Pergamon

Tetrahedron Letters 39 (1998) 5031–5034

TETRAHEDRON LETTERS

Preservation of the Fmoc protective group under alkaline conditions by using $CaCl_2$. Applications in peptide synthesis

Robert Pascal*[1] and Régine Sola

CNRS UPR 9023, Centre de Pharmacologie-Endocrinologie, Rue de la Cardonille, F-34094 Montpellier Cedex 5, France[2]

Received 13 February 1998; accepted 11 May 1998

Abstract

$CaCl_2$ is shown to dramatically increase the lifetime of the Fmoc protective group in alkaline iPrOH-H_2O but has little effect on hydrolytic processes. This enables efficient preparation of Fmoc-protected peptide segments by saponification of the corresponding *C*-terminal methyl or benzyl esters. Similarly, protected peptide segments prepared by Fmoc/tBu solid-phase synthesis can be selectively released from the support by $CaCl_2$-catalyzed alkaline hydrolysis of an *N*-acylurea-based linker. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: protecting groups; deblocking; peptides; solid-phase synthesis

In peptide chemistry, there is a need for procedures allowing the selective cleavage of carboxyl-protecting groups without removing the Fmoc groups (Fmoc = fluoren-9-ylmethoxycarbonyl). One application is the preparation of protected peptide segments for the convergent peptide synthesis that involves the Fmoc/tBu strategy. To date, the synthesis of such segments has been performed by using highly acid-labile linkers [1,2] or esters [3] in the solid-phase or solution methods, respectively. We report herein an alternative which involves alkaline release of the protected segment using $CaCl_2$ as an efficient additive to suppress Fmoc cleavage under basic conditions.

We previously demonstrated that alkaline cleavage of the Dpr(Phoc) linker (Dpr = L-2,3-diaminopropionic acid, Phoc = phenyloxycarbonyl, Scheme 1) can be catalyzed by $CaCl_2$ [4]. The increase in the reaction rate very likely results from two counteracting effects: *(i)* stabilization of the negatively charged tetrahedral transition state by coordination with Ca^{2+}, which facilitates the reaction, and *(ii)* reduction of the hydroxide ion concentration due to formation and incomplete dissociation of calcium hydroxide, which limits enhancement of the

[1] Fax: +33 4 67 54 24 32. E-mail: rpascal@ccipe.montp.inserm.fr

[2] This work was initiated at the Centre de Recherches de Biochimie Macromoléculaire, CNRS, 1919 Route de Mende, F-34293 Montpellier Cedex 5, France.

0040-4039/98/$19.00 © 1998 Elsevier Science Ltd. All rights reserved.
PII: S0040-4039(98)00994-0

Table 1
Effect of $CaCl_2$ on alkaline cleavage of the Fmoc group in Fmoc-Gly-NH_2 [a]

$[CaCl_2]$ / M	$t_{1/10}$ / h [b]	$t_{1/2}$ / h [b]
0	0.25	1.6
0.25	8	47[c]
0.5	11	71[c]

[a] 4 mM NaOH in iPrOH-H_2O 7:3, 20°C.
[b] Time required for a 10% ($t_{1/10}$) or a 50% ($t_{1/2}$) extent of the reaction of Fmoc-Gly-NH_2 (t_R =11.5 min) determined by HPLC: Brownlee Aquapore RP-300 column, buffer A, 0.1% aq. TFA; B, MeCN (0.06% TFA); linear gradient 20—62% B over 15 min; detection at 265 nm.
[c] Small amounts (< 10%) of Fmoc-Gly (t_R = 12.4 min) were observed.

rate. $CaCl_2$ had been proposed to act similarly during saponification of various acetate esters but, in this case, the latter effect prevails and the rate is slightly decreased [5,6].

Ca^{2+} catalysis should not occur in reactions proceeding through a very different mechanism. The base-catalyzed Fmoc cleavage involves β-elimination [7]. Under aqueous alkaline conditions, we reasoned that the rate of this reaction would therefore be markedly decreased in the presence of $CaCl_2$ because of the strong reduction of the hydroxide ion concentration. Furthermore, for compounds bearing both a carboxyl protection, hydrolyzed according to the mechanism described above, and Fmoc groups, two competing reactions would take place with a change in selectivity in favour of hydrolysis when using $CaCl_2$ as additive.

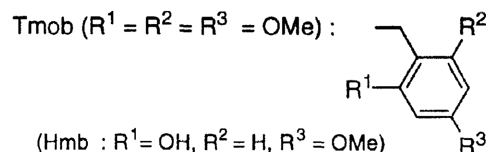
The increase of Fmoc group lifetime was checked initially with Fmoc-Gly-NH_2. This compound was submitted to alkaline treatment with or without $CaCl_2$ (Table 1). As expected, the effect of $CaCl_2$ on Fmoc cleavage is strong, unlike that obtained for saponification of the related methyl ester Fmoc-Gly-OMe. Thus, under identical experimental conditions, the latter quickly gave Fmoc-Gly-OH and the presence of $CaCl_2$ only induced a moderate decrease in rate ($t_{1/2}$ = 12.5 min with 0.25 M $CaCl_2$ instead of 3.25 min).[3]

Similar experiments were then carried out on Fmoc-Gly-Phe-Pro-OMe to illustrate the expected change in selectivity with peptides containing both Fmoc and ester protections.[4] Upon treatment of this compound (1 mM initial concentration) with 4 mM NaOH in iPrOH-H_2O 7:3 at 20.0°C, both the starting ester and its saponification product suffer Fmoc cleavage. Without $CaCl_2$, Fmoc-Gly-Phe-Pro-OH was thus observed to transiently accumulate with a 38% maximum yield after 1.5 h. In the presence of 0.5 M $CaCl_2$, this yield reached 88% (after 2 h) showing that it should be possible to efficiently isolate Fmoc-protected segments with a free carboxyl terminus from the corresponding esters. This was in fact achieved in the following experiment: purified Fmoc-Gly-Phe-Pro-OH was obtained in 85% yield (0.215 g) from its methyl ester (0.260 g) after treatment for 7 h with NaOH (1.2 equiv) added to 0.8 M $CaCl_2$ in

[3] Unlike Fmoc-Gly-NH_2, Fmoc-Gly-OMe or Fmoc-Gly-OH are not convenient models to study the effect of calcium salts on Fmoc cleavage because the rapid formation of the negatively charged carboxylate neighbouring group most probably inhibits the HO^- reaction with the Fmoc group. The saponification of Fmoc-Gly-OMe into Fmoc-Gly-OH was monitored by HPLC (t_R = 16.2 min and 14.2 min, respectively). HPLC conditions: Brownlee Aquapore RP-300 column, buffer A, 0.1% aq. TFA; B, MeCN (0.06% TFA); linear gradient 15—90% B over 25 min; detection at 220 and 265 nm.
[4] Fmoc-Gly-Phe-Pro-OMe was synthesized from Pro-OMe, HCl to which Boc-Phe and then Fmoc-Gly were coupled using standard protocols of peptide synthesis in solution. Saponification and Fmoc cleavage were monitored by HPLC analysis of Fmoc-Gly-Phe-Pro-OMe (t_R = 18.1 min) and Fmoc-Gly-Phe-Pro-OH (t_R = 16.5 min); see footnote 3 for HPLC conditions.

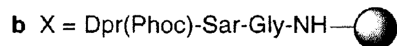
1 Fmoc-Val-Lys(Boc)-Lys(Boc)-Tyr(tBu)-Leu-Ala-X
2 Fmoc-Ser(tBu)-Tyr(tBu)-Ser(tBu)-Pro-Thr(tBu)-Ser(tBu)-Pro-X
3 Fmoc-Ser(tBu)-Pro-Thr(tBu)-Ser(tBu)-Pro-X
4 Fmoc-Asp(OtBu)-(Tmob)Gly-X

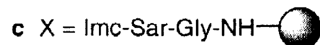
Tmob ($R^1 = R^2 = R^3$ = OMe) :



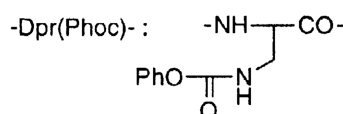
(Hmb : R^1= OH, R^2 = H, R^3 = OMe)

a X = OH

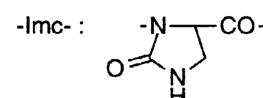




-Dpr(Phoc)- :



-Imc- :



Scheme 1.
Peptides and peptide-resins synthesized.
The resin is Expansin® (from Expansia, F-30390 Aramon, France). Peptide synthesis: N^{α}-Fmoc protected amino acids were used except for the linkage residues Dpr(Phoc), Sar and Gly which were introduced as N^{α}-Boc amino acids; 45—60 min coupling in DMF after preactivation with *O*-(benzotriazol-1-yl)-*N,N,N',N'*,-tetramethyluronium tetrafluoroborate or 1,3-diisopropylcarbodiimide/1-hydroxybenzotriazole, monitored with the ninhydrin or modified chloranil [18] test; cyclization of the linker [conversion of Dpr(Phoc) into Imc] with PhONa/PhOH in DMF and synthesis of *C*-terminal proline peptides as described in ref. [10]; conventional methods for Boc- and Fmoc-group removal, except for Fmoc removal in the presence of non-cyclized Dpr(Phoc) linker: morpholine-DMF 1:1, 35 min [10]. Release of protected peptides: 1.5—2.5 h treatments at room temperature with NaOH (1—2 equiv.) added to 0.8 M $CaCl_2$ in iPrOH-H_2O 7:3 repeated two or three times; extensive washing of the resin with MeOH.

iPrOH-H_2O 7:3 (10.6 cm^3) at room temperature.[5] This *C*-terminal Pro model was selected because *C*-terminal Gly or Pro segments are recommended for minimizing epimerization during the segment coupling steps of convergent peptide synthesis [1,2]. Furthermore, based on kinetic studies showing that *C*-terminal Pro or Val dipeptide methyl esters are saponified with the slowest rates [8], higher selectivity and consequently higher yields can be predicted for saponification of Fmoc-protected peptide esters containing other *C*-terminal residues.

The effect of $CaCl_2$ on the rate of benzyl ester saponification was also studied. Bz-Leu-Phe-OBn was selected because previous work had shown that this model permits ready determination of the extent of epimerization at the *C*-terminal residue[6] which may occur upon removal of the *C*-terminal protection (see Table 1 for the conditions of saponification and ref.[4] for those of HPLC analysis). As already noted with methyl esters, $CaCl_2$ only induced a moderate decrease in rate ($t_{1/2}$ = 50 min in the presence of 0.5 M $CaCl_2$ instead of 14 min without $CaCl_2$). Interestingly, it also reduced epimerization (0.3% Bz-Leu-D-Phe instead of 1%).

[5] The straightforward isolation and purification of the protected segments is one advantage of this new synthetic method: the hydrolysate was neutralized with 1 M AcOH, then evaporated *in vacuo*, and the solid residue was dissolved in MeOH; water was added to precipitate the product which was filtered and extensively washed with water; the crude peptide was recovered from the filter, by dissolution into MeOH and further evaporation of the solvent, with satisfactory purity (>80% by HPLC); contaminants resulting from limited Fmoc group cleavage (and unreacted starting ester too, in the case of ester saponification) were removed by silicagel liquid chromatography (eluent: CH_2Cl_2-MeOH-AcOH, 5—10% MeOH, 1% AcOH); residual AcOH was eliminated by dissolution of the product in MeOH followed by precipitation and washing with H_2O. All protected peptides displayed high purity by HPLC and gave the expected FAB-MS spectrum. Fmoc-Gly-Phe-Pro-OH (98.5%), *m/z* 542 ($M+H^+$); **1a** t_R 22.3 min (99%), *m/z* 1199 ($M+H^+$); **2a** t_R 22.8 min (99%), *m/z* 1240 ($M+H^+$); **3a** t_R 20.6 min (99%), *m/z* 878 ($M+H^+$); see footnote 3 for HPLC conditions.

[6] Diastereomers Bz-Leu-Phe and Bz-Leu-DPhe can be easily distinguished by HPLC analysis [4].

[7] This anchorage procedure allows the synthesis of *C*-terminal Pro segments without any loss at the dipeptide stage by diketopiperazine formation [10].

[8] A preliminary experiment was carried out without the Tmob protection under identical experimental conditions: the side-reaction occurred and was evidenced by the loss of tBu side-chain protection of Asp residue. The Tmob group was used instead of the related and more usual Hmb (Hmb = 2-hydroxy-4-methoxybenzyl) group (Scheme 1) because there is no simple way to protect the free hydroxyl group of the latter which might be susceptible to *O*-acylation during the subsequent segment coupling steps.

The applicability of the method to the solid-phase synthesis of protected segments was demonstrated by the selective cleavage of protected peptide-resins **1c**-**3c** and **4b** (Scheme 1). The syntheses were carried out on a hydrophilic polyacrylamide support with the Dpr(Phoc) linker [9] which was converted in most cases into its cyclic Imc (Imc = 2-oxo-imidazolidine-4-carboxylic acid) form compatible with Fmoc chemistry [10].[7] Purified segment **1a** was thus obtained in 72% overall yield (calculated from the alanine content of the starting resin) by cleavage of resin **1c** with a slight excess of NaOH added to 0.8 M $CaCl_2$ in iPrOH-H_2O 7:3 (90 min repeated twice) followed by purification.[5] Similarly, *C*-terminal Pro peptides **2a** and **3a** were prepared from resins **2c** and **3c**, respectively. However additional tools might be required for segments sensitive to mildly alkaline aqueous conditions. It is particularly necessary to circumvent the increased risk of aspartimide formation, especially for susceptible sequences [11] such as -Asp(OtBu)-Gly-. Peptide bond protections have been found to suppress this side-reaction if observed during Fmoc removal [12-14]. Their ability to provide good protection during the alkaline treatment was shown with the use of the Tmob (Tmob = 2,4,6-trimethoxybenzyl) protective group [15-17] in peptide-resin **4b**, since **4b** afforded the tBu-protected dipeptide **4a** only (FAB-MS *m/z* 648 $[M+H^+]$) upon cleavage with 1.2 equiv NaOH added to 0.8 M $CaCl_2$ in iPrOH-H_2O 7:3 (2.5 h, repeated twice).[8,5]

The method described here for the synthesis of protected segments in Fmoc chemistry may present advantages compared with those involving highly acid-labile protections at the *C*-terminus because *(i)* the risk of premature deblocking of the *C*-terminus or of side-chains is reduced, and *(ii)* it can offer an additional level of orthogonality. In addition, the easy saponification of unactivated esters made possible by Ca^{2+} catalysis in spite of a very low hydroxide ion concentration may find other useful applications in the field of peptide chemistry or elsewhere.

Acknowledgements. We thank Prof. F. Albericio for helpful suggestions, Drs. P. Jouin, J. Coste and G. Niel for critical reading of the manuscript, and Dr. S. L. Salhi for editorial assistance.

References

[1] Lloyd-Williams P, Albericio F, Giralt E. Chemical Approaches to the Synthesis of Peptides and Proteins. Boca Raton, FL: CRC Press, 1997 and references cited therein.
[2] Lloyd-Williams P, Albericio F, Giralt E. Tetrahedron 1993;49:11065-11133.
[3] Yue C, Thierry J, Potier P. Tetrahedron Lett. 1993;34:323-326.
[4] Pascal R, Sola R. Tetrahedron Lett. 1997;38:4549-4552.
[5] Caplow M. J. Am. Chem. Soc. 1965;87:5774-5785.
[6] Bell RP, Waind GM. J. Chem. Soc. 1950:1979-1983.
[7] Carpino LA. Acc. Chem. Res. 1987;20:401-407.
[8] Hoogwater DA, Peereboom M. Tetrahedron 1990;46:5325-5332.
[9] Sola R, Saguer P, David M-L, Pascal R. J. Chem. Soc., Chem. Commun. 1993:1786-1788.
[10] Sola R, Méry J, Pascal R. Tetrahedron Lett. 1996;37:9195-9198.
[11] Bodanszky M, Kwei JZ. Int. J. Pept. Protein Res. 1978;12:69-74.
[12] Quibell M, Owen D, Packman LC, Johnson T. J. Chem. Soc., Chem. Commun. 1994:2343-2344.
[13] Offer J, Quibell M, Johnson T. J. Chem. Soc., Perkin Trans. 1 1996:175-182.
[14] Packman LC. Tetrahedron Lett. 1995;36:7523-7526.
[15] Johnson T, Quibell M, Owen D, Sheppard RC. J. Chem. Soc., Chem. Commun. 1993:369-372.
[16] Johnson T, Quibell M, Sheppard RC. J. Pept. Sci. 1995;1:11-25.
[17] Clausen N, Goldammer C, Jauch K, Bayer E. In: Kaumaya PTP, Hodges RS, editors. Peptides: Chemistry, Structure and Biology. Kingswinford, UK: Mayflower Scientific Ltd., 1996:71-72.
[18] Vojkovsky T. Pept. Res. 1995;8:236-237.