A New and Efficient Approach for the Synthesis of Peptides Containing Aspartylglycyl Sequences

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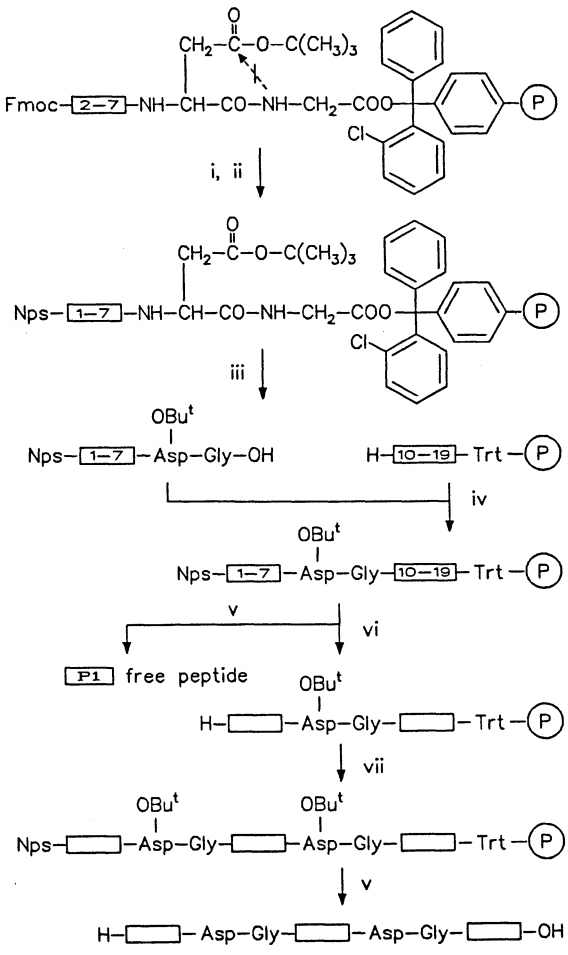
H-Asp(OBu^t)-Gly, coupled to the 2-chlorotrityl resin, does not undergo aspartimide cyclization upon further solid phase peptide elongation via the Fmoc strategy. Prolonged peptide fragments, side chain and *N*-terminally Nps-protected, are suitable intermediates for fragment condensations of long-chain Asp-Gly-containing peptides.

The ready conversion of aspartyl peptides to aminosuccinyl derivatives is one of the most disturbing side reactions in peptide synthesis.[1] This side reaction takes place predominantly, if the protected carboxyl residue of the Asp(OBzl) moiety is adjacent to amino acids with sterically less hindered side chains such as Gly and Ser[2] or occasionally Asn[3] and Phe.[4] The aspartimide moiety opens under certain conditions to form mainly ß-aspartyl peptides,[5] isoionic isomers of the desired α-aspartyl peptides, which are extremely difficult to separate by conventional methods of purification. The formation of such ß-aspartyl peptide bonds causes in many cases loss of inherent physiological activities.[6] Efforts therefore have been directed towards the elimination of this rearrangement. A widely used method is to introduce a sterically hindering voluminous protecting group for the ß-carboxy function of aspartic acid, like ß-cyclopentyl (Cpe),[7] ß-cyclohexyl (Chx),[8] ß-cycloheptyl (Chp), ß-cyclooctyl (Coc),[9] ß-menthyl (Men),[10] ß-adamantyl (Ada)[11] or ß-cyclododecyl (Cdo)[12] esters. Among these protecting groups, the cyclohexyl ester was widely applied because of its commercial availability. However, this protection did not avoid the side reaction completely. The presence of weakly acidic additives[13, 14] to the reaction mixtures or the cleavage reagents reduced the aspartimide formation to a low, but still detectable level.

As demonstrated,[15] treatment of an -Asp(OBzl)-Gly- sequence with 20% piperidine in DMF for 10 min resulted in 100% aspartimide formation. Preparing an -Asp(OChx)-Gly- sequence, the loss of the cyclohexyl group is appreciably reduced, but still significant, involving 2.5% of imide formation per deprotection step. For an -Asp(OBu^t)-Gly- sequence, an extent of 0.3% for the side reaction per deprotection cycle was observed, indicating that t-butyl protection is much more resistant to nucleophilic attack because of its poor leaving property. However, recent studies on the growth-blocking peptide (GBP) synthesis[6a] showed that the yield of the synthesis of the -Asp(OBu^t)^{16}-Gly^{17}- peptide was only 2.4%, indicating that approximately 90% of the peptide was cyclized using the Fmoc strategy. Similarly, our approach to synthesize the partial sequence of the nephritogenoside peptide[16] (H-Leu-Phe-Gly-Ile-Ala-Gly-Glu-Asp-Gly-Pro-Thr-Gly-Pro-Ser-Gly-Ile-Val-Gly-Gln-OH; **P1**; residue 1-19) using Fmoc-Asp(OBu^t)-OH as coupling amino acid, yielded 55% cyclized peptide. Therefore, t-butyl ester protection for the aspartic acid side chain using the Fmoc strategy is not generally reliable to prevent aspartimide formation, and a high yield of cyclization may occur, if the Asp-Gly sequence is far from the *N*-terminus.

The new approach which we have developed to avoid aspartimide formation is shown in Scheme 1 and includes the following steps: 1) synthesis of the *N*-terminal fragment (2-7) on the 2-chlorotrityl resin using Fmoc amino acids starting with the Asp(OBu^t)-Gly sequence, followed by coupling of the *N*-terminal amino acid, protected by a Nps group; 2) cleavage of the side chain and *N*-terminally Nps-protected fragment (1-9) from the resin and subsequent coupling to the *C*-terminal peptide (10-19) chlorotrityl resin; 3) TFA treatment, yielding the free peptide, or selective cleavage of the Nps group for subsequent peptide elongation via fragment condensation. The strategy banks on the following considerations: the Trt group of the 2-chlorotrityl resin[17] would be rigid and bulky enough to suppress aspartimide formation of Asp(OBu^t)-Gly attached to the support during the *N*-terminal fragment synthesis, and the other side reactions caused by conformational influence[13] can also be extensively eliminated by using the subsequent fragment condensation.

Based on these considerations, the *C*-terminal sequence H-Pro-Thr(Bu^t)-Gly-Pro-Ser(Bu^t)-Gly-Ile-Val-Gly-Gln-OH (**P2**)



Scheme 1. Synthetic approach of the Asp-Gly-containing peptide. i) 25% piperidine in DMF; ii) Nps-Leu-OH/TBTU/DIEA (1:1:1); iii) CH_2Cl_2/TFE/AcOH (7:2:1); iv) TBTU/DIEA (1:1); v) 50% TFA in CH_2Cl_2; vi) thiolysis; vii) protected *N*-terminal fragment/TBTU/DIEA (1:1:1).

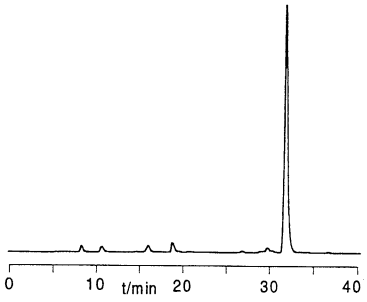


Figure 1. RP-HPLC of crude Nps-Leu-Phe-Gly-Ile-Ala-Gly-Glu(OBu^t)-Asp(OBu^t)-Gly-OH. column: Bio-Sil C_{18} (250×10 mm); solvent A: 0.1% TFA/H_2O, B: 0.1% TFA/CH_3CN; gradient elution: 0-1 min 70% A, 1-31 min 70-20% A; 31-40 min 20% A; flow rate: 2.0 ml/min; detection: 214 nm

was readily assembled by stepwise batch synthesis with Fmoc-amino acids on the 2-chlorotrityl resin. Couplings were performed employing a threefold molar excess of Fmoc-aa/TBTU/DIEA in DMF for 40 minutes. Fmoc deprotection was carried out using 25% piperidine in DMF for 20 min. The synthesis of the *N*-terminal fragment Nps-Leu-Phe-Gly-Ile-Ala-Gly-Glu(OBu^t)-Asp(OBu^t)-Gly-OH (**P3**) was started by coupling Fmoc-Gly-OH to the 2-chlorotrityl resin. During the synthesis, the α-amino functions were protected by Fmoc groups, except for the *N*-terminal leucine, for which Nps protection was used. Chain elongation proceeded well according to the procedure described above. Treatment with CH_2Cl_2/TFE/AcOH (7:2:1) released the desired protected peptide in >96% yield (RP-HPLC: Figure 1.) and showed no aspartimide formation. For condensation, the *N*-terminal fragment was coupled to the *C*-terminal fragment resin in DMF by TBTU/HOBt/DIEA. Cleavage of the assembled peptide from resin with CH_2Cl_2/TFE/AcOH (7:2:1) afforded the protected peptide Nps-Leu-Phe-Gly-Ile-Ala-Gly-Glu(OBu^t)-Asp(OBu^t)-Gly-Pro-Thr (Bu^t)-Gly-Pro-Ser(Bu^t)-Gly-Ile-Val-Gly-Gln-OH (**P4**)[18] in *ca.* 90% yield, from which by treatment with TFA the unprotected sequence (**P1**)[18] could be obtained.

For the purpose of *N*-terminal prolongation of the **P4**-chlorotrityl resin, optimal conditions for selective cleavage of the Nps group were investigated. Aspartimide formation and loss of chains accompanying the removal of the Nps protecting group were also evaluated. The results are summarized in Table 1. It shows that about 10% of chains are lost from the resin using thiourea/AcOH/MeOH. In the presence of other cleavage reagents, no loss of peptide chains was observed. Aspartimide formation was only detected upon 2-thiopyridone treatment. The most satisfactory cleavage reagent (neither toxicity nor formation of insoluble disulfides) proved to be 2-thiopyridone with an equivalent amount of acetic acid. Under these conditions, the Nps group can be quantitativly removed in a short time and neither aspartimide formation nor loss of chains were observed. The selective removal of the Nps group allows also to synthesize long-chain peptides including several aspartimide-sensitive sequences. A Nps-transfer, such as to the indole moiety of Trp during acidolysis, or other side reactions were also not observed.[19a-d]

In conclusion, we have outlined a new route for the synthesis of peptides containing aspartylglycyl sequences. Using the 2-chlorotrityl resin, *C*-terminal -Asp(OBu^t)-Gly- and *N*-terminally Nps-protected peptides can be assembled on the solid support via the Fmoc strategy without imide cyclization due to the bulkiness of the linker. Cleavage of the Nps-protected peptide from the resin yields synthons for fragment condensations of Asp-Gly-containing sequences, thereby efficiently suppressing the aspartimide formation.

Table 1. Treatment of **P4**-chlorotrityl resin with different Nps cleavage reagents and observed aspartimide formation resp. loss of chains

Cleavage reagent[a]	Time for complete cleavage (min)	Loss of chains[b]	Aspartimid formatiom[b]
1	480	/	6.7% (in 180 min)
2	45	/	/
3	25	/	/
4	40	/	/
5	15	10%	/

[a]Cleavage reagents: 1) 250 mg 2-thiopyridone in 1 ml DMF;[19a, b] 2) 250 mg 2-thiopyridone + 136 mg AcOH in 1 ml DMF;[19a, b] 3) 200 μl mercaptan + 250 mg imidazole in 1 ml DMF;[19c-e] 4) 100 mg 4-nitrothiophenol in 1 ml DMF;[19c-e] 5) 200 mg thiourea in 1 ml AcOH + 3 ml MeOH.[19f]
[b]For aspartimide formation and loss of chains from the resin during Nps cleavage, Fmoc-Glu(OBzl)-Asp(OBzl)-Gly-Pro-Thr(Bzl)-Gly-Pro-Ser(Bzl)-Gly-Ile-Val-Gly-Gln-2-chlorotrityl resin, highly susceptible to aspartimide formation, was chosen as a model compound. 5 mg peptide resin were exposed for a period of 70-180 min to various cleavage conditions, simulating about 4 cycles of Nps removal. Then the protected peptide was removed from the resin and analysed.

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