THERMAL STABILITY OF VASOPRESSIN-(7-9)-, AND OXYTOCIN-(7-9)-TRIPEPTIDE AMIDES*

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The heating of vasopressin-(7-9)-and oxytocin-(7-9)-tripeptide amides at 200°C leads to quick pyrolysis. The oxytocin tripeptide amide is considerably more stable than the analogous vasopressin peptides. The presence of c(Pro-Leu)** and c(Gly-Gly) was demonstrated by mass spectrometry and chemical methods in the reaction mixture after the pyrolysis of oxytocin-(7-9)-tripeptide amide. Mass spectrometry indicated the presence of c(Pro-Gly) in the reaction mixture. The N^{α},N^{ω}-dicapryl derivatives are suitable for mass spectrometry of vasopressin-(7-9)-tripeptide amides.

We have met several times earlier with the lability of vasopressin-(7-9)-tripeptide amides. The necessity of obtaining a more profound information on this phenomenon emerged both from our synthetic work and also from the fact that the properties of these tripeptide amides can contribute to the greater lability of the vasopressins compared to oxytocin. This necessity, however, became acute after prolyl-N^{ε}-benzyloxycarbonyl-D-lysyl-glycine amide (crystalline, well characterized, and uniform when subjected to thin-layer chromatography and electrophoresis) had afforded a mixture of two products after several months' storage over phosphorus pentoxide at room temperature. An analogous oxytocin peptide, stored in a common reagent wide mouth bottle under identical conditions, remained unaltered for several years. This paper reports studies on the behavior of tripeptide amides of the above described type at elevated temperature.

Three pairs of diastereoisomeric vasopressin tripeptide amides and oxytocin-(7-9)-tripeptide amide[1] were available for our study. All the vasopressin peptides contained 1/2 of molecule of crystal water (similarly to oxytocin tripeptide amide), were well characterized and uniform when subjected to thin-layer chromatography and paper electrophoresis. Their fundamental characteristics are given in Table I.

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** The nomenclature and abbreviations are identical with those used in the preceding paper. Unless stated otherwise, the amino acids used are of the L-series.

The fastest method of obtaining basic information on the behavior of these products at elevated temperatures was mass spectrometry. The first results were not encouraging. The molecular peak was missing in the spectrum of H-Pro-Lys(Z)-Gly-NH_2, the spectrum itself was not clear and difficult for interpretation (Fig. 1). This result was rather unexpected as the mass spectrometry of benzyloxycarbonyl peptides is known[2,3] and H-Pro-Leu-Gly-NH_2 gave a spectrum which could be interpreted

TABLE I

Vasopressin-(7-9) Tripeptide Amides H-Pro-X-Gly-NH_2

All peptides were prepared by stepwise synthesis from the carboxyl terminus. The syntheses will be described in the forthcoming communication.

X	L enantiomer		D enantiomer	
	m.p., °C	$[\alpha]_D^{25}$ c[a]	m.p., °C	$[\alpha]_D^{25}$ c[a]
Lys	70—73	−33·2° (0·85)	72—73	−11·9° (1·0)
Orn	92—97	−26·02° (1·0)	59—60	−12·34° (1·0)
Dab	95—100	−25·5° (0·95)	157—158	−13·6° (0·9)

[a] In methanol.

TABLE II

Dicapryl Derivatives of Vasopressin-(7—9) Tripeptides Cap-Pro-X(Cap)-GlyNH_2

X	Formula (mol. wt.)	M.p., °C	$[\alpha]_D^{25}$ (c 0·2 in methanol)	Calculated/Found		
				% C	% H	% N
D-Lys	$C_{29}H_{53}N_5O_5$	112—115	−31·2°	63·13	9·68	12·69
	(551·8)	139—140[a]		62·90	9·74	12·61
Lys		177—178	−64·1°	62·95	9·79	12·57
D-Orn	$C_{28}H_{51}N_5O_5$	115—118	−27·6°	62·54	9·56	13·02
	(537·7)	145—147[a]		62·37	9·59	12·88
Orn		182—183	−65·8°	62·38	9·47	12·93
D-Dab	$C_{27}H_{49}N_5O_5$	113—115	−11·1°	62·05	9·43	13·37
	(523·7)	179—179·5[a]		62·29	9·34	13·47
Dab		188—189	−62·9°	62·06	9·23	13·38

[a] The product melts at the lower temperature, then solidifies, and melts again.

easily (Fig. 2). In our subsequent work we followed two lines. We made a search for such derivatives of our tripeptides which would yield a molecular peak and would be suitable for rapid mass spectrometric identification at the same time. Further, we carried out chromatographic analysis of the reaction mixture after pyrolysis of oxytocin-(7-9)-tripeptide amide in order to identify chemically 2,5-piperazinediones whose presence was indicated by the mass spectrum of H-Pro-Leu-Gly-NH_2 (Fig. 2). We carried out the pyrolysis at the temperature of the ionic source of the mass spectrometer.

Permethylation[4] of H-Pro-D-Lys(Z)-Gly-NH_2 afforded a product whose spectrum showed the presence of a molecular peak. The latter was also found in the spectrum of Cap-Pro-D-Lys(Ac)-Gly-NH_2* (yet not in the spectrum of Cap-Pro-D-Lys(Z)-Gly-NH_2). This demonstrates that it is the benzyloxycarbonyl residue in ω-position which makes impossible the direct measurement of mass spectra of vasopressin tripeptide amides. The products of permethylation and of caprylation and acetylation,

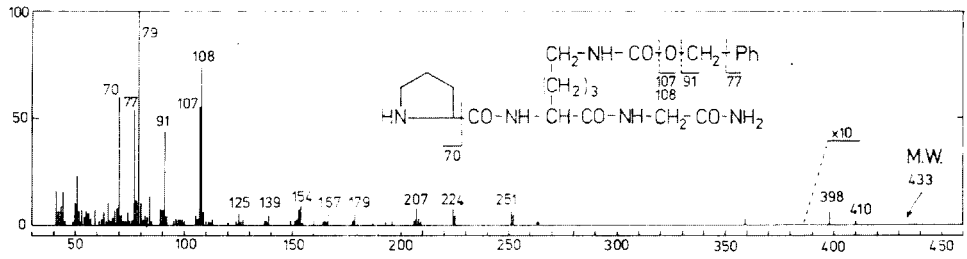

FIG. 1

Mass Spectrum of Prolyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-glycine Amide

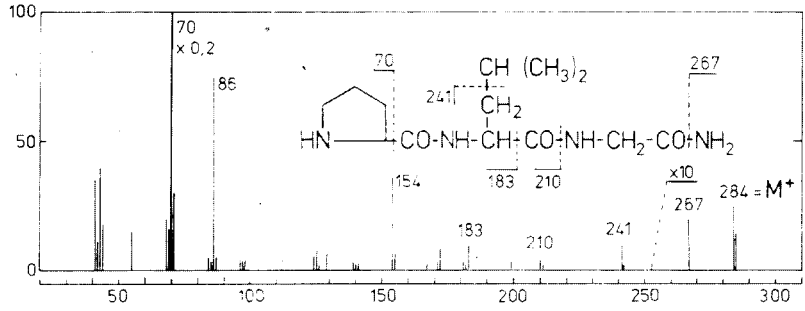

FIG. 2

Mass Spectrum of Prolyl-leucyl-glycine Amide

* Cap: $C_7H_{15}CO$—; Ac: CH_3CO—

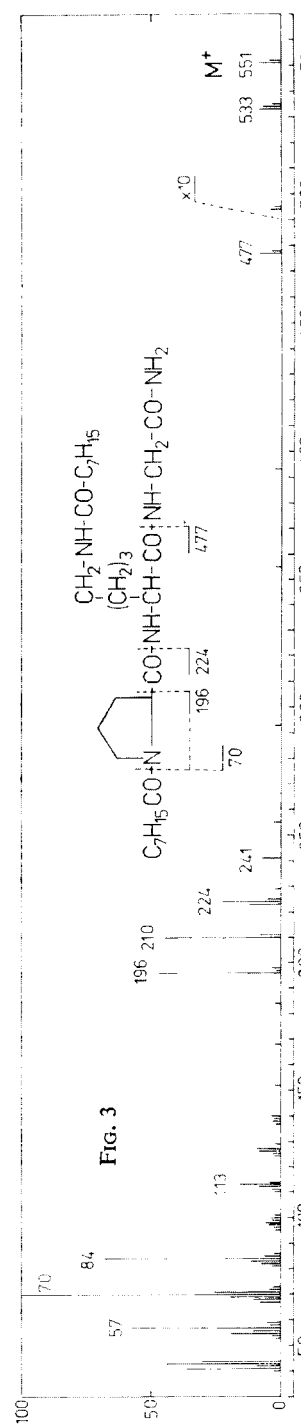
100
50
0
70
84
57
113
50
100
150
196 210
224
241
200
250
300
350
400
450
500
550
M+
551
533
x10
477
CH2-NH-CO-C7H15
(CH2)3
C7H15 CO-N
CO-NH-CH-CO-NH-CH2-CO-NH2
196 224
70
477

FIG. 3

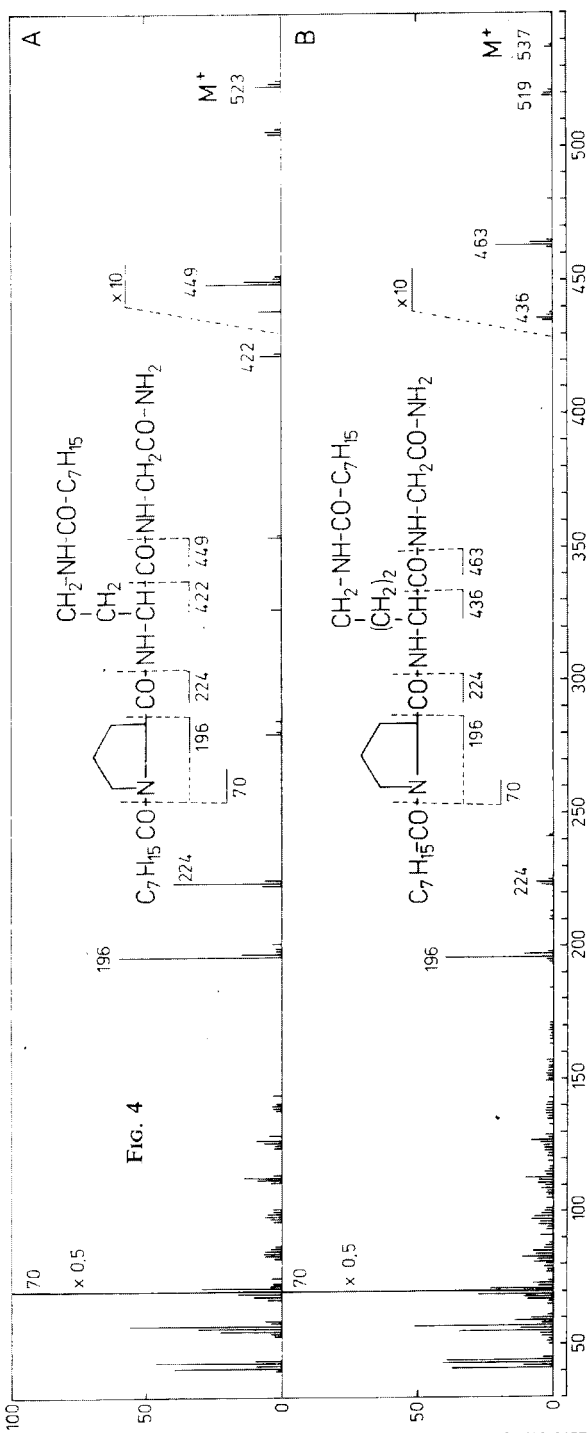
A
B
100
50
0
70
x 0,5
50
100
150
196
200
250
300
350
400
450
500
M+
523
519
537
x10
449
422
463
436
CH2-NH-CO-C7H15
CH2
(CH2)2
C7H15 CO-N
CO-NH-CH-CO-NH-CH2CO-NH2
224
196
70
422 449
436 463

FIG. 4

respectively, gave molecular peaks in their spectra but they were not suitable for the characterization of vasopressin tripeptide amides. N^{α}, N^{ω}-dicapryl derivatives, whose preparation is easy and quick (Table II), were found to be more appropriate. They afforded spectra which could be interpreted without difficulties (Fig. 3, 4).

After H-Pro-Leu-Gly-NH_2 had been heated at 200°C for one hour, the mass spectrum of the reaction mixture showed peaks corresponding particularly to c(Gly-Gly). The agreement of our finding with the reported[5,6] spectrum of c(Gly-Gly) is so convincing that we regard the presence of c(Gly-Gly) in the pyrolysate as proved. The thin-layer chromatography of the reaction mixture indicated the mixture of 4 products. The pyrolysate was resolved by partition chromatography on silica gel. The course of the separation was checked by thin-layer chromatography. The fastest fraction (A) (the fractions are designated A – D in order of decreasing mobility) contained c(Pro-Leu) (ref.[6]) according to mass spectrometry. This finding was in agreement with the amino acid composition of the fraction. Fraction B was ninhydrin-positive and showed the same mobility as the starting product when subjected to thin-layer chromatography. The mass spectrum of fraction C contained all the main products characteristic of c(Pro-Gly) and moreover peak *m/e* 86. The differences in the intensity ratios between the spectrum of fraction C and the spectrum of authentic c(Pro-Gly) (ref.[3,8]), together with the presence of peak *m/e* 86, are in accordance with the assumption that this spectrum is a result of superposition of mass spectra of c(Pro-Gly) and c(Pro-Leu) which contributes especially to the intensities of peaks *m/e* 70, 86, and 154. This assumption is supported also by the amino-acid composition of fraction C which shows, in addition to glycine (~70%), proline (~15%) and leucine (~15%). Fraction D, which was ninhydrin positive, corresponded to glycine amide by its mobility on thin-layer chromatography.

We made an effort to purify further fraction A and C by sublimation *in vacuo*. The product obtained by sublimation of the former fraction gave a mass spectrum which was identical with the spectrum of pure c(Pro-Leu) (ref.[6]) and an amino acid analysis corresponding to this 2,5-piperazinedione. We were not able to purify completely fraction C by sublimation. Even though the glycine content increased to 96%, the product still contained c. 2% of proline and 2% of leucine. The mass spectrum of fraction C (at a temperature of the ionic source equal to 170°C) corresponded to the spectrum of c(Pro-Gly) containing a small amount of c(Pro-Leu). A spectrum corresponding to c(Gly-Gly) and containing a little of c(Pro-Leu) was obtained when the

FIG. 3
Mass Spectrum of Caprylprolyl-N^{ε}-capryllysyl-glycine Amide

FIG. 4
Mass Spectrum of Caprylprolyl-N^{γ}-caprylα,γ-diaminobutyryl-glycine Amides (A) and its Ornithine Homologue (B)

temperature of the ionic source was 200°C. Hence, fraction C is a mixture of c(Gly--Gly), c(Pro-Gly), and c(Pro-Leu) in which c(Gly-Gly) is prevalent.

The vasopressin-(7-9)-tripeptide amides are distinctly more labile than the corresponding oxytocin tripeptides. This follows from our experience with their storage, from their mass spectrometric behavior, and from the results of their pyrolysis. The starting product practically disappeared from the reaction mixture after 40-min heating at 200°C of the vasopressin tripeptide amides; a considerable quantity of the starting product was present in the reaction mixture even after 1 h of heating of the oxytocin tripeptide.

The pyrolysis of H-Pro-Leu-Gly-NH_2 afforded three 2,5-piperazinediones, c(Pro--Leu), c(Pro-Gly), and c(Gly-Gly). The formation of 2,5-piperazinediones of the first two amino terminal acids of the peptide chain is well known[7-9]. Similarly, the rise of c(Gly-Gly) from glycine amide split off in the process of pyrolysis is expectable. The appearance of c(Pro-Gly) is less expectable and it is not obvious how it is formed.

EXPERIMENTAL

The mass spectra were measured on AEI-MS 902 spectrometer at 70 eV using a direct inlet system. The optical activity was determined both in an objective polarimeter (Polamat A, Zeiss, Jena, GDR) and in the ORD/UV5 (Jasco, Japan) apparatus. The melting points were measured on a Kofler block and are not corrected. Thin-layer chromatography was carried out on silica gel coated aluminum sheets (Silufol, Kavalier) in n-butanol–acetic acid–water (4 : 1 : 1, system A; 4 : 1 : 5, system B), and in n-butanol–formic acid–water (5 : 1 : 1, system C). The products to be analyzed were dried 8 h at 100°C and 0·1 Torr over phosphorus pentoxide.

Permethylation of Prolyl-N^{ε}-benzyloxycarbonyl-D-lysyl-glycine Amide

The reaction was carried out essentially as described in paper[4]. A solution of 43 mg of prolyl--N^{ε}-benzyloxycarbonyl-D-lysyl-glycine amide in 3 ml of dimethylformamide was treated with 1·5 g of Ag_2O and 2·5 ml of CH_3I. The mixture was stirred 3 days at room temperature, then diluted with 3 ml of dimethylformamide and filtered. Chloroform (80 ml) was added to the filtrates. The solution was washed 3-times with 100 ml of 5% solution of KCN, with water and dried by Na_2SO_4. The solvent was evaporated at low pressure and the residue was dried over P_2O_5 *in vacuo* to constant weight. When subjected to thin-layer chromatography (system A), it showed the presence of one main spot and of a weak, faster spot (c. 10%) of an impurity. Mass spectrum: M^+ 517 (0·33%); *m/e* (int., % of b.p.): 516 (0·30); 445 (9); 418 (11); 374 (2·5); 316 (2·0); 263 (6·0); 239 (2·0); 226 (2·0; 188 (3·5); 156 (9·7); 143 (5·2); 128 (100); 98 (10·3); 97 (9·0); 91 (30·3); 85 (8·0); 84 (11·5); 83 (8·0); 71 (10·0); 70 (7·5).

Caprylprolyl-N^{ε}-acetyl-D-lysyl-glycine Amide

A solution of prolyl-N^{ε}-benzyloxycarbonyl-D-lysyl-glycine amide (43 mg, c. 0·1 mM) in 0·5 ml of 4M-NaOH was treated with 20 mg (0·123 mM) of caprylic chloride. The mixture was stirred 30 min at room temperature, then diluted with 5 ml of water and extracted with ethyl acetate. The ethyl acetate solution was extracted with 3% HCl (3-times), water (twice), dried by Na_2SO_4, and the

solvent distilled off at reduced pressure. One ml of c. 35% solution of hydrogen bromide in glacial acetic acid was added to 28 mg (c. 0·05mM) of the dry residue. The mixture was set asside for 10 min at room temperature and then treated with an excess of ether. The precipitated hydrobromide was washed with ether and dried *in vacuo*. Next, 1 ml of 4M-NaOH and 10·2 mg (0·1 mM) of acetic anhydride were added and the mixture was shaken 30 min at room temperature. The neutral product was isolated as described above. It was subjected to mass spectrometry without any additional purification. Mass spectrum: M^+ 467 (0·05%), *m/e* (int., of b.p.): 410 (0·24); 393 (1·0); 283 (2·2); 241 (2·0); 224 (2·4); 196 (10·0); 126 (12·4); 113 (3·2); 84 (6·2); 70 (100).

Caprylprolyl-N$^\delta$-capryl-D-ornithyl-glycine Amide

The decarbobenzoxylation of prolyl-N$^\delta$-benzyloxycarbonyl-D-ornithyl-glycine amide (63 mg, c. 0·015 mM) was carried out as described for the preceding derivative. The dihydrobromide solution in 0·5 ml of 4M-NaOH was treated with 56 mg (a 15% excess) of caprylic chloride and the reaction mixture was stirred 30 min at rom temperature. The neutral product was isolated by the same procedure as in the preceding case. The dry residue crystallized after trituration with water. Yield 80·5 mg (quantitative). The yield after a two-fold crystallization from aqueous ethanol was 64·5 mg (80%). The mass spectrum is shown in Fig. 4. The dicapryl derivatives of the remaining tripeptide amides were prepared by the same procedure. The values of melting points, optical activities, and elemental analyses are given in Table II.

Chromatography of Product of Pyrolysis of Prolyl-leucyl-glycine Amide

Prolyl-leucyl-glycine amide (100 mg) was heated 60 min at 200°C in an open test tube. The brown-colored pyrolysate was dissolved in 1 ml of methanol and applied onto a silica gel column (25 × × 1 cm, particle size 60—100μ), equilibrated with system B (upper layer). The same system was used for elution. Fractions of 2 ml were collected at 5 min intervals. A total of 300 fractions were collected. The course of the separation was checked by thin-layer chromatography; detection was effected by ninhydrin and chlorination[10]. Fractions 85—135 and 211—225 were pooled, the solvents were distilled off under reduced pressure, and the crystalline, brown-tinged dry residues were dried *in vacuo* over P_2O_5. Fraction 85—135 was sublimed *in vacuo* and afforded chromatographically pure c(Pro-Leu). Amino-acid analysis[11]: Pro 0·98; Leu 1·03. Fraction C contained according to amino-acid analysis c. 70% of Gly, 15% of Pro, and 15% of Leu, after sublimation *in vacuo* c. 96% of Gly, 2% of Leu, and 2% of Pro. Its mass spectrum (temperature of ionic source 170°C) corresponded to c(Pro-Gly): M^+ 154 (42%); *m/e* 41 (100); 68 (35·5); 69 (45·2); 70 (74·2); 83 (93·5); 86 (35·5); 98 (27·4); 111 (46·8). The mass spectrum at the temperature of the ionic source 200°C corresponded to c(Gly-Gly): M^+ 114 (60·1); *m/e* 28 (100); 30 (96·1); 41 (48); 42 (60·1); 71 (49); 86 (13·7).

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