Self-indicating Activated Esters for Use in Solid Phase Peptide Synthesis. Fluorenylmethoxycarbonylamino Acid Derivatives of 3-Hydroxy-4-oxodihydrobenzotriazine

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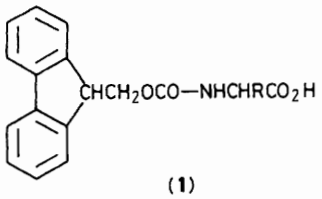
The title esters are effective acylating agents in solid phase peptide synthesis; completion of acylation is indicated by fading of the transient yellow colour produced by ionisation of the liberated hydroxy component.

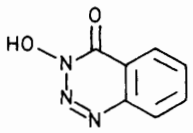
We have reported[1] that pentafluorophenyl esters[2] of fluorenylmethoxycarbonyl (Fmoc) amino acids (**1**) are efficient acylating agents in solid phase peptide synthesis under polar polyamide conditions.[3] Their use notably simplifies the conduct of solid phase synthesis by avoiding individual preactivation procedures, and provides a particularly simple solution to the problem of automatic peptide synthesiser design. However, the additional u.v. absorption introduced by the aryl ester and the liberated phenol makes quantitative spectroscopic monitoring[4] of the acylation step more difficult. This is especially the case when catalyst 1-hydroxybenzotriazole is added to the reaction mixture[1] to enhance further the reactivity of the pentafluorophenyl ester derivatives. We report now a new series of Fmoc-amino acid activated esters which are efficient in peptide synthesis and which offer an entirely new opportunity for non-destructive qualitative and quantitative monitoring of acylation reactions under continuous flow[4,5] conditions.

The favourable acylating properties of esters of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, (HODhbt) (**2**) were recognized by König and Geiger in 1970,[6] but no substantial application in solid phase synthesis has apparently been reported. We find that these esters of Fmoc-amino acids are very easily prepared and are generally stable crystalline solids,† most of which may be stored at low temperature for long periods without significant decomposition. Fmoc-Ile-ODhbt reacted with glycyl-polydimethylacrylamide resin[4] in dimethylformamide (DMF) at a rate closely similar to that of

† Details of melting points *etc.* may be obtained from us prior to full publication.



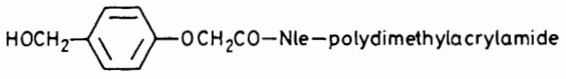
(1)



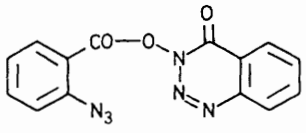
(2)

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

(3)



(4)



(5)

H.Val.Leu.Arg.Asn.Pro.Asp.Gly.Glu.Ile.Glu.Lys.Gly.OH

(6)

H.Ile.Ala.Glu.Ile.Gly.Ala.Ser.Leu.Ile.Lys.His.Trp.OH

(7)

H.Gly.Lys.Lys.Lys.Cys(Acm).Ser.Glu.Ser.Ser.Asp.Ser.Gly.Ser.Tyr.Gly.OH

(8)

Acm = *S*-acetamidomethyl

the corresponding symmetrical anhydride and about five times more rapidly than the uncatalysed pentafluorophenyl ester. During these and other early reactions, we observed that a transient bright yellow colour appeared on the resin during the acylation reaction, although the solution remained colourless in the absence of dissolved base. When acylation was complete the resin returned close to its initial off-white shade. We attribute this yellow colour to ionisation of liberated hydroxy component (**2**) by resin bound amino groups. Thus the Fmoc-amino acid activated ester provides both an effective acylating agent and a sensitive indicator of the presence of residual unreacted amino groups.

As before,[1] the efficiency of these new derivatives in solid phase peptide synthesis was tested by preparation of the difficult acyl carrier protein decapeptide sequence (**3**). The continuous flow variant[4,5] of the Fmoc-polyamide procedure was used. The polydimethylacrylamide resin was supported in rigid, macroporous kieselguhr particles[4,7] and was functionalised with a norleucine internal reference amino acid and the acid labile linkage agent as in (**4**). Esterification of the C-terminal Fmoc-glycine residue utilised the pentafluorophenyl ester derivative in the presence of 4-*N*,*N*-

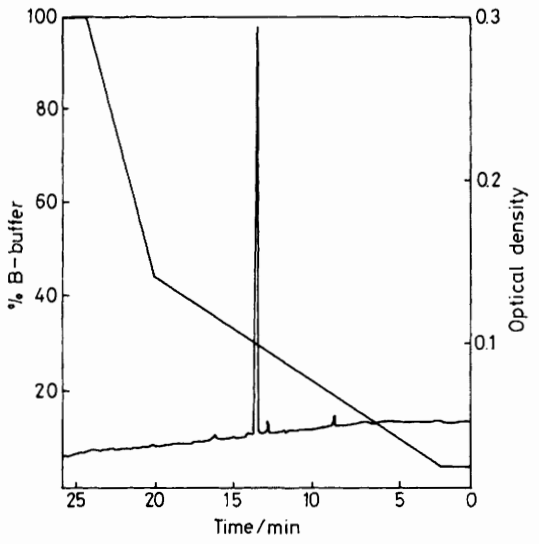


Figure 1. Analytical h.p.l.c. of total crude decapeptide on Aquapore RP-300. Reservoir A contained 0.1% aq. trifluoroacetic acid; B contained 90% acetonitrile, 10% A. After 2 min elution with 5% B, a linear gradient of 5—45% B was developed over 18 min and then 45—100% over 5 min; flow rate 1.5 ml/min. The effluent was monitored at 230 nm.

dimethylaminopyridine (DMAP) catalyst.‡ All peptide bond-forming reactions utilised the appropriate Fmoc-amino acid Dhbt ester (4 equiv.) in DMF. Urea was added to the reaction mixture[8] for incorporation of the final valine residue (see below). Fmoc groups were cleaved by 20% piperidine–DMF.

In this first experiment, the progress of the synthesis was followed by observing persistence of the initial yellow colouration of the column, although for safety acylation times were tentatively set considerably longer. The following very approximate times (in min, unless otherwise indicated) were noted§ for fading of the resin to its original off-white state with actual total reaction times in parentheses: Asn-Gly, 15 (35); Ile-Asn, 30 (65); Tyr-Ile, 18 (60); Asp-Tyr, 10 (40); Ile-Asp, 15 (40); Ala-Ile, 10 (40); Ala-Ala, 10 (40); Gln-Ala, 30 (130); and Val-Gln, 20 h (24 h). The exceptionally long reaction time noted for the final valine residue is in agreement with previous experience. After the addition of glutamine, photometric evidence for strong association of the peptide chains within the resin matrix was provided by slower release of dibenzofulvene–piperidine adduct during deprotection steps.[4,9]

The completed decapeptide was cleaved from the resin with 95% aqueous trifluoroacetic acid; detachment was 92%

‡ Remarkably, the oxodihydrobenzotriazine ester appears to be relatively ineffective in ester-forming reactions, even in the presence of DMAP catalyst. The pentafluorophenyl ester provides a convenient alternative to symmetrical anhydrides previously used for this step. With Fmoc-glycine pentafluorophenyl ester (5 equiv.) in the presence of DMAP (1 equiv.), esterification is complete in 1—2 h.

§ More recently, we have constructed a sensitive and accurate photometric system for monitoring resin-colour. The results obtained are generally consistent with visual estimation, except that both isoleucine residues now give approximately equal (36, 40 min) times for complete decolouration. With the more precise measurements now possible, we routinely allow a much shorter (10 min) safety factor after acylation is indicated as complete.

complete as judged by the glycine : norleucine analysis of residual resin. Unpurified decapeptide had amino acid analysis; Gly, 1.00; Asp, 1.91; Ile, 1.80; Tyr, 0.91; Ala, 1.87; Glu, 0.96; Val, 0.94. After h.p.l.c. purification (Figure 1; for conditions see ref. 1), the amino acid analysis was Gly, 1.00; Asp, 1.96; Ile, 1.92; Tyr, 0.95; Ala, 2.04; Glu, 0.98; Val, 0.98. A latter synthesis of the same sequence gave satisfactory results using only 2 equiv. of Dhbt ester except for the final valine (4 equiv., no urea). Satisfactory syntheses have also been achieved of the peptide sequences (**6**)—(**8**).

During the preparative work, we confirmed the observations of König and Geiger[6] that esters of (**2**) prepared with the aid of dicyclohexylcarbodiimide (DCCI) may be accompanied by the by-product (**5**). Traces of (**5**) may easily be detected by h.p.l.c. [*e.g.* Fmoc·Gly·ODhbt and (**5**) emerge at 26.8 and 24.3 min respectively on Aquapore 300 using a gradient of 0—100% B (see caption Figure 1) in 40 min]. All Fmoc-amino acid derivatives should therefore be rigorously purified before use in solid phase synthesis since the azidobenzoate (**5**) is an effective chain terminating agent. In some early experiments, traces of contaminating azidobenzoyl peptides were detected. Formation of (**5**) is minimised by preparation of the active esters in a non-polar solvent (tetrahydrofuran) rather than in polar DMF, although the latter is to be preferred for the less soluble, side chain reactive amino acids, asparagine and glutamine. Almost complete suppression of (**5**) is obtained by preformation of the Fmoc-amino acid–DCCI adduct 4 min before addition of (**2**).

We conclude that Fmoc-amino acid Dhbt esters are valuable alternatives to other acylating species previously employed in solid phase synthesis. They are easily prepared, generally crystalline and apparently stable to storage,¶ yet are exceptionally reactive towards nitrogen nucleophiles. Their very favourable racemisation-resistant properties were established by König and Geiger.[6] They provide a unique opportunity for continuous, non-invasive monitoring of solid phase synthesis‖ with potential for automation. We shall report shortly on the design and operation of a fully automated peptide synthesiser utilising Dhbt esters in which acylation times are established individually for each coupling reaction as the synthesis proceeds.

We are grateful to Mr R. Cotton (ICI Pharmaceuticals Division) who first suggested to us that these esters might be useful alternatives to pentafluorophenyl derivatives in solid phase synthesis.

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¶ The long-term stability of these esters is not yet known. We recommend storage at low temperature (−20 °C), especially for the derivatives of asparagine, glutamine, and arginine.

‖ Patent applied for.