A Sensitive, General Method for Quantitative Monitoring of Continuous Flow Solid Phase Peptide Synthesis

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A quantitative procedure for monitoring continuous flow solid phase peptide synthesis has been developed based on the colour reaction of residual amino groups with Bromophenol Blue; the procedure allows automated feedback control of syntheses.

Sensitive, quantitative analytical techniques for following the progress of peptide bond formation would be particularly valuable in solid phase peptide synthesis.[1] Introduction of the fluoren-9-ylmethoxycarbonyl (Fmoc) amino protecting group[2,3] and development of continuous flow techniques which enabled the concentration of fluorenyl derivatives in the reagent stream to be continuously measured[4] were significant steps in this direction. A further advance was the use of 2,3-dihydro-3-hydroxy-4-oxobenzotriazine (**1**) as a sensitive colour indicator for resin-bound amino groups.[5] The triazine (**1**) was liberated when Fmoc-amino acid dihydrobenzotriazinyl esters (**2**) were used as acylating agents;[5,6] the technique was thus completely non-invasive† and the yellow colour developed on the solid amino resin was easily measured and computer interpreted.[7] This provided the first fully automated system for solid phase peptide synthesis in which progress was determined by feedback analytical control.[7] We describe herein a remarkably sensitive alternative technique using the acid–base indicator Bromophenol Blue, which is likely to be completely general and to have significant advantages.

Krchnak, Lebl, and co-workers[8] showed that Bromophenol Blue is a sensitive indicator for residual amino groups under the conditions of the Merrifield technique (polystyrene resins suspended in methylene chloride). We found, however, that the indicator did not bind to amino-polydimethylacrylamide resin in dimethylformamide (DMF), the current conditions for continuous flow synthesis.[1] The resin (and solution) acquired only a pale blue colouration which was rapidly lost by washing with further DMF. In contrast, the indicator bound very strongly in the presence of weakly acidic 1-hydroxybenzotriazole (HOBt), imparting a deep bluish red colour (by transmitted light) to the resin while the solution remained colourless. The colour was discharged on acylation of the amino groups. Because of the very high extinction coefficient of ionised Bromophenol Blue ($\lambda_{max.}$ 600 nm, $\varepsilon_{max.}$ >90 000 in aqueous solution), only very small amounts of the indicator were needed. In the experiments described below, less than 0.2% of Bromophenol Blue relative to total initial amino

† *I.e.*, the technique did not require interruption of the synthesis for removal of samples or addition of special reagents. Others have used the word differently.[8]

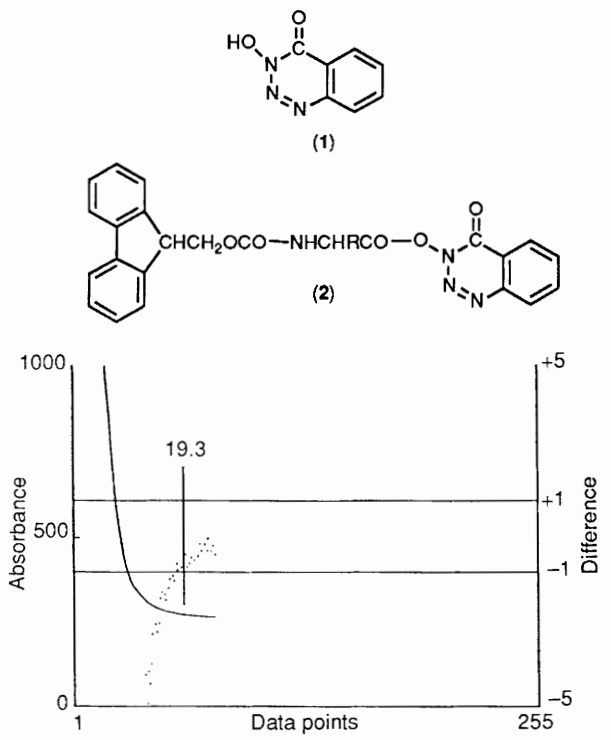

Figure 1. Coupling of Fmoc-Phe-OPfp to leucyl-resin in the synthesis of leucine-enkephalin (**3**). The controller was programmed to collect a maximum of 255 absorption readings at 20 s intervals. A rolling average[7] of six readings is plotted on a vertical scale of 0—1000 (solid line). Differences between successive data points are plotted on a vertical scale of −5 to +5 (dots). The computer determined end point is marked by the vertical line. Total reaction time 25.3 min.

group content was added, eliminating any possibility of significant side reactions caused by the indicator itself.

The following general technique was developed for continuous flow synthesis using Fmoc-amino acid pentafluorophenyl esters[9] with automatic feedback termination of acylation reactions. The computer-controlled flow synthesiser[9] was modified by addition of two extra reagent bottles and associated valving. The first contained 1% HOBt (0.065 M) in DMF; the second Bromophenol Blue (50 μM) in this solution. The solid phase photometer[7] was modified by replacement of the 440 nm narrow band pass filter by one transmitting maximally at 570 nm‡ and was used with a narrow cylindrical reactor column§ placed with its lower part in the light path. Immediately before the acylation step, DMF in the recirculation loop (resin-filled reactor column, pump, UV flow cell, and connecting tubing) was displaced automatically by 1% HOBt–DMF. For a typical column containing about 1 g (0.1 mmol) of resin,¶ the volume of reagent is *ca.* 8 ml,

‡ The filter used was from the ninhydrin colorimeter of an amino acid analyser. A four-fold increase in sensitivity should be obtained by use of a 600 nm narrow bandpass filter.

§ A simple 6 mm i.d. cylindrical column (Omnifit) may be used for small quantities of resin, or a two part cylindrical reactor in which the bulk of the resin is contained in a larger diameter column fused or joined by PTFE tubing to the smaller optically sensed part. The rectangular cross-section flow reactors used earlier[7] were shown to have poor flow characteristics by observing their behaviour with Bromophenol Blue, and are no longer recommended.

¶ For economy, most of the trial experiments used only 0.2—0.3 g of commercial Fmoc-leucine resin for which the total recirculating volume is about 6 ml.

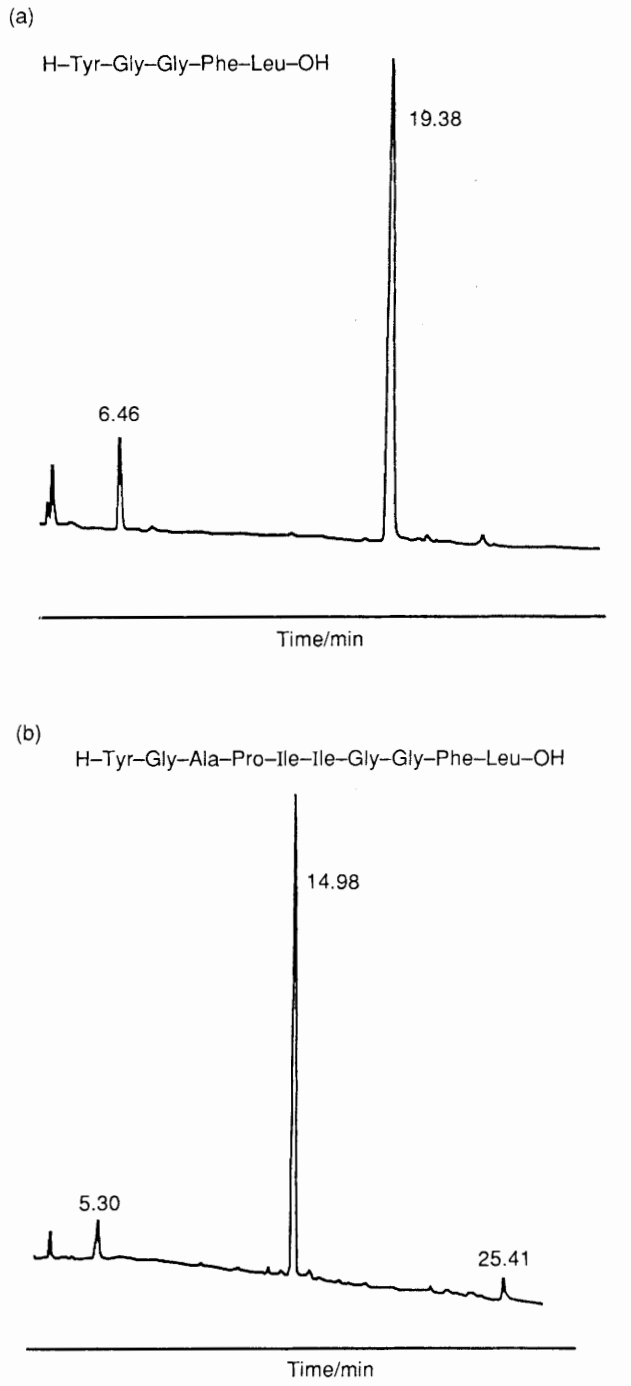

Figure 2. (a) HPLC profile of the total crude product from Bromophenol Blue-monitored synthesis of leucine-enkephalin (**3**). HPLC conditions: solvent A, 0.1% aq. trifluoroacetic acid; B, acetonitrile containing 10% A. A gradient of 10—40% B was run over 30 min. The effluent was monitored at 230 nm. (b) HPLC profile of the total crude decapeptide (**4**). Conditions as (a) except that the HPLC gradient was 10—90% B over 25 min.

Tyr-Gly-Gly-Phe-Leu (**3**)
22 15 21 19 min

Tyr-Gly-Ala-Pro-Ile-Ile-Gly-Gly-Phe-Leu (**4**)
18 14 33 21 48 26 min

Met-Gln-His-Phe-Arg-Trp (**5**)

Gln-Val-Ile-Ile-Thr-Asp-Asp-Tyr-OH (**6**)

containing *ca.* 0.5 mmol of HOBt. The HOBt reagent was followed immediately by 50 μM Bromophenol Blue (1 ml, 0.05 μmol, 0.05%) which was introduced at the bottom of the reactor column by reversing the normally downward liquid flow. It formed a deep blue diffuse band about 1 cm high at the base of the column, the rest of the resin remaining colourless. After rinsing on with further HOBt–DMF, the Fmoc-amino acid pentafluorophenyl ester dissolved in the same solvent mixture was pumped into the top of the reactor and recirculated as usual. In all cases the initial concentration of acylating agent was maintained within the normal range for continuous flow synthesis of 0.05—0.1 M by use of 0.5 mmol aliquots of each Fmoc-amino acid pentafluorophenyl ester, approximately equivalent to the amount of catalyst present.

The pentapeptide leucine-enkephalin (**3**) was used as a simple test sequence. A typical output of the solid phase photometer is shown in Figure 1. The photometer was adjusted so that output was over-scale in the initial phase of the reaction, and only the later stages were recorded. The trace falls steadily, plateauing when the reaction is complete. A simultaneous plot of the differences between successive data points was arbitrarily set to indicate completion when five successive points fell within the ±1 delimiters shown.[7] Reaction times indicated beneath (**3**) were obtained in this first enkephalin synthesis. Following previous practice,[7] an additional safety margin of 30% of the elapsed reaction period was allowed in this experiment before acylation was terminated by the controlling computer. The unpurified synthetic product was of excellent quality (Figure 2).

In a later experiment, the variety of couplings was increased by synthesis of the decapeptide (**4**) (Figure 3). The range of reaction times obtained were as expected for the more hindered couplings, and confirmed that decolourisation is not due to acylation of the indicator itself. The solid phase photometer proved to be very much more sensitive than the eye. In all the cases observed, visual decolourisation of the resin was complete when the photometer output had fallen to 500—600 units (Figure 1), corresponding to reaction times less than half of those determined automatically.

Applicability of the monitoring procedure to a wider range of amino acid types was tested with the ACTH-related sequence (**5**) (ACTH = adrenocorticotropic hormone). No difficulties were experienced with Fmoc-His(Boc)-OPfp (this amino acid derivative failed with the earlier monitoring procedure[7] based upon the benzotriazinyl esters (**2**) since Fmoc-His(Boc)-ODhbt is itself bright yellow), nor with Fmoc-Gln-OPfp or Fmoc-Arg(Mtr)-ODhbt where slow drift may occur due to interaction of the side-chains with activated carboxyl groups (OPfp = pentafluorophenyl ester, ODhbt = dihydrobenzotriazinyl ester). The general applicability of Dhbt esters in conjunction with quantitative bromophenol blue monitoring was confirmed with the threonine residue in the sequence (**6**).

We conclude that the use of Bromophenol Blue indicator in solid phase synthesis introduced by Krchnak and Lebl[8] provides an excellent and important basis for reaction monitoring and automated control in the continuous flow technique. Other indicators of similar pK_a probably may be used similarly, but the absorption maximum of Bromophenol Blue lies in a convenient part of the spectrum where the photometric system is relatively insensitive to ambient light. The new technique is likely to be general for a wide range of acylating species. It is invasive, but the additional reagents required are easily added automatically. The very small quantities of indicator used make it very unlikely that significant side reactions will ensue. The second reagent, hydroxybenzotriazole, is frequently a normal component of acylation mixtures, and is widely regarded as beneficial.

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