Pergamon

Tetrahedron Letters 43 (2002) 7717–7719

TETRAHEDRON LETTERS

Peptide coupling of unprotected amino acids through in situ *p*-nitrophenyl ester formation

Paul Gagnon, Xicai Huang,† Eric Therrien and Jeffrey W. Keillor*

Département de chimie, Université de Montréal, CP 6128, Succursale centre-ville, Montréal, Québec, Canada H3C 3J7

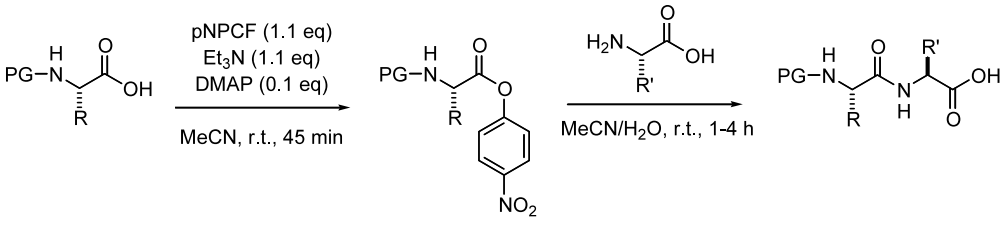
Received 16 May 2002; revised 24 July 2002; accepted 30 August 2002

Abstract—Several series of dipeptides and tripeptides were prepared via an activation–coupling method involving the in situ formation of a *p*-nitrophenyl ester of an (*N*-protected) amino acid, followed by coupling with an unprotected amino acid in partially aqueous solutions. The resulting peptide is easily isolated by precipitation. In general, the yields obtained are good to excellent and racemization is minimal. This method is particularly advantageous with respect to its simplicity and lack of obligatory side chain protection/deprotection steps. © 2002 Elsevier Science Ltd. All rights reserved.

Small peptides are important biomolecules and many have therapeutic value. Unlike large peptides that are commonly isolated from natural sources or produced through recombinant techniques, small peptides are usually prepared using organic synthetic methods.[1] There exist many different methods for peptide coupling, most of which require prior protection and subsequent deprotection of various amino acid functional groups.[2] One method that remains popular since its introduction by Bodanszky in 1955 involves the activation of carboxylate groups through the formation of *p*-nitrophenyl esters, which can typically be isolated as stable solids.[3] The activation of the carboxylate afforded by the *p*-nitrophenyl ester group is sufficiently strong to favor efficient peptide coupling with most amino acids. Furthermore, it is sufficiently mild to disfavor undesired side reactions with amino acid side-chain functional groups or adventitious water. Herein we report a coupling method based on the in situ formation of *p*-nitrophenyl esters of *N*-protected amino acids and the reaction of these appropriately activated esters in aqueous/organic solvent mixtures with unprotected amino acids. This simple two-step process allows the rapid and efficient peptide coupling and is broadly applicable.

In the first step, the esterification of *N*-protected amino acids is easily effected through a protocol similar to one previously reported by Sunggak and Kim.[4] As shown in Scheme 1, an *N*-protected amino acid is allowed to react with *p*-nitrophenyl chloroformate (pNPCF) in the presence of triethylamine and 4-dimethylaminopyridine (DMAP). The general procedure is as follows: To a solution of 2.0 mmol of PG-Xaa in 100 mL of acetonitrile was added 0.31 mL (2.2 mmol) of Et_3N. After cooling in an ice bath, 0.44 g (2.2 mmol) of pNPCF was added. After 5 min, 24 mg (0.2 mmol) of DMAP was added. The reaction was then stirred for 50 min.

It has been suggested that the intermediate product of this reaction is the mixed anhydride formed upon dis-



Scheme 1. In situ *p*-nitrophenyl ester formation and coupling.

* Corresponding author. Tel.: +1-514-343-6219; fax: +1-514-343-7586; e-mail: keillorj@chimie.umontreal.ca

† Present address: Medicinal Chemistry Department, Conjuchem, Montréal, Canada.

PII: S0040-4039(02)01840-3

placement of the chloride of pNPCF by the amino acid carboxylate group. Presumably, this unstable mixed anhydride then undergoes an intramolecular condensation reaction to give the more stable *p*-nitrophenyl ester with the expulsion of an equivalent of CO_2. However, the mechanism has not been thoroughly explored.[4] The resulting *p*-nitrophenyl ester is formed cleanly and in excellent yield. The reaction can be carried out in various polar organic solvents, and in the presence of different bases, of which DMAP appears to be the most efficient. The ester can be isolated and purified, or used directly in the following coupling step.

The coupling reaction shown in the second step of Scheme 1 can be performed using many different unprotected amino acids, dissolved in partially aqueous solvent mixtures in the presence of Et_3N as a base. The general procedure for coupling is as follows: A clear solution was prepared of 8.0 mmol of free amino acid (Xaa′) in 100 mL of distilled water, and 1.12 mL (8.0 mmol) of Et_3N. The ice bath was removed from the activation solution and the solution of the second amino acid was added to it dropwise with stirring over 5 min, and the solution was allowed to react for an additional 100 min. The acetonitrile was then removed under reduced pressure. To the remaining solution, water was added to a final volume of 150 mL. This solution was acidified with 6 M HCl to pH $\sim$2 and the peptide product was allowed to precipitate at 4°C. The solution was filtered and the remaining solid was rinsed with 80 mL of 1 M HCl. The solid was dried under vacuum for 24 h to give a white powder.

Although the dipeptide products can be purified by chromatography, they can often be isolated more easily by precipitation, after removal of the organic solvent by rotary evaporation and acidification of the aqueous solution. The use of Cbz and Fmoc as N-terminal protecting groups particularly facilitates the isolation and purification of the peptide products through precipitation. However, since the Fmoc group is known to be slightly labile under basic conditions,[5] the use of the Cbz protecting group, given the presence of Et_3N during the coupling step, is preferable.

Many different aprotic organic solvents were found to be suitable for the activation step and several different organic solvents can be used with water for the coupling step. Acetonitrile was found to be particularly well-suited to this method since it is compatible with the activation reaction, miscible with water for the coupling step and easily removed under reduced pressure.

Shown in Table 1 are some of the many peptides prepared according to this method. In all cases the desired peptide was obtained rapidly and easily, but the yields were found to vary according to the different amino acids used. The first four entries of Table 1 clearly show that amino acids with nucleophilic, basic or bulky side chains may all be coupled using this method. However, it should be noted that the activation of glutamine as a *p*-nitrophenyl ester may be accompanied by the formation of other by-products. This is not surprising, since activated glutamine has been shown previously to be susceptible to such side reactions; it has been suggested previously that one of the by-products formed could be the corresponding glutarimidine.[6]

Table 1. Yields of some di- and tripeptides synthesized herein

Peptide	Yield (%)
Cbz-L-Phe-L-Cys	98[a]
Cbz-L-Phe-L-His	88[a]
Cbz-L-Phe-L-Ser	67[a]
Fmoc-L-Phe-L-Phe	82[a]
Cbz-L-Gln-L-Ala	66[a]
Cbz-L-Gln-L-Leu	70[a]
Cbz-L-Gln-L-Phe	79[a]
Cbz-L-Gln-L-Val	44[a]
Cbz-L-Phe-L-Phe-L-Val	66[a,b]
Boc-L-Gln-Gly	35[c]
Cbz-L-Gln-Gly	65[c]
Cbz-L-Gln-L-Ser	31[c]
Cbz-L-Gln-Gly-Gly	30[b,c]
Cbz-Gly-L-Gln-Gly	15[b,c]
Cbz-L-Gln-L-Leu	71[b,c]
Cbz-Gly-L-Leu-Gly	43[b,c]

[a] Purified by precipitation.
[b] Overall yield for two activation/coupling and purification steps.
[c] Purified by chromatography.

The tripeptides shown in Table 1 were obtained by first making the Cbz-dipeptide by the above method, and then coupling with the third free amino acid. Although the coupling of Cbz-dipeptide with free amino acid remains efficient, the reported yields for the purified tripeptides are typically lower due to loss of material during purification.

Given the mild conditions of this coupling method, racemization was not anticipated to be a problem and was in fact determined to be negligible by several different methods. Firstly, the di- and tripeptides prepared by this method were shown by NMR spectroscopy to be diastereomerically pure, as confirmed by doping the samples with authentic diastereomer. Furthermore, analysis of crude reaction product mixtures by chiral HPLC also showed that negligible racemization had taken place. Finally, analysis of reaction product mixtures by capillary electrophoresis using a chiral mobile phase[7] verified the diastereomeric purity of the dipeptide products. The authenticity and purity of the peptide products were also established by comparison of some of their physical properties with values reported in the literature.[8–12] This comparison is summarized in Table 2.

This rapid method of activation and coupling with free amino acids is not without limitations. The side chain hydroxyl group of serine was found to react with pNPCF leading to a mixture of products.[13] Furthermore, the activation of aspartic acid or glutamic acid as *p*-nitrophenyl esters is not selective for the α-carboxyl-

Table 2. Comparison of physical properties of synthesized peptides to literature values

Peptide	α_D		mp (°C)	
	Exp.	Lit.	Exp.	Lit.
Cbz-L-Phe-L-His	−8.0 (1.0, DMF)	−5.1[a] (1.0, DMF)	201–203	205–207[a]
Cbz-L-Phe-L-Ser	−3 (2.0, DMF)	−2.6[b] (1.0, DMF)	148–149	147–148[c]
Cbz-L-Gln-Gly	−3.9 (1.0, DMF)	−3.0[d] (1.0, DMF)	183–184	182–183[d]
Cbz-L-Gln-L-Ala	−1.5 (1.5, DMF)	−1.5[a] (1.5, DMF)	218–219	219–220[a]
Cbz-L-Gln-L-Phe	4.7 (1.0, DMF)	5.2[a] (1.0, DMF)	198–199	199–200[a]
Cbz-L-Gln-L-Val	3 (1.1, 95% HOAc)	1.4[e] (1.1, 95% HOAc)	191–192	193–194[e]

[a] Reference 8.
[b] Reference 9.
[c] Reference 10.
[d] Commercially available material (Sigma-Aldrich).
[e] Reference 12.

ate group. Finally, the coupling reaction with free lysine is not selective for the α-amino group, and can also take place with the ε-amino group. Although these features may be exploited for the synthesis of branched peptides, they are undesirable for the typical synthesis of linear, unbranched peptides, and indicate the necessity of side-chain protecting groups for these amino acids. However, overall this method offers the distinct advantages of being simple, rapid and practical. Many di-, tri- and tetrapeptides[14] can be made quickly, without the requirement of any additional protection and deprotection steps. As a result, they can thus be prepared in overall yields that compare favorably to other peptide coupling methods. The key feature of this method is that the in situ formation of *p*-nitrophenyl esters apparently affords the appropriate level of activation of the C-terminal carboxylate group to allow efficient subsequent aminolysis, without rendering the activated amino acid susceptible either to adventitious hydrolysis, racemization or reaction with side chain functional groups. Finally, this method may prove to be useful in solid-phase peptide synthesis. Preliminary experiments[14] have shown that crude reaction mixtures of *p*-nitrophenyl esters of *N*-protected amino acids prepared by this method can be used in standard Fmoc (C→N) solid-phase peptide synthesis. Moreover, an adaptation of this method may be highly amenable to (N→C) solid-phase peptide synthesis. If the N-terminal amino acid were anchored to a water compatible resin, the peptide could be lengthened through successive rounds of activation and coupling, without the necessity of intermediate deprotection steps.[15]

Supporting information available

Analytical data for all dipeptides and tripeptides.

Acknowledgements

We acknowledge the Natural Sciences and Engineering Research Council (NSERC) and the Université de Montréal for their financial support. PG also thanks the Fonds pour les chercheurs et à l'aide de la recherche (FCAR) for a postgraduate scholarship.

References

1. Dutta, A. S. In *Amino Acids, Peptides and Proteins*; Davies, J. S., Ed.; Royal Society of Chemistry: Cambridge, UK, 1999; Vol. 30, Chapter 3, pp. 163–284.
2. Jones, J. *The Chemical Synthesis of Peptides*; Clarendon Press: Oxford, UK, 1991.
3. Bodanszky, M. *Nature* **1955**, *175*, 685.
4. Sunggak, K. L. J. I.; Kim, Y. C. *J. Org. Chem.* **1985**, *50*, 560.
5. Bodanszky, M.; Deshmane, S.; Martinez, J. *J. Org. Chem.* **1979**, *44*, 1622.
6. Bodanszky, A.; Bodanszky, M.; Chandramouli, N.; Kwei, J. Z.; Martinez, J.; Tolle, J. C. *J. Org. Chem.* **1980**, *45*, 72.
7. Huang, B.; Huang, X.; Waldron, K.; Keillor, J. W., unpublished observations.
8. Kemp, D. S.; Wrobel, S. J., Jr.; Wang, S.-W.; Bernstein, Z.; Rebek, J., Jr. *Tetrahedron* **1974**, *30*, 3969.
9. Bodanszky, M.; Sheehan, J. T.; Ondetti, M. A.; Lande, S. *J. Am. Chem. Soc.* **1962**, *85*, 991.
10. Wharton, C. W. *Biochem. J.* **1974**, *143*, 575.
11. Clayton, D. W.; Farrington, J. A.; Kenner, G. W.; Turner, J. M. *Peptides* **1957**, 1399.
12. Klieger, E.; Gibian, H. *Liebigs Ann. Chem.* **1961**, 183.
13. This mixture was observed in the synthesis of Fmoc-L-Ser-L-Phe, but not Cbz-L-Ser-Gly.
14. Huang, X.; Keillor, J. W., unpublished observations.
15. Thieret, N.; Guibé, F.; Albericio, F. *Org. Lett.* **2000**, *2*, 1815.