0.28 mmol) in a round bottomed flask for 45 min at room temp. The resin was filtered off, washed well with DMF, t-pentyl alcohol, acetic acid, t-pentyl alcohol, DMF, and ether, and dried *in vacuo*. The resin was resuspended in DMF (6 ml) and acetylated with acetic anhydride (51 μl, 0.55

mmol) and dimethylaminopyridine (34.2 mg, 0.28 mmol) for 30 min, then filtered off, washed well, and dried as before. The resin was packed in DMF into the reactor column shown in Figure 1 (Omnifit, type 446111, modified by flattening to a thickness of about 4 mm for *ca.* 2 cm just below the centre of the column).

The reactor column was attached to the automatic continuous flow synthesizer[12] fitted with the solid phase photometric system described above. The recirculating solution was also monitored at 340 nm (acylation) and at 312 nm (deprotection reactions) using a 1 mm* flow cell.

Assembly of the nonadecapeptide sequence using Fmoc-amino acid dihydro-oxobenzotriazinyl esters (initially 0.56 mmol, two-fold excess) followed the reaction protocols already described.[1] Except where stated otherwise, acylation reactions were terminated automatically 10 min after reaction was complete as indicated by the solid phase monitoring system (see text). The following *total* reaction times were recorded: Tyr-2, 37 min; Ser-3, 27 min; Lys-4, 36 min; Gly-5, 29 min; Leu-6, 32 min; Asp-7, 36 min; Glu-8, 34 min; Glu-9, 36 min; Val-10, incomplete at 45 min (see Figure 5), terminated manually after 17 h (overnight); Val-10 (repeat), 22 min (Figure 6); Thr-11, incomplete at 45 min, terminated manually after 154 min; Thr-11 (repeat), 22 min; Gly-12, 28 min; Asp-13, 33 min; Val-14, incomplete at 45 min, terminated manually at 100 min; Asp-15, 38 min; Val-16, 38 min; Glu-17, 44 min; Asp-18, 42 min; Asp-19, 43 min.

A sample (101.4 mg) of the dried, final peptide resin was treated with 95% aqueous trifluoroacetic acid for 1.5 h, the resin was filtered, washed with further trifluoroacetic acid, and the combined filtrates evaporated [Found: Gly, 3.06; Tyr, 1.05; Ser, 0.96; Lys, 1.00 (*cf.*, 3.13 μmol, equivalent to 6.34 mg of peptide); Leu, 0.96; Asp, 4.92; Glu, 3.12; Val, 2.95; Thr, 0.93]. The h.p.l.c. of this total crude product is shown in Figure 8. Peaks 1—3 from this chromatogram were collected, [Found: (peak C) Gly, 3.02; Tyr, 0.84; Ser, 0.95; Lys, 1.00 (*cf.*, 1.20 μmol, 2.43 of peptide); Leu, 1.00; Asp, 4.63; Glu, 3.03; Val, 2.55; Thr, 0.92]. Crude peptide solution corresponding to about 100 mg of dried peptide resin was applied to a column (12.6 cm × 1 cm diam.) of Whatman DE52 anion exchange resin packed in 0.01M ammonium hydrogen carbonate and eluted with a linear gradient of 0.1M (pH 7.22) to 0.5M (pH 8.22) ammonium hydrogen carbonate, flow rate 0.5 ml min^{-1}. The effluent was monitored at 230 nm and 3 ml fractions were collected. The very major peak eluting at fractions 77—100 was collected in two parts (fractions 77—87 and 88—96) and identified by h.p.l.c. but not quantified. The analytical h.p.l.c. profile of fractions 88—96 is shown in Figure 9; that from the earlier fractions 77—87 was marginally less pure.

* The thicker path length 1 mm flow cell is now strongly preferred over the 0.1 mm cell used earlier.[9] The high concentrations prevent measurement at $\lambda_{max.}$ in either case, and the thin cell generates unnecessarily high back pressure and in recirculating mode, may impede the liquid inlet action of the reciprocating pump.

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