Chemical Modification of Arginine by Nitromalondialdehyde. Synthesis and Properties of δ-(5-Nitro-2-pyrimidyl)ornithyl Derivatives*

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ABSTRACT: The reaction of nitromalondialdehyde sodium salt with arginine in aqueous alkaline media results in the quantitative formation of δ-(5-nitro-2-pyrimidyl)ornithine. The method has been applied to the synthesis, in high yields, of several δ-(5-nitro-2-pyrimidyl)ornithyl derivatives from the corresponding arginine analogs and to the selective modification of the *S*-carboxymethyl B chain of insulin. The amide bonds involving the carboxyl group of the modified arginyl residues were not susceptible to hydrolysis by trypsin; however it was possible to regenerate trypsin digestion by mild reduction with sodium borohydride of the aromatic nitropyrimidyl nucleus to 1,4,5,6-tetrahydropyrimidyl derivative. Therefore these combined reactions may be of use in sequence analysis of proteins.

It is well known that the reaction of a number of aromatic aldehydes with amino acids leads to the formation of the corresponding crystalline Schiff bases (McIntire, 1947; Heyl *et al.*, 1948); furthermore dialdehydes, such as glutaraldehyde, can react bifunctionally with proteins to give inter- and intramolecular cross-linking (Quiocho and Richards, 1964). The condensation products formed between simple carbonyl compounds and primary amino groups are usually unstable and easily dissociated; however in the case of the reaction between dicarbonyl compounds and guanidyl derivatives, there is the possibility of stabilization of the condensation products by the formation of heterocyclic rings. On these bases it was recently reported the application of 1,2- and 1,3-dicarbonyl compounds for the chemical modification of arginine in proteins (King, 1966; Toi *et al.*, 1967; Yankeelov *et al.*, 1968). Such a modification is desirable for a number of purposes: to limit the hydrolytic action of trypsin on a protein to the lysyl bonds, to chemically modify the arginyl groups of several inhibitors of proteolitic enzymes (Liu *et al.*, 1968) and also in connection with the definition of the active sites of biologically active proteins (Grossberg and Pressman, 1968). Because it remains protonated within the pH range of enzyme stability and because of its inherent resonance stabilization, the guanidyl group of arginine is chemically distinct from other basic groups in proteins. The reagent of choice should react specifically and quantitatively with guanido groups to form a derivative completely stable at the optimum pH for the reaction; the reaction should also be reversible in order to restore the original properties of the modified group, principally in connection with the susceptibility to tryptic digestion.

Recently the reactivity and structure of malondialdehyde and its derivatives have received some attention (Ulbricht and Price, 1957; Kwon and Brown, 1965; Crawford *et al.*, 1966; Chio and Tappel, 1969); most of these compounds do not very readily form condensation products such as dianils or pyrimidines, however NMA[1], with a strongly electron-withdrawing group, is significantly more reactive. NMA is a useful reagent, particularly for the synthesis of a variety of carbocyclic and heterocyclic nitro compounds which would be difficult to prepare by any alternative procedure (Fanta and Stein, 1960). It was shown that it gives mono- and dicondensation products with several amino derivatives (Fanta and Hedman, 1956; Hale and Brill, 1912a) and the reaction with guanidine, in the presence of piperidine and at room temperature, leads to the formation of 2-amino-5-nitropyrimidine in about quantitative yield (Hale and Brill, 1912b). Nevertheless, the first condensation step takes place more readily than the second since the monocondensation product is largely stabilized through intramolecular formation of a chelate ring with the nitro group.

As part of a program concerned with the study of new applications of NMA to the protein chemistry, we have investigated the possibility of selective and reversible chemical modification of arginine in polypeptidic chains. In the present paper the effect of various reaction conditions on the condensation of NMA with arginine and related model compounds is studied, in order to establish a procedure resulting in an optimum yield of the pyrimidine; furthermore the properties of the modified derivatives are investigated.

Experimental Section

Materials. The following materials were used as purchased: DL-arginine monohydrochloride, DL-ornithine monohydrochloride, and α-acetyl-L-arginine (Fluka, Switzerland). Trypsin was obtained from Worthington Biochemical Corp.; BAPA and BAA were products of Nutritional Biochemicals. The reduced *S*-carboxymethyl B chain of insulin was obtained from the Mann Research Laboratories (lot R-2209A). 2-

* From the Institute of Organic Chemistry, University of Padova, Padova 35100, Italy. *Received September 17, 1970.* Part of the material was taken from thesis of G. M. B., submitted to the Graduate Division of the University of Padova in partial fulfillment of Dr. degree. This investigation was supported in part by Grant from the National Research Council of Italy.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: NMA, nitromalondialdehyde sodium salt; DMF, *N,N*-dimethylformamide; NPm, 5-nitro-2-pyrimidyl; BAPA, α-benzoylarginine *p*-nitroanilide; BAA, α-benzoylargininamide.

Chloro-5-nitropyrimidine was prepared following the procedure described in an earlier paper (Signor *et al.*, 1963). Sodium nitromalondialdehyde monohydrate (Fanta, 1952) was prepared from mucobromic acid which in turn was synthetized according to the method previously described (Allen and Spangler, 1947); the reagent was stored in an evacuated desiccator in the cold. All other chemicals were obtained from "Carlo Erba" (Milano) and were of reagent grade.

Methods. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Prior to analysis the compounds were dried *in vacuo* over phosphorus pentoxide at 45°; microanalyses were performed by the Microanalytical Laboratory of the Institute of Organic Chemistry (Padova, Italy). Thin-layer chromatography was carried out on silica gel G using the ascending technique; paper chromatograms were run on Whatman No. 1 paper. The solvents used were (1) 1-butanol–acetic acid–water (4:1:5, v/v) and (2) chloroform–methanol (95:5, v/v). Detection of compounds was generally achieved visually or with an ultraviolet lamp. Ultraviolet spectra were recorded on a Perkin-Elmer Model 124 spectrophotometer in 1% sodium bicarbonate solution, except otherwise specified. Amino acid analyses were carried out on an Erba Model 3A27 amino acid analyzer utilizing the Spackman–Stein–Moore (1958) system. Digestion with trypsin of BAPA and its modified derivative was carried out according to the procedure described by Roman and Favilla (1963). The extent of tryptic hydrolysis for BAA and related compounds was followed at constant pH by the use of a Radiometer TTT-1 titrator with a combination electrode GK2021 (Radiometer, Copenhagen, Denmark). The electrode vessel was a water-jacketed glass container; the temperature of the circulating water was controlled to ±1° by a thermostat and the solutions mechanically stirred during reaction (Bergmann *et al.*, 1939).

*α-NPm-*DL*-arginine* (*I*). A solution of 4 mmoles of 1% standard carbonate-free potassium hydroxide in absolute methanol was added under an atmosphere of dry nitrogen to 2 mmoles of the crystalline DL-arginine hydrochloride. The amino acid was stirred with gentle warming, until it had completely dissolved in the methanol–KOH solution. After removal of potassium chloride by filtration, the solution was cooled to 0–5° and 2 mmoles of 2-chloro-5-nitropyrimidine dissolved in 5 ml of absolute methanol were rapidly added. Immediately the color of the solution changed to deep yellow and the pH decreased to about 6. The reaction was allowed to come to completion by stirring at room temperature over a 30-min period. On standing, yellow crystals separated, which were recrystallized from methanol, to give a 90% yield, mp 287–288° dec.

Anal. Calcd for $C_{10}H_{15}N_7O_4$: C, 40.40; H, 5.05; N, 32.99. Found: C, 40.17; H, 4.98; N, 32.61.

*δ-NPm-*DL*-ornithine* (*II*). Copper carbonate was added to a boiling solution of 0.5 g of DL-ornithine hydrochloride (3 mmoles) in 10 ml of water; the excess copper carbonate was filtered and washed with 2–3 ml of water. Excess sodium bicarbonate was added, followed by a solution of 1.3 g of 2-chloro-5-nitropyrimidine (8 mmoles) in 20 ml of ethanol. The mixture was shaken for two hours at room temperature and the yellow-green precipitate filtered and washed with water, ethanol, and ether. It was then suspended in 5 ml of water and the copper ion removed by bubbling a stream of hydrogen sulfide through the cooled solution. A trace of charcoal was added and the mixture filtered rapidly. The filtrate was taken to dryness *in vacuo* and the residue crystallized from water to give a 70% yield, mp 249–250° dec. *Anal.* Calcd for $C_9H_{13}N_5O_4$: C, 42.35; H, 5.09; N, 27.45. Found: C, 41.87; H, 5.05; N, 27.12.

*α-Acetyl-δ-NPm-*L*-ornithine* (*III*). To a solution of 2.2 g of *N*-acetyl-L-arginine (0.01 mole) in 15 ml of 1 M sodium hydroxide at room temperature was added a solution of 2.8 g of NMA (0.02 mole) in 15 ml of water with occasional shaking. After stirring for a further 30 min, the mixture was cooled and acidified to congo red with 2 M hydrochloric acid; the yellow solid which separated was filtered and washed with cold dilute hydrochloric acid; yield 70%, mp 187°. *Anal.* Calcd for $C_{11}H_{15}N_5O_5$: C, 42.99; H, 4.88; N, 22.80. Found: C, 43.03; H, 4.95; N, 22.64.

*α,δ-Di-NPm-*DL*-ornithine* (*IV*). FROM NMA. To a solution of I (0.48 g, 1.6 mmoles) in 20 ml of water containing piperidine (0.5 ml), was slowly added, under stirring, a solution of 0.25 g of NMA (1.8 mmoles) in 10 ml of water. After standing overnight at room temperature, the solution was acidified with 2 M hydrochloric acid to pH 2. The yellow solid which separated was filtered and crystallized from ethanol; yield 70%, mp 242–244° dec. *Anal.* Calcd for $C_{13}H_{14}N_8O_6$: C, 41.27; H, 3.70; N, 29.62. Found: C, 40.96; H, 3.65; N, 29.22.

FROM 2-CHLORO-5-NITROPYRIMIDINE. DL-Ornithine hydrochloride (0.49 g) and sodium bicarbonate (1.0 g) were dissolved in 15 ml of water and mixed under stirring at room temperature with a solution of 2-chloro-5-nitropyrimidine (0.96 g) in 20 ml of ethanol. After 2 hr at 40°, the mixture was concentrated at 30° under reduced pressure and then acidified with 2 M hydrochloric acid. The solid collected by filtration was twice crystallized from ethanol–water; yield 90%, mp 242–244° dec. *Anal.* Calcd for $C_{13}H_{14}N_8O_6$: C, 41.27; H, 3.70; N, 29.62. Found: C, 41.23; H, 3.78; N, 29.17.

*α-Benzoyl-δ-NPm-*DL*-ornithine p-Nitroanilide* (*V*). A solution of BAPA·HCl (1.0 g) in aqueous dimethylformamide (80%, 30 ml) containing piperidine (0.5 ml) was slowly added to a solution of NMA (0.4 g) in water (20 ml) with stirring over a 15-min period. After standing overnight at room temperature, the solution was concentrated under reduced pressure during which a crystalline solid separated. The mixture was acidified with 2 M hydrochloric acid, filtered and the product collected, washed several times with ice-cold water; yield 70%, mp 203–205°. *Anal.* Calcd for $C_{22}H_{20}N_7O_6$: C, 55.23; H, 4.18; N, 20.50. Found: C, 55.37; H, 4.12; N, 20.62.

*α-Benzoyl-δ-NPm-*DL*-ornithinamide* (*VI*). The derivative was prepared in aqueous solution in the same manner as described for V and it was recrystallized from water; yield 70%, mp 199°. *Anal.* Calcd for $C_{16}H_{18}N_6O_4$: C, 53.44; H, 5.10; N, 23.56. Found: C, 52.96; H, 5.06; N, 23.47.

*α-Benzoyl-δ-(5-nitro-1,4,5,6-tetrahydro-2-pyrimidyl)-*DL*-ornithinamide* (*VII*). Compound VI (0.36 g) and sodium borohydride (0.038 g) were intimately mixed and 2 ml of absolute ethanol were added under stirring; an exothermic reaction set in. After a short time the product separated from the liquid reaction mixture; it was mixed with 6 ml of cold water, filtered, washed with cold water, and dried over phosphorus pentoxide; yield 70%, mp 174–176°. *Anal.* Calcd for $C_{16}H_{22}N_6O_4$: C, 53.06; H, 6.09; N, 23.14. Found: C, 52.52; H, 6.18; N, 22.82.

Modification of S-Carboxymethyl B Chain of Insulin. The peptide (approximately 3.0 mmoles) was dissolved in 1.0 ml of 1 M NaOH and then it was added to 1 ml of water containing 10 mg of NMA (70 mmoles). After 2 hr the reaction was terminated by adding 6 M HCl to pH 3–4 and the mixture passed through a Sephadex G-25 column (60 × 1.5 cm)

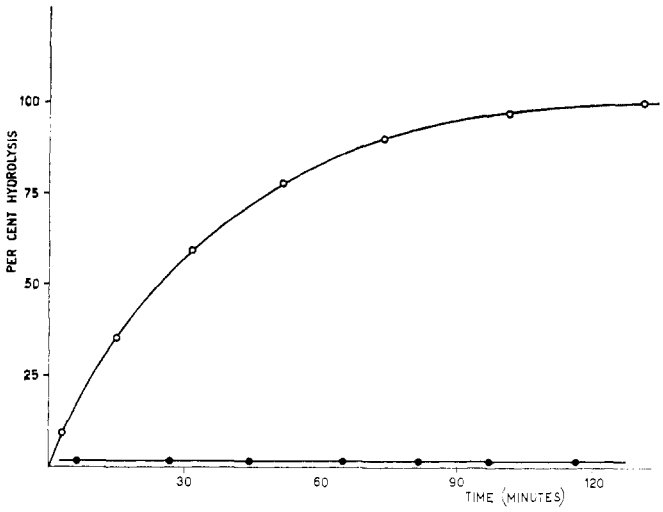


FIGURE 1: Tryptic digestion of BAPA (○) and of α-benzoyl-δ-NPm-ornithine *p*-nitroanilide (●). The experiments were performed at pH 8.2 in diethylformamide–0.05 M Tris buffer (1:100, v/v), containing 0.02 M $CaCl_2$; temperature 37 ± 1°. The relative concentrations of substrate and enzyme were 10^{-3} and 2×10^{-7}, respectively (100:1, w/w). The optical density was measured by a Perkin-Elmer Model 139 spectrophotometer at 410 mμ.

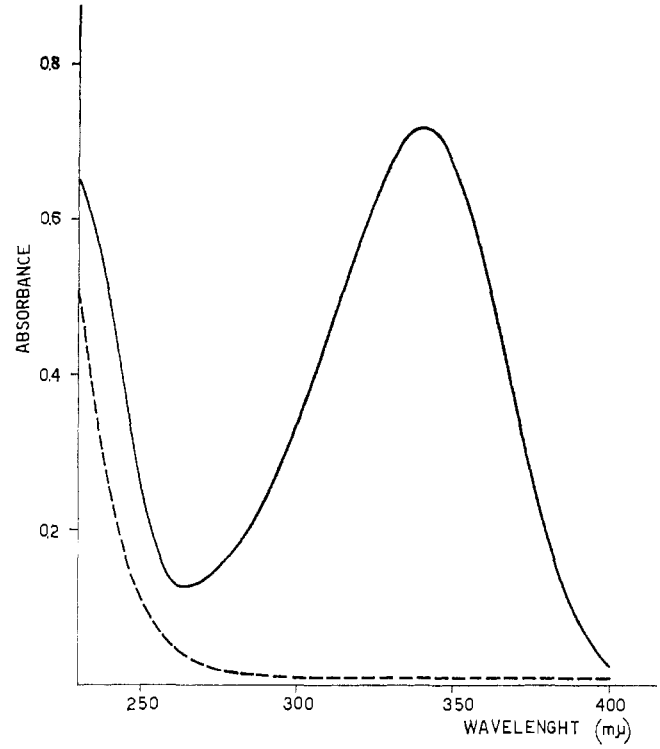


FIGURE 2: Ultraviolet absorption spectra of α-benzoyl-δ-NPm-ornithinamide (solid line) and α-benzoyl-δ-(5-nitro-1,4,5,6-tetrahydro-2-pyrimidyl)ornithin amide (dashed line). The spectra were recorded on a Perkin-Elmer Model 124 spectrophotometer against 1% $NaHCO_3$ in which they were dissolved at a concentration of 5×10^{-5} M.

at a flow rate of 50 ml/hr. The eluent was 50% formic acid. The first peak containing the modified peptide was pooled and lyophilized. To investigate the homogeneity of the modified peptide, a sample of about 3 mg was chromatographed on a Sephadex G-75 column (40 × 0.9 cm) equilibrated with 50% formic acid. The flow rate was 15 ml/hr and 2-ml fractions were collected.

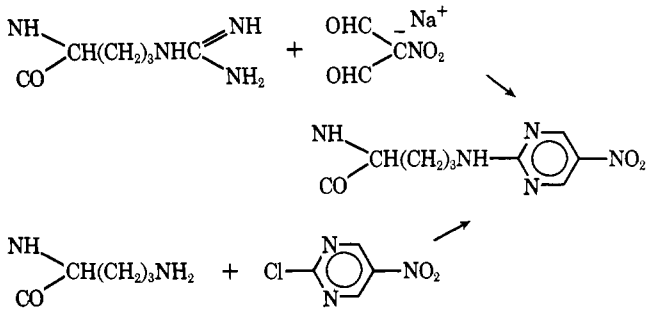
Results

NMA was prepared in somewhat variable yield (about 40%) from mucobromic acid. Ultraviolet spectra of aqueous solutions of NMA at various pH clearly showed that the conjugate acid is present at the equilibrium only in strong acid. Preliminary examination of the ability of NMA to modify the guanido group, using as model substrate α-acetyl-L-arginine, demonstrated that the reaction does not occur in acidic and neutral solutions. It was found however that the condensation reaction can be carried out rapidly at 0–5° in alkaline solution and the optimum pH range was found to be between 12 and 14. The reaction of NMA and α-acetyl-L-arginine in 1 M sodium hydroxide solution and at room temperature was found to be complete in about 15 min; the progress of the reaction was followed conveniently by amino acid analysis of the reaction mixtures, determining at various interval times the free arginine recovered after acid hydrolysis. The ease with which δ-NPm-ornithyl derivatives can be obtained, permitted the quantitative preparation of a variety of compounds, all the derivatives exhibit an absorption maximum near 335 mμ and the average molar extinction coefficient is 16,000.

Arginine itself can be converted smoothly in aqueous alkaline solution into a new aromatic α-amino acid; amino acid analysis of the final reaction mixture on the 15-cm column with the regular pH 5.28 buffer (Spackman *et al.*, 1958) demonstrated that arginine was absent and was replaced by a component eluted at the column front. The product was shown to be δ-NPm-ornithine by an independent synthesis. Reaction of the cuprous complex of ornithine with 2-chloro-5-nitropyrimidine, followed by treatment with hydrogen sulfide, yielded a compound identical with the condensation product of NMA and arginine as judged by chromatography in different solvent systems. It was possible to isolate a common derivative, namely α,δ-diNPm-ornithine by further condensation of the two compounds with 2-chloro-5-nitropyrimidine. The above results clearly show that in these conditions NMA specifically reacts with the guanido group of arginine, the amino function remaining unaffected. δ-NPm-ornithine was not stable under the conditions commonly used for the hydrolysis of proteins. Amino acid analysis of the product which had been heated in 6 M HCl at 110° for 24 hr, gave a mixture of the starting material and ornithine; therefore, in these conditions, cleavage at the 2 position of the pyrimidine nucleus occurs.

The reaction of BAPA and NMA was carried out in aqueous dimethylformamide, because of the low solubility of the starting material. The structure of the modified derivative was demonstrated on the basis of chemical evidences; after complete acid hydrolysis, ornithine and *p*-nitroaniline were recovered. The modified compound V is not hydrolyzed by trypsin, as judged by comparing the rates of *p*-nitroaniline liberation for BAPA and its δ-NPm derivative (Figure 1). Any attempt to obtain the reduced derivative of V were unsuccessful because of the high sensitivity of the *p*-nitroanilide function to borohydride attack (Severin and Schmitz, 1962; Kaplan, 1964). On the other hand, using BAA as starting material, it was possible to synthetize the modified compound VI and the corresponding reduced derivative VII; it was observed that the operative conditions did not affect the amide bond and are probably too mild even for

SCHEME I



the possible reduction of disulfide bridges in proteins (Kress and Laskowski, 1967; Light and Sihna, 1967). In Figure 2 the ultraviolet spectra of VI and VII are reported; as can be seen the extent of reduction may be conveniently followed by ultraviolet spectrophotometry since the tetrahydropyrimidyl compound does not absorb in the ultraviolet region between 250 and 400 mμ, widely differing from the heteroaromatic parent derivative. It was observed that the modified substrate VI is not sensible to trypsin, while the reduced derivative VII is readily hydrolyzed and is even more susceptible to tryptic attack than the parent arginyl compound (Figure 3).

The reaction has been applied to the modification of arginine in the *S*-carboxymethyl B chain of insulin which has phenylalanine as N-terminal amino acid and contains one arginyl and one lysyl residue. The derivative obtained by reaction with NMA in alkaline solution, after purification by gel filtration, was examined by two different procedures. The ultraviolet spectra of the modified peptide in 0.1 M NaOH were similar to the spectra of the model NPm derivatives with a strong absorption band near 335 mμ; a single NPm group per mole of peptide was calculated using an average value of 16,000 for the molar extinction coefficient. The results of the amino acid composition after acid hydrolysis of the polypeptide chain showed the complete disappearance of arginine and the presence of one residue of ornithine per mole of peptide.

Discussion

Reversible methods for the chemical modification of proteins are very useful in studies of structure–function relationships. Another advantage of reversible modifications is that they can be used for the limited degradation of proteins to large peptides, the usual first step in sequence analysis. The currently used methods for the chemical modification of arginine in proteins do not consent the possibility of restoring the original guanido function; to overcome this difficulty we suggest a different approach to the problem, namely the reduction of 5-nitro-2-pyrimidyl compounds with sodium borohydride. The purpose of this study was to demonstrate that tryptic digestion is inhibited by modification of the guanido function with NMA and can be restored by further reduction with sodium borohydride.

NMA has favorable properties as far as is concerned with its use in protein and amino acid chemistry, mainly its high solubility in water and the high molar extinction coefficient of its heteroaromatic derivatives. NMA itself is unstable but is readily isolated and stored in the form of the easily recrystallizable sodium salt, in which the nitromalondialdehyde anion is stabilized by resonance involving distribution of the negative charge among all the oxygen atoms. The stoichiometry of the reaction between NMA and arginyl groups is shown in Scheme I; the formation of isomeric products was not observed in the aforementioned conditions. The alternative route, using the coupling reaction between ornithyl groups and 2-chloro-5-nitropyrimidine, is also reported. The presence of a nitro group on the central carbon atom of the parent malondialdehyde makes it possible to rapidly perform the condensation reaction either in 0.5 M aqueous piperidine or in 0.5–1.0 M sodium hydroxide solution; these conditions are mild enough to avoid any hydrolytic cleavage of peptide bonds but are probably not suitable for the retention of biological activity of several enzymes.

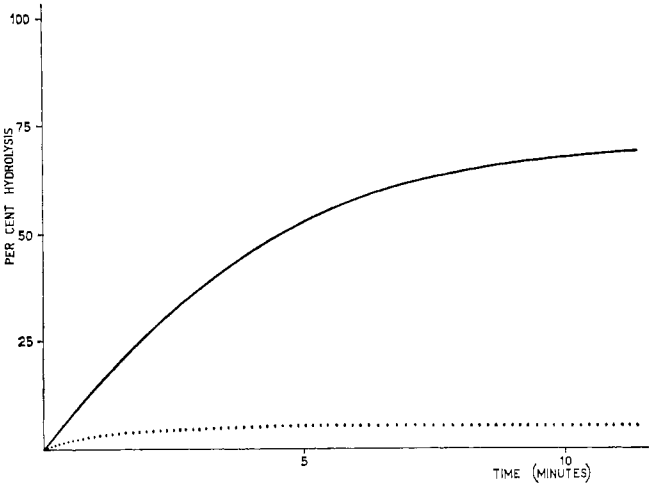


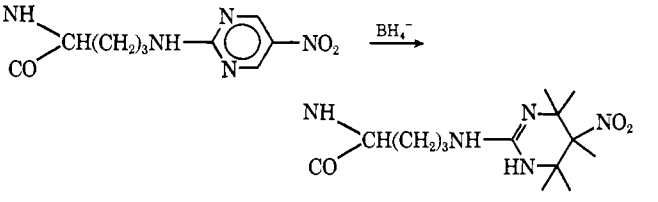
FIGURE 3: Base uptake during tryptic digestion of α-benzoyl-δ-NPm-ornithinamide (dotted line) and α-benzoyl-δ-(5-nitro-1,4,5,6-tetrahydro-2-pyrimidyl)ornithinamide (solid line). The samples were dissolved in water at a concentration of 1.4×10^{-3} M, 0.02 M, in $CaCl_2$, at 25 ± 1° and the pH was maintained at 8.2 by the automatic addition of 0.1 M NaOH (Radiometer TTT-1 titrator).

It was possible in these conditions to convert quantitatively and selectively the arginine residue of the *S*-carboxymethyl B chain of insulin into δ-NPm-ornithine residue. It is therefore expected that the reaction between NMA and proteins can, in general, be controlled in much the same way as the reactions involving small molecules. No side reactions of the α- and ε-amino groups should take place at high pH values where the reaction of the guanido function is favored.

The formation of a pyrimidine with a nitro group in the 5 position is of paramount importance not only for the region of ultraviolet absorption of the corresponding derivatives, but also for the possibility of a mild reduction of the heteroaromatic nucleus by the use of metal hydrides (Scheme II).

The reduction by sodium borohydride of 5-nitropyrimidines to tetrahydropyrimidines was recently reported for a model

SCHEME II



5-nitro-2-pyrimidylthio derivative (Bordignon *et al.*, 1970) and follows an entirely analogous path to that observed for the reduction of 3,5-dinitro-2-pyridyl compounds (Signor and Bordignon, 1968); furthermore 5-nitro-2-pyrimidylamino derivatives have also been shown to undergo reduction by sodium borohydride at the pyrimidyl nucleus to yield tetrahydropyrimidines (A. Signor, unpublished results).

The most interesting results are related to the behavior of the NPm and corresponding reduced derivatives to tryptic hydrolysis. The observation that trypsin does not split the amide bond adjacent to a nitropyrimidylated ornithyl residue might have been expected, since basicity and polarity of the guanido function are deeply changed; the restored sensibility to tryptic hydrolysis after reduction with sodium borohydride, is in agreement with structure and chemical properties of the tetrahydropyrimidyl compounds which are strictly similar to those of the parent arginine.

On these bases, it is conceivable that the nuclear reduction of δ-NPm-ornithyl derivatives potentially converts the reaction of NMA with guanidyl groups into a reversible technique for the chemical modification of arginine in proteins.

Acknowledgments

The authors thank Dr. E. Celon for microanalyses, Mr. E. De Menego and Mrs. R. Mazzanti for valuable technical assistance.

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