Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 742–748
Published online 13 September 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.885

JOURNAL OF PEPTIDE SCIENCE

1,4-Diazepine-2,5-dione ring formation during solid phase synthesis of peptides containing aspartic acid β-benzyl ester

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Received 12 April 2007; Revised 15 May 2007; Accepted 15 May 2007

Abstract: The Fmoc-based SPPS of H-Xaa-Asp(OBzl)-Yaa-Gly-NH_2 sequences results in side reactions yielding not only aspartimide peptides and piperidide derivatives, but also 1,4-diazepine-2,5-dione-peptides. Evidence is presented to show that the 1,4-diazepine-2,5-dione derivative is formed from the aspartimide peptide. The rate of this ring transformation depends primarily on the tendency to aspartimide and piperidide formation, which is influenced by the nature of the amino acid following the aspartic acid β-benzyl ester (Xaa). However the bulkiness of the amino acid side chain preceeding the aspartic acid β-benzyl ester (Yaa) is also important. Under certain conditions the 1,4-diazepine-2,5-dione peptide derivative may even be formed dominantly, which is a highly undesirable side reaction in peptide synthesis, but which provides a new way for the synthesis of diazepine peptide derivatives with targeted biological or pharmacological activity. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: 1,4-diazepine-2,5-dione-peptides; aspartimide; SPPS; Fmoc technique

INTRODUCTION

In the synthesis of a thrombin inhibitor – RGD peptide conjugate [Ilaš J, Kikelj D, Süli-Vargha H, Hudecz F, in preparation manuscript], as a first attempt the β-carboxyl group of aspartic acid was protected with benzyl ester. H-Cys(Acm)-Arg(Tos)-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 was prepared by Fmoc-technique on Rink-amide resin. The molecular mass of the main product cleaved from the resin did not correspond to the expected molecular mass of the peptide, showing that amino acid coupling had been terminated after the second glycine residue. The NMR spectrum of the product indicated that 1,4-diazepine-2,5-dione derivative of Cys(Acm)-Gly-NH_2 1 had been formed, hindering the further elongation of the peptide chain.

The danger of using aspartic acid β-benzyl ester in peptide synthesis is well known, since aminosuccinyl peptides can be formed in both basic [1] and in acidic condition [2]. In the latter case the conversion of the aminosuccinyl peptides H-Leu-Asu-Ala-NH_2 and H-Pro-Asu-Gly-NH_2 to their piperazine-2,5-dione derivative was observed in 3 days in ethanol–water mixture containing TEA [2]. The formation of aspartimide in Fmoc-based solid phase peptide synthesis has been discussed in detail, however there is no mention of 1,4-diazepine-2,5-dione ring closure [3,4,5]. The transformations of aspartimide-peptides are shown on Figure 1.

The aim of the present work was to investigate the structural features in peptides leading to the 1,4-diazepine-2,5-dione ring formation and to clarify the mechanism of this transformation. Model peptides were designed on the basis of the Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 sequence where the diazepine ring closure was observed. Substitutions were performed in two places, and amino acids preceeding or following the aspartic acid were changed in order to investigate the role of the amino acid side chain in this transformation.

MATERIALS AND METHODS

Chemicals and Equipment

Boc-Gly-OH, Z-Gly-OH, solvents and reagents, unless otherwise stated, were of analytical grade, obtained from Reanal Fine Chemical Works (Budapest, Hungary). DIC, HOBt, DIEA, piperidine were purchased from Fluka (Hungary), and Fmoc-amino acids, Rink amide MBHA and Wang resins from Novabiochem (Merck Kft, Budapest, Hungary). Analytical RP-HPLC was run on a Knauer instrument with a Phenomenex Jupiter C18 5 μm, 300 Å, (150 × 4.6 mm) column, using a linear gradient (1 → 60% B, 30 min); A: 0.1% TFA in water, B: 0.08% TFA in acetonitrile, flow rate 1ml/min. Electrospray ionization mass spectrometry was performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bremen, Germany). NMR spectra were recorded in DMSO-d_6 solution on a Bruker DRX 500 spectrometer at 500 (^{1}H), 125 (^{13}C) and 50 (^{15}N) MHz, with the deuterium signal of the solvent. Each assignment is based on 2D-HMQC and 2D-HMBC spectra obtained by using the standard Bruker pulse programs INV4GS and INV4GSLPLRND, respectively.

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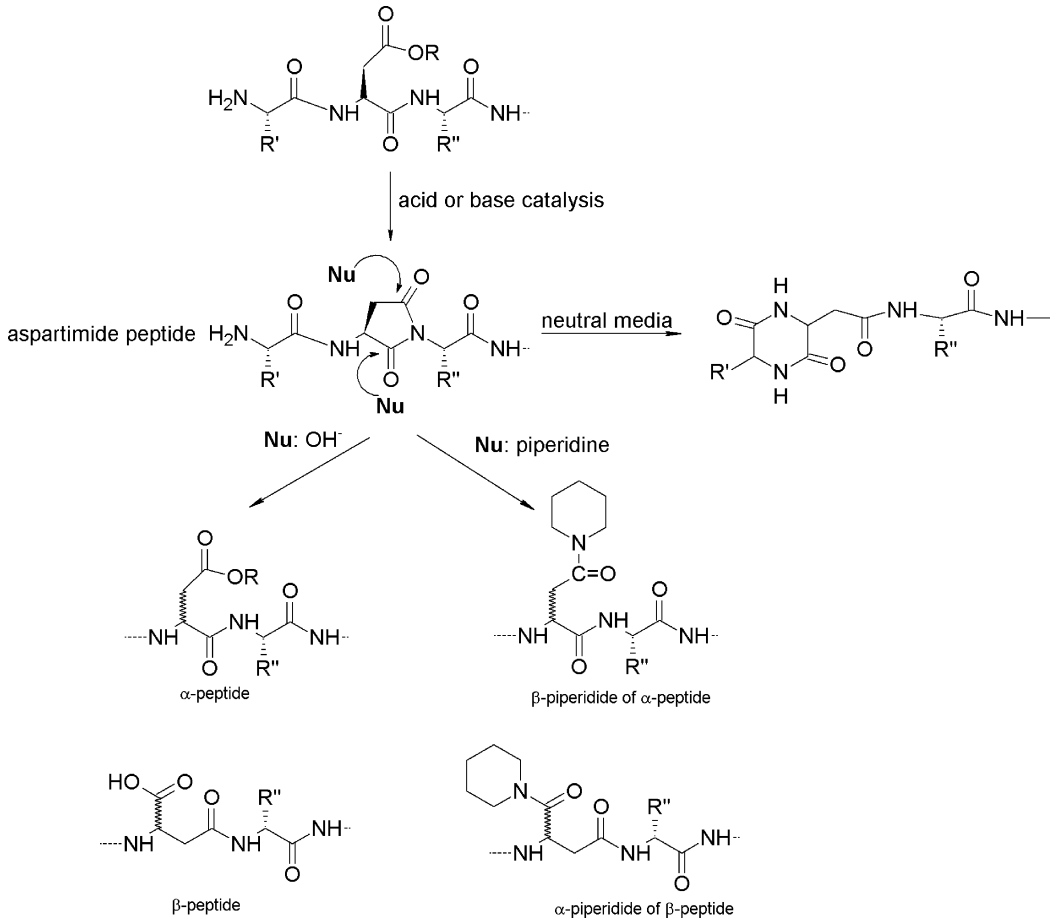


Figure 1 The transformations of aspartimide peptides 172 × 151 mm (300 × 300 DPI).

Peptide Synthesis

Peptides were synthesized manually by the Fmoc technique using DIC/HOBt as coupling reagents. The standard protocol for Fmoc cleavage consisted of two treatments (2 and 18 min) with 20% piperidine in DMF. The peptides with a free amino terminus and with a Z or Boc protecting group on the amino terminus were cleaved from the resin with TFA/TIS/water mixture during 2 h, the solution was poured into ether and the precipitate was separated by centrifugation. For MS and HPLC data of the peptides see Table 1.

NMR Measurements

1,4-Diazepine-2,5-dion derivative of Cys(Acm)-Gly-NH_2 [*N*-(3-(acetamidomethylthio)-1-(2-amino-2-oxoethylamino)-1-oxopropan-2-yl)-3,7-dioxo-1,4-diazepane-5-carboxamide].

^{1}H NMR: δ 8.47 (1H, t, $J = 7.5$ Hz, $-NH^{16}-CO-$), 8.40 (1H, d, $J = 8.4$ Hz, $-NH^{3}-CO-$), 8.22 (1H, t, $J = 6.0$ Hz, $-NH^{6}-CO-$), 8.06 (1H, brs, $-NH^{10}-CO-$), 7.91 (1H, d, $J = 1.7$ Hz, $-NH^{7}-CO-$), 7.10 and 7.04 (2H, 2 × 1 s, $-CO-NH_2$), 4.44 (1H, td, $J = 8.4$ and 5.0 Hz, $-CH^{4}-$), 4.23–4.19 (2H, ABX, $J_{AB} = 13.5$ Hz, $J_{AX} = J_{BX} = 6.4$ Hz, $-S-\underline{CH_2}-HN-CO$), 4.11 (1H, td, $J = 6.2$ and 1.7 Hz, $-CH^{1}-$), 3.72 (2H, d, $J = 1.3$ Hz, CH_2^9), 3,62–3.59 (2H, ABX, $J_{AB} = 16.7$ Hz, $J_{AX} = J_{BX} = 6.0$ Hz, $-NH-\underline{CH_2}-CO-NH_2$), 2.93–2.71 (2H, ABX, $J_{AB} = 13.9$ Hz, $J_{AX} = 5.0$ Hz, $J_{BX} = 8.4$ Hz, $-CH-\underline{CH_2}-S-$), 2.67–2.57 (2H, ABX, $J_{AB} = 15.2$ Hz, $J_{AX} = J_{BX} = 6.2$ Hz, CH_2^{12}), 1.83 (3H, s, $CO-\underline{CH_3}$). ^{13}C NMR: δ 171.55 (C^{22}), 171.18 (C^5), 170.54 (C^2), 170.44 (C^{25}), 168.78 (C^8), 166.83(C^{11}), 53.75 (C^4), 52.23 (C^1), 45.38 (C^9), 42.94 (C^{21}), 41.33 (C^{15}), 38.81 (C^{12}), 33.22 (C^{13}), 23.45 (C^{27}).

1,4-diazepine-2,5-dion derivative of Ala–Gly–NH_2 [*N*-(1-(2-amino-2-oxoethylamino)-1-oxopropan-2-yl)-3,7-dioxo-1,4-diazepane-5-carboxamide].

^{1}H NMR: δ 8.34 (1H, d, $J = 7.3$ Hz, $-NH^{3}-CO-$), 8.15 (1H, t, $J = 6.2$ Hz, $-NH^{6}-CO-$), 8.10 (1H, brs, $-NH^{10}-CO-$), 7.99 (1H, brs, $-NH^{7}-CO-$), 7.06 and 7.01 (2H, 2 × 1 s, $-CO-NH_2$), 4.19 (1H, q, $J = 7.1$ Hz, $-CH^{4}-$), 4.13 (1H, t, $J = 6.3$ Hz, $-CH^{1}-$), 3.58 (2H, d, $J = 6.0$ Hz, $-\underline{CH_2}-CO-NH_2$), 3.75–3.71 (2H, AB, $J_{AB} = 16.0$ Hz,, $-CH_2^9$), 2.66–2.53 (2H, ABX, $J_{AB} = 15.2$ Hz, $J_{AX} = 6.4$ Hz $J_{BX} = 6.0$ Hz, CH_2^{12}), 1.22 (3H, d, $J = 7.1$ Hz, $CH-\underline{CH_3}$). ^{13}C NMR: δ 173.3 (C^5), 171.8 (C^{19}), 170.3 (C^2), 168.8 (C^8), 166.9(C^{11}), 59.8 (C^4), 52.1 (C^1), 45.5 (C^9), 42.6 (C^{18}), 38.8 (C^{12}), 18.5 (C^{13})

Aspartimide and Diazepine-Peptide Formation in Solution

H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 was left in 20% piperidine/DMF solution for 20 min at room temperature,and then the solution was concentrated *in vacuo*, triturated with ether and the precipitate was separated by centrifugation and applied onto the HPLC column.

DOI: 10.1002/psc

Table 1 Product analysis after Fmoc-based SPPS of peptides containing aspartic acid β-benzyl ester (*negligible amount)

Peptide plan	M calc.	Product analysis	$[M+H]^+$ found	t_r HPLC (min)
1 H-Gly-Cys(Acm)-Arg(Tos)-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{40}H_{58}N_{12}O_{12}S_3$: 994.3	⌐Gly-Asp-Cys(Acm)-Gly-NH_2	403.1	6.5
1a H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 from Boc-Gly-Asp(OBzl)-Cys(Acm)-Gly-P (Rink amide)	$C_{21}H_{30}N_6O_7S$: 510.2	⌐Gly-Asp-Cys(Acm)-Gly-NH_2	403.1	6.5
		H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2	511.2	15.4
1b H-Gly-Asp(OBzl)-Cys(Acm)-Gly-OH from Fmoc-Gly-Asp(OBzl)-Cys(Acm)-Gly-P (Wang)	$C_{21}H_{31}N_5O_8S$: 511.2	⌐Gly-Asp-Cys(Acm)-Gly-NH_2	404.1	6.2
		H-Gly-Asp(Pip)-Cys(Acm)-Gly-OH	489.2	9.8 and 11.5
2 H-Gly-Asp(OBzl)-Leu-Gly-NH_2	$C_{21}H_{31}N_5O_6$: 449.2	⌐Gly-Asp-Leu-Gly-NH_2	342.2	9.6
3 H-Gly-Asp(OBzl)-Gly-Gly-NH_2	$C_{17}H_{23}N_5O_6$: 393.2	⌐Gly-Asp-Gly-Gly-NH_2	286.0	4.8
		H-Gly-Asp(Pip)-Gly-Gly-NH_2	371.2	7.8 and 8.8
		*H-Gly-Asp-Gly-Gly-NH_2	304.1	4.6
4 H-Gly-Asp(OBzl)-Ala-Gly-NH_2	$C_{18}H_{25}N_5O_6$: 407.2	⌐Gly-Asp-Ala-Gly-NH_2	300.1	4.8
		H-Gly-Asp(Pip)-Ala-Gly-NH_2	385.2	7.8 and 8.8
		*H-Gly-Asp-Ala-Gly-NH_2	318.1	4.5
5 H-Gly-Asp(OBzl)-Ile-Gly-NH_2	$C_{21}H_{31}N_5O_6$: 449.2	H-Gly-Asp(OBzl)-Ile-Gly-NH_2	450.2	18.5

DOI: 10.1002/psc

Table 1 (*Continued*)

Peptide plan	M calc.	Product analysis	$[M+H]^+$ found	t_r HPLC (min)
6 H-Ala-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{22}H_{32}N_6O_7S$: 524.2	⌐Ala-Asp-Cys(Acm)-Gly-NH_2	417.1	7.7
		H-Ala-Asp(Pip)-Cys(Acm)-Gly-NH_2	502.2	10.3
7 H-Leu-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{25}H_{38}N_6O_7S$: 566.3	⌐Leu-Asp-Cys(Acm)-Gly-NH_2	459.2	11.6
		H-Leu-**Asu**-Cys(Acm)-Gly-NH_2	459.2	10.7
		H-Leu-Asp(Pip)-Cys(Acm)-Gly-NH_2	544.3	14.2
8 H-Val-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{24}H_{36}N_6O_7S$: 552.2	⌐Val-Asp-Cys(Acm)-Gly-NH_2	445.2	10.2
		H-Val-**Asu**-Cys(Acm)-Gly-NH_2	445.2	9
		H-Val-Asp(Pip)-Cys(Acm)-Gly-NH_2	530.3	12.7
9 H-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{19}H_{27}N_5O_6S$: 453.2	H-Asp(OBzl)-Cys(Acm)-Gly-NH_2	454.2	15
		H-**Asu**-Cys(Acm)-Gly-NH_2	346.1	5.5
10 H-Asp(OBzl)-Leu-Gly-NH_2	$C_{19}H_{28}N_4O_5$: 392.2	H-Asp(OBzl)-Leu-Gly-NH_2	393.4	18
		H-**Asu**-Leu-Gly-NH_2	285.1	9.4
11 Z-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2	$C_{29}H_{36}N_6O_9S$: 644.2	Z-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2	645.3	26.8
		Z-Gly-**Asu**-Cys(Acm)-Gly-NH_2	537.2	19
		H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2	511.2	15.8

DOI: 10.1002/psc

RESULTS AND DISCUSSION

The peptides were synthesized on a Rink amide resin by the Fmoc technique (DIC/HOBt coupling agent); for the cleavage of the Fmoc protecting group 20% piperidine in DMF was used in all cases. After removal from the resin with the usual TFA cleavage procedure, the products were analyzed by HPLC. The peaks on the chromatogram were separated with semipreparative HPLC and identified by mass spectrometry (Table 1) and, when necessary by NMR.

In the Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 sequence Cys(Acm) was substituted (i) for Leu to test the effect of the neutral leucine side chain instead of the Acm group **2**; (ii) for Gly lacking a side chain **3**; (iii) for Ala having a small methyl group **4** and for the sterically more hindered Ile **5**. The first Gly was changed for Ala **6**; for Leu **7** and for Val **8** in order to check the effect of steric hindrance in this place. To investigate the rate of succinimide ring formation after only one usual piperidine treatment for Fmoc removing, two tripeptides Asp(OBzl)-Cys(Acm)-Gly-NH_2 **9** and Asp(OBzl)-Leu-Gly-NH_2 **10** were synthesized, too.

The HPLC diagram of the crude product after the abortive synthesis of H-Cys(Acm)-Arg(Tos)-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 is shown in Figure 2, the first peak was isolated and its structure was determined by mass spectrometry and NMR measurements as the 1,4-diazepine-2,5-dione derivative of Cys(Acm)-Gly-NH_2.

In Figure 3 some typical HPLC diagrams of the crude model peptides are shown. The chromatograms of **1**, **2**, **3**, **4**, **6** are of similar character (**4, 6** are shown; A, B). After separation, the first peak according to mass spectrometry always proved to be the appropriate 1,4-diazepine-2,5-dione derivative of the peptide (in case **4** also proved by NMR). The second peak in **6,** and the second and the third peaks in **1**, **3** and **4** are aspartic acid piperidides, presumably α- and β-isomers. There is a dramatic change in the case of **5**, namely H-Gly-Asp(OBzl)-Ile-Gly-NH_2 is the main product (>90%, Figure 3(C)). On the chromatogram of H-Leu-Asp(OBzl)-Cys(Acm)-Gly-NH_2 and H-Val-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (Figure 3(D), (E)), the first and the second peak have the same mass, the first one is ninhydrine positive, so it must be the aspartimide derivative and the second the diazepine derivative, while the third was identified as piperidide. On the chromatogram of the tripeptides **9** and **10** (Figure 3(F), (G)), the first peak is aspartimide peptide formed –in accordance with literature data [3] –in greater extent, when Asp(OBzl) was followed by Cys(Acm), and the second peak is in both cases the expected H-Asp(OBzl)-Cys(Acm)-Gly-NH_2 and H-Asp(OBzl)-Leu-Gly-NH_2, respectively.

These resuslts strongly suggest that 1,4-diazepine-2,5-dione derivatives were not formed by direct cyclization from the terminal amino group of the peptide and the β-benzyl ester group of aspartic acid, but rather from previously formed aspartimide intermediates (Scheme 1). This hypothesis is supported by the

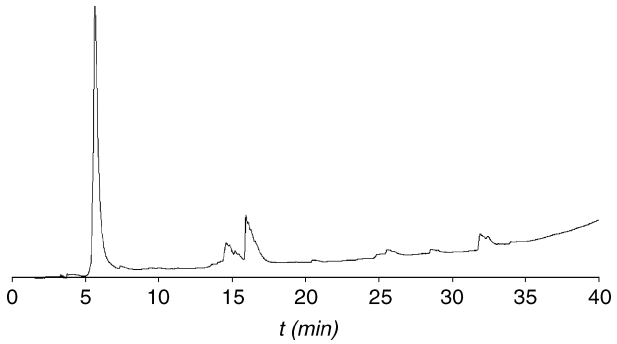


Figure 2 The HPLC diagram of the abortive synthesis of H-Cys(Acm)-Arg(Tos)-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (crude product). 167 mm (96 × 96 DPI).

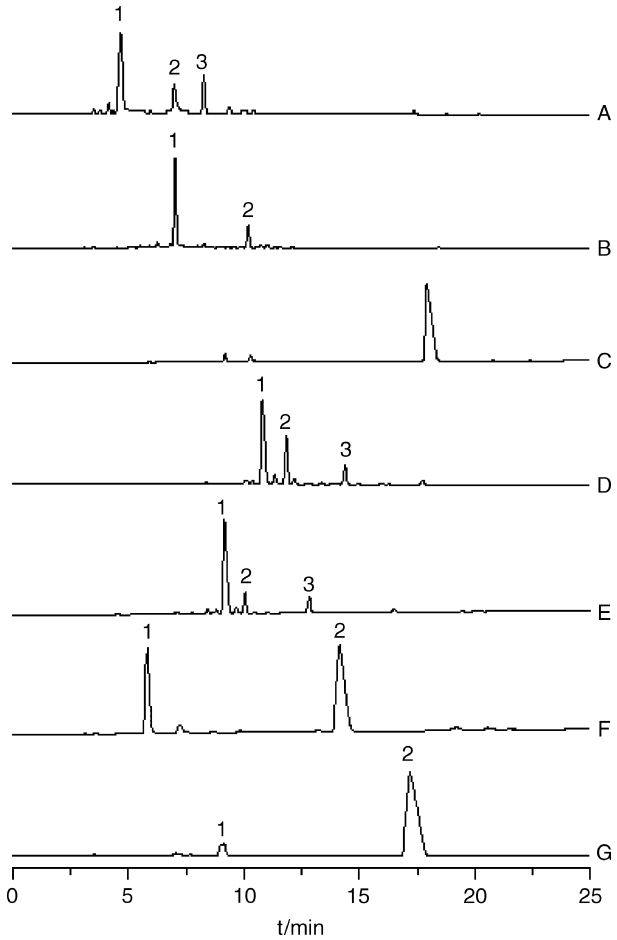


Figure 3 Typical analytical HPLC profiles of crude products obtained after Fmoc-based SPPS of H-Xaa-Asp(OBzl)-Yaa-Gly-NH_2 sequence (see also Table 1): A, H-Gly-Asp(OBzl)-Ala-Gly-NH_2 (4); B, H-Ala-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (6); C, H-Gly-Asp(OBzl)-Ile-Gly-NH_2 (5); D, H-Leu-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (7); E H-Val-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (8); F, H-Asp (OBzl)-Cys(Acm)-Gly-NH_2 (9); G, H-Asp(OBzl)-Leu-Gly-NH_2 (10).

DOI: 10.1002/psc

observation of Bodánszky and Kwei [1], who previously reported that the time required for base-catalyzed formation of aminosuccinyl derivatives of β-benzylaspartyl peptides is by far the longest when aspartic acid β-benzyl ester is followed by Ile ($t_{1/2}$ 730 h for Boc-Asp(OBzl)-Ile-, 3 h for Boc-Asp(OBzl)-Gly-) [1]. The successful synthesis of H-Gly-Asp(OBzl)-Ile-Gly-NH_2 **5** indicates that if the aspartimide ring formation is hindered by Ile, no 1,4-diazepine-2,5-dione derivative can be formed. The rate of diazepine ring formation from aspartimide peptide is the highest, when Gly or Ala is preceeding, and Cys(Acm) or Leu is following aspartic acid β-benzyl ester (i.e. Figures 2 and 3(B)).

The aspartimide mediated 1,4-diazepine-2,5-dione ring closure is also supported by the fact that although after the piperidine treatment of Fmoc-Asp(OBzl)-Cys(Acm)-Gly-resin, the crude product obtained from the resin contained a significant amount, about 40% of aspartimide (Figure 3(F)). When this aspartimide peptide was elongated with a further Fmoc-amino acid and treated for the second time with piperidine, after cleavage from the resin, the crude product contained beside piperidides only the 1,4-diazepine-2,5-dione derivative (Figure 3(B)). It is interesting that piperidide formátion is dependent on the properties of the amino acid side chain following the aspartic acid in the sequence in decreasing order: Gly ≈ Ala > Cys(Acm) > Leu.

For further investigation of the transformation of aspartimide peptides to diazepine peptides, other combinations of side chain protecting groups were also tested. Boc-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 was synthesized on Rink amide resin by Fmoc technique. In this case, after the incorporation of aspartic acid β-benzyl ester only one piperidine treatment was applied. After TFA mediated Boc removal and cleavage from the resin, in the HPLC chromatogram (Figure 4(b)) of the crude product **1a** two peaks were identified

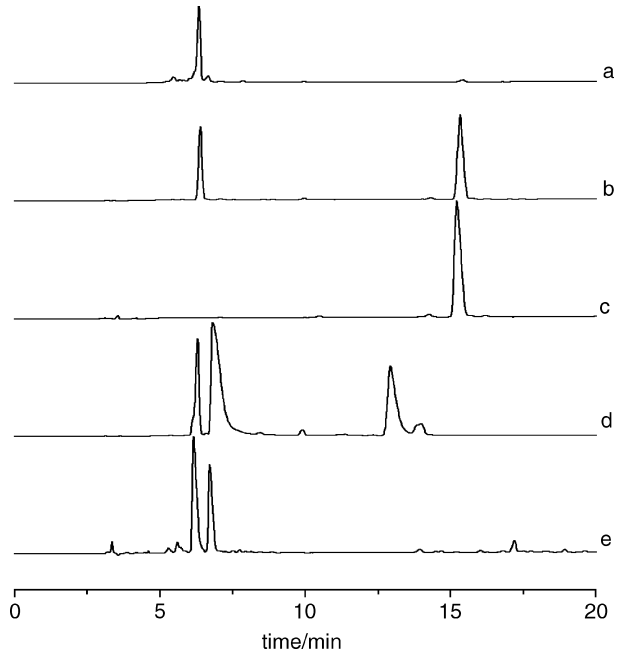
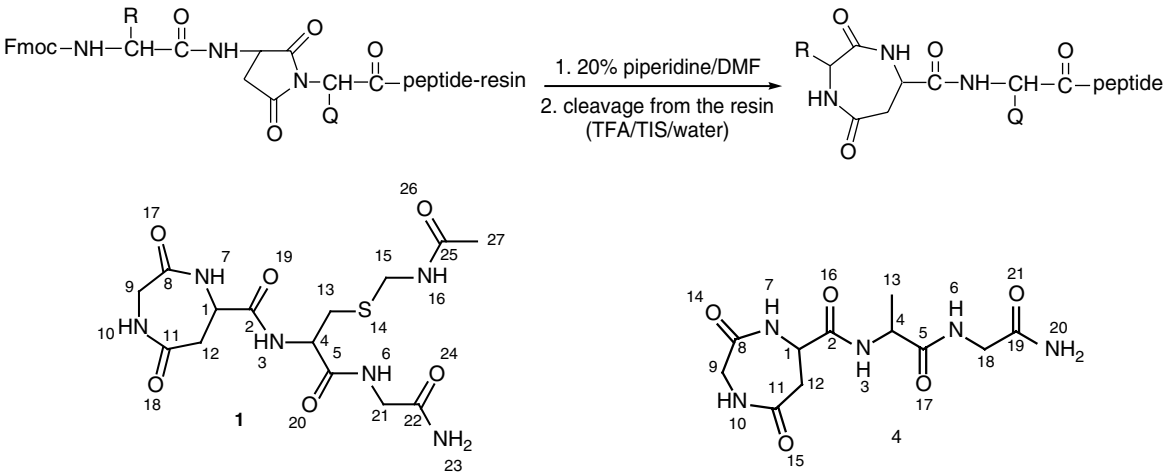


Figure 4 Product identification after the treatment of H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 with 20% piperidine/DMF. (a) 1,4-diazepine-2,5-dione derivative of Cys(Acm)-Gly-NH_2; (b) crude 1a, 1,4-diazepine-2,5-dione derivative of Cys(Acm)-Gly-NH_2 and H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 (see Table 1); (c) purified H-Gly-Asp(OBzl) -Cys(Acm)-Gly-NH_2; (d) H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 after 20% piperidine/DMF treatment; (e) Z-Gly-Asu-Cys(Acm)-Gly-NH_2 treated with liquid HF. 215 mm (150 × 150 DPI).

by mass spectrometry, the expected H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 at t_r 16 min and 1,4-diazepine-2,5-dione derivative of -Cys(Acm)-Gly-NH_2 with the same retention time as that identified already by NMR in case of **1**. It is worth of mentioning that the ratio of the peptide and the diazepine derivative is very



Scheme 1 Ring transformation in aspartimide peptides; the rate of transformation depends on the side chains R and Q, respectively (see text).

DOI: 10.1002/psc

similar to that of H-Asp(OBzl)-Cys(Acm)-Gly-NH_2 and H-Asu-Cys(Acm)-Gly-NH_2 shown on Figure 3(F). Since in the last step of the synthesis and during the cleavage of Boc-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 from the resin there are no basic conditions, the aminosuccinyl ring must have been transformed to the diazepine ring.

Z-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 **11** was synthesized and cleaved from the resin as above and in the HPLC chromatogram of the crude product three peaks were identified by mass spectrometry, Z-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 at t_r 27 min, Z-Gly-Asu-Cys(Acm)-Gly-NH_2 (the main component) at t_r 19 min, and H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 t_r 15.5 min (indicating that the 2 h TFA treatment cleaved the benzyloxycarbonyl group in some extent). Z-Gly-Asu-Cys(Acm)-Gly-NH_2 was isolated and treated with liquid HF to use the deprotected aspartimid peptide as a control (on Figure 4(e) it can be seen that beside the aspartimide peptide the diazepine ring has also been formed).

In our last experiment the purified H-Gly-Asp(OBzl)-Cys(Acm)-Gly-NH_2 was treated with 20% piperidine/DMF in solution. It can be seen on Figure 4 with the aid of controls that the peptide is converted to the diazepine (t_r 6.3 min, a) to the aspartimide (t_r 6.8 min, e), and to the piperidide derivatives (t_r 12.8 and 14 min) simultanously. The transformation is similar to that in the course of the solid phase synthesis, albeit the ratio of the reaction products is different. The explanation for this difference may lie in conformational properties; the peptides on solid support are not as flexible as in solution and thus the arrangement of the functional groups participitating in the reaction may be more favorouble for ring than for piperidide formation on solid phase.

CONCLUSION

It was found that using aspartic acid β-benzyl ester in Fmoc-based SPPS does not only involve the risk of the formation of aspartimide peptide as it has already been well known, but also its further transformation to a 1,4-diazepine-2,5-dione-peptide derivative. The possibility of this ring transformation is dependent on two factors. One is the tendency of the aspartimide ring formation in the peptide and its readiness to turn into the piperidide, which is influenced by the amino acid following the aspartic acid, while the other factor is the sterie hindrance exhibited by the amino acid side chain preceeding the aspartic acid. Attention was drawn to the fact that under certain conditions the 1,4-diazepine-2,5-dione-peptide derivative may even be formed dominantly. This is a highly undesirable side reaction in peptide synthesis; however at the same time it provides a new way for the synthesis of certain diazepine peptide derivatives with targeted biological or pharmacological activity.

Acknowledgements

We thank Antal Csámpai for the NMR measurements and Irén Gali for the skilled technical assistance.

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