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The Hydrolysis of Primary Amide Groups in Asn/Gln-containing Peptides

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Abstract—Peptides Boc-Ala-Asn/Gln-OH and Boc-Asn/Gln-Ala-OH were saponified with barium hydroxide to corresponding Asp/Glu-containing peptides. Under the conditions of saponification, Boc-Asn-Ala-OH additionally afforded Boc-Asp-OH, isopeptide Boc-Asp(Ala)-OH, and Boc-NHSuc > Ala-OH, with the third being the key intermediate in these transformations. Boc-Asp(OMe)-Ala-OMe underwent similar transformations under treatment with diazomethane or triethylamine. Saponification with barium hydroxide was accompanied by a high epimerization of *N*-terminal amino acid residues, whereas the products of the diazomethane treatment of Boc-Asp(OMe)-Ala-OMe had a low degree of epimerization.

Key words: asparagine; aspartic acid; glutamine; glutamic acid; peptides, epimerization, rearrangement; primary amides, selective saponification

INTRODUCTION

It is well known that the three-dimensional structures of proteins and large (over 30 amino acid residues) polypeptides (i.e., their conformations in solution) are determined by noncovalent interactions: hydrogen bonding, electrostatic interactions of charged groups, and interactions with solvent molecules. Shorter biologically active peptides have less certain conformations in solution, but they are determined by the same causes. On the other hand, the biologically active structures of these peptides depend mainly upon noncovalent interactions with receptor molecules. The Asn and Gln residues are interesting, because their side chains end in amide groups and can mimic (especially in the case of Asn) the peptide backbone [1]. The geometries of Asp and Glu residues are similar to those of Asn and Gln; however, the negative charges at biological pH values help them both specifically affect the structure of biologically active peptides and bind some cations (e.g., Ca^{2+}) [1].

Therefore, a simple and efficient method for the transformation of the primary amide functions of Asn and Gln into the carboxylic functions of Asp and Glu in the composition of peptides and proteins is desirable. It can be hoped that such a transformation will provide new opportunities in the conformational studies of peptides and in the preparation of new peptide derivatives with enhanced or new biological activities.

In 1954–1957, Sondheimer and Holley [2, 3] studied the alkaline treatment of N^{α}-acyl derivatives of asparagine and glutamine esters and found that this reaction results in high yields of the corresponding cyclic imides, which can be cleaved by alkalis to mixtures of partially or fully racemic normal and iso monoamides. However, there has recently been described a successful chemical modification of the glycopeptide antibiotic eremomycin at the Asn side chain, the significant stage of which was the barium hydroxide saponification of the primary amide function of Asn residue and the formation of a biologically active Asp-derivative [4]. This prompted us to begin the study of conversions of Asn and Gln residues in peptides by the example of simple dipeptide derivatives.

RESULTS AND DISCUSSION

We chose the simplest dipeptides suitable for our purposes: Boc-Asn-Ala-OH (**IVa**), Boc-Ala-Asn-OH (**VIIIa**), Boc-Gln-Ala-OH (**XIa**), and Boc-Ala-Gln-OH (**XIVa**). They were synthesized through the coupling of the corresponding *p*-nitrophenyl ester [Boc-Asn-ONp (**I**), Boc-Ala-ONp (**V**), or Boc-Gln-ONp (**IX**)] with the appropriate amino acid derivative [TosOH · H-Ala-OBzl (**II**), HBr · H-Asn-ONb (**VI**), or TFA · H-Gln-OBzl (**XII**)] in DMF in the presence of triethylamine and HOBt (1 equiv each) followed by hydrogenolysis of the benzyl protecting group (see the Experimental section and Table 1). The corresponding methyl esters (**IVb**), (**VIIIb**), (**XIb**), and (**XIVb**) were prepared by diazomethane treatment of the carboxyl unprotected peptides.

Peptides (**IVa**), (**VIIIa**), (**XIa**), and (**XIVa**) were subjected to saponification with a saturated (i.e., approximately 0.2 M) aqueous solution of barium

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Table 1. The synthesis of N^{α}-Boc-protected dipeptides

Starting amino acid or active ester	Starting amino component	Reaction product	Yield, %	Mp, °C	R_f or *RT*	EI MS, *m/z* (*I*, %)
Boc-Asn-OnNp (**I**)	TsOH · H-Ala-OBzl (**II**)	Boc-Asn-Ala-OBzl (**III**)	73	142–143	0.37[a]	394 (1) $[M + H]^+$, 340 (5.5), 203 (21), 189 (55)
(**III**)	H_2/Pd	Boc-Asn-Ala-OH (**IVa**)	93	190–191 (decomp.)	0.48[b]	
(**IVa**)	CH_2N_2	Boc-Asn-Ala-OMe (**IVb**)	~100	Ï‡ÒÎÓ	4.38 min[c]	320 (10.7) $[M + 3H]^+$, 303 (3.5), 264 (56), 229 (53), 184 (57), 131 (100)
Boc-Ala-ONp (**V**)	HBr · H-Asn-ONb (**VI**)	Boc-Ala-Asn-ONb (**VII**)	84	143–145	0.30[a]	440 (5.6) $[M + 2H]^+$, 384 (13), 366 (11), 282 (11), 233 (63), 214 (81), 144 (93), 136 (83)
(**VII**)	H_2/Pd	Boc-Ala-Asn-OH (**VIIIa**)[d]	77	Amorphous solid	0.33[b]	
(**VIIIa**)	CH_2N_2	Boc-Ala-Asn-OMe (**VIIIb**)	~100	oil	3.84 min[c]	320 (3.9) $[M + 3H]^+$, 303 (24), 300 (10), 278 (29), 246 (54), 227 (86), 200 (28), 144 (85), 127 (69)
Boc-Gln-ONp (**IX**)	TsOH · H-Ala-OBzl (**II**)	Boc-Gln-Ala-OBzl (**X**)	61	132–134.5	0.40[a]	408 (5.5) $[M + H]^+$, 353 (33), 336 (9), 218 (14), 202 (50), 201 (41), 145 (67), 101 (83)
(**X**)	H_2/Pd	Boc-Gln-Ala-OH (**XIa**)	~100	Amorphous solid	0.51[b]	
(**XIa**)	CH_2N_2	Boc-Gln-Ala-OMe (**XIb**)	~100	oil	4.52 min[c]	334 (13) $[M + 3H]^+$, 280 (83), 262 (45), 244 (53), 218 (39), 202 (83), 181 (74), 145 (93)
Boc-Ala-ONp (**V**)	TFA · H-Gln-OBzl (**XII**)	Boc-Ala-Gln-OBzl (**XIII**)	64	136–138	0.22[a]	408 (29) $[M + H]^+$, 352 (80), 336 (69), 310 (32), 294 (22), 277 (38), 267 (99), 264 (39)
(**XIII**)	H_2/Pd	Boc-Ala-Gln-OH (**XIVa**)	~100	Amorphous solid	0.48[b]	
(**XIVa**)	CH_2N_2	Boc-Ala-Gln-OMe (**XIVb**)	~100	oil	3.84 min[c]	334 (6.9) $[M + 3H]^+$, 280 (28), 262 (41), 244 (21), 215 (24), 202 (47), 187 (100), 144 (75)
Boc-Glu(OBzl)-OH · DCHA (**XV**)	TsOH · (H-Ala-OBzl (**II**)	Boc-Glu(OBzl)-Ala-OBzl (**XVI**)	83	oil	0.55[e]	500 (56) $[M + 2H]^+$, 499 (47) $[M + H]^+$, 498 (52) $[M]^+$, 441 (78), 425 (51), 400 (29), 296 (76), 268 (50), 252 (52), 238 (76), 192 (93)
(**XVI**)	H_2/Pd	Boc-Glu-Ala-OH (**XVII**)	~100	oil	0.72[b]	
(**XVII**)	CH_2N_2	Boc-Glu(OMe)-Ala-OMe (**XVIII**)	~100	oil	0.15[g] 8.41 min[c]	349 (30) $[M + 3H]^+$, 348 (31) $[M + 2H]^+$, 294 (56), 278 (58), 263 (53), 244 (55), 234 (46), 217 (72), 211 (38), 188 (46), 181 (87), 159 (73), 116 (87)
Boc-Asp(OBzl)-OH (**XIX**)	TsOH · H-Ala-OBzl (**II**)	Boc-Asp(OBzl)-Ala-OBzl (**XX**)	79	oil	0.63[e]	485 (16) $[M + H]^+$, 484 (10) $[M]^+$, 428 (52), 412 (51), 384 (4), 351 (16), 323 (82), 283 (82), 253 (34), 224 (88), 215 (62), 185 (82), 177 (100)
(**XX**)	H_2/Pd	Boc-Asp-Ala-OH (**XXI**)	~100	oil	0.56[b]	
(**XXI**)	CH_2N_2	Boc-Asp(OMe)-Ala-OMe (**XXII**)	~100	oil	0.56[f] 11.46 min[h]	336 (31) $[M + 4H]^+$, 281 (37), 263 (61), 248 (69), 236 (18), 229 (48), 218 (55), 203 (67), 199 (65), 185 (58), 173 (47), 167 (60), 146 (76)

[a] Chromatographic system: 47 : 3 $CHCl_3$–MeOH. [b] Chromatographic system: 15 : 5 : 1 $CHCl_3$–90% MeOH–AcOH. [c] Elution with 50% MeOH, 0.5 ml/min. [d] This product was purified by column chromatography on a silica gel column (7.5 g) eluted with a 20 : 5 : 1 $CHCl_3$–90% MeOH–AcOH system. [e] Chromatographic system: 49 : 1 $CHCl_3$–MeOH. [f] Chromatographic system: 1 : 1 benzene–ether, double development. [g] Chromatographic system: 1 : 2 benzene–ether. [h] Elution with 40% MeOH, 0.75 ml/min.

Table 2. The saponification of dipeptides (**IVa**), (**VIIIa**), (**XIa**), and (**XIVa**) with a saturated barium hydroxide solution at 37°C followed by diazomethane treatment (experiments 1–4) and the isomerization of (**XXII**) and (**XXV**) under treatment with diazomethane or 1.5 M triethylamine (experiment 5)

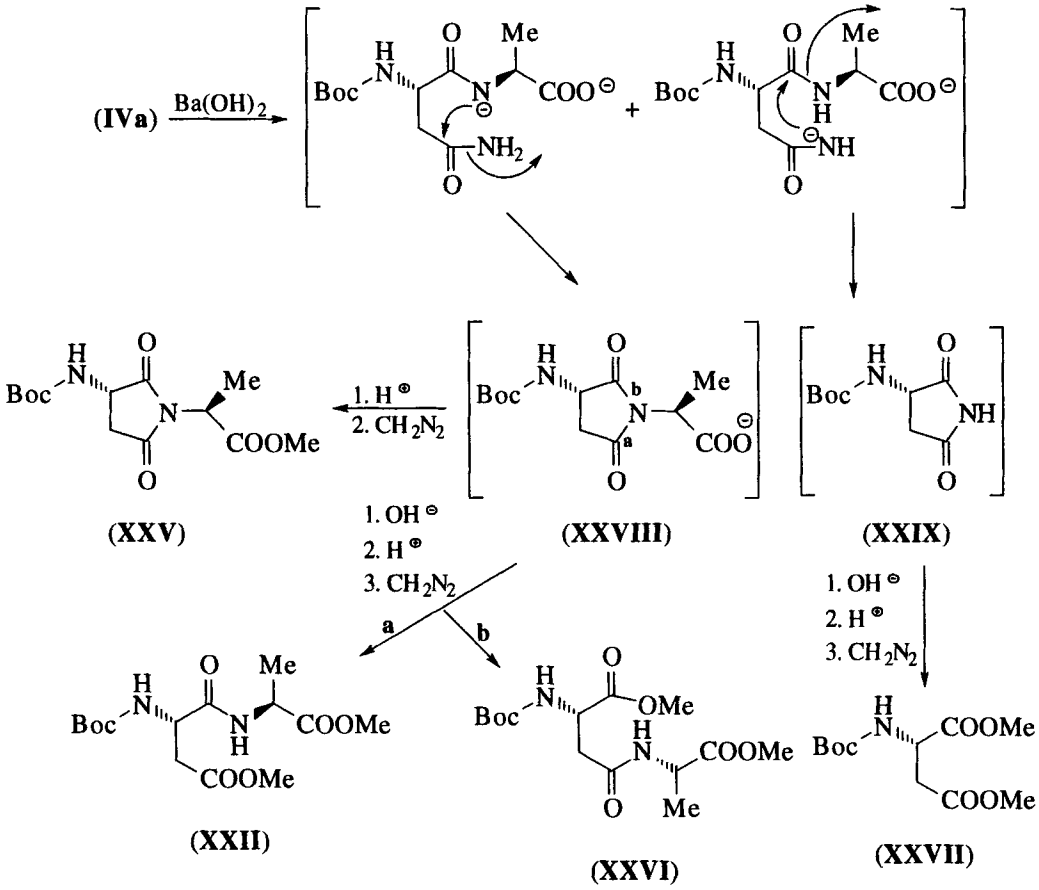
Exp. no.	Starting dipeptide	Reaction time, h	Reaction product(s)	Analysis conditions	Yield, %	Identification
1	(**VIIIa**)	96[a]	Boc-Ala-Asp(OMe)-OMe (**XXIII**)	HPLC[b], *RT* 5.95 min	59	EI MS
			Boc-Ala-Asn-OMe (**VIIIb**)		20	HPLC and EI MS
2	(**XIa**)	42	Boc-Glu(OMe)-Ala-OMe (**XVIII**)	HPLC[b], *RT* 8.41 min	64	TLC, EI MS, and the comparison with an independent sample
3	(**XIVa**)	42	Boc-Ala-Glu(OMe)-OMe (**XXIV**)	HPLC[b], *RT* 7.58 min	69	EI MS
4	(**IVa**)	40	Boc-Asp(OMe)-Ala-OMe (**XXII**)	TLC[c]	11	TLC and EI MS; the comparison with an independent sample for (**XXII**)
			Boc-NHSuc > Ala-OMe (**XXV**)		20	
			Boc-Asp(AlaOMe)-OMe (**XXVI**)		38	
			Boc-Asp(OMe)-OMe (**XXVII**)		12	
5	(**XXII**)[d, e] or (**XXV**)[e]	1 or 1.5–18	(**XXII**)	TLC[c]	47	TLC and the comparison with samples from experiment 4
			(**XXV**)		33	
			(**XXVI**)		16	

[a] After 40-h hydrolysis, the yield of (**XXIII**) was 31%, and the conversion of starting (**VIIIa**) was 50%.
[b] Separon SGX C18 glass column (7 μm, 3 × 150 mm); 50% MeOH, flow rate 0.50 ml/min; detection at 210 nm.
[c] A 3 : 2 benzene–ether system, triple development. R_f values: (**XXII**) 0.52; (**XXV**) 0.44; (**XXVI**) 0.24; and (**XXVII**) 0.80.
[d] Approximately 0.7 M CH_2N_2 in a methanol–ether mixture at 30°C.
[e] 1.5 M TEA in methanol.

hydroxide at 37°C for 40–96 h. The reaction mixtures were separated from the cations with excess Dowex 50 resin (H^+-form), and the resulting carboxyl-containing products were converted to methyl esters by the treatment with diazomethane. The esters were separated by preparative HPLC or TLC in order to measure their mass spectra and yields (see Table 2). The results of these experiments show that the dipeptides (**VIIIa**), (**XIa**), and (**XIVa**) are smoothly converted into the corresponding dipeptide derivatives of aspartic and glutamic acids (**XXIII**), (**XVIII**), (**XXIV**) (experiment nos. 1–3; the racemization degree of these products is discussed below). We observed only one reaction product in all three cases but, in order to be sure, we confirmed the structure of (**XVIII**) by a counter synthesis from Boc-Glu(OBzl)-OH · DCHA (**XV**) and TsOH · H-Ala-OBzl (**II**) (see Table 1 and the Experimental section).

However, the alkaline transformation of (**IVa**) proceeded less specifically and resulted in four products (**XXII**), (**XXV**), (**XXVI**), and (**XXVII**) (see Table 2, experiment 4, and Scheme 1). Their structures were confirmed by mass spectra and amino acid analyses; (**XXII**) was also synthesized by the coupling of Boc-Asp(OBzl)-OH (**XIX**) and TosOH · H-Ala-OBzl (**II**) followed by hydrogenolysis and diazomethane treatment (see Table 1). Interestingly, the yields of succinimide derivative (**XXV**), isopeptide (**XXVI**), and even dimethyl ester of Boc-aspartic acid (**XXVII**) exceeded the yield of normal Boc-dipeptide (**XXII**). This suggests that the reaction probably proceeds through the subtraction of protons from the secondary or primary amide groups, the splitting off of ammonia or alanine molecules, and the subsequent cyclization to intermediates (**XXVIII**) and (**XXIX**). The alkaline opening of the succinimide ring of (**XXVII**) in either position, the subsequent saponification of its amide group, and methylation result in dimethyl ester of Boc-aspartic acid (**XXVIII**) (yield 12%). The major part of (**XXVIII**) undergoes alkaline hydrolysis on bond **a** or **b** giving rise to normal peptide (**XXII**) (yield 11%) and isopeptide (**XXVI**) (yield 38%), respectively. The nonhydrolyzed (**XXVIII**) is protonated on its carboxyl anion during the process of treatment and methylated with diazomethane to form succinimide derivative (**XXV**) (yield 20%).

To gain greater insight into the mechanism of these reactions, we studied some transformations of Boc-Asp-Ala-OH (**XXI**) (see Scheme 2). The treatment of (**XXI**) with excess diazomethane in 4 : 1 ether–methanol for 1 h at 30°C resulted in a mixture of (**XXII**), (**XXV**), and (**XXVI**), and only its treatment for 3 min at 0°C led almost exclusively to (**XXII**). We also found that methanol, as the solvent, is a necessary prerequisite of this isomerization reaction, since, in ethyl acetate, a similar treatment of (**XXI**) with an ether solution of diazomethane both for 4 min at 0°C and for 1 h at 30°C led exclusively to diester (**XXII**). However, a stronger



Scheme 1. Alkaline hydrolysis of Boc-Asn-Ala-OH (**IVa**).

base, sodium hydride (3-molar excess in THF), ensured the conversion of (**XXII**) into succinimide derivative (**XXV**) in a 60% yield.

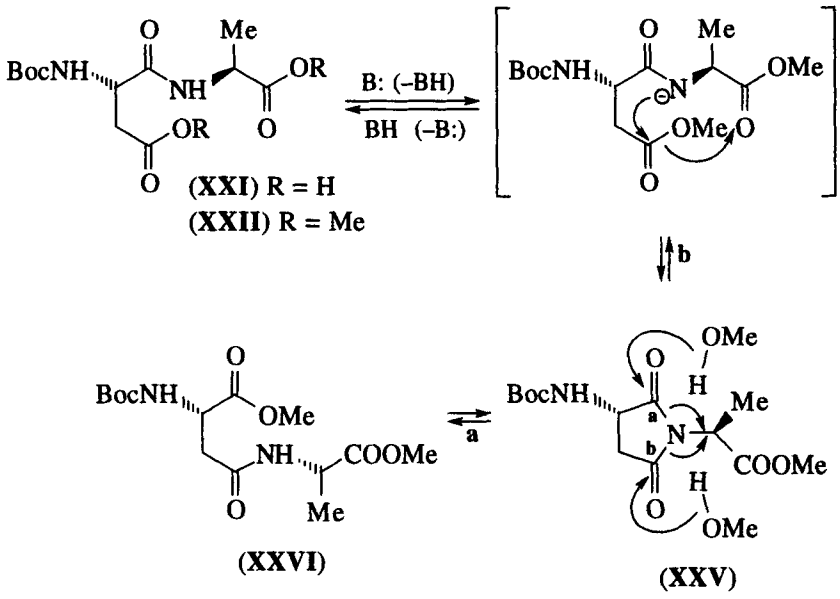
We also studied the formation of the equilibrium mixture of (**XXII**), (**XXV**), and (**XXVI**) directly from diester (**XXII**) and succinimide derivative (**XXV**) under the action of bases (see Table 2, experiment 5). Diazomethane in ethyl acetate or dimethylformamide brought about no changes in (**XXII**), whereas the action of 1.5 M triethylamine in methanol or diazomethane excess in the methanol–ether mixture led to the formation of this equilibrium mixture. The same mixture resulted from (**XXV**) under the action of 1.5 M triethylamine in methanol. A consideration of these facts allowed us to suggest the possible mechanism of these conversions presented in Scheme 2. An initial deprotonation of the amide group, for which even the weak basicity of 0.6–0.8 M diazomethane is sufficient, leads to the splitting off of a methoxyl anion and the formation of (**XXV**). The subsequent concerted addition of methanol (methoxyl) to bond **a** or **b** gives isopeptide (**XXVI**) or normal peptide (**XXII**), respectively.

The epimerization of the α-chiral center of amino acid residues is one of the most serious problems in peptide chemistry. It was considered in detail in the review by Kemp [5], who compiled the results of its study with various coupling methods used in peptide synthesis. However, the methodology of the analysis of chiral purity of amino acid residues in peptides described in this review underwent a revolutionary change after the introduction of capillary Chirasil–Val columns, which can separate *D*- and *L*-epimers of almost all common amino acids in one gas-chromatographic run (cf., e.g., [6, 7]).

In accordance with this technique, we studied the racemization degree of amino acid residues in our peptides after their conventional acidic hydrolysis (6 N HCl, 24 h, 106°C). The free amino acids in the hydrolysates were transformed into *N*-trifluoroacetyl derivatives of methyl esters before gas chromatography. The racemization degree (*R*) of an amino acid residue was calculated according to the formula

$$R = 200A_D/(A_D + A_L), \%,$$

where A_D and A_L are the areas of the chromatographic peaks of *D*- and *L*-enantiomers of the corresponding amino acid derivative.



Scheme 2. Isomerization of (**XXII**) and (**XXV**) with bases.

One can see from Table 3 that the synthetic peptides (**IVa**), (**VIIIa**), (**XIa**), and (**XIVa**) (experiment nos. 2, 5, 7, and 9) had a sufficiently low racemization degree and their treatment with diazomethane (cf., e.g., experiment 3) left the racemization practically unaffected. However, in complete accordance with [5], the alkaline treatment of these peptides resulted in a high epimerization of their *N*-terminal residues while affecting their *C*-terminal residues significantly less. The racemization degree of *N*-terminal residues was especially high (up to 46–54%) in the Asp-containing saponification products of (**IVa**) and (**XXIII**) (experiments 1 and 4), whereas the saponification products of Gln-containing peptides (**XVIII**) and (**XXIV**) had a less pronounced racemization degree (15–22%, experiments 6 and 8). Note also that the *C*-terminal residues were also affected to a greater degree in Asn-containing than in Gln-containing peptides (cf. experiments 1 and 4 with experiments 6 and 8).

CONCLUSIONS

We found that the Asn/Gln-containing dipeptides can be hydrolyzed by barium hydroxide to the corresponding Asp/Glu-containing dipeptides. However, both amino acid residues in the resulting dipeptides

Table 3. The racemization degree (*R*, %) of amino acid residues in peptides (for the conditions of gas chromatography, see the Experimental section)

Exp. no.	Peptide	Ala[a]	Asp[b]	Glu[c]
1	The Ba(OH)2-hydrolysis products of Boc-Asn-Ala-OH (**IVa**)	3.0	54.0	–
2	Boc-Asn-Ala-OH (**IVa**)	2.4	5.7	–
3	The products of rigorous CH_2N_2-treatment of Boc-Asp(OMe)-Ala-OMe (**XXII**)[d]	2.1	6.2	–
4	Boc-Ala-Asp(OMe)-OMe (**XXIII**)[e]	46	10	–
5	Boc-Ala-Asn-OH (**VIIIa**)	2.2	4.7	–
6	Boc-Glu(OMe)-Ala-OMe (**XVIII**)[e]	2.1	–	21.9
7	Boc-Gln-Ala-OH (**XIa**)	2.0	–	2.9
8	Boc-Ala-Glu(OMe)-OMe (**XXIV**)[e]	14.9	–	4.5
9	Boc-Ala-Gln-OH (**XIVa**)	1.6	–	3.1

[a] *RT* 7.56 min (*D*) and 8.27 min (*L*).
[b] *RT* 16.86 min (*D*) and 17.09 min (*L*).
[c] *RT* 20.92 min (*D*) and 21.42 min (*L*).
[d] 0.8 M CH_2N_2 in 4 : 1 ether–methanol at 30°C for 1 h.
[e] Obtained by the $Ba(OH)_2$-hydrolysis and subsequent methylation with diazomethane (see Table 2).

become partially racemized. The racemization degree is especially high in the case of Asn-containing peptides. When Asn is *N*-terminal, the saponification reaction is accompanied by the formation of a succinimide derivative, which undergoes subsequent cleavage of the peptide chain to give Asp-containing isodipeptide. Nevertheless, the barium hydroxide saponification of Gln-containing peptides seems to be promising for their modification in some specific cases. The same treatment of Asn-containing peptides is unlikely to prove useful, but, in this case, our results reveal another possibility: the isomerization of normal Asp-Xaa-containing peptides to Asp(Xaa)-isopeptides upon the action of bases; this might be interesting in some specific cases.

EXPERIMENTAL

Boc-Asn-ONp (**I**), Boc-Ala-ONp (**V**), Asn-ONb hydrobromide (**VI**), Boc-Gln-ONp (**IX**), and Boc-Glu(OBzl)-OH dicyclohexylamine salt (**XV**) were from Reanal (Hungary) and Ala-OBzl *p*-toluenesufonate (**II**) was from NPO IREA (Russia).

Melting points were measured on a Boetius hot plate (Germany) and were not corrected. Silica gel 60 (40–63 μm, Merck) was used for preparative column chromatography. TLC was performed on sheets precoated with Silica gel 60 F_{254} (Merck). Spots of substances were visualized under UV light or by spraying with 0.8% $KMnO_4$, conc. H_2SO_4, or a standard ninhydrin solution. For HPLC, a Pye Unicam modular instrument consisting of an LC-XPD pump, an LC-UV detector, and Rheodyne injector was used. HPLC column was a glass Separon SGX C18 (7 μm, 3 × 150 mm, Tessek, Czech Republic) eluted with 50% methanol with a rate of 0.50 ml/min; detection was at 210 nm.

A Carlo Erba 5360 capillary gas chromatograph equipped with a flame-ionization detector, cold-on-column injector, and Chirasil-Val fused silica column (film thickness 0.15 μm, internal diameter 0.3 mm, and length 50 m) was used for GC. The carrier gas was hydrogen at 105 kPa inlet pressure, and the temperature program was linear from 50 to 200°C at a rate of 5°C/min.

Electron impact mass spectra (EI MS, 70 eV) were obtained on a Varian MAT 44 mass spectrometer at the direct inlet of samples into the ion source. Plasma desorption mass spectra (PD MS) were measured on a spectrometer from Sumy (Ukraine) under ionization by the ^{252}Cf scission products.

Glutamine *O*-benzyl ester trifluoroacetate (XII). Boc-Gln-OH obtained from glutamine as described in [8] (mp 119–120°C, lit. mp 116–118°C [9]) was transformed into Boc-Gln-OBzl (mp 110–112°C, lit. mp 109–110°C [10]) as described in [10]. This derivative (1.4 g, 4.17 mmol) was treated with trifluoroacetic acid (11 ml) for 30 min at 24°C. The reaction mixture was evaporated, and the residue was crystallized at –12°C. The crystals were triturated with diethyl ether and filtered to give (**XII**), yield 1.097 g (75%); mp 105.5–107°C; R_f 0.20 (20 : 5 : 1 chloroform–90% methanol–acetic acid); PD MS, *m/z*: 237 $[M + H]^+$ (here, *M* is the molecular mass of Gln-OBzl).

Preparation of N^{α}-Boc-protected dipeptides from amino acid *p*-nitrophenyl esters (general procedure). A Boc-derivative of *p*-nitrophenyl ester of alanine, asparagine, or glutamine (0.55 mmol) and a *p*-toluenesulfonate, trifluoroacetate, or hydrobromide of amino acid benzyl or *p*-nitrobenzyl ester (0.50 mmol) (see Table 1) were dissolved in anhydrous DMF (2 ml) and treated with triethylamine (0.50 mmol) and HOBt (0.50 mmol). The reaction mixture was kept for 24 h at room temperature, evaporated, and dissolved in 1 : 1 ethyl ether–ethyl acetate mixture. The solution was successively washed with water, 1 N HCl, a saturated solution of $NaHCO_3$, and water; dried with sodium sulfate; and evaporated. The residue was crystallized from diethyl ether, and the crystals were filtered, washed with diethyl ether, and dried in a vacuum. The resulting *N*-Boc-protected benzyl or *p*-nitrophenyl ester (**III**), (**VII**), (**X**), or (**XIII**) (0.25 mmol), which contained no admixture of *p*-nitrophenol, was dissolved in mathanol (2 ml) and added to 10% PdO/C catalyst (37 mg) preliminarily hydrogenated in methanol (3 ml) under stirring on a magnetic stirrer at room temperature and atmospheric pressure for 1 h. The hydrogenolysis of the dipeptide was continued for 3 h, the catalyst was filtered off and washed with methanol, and the combined filtrates were evaporated to give the corresponding chromatographically pure dipeptide (**IVa**), (**VIIIa**), (**XIa**), or (**XIVa**). These were also characterized as methyl esters (**IVb**), (**VIIIb**), (**XIb**), or (**XIVb**) obtained by the treatment with 0.6–0.8 M diazomethane in diethyl ether of a methanolic solutions of the dipeptides for 1 h at 30°C (see Table 1).

Boc-Asp(OBzl)-Ala-OBzl (XX). Boc-Asp(OBzl)-OH (**XIX**) (161.5 mg, 0.5 mmol; prepared according to the procedure in [9]) and TosOH · H-Ala-OBzl (**II**) (175.5 mg, 0.5 mmol) were dissolved in anhydrous DMF (2 ml) and treated with triethylamine (50.5 mg, 0.5 mmol), HOBt (67.5 mg, 0.5 mmol), and DCC (113 mg, 0.5 mmol) at 0°C. The reaction mixture was slowly heated to 30°C, kept for 3 h at this temperature and for 16 h at 5°C, and filtered from the dicyclohexylurea precipitate. The precipitate was washed with ethyl acetate, and the combined filtrates were evaporated to a small volume and diluted with a 1 : 1 diethyl ether–ethyl acetate mixture. This solution was washed with 1 N HCl, water, saturated $NaHCO_3$, and water, dried with sodium sulfate, and evaporated. The residue was chromatographed on a silica gel column (8 g) eluted with a 1 : 49 methanol–chloroform mixture. The yield of (**XX**) was 191.5 mg (79%); see Table 1 for its characteristics.

Boc-Asp-Ala-OH (XXI) and Boc-Asp(OMe)-Ala-OMe (XXII) were obtained by hydrogenolysis of

(**XX**) followed by treatment of the intermediate (**XXI**) with diazomethane for 3 min at 0°C in a 4 : 1 ether–methanol mixture or for 4 min at 0°C in ethyl acetate according to the procedures described above.

Boc-Glu(OBzl)-Ala-OBzl (XVI), Boc-Glu-Ala-OH (XVII), and Boc-Glu(OMe)-Ala-OMe (XVIII). Boc-Glu(OBzl)-OH dicyclohexylamine salt (**XV**) (259.4 mg, 0.5 mmol) was suspended in ethyl acetate (3 ml) and shaken with 1 N $NaHSO_4$ (0.6 ml) until the complete disappearance of the crystals. The aqueous layer was separated and extracted twice with ethyl acetate. The combined organic solution was washed with water, dried with anhydrous sodium sulfate, and evaporated. The resulting oily Boc-Glu(OBzl)-OH (yield 91%) was coupled with (**II**) as described above. The resulting (**XVI**) was hydrogenolysed to (**XVII**) and then methylated with diazomethane (for 3 min at 0°C or for 1 h at 30°C in a 4 : 1 ether–methanol mixture) to give (**XVIII**) (see Table 1).

Saponification of peptides with barium hydroxide. A solution of dipeptide (**IVa**), (**VIIIa**), (**XIa**), or (**XIVa**) (10 μmol) in a saturated solution of barium hydroxide (0.3 ml) was kept at 37°C for the time specified in Table 2 and then treated with Dowex 50 (H^+) at room temperature to adjust the pH value to approximately 2–3. The resin was filtered off and washed with distilled water and ethanol, and the combined filtrate was evaporated in a vacuum. The residue was dissolved in methanol (200 μl), the solution was treated with excess diazomethane (800 μl of ~0.6–0.8 M ethereal solution) and kept for 3 min at 0°C in the case of (**IVa**) or for 1 h at 30°C in the case of (**VIIIa**), (**XIa**), or (**XIVa**). The reaction products were analyzed, separated by preparative TLC or HPLC, and identified as described in Table 2:

Boc-Ala-Asp(OMe)-OMe (XXIII); HPLC: *RT* 5.95 min; EI MS, *m/z* (*I*, %): 336 (13) $[M + 4H]^+$, 335 (25) $[M + 3H]^+$, 334 (28) $[M + 2H]^+$, 333 (28) $[M + H]^+$, 332 (28) $[M]^+$, 305 (27), 302 (27), 288 (33), 280 (40), 263 (68), 242 (32), 239 (100), 237 (64), 203 (6), 160 (12), 144 (35), 128 (12), 113 (14).

Boc-Glu(OMe)-Ala-OMe (XVIII); HPLC: *RT* 8.41 min; TLC: R_f 0.15 (1 : 2 benzene–ether); EI MS, *m/z* (*I*, %): 349 (30) $[M + 3H]^+$, 348 (31) $[M + 2H]^+$, 294 (56), 278 (58), 263 (53), 244 (55), 234 (46), 217 (72), 211 (38), 188 (46), 181 (87), 159 (73), 116 (87).

Boc-Ala-Glu(OMe)-OMe (XXIV); oil; HPLC: *RT* 7.58 min; EI MS, *m/z* (*I*, %): 348 (6.3) $[M + 2H]^+$, 347 (2.1) $[M + H]^+$, 346 (1.0) $[M]^+$, 295 (60), 278 (79), 264 (51), 244 (24), 234 (37), 217 (59), 215 (51), 203 (68), 187 (38), 181 (44), 174 (70), 160 (21), 144 (84), 142 (87).

Boc-Asp(OMe)-Ala-OMe (XXII); oil; HPLC (40% methanol, 0.75 ml/min): *RT* 11.46 min; TLC: R_f 0.56 (1 : 1 benzene–ether, double development), R_f 0.52 (3 : 2 benzene–ether, triple development); EI MS, *m/z* (*I*, %): 336 (31) $[M + 4H]^+$, 281 (37), 263 (61), 248 (69), 236 (21), 229 (48), 218 (55), 203 (67), 199 (65), 185 (58), 173 (47), 167 (60), 146 (76).

Boc-NHSuc>Ala-OMe (XXV); oil; TLC: R_f 0.63 (1 : 1 benzene–ether, double development), R_f 0.44 (3 : 2 benzene–ether, triple development); EI MS, *m/z* (*I*, %): 304 (28) $[M + 4H]^+$, 303 (14) $[M + 3H]^+$, 274 (4), 260 (8), 244 (74), 229 (84), 214 (77), 201 (71), 195 (71), 185 (79), 172 (47), 167 (83), 140 (72).

Boc-Asp(AlaOMe)-OMe (XXVI); oil; TLC: R_f 0.33 (1 : 1 benzene–ether, double development), R_f 0.24 (3 : 2 benzene–ether, triple development); EI MS, *m/z* (*I*, %): 335 (21) $[M + 3H]^+$, 334 (14) $[M + 2H]^+$, 304 (6), 278 (99), 263 (94), 248 (77), 229 (69), 219 (83), 200 (67), 185 (57), 173 (96), 167 (61), 145 (71).

Boc-Asp(OMe)-OMe (XXVII); oil; TLC: R_f 0.80 (3 : 2 benzene–ether, triple development); EI MS, *m/z* (*I*, %): 204 (23) $[M - Bu^t]^+$, 203 (26) $[M - COOCH_2]^+$, 189 (7), 174 (13), 160 (25), 146 (52), 128 (12), 113 (12), 103 (100).

Transformations of (XXII). a. Treatment with diazomethane. Approximately 0.7 M ethereal solution of diazomethane (0.8 ml) was added to a solution of (**XXII**) (3.32 mg, 10 μmol) in methanol (0.2 ml). The mixture was kept for 1 h at 30°C and evaporated. The residue was separated by preparative TLC in a 3 : 2 benzene–ether system. Starting (**XXII**), (1.56 mg, 47%), succinimide derivative (**XXV**) (0.99 mg, 33%), and isopeptide (**XXVI**) (0.54 mg, 16%) were isolated from zones with R_f 0.52, 0.44, and 0.24, respectively.

b. Treatment with triethylamine. Triethylamine (29 mg, 0.29 mmol) was added to a solution of (**XXII**) (3.32 mg, 10 μmol) in methanol (0.2 ml), and the mixture was kept for 1.5 h at 30°C. The same product mixture as in experiment **a** was obtained. When carrying out the reaction in ethyl acetate or DMF, no conversions of the starting (**XXV**) were observed.

c. Treatment with sodium hydride. A 75% suspension of sodium hydride in paraffin oil (1.0 mg) was added to a solution of (**XXII**) (3.32 mg, 10 μmol) in anhydrous THF (0.4 ml) and the mixture was stirred for 1 h at 30°C and quenched with acetic acid (20 μl). The major reaction product, succinimide derivative (**XXV**), was isolated by preparative TLC in a yield of 1.82 mg (60%).

Transformations of (XXV). A solution of (**XXV**) (1.5 mg, 5 μmol) in methanol (0.1 ml) was treated with triethylamine under the conditions of experiment **b**. The resulting product mixture was the same as described in experiments **a** and **b**. When the duration of the reaction increased to 18 h, the product ratio did not change.

Acidic hydrolysis of peptides and preparation of samples for GC. Peptide samples were hydrolyzed with 6 N HCl in sealed evacuated ampules for 24 h at 106°C, and the hydrolysates were evaporated in a vacuum. The residue was successively treated with 4 M

HCl in methanol (2 h at 60°C) and a 10% solution of trifluoroacetic anhydride in chloroform (15 min at room temperature). The samples were then analyzed by GC as described above.

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