Fmoc-Amino Acid Active Esters in Solid Phase Peptide Synthesis using *p*-Alkoxybenzyl Alcohol Resin: Synthesis of [Leu-NH_2]^5-enkephalin

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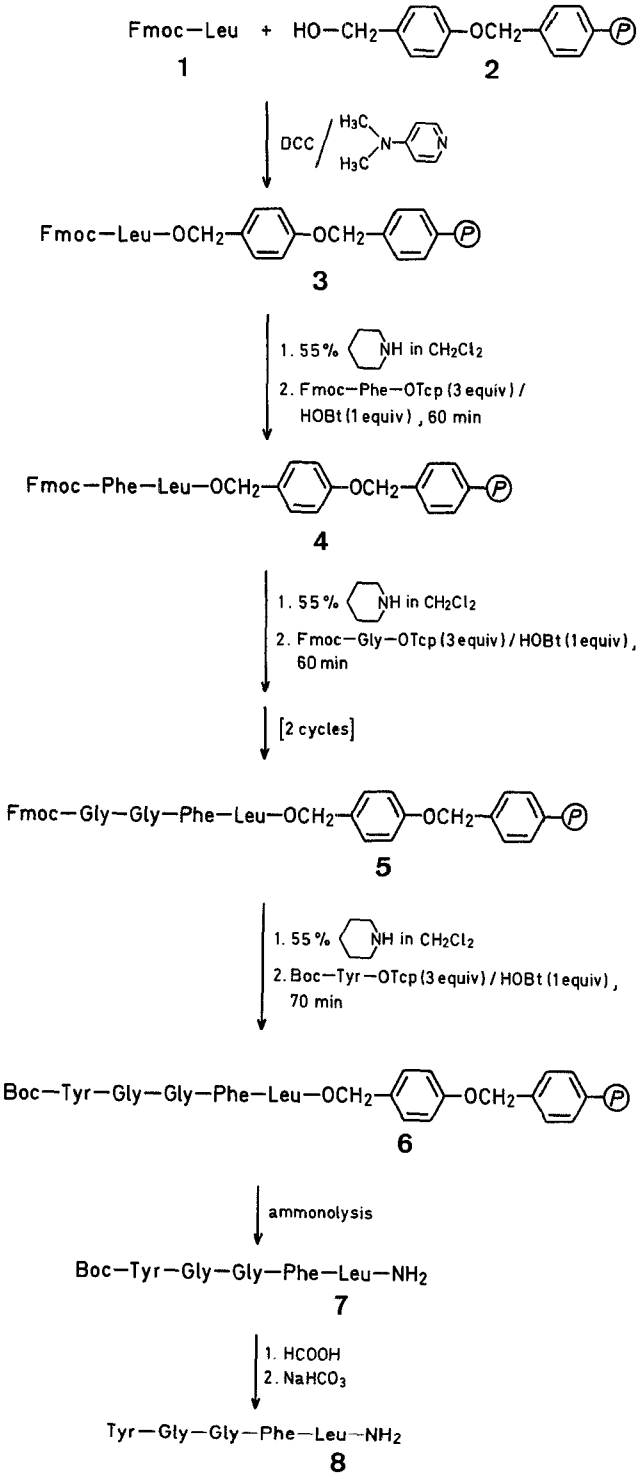
Coupling of Boc-amino acid active esters on the solid phase using the conventional chloromethylated Merrifield resin can be satisfactorily achieved in presence of 1-hydroxybenzotriazole (HOBt) as demonstrated by our syntheses of oxytocin[1] and bradykinin[2]. The use of *t*-butyloxycarbonyl (Boc) group for *N*-protection of amino acids necessitates acidolysis for deprotection which has to be repeated after incorporation of successive Boc-amino acids into the peptide chain on the solid phase and this has a deleterious effect leading to an impure product at the end[3].

The introduction of the base labile fluorenylmethyloxycarbonyl (Fmoc) group[4,5,6] for *N*-protection obviates the need for acidolysis. The milder conditions employed for removal of Fmoc group, such as treatment with piperidine, offer certain advantages in the solid phase synthesis of peptides as shown by the synthesis of somatostatin[7] and human *β*-endorphin[8]. For the former synthesis[7], a *p*-alkoxybenzyl alcohol resin[9] was used whereas in the latter a polyamide resin was employed. Both methods have generally involved use of Fmoc-amino acid anhydrides in large excess (6 fold) for incorporation of successive amino acids.

This report describes the use of Fmoc-amino acid trichlorophenyl (OTcp) esters in the presence of 1-hydroxybenzotrizole for the solid phase peptide synthesis in a manner similar to the use of Boc-amino acid active esters reported earlier[1,2]. The Fmoc-amino acid trichlorophenyl esters are crystalline solids that are easily prepared and stored.

For the present synthesis of the opioid peptide, [Leu-NH_2]^5-enkephalin **(8)**, the *p*-alkoxybenzyl alcohol resin[9] was used. The first amino acid Fmoc-Leu **(1)** was anchored to the resin **(2)** by the procedure of Ref.[7]. The resultant product **3** was deprotected using 55% piperidine in dichloromethane and then reacted with Fmoc-Phe-OTcp (3 equiv) in presence of 1-hydroxybenzotriazole (1 equiv) for 60 min. The completion of the reaction was monitored by Kaiser's test[10]. Deprotection of **4** with piperidine was then effected and this was followed by introduction of Fmoc-Gly-OTcp as described above. This reaction also was over in 60 min. A second glycine unit was similarly introduced (→**5**) and the final amino acid unit was introduced as Boc-Tyr-OTcp (→**6**) which took 70 min for complete reaction. The protected peptide resin **6** was sub-



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jected to ammonolysis and the product after purification furnished Boc-Tyr-Gly-Gly-Phe-Leu-NH_2 **(7)** in 76% yield. Removal of the Boc group from the protected peptide **7** by treatment with 98–100% formic acid afforded the hygroscopic Tyr-Gly-Gly-Phe-Leu-$NH_2 \cdot HCOOH$ in 90% yield. Neutralisation of the formate with dilute sodium hydrogen carbonate solution yielded Tyr-Gly-Gly-Phe-Leu-NH_2 **(8)** in 70% yield.

The main advantages of the present synthesis are: (a) completion of the coupling reaction with 3 equiv of Fmoc-amino acid trichlorophenyl esters in presence of 1-hydroxybenzotriazole in about 60 min; (b) the yield of the final product is good and needs very little purification; (c) the peptide anchored to the *p*-alkoxybenzyl alcohol resin can be conveniently released by ammonolysis. In earlier reports[7,9] using this resin, the final peptide resins were treated with trifluoroacetic acid to liberate the free, *C*-terminal carboxy peptides.

Apart from the active esters used in this synthesis, several other Fmoc-amino acid trichlorophenyl esters have been prepared by us as crystalline solids and have been used in the synthesis of analogues of [Leu-NH_2][5]-enkephalin and oxytocin by the procedure described in this paper.

Fmoc-Phe-OTcp:
This compound is prepared using Fmoc-Phe (3.87 g, 10 mmol), trichlorophenol (2.37 g, 12 mmol), tetrahydrofuran (30 ml) and dicyclohexylcarbodiimide (2.47 g, 12 mmol), following the procedure described below for Fmoc-Gly-OTcp. The product is recrystallised from absolute alcohol; yield 5.1 g (90%); m.p. 186.5 °C; R_fA: 0.95; $[\alpha]_D^{25}$: −55° (*c* 1, DMF).

$C_{30}H_{22}Cl_3NO_4$	calc.	C 63.54	H 3.88	N 2.47
(566.5)	found	63.52	3.99	2.40

Fmoc-Gly-OTcp:
A solution of Fmoc-Gly (2.97 g, 10 mmol) and 2,4,5-trichlorophenol (2.37 g, 12 mmol) in tetrahydrofuran (30 ml) is cooled in an ice bath and stirred with dicyclohexylcarbodiimide (2.47 g, 12 mmol) for 4 h at that temperature. After stirring of the mixture overnight at that temperature, the liberated dicyclohexylurea is filtered off and the filtrate is evaporated in vacuo. The residue is purified from absolute ethanol; yield 4.37 g (92%); m.p. 146 °C; R_fA: 0.91.

$C_{23}H_{16}Cl_3NO_4$	calc.	C 57.92	H 3.35	N 2.93
(476.5)	found	58.21	3.28	2.85

Fmoc-Leu-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin (3):
Fmoc-Leu (**1**; 1.2 g, 3.3 mmol) is reacted with *p*-alkoxybenzyl alcohol resin (**2**; 2.0 g; prewashed with dichloromethane) using dicyclohexylcarbodiimide (0.66 g, 3.3 mmol) and *p*-dimethylaminopyridine (0.36 g, 3.6 mmol) in 4:1 dichloromethane/dimethylformamide (40 ml) according to Ref.[7] to afford **3**; yield: 2.25 g; leucine content: 0.41 mmol/g of resin.

Fmoc-Phe-Leu-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin (4):
Fmoc-Leu-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin (**3**; 2.25 g) is subjected to the following cycles using 20 ml solvent for each operation.

Step	Reagent	Time (min)
1	CH_2Cl_2 (3×)	2
2	Deblocking with 55% piperidine in CH_2Cl_2 (1×)	40
3	CH_2Cl_2 (3×)	2
4	DMF (2×)	2
5	Dioxane:Water, 2:1 (3×)	5
6	DMF (2×)	2
7	CH_2Cl_2 (2×)	2
8	DMF (2×)	2

The resin is next shaken with Fmoc-Phe-OTcp (1.39 g, 2.46 mmol, 3 equiv) in dimethylformamide (10 ml) and 1-hydroxybenzotriazole (0.11 g, 0.82 mmol, 1 equiv) for 60 min when the reaction is complete as judged by Kaiser's test[10].

Boc-Tyr-Gly-Gly-Phe-Leu-OCH_2-C_6H_4-OCH_2-C_6H_4-Resin (6):
The procedure described in the previous preparation is repeated to introduce the subsequent three amino acid units. For the incorporation of each glycine unit, Fmoc-Gly-OTcp (1.16 g, 2.46 mmol, 3 equiv) and 1-hydroxybenzotriazole (0.11 g, 0.82 mmol, 1 equiv) in dimethylformamide (10 ml) are employed, the duration of each coupling being 60 min. The final amino acid is introduced using Boc-Tyr-OTcp (1.13 g, 2.46 mmol, 3 equiv) in dimethylformamide (10 ml) and 1-hydroxybenzotriazole (0.11 g, 0.82 mmol, 1 equiv), and the completion of this reaction required 70 min. The peptide resin is then subjected to the following cycle of washings, each of 2 min duration: dimethylformamide (3×20 ml), dichloromethane (3×20 ml), isopropanol (3×20 ml), and ether (3×20 ml), and dried over phosphorus pentoxide in vacuo; yield: 2.5 g.

Boc-Tyr-Gly-Gly-Phe-Leu-NH_2 (7):
The protected peptide resin **6** (2.5 g) is suspended in absolute methanol (100 ml) and allowed to swell for 2 h. The suspension is then cooled to −10 °C, stirred, and dry ammonia is bubbled till the solution is saturated. After stirring at 0 °C for a further 72 h, the solvent is removed in vacuo. To eliminate the last traces of ammonia, the residue is treated with absolute methanol (20 ml) and evaporated in vacuo. This operation is repeated once more and then the residue is extracted with hot absolute methanol (2×75 ml). The solvent is removed in vacuo and the product is heated under reflux with ethyl acetate (10 ml), then cooled. The separated solid is recrystallised from absolute methanol/ether; yield: 0.41 g (76% based on the amount of leucine esterified to the resin); m.p. 173–174 °C; R_fB: 0.61; $[\alpha]_D^{25}$: −17° (*c* 1.0, DMF); amino acid analysis: Gly 2.17, Leu 1.00, Tyr 0.99, Phe 1.03.

$C_{33}H_{46}N_6O_8$	calc.	C 60.54	H 7.03	N 12.84
(654.8)	found	60.87	7.48	13.02

Tyr-Gly-Gly-Phe-Leu-$NH_2 \cdot HCOOH$:
A solution of peptide **7** (0.065 g) in 98–100% formic acid (2.0 ml) is kept at room temperature for 3 h and then the formic acid is removed in vacuo. To the residue anhydrous ether (30 ml) is added and the precipitated product is purified from methanol/ether; yield: 0.055 g (90%); hygroscopic solid; R_fC: 0.46; R_fD: 0.90.

Tyr-Gly-Gly-Phe-Leu-NH_2 (8):
The formate (0.055 g) from the previous experiment is dissolved in water (1.0 ml) and mixed with an aqueous solution of 2% sodium hydrogen carbonate (3.0 ml). It is shaken for 20 min and then extracted with ethyl acetate (3×40 ml). The organic layer is washed with water (2×10 ml), and dried in vacuo; yield: 0.035 g (70%); m.p. 120–126 °C; R_fC: 0.63; R_fD: 0.79; $[\alpha]_D^{25}$: +14° (*c* 1.0, 95% AcOH); identical with an authentic sample[11].

T.L.C. analyses were carried out using silica gel G plates in the solvent systems: chloroform/methanol/acetic acid (40:2:1), chloroform/methanol/acetic acid (40:5:5), and *n*-butanol/acetic acid/water (4:1:5, upper phase), and the R_f values are expressed as R_fA, R_fB, and R_fC, respectively. Paper chromatography was carried out using the solvent system, *n*-butanol/acetic acid/water (4:1:5, upper phase), and the R_f value is expressed as R_fD.

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11 Authentic sample kindly supplied by Prof. V. J. Hruby, University of Arizona, Tucson, Arizona, U.S.A.