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AN EFFICIENT METHOD FOR RACEMIZATION FREE ATTACHMENT OF 9-FLUORENYLMETHYLOXYCARBONYL-AMINO ACIDS TO PEPTIDE SYNTHESIS SUPPORTS

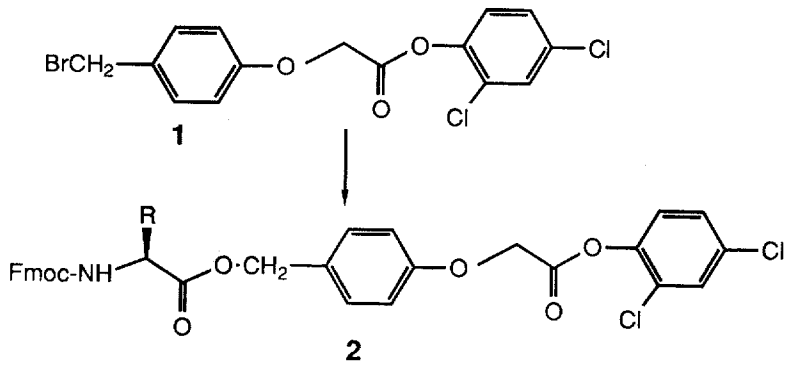
Michael S. Bernatowicz*, Thomas Kearney, Richard S. Neves and Hubert Köster
Peptide Research Laboratory, MilliGen/Biosearch Division - Millipore Corporation
186 Middlesex Turnpike, Burlington, MA 01803

Summary: An efficient, general, and racemization free method of covalently attaching N α-Fmoc protected amino acids to solid supports for peptide synthesis is described. The process involves the preparation of 2,4-dichlorophenyl-N α-Fmoc-aminoacyl-4-oxymethyl-phenoxy acetates which can be used to directly and efficiently acylate amine functionalized polymers.

The process by which N α-protected amino acids are anchored to solid supports and the nature of the anchoring linkage ultimately contribute to the yield and purity of the final products produced by solid phase peptide synthesis. When the N α-9-fluorenylmethyloxycarbonyl (Fmoc) protecting group strategy is used for peptide synthesis, the C-terminal amino acid is generally attached by activation of the carboxyl group and its subsequent esterification to 4-alkoxybenzyl alcohol functionalized polymers[1-3]. In order to obtain useful levels of polymer functionalization by such a process, it is necessary to utilize a catalyst such as 4-dimethylaminopyridine (DMAP) for acylation of the polymer bound hydroxyl group[1]. Such methods suffer from a number of disadvantages owing to the ability of DMAP to promote racemization[1,4] and produce anchored dipeptide byproducts[5,6]. Furthermore, some N α-Fmoc-amino acid symmetrical anhydrides have poor solubility properties which give rise to inefficient esterification. Although alternate methods have been investigated to overcome these drawbacks[6-8], none is completely satisfactory for industrial scale production because of any combination of a variety of factors: commercial unavailability, instability, or expense of starting reagents, requirements for long reaction times, inefficient esterification, or lack of demonstrated generality. The need for improved methodology has led to the developments reported here.

An attractive method for attachment of the C-terminal N α-Fmoc-amino acid to resins functionalized with the acid labile 4-oxymethylphenoxacetyl linkage[9] utilizes novel 2,4-dichlorophenyl-N α-Fmoc-aminoacyl-4-oxymethylphenoxy acetates 2, prepared by Scheme 1, to directly acylate amine-functionalized polymers. The preparation of 2 was straightforward: 4-methylphenoxyacetic acid was brominated by the action of N-bromosuccinimide and 2,2'-azobisisobutyronitrile in refluxing chloroform[10] to give 4-bromomethylphenoxyacetic acid in 36-55% yield as a white crystalline compound

Scheme 1



(mp.121 -3°C)[11]. This bromo acid was converted to its 2,4-dichlorophenyl active ester derivative 1 by reaction with dicyclohexylcarbodiimide and 2,4-dichlorophenol in ethyl acetate at 0°C. The active ester 1 was obtained in 85% yield after crystallization from ethyl acetate/hexanes (mp. 114-6°C)[12]. Displacement of bromide from active ester 1 by Nα-Fmoc-amino acid anions generated with diisopropylethylamine in dimethylformamide[13] occurred cleanly[14] (2-3 hr room temp.) to give intermediates 2 in good yields and high purity (^{1}H NMR, TLC) for all Nα-Fmoc-amino acids thus far examined[15]. Representative examples of derivatives 2 prepared are given in Table 1. All derivatives 2 could be obtained as easily handled, very pure solids by crystallization or precipitation from an appropriate solvent or solvent pair. In addition, all derivatives 2 were soluble in dimethylformamide at concentrations sufficient to allow for rapid and efficient acylation of a variety of amine-functionalized polymers useful for peptide synthesis.

Table 1

Nα-Fmoc-Amino Acid Derivative 2	% Yield	mp. (°C)
Ala	99	120-2
Asn	75	amorphous solid
Val	89	74-7
Glu(^{t}Bu)[a]	84	61-5
Ser(^{t}Bu)[a]	78	90-5
Met	75	92-5
Lys(Boc)[a]	90	75-8
Trp	80	136-9
Pro	74	62-5

[a]Abbreviations: ^{t}Bu, t-butyl; Boc, t-butyloxycarbonyl

Preliminary experiments showed that all derivatives 2 were readily coupled to ethylenediamine-functionalized (0.24 mmole NH_2/g) Pepsyn K™ (polyamide-kieselguhr) resin[17] using 2.0-3.0 equivalents of 2 in the presence of 0.7 equivalents DMAP[18] in dimethylformamide (2-3 hr reaction at room temp.). Representative results from these experiments are summarized in Table 2. More recent experiments have shown that

Table 2

Pepsyn K™ resin Derivative	Product Substitution Level[16] (mmole Fmoc/g)
Ala	0.187
Phe	0.196
Pro	0.213
Leu	0.195
Tyr(^{t}Bu)[a]	0.190
Lys (Boc)[a]	0.191
Trp	0.175
Val	0.184

[a]Abbreviations: See Table 1.

comparable, and in some cases better, results were obtained when 3.0 equivalents of 2 were used and 3.0 equivalents of pyridine were substituted for DMAP. For example, coupling of Fmoc-Cys(Trt) derivative 2 under the former conditions yielded resin with a substitution of 0.15 mmole Fmoc/g whereas under the latter conditions a substitution of 0.19 mmole Fmoc/g was obtained. Aminomethyl and p-methylbenzhydrylamine functionalized polystyrene resins as well as amine-functionalized polymeric membranes (Millipore Corp., Bedford, MA) useful for peptide synthesis[19] have also been efficiently coupled to derivatives 2 by this procedure.

In order to ascertain the extent of amino acid racemization induced by this process, amino acids were isolated from resins derived from 2 and reacted with GITC, a reagent that produces HPLC resolvable diastereomers from D,L-amino acid mixtures[20]. In all cases thus far examined, no racemization (<0.1% D-isomer) was observed for the products derived from 2. In contrast, this analytical procedure detected 2% D-Phe from a resin produced by sym-anhydride/DMAP coupling of Fmoc-L-Phe-OH to a 4-alkoxybenzyl alcohol support, a result consistent with the literature[1].

In addition to eliminating the problem of racemization, the process described here eliminates the possibility of obtaining resin bound dipeptide side products[5,6] and thereby would be expected to ultimately provide purer synthetic target peptides.

Furthermore, the process is straightforward, convenient, as well as attractive and practical from the standpoint of utilizing readily available, inexpensive starting materials for the synthesis of 1.

Results obtained using intermediates 2 for the synthesis of a variety of peptides have been detailed elsewhere[19].

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10. Although the bromination reaction gave comparable results using CCl_4 as solvent, the limited solubility of 4-methylphenoxyacetic acid made its use impractical for large (>100g) scale reactions.
11. ^{1}H NMR (300 MHz, $CDCl_3$, ppm δ relative to tetrametylsilane): 4.41 (s, 2H), 4.62 (s, 2H), 6.24 (broad s, 1H) 6.82 (d, J=8.7 Hz, 2H), 7.28 (d, J=8.7 Hz, 2H).
12. ^{1}H NMR (300 MHz, $CDCl_3$, ppm δ relative to tetramethylsilane): 4.51 (s, 2H), 4.98 (s, 2H), 6.97 (d, J=9.3 Hz, 2H), 7.12 (d, J=9.3 Hz, 1 H), 7.28-7.4 (m, 3H, includes 7.47 d, J=9.3 Hz, 2H), 7.48 (d, J=2 Hz, 1H).
13. The esterification reaction providing 2 could also be mediated using preformed N α-Fmoc-amino acid-cesium salts [(a) Columbo, R.; Atherton, E.; Sheppard, R.C.; Woolley, V.; Int. J. Peptide Protein Res., 1983, **21**, 118. (b) Gisin, B.F.; Helv. Chim. Acta 1973, 56, 1476. (c) Wang, S.; Gisin, B.F.; Winter, D.P.; Makofske, R.; Kulesha, I.D.; Tzougraki, C.; and Meienhofer, J.; J. Org. Chem., 1977, **42**, 1286.] or by reaction of N α-Fmoc-amino acids and 2 in the presence of potassium fluoride [(d) Horiki, K.; Igano, K.; Inouye, K.; Chem. Lett., 1978, 165].
14. When other active ester derivatives of 4-bromomethylphenoxyacetic acid (p-nitrophenyl, pentafluorophenyl, N-succinimidyl) were employed for reaction with Fmoc-amino acid anions, transesterification with the Fmoc-amino acid was observed. Thus when Fmoc-Ala-OH was reacted with the p-nitrophenyl ester of 4-bromomethyl-phenoxyacetic acid, a substantial amount (~30%) of Fmoc-Ala-p-nitrophenyl ester was formed as a side product.
15. All N α-Fmoc-L-amino acids currently commercially available have been converted to linker derivatives 2.
16. Substitution level determined spectrophotometrically based on Fmoc-derived chromophore liberated upon treatment with piperidine (ε_{301} = 7,800 in 4% piperidine in CH_2Cl_2).
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