# CLEAVAGE OF DINITROPHENYL SIDE CHAIN PROTECTING GROUP OF HISTIDINE UNDER FMOC- DEPROTECTION CONDITIONS DURING THE SYNTHESIS OF THE PEPTIDE GLY-HIS-ALA-LEU-GLY

✉ Hilda E Garay, Luis J González, Luis J Cruz, Regla C Estrada and Osvaldo Reyes

*Division of Physical Chemistry, Center for Genetic Engineering and Biotechnology, PO Box 6162, Havana 10600, Cuba. E-mail: osvaldo.reyes@cigb.edu.cu*

**ABSTRACT**

The imidazole ring of histidine has caused problems in solid-phase peptide synthesis. The basic feature of this group requires its protection in order to avoid racemization. The cleavage of the dinitrophenyl (Dnp), a protecting group of the imidazole ring of histidine, under 9-fluoroenylmethoxycarbonyl (Fmoc-) deprotection conditions (20 % piperidine in dimethylformamide (DMF) is reported. This Dnp cleavage becomes particularly important if the combined use of Boc/Bzl and Fmoc/tBu strategies is required in peptide synthesis. In order to demonstrate the Dnp cleavage, a pentapeptide (GHALG) was synthesized by the Solid-Phase method. The cleavage of the Dnp group was carried out by the standard procedure and by the treatment of 20 % piperidine in DMF. Pauly's test, high performance liquid chromatography and mass spectrometry were used to demonstrate the Dnp cleavage. The amino acid analysis showed 84 % of the Dnp cleavage.

*Key words:* solid phase peptide synthesis, side reaction, dinitrophenyl cleavage, histidine, Dnp

*Biotecnología Aplicada 1997;14:193-195*

**RESUMEN**

El grupo imidazol de la cadena lateral de la histidina ha causado problemas en la síntesis en fase sólida de péptidos. El carácter básico de este grupo hace necesario su protección para evitar reacciones colaterales durante la síntesis. En nuestro trabajo se reporta la eliminación del dinitrofenilo (Dnp), grupo protector del imidazol de la histidina, bajo las condiciones de desprotección del grupo Fmoc- (20 % piperidina/DMF). Esta reacción colateral de eliminación del Dnp bajo las condiciones de desprotección del Fmoc- es perjudicial cuando se combinan las dos estrategias Boc/Bzl y Fmoc/t-But en la síntesis de péptidos. Para demostrar la eliminación del Dnp bajo las condiciones de eliminación del Fmoc-, se sintetizó un pentapétido (GHALG) en fase sólida. Se eliminó el Dnp utilizando el procedimiento convencional y por tratamiento con 20 % piperidina/DMF. La eliminación del Dnp con 20 % piperidine/DMF se demostró por la prueba de Pauly, por cromatografía líquida de alta resolución y por espectrometría de masas. El análisis de aminoácidos mostró un 84 % de eliminación del Dnp.

*Palabras claves:* síntesis de péptidos en fase sólida, reacción colateral, eliminación del dinitrofenilo, histidina, Dnp

## Introduction

Peptide synthesis has emerged as one of the most powerful tools in biochemical, pharmacological, immunological, and biophysical laboratories. During solid phase peptide synthesis, the protection of the reactive amino acid side chains is required in order to obtain the correct sequence.

The imidazole ring of histidine has caused problems in solid-phase peptide synthesis. The basic character of this group requires its protection in order to avoid racemization (1). On the other hand, an unprotected imidazole ring is generally acylated during the coupling reaction, resulting in branched peptide chains. Furthermore, acylimidazoles are good acylating agents and can promote the acyl-transfer side reaction to other nucleophilic sites on the peptide, resulting in several by-products and amino acid insertions (2, 3).

✉ Corresponding author

The amino group of the imidazole ring can be blocked by several protecting groups, such as: $N^{im}$-p-toluenesulfonyl (Tos), $N^{im}$-benzyl (Bzl), $N^{im}$-2.4-dinitrophenyl (Dnp), $N^{im}$-t-butyloxycarbonyl (Boc) and others (4). The Dnp group suppresses the weak basic feature of the imidazole ring and therefore avoids the side reactions caused by this characteristic (5).

The Dnp group can be cleaved by thiolysis (6) with thioglycolic acid, 2-mercaptoethanol, dithiotreitol or thiophenol and other nucleophiles (7) such as hidrazine, thiocyanates ($SCN^-$), thiolates ($RS^-$). We found that the Dnp group can also be removed by 20 % piperidine in DMF, the deprotection conditions used to cleave the Fmoc- protecting group of α-amino acids during the Fmoc/tBu synthesis. This Dnp cleavage becomes particularly important if the

1. Pessi A, Mancini V, Filtri P, Chiappinelli L. Side reactions in solid phase synthesis of histidine-containing peptides. Int J Peptide Protein Res 1992; 39:58-62.

2. Fields GB, Noble RL. Solid phase synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Peptide Protein Res 1990;35:173-174.

3. Kusunoki M, Nakagawa S, Seo K, Hamana T, Fukuda T. A side reaction in solid phase synthesis. Insertion of glycine residues into peptide chains. Int J Peptide Protein Res 1990;36:381-386.

4. Barany G, Merrifield RB. The peptides: analysis, synthesis, biology. Gross E and Meienhofer J, editors. Academic Press, New York 1980;2:179-189.

5. Bodanszky M. Principles of peptide synthesis. Springer-Verlag, Berlin Heidelberg, Inc. Second edition 1993:154.

combined use of Boc/Bzl and Fmoc/tBu strategies is required. For example, to obtain the side chain of lysine modified by acetylation, biotinylation or with lipoic acid, the Boc-Lys (Fmoc)-OH derivative can be used. In this case, when the Fmoc- group is removed to acylate the side chain of lysine, the Dnp protecting group of histidine can be removed, the free imidazole ring can be acylated, and can promote the acyl transfer side reaction. Another example could be the synthesis of a multiple antigen peptide containing two different epitopes (8). In this case, the Boc-strategy is used for the first epitope synthesis and the Fmoc- strategy for the second epitope synthesis. If Dnp is used as the imidazole protecting group of histidine, to synthesize the first peptide, it can be removed during the Fmoc- synthesis of the second peptide.

In order to demonstrate the cleavage of the Dnp group by 20 % piperidine in DMF, a peptide was synthesized and the amino acid analysis was used to quantify the Dnp cleavage.

## Materials and Methods

### Peptide synthesis

A pentapeptide (GHALG) was synthesized by the Solid-Phase method to demonstrate the cleavage of the Dnp group by 20 % piperidine in DMF (9). Synthesis was performed on a 4-methylbenzhydrylamine (MBHA) resin (1 mmol/g, 100-200 mesh), using N-α-Boc-protection for amino acids and Dnp as a protecting group for the histidine side-chain. Coupling reactions were carried out using diisopropylcarbodiimide. The Boc deprotection was carried out by 37.5 % trifluoroacetic acid (TFA) in dichloromethane (DCM) during 30 min. Neutralization was accomplished by three successive two-min treatments with 5 % diisopropylethylamine (DIEA) in DCM. After the last coupling, and before the N-α-Boc-terminal deprotection, the peptide-resin was divided into two identical parts. One was treated with 20 % thiophenol in DMF, following the standard procedure (10) to cleave the Dnp group: the Boc-protected peptide-resin was treated with 20 % thiophenol in DMF twice during 1 h, and washed with DMF, 2-propanol and DCM ten times for 1 min each. The other was treated with 20 % piperidine in DMF for 30 min. The peptide-resin was cleaved with hydrogen fluoride (HF) (90 %): Anisole (10 %) during 1 h at 0 °C and the peptide was extracted in 30 % acetic acid.

### High performance liquid chromatography purification

The crude peptides obtained from each experimental procedure were analyzed by reverse-phase high performance liquid chromatography (HPLC) using an RP-Vydac (4.6 x 150 mm) column with a linear gradient from 0 % to 40 % of TFA/acetonitrile (0.05 %) during 30 min.

### FAB mass spectrometry

The eluted fractions in both chromatograms were characterized by Fast Atom Bombardment (FAB) mass spectrometry. The FAB spectra of peptides were obtained on a JMS-HX110HF two-sector mass spectrometer (JEOL, Japan), equipped with a FAB ion source and the data-acquisition system JMA DA-5000. The samples were dissolved in m-nitrobenzyl alcohol on the probe tip and bombarded with a Xenon beam accelerated at 6 kV in the ion source. The $(M+H)^+$ ions were accelerated at -10 kV and detected with a secondary electron multiplier. The resolution was set to 1000.

### Pauly's test

Pauly's test (11) was used to evaluate the deprotection of a imidazole nucleus of histidine, since the imidazole coupled with diazotized sulphanilic acid yields a red product. On the contrary, the blocked imidazole does not react. Cold equal parts of 5 % sodium nitrite in $H_2O$ and sulphanilic acid (0.5 % in diluted HCl) were mixed (mix No. 1). One milliliter of 10 % sodium carbonate was added to 1 mL of the sample (mix No. 2). Finally, 1 mL of mix No. 2 was added to 1 mL of mix No. 1.

### Amino-acid analysis

The samples were hydrolyzed for 24 h at 110 °C with 6N HCl. Norleucine was used as an internal standard for quantification.

## Results and Discussion

Peak No. 1 of Figure 1A has the same retention time (14.4 min) as peak No. 1 in Figure 1B. These are the peptides with the correct sequence because there

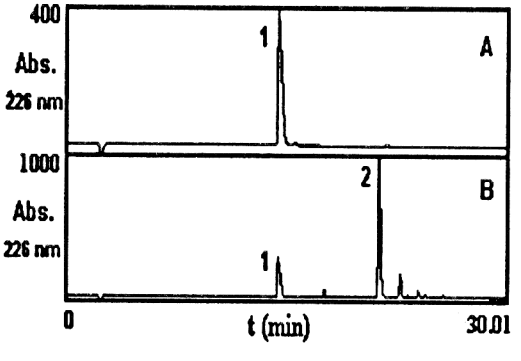


Figure 1. A) HPLC profile of the peptide treated with 20 % thiophenol in DMF; B) HPLC profile of the peptide treated with 20 % piperidine in DMF. Column: RP-C18 Vydac (4.6 x 150 mm). Linear gradient: 0 % to 40 % of TFA/acetonitrile (0.05 %) during 30 min.

6. Shaltiel S. Thiolysis of some dinitrophenyl derivatives of amino acids. Biochem Biophys Res Commun 1967;29: 178-183.

7. Greene TW, Wuts PGM. Protective groups in organic synthesis. John Wiley & Sons Inc. Second edition 1991:449-450.

8. Tam JP, Yi-An Lu. Vaccine engineering: enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. Proc Natl Acad Sci USA 1989;86:9084-9088.

9. Houghten RA, de Graw ST, Bray MK. Simultaneous multiple peptide synthesis: The rapid preparation of large numbers of discrete peptides for biological, immunological and methodological studies. BioTechniques 1986;4: 522-526.

10. Applied Biosistems Inc. Introduction to cleavage techniques. 1990.

11. Morrow, Stewart J, Dillaha, Young J. Solid phase peptide synthesis. Pierce Chemical Company, Rockford, Illinois. Second edition, 1983:114.

were identities between the $[M+H]^+$ experimental and theoretical mass (m/z 453.2 Da) (Figure 2A). Pauly's qualitative test of both peaks was positive, which evidenced the presence of a free imidazole ring of histidine. The presence of peptides with free imidazole rings (Figure 1B) evidences the cleavage of the Dnp group with 20 % piperidine in DMF.

The FAB mass spectrum (Figure 2B) of peak No. 2 in Figure 1B showed a signal at m/z 619.3 Da. This signal was 166 Da higher than the theoretical mass (m/z 453.2 Da) and this increase corresponded with the mass of the Dnp molecule. Besides, Pauly's qualitative test of this peak was negative, which evidenced Dnp-imidazole protection.

In order to quantify the Dnp cleavage by 20 % piperidine in the DMF treatment, peak No. 1 in Figure 1B, corresponding to the peptide with the free imidazole ring and peak No. 2, corresponding to the peptide with the Dnp blocked imidazole (Figure 1B), were collected and quantified by amino acid analysis. This analysis showed 84 % Dnp cleavage.

The cleavage of the Dnp group was demonstrated by the treatment of 20 % piperidine in DMF. The resulting cleavage was 84 %.

*Received in December, 1996. Accepted for publication in April, 1997.*

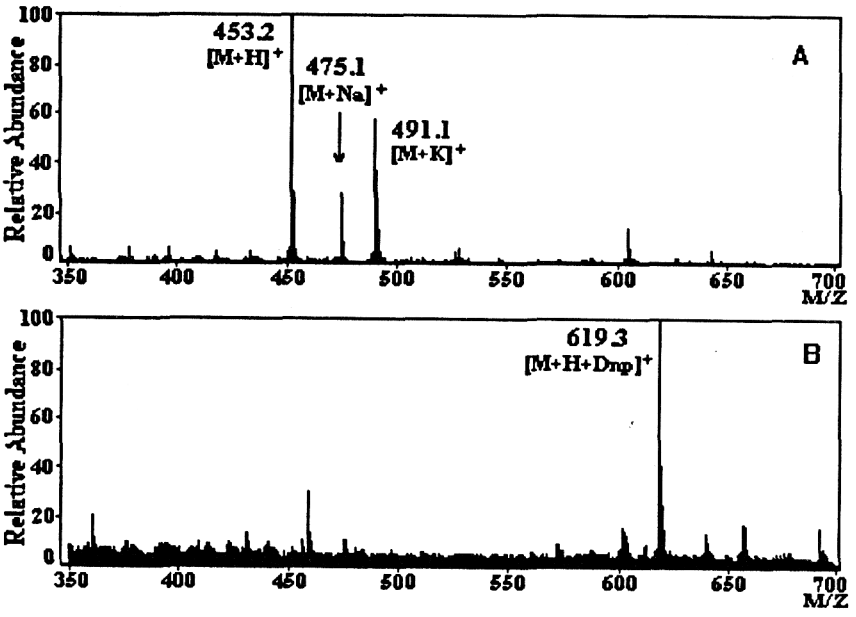


Figure 2. A) Mass spectra of peak No. 1 on Figure 1A and 1B; B) mass spectra of peak No. 2 on Figure 1B. The samples were dissolved in m-nitrobenzyl alcohol on the probe tip and bombarded with a Xenon beam accelerated at 6 kV in the ion source. The $(M+H)^+$ ions were accelerated at -10 kV and detected with a secondary electron multiplier. The resolution was set to 1,000.