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65. Ammonolysis of Nitroarginine and Nitroarginine-Containing Peptides; Some Side Reactions

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(25. I. 74)

Summary. Ammonolysis of nitroarginine-containing peptides leads to the formation of ornithine-containing side products. The structure of these side products has been deduced from model experiments. This side reaction can be avoided by using the N^G-tosyl or the N^G-dicarbobenzoxy protecting groups.

Nitroarginine, an important N^G-substituted derivative of arginine, was first introduced into peptide synthesis by *Bergmann et al.* [1] and for many years arginyl

peptides were synthesized primarily with the use of nitroarginine derivatives [2]. Although these compounds proved to be very useful intermediates, some difficulties were encountered during the hydrogenolytic removal of the nitro group and the formation of side products was observed in several cases [2] [3]. Nevertheless nitroarginine derivatives were used in the recent synthetic work on luteinizing hormone releasing hormone (LH–RH) and its structural analogs by several groups [4–10] including our own. During this work [11] we came across some peculiarities of nitroarginine-containing analogs which led to the investigation here reported.

We synthesized the LH–RH by classical and solid phase methods. In the solid phase approach the peptide was prepared using dicyclohexylcarbodiimide as coupling agent and the *t*-butyloxycarbonyl group for the amino protection of all amino acids, except pyroglutamic acid, the latter being introduced as pentachlorophenylester. For the protection of the side chain functions the benzyl group was selected for serine and tyrosine, the dinitrophenyl group for histidine and the nitro group for arginine. The protected decapeptide was cleaved from the resin by ammonolysis in methanol/DMF at room temperature for about 60 hours. Subsequent deprotection either by catalytic hydrogenation or by treatment with liquid HF provided crude LH–RH which was purified by gel filtration on Sephadex G-25 and column chromatography on CM-cellulose using ammonium acetate as eluent. The product obtained seemed to be pure and homogeneous as judged by thin layer chromatography on silicagel and cellulose plates in various solvent systems. However, comparison with LH–RH made by classical solution methods [11] revealed slightly shifted Rf-values in some systems, although no difference in activity was detectable in biological tests. By amino acid analysis an unusually high value was found for ornithine in the LH–RH made by the solid phase procedure. Enzymatic degradation of the two preparations with chymotrypsin yielded identical fragments except that in the solid phase LH–RH preparation an additional fragment was observed besides the expected C-terminal pentapeptide.

These findings suggested that the solid phase LH–RH was contaminated with considerable amounts of hard-to-separate [Orn^8]–LH–RH. Furthermore the results suggested that the partial conversion of nitroarginine occurred during ammonolysis and/or during the deprotection, since amino acid analysis of resin bound peptide gave no unusually elevated values for ornithine. In order to evaluate the influence of the deprotection procedure, the protected decapeptide was cleaved from the resin by transesterification [12–15] followed by deprotection either by liquid HF or by catalytic hydrogenation prior to ammonolysis and purification. Amino acid analysis revealed correct values for arginine and only minute values for ornithine, which could even be due to effects already described by *Iselin* [3] and recently by *Blake et al.* [16] and *Yamashiro et al.* [17]. Thus, these results indicate that the considerable conversion of nitroarginine into ornithine was due to ammonolysis of the nitro protected compound.

In order to confirm these findings several nitroarginine-containing compounds, prepared by classical or solid phase methods, were submitted to ammonolysis and subsequently examined by amino acid analysis as shown on Table 1.

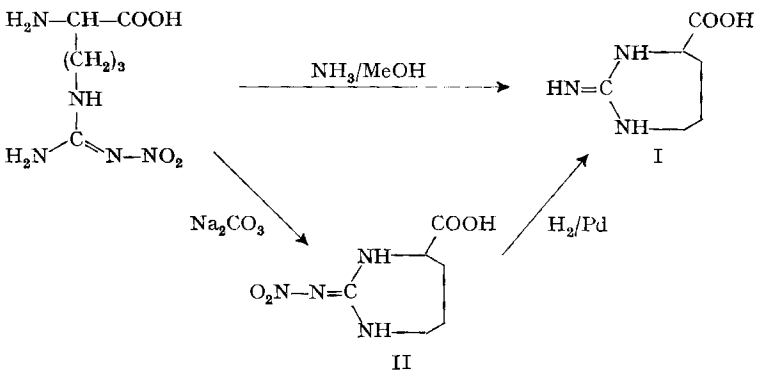
The results summarized in Table 1 clearly show that the conversion of nitroarginine is in fact due to ammonolysis, regardless of the method of synthesis and the

Table 1

		A	B	C	D	E	F
acid hydrolysis	Arg	21%	19%	19%	18%	18%	12%
	Arg(NO_2)	66%	71%	72%	71%	73%	83%
	Orn	13%	10%	9%	11%	9%	5%
ammonolysis	Arg	16%	16%	17%	16%	20%	21%
for *24 hrs*, 25°;	Arg(NO_2)	60%	58%	62%	61%	65%	56%
acid hydrolysis	Orn	24%	26%	21%	23%	15%	23%
ammonolysis	Arg	20%	22%	22%	16%	23%	18%
for *72 hrs*, 25°;	Arg(NO_2)	27%	25%	32%	37%	29%	44%
acid hydrolysis	Orn	53%	53%	46%	47%	48%	38%

A = pGlu-His(DNP)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-resin[1])
B = Z-Arg(NO_2)-Pro-Gly-NH_2 C = Z-Pro-Arg(NO_2)-Gly-NH_2
D = pGlu-Arg(NO_2)-OH E = pGlu-Arg(NO_2)-OCH_3
F = Boc-Arg(NO_2)-OH

sequence and independent of the amino and carboxyl substituents of nitroarginine, although the amount of ornithine formed as function of time varies slightly with each individual compound. Furthermore it is obvious that the reaction takes place within time intervals and under conditions which are comparable to those mentioned in the literature [5–7] [10] [18–22]. Thus it seems clear that whenever nitroarginine-containing peptides cannot be ammonolyzed under very mild conditions and within short reaction times, ornithine-containing compounds must be expected and eventually tedious purifications must be performed. Since the conversion of nitroarginine into ornithine can occur either directly or *via* an ammonolytically formed intermediate, we submitted several nitroarginine-containing peptides to ammonolysis and tried to characterize all the different products. However, the attempts to separate and isolate these products were not very successful and the results obtained were rather ambiguous.



[1]) Abbreviations according to: IUPAC-IUB Commission, Biochemistry *5*, 2485 (1966); *ibid. 6*, 362 (1967).

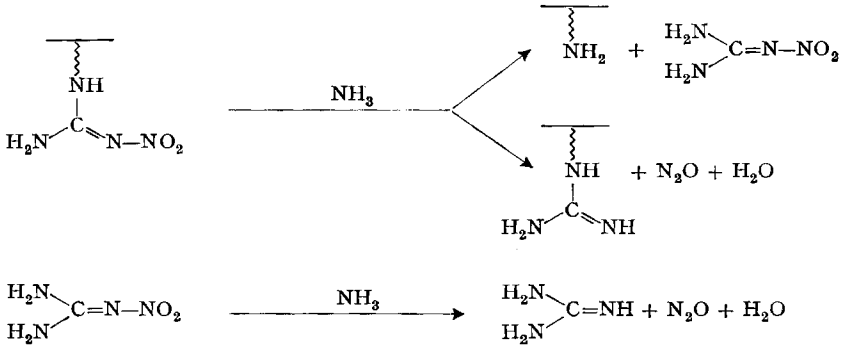
Z = benzyloxycarbonyl, Boc = *t*-butyloxycarbonyl, DNP = 2,4-dinitrophenyl, Bzl = benzyl.

Therefore, as a simple model compound, nitroarginine was submitted to ammonolysis in methanol at room temperature for 6 days. Under these conditions essentially one product was obtained besides small amounts of nitroarginine, arginine and ornithine. The crystalline product was identified by microanalysis, amino acid analysis, NMR. and IR. as 2-imino-4-carboxy-1,3-diazacycloheptane (I), a compound which *Paul et al.* [23] obtained upon catalytic hydrogenation of 2-nitrimino-4-carboxy-1,3-diazacycloheptane (II). The latter was obtained by heating nitroarginine in aqueous sodium carbonate for about 2 hours. Thus, by following the method of *Paul et al.*, I was synthesized and compared with the compound prepared by ammonolysis of nitroarginine. All physical and chemical properties of the two preparations were identical.

From the experiments carried out with nitroarginine and ammonia it is not clear whether or not I is formed *via* II. Both possibilities can be understood if the carbon in the nitroguanidino group is regarded as being an activated carbon such as that of a carbonyl group.

In this experiment, since only small amounts of arginine and ornithine are formed, the intramolecular reaction clearly dominates the intermolecular reaction. However, the formation of a cyclic structure similar to 2-imino-4-carboxy-1,3-diazacycloheptane in nitroarginine-containing peptides seems unlikely, since a rather unfavourable tertiary amino group in the peptide backbone would have to be created. In addition acid hydrolysis of compound I under identical conditions as described in Table 1 yielded not only arginine and ornithine but also citrulline. It therefore seemed indicated to investigate the reaction of a simple amino protected nitroarginine derivative with ammonia. For this purpose N-acetyl-nitroarginine was submitted to ammonolysis in methanol/DMF at room temperature for 10 days.

From the reaction mixture, which consisted of 5 major components as judged by thin layer chromatography, the following compounds were isolated by extraction with organic solvents and by ion exchange chromatography on Amberlite CG-50 and Dowex 50: nitro-guanidine, guanidine as its acetate, N-acetyl-arginine, N-acetyl-ornithine and considerable amounts of the starting material N-acetyl-nitroarginine. The compounds were identified and characterized by their physical properties and by comparison with authentic samples. A compound with a cyclic structure similar to compound I could not be detected, indicating that the simple amino substituent completely inhibits ring formation by ammonolysis.



On the basis of these experiments, the following conclusions seem justified:

Ammonolysis of nitroarginine-containing peptides leads to the formation of ornithine-containing side products. This conversion is accompanied by a second side reaction, namely the deprotection of nitroarginine by elimination of the nitroamino group. In both reactions the reactive centre which is attacked by ammonia is the activated carbon in the nitroguanidino group; the carbon in the guanidino group does not react, as was shown in experiments of arginine with ammonia where no conversion into ornithine could be detected. Thus the formation of guanidine is not due to the primary reaction, but due to a secondary reaction of the nitroguanidine with ammonia.

Of further interest was the question whether other protected arginyl peptides like N^G-tosyl or N^G-dicarbobenzoxy arginyl peptides undergo similar side reactions caused by ammonolysis. The reaction of ammonia with nitroarginine peptides is based on the reactivity of the nitroguanidino carbon adjacent to the electron withdrawing nitroamino group. In contrast, N^G-tosyl and N^G-dicarbobenzoxy groups do not act as strongly electron-withdrawing groups on the guanidino carbon, and in addition both groups are rather bulky. Thus a conversion of these protected arginyl derivatives into ornithine derivatives seems unlikely. In order to prove this, several arginyl peptides, – identical in sequence, but with different arginine protecting groups –, were treated with ammonia and subsequently examined by amino acid analysis as summarized in Table 2.

Table 2

		Z-Arg(NO_2)-Pro-GlyNH_2	Z-Arg(Tos)-Pro-GlyNH_2	Z-Arg(Z_2)-Pro-GlyNH_2	H-Arg-Pro-GlyNH_2
acid hydrolysis	Arg	19%	99%	99%	100%
	Arg(NO_2)	71%			
	Orn	10%	1%	1%	–
ammonolysis	Arg	16%	100%	99%	100%
24 hrs at 25°;	Arg(NO_2)	58%			
acid hydrolysis	Orn	26%	–	1%	–
ammonolysis	Arg	22%	99%	99%	100%
72 hrs at 25°;	Arg(NO_2)	25%			
acid hydrolysis	Orn	53%	1%	1%	–

From these results it is evident that whenever a protected arginyl peptide must be submitted to ammonolysis, the N^G-tosyl or N^G-dicarbobenzoxy groups should be chosen for the protection of arginine. If for any reason the nitro-protecting group cannot be avoided, the peptide should be deprotected prior to ammonolysis.

Experimental Part

Amino acid analysis was performed on a *Beckmann* Unichrom amino acid analyzer. The samples were hydrolyzed in evacuated tubes with 6 N HCl at 110° for 24 h according to *Spackman et al.* [24]. The ratio of arginine, nitroarginine and ornithine is expressed in per cent of the total amount of arginine derivatives found. In no case was loss of arginine-containing derivatives encountered as judged by comparison with other amino acids in the molecule.

Ammonolysis was carried out with absolute methanol saturated with anhydrous ammonia at about $-5°$. 10N solutions were prepared in order to obtain standardized conditions. The reactions were performed with 50 mg of compound in 10 ml of the ammonia solution in tightly stoppered vessels at room temperature. After the reaction the samples were evaporated, dried overnight *in vacuo* over P_2O_5 and submitted to amino acid analysis.

All nitroarginine-containing compounds were either prepared in our laboratories [11] or purchased from *Fluka AG*, Buchs. They were of L-configuration and prior to use all were tested by means of amino acid analysis and thin layer chromatography in different systems.

Melting points were performed on a *Büchi* melting point apparatus and are uncorrected. The IR. spectra were recorded on *Beckmann* instruments and the NMR. spectra on a *Varian* A-60 apparatus.

2-Imino-4-carboxy-1,3-diazacycloheptane (*I*). L-Nitroarginine (5 g) was suspended at $-10°$ in 250 ml of 10N NH_3/methanol in a tightly stoppered round bottomed flask. Under permanent stirring the reaction mixture was kept at room temperature for 6 days. Upon evaporation to dryness the residue was dissolved in hot water. After filtration and addition of methanol to the filtrate 1.65 g of pure 2-imino-4-carboxy-1,3-diazacycloheptane were obtained. Upon column chromatography of the mother liquor on silicagel using propanol/water 7:3 as eluent a second crop of 1.2 g could be crystallized. Yield: 2.85 g (79%) m.p.: 328° (dec.) (Lit: 323°) $[\alpha]_D^{25} + 55.4°$ (H_2O, $c = 1$) (Lit: $+54°$).

$C_6H_{11}N_3O_2$	Calc.	C 45.85	H 7.05	N 26.74%
(157.17)	Found	,, 45.56	,, 7.01	,, 26.67%

Ammonolysis of N-acetyl-L-nitroarginine. 2.5 g of N-Acetyl-L-nitroarginine were dissolved in 40 ml of absolute DMF and cooled to $-10°$. After addition of 200 ml 10N NH_3/methanol the reaction mixture was kept at room temperature in a tightly stoppered round bottomed flask for 10 days. Upon evaporation to dryness a yellow oil (4.0 g) was obtained, which consisted of 5 components as judged by thin layer chromatography in different systems. This residue was dissolved in water and extracted with ethylacetate.

From the ethylacetate layer a homogeneous product (0.1 g) was obtained, which could be crystallized from hot water to yield pure nitroguanidine. m.p.: 243–244° (Lit: 246–247°).

$CH_4N_4O_2$ (104.07) Calc. C 11.54 H 3.87 N 53.83% Found C 11.31 H 3.98 N 53.45%

The residue from the aqueous layer (3.85 g) was submitted to ion exchange chromatography on a column (2.5 × 28 cm) of Amberlite CG-50 200–400 mesh using water as eluent at the beginning, followed by a gradient of dilute acetic acid (0–0.4M). This chromatography yielded one fraction consisting of a mixture of early emerging components and two fractions consisting of pure products.

The earlier pure fraction (0.4 g), which was eluted by water was dissolved in methanol, decoloured by a little charcoal and filtered. Upon addition of ethylacetate to the filtrate N-acetyl-arginine crystallized (0.25 g). m.p.: 265–266° (dec.) (Lit: 269–270°).

$C_8H_{16}N_4O_3$ (216.24) Calc. C 44.44 H 7.46 N 25.91% Found C 44.29 H 7.48 N 25.49%

The second compound from this chromatography, obtained from the acidic fractions (0.92 g), was dissolved in methanol and separated from the insoluble. Upon evaporation the residue was crystallized from ethanol/ethylacetate to yield pure guanidine acetate (0.6 g). m.p.: 228–229° (Lit: 229–230°).

$CH_5N_3 \cdot C_2H_4O_2$	Calc.	C 30.25	H 7.62	N 35.28%
(119.12)	Found	,, 30.25	,, 7.76	,, 35.44%

The fraction from the Amberlite chromatography consisting of a mixture of early emerging components (2.2 g) was further separated by ion exchange chromatography on a column (1.7 × 8.4 cm) of Dowex 50 W 200–400 mesh using water as eluent. This yielded essentially pure starting material N-acetyl-nitroarginine (1.7 g), which was not worked up further.

By subsequently eluting the column with diluted ammonia solution (2.5%) a further compound was obtained. After decolouring with a little charcoal and filtration, the solution was evaporated to dryness. Upon trituration with ethanol N-acetyl-ornithine solidified (0.1 g).

$C_7H_{14}N_2O_3 \cdot 0.5\,H_2O$	Calc.	C 45.89	H 8.25	N 15.29%
(183.21)	Found	,, 46.12	,, 8.46	,, 15.28%

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66. Über stereoselektive Synthesen und Reaktionen von 5,6-Epoxy-perhydroisoindolinen

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(11. I. 74)

Summary. Stereo-selective syntheses of 2,5,6-trisubstituted perhydroisoindolines are achieved by preparation of the three diastereoisomeric 5,6-epoxy-perhydroisoindolines and their reactions with nucleophiles, *e.g.* amines. The transformation of *trans*-amino alcohols obtained in this way into *cis*-amino alcohols is described.

Im Rahmen unserer Arbeiten über nicht-aromatische N-Heterocyclen beschäftigten wir uns vor einiger Zeit mit der stereoselektiven Synthese von 2,5,6-trisubstituierten Perhydroisoindolinen. In der vorliegenden Arbeit beschreiben wir den