Tetrahedron Letters, Vol. 34, No. 26, pp. 4201-4204, 1993
Printed in Great Britain

0040-4039/93 $6.00 + .0
Pergamon Press Ltd

A Convenient Method for Synthesis of Fmoc-Amino Acid p-Nitroanilides Based on Isobutyl Chloroformate as Condensation Agent[1].

Hinyu Nedev, Hanitra Naharisoa and Tomasz Haertlé*.

Groupe Protéines, LEIMA, Institut National de la Recherche Agronomique BP 527, 44026 Nantes, FRANCE.

Key words: Mixed anhydride method, isobutyl chloroformate, FMOC-amino acids, p-nitroanilides, chromogenic protease substrates.

Abstract: Mixed anhydride method with condensation agent isobutyl chloroformate is described for obtaining a series of p-nitroanilides of FMOC-Pro-, FMOC-Ala-, FMOC-Leu-, FMOC-Ile-, FMOC-Trp-, FMOC-Asp(OtBu)-, FMOC-Gln(Trt)-, FMOC-Hyp- and FMOC-Hyp(OtBu)- in good yields and in an optical pure form. The use of other activating agents for obtaining p-nitroanilides is discussed.

It is known that amino acid p-nitroanilides (-pNA) are used as chromogenic substrates for the determination of the activity of proteolytic enzymes[2]. The synthesis of p-nitroanilides is relatively difficult because of the low nucleophilicity of p-nitroaniline. The published reports concerning the use of mixed anhydride method for obtaining of the amino acid p-nitroanilides are often contradictory. On the one hand Erlanger et. al.[3] reported that the attempts to synthesize Z-Lys(Z)-p-nitroanilide via isobutyl chloroformate were unsuccessful. Okada et al.[4] described that the mixed anhydride method does not yield the the desired reaction product Z-Val-pNA. On the other hand the successful synthesis of different BOC-protected amino acid p-nitroanilides by the mixed anhydride method using isobutyl chloroformate[5] or pivaloyl chloride[6], have been recently reported. The use of isobutyl chloroformate, however, as activating agent for the synthesis of FMOC-protected amino acid p-nitroanilides has not been examined until now. In this study we have applied the mixed anhydride method[7] for synthesis of the p-nitroanilides of FMOC-Pro-, FMOC-Ala, FMOC-Leu-, FMOC-Ile-, FMOC-Trp-, FMOC-Asp(OtBu)-, FMOC-Gln(Trt)-, FMOC-Hyp-, FMOC-Hyp(OtBu)-. The yields vary usually between 50 and 86% after recrystallization from the appropriate solvents.

The obtaining of the abovementioned p-nitroanilides was achieved by the following scheme:

$$\text{Fmoc-AA-OH} + \text{i-BuOCOCl} \xrightarrow[-15^{\circ}C]{\text{NMM}} \text{Fmoc-AA-OCOi-Bu} \xrightarrow{NH_2\text{-}C_6H_4\text{-}NO_2(p)} \text{Fmoc-AA-NH-}C_6H_4\text{-}NO_2(p)$$

AA = Pro-, Ala-, Leu-, Ile-, Trp-, Asp(OtBu)-, Gln(Trt)-, Hyp-, Hyp(OtBu)-

After suitable deprotection they can be used as synthons for the synthesis of more complicated chromogenic substrates.

The synthesis of the synthon FMOC-Pro-pNA by the $POCl_3$ method, recently described by Rijkers et al.[8] gave, after recrystallisation, 53% of the model compound versus 63%, when the mixed anhydride method was used.

In order to make an other model synthon - FMOC-Val-pNA, we have tried to use other coupling methods employing different activating agents such as 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (IIDQ), diphenylphosphoryl azide and carbonyl diimidazole. When the last two activating agents were used, the desired reaction product could not be isolated in pure form, nor in sufficient amount. The IIDQ method gave better results, but significant racemization took place in this case. The yields and optical purity of FMOC-Val-pNA were almost identical when $POCl_3$ or isobutyl chloroformate were used as condensation agents. The properties of FMOC-amino acid p-nitroanilides prepared by the mixed anhydride method are summarized in Table 1.

TABLE 1. Yields and Physical Constants of the FMOC-Amino Acid p-Nitroanilides Obtained by Mixed Anhydride Method:

Compound	Yield %	M.P. (°C)	R_f a)	R_f b)	R_f c)	$[\alpha]_D$ DMF, c=0.5	Mass spectra $[M.M.+NH_4]^+$ calc./found
FMOC-Pro-pNA[d)]	53	186-187	0.86	0.77	0.75	-61	-
FMOC-Pro-pNA	63	185-187	0.86	0.77	0.75	-58	475.5/475
FMOC-Ala-pNA	66	183-185	0.79	0.61	0.59	-40	450.4/449
FMOC-Leu-pNA	58	187-190	0.89	0.81	0.86	-28	491.5/491
FMOC-Ile-pNA	57	214-218	0.90	0.83	0.88	-16	491.5/491
FMOC-Trp-pNA	61	188-190	0.81	0.61	0.66	+24	564.6/564
FMOC-Asp(OtBu)-pNA	70	amorph.	0.91	0.84	0.88	-32	549.5/549
FMOC-Gln(Trt)-pNA	86	131-136	0.94	0.79	0.81	-20	748.8/748
FMOC-Hyp-pNA	50	208-209	0.77	0.52	0.31	-61	491.5/491
FMOC-Hyp(OtBu)-pNA	52	122-126	0.95	0.80	0.74	-41	547.6/547

a) chloroform/methanol/water = 65/25/4; b) chloroform/methanol/acetic acid =85/10/5; c) chloroform/methanol = 9/1;
d) obtained by the $POCl_3$ method.

EXPERIMENTAL:

All melting points were determinated with a Reichert melting point apparatus and are uncorrected. FMOC- amino acids were obtained from Applied Biosystems. Thin-layer chromatography was performed on Merck Kieselgel 60 F254 plates in three different systems. Spots were visualized under UV light, or after exposure to iodine vapours. Optical rotations were measured on polarimeter Optical Activity, model AA-10 with 2.0 dm cell in DMF at ambient temperature. The structures were confirmed by mass spectra, recorded by the chemical ionization mode (ammonia used as reagent gas) on NERMAG R10 - 10 C mass spectrometer.

General Procedure for Preparation of N^{α}-Fluorenylmethyloxycarbonyl Protected Amino Acid p-Nitroanilides:

1.12 mmol of the respective N^{α}-fluorenylmethyloxycarbonyl protected amino acid was dissolved in 1 ml tetrahydrofurane. After addition of N-methylmorpholine (124 µl, 1.12 mmol) and cooling to -15° C, isobutyl chloroformate (150 µl, 1.12 mmol) was added. Subsequently the solution was stirred 5 min at 0° C and cooled again to -15° C, afterwards p-nitroaniline[9] (155 mg, 1.12 mmol) dissolved in 1 ml tetrahydrofurane was added dropvise. The reaction mixture was stirred during 1 h at 0° C, and after stirring at room temperature overnight the solvent was evaporated. The product was dissolved in ethyl acetate and washed 5 fold with 2 M hydrochloric acid[10], 0.5 M sodium hydrogen carbonate and saline. The organic layer was dried over anhydrous magnesium sulphate and the respective FMOC-amino acid p-nitroanilides were recrystallized from appropriate solvents[11]. In some cases, if necessary, column chromatography was performed prior to recrystallization[12].

In summary, the successful use of the mixed anhydride method with isobutyl chloroformate as activating agent is described for the synthesis of FMOC-amino acid p-nitroanilides. The above method is comparable with the $POCl_3$ method[8,13], used for obtaining of BOC- and some FMOC-protected amino acid p-nitroanilides. The presented method affords the desired chromogenic amino acid derivatives conveniently, in good yields and has the advantage of using milder activation agent - isobutyl chloroformate.

Acknowledgement:

We thank the National Institute of Agronomical Research for the financial support. Thanks are also due to Dr. François Metro of this Institute for the mass spectra measurements.

References and notes:

1. The abbreviations used in this paper are those reccomended by IUPAC - IUB: *Eur. J. Biochem.* **381**, 9-37, (1984). All amino acid symbols denote L-configuration.
2. Hemker, H.C. ed. "*Handbook of Synthetic Substrates for the Coagulation and the Fibrinolytic System*" Martinus Nijhoff Publishers, Boston, 1983.
3. Erlanger, B., Kokowsky, N. and Cohen, W., *Arch. Biochem. Biophys.*, **95**, 271-278, (1961).
4. Okada, Y., Tsuda, Y., Nagamatsu, Y. and Okamoto, U., *Int. J. Peptide Protein Res*, **17**, 560-564, (1981).
5. Barth, A., Mager, H., Fischer, G., Neubert, K. and Schwarz, G., *Acta Biol. Med. Germ.*, **39**, 1129-1142, (1980).
6. Noda, K., Oda, M., Sato, M. and Yoshida, N., *Int. J. Peptide Protein Res*, **36**, 197-200, (1990).
7. Vaughan, J.R. and Osato, R.L., *J. Amer. Chem. Soc.*, **73**, 3547, (1951); ibid. **74**, 676, (1952).

8. Rijkers, D.T.S., Hemker, H.C., Nefkens, G.L.H. and Tesser, G.I., Poster No 8, 22nd European Peptide Symposium, Interlaken, Switzerland (1992).
9. Recrystallized twice from methanol.
10. 2 M hydrochloric acid solution was used for removing the unreacted p-nitroaniline from the ethyl acetate solution, except for FMOC-Asp(OtBu)-pNA and FMOC-Gln(Trt)-pNA, where molar hydrochloric acid was repeatedly used.
11. The p-nitroanilides of FMOC-Pro-, FMOC-Ala-, FMOC-Leu-, FMOC-Hyp- and FMOC-Hyp(OtBu)- were recrystallized from ethyl acetate/hexane; FMOC-Trp-pNA from chloroform/hexane (3/1). FMOC-Asp(OtBu)-pNA and FMOC-Gln(Trt)-pNA were precipitated twice with ethyl acetate/hexane.
12. FMOC-Ile-pNA was purified by flash chromatography on Silicagel Type 60 - Aldrich; 2x30 cm column using 100 ml chloroform and 200 ml 2% methanol in chloroform as eluents. After removal of the solvent under reduced pressure, the pure product was recrystallized from ethyl acetate.
13. Rijkers, D.T.S., Hemker, H.C., Nefkens, G.L.H. and Tesser, G.I., *Recl. Trav. Chim. Pays-Bas*, **110**, 347-348, (1991).

(Received in France 21 April 1993; accepted 4 May 1993)