# The Preparation and Properties of some 2,4-Dinitrophenyl Dipeptides and 2,4-Dinitrophenyl Amides

by

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## Abstract

2,4-Dinitrophenyl glycyl-L-tryptophan, 2,4-dinitrophenyl glycyl-L-proline, 2,4-dinitrophenyl glycyl-L-phenylalanine, 2,4-dinitrophenyl glycyl-L-glutamic acid, 2,4-dinitrophenyl glycyl amide, 2,4-dinitrophenyl glycyl anilide, 2,4-dinitrophenyl glycyl-p-toluide, 2,4-dinitrophenyl-L-alanyl amide, 2,4-dinitrophenyl-L-alanyl anilide, 2,4-dinitrophenyl-L-alanyl-p-toluide were prepared in order to studies their properties. For the 2,4-dinitrophenyl dipeptides, two methods of preparation were used, each giving an identical product. (2,4-Dinitrophenyl glycyl-L-glutamic acid was difficult to recrystallize when prepared from 2,4-dinitrophenyl glycyl chloride.) The melting points of the above compounds were determined on a Gallenkamp melting point apparatus. No racemization occurred when the 2,4-dinitrophenyl dipeptides were prepared from 2,4-dinitrophenyl glycyl chloride.

Ultraviolet absorption spectra of these substances were obtained in 1 cm silica cuvettes with a Beckman DK spectrophotometer using $5X10^{-5}$ molar sodium bicarbonate solution or in 95% ethanol. Their infrared spectra were measured with a Model No. 21 Perkin-Elmer spectrophotometer (equipped with sodium chloride optics) using Nujol mulls. Molar rotations $[M]_D$ were calculated as specific rotations multiplied by the molecular weight and divided by 100. Specific rotations were obtained in a Kern Full Circle polarimeter, using a two-decimeter tube and adjusting the concentration (in 4% sodium

bicarbonate solution, 95% ethanol, acetone, or glacial acetic acid), so that the angular rotation was always more than 0.2° and usually between 0.5 to 1.0°.

The behaviour of the 2,4-dinitrophenyl dipeptides on thin-layer chromatography and their electrophoretic behaviour were studied; both methods gave good separation of the above 2,4-dinitrophenyl dipeptides.

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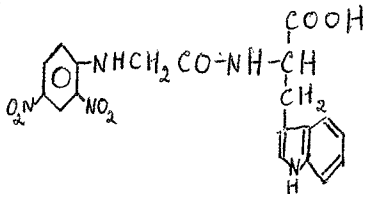
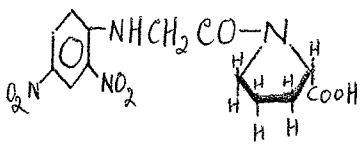
## Introduction

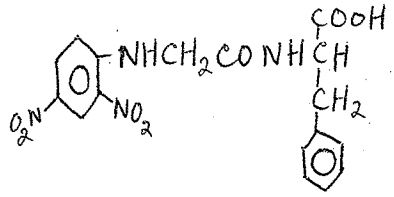
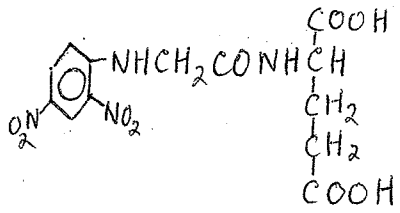
The method most widely employed for the identification of N-terminal amino acids of proteins and peptides was elaborated by Sanger(1) and is based on the reaction of N-terminal $\alpha$-amino groups with 2,4-dinitrofluorobenzene(FDNB)[1].

The present study was undertaken in order to obtain more knowledge about the properties of 2,4-dinitrophenyl(DNP)[2] dipeptides. The DNP-glycyl and DNP-L-alanyl amides also have been prepared for the first time in order to obtain further means of characterising the respective DNP acid chlorides.

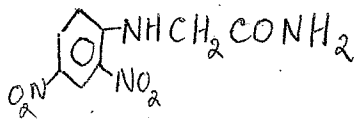
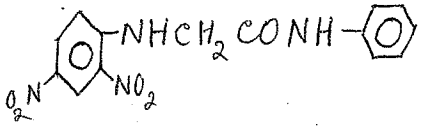
The melting points, neutral equivalents, molar rotations, paper electrophoretic behaviour, infrared and ultra-violet absorption spectra, and thin-layer chromatography behaviour were determined for four DNP-dipeptides whose structural formulae are shown below.

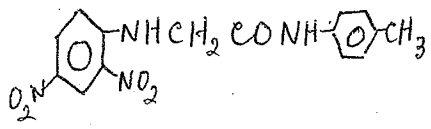
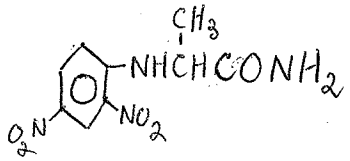
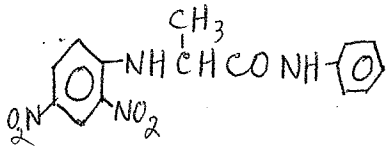
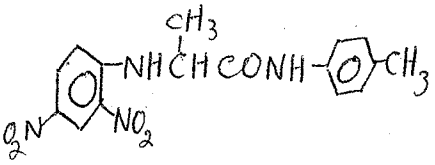
| DNP-dipeptides | Structure |
|---|---|
| DNP-glycyl-L-tryptophan |  |
| DNP-glycyl-L-proline |  |

(1) FDNB will be used to designate 1-fluro-2,4-dinitrobenzene.
(2) DNP will be used to designate the 2,4-dinitrophenyl radical.

| DNP-dipeptide | Structure |
|---|---|
| DNP-glycyl-L-phenylalanine |  |
| DNP-glycyl-L-glutamic acid |  |

In addition, the melting point, the molar rotation, infrared and ultra-violet absorption spectra were determined for all DNP-glycyl and DNP-L-alanyl amides whose structural formulae are shown below.

| DNP-amide | Structure |
|---|---|
| DNP-glycyl amide |  |
| DNP-glycyl anilide |  |

| DNP-amide | Structure |
|---|---|
| DNP-glycyl-p-toluide |  |
| DNP-L-alanyl amide |  |
| DNP-L-alanyl anilide |  |
| DNP-L-alanyl-p-toluide |  |

IN order to study the properties of these DNP-derivatives, it was first necessary to prepare them. For the DNP-dipeptides, two methods of preparation were used. For the others, the DNP-acid chloride was reacted with ammonia or the appropriate amine.

~~(1) FDNB will be used to designate 1-fluro-2,4-dinitrobenzene.~~

~~(2) DNP will be used to designate the 2,4-dinitrophenyl radical.~~

## Literature Review

For the purpose of identification and estimation of the N-terminal amino acid residues of peptides and proteins, DNP-derivatives of amino acids have been prepared by many workers since 1910, in the field of end group and amino acid analysis.

In 1910, DNP-derivatives of glycine, DL-alanine, DL-valine, DL-leucine and L-asparagine were first prepared by Abderhalden and Blumberg,(2,3) using 1-chloro-2,4-dinitrobenzene to react with the free amino group in aqueous sodium bicarbonate solution. In 1934, Saunders(4) prepared a derivative by the action of 1-chloro-2,4-dinitrobenzene on cysteine, which he reported was difficult to crystallize, and showed that 1-chloro-2,4-dinitrobenzene reacts more readily with -SH groups than with amino groups.

Later, F. Sanger(1) found that FDNB was a more suitable reagent, capable of reacting quantitatively at room temperature with the free amino groups of proteins and peptides. The procedure comprises shaking the amino acid with a twofold excess of FDNB and an equal weight of sodium bicarbonate in 67% ethanol(by volume) for two hours at room temperature, followed by evaporation of the ethanol, dilution with water, and extraction of the excess FDNB with ether. Acidification then yields the required DNP-amino acids.

The crystalline DNP-derivatives of many L-amino acids were prepared for the first time by Krishnarau R. Rao and H. A. Sober (5) in 1953. The method used was that employed by Sanger(1).

They found that the melting points of the DNP-derivatives of optically active amino acids differed in many instances from those of the racemic form and that the molecular rotation of the optically active derivative was much larger than that of the parent amino acid and peptide.

In 1954, Anthony L. Levy and David Chung(6) found a simplified procedure for the synthesis of DNP-amino acids by modifying Sanger's original method. They found that several advantages result from using aqueous solutions at a slightly elevated temperature(40°) and from employing only an equivalent amount of FDNB. A more rapid reaction can be achieved, ethanol evaporation and extraction of excess FDNB can be eliminated and a pure product results with greater economy of reagents.

W. A. Schroeder and Joann Le Gette(7), and W. A. Schroeder and L. R. Honnen(8) in 1953 reported the preparation of a few DNP-peptides, to study their behaviour in column chromatography. Yields and other properties were not reported.

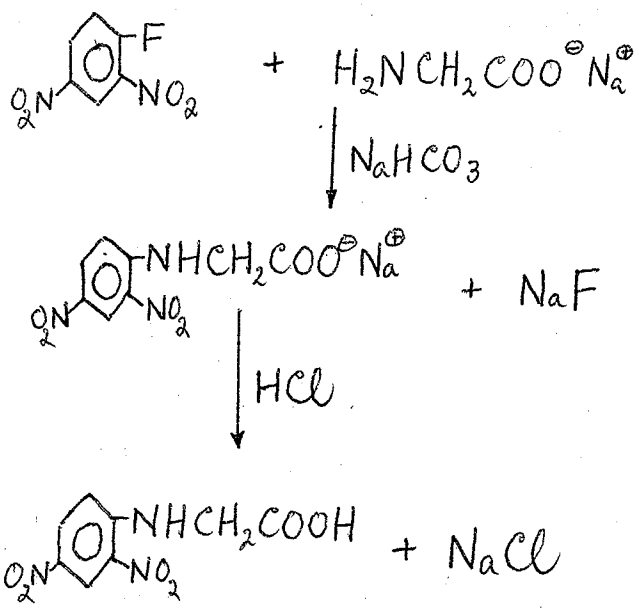
The first systematic study of the preparation and properties of DNP-peptides was undertaken by J. H. Loudfoot and J. E. Kruger (9) and reported in 1963.

## Experimental and Results

### Preparation of Starting Materials

#### 2,4-Dinitrophenyl Glycine

DNP-glycine was required for the preparation of DNP-glycyl chloride. It was prepared by the method of Sanger(1), except that one equivalent FDNB was used per equivalent of amino acid, as reported by Levy and Chung(6). The equations for the overall reaction are as follows:




Materials:

| | |
|---|---|
| Glycine | 2.00gm (0.026mole) |
| FDNB | 5.00gm (0.026mole) |
| Sodium bicarbonate | 21.8gm (0.26 mole) |
| Water | 180ml |
| Ethanol (95%) | 210ml |

Glycine and sodium bicarbonate were dissolved in the water

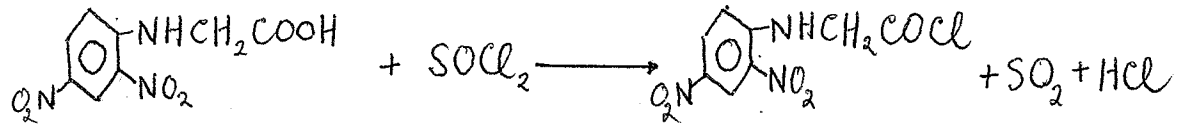
and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature, then concentrated to remove ethanol by vacuum distillation below 40°; the residue was dissolved in water and acidified with concentrated hydrochloric acid to pH 2, which precipitated a yellow crystalline solid. The mixture was refrigerated overnight to precipitate additional product. The crystals were filtered with suction, washed with ice cold water to remove excess hydrochloric acid, stored for 24 hours in a vacuum desicator, and heated at 100° to constant weight.

The yield of product was 6.20 gm(96.2% of theory).

The melting point was 205-206° with decomposition. (literature 205°(2,9,10), 206°(12).)

## 2,4-Dinitrophenyl Glycyl Chloride

DNP-glycyl chloride was required as a starting material for one of the methods of preparing DNP-glycyl peptides, also for the preparation of DNP-glycyl amides. It was prepared by the method of J. H. Loudfoot and J. E. Kruger(9, 10). The overall equation is shown below:




**Materials:**

| | |
|---|---|
| DNP-glycine | 1.205gm (0.005 mole) |
| Thionyl chloride | 10ml |

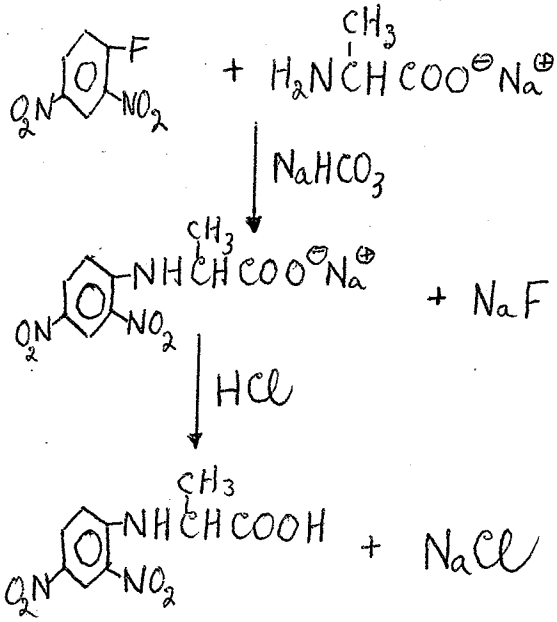
Glycine and thionyl chloride were heated under reflux on a water bath until a clear solution was obtained(20-40 minutes). The solution was then heated for an additional 30 minutes, and the excess thionyl chloride was removed by vacuum distillation using a water bath. Large yellow, needlelike crystals were formed. Throughout the preparation, a calcium chloride tube was attached to the condenser in order to exclude moisture.

The yield of product was 1.28gm (99%) of theory).

The melting point was 129-129.5° (Literature 129-129.5°(9, 10).)

## 2,4-Dinitrophenyl-L-Alanine

DNP-L-alanine was required for the preparation of DNP-L-alanyl chloride. The equations for the overall reaction are as follows:



Materials:

| | |
|---|---|
| L-Alanine | 0.89gm. (0.01 mole) |
| FDNB | 1.86gm. (0.01 mole) |
| Sodium bicarbonate | 8.40gm. (0.10 mole) |
| Water | 70 ml. |
| Ethanol(95%) | 90 ml. |

The preparation was carried out in the same manner as DNP-glycine prepared from the FDNB and glycine. The yellow crystalline product was usually of such purity as not to required recrystallization. For some preparations, the crude product was recrystallized from dilute ethanol.

The yield of product was 2.44gm (95.7% of theory).

The melting point was 178-179°.(Literature 178°(2, 12), 173°(11), 177°(5).)

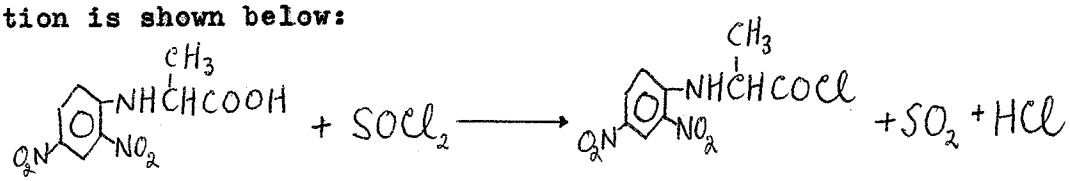
$[\alpha]_D^{22}$ = +16.4°, $[M]_D$ = +41.8° (c= 2% in 95% ethanol)

$[\alpha]_D^{22}$ = -12.5°, $[M]_D$ = +31.85° (c= 3% in acetone)

(Literature $[\alpha]_D^{20}$ = +14.3° (c= 2% in 6N HCl) (22), $[M]_D$ = +367° in N NaOH, $[M]_D$ = +39° in glacial acetic acid (5).)

## 2,4-Dinitrophenyl-L-Alanyl Chloride

DNP-L-alanyl chloride was required as a starting material for the preparation of DNP-L-alanyl amides. The overall equation is shown below:




Materials:

| | |
|---|---|
| DNP-L-alanine | 1.02gm (0.004 mole) |
| Thionyl chloride | 10 ml |

The preparation was carried out in the same manner as DNP-glycyl chloride from DNP-glycine. The product was a red-brown liquid. The yield was 1.08 gm(99% of theory).

## Preparation of 2,4-Dinitrophenyl Dipeptides

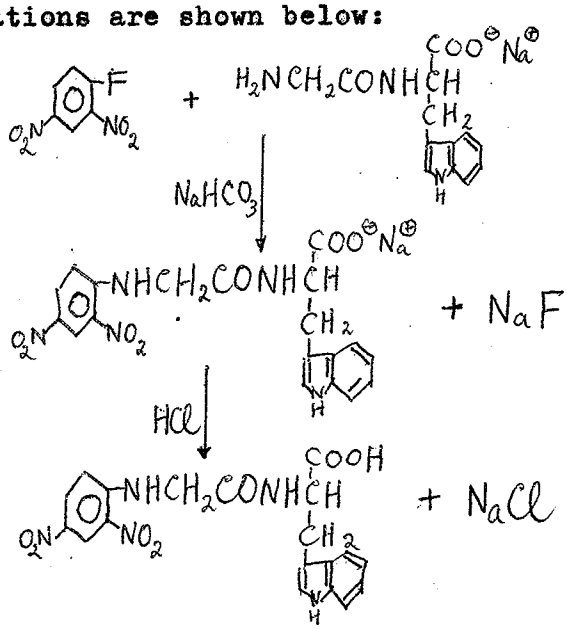
Two methods of preparing DNP-glycyl peptides are reported here as reported by J. H. Loudfoot and J. E. Kruger(9, 10).

1) By reacting FDNB with the pertinent dipeptide.
2) By reacting DNP-glycyl chloride with the pertinent amino acid.

## 2,4-Dinitrophenyl Glycyl-L-Tryptophan

### From the Dipeptide

DNP-glycyl-L-tryptophan was prepared by the method employed by Sanger(1), except one equivalent of FDNB was used for per equivalent glycyl-L-tryptophan as reported by Levy and Chung(6). The overall equations are shown below:



Materials:

| | |
|---|---|
| Glycyl-L-tryptophan | 0.261gm (0.001 mole) |
| FDNB | 0.186gm (0.001 Mole) |
| Sodium bicarbonate | 0.746gm (0.009 mole) |
| Water | 13 ml. |
| Ethanol | 16 ml. |

Glycyl-L-tryptophan and sodium bicarbonate were dissolved in the water and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature, then concentrated to remove ethanol by vacuum distillation below 40°; the residue was dissolved in water and acidified with concentrated hydrochloric acid to pH 2, which precipitated an orange crystalline solid. The mixture was then refrigerated overnight to precipitate additional product. The crystals were filtered with suction, washed with ice cold water to remove excess hydrochloric acid, stored for 24 hours in a vacuum desicator and heated at 100° to constant weight.

The crude product was dissolved in hot ethanol, adding hot water to cause turbidity and cooling slowly. The mixture then left in the refrigerator for a few hours, and the yellow crystals were then filtered with suction and dried at 100° to constant weight.

The yield of the yellow product was 0.286gm. (67% of theory)

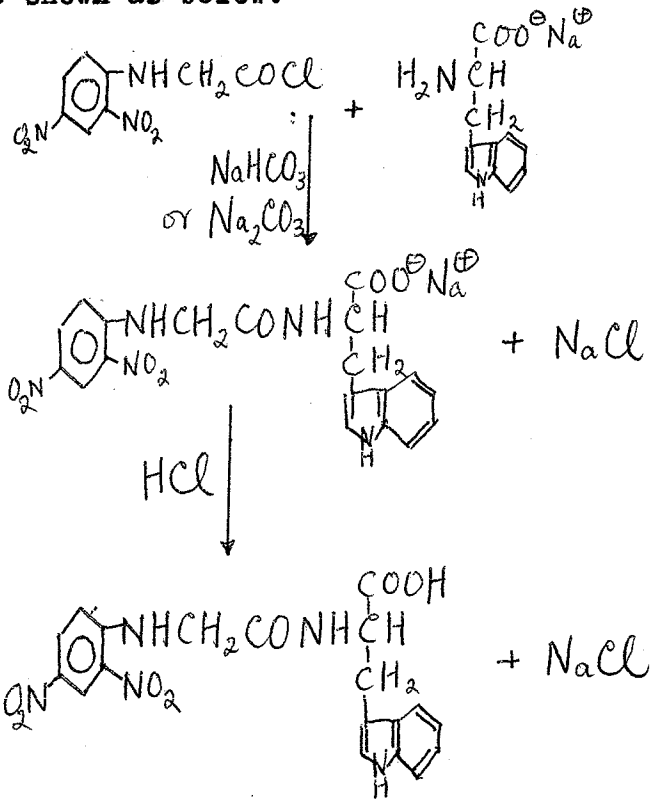
The melting point was 233-234° with decomp. and 190-227° when mixed with DNP-glycine.

$[\alpha]_D^{22} = +20°$, $[M]_D = +85.4°$ (c= 0.5% in 95% ethanol)

$[\alpha]_D^{22} = +26.2°$, $[M]_D = +112.0°$ (c= 0.5% in acetone)

**From DNP-glycyl Chloride**

L-Tryptophan and DNP-glycyl chloride were reacted together in sodium bicarbonate or sodium carbonate medium, followed by acidification with hydrochloric acid. The equations for the overall reaction are shown as below:




**Materials:**

| | |
|---|---|
| DNP-glycyl chloride | 0.519gm. (0.002 mole) |
| L-Tryptophan | 0.408gm. (0.002 mole) |
| Sodium bicarbonate | 1.67 gm. (0.02 mole) |
| Water | 30 ml. |
| Ethanol | 10 ml. |

L-Tryptophan and sodium bicarbonate were dissolved in the water and to this slowly added a solution of DNP-glycyl chloride

in benzene over a two hours period with constant stirring at ice-bath temperature. Stirring at ice-bath temperature was continued a further two hours. The mixture was then transferred to a separatory funnel and allowed to stand two hours for separation of the layers. The lower aqueous layer was removed and acidified with 4 c. c. of concentrated hydrochloric acid, which precipitated an orange coloured solid. In order to precipitate the product which remained in solution, the mixture was then left overnight in the refrigerator. The crystals were then filtered with suction, and washed with ice-cold water to remove excess hydrochloric acid. The product was dried by storing overnight in a desicator and heating at 100° to constant weight.

The orange crude product melted below 130°. Recrystallization was effected by dissolving the crystals in hot ethanol, adding hot water to cause turbidity and cooling slowly. The mixture then left in the refrigerator for a few hours, and the yellow crystals were then filtered with suction and dried to constant weight.

The yield of product was 0.58 gm. (67.5% of theory).

The melting point was 233-234°, 233-234° when mixed with DNP-glycyl-L-tryptophan prepared from the dipeptide, and 190-225° when mixed with DNP-glycine.

$[\alpha]_D^{22} = +20.0^\circ$ $[M]_D = +85.4^\circ$ (c= 0.5% in 95% ethanol)

$[\alpha]_D^{22} = +26.5^\circ$ $[M]_D = +113.0^\circ$ (c= 1.5% in acetone)

$[\alpha]_D^{22} = -60.0^\circ$ $[M]_D = -256.0^\circ$ (c= 0.2% in 4% sodium bicarbonate)

The neutral equivalent was 434.38, theoretical molecualar weight was 427.

Analysis:

Based on $C_{19}H_{17}O_7N_5$ M.W. = 434.38.

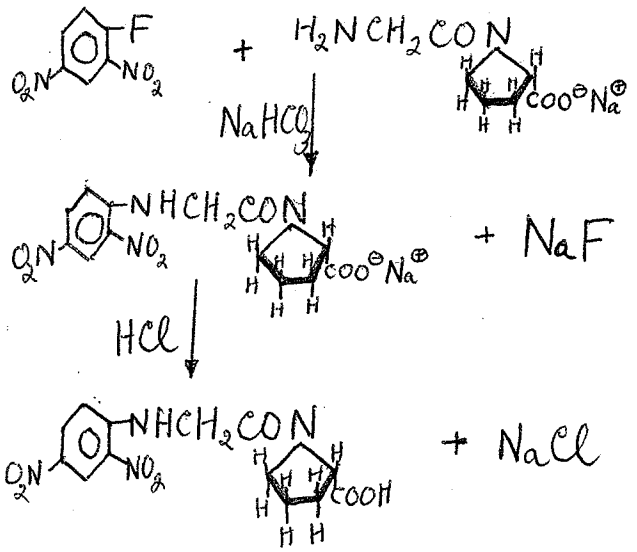
| | | | |
|---|---|---|---|
| Calculated: | C= 53.40% | H=4.00% | N=16.39% |
| Found: | C=53.40% | H=3.89% | N=16.00% |

All analyses were performed by Geller Laboratories, Charleston, West Virginia.

## 2,4-Dinitrophenyl Glycyl-L-Proline

From the Dipeptide

The equations for the overall reaction are as follows:




Materials:

| | |
|---|---|
| Glycyl-L-proline | 0.172 gm. (0.001 mole) |
| FDNB | 0.186 gm. (0.001 mole) |
| Sodium bicarbonate | 0.84 gm. (0.01 mole) |
| Water | 10 ml. |
| Ethanol | 14 ml. |