Pergamon

Tetrahedron Letters, Vol. 35, No. 52, pp. 9689-9692, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)02151-1

Aspartimide Formation in Base-driven 9-Fluorenylmethoxycarbonyl Chemistry

Yan Yang[1], William V. Sweeney[1], Klaus Schneider[2], Susanna Thörnqvist[2], Brian T. Chait[2], and James P. Tam[3]*

[1] Department of Chemistry, Hunter College of CUNY, New York, NY 10021, USA.

[2] Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY 10021, USA.

[3] Department of Microbiology and Immunology, Vanderbilt University, A5119 MCN, Nashville, TN 37232, USA.

Abstract: Aspartimide and its piperidine adduct formed between -Asp-Asn- has been found during an Fmoc-based synthesis of an EGF-like domain in a blood coagulation factor. Model studies with seven susceptible -Asp-X- pentapeptides show that -Asp(Ot-Bu)-Asn(Trt)- and -Asp(Ot-Bu)-Gly- are the most problematic sequences.

The formation of aspartimide is a side reaction often observed in the synthesis of aspartic acid-containing peptides either in solution or in solid phase with t-Boc (tert-butyloxycarbonyl) chemistry[1]. It is well known to be sequence dependent and occurs under either strongly acidic or basic conditions. Aspartimide formation is known to occur readily with Asp-Gly or Asp-Ser in strong acids such as HF or CH_3SO_3H. Similarly these sequences are also susceptible to base-catalyzed aspartimide formation during synthesis using benzyl protecting groups[2,3]. Use of electron donating or sterically hindered side chain protecting groups for aspartic acid, for example cyclohexyl ester or t-butyl ester in solid phase peptide synthesis, has been recommended as a practical approach to circumvent aspartimide formation[4,5]. Neither of these branched-chain esters are thought to be subject to appreciable base-catalyzed aspartimide formation. Thus, the common belief[6] is that aspartimide formation does not pose any problem in the base-driven Fmoc (9-fluorenylmethoxycarbonyl) chemistry since the very hindered t-butyl group is used for side-chain protection. Contrary to this conventionally held view, we have found aspartimide formation to be a significant side reaction during the synthesis[7] of the C-terminal EGF-like domain from human blood coagulation factor X[8] with the following sequence:

LFTRKLCSLDNGDCDQFCHEEQNSVVCSCARGYTLADNGKACIPTGPY

1 48

The first attempt at synthesis of this peptide on a synthesizer using Fmoc chemistry failed. The resulting cleavage product was found to be a mixture containing no detectable amount of the desired peptide. An attempt to manually synthesize the peptide also failed. To determine where the problematic sequence was, a stepwise detection method was then employed while performing manual synthesis.

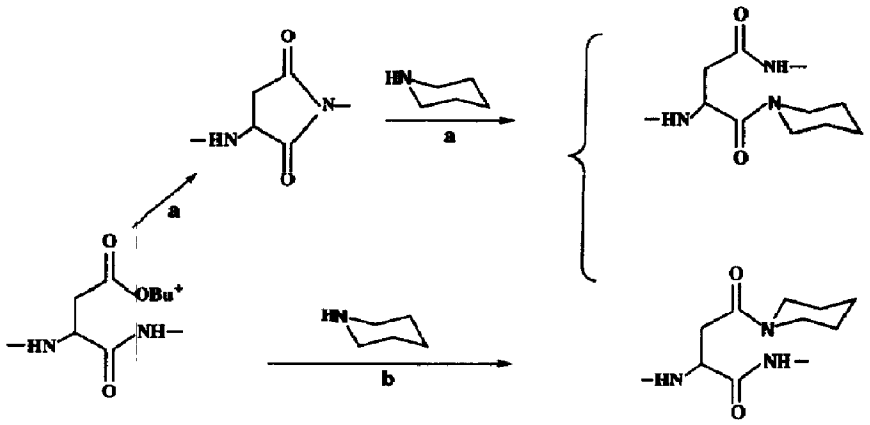

Fig. 1. Two possible pathways for piperidine addition to the aspartimide formed between -Asp(OtBu)-Asn(Trt)- or -Asp(OtBu)-Gly-.

Resin samples were taken after each coupling step, and the peptide fragments generated by TFA cleavage[9] were examined by HPLC and mass spectrometry (MS) for identification of products. HPLC profiles[10] indicated that the synthesis from the C-terminal proceeded well up to the 11th residue. However, after the 13th residue a new peak appeared in the HPLC profile. The new peak grew with each additional coupling, so that after the 23rd residue had been coupled it exhibited the same area as the peak from the desired peptide. The mass of the peptide corresponding to this new peak was 67u larger than that of the expected peptide. This mass difference suggested that the unknown peak was caused by aspartimide formation (-18u) and subsequent ring opening by piperidine addition (+85u). For the crude peptide containing 12 residues, a small peak with mass 18u lower than that of the target peptide indicated aspartimide formation. This finding supports the possibility that a piperidine adduct was created from the nucleophilic attack at the aspartimide formed between Asp and Asn, the 12th and 11th residues from the C-terminus (Fig. 1). An alternative pathway for the formation of the piperidine adduct by a direct substitution reaction can not be excluded (Fig. 1).

Proton NMR studies were consistent with the presence of a piperidine adduct in the peptide. Two NMR samples were prepared from 17 mer peptides corresponding to the desired peptide and the unknown peptide with a 67u larger mass. The proton NMR spectrum of the unknown peptide showed increased peak intensity at 1.6 and 3.6 ppm relative to the spectrum of the desired peptide. Since the spectrum of acetylpiperidine displays peaks of similar chemical shift, this suggested the difference between the two peptides was a piperidine adduct.

To investigate which amino acids in the sequence -Asp(Ot-Bu)-X- are susceptible to the aspartimide side reaction, seven model pentapeptides were synthesized: Lys(Boc)-Ala-Asp(Ot-Bu)-X-Ala, where X was Ala, Asn(Trt), Gly, Gln (Trt), His (Trt), Ser(t-Bu),Thr (t-Bu). The -Ala-Asp(Ot-Bu)-X- portion of pentapeptides was designed to mimic the sequence -Ala-Asp-Asn-, the 13th, 12th, and 11th amino acids from the C-terminal

Table 1. Occurrences of Aspartimide Formation in Fmoc- and Boc- Chemistry

Susceptible residue	Aspartimide formation[1] Fmoc chemistry[2] -Asp(OtBu)-	Boc chemistry[3] -Asp(OBzl)-	
Ala	+[4]	+	(12,13)
Asn	+++[5]	++	(4)
Gly	+++[5]	+++	(5,12,14,15,16)
Gln	+[4]	-	-
His	-	++	(17)
Ser	-	+++	(12,18)
Thr	-	++	(18)

1. Arbitrary scale. 2. This work. 3. Data from references in parentheses.
4. No piperidine adduct was observed. 5. Piperidine adduct was observed.

in the EGF-like peptide mentioned previously. The formation of aspartimide and its piperidine adduct in these model peptides was monitored using HPLC[10] and MS analysis of the crude peptides. Aspartimide formation was also found to increase with piperidine concentration. To reduce the amount of aspartimide in a lengthy synthesis, the pentapeptide resins were treated for 4 hours with 10% piperidine in DMF(v/v) rather than 20% piperidine to obtain milder deprotection conditions. The amount of aspartimide and piperidine adduct formed after four hours incubation with 10% piperidine, as determined by HPLC, was between 15 to 25 % for -Asp(Ot-Bu)-Asn(Trt)- and -Asp(Ot-Bu)-Gly-[11]. A smaller amount of aspartimide formation, without piperidine adduct formation, was observed for the -Asp(Ot-Bu)-Ala- and Asp(Ot-Bu)-Gln(Trt)- sequences.

A comparison of aspartimide formation in base-driven Fmoc and in acid-driven Boc chemistry is shown in Table 1. The nature of the amino acid following aspartic acid influences not only the rate of aspartimide formation, but also the formation of piperidine adducts. In Boc chemistry Ser is a susceptible residue. However in Fmoc chemistry, when Ser is protected by t-butyl, there is no significant side reaction. Asp-Asn and Asp-Gly are susceptible to aspartimide formation in both Fmoc and Boc approaches.

ACKNOWLEDGMENTS

Part of the peptide synthesis was performed at The Rockefeller University using facilities generously provided by Dr. R.B. Merrifield. This work was supported in part by US PHS grants HL41935 (J.P.T. and W.V.S.), CA 36544 (J.P.T.), and RR00862, GM38274 (B.T.C.), and by US PHS grant RR03037 to the Hunter College Synthesis and Sequence Facility. A preliminary report of part of this work was presented at the Eighth Symposium of The Protein Society (San Diego, USA, July 1994)[19].

REFERENCES AND NOTES

1. Barany, G.; Merrifield, R.B.: Solid-phase peptide synthesis. In *The Peptides, Vol 2*; Gross, E.; Meienhofer, J. Eds.; Academic Press: New York, 1979; pp.193-196.
2. Bodanszky, M.; Kwei, J.Z. *Int. J. Peptide Protein Res.* **1978**, 12, 69-74.
3. Bodanszky, M.; Tolle, J.C.; Deshmane, S.S.; Bodanszky, A. *Int. J. Peptide Protein Res.* **1978**, 12, 57-68.
4. Tam, J.P., Rieman, M.W.; Merrifield, R.B., *Peptide Res.*, **1988**, 1, 6-18.
5. Blake, J. *Int. J. Peptide Protein Res.* **1979**, 13, 418-425.
6. There is only one previous mention of this side reaction in Fmoc chemistry. See reference 15.
7. Side-chain protecting groups were as follows: Asp and Glu by Ot-Bu, Ser, Thr and Tyr by t-Bu, Asn, Gln and His by Trt, Lys by Boc, Arg by Pmc, and Cys by either Trt or Acm. A 4-(hydroxymethyl)phenoxymethyl-Copoly styrene resin (HMP resin/Wang resin) with a substitution of 0.88 mmol/g was used. Stepwise coupling of Fmoc amino acids using DCC/HOBt was performed first, followed by TBTU as a second coupling when necessary. Deblocking the NH_2-terminal Fmoc protecting group with 20% piperidine in DMF (v/v) was typically carried out for 20 min.
8. Leytus, S.P.; Foster, D.C.; Kurachi, K.; Davie, E.W. *Biochem.* **1986**, 25, 5098-5102.
9. A micro-scale TFA Cleavage method was used. The cleavage mixture used for peptides containing Trt protecting groups was: 0.25 ml 1,2-ethanedithiol, 0.25 ml H_2O and 9.5 ml TFA.
10. C18 reverse phase HPLC was performed. (1) For EGF-like peptides: Buffer A contained 5% acetonitrile in 0.045% TFA. Buffer B contained 60% acetonitrile in 0.037% TFA. Peptides were eluted with a 10-40% buffer B linear gradient over 30 min at 1.5 ml/min, monitored at 220 nm. (2) For model peptides: Buffer A contained H_2O in 0.045% TFA. Buffer B contained 60% acetonitrile in 0.037% TFA. the model peptides were eluted with 100% buffer A for 3 minutes and 0-22% buffer B linear gradient over 22 min at 1.5 ml/min, monitored at 220 nm.
11. The extent of formation of aspartimide and piperidine adduct between Asp and Asn (or between Asp and Gly) is less pronounced in the model peptide studies than in the synthesis of EGF-like peptide containing -Asp-Asn-.
12. Yang, C.C.; Merrifield, R.B. *J. Org. Chem.* **1976**, 41, 1032-1041.
13. Marshall, G.R.; Merrifield, R.B. *Biochem.* **1965**, 4, 2394-2401.
14. Wang, S.S.; Yang, C.C.; Kulesha, I.D.; Sonenberg, M.; Merrifield, R.B. *Int. J. Peptide Protein Res.*,**1974**, 6, 103-109.
15. Nicolás, E.; Pedroso, E.; Giralt, E. *Tetrahedral Lett.*. **1989**, 30, 497-500.
16. Ondetti, M.A.; Deer, A.; Sheehan, J.T.; Pluscec, J.; Kocy, O. *Biochem.* **1968**, 7, 4069-4075.
17. Baba, T.; Sugiyama, H.; Seto, S. *Chem. Pharm. Bull. Japan* **1973**, 21, 207-209.
18. Suzuki, K.; Endo, N. *Chem. Pharm. Bull. Japan* **1978**, 26, 2269-2274.
19. Yang, Y; Sweeney, W.V.; Thörnqvist, S.; Schneider, K.; Chait, B.T.; Tam, J.P. In *Techniques in Protein Chemistry VI*. Crabb, J. Eds.; Academic Press: New York, In press.

(*Received in USA* 22 *August* 1994; *revised* 25 *October* 1994; *accepted* 27 *October* 1994)