Tetrahedron Vol. 49, No. 20, pp. 4141-4146, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00
Pergamon Press Ltd

Studies on the Acylation of Hydroxy-Functionalized Resins Using Fmoc Amino Acids Activated Using Diisopropylcarbodiimide/HOBt or as Acid Fluorides[1]

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(*Received in USA* 16 *March* 1993)

Abstract: The data presented suggests that esterification of hydroxy-functionalized resins with Fmoc-protected amino acids proceeds best in low polarity solvents such as CH_2Cl_2 and THF and that pyridine, as co-solvent, is a suitable acylation catalyst. This has been demonstrated using DIC/HOBt activation, as well as Fmoc-protected amino acid fluorides which are excellent reagents for rapid, racemization-free acylation of hydroxy-functionalized resins used in solid phase peptide synthesis.

INTRODUCTION

In recent years a number of methods have been developed for acylating hydroxy-functionalized resins (such as the widely used *p*-alkoxybenzyl alcohol polystyrene resin[4]) used in solid phase peptide synthesis with Fmoc-protected amino acids (see Table 1). The acylation reaction introduces the C-terminal amino acid of the peptide sequence, therefore the success of any peptide synthesis depends on its efficiency. The conditions employed in an esterification reaction are substantially different from those used in the stepwise, amino-acylation reactions used in sequential solid-phase peptide synthesis. However, high yields and retention of optical purity are essential requirements.

DISCUSSION

A study of the methods used for resin hydroxyl esterification (Table 1) indicates that the means of carboxyl activation is less important than the solvent of the reaction. The most striking example of this is the successful use of Fmoc-protected amino acid chlorides by Akaji *et al.*[8] (Table 1, entry 4) in contrast with the failure of the same method as described by Grandas *et al.*[7] (Table 1, entry 5). The only difference between these two methods is the choice of solvent: Akaji *et al.* used CH_2Cl_2 (containing 40% pyridine), whereas Grandas *et al.* used DMA (containing pyridine or NMM). Similarly the DCC mediated esterifications catalyzed by DMAP were superior when carried out in CH_2Cl_2/DMF (3:1) (Table 1, entry 2), when compared with DMF or DMA (Table 1, entries 1 and 3).

From these results it seemed that a low polarity solvent would be superior in the acylation reaction with regard to both reaction yield and racemization. In an initial experiment (see Table 2, entry 14), FmocPheOH was

Table 1. Methods for Esterification of Hydroxy-Functionalized Resins

Reagents (Stoichiometries)	Solvent	Reaction Time (Temperature)	Yields	D-Amino Acids	Reference
1. FmocAA-OH (2)/DCC (2)/ HOBt(4)/DMAP(<2)	DMF	18 h (0°C)	NA	0.7%	5
2. FmocAA-OH (1.5)/DCC (1.2)/ DMAP(1.2)	DMF/CH_2Cl_2 (1:3)	20 h (-20 to 0°C)	~80%	0.2-0.5%	6
3. FmocAA-OH (4)/DCC (4)/ HOBt(3)	DMA	17 h (RT)	~80%	<0.5%	7
4. FmocAA-Cl (5)	CH_2Cl_2/ pyridine	1 h (RT)	~100%	<0.5%	8
5. FmocAA-Cl (5)/pyridine (3) or NMM (3)	DMA	3 h (RT)	14-38%	NA	7
6. FmocAA-OH (2)/DCBC (2)/ pyridine (3)	DMF	15-20 h (RT)	55-66%	0.1-0.7%	9
7. FmocAA-OH(2)/MSNT (2)/ MeIm (1.5)	CH_2Cl_2	0.5 h (RT)	72-100%	0-0.6%	10

pre-activated using DIC and HOBt in CH_2Cl_2/DMF (9:1), divided into three equal portions and added to:

(a) resin in the presence of pyridine (as co-solvent)

(b) resin in the presence of DMAP (as ca. 30 mol % catalyst)

(c) resin in CH_2Cl_2

The results of this experiment demonstrated that, under these conditions, pyridine was equally as effective as DMAP in obtaining good yields with low levels of racemization. In the absence of either pyridine or DMAP the reaction yield was only 5% after 2 hours. In subsequent experiments the CH_2Cl_2/DMF solvent combination was replaced with THF. This is a superior solvent (relative to CH_2Cl_2) towards most Fmoc-protected amino acids as well as HOBt, and is similarly non-polar (dielectric constant 7.6, compared with CH_2Cl_2 9.08, and DMF 36.7[11]).

Reactions carried out in the presence of pyridine were of reasonable (>60%) yield, and comparable to those catalyzed by DMAP (within 5%). The most notable exceptions were, as expected, the amide-containing amino acids, asparagine and glutamine, though convenient alternatives for the preparation of peptides containing these C-

Table 2. Comparison of Pyridine and DMAP as Acylation Catalysts

AminoAcid	DIC/HOBt/Py		DIC/HOBt/DMAP	
	Yield	% D[a]	Yield	% D[a]
1. FmocAlaOH	66	<0.1	75	0.6
2. FmocArg(Pmc)OH	37	2.2	13	ND[c]
3. FmocArg(Pmc)OH[b]	43	ND[c]	18	ND[c]
4. FmocAsnOH	36	4.7	44	10.2
5. FmocAsp(*t*-Bu)OH	79	4.3	84	5.7
6. FmocCys(Tr)OH	83	ND[c]	89	ND[c]
7. FmocGlnOH	32	1.5	15	1.3
8. FmocGlu(*t*-Bu)OH	55	0.9	73	0.6
9. FmocHis(Tr)OH	77	6.9	85	5.3
10. FmocIleOH	59	<0.1	57	<0.1
11. FmocLeuOH	72	0.7	71	1.0
12. FmocLys(Boc)OH[d]	66	0.8	71	1.0
13. FmocMetOH	81	1.0	84	1.4
14. FmocPheOH[d]	75	<0.1	80	2.0
15. FmocPheOH	69	<0.1	65	<0.1
16. FmocProOH	38	<0.1	65	<0.1
17. FmocSer(*t*-Bu)OH	74	2.4	70	4.5
18. FmocThr(*t*-Bu)OH	29	<0.1	56	<0.1
19. FmocTrpOH	73	ND[c]	77	ND[c]
20. FmocTyr(*t*-Bu)OH	79	0.6	83	0.8
21. FmocValOH	78	<0.1	69	0.8

[a] % D-Amino acid present, as determined by Marfey method[14] (see text); [b] Activated *in situ*; [c] Not determined; [d] Solvent 10% DMA in CH_2Cl_2

terminal amino acids exist (via the amide-generating resins[12]). FmocArg(Pmc)OH[13] also gave a poor yield (table 2, entry 2), undoubtedly due to cyclization of the activated intermediate. *In situ* activation of this derivative afforded only slightly improved results (table 2, entry 3), though the use of pyridine is more effective than DMAP. Of the hindered, β-branched amino acids, FmocValOH and FmocIleOH gave good results after extended reaction times, but FmocThr(*t*-Bu)OH did not react well in the presence of pyridine even after 4 days.

Optical purity of the resin-bound amino acid was determined by the Marfey method,[14] whereby the derivatized resin was hydrolysed (6N HCl, 110 °C, 6 - 18 h), and the amino acid derivatized using Nα-(2,4-dinitro-

5-fluorophenyl)-l-alaninamide, and optical purity of the resulting diastereomers assessed by HPLC. In all cases examined (Table 2) the levels of D-amino acid are comparable between the two acylation methods, though the presence of DMAP may slightly enhance levels of racemization relative to reactions carried out using pyridine.

Use of Fmoc-Protected Amino Acid Fluorides

The recent introduction of Fmoc-protected amino acid fluorides,[15,16] in which *t*-butyl side chain protecting groups have been shown to be stable, prompted us to investigate the use of these derivatives for esterification of the C-terminal amino acid with *p*-alkoxybenzyl alcohol resin.

Table 3. Derivatization of *p*-Alkoxybenzyl Alcohol Resin using Amino Acid Fluorides

Amino Acid	Final Resin		
	Substitution[a]	% Yield	% D Amino Acid
1. FmocAsp(*t*-Bu)F	0.645	92	1.9
2. FmocGlu(*t*-Bu)F	0.608	88	1.4
3. FmocLeuF	0.599	82	<0.1
4. FmocIleF	0.429	59	<0.1
5. FmocLys(Boc)F	0.591	88	3.5
6. FmocMetF	0.678	94	1.5
7. FmocSer(*t*-Bu)F	0.563	79	0.5
8. FmocThr(*t*-Bu)F[c]	0.521	74	<0.1
9. FmocTrpF	0.638	92	ND[b]
10. FmocTyr(*t*-Bu)F	0.539	87	2.2
11. FmocValF[c]	0.583	79	<0.1

[a] *p*-Alkoxybenzyl alcohol resin, 0.97mmol g^{-1} as starting material; [b] Not determined; [c] FmocValF and FmocThr(*t*-Bu)F were allowed to react with the resin for 6h: all others were left for 2h.

Results presented in Table 3 show that esterification of the hydroxyl group by Fmoc-protected amino acid fluorides proceeds rapidly, in good yield, and with minimum racemization, using pyridine and CH_2Cl_2 as solvents. Amino acids bearing *t*-butyl-based side chain protecting groups were incorporated, as well as acid-sensitive amino acids such as tryptophan and methionine. Thus the acid chloride method developed by Akaji *et al.*[8] can be extended by the use of amino acid fluorides. Again, the hindered amino acid fluorides, FmocIleF, FmocThr(*t*-Bu)F, and FmocValF, required longer reaction times for complete reaction. In some instances the preparation of the acid

fluoride proved to be a limiting factor: FmocArg(Pmc)F underwent cyclization to the lactam; FmocCys(Tr)F and FmocHis(Tr)F suffered premature detritylation.

CONCLUSIONS

Fmoc-protected amino acids may be conveniently attached to *p*-alkoxybenzyl alcohol resin by the use of standard DIC/HOBt activation in the presence of pyridine in a low polarity solvent such as THF, or CH_2Cl_2 (containing minimal quantities of DMF or DMA to solubilize the Fmoc-protected amino acid and HOBt). This method is convenient, simple, reliable, high yielding and proceeds with minimal racemization. Pyridine, present as a co-solvent, is generally as effective as catalytic DMAP in mediating this reaction, but slightly longer reaction times (e.g. 4-6 hours) may be optimal. Similarly, amino acid fluorides are effective acylating agents in CH_2Cl_2/pyridine mixtures. It seems likely that in all methods published to date, low polarity solvents and the intermediacy of acyl-pyridinium species promote the successful acylation of hydroxy-functionalized resins.

EXPERIMENTAL

Fmoc-protected amino acids and *p*-alkoxybenzyl alcohol resin (0.97 mmol g^{-1})were purchased from Bachem Bioscience Inc., Philadelphia, PA, and were checked by HPLC prior to use. UV spectroscopy was carried out on a Beckman DU-64 spectrophotometer in quartz glass cells using spectroscopic grade MeOH as solvent. UV absorbance was recorded over the range 310-290 nm, with determination of the dibenzofulvene-piperidine adduct at 301 nm, $\varepsilon = 7200$ mol^{-1} dm^3 cm^{-1}. Optical purity analysis of Fmoc-protected amino acid derivatized resins was carried out by the Marfey method.[14] Yields are calculated from the theoretical maximum substitution of the resin.

General Procedure for the Acylation of Resins.

A. DIC/HOBt method.

Fmoc-protected amino acid (1.0 mmol) and HOBt (1.1-1.2 mmol) were dissolved in 2 mL dry THF in a 25 mL round bottom flask. A 0.5 M solution of DIC in THF (2.8 mL) was added and the reaction stirred at RT for 20 min. Meanwhile two *ca.* 8 mL shaker vessels for manual solid phase synthesis were each charged with *p*-alkoxybenzyl alcohol resin (155 mg, 150 μmol) and washed with DMF (2 x 3 mL) and CH_2Cl_2 (2 x 3 mL). The first resin sample was then suspended in pyridine (1.6 mL) and exactly half of the activated amino acid (2.4 mL) added. The second resin sample was suspended in THF (1.6 mL) and DMAP (6 mg, 50 μmol) added, followed by half of the activated amino acid derivative (2.4 mL). The contents of the two vessels were mixed by gentle inversion over 2 h, then washed with CH_2Cl_2 (6 x 3 mL).

B. Amino Acid Fluoride Method.

Fmoc-protected amino acid fluoride (3-5 equiv.) was added to the washed *p*-alkoxybenzyl alcohol resin (155 mg, 150 μmol) and mixed by gentle inversion in CH_2Cl_2 (2.4 mL) and pyridine (1.6 mL). After 2 h the resin was washed with CH_2Cl_2 (6 x 3 mL) and the substitution determined.

Determination of Resin Substitution.

A sample of derivatized resin (ca. 5-15 mg) was removed, washed with 40% MeOH in CH_2Cl_2 on a sintered funnel, and dried under suction for 5-10 min. The sample was weighed, then the Fmoc group was cleaved using a solution of 20% piperidine in DMF (X mL) for 15-20 min. After allowing the resin to settle, a sample (Y mL) of the supernatant was diluted to Z mL with MeOH and UV absorbance recorded at 301 nm. Substitution (in mmol g^{-1}) was determined according to the equation:

$$\text{substitution} = \frac{\text{absorbance x Z x X}}{\text{7200 x weight x Y}} \text{ x } 1000$$

The absorbance for the dibenzofulvene-piperidine adduct (λ = 301 nm: ε = 7200 mol^{-1} dm^3 cm^{-1}) was determined experimentally from Fmoc-protected amino acids. In typical experiments X = 2 mL, Y = 1 mL, and Z = 25 mL.

ACKNOWLEDGEMENT

The authors wish to thank Ken Cornelius for optical purity determinations.

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