THE UNIVERSITY OF MANITOBA

THE PREPARATION AND PROPERTIES OF 2,4-DINITROPHENYL-PEPTIDES, AND THE POSSIBLE USE OF THESE COMPOUNDS TO DETERMINE PEPTIDE AND PROTEIN MOLECULAR WEIGHTS BY SIMPLE MEANS.

by

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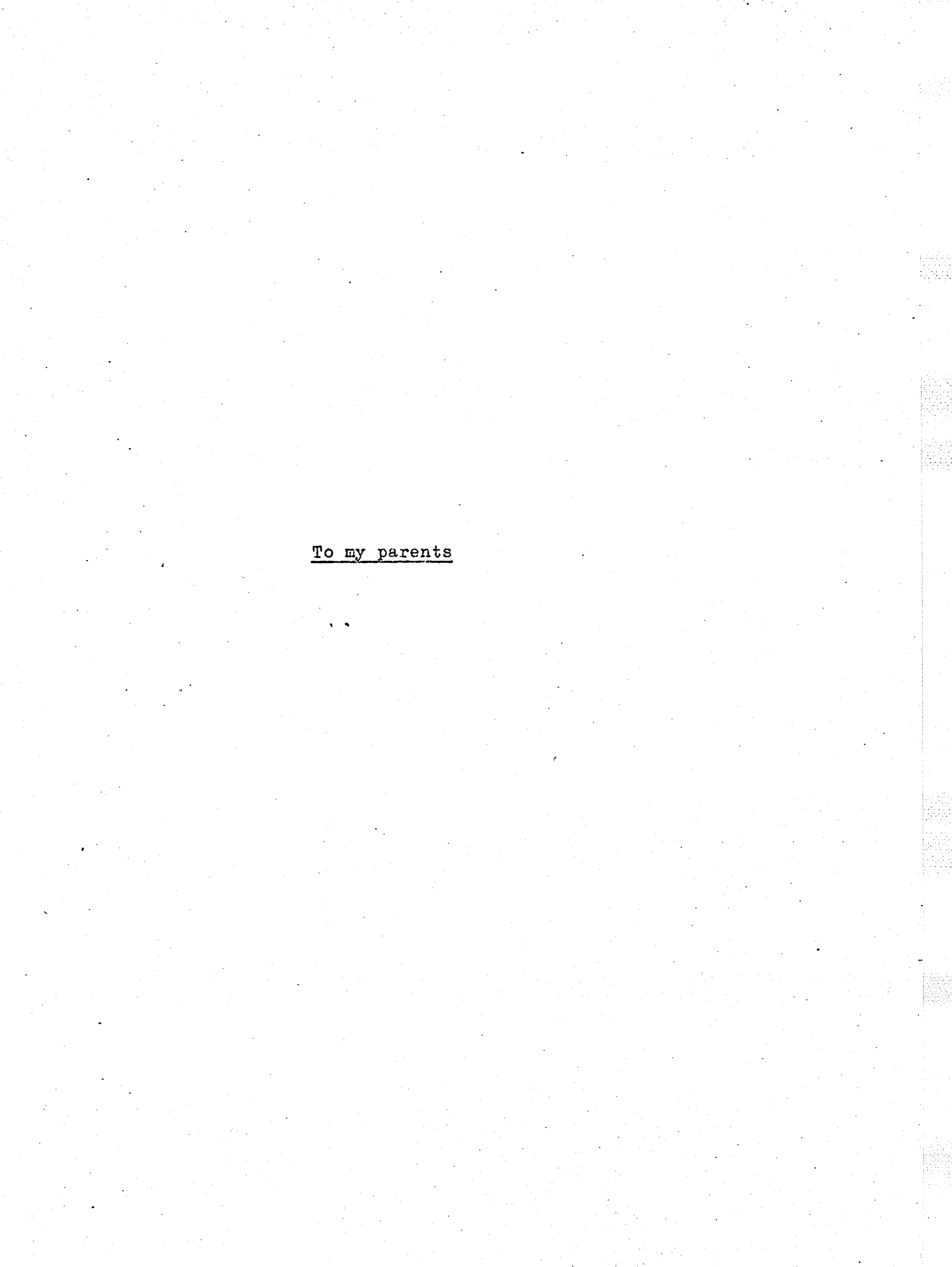

To my parents

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Abstract

Separation and identification of intact peptides in the form of their 2,4-dinitrophenyl (DNP) derivatives has been studied, using thin layer chromatography. Thin layer chromatography has been found very effective for this purpose. Fifteen DNP-dipeptides and six DNP-tripeptides were used for these studies. The molar extinction coefficients of the above DNP-peptides were calculated.

The possibility of using freezing point depression as a means of calculating the molecular weights of intact peptides (as their DNP-derivatives) has also been studied, testing the method on five DNP-dipeptides, three DNP-tripeptides and DNP-poly-L-valine. The method has been shown suitable for direct molecular weight determination of the dipeptide and tripeptide derivatives to $\pm$ 2% of the correct value (frequently much closer) down to 5×10^{-3} molal concentration, representing some 1.5×10^{-4} mole of DNP-peptide handled. When applied to a mixture of the test DNP-peptides (representing some twenty amino acid residues), the molality of the solution was found to be within $\pm$ 1.5% of the correct value. The method has been found unsuitable for direct molecular weight determination of intact poly-L-valine (as DNP-poly-L-valine). Instead, the molecular weight of intact poly-L-valine (as DNP-Poly-L-valine) was determined spectrometrically (a); the DNP-poly-L-valine was then hydrolysed completely, the DNP-L-valine was separated, and the molecular weight of the original polymer calculated indirectly (b) by spectrometric determination of the amount of DNP-L-valine obtained, and (c) from freezing point depression of a solution of the dinitrophenylated hydrolysate. Results from (b) differed from (a) by one or more amino acid residues; results from (c) were consistent

with those from (a) to within about half an amino acid residue.

Because the DNP-peptides required for these studies are not available commercially and have not been previously prepared, their preparation had to be undertaken. Coupling the DNP-L-amino acyl chlorides with sodium salts of amino acids in aqueous medium was found unsuitable for the compounds studied (poor yield, poor quality), therefore alternative means of preparation were necessary. Hydrolysis of the corresponding ethyl esters was found satisfactory. Three methods of preparing these esters were studied. Since the esters were also unknown compounds, their preparation and the preparation of various intermediates (acid chlorides, hydrazides) required for their synthesis also had to be undertaken.

Throughout the thesis, individual amino acids and peptides are mentioned by their full name, but in tables and in discussion, abbreviations are used for the amino acid residues in naming peptides (i. e., glycine = gly, alanine = ala, valine = val, leucine = leu, isoleucine = Ileu, phenylalanine = phe, tryptophan = try).

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Part A

Introduction

Identification of N-terminal amino acids of proteins and peptides by reaction of N-terminal α-amino groups and other free amino groups with 1-fluoro-2,4-dinitrobenzene (FDNB) was elaborated by Sanger (1). The dinitrophenylated protein (DNP-protein) could then be hydrolyzed in strong acid to yield a mixture of amino acids and 2,4-dinitrophenylamino acids (DNP-amino acids). The latter were relatively resistant to hydrolysis, and could be separated and identified, therefore the N-terminal amino acid residues which bore the free amino group in the original protein molecule could be identified.

The DNP-amino acids produced by hydrolysis of the DNP-protein have been studied extensively. Various DNP-amino acids have been prepared by many workers (1-5); the characteristics of those synthetic DNP-amino acids were first recorded in 1910 by Abderhalden and Blumberg. A number of the optically active DNP-L-amino acids were prepared for the first time by Rao and Sober (6), Levy and Chung (7) and Fraenkel-Conrat, Harris and Levy (8). However, much less attention has been given to the DNP-peptides which are formed either by partial hydrolysis of a DNP-protein or by the direct dinitrophenylation of the partial hydrolysates of proteins.

The purpose of the present work was to prepare and purify some DNP-dipeptides and DNP-tripeptides, in order to study the possibility of separating and identifying intact peptides and of determining molecular weights of peptide fragments in solution by freezing point depression principles. Direct dinitrophenylation of the dipeptides and tripeptides gave the desired DNP-peptides in fairly good quantity and quality, but the dipeptides and tripeptides are much more expensive

and less readily available than the amino acids, therefore, other methods are required to prepare the DNP-peptides.

Coupling of an amino acid in aqueous solution (mildly basic) with a DNP-L-aminoacyl chloride has been useful (9) (equation 1), but presented difficulties in this work. DNP-L-valyl chloride, DNP-L-leucyl chloride and DNP-L-isoleucyl chloride were hydrolysed extensively, giving poor yields of DNP-peptide; DNP-L-phenylalanyl chloride was hydrolysed so extensively that it could not be used for this method of synthesis. Hydrolysis of DNP-peptide ethyl esters was subsequently found to be a more suitable means of obtaining the DNP-peptides.

<u>Equation 1</u>

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COCl + H_2NCH(R')COONa \xrightarrow{Na_2CO_3} O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)CONHCH(R')COONa + NaCl$$

Three methods of preparing the required DNP-peptide ethyl esters were studied, viz:- (a) Reacting the DNP-L-aminoacyl chloride with an amino acid ester in presence of a tertiary base in a non-aqueous solvent (10) (equation 2); (b) The method of Bossard et al (11) (equation 3); (c) The carbodiimide method of Sheehan and Hess (12, 13) (equation 4). Method (a) could not be used for preparing the

DNP-L-tryptophyl peptide esters, because attempted conversion of DNP-L-tryptophan to its chloride only resulted in tar formation. Bossard's method (b) involves formation of N,N-dimethyl chloroformimidium chloride which Zaoral and Arnold (14) showed to be suitable for peptide synthesis. It converts the DNP-L-amino acid to its chloride in dimethylformamide solution, which is then added without isolation to a dimethylformamide solution of amino acid ester containing two moles of tertiary base. This method was suitable for making the DNP-L-tryptophyl esters and also those which had been made by (a). The carbodiimide method (c) of Sheehan and Hess (12, 13) has been used frequently in peptide synthesis and was used in this work to prepare DNP-peptide esters from a DNP-amino acid or DNP-peptide and an amino acid ester in a non-aqueous solvent. Contamination of a peptide by dicyclohexyl urea, frequently most difficult to remove and proving to be a considerable obstacle to purification of product has been reported in the literature (15, 16), and resolved by using instead 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate (16). Such difficulty was encountered only in preparing DNP-glycyl glycine ethyl ester and DNP-L-tryptophyl glycine ethyl ester, and in preparing the DNP-tripeptides. In all other cases the use of dicyclohexyl carbodiimide as condensing agent was suitable; to prepare the two DNP-dipeptide ethyl esters mentioned and the DNP-tripeptide esters, the difficulty was resolved by using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate; no advantage in its use was found when used to prepare those compounds which were prepared satisfactorily from dicyclohexyl carbodiimide.

Equation 2

$$2,4\text{-}(O_2N)_2C_6H_3\text{-}NH\overset{R}{\overset{|}{C}}HCOCl + H_2N\overset{R'}{\overset{|}{C}}HCOOC_2H_5 \xrightarrow{(C_2H_5)_3N}$$

$$2,4\text{-}(O_2N)_2C_6H_3\text{-}NH\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOOC_2H_5 + (C_2H_5)_3N\cdot HCl$$

Equation 3

$$(H_3C)_2N-\overset{O}{\overset{\|}{C}}-H + SOCl_2 \longrightarrow \left[(H_3C)_2N=C\begin{matrix}Cl\\H\end{matrix}\right]^{\oplus} Cl^{\ominus} + SO_2$$

$$\xrightarrow{2,4\text{-}(O_2N)_2C_6H_3\text{-}NH\overset{R}{\overset{|}{C}}HCOOH} 2,4\text{-}(O_2N)_2C_6H_3\text{-}NHCHCOCl + (H_3C)_2N\overset{O}{\overset{\|}{C}}-H + HCl$$

$$\xrightarrow[2(C_2H_5)_3N]{H_2N\overset{R'}{\overset{|}{C}}HCOOC_2H_5} 2,4\text{-}(O_2N)_2C_6H_3\text{-}NH\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOOC_2H_5 + 2\ (C_2H_5)_3N\cdot HCl$$

Equation 4

$$\text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)COOH} + C_6H_{11}\text{-N=C=N-}C_6H_{11} \longrightarrow \text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)CO-O-C(=N-}C_6H_{11})\text{-NH-}C_6H_{11}$$

Path A, via $H_2NCH(R')COOC_2H_5$:

$$\text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)CONHCH(R')COOC}_2H_5 + C_6H_{11}\text{-NH-CO-NH-}C_6H_{11}$$

Path B, via $\text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)COOH}$:

$$\left(\text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)CO}\right)_2O + C_6H_{11}\text{-NH-CO-NH-}C_6H_{11}$$

then, via $H_2NCH(R')COOC_2H_5$:

$$\text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)CONHCH(R')COOC}_2H_5 + \text{2,4-}(O_2N)_2C_6H_3\text{-NHCH(R)COOH}$$

Because the azide coupling method first developed by Curtius (17-25) has often proved so satisfactory for peptide synthesis, preparation of the DNP-tripeptide esters by this method was attempted. Use of this method required preparation of the appropriate DNP-dipeptide hydrazide by hydrazinolysis of the corresponding DNP-dipeptide ethyl ester in alcoholic solution. Sometimes the solution only needed to stand a short time at room temperature, in other cases longer reaction times, higher temperatures and a large excess of hydrazine hydrate was required. The reactions can be expressed in equation (5).

Equation 5

$$(O_2N)(NO_2)C_6H_3NHCH(R)CONHCH(R')COOC_2H_5$$

$$\downarrow H_2NNH_2$$

$$(O_2N)(NO_2)C_6H_3NHCH(R)CONHCH(R')CONHNH_2 + C_2H_5OH$$

$$\downarrow HNO_2$$

$$(O_2N)(NO_2)C_6H_3NHCH(R)CONHCH(R')CON_3 + 2\,H_2O$$

$$\downarrow H_2NCH(R'')COOC_2H_5$$

$$(O_2N)(NO_2)C_6H_3NHCH(R)CONHCH(R')CONHCH(R'')COOC_2H_5 + HN_3$$

The DNP-dipeptide hydrazides were rather insoluble, easily crystallizable compounds and very stable.

In order to prepare the azides, the corresponding hydrazides were dissolved in a mixture of acetic acid and hydrochloric acid. After cooling to about -10°, the calculated amount of sodium nitrite was added in the form of a concentrated aqueous solution. Conversion of the hydrazides to the azides occurs almost instaneously. The acid solution was diluted with ice-water and the azide was extracted with ethyl acetate, the organic layer was then washed with ice-cold sodium bicarbonate solution to remove the acids and was dried prior to the reaction with the amino acid ester. All operations had to be carried out in a rapid sequence and at low temperatures.

The coupling step followed immediately the preparation of the azide, and required 24-48 hours at 5° for coupling to be completed.

Side reaction with the azide method was observed, with formation of urea derivatives during the coupling reaction. This may be due to a Curtius rearrangement of the azide to isocyanate, which would then react with the added amino component to form the corresponding urea derivatives (21, 26-28). The azide coupling method was found to be less satisfactory for preparing the DNP-tripeptide esters. The reactions can be expressed in equation (6).

The free DNP-dipeptides were obtained by alkaline hydrolysis of the corresponding esters (29). The hydrolysis was carried out with a small excess of aqueous alkali in absolute alcohol solution for 4 to 8 hours at room temperature. The base was added in small portions. Lower temperatures and longer reaction times were required in some cases.

Equation 6

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\underset{}{CH}(R)CONHCH(R')C(=O)\text{-}N=\overset{\oplus}{N}=\overset{\ominus}{N}$$

$$\downarrow -N_2$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)CONHCH(R')C(=O)\text{-}\ddot{\underset{\cdot\cdot}{N}}$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)CONHCH(R')\text{-}N=C=O$$

$$\downarrow H_2NCH(R'')COOC_2H_5$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)CONHCH(R')NHCONHCH(R'')COOC_2H_5$$

Literature Survey

Methods of N-terminal residue analysis

As early as 1910, Abderhalden and Blumberg (2) used 1-chloro-2,4-dinitrobenzene (CDNB) in a sodium bicarbonate medium to prepare a number of amino acid derivatives.

$$O_2N\text{-}C_6H_3(NO_2)\text{-}Cl + H_2NCH(R)COOH \longrightarrow O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COOH + HCl$$

Subsequent attempts to use CDNB for the identification of N-terminal amino acids of a protein or peptide were unsuccessful. In 1923, Abderhalden (30) attempted to use CDNB for the detection of free amino groups in a partial hydrolysate of silk fibroin, but did not meet with much success, chiefly owing to the presence of anhydrides in the hydrolysate and the difficulties of separating the products. Furthermore, heat was required to react the CDNB with the amino groups in sodium bicarbonate solution, causing some hydrolysis of the protein. Sanger (1) in 1945 replaced CDNB by 1-fluoro-2,4-dinitrobenzene (FDNB) which reacted smoothly with proteins and peptides at room temperature in alcohol-aqueous bicarbonate mixture with the formation of 2,4-dinitrophenyl (DNP) derivatives. The dinitrophenylation of N-terminal amino acid residues then played a central role in the elucidation of the structure of the peptide chains of insulin, and became a well-established procedure in protein chemistry.

Sanger's method for the identification and estimation of the N-terminal residue as applied to insulin consisted of three stages. The protein was treated with FDNB, hydrolyzed, and the resulting coloured compounds separated chromatographically. Identification

of these was based on band rates, and was confirmed by mixed chromatograms. Secondly, knowing which DNP-derivatives were present, the amount of each could be assessed with reasonable accuracy by separating the fraction quantitatively and estinating the material present colorimetrically, using the pure DNP-amino acid as a standard. Thirdly, the whole operation was carried out on a large scale, so that the DNP-amino acids could be isolated and satisfactorily characterized.

Although Sanger's method has been used extensively for the N-terminal analysis of peptides and proteins, four major factors have created special problems for many workers. First, it was found that on hydrolysing the DNP-protein, some DNP-amino acids are broken down by hot hydrochloric acid, (Table 1), therefore, correction factors had to be introduced for their instability, and were based on the recovery of these compounds after various periods of hydrolysis.

Table 1

Approximate breakdown of DNP-amino acid on acid hydrolysis*.

Compounds†	Per cent break down
Bis-DNP-cystine	100
DNP-glycine	50
DNP-hydroxyproline	50
DNP-phenylalanine	50
DNP-proline	50
DNP-tryptophan	100
DNP-tyrosine	50

* 16 hours with 12 N HCl at 105

† DNP-amino acids not included are altered to the extent of 25% or less under these conditions.

Secondly, most of the DNP-amino acids lost the dipolar ion character of the parent amino acids due to the N-substitution, and were soluble in ether, hence ether extraction from the protein hydrolysate was satisfactory in these cases. However, some N-terminal amino acids such as α-mono-DNP-arginine, di-DNP-histidine, α-mono-DNP-lysine, α-mono-DNP-ornithine, α-mono-DNP-diaminobutyric acid and non N-terminal amino acids such as ε-mono-DNP-lysine, imidazole-DNP-histidine (colourless), O-DNP-tyrosine (colourless), S-DNP-cysteine (colourless), δ-mono-DNP-ornithine and γ-mono-DNP-diaminobutyric acid are insoluble in ether, remained in the aqueous layer, and had to be extracted by some suitable solvent (i. e. 2-butanol/ ethyl acetate (1:1).

The third factor was the effect of the reagent on the various groups in the protein. FDNB reacted not only with the free amino groups, but also with the imino group of proline, the phenolic group of tyrosine, the sulfhydryl group of cysteine, and the imidazole group of histidine. The native protein generally exists in a coiled or folded conformation, in which the free reactive groups on the α- or ω-carbon atoms may be all or only partly exposed to the FDNB, varying with different proteins. However, when the protein is fully denatured, all the available groups will react with the reagent.

Finally, the fourth factor was the stability of the peptide linkages during the reaction between protein and FDNB. If the

protein is not completely stable during the dinitrophenylation, the internal bonds open to yield new reactive amino groups to the reagent, and hence together with the true N-terminal DNP-amino acid, there will appear one or more DNP-amino acids originating from the points of breakage in the protein molecule. The stoichiometry of the results obtained is often of help in this problem, for if very close to one residue of DNP-amino acid is obtained per mole of protein, together with only traces of other DNP-amino acids, it may assumed the peptide linkage is stable. If, However, appreciable amounts of two or more DNP-amino acids are obtained per mole of protein, the problem of deciding whether the results are valid and representative of two or more N-terminal peptide chains in the molecule, or whether they may spring from secondary causes, could well be difficult to solve.

Preparation of DNP-Amino Acids

The FDNB procedure has required the use of reference standards; the DNP-amino acids derived from the protein have to be compared with the synthetic DNP-amino acids. Many workers used different methods to prepare DNP-amino acids. Inasmuch as pure L-amino acids were not available in quantity before 1950, only DNP-DL-amino acids have been prepared as reference standards. However, the DNP-amino acids isolated from acidic or neutral digests of proteins would be expected to be of the L-variety, and it is better to prepare the DNP-L-amino acids as reference standard; this not only eliminates doubt on the chromatographic criteria comparison, but also permits

measurement of specific rotation as another criterion of comparison. For these reasons, Rao and Sober (6) prepared a number of optically active DNP-L-amino acids by Sanger's method. The procedure of the method is described as follows:

They shook the amino acid with FDNB in the presence of a slight excess of sodium bicarbonate for 2 to 5 hours in 50% ethanol at room temperature. (it is recommanded that this reaction as well as all stages of the preparation be carried out in the dark). The alcohol was removed at room temperature and the excess of FDNB extracted by shaking three times with ether. The aqueous solution was acidified with 6 N hydrochloric acid, and the precipitated DNP-amino acid washed repeatly with small quantities of ice-cooled water.

One year later, Levy and Chung (7) suggested a modified method for preparing DNP-amino acids. They found that several advantages result from working in an aqueous solution at slightly elevated temperature (40°) and by using only an equivalent amount of FDNB, a more rapid reaction could be achieved, ethanol evaporation and extraction of excess FDNB could be eliminated, a pure product resulting with greater economy of reagents. Their procedure is described below:

The amino acid (10 m moles) and anhydrous sodium carbonate (2 gm.) were dissolved in 40 ml. of water at 40°, FDNB (10 m moles) was added, and the mixture vigorously agitated, the temperature being maintained at 40°. The small drops of FDNB in suspension disappeared after 30-90 minutes marking the end of the reaction. The orange solution

was acidified with concentrated hydrochloric acid (3 ml) to pH 2, the precipitated DNP-amino acid washed repeatly with small amounts of ice-cold water, and was recrystallized by special solvent mixtures.

Dinitrophenylation of an amino acid mixture

Various methods of dinitrophenylating the amino acid mixture from the protein hydrolysate have been described. The reaction can be carried out in either an aqueous or an aqueous alcoholic medium.

Coupling in aqueous medium: According to Levy et al (31) and later modified by Wallenfels (32), the dry hydrolysate (2-5 mg) was dissolved in 2 ml CO_2-free water at room temperature with vigorous stirring. An aliquot (1.2 ml) was pipetted into a small reaction vessel equipped with a magnetic stirrer, was diluted with 1.8 ml CO_2-free water, 0.1 ml 3.1 N KCl was added, and the solution was heated to $40 \pm 0.1^{\circ}$. The pH was maintained at 8.9 by the addition of 0.2 N NaOH with vigorous stirring. Approximately 0.1 ml FDNB was added in a small excess in the absence of light, and the pH held at 8.9 for 100 minutes by means of an autotitrator. The reaction kinetics can be followed by automatic recording of the alkali uptake. After the reaction was terminated, the excess FDNB was removed by extracting twice with 5 ml each of peroxide free ether, and the water soluble DNP-amino acids extracted repeatedly with sec-butanol/ethyl acetate (1:1).

Fraenkel-Conrat and Singer (33) have also described a coupling reaction in 5% carbonate buffer at pH 9.3 during 3 hours at 40°.

The quantity of FDNB was 1.5 μl per 2 mg of amino acids.

Coupling in alcohol solution: According to Fraenkel-Conrat and Singer (33) and Lucas et al (34), the dry hydrolysis residue (3-5 mg) was dissolved in 5 ml water. This solution was treated with 100 mg $NaHCO_3$ and a solution of 100 mg FDNB in 8 ml ethanol. The single-phase mixture obtained was allowed to stand for 3 hours at room temperature in darkness. The excess of FDNB was extracted with ether after evaporation of most of the alcohol, then acidified with concentrated hydrochloric acid to pH 1-2, and extracted five times with peroxide-free ether. The residual aqueous phase was extracted repeatedly with sec-butanol/ethyl acetate (1:1).

Biserte et al (35) carried out the reaction with FDNB in an aqueous alcoholic medium according to the following technique. The hydrolysate (10 mg) was dissolved in 5 ml of double distilled water, which was brought to and maintained at 40°. The solution was adjusted to pH 9 with N/15 NaOH and 0.2 ml of FDNB was added, the solution was stirred for 15 minutes at 40° while the pH was maintained at 9. Ten ml of absolute alcohol were added and stirring was continued for 90 minutes at 40°, the pH still being kept at 9. After the reaction, the alcohol was removed by evaporation in a current of cold air and the excess of FDNB extracted several times with peroxide-free ether. The solution was acidified (1 ml of concentrated hydrochloric acid) and again extracted with peroxide-free ether, then with ethyl acetate. The residual aqueous phase was extracted with a mixture of equal parts of ethyl acetate and sec-butanol.

Dinitrophenylation of free amino acids in biological specimens is also described by many workers. Amino acids in urine, blood and sperm were dinitrophenylated in alcohol solution with a carbonate buffer at pH 8.8 for 1 hour at 40° (36-38).

Dinitrophenylation of a protein

Dinitrophenylation of a protein was first described by Sanger (1). Coupling could be effected with native, denatured or oxidized proteins. The protein (0.5 gm) and sodium bicarbonate (0.5 gm) were dissolved in 5 ml of water; to the solution were added 10 ml of a 5% (V/V) ethanol solution of FDNB, and the mixture was agitated mechanically for 2 to 3 hours in the dark at room temperature. It requires a prolonged period of agitation (48 hours at 40°, 72 hours at 20°) and repeated additions of sodium bicarbonate and FDNB for insoluble proteins. After complete dinitrophenylation, the solution was acidified, the precipitated DNP-protein was centrifuged and washed with water, acetone and ether.

Levy and Li (39) have described a coupling reaction in aqueous medium maintained at pH 8 by means of an autotitrator. The protein (0.2 gm) was dissolved in 3 ml of 0.1 M potassium chloride at 40°. The pH was adjusted and maintained at 8 by addition of 0.05 N KOH. After the addition of FDNB (0.1 ml), the solution was agitated vigorously for 2 hours. After complete dinitrophenylation, the solution was acidified, the precipitated DNP-protein centrifuged and washed with water, acetone and ether.

According to Phillips (40), a more satisfactory yield of terminal groups is obtained by carrying out the dinitrophenylation in a solution made from potassium bicarbonate and guanidine hydrochloride. The protein was dissolved in a solution of 6 M guanidine (protein concentration 20 mg/ml), and to this solid potassium bicarbonate was added in a concentration of 10-15 mg/ml and FDNB in a concentration of 0.05-0.1 ml/ml. The mixture was stirred for 6 to 24 hours at 20°, acidified and diluted with three volumes of water. The precipitated DNP-protein was centrifuged and washed with water, acetone and ether.

Dinitrophenylation of a peptide

In practice the dinitrophenylation of a peptide may be carried out in exactly the same way as that of a protein. However, certain methods are especially suitable for this coupling reaction. According to Sanger and Thompson (41), substitution of trimethylamine for sodium bicarbonate results in a diminution of the ionisation of the medium; moreover, this reagent can be conveniently removed afterwards. The peptide (0.2 μmole) was dissolved in 0.1 ml of 1% trimethylamine. A solution of 10 μl of FDNB in 0.2 ml of ethanol was added, after 2 hours contact, a few drops of water and of the trimethylamine solution were added and the excess of FDNB was removed by extraction three times with ether. The solution was evaporated in vacuum to dryness.

To reduce the formation of dinitrophenol, Lockhart and Abraham (42) replaced trimethylamine by trimethyl ammonium carbonate and

proceeded as follows: The peptide (50-150 μg) was dissolved in 0.1 ml 1.5% (g/v) trimethyl ammonium carbonate solution (pH 9.3), 0.2 ml of a 5% alcohol solution of FDNB was added, and the mixture allowed to stand in darkness for 2½ hours; the ethanol was evaporated in vacuum, the product treated with 0.24 ml trimethyl ammonium carbonate solution and 1 ml ether, followed by mixing with a vibromixer, centrifuging in order to separate the liquid phase, separation of the ether (discard) and evaporation of the aqueous solution in vacuum to dryness.

Waley (43) suggested a method somewhat similar to the foregoing with a buffer of trimethylamine carbonate obtained by treatment of a 6% (v/v)solution of trimethylamine with carbon dioxide until alkaline to phenol red, but neutral to phenolphthalein. Under these conditions, the dinitrophenylation was conducted at a slightly lower pH than in the method of Lockhart and Abraham. A small amount of dinitrophenol was formed.

Regeneration of amino acids from their DNP-derivatives

During the early studies of chromatographic separation of DNP-amino acids, it was necessary to establish their identity by reconversion of DNP-amino acids to free amino acids. Several methods were developed. Mills (44) first brought about regeneration by heating the DNP-derivatives in dilute sulfuric acid containing hydrogen peroxide; 2,4-dinitrophenol was produced as a by-product. This method was not well adapted to the micro scale; a more satisfactory technique involved heating the compound with saturated

baryta water in a sealed tube. All the common occurring amino acids were recovered in this way, except histidine and cystine, of which very small yields are obtained. DNP-arginine after hydrolysis gives rise to several extraneous ninhydrin-positive substances, and the recovered threonine was also accompanied by an artefact. Lowther (45) modified Mills method by heating the DNP-derivatives with ammonia in a sealed tube, and found that all amino acids were recovered, but the yields were very poor. In 1959, Liebold and Braunitzer (46) treated the DNP-amino acids with hydriodic acid at 100° and recovered the free amino acids in yields up to 80%. However, the hydroxyl groups of serine and threonine were removed in this reaction; furthermore, some amino acids were recovered in a poor yield, and the method was not suitable for DNP-peptides. Therefore, Fasold and his coworkers (47, 48) regenerated the free amino acids by hydrogenation. The hydrogenation of the DNP-amino acids and DNP-peptides was performed in glacial acetic scid or methanol using a large excess of the platinum catalyst. The previously blocked amino groups were obtained in the free form in yields up to 90%. DNP-methionine was split only in the form of its sulfone, and DNP-cysteine was split as the sulfonic acid derivative. A very simple method was recommended by Macek (49), in which the dry sample of DNP-amino acids was dissolved in anhydrous hydrazine and heated for 1 hour at 80°, the free amino acids were recovered.

The advantage of converting the DNP-compoundsto the amino acids

was that the chromatographic behavior of the acids was well-known and more reliable than that of the DNP-compounds. However, as further work with the DNP-derivatives progressed, separations became better and the regeneration of amino acids became unnecessary.

Chromatographic separation of DNP-derivatives

Sanger's original work on the free amino groups of insulin (1) included an attempt to separate the DNP-amino acids obtained on complete hydrolysis of the DNP-protein. He employed three chromatographic procedures. The separation of DNP-derivatives by adsorption chromatography was not very successful, since decomposition of the compounds occurred on magnesium oxide or alumina columns. The use of partition chromatography on filter paper or starch was not very satisfactory, due to "tailing" of the spots or bands. The most successful separations were obtained with column partition chromatography using a stationary aqueous phase adsorbed on the silica gel and a moving organic phase. Mono amino acid derivatives are well separated by using glycol or aqueous ethanol or acetone as the stationary phase, and a non-polar solvent in equilibrium with it as the moving phase. However, the success of this method was highly dependent on the batch of silica gel used. In an attempt to resolve this difficulty, Blackburn (50) and Middlebrooke (51) modified the method by using concentrated phosphate buffered columns of silica gel. By varying pH, they found that the rate of movement of a band of a given DNP-amino acid could be varied within wide limits, the higher pH giving the slower rate. No significant

variation occurred in the rate of movement of the band of a given DNP-amino acid between different batches of silica gel, and the difficultly in preparing a suitable gel did not arise. Also by using a single solvent with columns buffered to different pH values, the number of DNP-amino acids which can be separated is greater than on the unbuffered column.

Green and Kay (52) described a method for the separation and identification of sixteen ether-soluble DNP-amino acids by "adsorption" chromatography on silicic acid "Celite". DNP-amino acids were rather strongly adsorbed on prewashed silicic acid columns and formed compact zones which moved at a reasonable rate when developed with glacial acetic acid in petroleum ether (ligroin). This method had the advantage of being much faster than the partition methods, and fewer chromatograms were required for the complete resolution of a mixture. Satisfactory results were obtained on different batches of silicic acid from several sources.

One of the first chromatographic investigations of DNP-peptides arose as a result of the studies made on the partial hydrolysates of gelatin. This study showed that the separation of unknown peptides would be greatly facilitated by a study of the chromatographic behavior of known DNP-peptides. According to Schroeder et al (55, 56), a variety of known DNP-peptides were chromatographed and they were able to deduce some generalizations which permitted the prediction of the chromatographic behavior of known DNP-peptides. This aided in the identification of tentatively identified DNP-peptides through the comparison of determined and predicted behavior.

In addition to the above methods, a variety of other techniques for the separation of mixtures of DNP-amino acids have been described in the literature. Paper chromatography has been used extensively. Early workers found difficulty in the separation and identification of the compounds because of tailing of the spots, but Blackburn and Lowther (55) observed that DNP-amino acids were successfully separated on buffered one-dimensional paper chromatograms. The paper is soaked in phthalate buffer and dried at room temperature, compact spots without "tailing" and possessing characteristic rates (R_f values) being formed in suitable solvents.

Instead of using a phthalate buffer system, Pairent and Williamson (56) separated the DNP-amino acids on a sulfate-phthalate buffer system. They found that the separation of the DNP-amino acids was essentially the same as with 1.5 M phosphate buffer, but a more distinct spot with sharper boundaries was obtained for most of the DNP-amino acids.

Two dimensional paper chromatography employing a "toluene" solvent and 1.5 M phosphate buffer (57, 58) is considered the most satisfactory for the separation of ether soluble DNP-amino acids normally obtained from proteins. The DNP-amino acids are subjected to ascending chromatography in the first direction with the toluene-pyridine-chloroethanol-0.8 M ammonia (5:1.5:3:3) mixture, and descending chromatography with 1.5 M pH 6 phosphate in the second direction. Most of the ether soluble DNP-amino acids can be separated by this method, with the exception of dicarboxylic DNP-amino acids (DNP-aspartic acid, DNP-glutamic acid). These two derivatives can be

separated by means of a second chromatogram, using the "toluene" system in the first dimension and a phosphate buffer of higher concentration (2.5M) in the second dimension. However, the "toluene" solvent system has been criticized on two points. First, it is not convenient to use this solvent in laboratories where either nitrogen or colorimetric ninhydrin determination are being carried out routinely. Secondly, the two phase "toluene" system requires several hours of equilibration before use and then the organic phase used for the development of the chromatogram is not stable.

Braunitzer (59) has replaced the toluene-pyridine-chloroethanol mixture with the n-butyl alcohol-0.1% ammonia (1:1) system, and achieved satisfactory resolution of all DNP-amino acids by two dimensional chromatography in combination with 1.5 M phosphate.

Phillips (40) has also suggested a two dimensional procedure in which 2-butanol or tert-amyl alcohol or 2-methyl butan-2-ol saturated with 0.05 M phthalate buffer of pH 6 is used in the first dimension, and 1.5 M phosphate buffer of pH 6 is used in the second dimension.

In 1961, Brenner et al (60) first introduced an improved method for the identification of DNP-amino acid by application of thin layer chromatography using Silica G. They found the water soluble DNP-amino acids can be chromatographed most favorably with solvents which consist of a mixture of an alcohol (n-propanol or n-butanol) and ammonia solution. The ether soluble DNP-amino acids are separated by two dimensional chromatography on Silica G layer, the "toluene" solvent of Biserte et al (57) being used in the first dimension, and the solvent systems of chloroform, alcohol, glacial acetic acid or

benzene (toluene), pyridine, glacial acetic acid in the second dimension. This method has considerable advantages over paper chromatography, giving excellent sharpness of separation, high sensitivity and great speed.

Since then, thin layer chromatography has become increasingly popular for the rapid separation and identification of DNP-amino acids. Instead of Silica gel G, several other adsorbents were used. Fittkau et al (61) used Supergel layers and Munier et al (62, 63) used cellulose layers, all giving good separation. Recently, studies have been published dealing with thin-layer chromatography of ether-soluble DNP-compounds on polyamide. Wang et al (64, 65) described an excellent separation of 31 DNP-amino acids together with 2,4-dinitrophenol and 2,4-dinitroaniline by polyamide or by polyester film supported polyamide layer chromatography. They found the polyamide layer, especially with the polyester film supported, gave less diffuse spots and better separation than the Silica gel G.

Photosensitivity of DNP-compounds

The instability of DNP-amino acids when exposed to light was first pointed out by Sanger (66) and Blackburn (50), who found that DNP-amino acids tended to decompose if exposed to sunlight on the column. Solutions of DNP-amino acids in solvents such as chloroform also tended to decompose when exposed for long periods to light, particularly sunlight. The solutions then gave rise to additional bands on the chromatogram. Mills (67) suggested that during the

course of the work, it is necessary to protect the DNP-amino acids from light at all stages of their preparation and separation. Rao and Sober (6) reported that the yields of DNP-glutamic acid and DNP-aspartic acid were increased if light were excluded at all stages of preparation.

Photodecomposition of DNP-amino acids has been studied by Akabori et al (68), who showed that several α-DNP-amino acids in aqueous solution were decomposed by light at about the same rate, but did not identify the products. They found that ε-DNP-lysine was photostable. Pollara and von Korff (69) using ^{14}C-labelled DNP-amino acids, observed that many of these compounds in the solid state are decarboxylated under the influence of light and converted into the corresponding DNP-alkylamines.

Russel (70-72) irradiated a solution of DNP-L-leucine in dilute sodium bicarbonate solution. On extracting the solution with ethyl acetate, he obtained an unknown compound which he later proved to be 4-nitro-2-nitrosoaniline. The same compound was also obtained from an irradiated solution of DNP-glutamic acid; subsequent acidification and extraction of the aqueous phase furnished 3-formyl propionic acid, isolated in 70% yield as 2,4-dinitrophenyl hydrazone. The reaction scheme is shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(COOH)CH_2CH_2COOH \xrightarrow{h\nu} O_2N\text{-}C_6H_3(NO_2)\text{-}NH_2 + OCHCH_2CH_2COOH + CO_2$$

Further work on DNP-L-leucine showed that irradiation produced carbon dioxide and 3-methyl butyraldehyde in addition to 4-nitro-

2-nitrosoaniline. The reaction may be shown as below:

$$O_2N-C_6H_3(NO_2)-NHCH(COOH)CH_2CH(CH_3)CH_3 \xrightarrow{h\nu} O_2N-C_6H_3(NO)-NH_2 + OCHCH_2CH(CH_3)CH_3 + CO_2$$

DNP-L-leucine was rapidly decomposed by light at any pH, 4-nitro-2-nitrosoaniline was formed only at pH 6 or above. The rate of reaction in dilute solution was independent of the concentration, so that the process is an intramolecular oxidation-reduction accompanying decarboxylation.

The reaction was found fairly general for DNP-α-amino acids, although not all decomposed in the same way. A large group formed 4-nitro-2-nitrosoaniline in high yield, some others gave a lower yield of 4-nitro-2-nitrosoaniline, together with other (unidentified) products, and a few decomposed without formation of the nitros compound. The presence in the amino acid side chain of a group such as hydroxyl, sulfoxide, etc. permits side reaction, so the usual product was formed to a smaller extent or not at all. Similar compounds, in which the amino group was not in the α-position or the carboxyl group was not free, were relatively stable to light. DNP-peptides are decomposed much more slowly than α-DNP-amino acids.

The unidentified compound from the irradiated DNP-amino acid solution was identified by Neadle and Pollitt (73), as a 2-substituted-6-nitro-benzimidazole-1-oxide. The formation of this compound by photolysis at a suitable pH appears to be a general reaction for DNP-α-amino acids with an α-hydrogen atom. The reaction scheme

is shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COOH \xrightarrow[-CO_2,\,-RCHO]{h\nu} O_2N\text{-}C_6H_3(NO)\text{-}NH_2 \quad (I)$$

$$\downarrow h\nu,\ -CO_2,\ -H_2O$$

$$(II) \rightleftharpoons (III)$$

(I)

(II)

(III)

In neutral aqueous solution, they exist in the N-protonated form (II), whereas in ethanol and a variety of other organic solvents, they exist in the O-protonated form (III).

Many α-DNP-amino acids undergo photolysis in aqueous solution to give a mixture of mainly 4-nitro-2-nitrosoaniline and 2-substituted-6-nitro-benzimidazole-1-oxides are stable to further visible irradiation, and the 4-nitro-2-nitrosoaniline slowly undergoes secondary photolytic reactions. The factors which influence the proportions of the different products formed by the photolysis of DNP-amino acids have been investigated. The nature of the light source is not critical as long as the main absorption band at ca 360 mμ is activated. The temperature of the photolysis mixture

has a slight effect on the proportions of the products, but the main factor is the pH of the reaction mixture, the products varying with pH in an unusually complicated manner.

Experimental and Results

Preparation of starting materials

2,4-Dinitrophenyl-L-amino acids

These were required as starting materials for conversion to acid chlorides (9), for reaction with N,N-dimethyl chloroformimidium chloride in Bossard's method (11), and for use in Sheehan's carbodiimide method (12, 13), and were prepared by Levy and Chung's (7) modification of Sanger's method (1).

$$2,4\text{-}(O_2N)_2C_6H_3F + H_2N\overset{R}{C}HCOO^{\ominus}Na^{\oplus} \xrightarrow{NaHCO_3} 2,4\text{-}(O_2N)_2C_6H_3NH\overset{R}{C}HCOO^{\ominus}Na^{\oplus} + NaF$$

$$\xrightarrow{HCl} 2,4\text{-}(O_2N)_2C_6H_3NH\overset{R}{C}HCOOH + NaCl$$

Table 1. Structural formulae of DNP-L-amino acids

$2,4\text{-}(O_2N)_2C_6H_3$-NHCH(R)COOH	R	$2,4\text{-}(O_2N)_2C_6H_3$-NHCH(R)COOH	R
Glycine	H-	Isoleucine	$CH_3CH_2CH(CH_3)$-
Alanine	CH_3-	Phenylalanine	$C_6H_5CH_2$-
Valine	$CH_3CH(CH_3)$-	tryptophan	indol-3-yl-CH_2-
Leucine	$CH_3CH(CH_3)CH_2$-		

Materials:

L-Amino acid	0.01 mole
FDNB	0.01 mole (1.86 gm.)
Sodium bicarbonate	0.03-0.1 mole (2.58-8.6 gm.)
Water	100 ml.
Ethanol (95%)	125 ml.
Hydrochloric acid	3-10 ml.

L-Amino acid and sodium bicarbonate were dissolved in water and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature. The solution was evaporated at room temperature until only a small amount of solvent remained. The residue was dissolved in water and the solution filtered to remove the insoluble substances, then acidified with concentrated hydrochloric acid to pH 2, which precipitated a yellow solid. The mixture was refrigerated overnight to precipitate additional product. The crystals were filtered with suction, washed with ice water to remove excess hydrochloric acid and dried in a vacuum desiccator.

The crude product DNP-L-amino acids were purified by the following methods.

For DNP-glycine and DNP-L-alanine, the crude product was dissolved in a small amount of ethanol, water added until the solution just turned cloudy, and then refrigerated overnight. The crystals were filtered with suction, stored for 24 hours in a vacuum desiccator, and heated at 100° to constant weight.

For DNP-L-valine, DNP-L-leucine, DNP-L-isoleucine and DNP-L-

phenylalanine, the crude product was dissolved in a large volume of acetone and the solution dried over anhydrous sodium sulfate. After filtration, the solution was concentrated to a small volume. An equal volume of benzene was added to the acetone solution and the DNP-L-amino acid precipitated by adding an excess of petroleum ether (b.p. 30-75°). The derivative is dried in a current of air, dissolved in ether and precipitated with petroleum ether. The ether-petroleum ether procedures may be repeated several times, until the DNP-L-amino acid crystallizes at a low temperature. The crystals were filtered with suction, stored for 24 hours in a vacuum desiccator and heated at 100° to constant weight. (except for DNP-L-leucine, which was heated at 80° to constant weight.)

For DNP-L-tryptophan, the crude product was dissolved in acetone, ether added until the solution just turned cloudy, and then refrigerated overnight. The crystals were filtered with suction, stored for 24 hours in a vacuum desiccator, and heated at 100° to constant weight.

Light was excluded at all stages of preparation and purification.

A Gallenkamp melting point apparatus was used to determine the melting point which was uncorrected.

Melting points and specific rotations in 95% ethanol were the same as those reported in the literature. Yield and melting point of the products are summarized in Table 2, specific rotations in 95% ethanol and also in dimethylformamide and glacial acetic acid are summarized in Table 3, as are also the calculated molecular rotations.

Table 2

Yield and melting point of DNP-L-amino acids

Compound	Weight of amino acid (mole)	Weight of FDNB (mole)	Weight of $NaHCO_3$ (mole)	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C
DNP-glycine	0.01	0.01	0.100	94-98	201-205	92-94	205-206
DNP-L-alanine	0.01	0.01	0.100	92-97	173-175	90-95	178-179
DNP-L-valine	0.01	0.01	0.075	93-97	130-134	90-94	133-134
DNP-L-leucine	0.01	0.01	0.030	92-95	53-65	58-65	98-99
DNP-L-isoleucine	0.01	0.01	0.075	94-98	112-113	90-96	113-114
DNP-L-phenylalanine	0.01	0.01	0.100	94-96	170-175	82-88	192-193
DNP-L-tryptophan	0.01	0.01	0.050	95-98	221-223	92-95	222-223

Table 3

Specific rotations and molecular rotations of DNP-L-amino acids in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	Ethanol		Dimethylformamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-alanine	+17.5°	+44.6°	-29.3°	-74.4°	+19.3°	+49.2°
DNP-L-valine	-12.4°	-35.1°	-89.8°	-254.4°	-26.8°	-75.9°
DNP-L-leucine	-28.0°	-83.3°	-77.8°	-231.3°	-48.9°	-145.4°
DNP-L-isoleucine	-21.3°	-63.3°	-95.9°	-285.1°	-30.4°	-90.4°
DNP-L-phenylalanine	-87.2°	-288.9°	-120.0°	-397.6°	-97.0°	-321.4°
DNP-L-tryptophan	-286.2°	-1059.9°	-211.2°	-782.1°	-196.7°	-728.4°

Optical rotation

The specific rotation $[\alpha]_D^{22}$ was determined in 1-2% solution in 95% ethanol, dimethylformamide and glacial acetic acid on samples previously dried at 100° to constant weight. The values were calculated from the well known relation:

$$\text{Specific rotation } [\alpha]_D^{22} = \frac{\alpha \times 100}{l \cdot c}$$

Where α is the observed rotation in degrees.

l is the length of the polarimeter tube in decimeters.

c is the concentration os samples (grams/100 ml of solution).

Molar rotations $[M]_D^{22}$ equal to specific rotations $[\alpha]_D^{22}$ multiplied by the molecular weight and divided by 100.

The measurements were made by using a Kern Full Circle polarimeter and a two decimeter tube.

2,4-Dinitrophenyl-L-amino acid chlorides

These were required for attempting preparation of DNP-dipeptides (9) and DNP-dipeptide ethyl esters. They were prepared by the method of Loudfoot and Kruger (9) and characterized by conversion to their amides, anilides and p-toluidides. The general reactions for the overall reaction is shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COOH + SOCl_2 \longrightarrow O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COCl + SO_2 + HCl$$

Materials:

DNP-L-amino acid	0.005 mole
Thionyl chloride	10 ml.

DNP-L-amino acid and thionyl chloride were heated under reflux on an oil bath at 80° until a clear solution was obtained (10-30 minutes). The solution was then heated at 80° for an additional 90 minutes, and the excess thionyl chloride removed by vacuum distillation at 50°. Except for DNP-glycyl chloride, red-brown liquids were formed, which were used for the preparation of DNP-amino acid derivatives without further purification. DNP-L-tryptophan fails to yield the acid chloride due to destruction of DNP-L-tryptophan in thionyl chloride even at a lower temperature. Throughout the preparation, a calcium chloride tube was attached to the condenser in order to exclude moisture.

The yield of the products are summarized in Table 4.

Table 4

Yield of DNP-L-amino acid chlorides

Compound	Yield (%)
DNP-glycyl chloride	95-99
DNP-L-alanyl chloride	98-99
DNP-L-valyl chloride	95-98
DNP-L-leucyl chloride	97-99
DNP-L-isoleucyl chloride	95-98
DNP-L-phenylalanyl chloride	96-99

Characterization of DNP-L-amino acid chlorides

The acid chlorides from DNP-L-valine, DNP-L-leucine, DNP-L-isoleucine, and DNP-L-phenylalanine were converted to their amides, anilides and p-toluidides as was DNP-L-alanyl chloride (9). Yields, melting point and recrystallizing solvent for the amides are listed in Table 5, for the anilides in Table 6, and for the p-toluidides in Table 7. Specific rotations and molecular rotations of all these derivatives in 95% ethanol, dimethylformamide, acetone and glacial acetic acid are listed in Table 8. Results of the elemental analyses of these derivatives (performed by Organic Microanalyses, Montreal, Quebec; Geller Laboratories, Charleston, West Virginia or Chemalytics, Tempe, Arizona.) are summarized in Table 9.

Table 5

Yield and melting point of DNP-L-amino acid amides

Compound	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C
DNP-L-valyl amide	94-98	166-170	88-93	171-172
DNP-L-leucyl amide	92-95	109-124	86-90	128-129
DNP-L-isoleucyl amide	92-96	175-180	89-94	183-184
DNP-L-phenylalanyl amide	82-88	180-182	80-84	181-182

Table 6

Yield, melting point and recrystallizing solvent of DNP-L-amino acid anilides

Compound	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C	Solvents for recrys.
DNP-L-val anilide	90-94	216-220	83-88	222-223	E-W
DNP-L-leu anilide	66-75	150-165	52-64	170-171	M
DNP-L-Ileu anilide	88-93	208-210	84-88	210-211	E-W
DNP-L-phe anilide	77-84	203-205	69-78	206-207	E

Table 7

Yield, melting point and recrystallizing solvent of DNP-L-amino acid p-toluidides

Compound	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C	Solvents for recrys.
DNP-L-val-p-toluidide	90-93	181-185	84-89	188-189	E-W
DNP-L-leu-p-toluidide	90-92	115-130	81-86	131-132	E-W
DNP-L-Ileu-p-toluidide	92-95	202-204	88-92	203-204	E-W
DNP-L-phe-p-toluidide	78-86	201-203	69-76	203-204	E

Where E-W = ethanol-water; E= ethanol.

Table 8

Specific rotations and molecular rotations of DNP-L-amino acid amides, anilides and p-toluidides in 95% ethanol, dimethylformamide, acetone and glacial acetic acid

Compound	95% ethanol		Dimethylformamide		Acetone		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-val amide	+85.1°	+240.2°	+27.7°	+71.2°	+45.3°	+127.9°	+128.7°	+363.3°
DNP-L-val anilide	-----	------	+108.3°	+388.1°	+113.8°	+407.8°	+203.5°	+729.3°
DNP-L-val-p-toluidide	-----	------	+105.0°	+391.0°	+111.7°	+416.0°	+200.0°	+744.8°
DNP-L-leu amide	+80.0°	+237.0°	+12.6°	+37.3°	+38.3°	+113.5°	+120.8°	+357.9°
DNP-L-leu anilide	+131.4°	+489.3°	+78.8°	+293.4°	+100.3°	+373.5°	+182.4°	+679.2°
DNP-L-leu-p-toluidide	+118.6°	+458.3°	+92.1°	+355.9°	+102.9°	+397.6°	+210.4°	+813.0°
DNP-L-Ileu amide	+66.9°	+198.2°	+17.1°	+50.7°	+29.5°	+87.4°	+120.1°	+355.8°
DNP-L-Ileu anilide	-----	------	+107.8°	+401.4°	+105.7°	+393.6°	+175.7°	+654.3°
DNP-L-Ileu-p-toluidide	-----	------	+120.8°	+382.8°	+104.6°	+389.5°	+195.0°	+726.2°
DNP-L-phe amide	-67.5°	-223.0°	-105.3°	-347.8°	-98.2°	-324.4°	+25.9°	+85.6°
DNP-L-phe anilide	-----	------	-23.2°	-94.3°	-20.0°	-83.1°	+98.3°	+399.5°
DNP-L-phe-p-toluidide	-----	------	-27.0°	-113.5°	-22.0°	-92.5°	+91.6°	+385.1°

Table 9

Results of the elemental analyses of DNP-L-amino acid amides, anilides and p-toluidides

Compound	Formula	Theory (%)			Found (%)		
		C	H	N	C	H	N
DNP-L-val amide	$C_{11}H_{14}N_4O_5$	46.81	5.00	19.85	46.79	4.83	19.60
DNP-L-val anilide	$C_{17}H_{18}N_4O_5$	56.98	5.06	15.64	56.96	4.94	15.40
DNP-L-val-p-toluidide	$C_{18}H_{20}N_4O_5$	58.06	5.41	15.05	57.95	5.30	14.85
DNP-L-leu amide	$C_{12}H_{16}N_4O_5$	48.65	5.44	18.91	48.88	5.30	18.97
DNP-L-leu anilide	$C_{18}H_{20}N_4O_5$	57.90	5.40	15.00	58.60	5.47	14.75
DNP-L-leu-p-toluidide	$C_{19}H_{22}N_4O_5$	59.06	5.74	14.50	59.45	5.73	14.73
DNP-L-Ileu amide	$C_{12}H_{16}N_4O_5$	48.65	5.44	18.91	49.07	5.50	19.06
DNP-L-Ileu anilide	$C_{18}H_{20}N_4O_5$	57.90	5.40	15.00	58.41	5.30	14.92
DNP-L-Ileu-p-toluidide	$C_{19}H_{22}N_4O_5$	59.06	5.74	14.50	59.28	5.51	14.37
DNP-L-phe amide	$C_{15}H_{14}N_4O_5$	54.54	4.27	16.96	54.33	4.34	16.87
DNP-L-phe anilide	$C_{21}H_{18}N_4O_5$	62.06	4.46	13.79	61.46	4.25	13.91
DNP-L-phe-p-toluidide	$C_{22}H_{20}N_4O_5$	62.85	4.80	13.33	62.60	4.67	13.54

Preparation of DNP-dipeptide esters

Three methods of preparing DNP-dipeptide esters were employed, each giving a satisfactory result.

(1) By reacting DNP-L-amino acid chloride with the ethyl ester of the pertinent amino acid (10).

(2) By reacting DNP-L-amino acid with the ethyl ester of the pertinent amino acid in the presence of N,N-dimethyl chloroformimidium chloride (11).

(3) By reacting DNP-L-amino acid with the ethyl ester of the pertinent amino acid in the presence of N,N-dicyclohexylcarbodiimide (12, 13).

Acid chloride method

DNP-L-amino acid chloride was coupled with the amino acid ethyl ester in the presence of a tertiary base (i.e. triethylamine). The general equation for the overall reaction is shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{\overset{|}{C}}HCOCl + H_2N\overset{R'}{\overset{|}{C}}HCOOC_2H_5 + (C_2H_5)_3N$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOOC_2H_5 + (C_2H_5)_3N\cdot HCl$$

Materials:

DNP-L-amino acid chloride	0.002 mole
L-Amino acid ethyl ester hydrochloride	0.002 mole
Triethylamine	0.004 mole (0.404 gm.)
Tetrahydrofuran	50 ml.
(or anhydrous ether)	100 ml.

L-Amino acid ester hydrochloride and triethylamine were dissolved in 30 ml. of tetrahydrofuran (or 60 ml of ether); to this a solution of DNP-L-amino acid chloride in 20 ml. of tetrahydrofuran (or 40 ml. of ether) was added and the solution stirred for 16 to 24 hours at room temperature in the absence of light. After the reaction was completed, the solvent was evaporated at room temperature and the residue extracted with ethyl acetate (3 X 25 ml.). The ethyl acetate solution was washed successively with N HCl (1 X 35 ml.), water (2 X 25 ml.), saturated $NaHCO_3$ (3 X 25 ml.) and water (2 X25 ml.). After drying the solution over anhydrous sodium sulfate, the solvent was evaporated at room temperature and the residue was purified by recrystallized with appropriate solvents.

The yield, melting point, and the solvents of recrystallization of the products are summarized in Table 10.

The specific rotations and molecular rotations of the above DNP-dipeptide esters in 95% ethanol, dimethylformamide, and glacial acetic acid are summarized in Table 11.

Table 10

Yield, melting point and
recrystallizing solvent of DNP-dipeptide ethyl esters

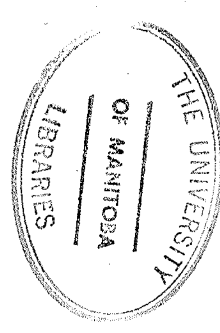
Compound	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C	Solvents for recrys.
DNP-gly-gly ethyl ester	78-83	110-180	42-50	201-202	E
DNP-gly-L-leu ethyl ester	86-90	101-112	73-78	114-115	M-W
DNP-gly-L-phe ethyl ester	91-95	154-156	85-90	157-158	E
DNP-L-ala-L-phe ethyl ester	88-92	170-174	82-88	178-179	E
DNP-L-val-gly ethyl ester	76-80	163-165	67-72	175-176	E-W
DNP-L-val-L-leu ethyl ester	89-93	113-117	82-88	120-121	E-W
DNP-L-val-L-phe ethyl ester	90-94	165-166	81-88	171-172	E-W
DNP-L-leu-gly ethyl ester	82-85	85-102	52-56	131-132	P-W
DNP-L-leu-L-leu ethyl ester	88-92	90-100	85-90	109-110	E-W
DNP-L-leu-L-phe ethyl ester	89-94	134-136	82-90	135-136	E-W
DNP-L-Ileu-gly ethyl ester	58-66	162-165	52-60	170-171	E-W
DNP-L-Ileu-L-leu ethyl ester	91-94	129-131	81-87	133-134	E-W
DNP-L-Ileu-L-phe ethyl ester	86-89	152-153	77-82	155-156	E-W
DNP-L-phe-gly ethyl ester	77-82	110-130	55-64	137-138	E-W
DNP-L-phe-L-leu ethyl ester	88-94	110-130	84-88	135-136	E-W
DNP-L-phe-L-phe ethyl ester	89-94	183-185	83-90	187-188	E-W

Where E = ethanol; E-W = ethanol-water; M-W = methanol-water; P-W = n-propanol-water.

Table 11

Specific rotations and molecular rotations of DNP-dipeptide ethyl esters in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-gly-L-leu ethyl ester	-33.9°	-129.6°	-18.3°	-70.0°	-20.4°	-78.0°
DNP-gly-L-phe ethyl ester	-----	------	-9.6°	-40.0°	-4.5°	-18.7°
DNP-L-ala-L-phe ethyl ester	-----	------	+62.5°	+269.0°	+162.6°	+699.9°
DNP-L-val-gly ethyl ester	+88.6°	+326.4°	+31.4°	+115.7°	+156.0°	+574.6°
DNP-L-val-L-leu ethyl ester	+57.8°	+245.3°	+36.2°	+153.7°	+125.0°	+530.6°
DNP-L-val-L-phe ethyl ester	+36.3°	+166.4°	+30.4°	+139,4°	+118.2°	+541.9°
DNP-L-leu-gly ethyl ester	+79.4°	+303.6°	+25.0°	+95.6°	+142.2°	+543.7°
DNP-L-leu-L-leu ethyl ester	+60.0°	+263.1°	+17.2°	+75.4°	+128.7°	+564.3°
DNP-L-leu-L-phe ethyl ester	+41.2°	+194.7°	+16.6°	+78.4°	+117.1°	+553.3°
DNP-L-Ileu-gly ethyl ester	+75.8°	+289.8°	+21.8°	+83.4°	+144.0°	+550.6°
DNP-L-Ileu-L-leu ethyl ester	+46.6°	+250.9°	+26.9°	+144.8°	+124.8°	+672.0°
DNP-L-Ileu-L-phe ethyl ester	-----	------	+19.5°	+92.1°	+111.5°	+526.8°
DNP-L-phe-gly ethyl ester	-24.3°	-101.2°	-61.7°	-256.9°	+64.0°	+266.5°
DNP-L-phe-L-leu ethyl ester	-49.0°	-231.5°	-59.7°	-282.1°	+77.6°	+366.7°
DNP-L-phe-L-phe ethyl ester	-----	------	-59.7°	-302.4°	+66.2°	+335.3°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Modified acid chloride method

The N,N-dimethyl chloroformimidium chloride which is prepared from dimethyl-formamide and thionyl chloride is reacted with DNP-L-amino acid at -5° in dimethyl-formamide. Without isolation, the acid chloride is added to the amino component in presence of 2 equivalents of base. The general equations for the overall reaction are shown as below:

$$(H_3C)_2N-\overset{O}{\overset{\|}{C}}-H + SOCl_2$$

$$\downarrow$$

$$\left[(H_3C)_2N=C\begin{matrix}Cl \\ H\end{matrix} \right]^{\oplus} Cl^{\ominus} + SO_2$$

$$\downarrow \; O_2N-C_6H_3(NO_2)-NHCH(R)COOH$$

$$O_2N-C_6H_3(NO_2)-NHCHCOCl + (H_3C)_2N\overset{O}{\overset{\|}{C}}-H + HCl$$

$$\downarrow \; H_2NCH(R')COOC_2H_5,\; 2(C_2H_5)_3N$$

$$O_2N-C_6H_3(NO_2)-NHCH(R)CONHCH(R')COOC_2H_5 + 2\,(C_2H_5)_3N\cdot HCl$$

Materials:

DNP-L-amino acid	0.002 mole
L-Amino acid ethyl ester hydrochloride	0.002 mole
Thionyl chloride	0.002 mole (0.238 gm.)
Dimethylformamide	20 ml.
Triethylamine	0.006 mole (0.606 gm.)

Thionyl chloride was dissolved in 10 ml. dimethylformamide, the solution kept at -10° for 2 hours, DNP-L-amino acid added and the mixture was cooled at -10° for another 2 hours. A solution of freshly prepared L-amino acid ethyl ester (prepared by stirring 1 equivalent of the ester hydrochloride and 3 equivalents of triethylamine in dimethylformamide) was added, the mixture kept at 5° for 72 hours with occassional shaking, then stirred at room temperature for another 24 hours. At the end of the reaction, the solution was diluted with 50 ml. of water, and extracted with ethyl acetate (3 X 25ml.). The organic layer was washed successively with N HCl (1 X 25 ml.), water (2 X 25ml.), saturated $NaHCO_3$ (3 X25ml.) and water (2 X 25ml.). After drying the solution over anhydrous sodium sulfate, the solvent was evaporated at room temperature and the residue purified by recrystallizing with appropriate solvents.

Yield, melting point and solvents of recrystallization of the products are summarized in Table 12.

Specific rotations and molecular rotations of the above esters in 95% ethanol, dimethylformamide and glacial acetic acid are summarized in Table 13.

Table 12

Yield, melting point and recrystallizing solvent of DNP-dipeptide ethyl esters

Compound	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C	Solvents for recrys.
DNP-gly-gly ethyl ester	77-80	202-205	70-75	201-202	E
DNP-gly-L-leu ethyl ester	77-84	112-114	73-78	114-115	E-W
DNP-gly-L-phe ethyl ester	87-90	152-157	83-87	157-158	E
DNP-L-ala-L-phe ethyl ester	85-88	176-178	80-83	178-179	E
DNP-L-val-gly ethyl ester	78-83	171-172	73-78	175-176	E-W
DNP-L-val-L-leu ethyl ester	80-82	114-116	71-76	120-121	E-W
DNP-L-val-L-phe ethyl ester	86-90	170-172	76-82	171-172	E-W
DNP-L-leu-gly ethyl ester	72-76	131-132	70-73	131-132	P-W
DNP-L-leu-L-leu ethyl ester	82-85	107-108	76-80	109-110	E-W
DNP-L-leu-L-phe ethyl ester	87-91	133-134	80-85	135-136	E-W
DNP-L-Ileu-gly ethyl ester	81-83	169-170	72-77	170-171	E-W
DNP-L-Ileu-L-leu ethyl ester	77-82	130-131	69-75	133-134	E-W
DNP-L-Ileu-L-phe ethyl ester	83-86	152-154	73-79	155-156	E-W
DNP-L-phe-gly ethyl ester	71-74	125-134	60-65	137-138	E-W
DNP-L-phe-L-leu ethyl ester	81-86	131-134	74-80	135-136	E-W
DNP-L-phe-L-phe ethyl ester	81-84	184-185	70-76	187-188	E-W
DNP-L-try-gly ethyl ester	85-88	195-198	73-80	196-197	E-W
DNP-L-try-L-leu ethyl ester	89-93	125-130	78-86	137-138	E-W
DNP-L-try-L-phe ethyl ester	73-79	226-230	67-72	233-234	A-W

Where E = ethanol; E-W = ethanol-water; P-W = n-propanol-water; A-W = acetone-water.

Table 13

Specific rotations and molecular rotations of DNP-dipeptide ethyl esters in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-gly-L-leu ethyl ester	-33.8°	-129.2°	-18.4°	-70.4°	-20.5°	-78.4°
DNP-gly-L-phe ethyl ester	-----	------	-9.6°	-40.0°	-4.8°	-20.0°
DNP-L-ala-L-phe ethyl ester	-----	------	+62.6°	+269.4°	+162.4°	+699.0°
DNP-L-val gly ethyl ester	+88.6°	+326.4°	+31.5°	+116.0°	+156.0°	+574.6°
DNP-L-val-L-leu ethyl ester	+57.3°	+243.2°	+36.4°	+154.5°	+162.4°	+689.3°
DNP-L-val-L-phe ethyl ester	+35.8°	+164.1°	+30.3°	+138.9°	+118.0°	+541.0°
DNP-L-leu-gly ethyl ester	+79.0°	+302.1°	+25.2°	+96.4°	+142.5°	+544.9°
DNP-L-leu-L-leu ethyl ester	+59.5°	+260.9°	+17.2°	+75.4°	+128.5°	+563.5°
DNP-L-leu-L-phe ethyl ester	+43.1°	+195.1°	+16.5°	+78.0°	+117.0°	+552.8°
DNP-L-Ileu-gly ethyl ester	+75.7°	+289.5°	+22.0°	+84.1°	+144.0°	+550.6°
DNP-L-Ileu-L-leu ethyl ester	+47.0°	+253.1°	+26.6°	+143.2°	+125.1°	+637.7°
DNP-L-Ileu-L-phe ethyl ester	-----	------	+19.5°	+92.1°	+111.6°	+527.3°
DNP-L-phe-gly ethyl ester	-24.2°	-100.8°	-61.5°	-256.1°	+64.2°	+267.3°
DNP-L-phe-L-leu ethyl ester	-48.5°	-229.2°	-60.0°	-283.5°	+77.5°	+366.2°
DNP-L-phe-L-phe ethyl ester	-----	------	-59.5°	-301.4°	+66.4°	+336.3°
DNP-L-try-gly ethyl ester	-----	------	-150.6°	-685.9°	+47.0°	+214.1°
DNP-L-try-L-leu ethyl ester	-----	------	-174.3°	-891.6°	+63.5°	+324.8°
DNP-L-try-L-phe ethyl ester	-----	------	-153.5°	-837.4°	+84.1°	+458.8°

Carbodiimide method

The two components, one containing a free carboxyl group (i.e. DNP-L-amino acid) and the other a free amino group (i.e. L-amino acid ethyl ester) couple directly and rapidly in high yield on treatment with N,N-dicyclohexyl-carbodiimide at room temperature. The general equations for the overall reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)COOH + H_2NCH(R')COOC_2H_5 + C_6H_{11}\text{-}N{=}C{=}N\text{-}C_6H_{11}$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NHCH(R)CONHCH(R')COOC_2H_5 + C_6H_{11}\text{-}NH\text{-}CO\text{-}NH\text{-}C_6H_{11}$$

Materials:

DNP-L-amino acid	0.002 mole
L-Amino acid ethyl ester hydrochloride	0.002 mole
Triethylamine	0.002 mole (0.202gm.)
N,N'-Dicyclohexylcarbodiimide	0.0024 mole(0.495gm.)
Anhydrous ether	125 ml.

DNP-L-amino acid was dissolved in 50 ml. anhydrous ether, and to this a solution of freshly prepared L-amino acid ethyl ester in ether (prepared by stirring 1 equivalent of the ester hydrochloride with 1 equivalent of triethylamine in ether) was added, followed

by a solution of dicyclohexyl-carbodiimide in 50 ml. ether. The mixture was stirred at room temperature for 72 hours in the absence of light. At the end of the reaction, the solvent was evaporated at room temperature and the residue was extracted with ethyl acetate (3 X 25ml.), and the ethyl acetate solution washed successively with N HCl (1 X 25ml.), water (2 X 25ml.), saturated $NaHCO_3$ (3 X 25ml.) and water (3 X 25ml.). After drying over anhydrous sodium sulfate, the solvent was evaporated at room temperature, and the residue was purified by recrystallizing with appropriate solvents.

Purification of DNP-glycyl-glycine and DNP-L-tryptophyl-glycine was rather difficult due to a large excess of the dicyclohexyl urea present, but this was resolved by using instead 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate; yields up to 70% were obtained in both cases.

Yield, melting point and solvents of recrystallization of the products are summarized in Table 14.

Specific rotations and molecular rotations of the above esters in 95% ethanol, dimethylformamide and glacial acetic acid are summarized in Table 15.

Analytical results for the above dipeptide esters are summarized in Table 16.

<u>Preparation of DNP-dipeptides</u>

Three methods of preparing DNP-dipeptides were employed:

(1) Hydrolyzing the DNP-dipeptide esters with N aqueous sodium hydroxide in 95% ethanol.

(2) Reacting the DNP-L-amino acid chlorides with the pertinent amino acids (9).

(3) Reacting the dipeptides directly with FDNB.

Table 14

Yield, melting point and recrystallizing solvent of DNP-dipeptide ethyl esters

Compounds	Crude yield %	Crude M.P. °C	Pure yield %	Pure M.P. °C	Solvents for recrys.
DNP-gly-gly ethyl ester	96-99	110-190	35-42	201-202	E
DNP-gly-L-leu ethyl ester	90-94	80-105	71-75	114-115	M-W
DNP-gly-L-phe ethyl ester	98-101	154-157	88-92	157-158	E
DNP-L-ala-L-phe ethyl ester	98-104	149-155	90-93	177-178	E
DNP-L-val-gly ethyl ester	106-114	165-170	89-92	173-175	E-W
DNP-L-val-L-leu ethyl ester	101-108	115-120	92-96	119-121	E-W
DNP-L-val-L-phe ethyl ester	115-120	160-165	93-96	167-169	E
DNP-L-leu-gly ethyl ester	76-83	105-130	56-60	131-132	P-W
DNP-L-leu-L-leu ethyl ester	92-96	85-100	80-85	108-110	E-W
DNP-L-leu-L-phe ethyl ester	101-105	124-126	89-92	134-136	E
DNP-L-Ileu-gly ethyl ester	98-102	157-159	80-84	166-168	E
DNP-L-Ileu-L-leu ethyl ester	106-110	124-130	74-82	133-134	E
DNP-L-Ileu-L-phe ethyl ester	103-107	147-151	65-72	155-156	E
DNP-L-phe-gly ethyl ester	93-96	125-150	52-60	137-138	E
DNP-L-phe-L-leu ethyl ester	88-94	120-145	62-66	135-136	E
DNP-L-phe-L-phe ethyl ester	95-99	183-184	80-85	186-188	E
DNP-L-try-gly ethyl ester	115-121	85-100	54-60	195-197	E
DNP-L-try-L-leu ethyl ester	125-130	80-100	45-52	137-138	M
DNP-L-try-L-phe ethyl ester	78-85	220-230	67-72	233-234	A-W

Where E = ethanol; E-W = ethanol-water; M = methanol; M-W = methanol-water; P-W = n-propanol-water; A-W = acetone-water.

Table 15

Specific rotations and molecular rotations of DNP-dipeptide ethyl esters in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-gly-L-leu ethyl ester	-33.7°	-128.9°	-18.6°	-71.1°	-20.2°	-77.2°
DNP-gly-L-phe ethyl ester	-----	------	-9.7°	-40.4°	-4.6°	-19.2°
DNP-L-ala-L-phe ethyl ester	-----	------	+62.2°	+267.7°	+163.0°	+701.6°
DNP-L-val-gly ethyl ester	+89.0°	+327.8°	+31.8°	+117.1°	+155.6°	+573.2°
DNP-L-val-L-leu ethyl ester	+58.2°	+247.0°	+36.2°	+153.7°	+162.8°	+689.9°
DNP-L-val-L-phe ethyl ester	+36.8°	+168.7°	+30.0°	+137.5°	+118.5°	+543.3°
DNP-L-leu-gly ethyl ester	+79.2°	+302.8°	+25.5°	+97.5°	+142.2°	+543.7°
DNP-L-leu-L-leu ethyl ester	+59.8°	+262.2°	+17.5°	+76.7°	+129.0°	+565.7°
DNP-L-leu-L-phe ethyl ester	+42.0°	+198.4°	+16.5°	+78.0°	+116.8°	+551.9°
DNP-L-Ileu-gly ethyl ester	+76.5°	+292.5°	+21.6°	+82.6°	+143.5°	+548.7°
DNP-L-Ileu-L-leu ethyl ester	+47.1°	+253.6°	+26.6°	+143.2°	+125.0°	+673.1°
DNP-L-Ileu-L-phe ethyl ester	-----	------	+19.6°	+92.6°	+112.1°	+529.7°
DNP-L-phe-gly ethyl ester	-24.5°	-102.0°	-61.5°	-256.0°	+64.0°	+266.5°
DNP-L-phe-L-leu ethyl ester	-48.9°	-231.1°	-60.2°	-284.4°	+77.2°	+364.8°
DNP-L-phe-L-phe ethyl ester	-----	------	-60.6°	-307.0°	+66.6°	+337.3°
DNP-L-try-gly ethyl ester	-----	------	-151.2°	-688.6°	+46.8°	+213.1°
DNP-L-try-L-leu ethyl ester	-----	------	-174.9°	-894.7°	+63.6°	+325.3°
DNP-L-try-L-phe ethyl ester	-----	------	-153.0°	-834.7°	+83.7°	+456.6°

Table 16

Results of the elemental analyses of DNP-dipeptide ethyl esters

Compound	Formula	Theory (%)			Found (%)		
		C	H	N	C	H	N
DNP-gly-gly ethyl ester	$C_{12}H_{14}N_4O_7$	44.17	4.33	17.17	44.33	4.39	17.16
DNP-gly-L-leu ethyl ester	$C_{16}H_{22}N_4O_7$	50.62	5.85	14.72	50.26	5.78	14.65
DNP-gly-L-phe ethyl ester	$C_{19}H_{20}N_4O_7$	54.81	4.84	13.46	54.43	4.81	13.31
DNP-L-ala-L-phe ethyl ester	$C_{20}H_{22}N_4O_7$	55.81	5.15	13.02	55.69	5.15	12.88
DNP-L-val-gly ethyl ester	$C_{15}H_{20}N_4O_7$	48.91	5.47	15.21	49.06	5.14	15.11
DNP-L-val-L-leu ethyl ester	$C_{19}H_{28}N_4O_7$	53.76	6.65	13.20	53.87	6.25	13.14
DNP-L-val-L-phe ethyl ester	$C_{22}H_{26}N_4O_7$	57.63	5.71	12.22	57.71	5.46	12.20
DNP-L-leu-gly ethyl ester	$C_{16}H_{22}N_4O_7$	50.62	5.85	14.72	50.15	5.71	14.71
DNP-L-leu-L-leu ethyl ester	$C_{20}H_{30}N_4O_7$	54.78	6.90	12.78	54.86	6.82	12.81
DNP-L-leu-L-phe ethyl ester	$C_{23}H_{28}N_4O_7$	58.47	5.97	11.86	58.68	5.85	11.63
DNP-L-Ileu-gly ethyl ester	$C_{16}H_{22}N_4O_7$	50.62	5.85	14.72	50.44	5.63	14.73
DNP-L-Ileu-L-leu ethyl ester	$C_{20}H_{30}N_4O_7$	54.78	6.90	12.78	54.80	6.64	12.88
DNP-L-Ileu-L-phe ethyl ester	$C_{23}H_{28}N_4O_7$	58.46	5.97	11.86	58.29	5.81	11.89
DNP-L-phe-gly ethyl ester	$C_{19}H_{20}N_4O_7$	54.80	4.84	13.46	55.26	4.89	13.34
DNP-L-phe-L-leu ethyl ester	$C_{23}H_{28}N_4O_7$	58.46	5.97	11.86	58.41	6.15	11.81
DNP-L-phe-L-phe ethyl ester	$C_{26}H_{26}N_4O_7$	61.65	5.17	11.06	61.84	5.04	11.03
DNP-L-try-gly ethyl ester	$C_{21}H_{21}N_5O_7$	55.38	4.65	15.38	55.70	4.48	15.34
DNP-L-try-L-leu ethyl ester	$C_{25}H_{29}N_5O_7$	58.70	5.71	13.69	58.39	5.32	13.72
DNP-L-try-L-phe ethyl ester	$C_{28}H_{27}N_5O_7$	61.65	4.99	12.84	61.81	4.87	12.80

Alkaline hydrolysis method

Sixteen DNP-dipeptides were prepared by the hydrolysis of the corresponding ethyl ester in 95% ethanol with N aqueous sodium hydroxide. For most of the DNP-dipeptide esters, the hydrolysis is carried out in ethanol with a small excess of alkali for 4 to 8 hours at room temperature, but for some esters, they required 24 to 48 hours at 0° C. The general equations for the overall reaction are shown as below:

$$2,4\text{-}(O_2N)_2C_6H_3\text{-}NHCH(R)CONHCH(R')COOC_2H_5 + NaOH$$

$$\downarrow$$

$$2,4\text{-}(O_2N)_2C_6H_3\text{-}NHCH(R)CONHCH(R')COO^{\ominus}Na^{\oplus} + C_2H_5OH$$

$$\downarrow HCl$$

$$2,4\text{-}(O_2N)_2C_6H_3\text{-}NHCH(R)CONHCH(R')COOH + NaCl$$

Materials:

DNP-dipeptide esters	0.001 mole
N NaOH	1.2 ml.
95% ethanol	20 ml.

DNP-dipeptide ethyl ester was dissolved in 20 ml. ethanol, and to this N NaOH was added in small portions. The solution was stirred at room temperature for 4 to 8 hours or in an ice-water bath for 24 to 48 hours. After the reaction

was completed, (as judged by formation of a clear solution on addition of a drop of this solution to a large excess of water.) the solution was diluted with 50 ml. of water, 5 ml. saturated $NaHCO_3$ added, and washed twice with 25 ml. ethyl acetate. The aqueous layer was then acidified with concentrated hydrochloric acid to pH 2, and the DNP-dipeptide was extracted with ethyl acetate (3 X 25 ml.). The organic layer was washed with water (2 X 25 ml.), and dried over anhydrous sodium sulfate. The solvent was evaporated at room temperature and the residue purified by recrystallization from ethanol-water.

Reaction times and temperatures, together with yield and melting point of products are summarized in Table 17.

Specific rotations and molecular rotations of the above DNP-dipeptides in 95% ethanol, dimethylformamide and glacial acetic acid are summarized in Table 18.

Acid chloride method

Because DNP-L-tryptophan does not yield the acid chloride and the DNP-L-phenylalanyl chloride only produced the hydrolysis product, only ten DNP-dipeptides were prepared by reacting the DNP-L-amino acid chloride with the L-amino acid in sodium carbonate medium, followed by acidification with hydrochloric acid. The general equations for the overall reaction are shown as below:

Table 17

Reaction time, temperature, yield and melting point of DNP-dipeptides

Compound	Time (hr)	Temp. (°C)	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP--L-ala-L-phe	8	room	82-85	205-210	72-76	214-215
DNP-L-val-gly	24	0	75-80	208-215	68-72	226-227
DNP-L-val-L-leu	24	0	83-88	------	70-74	146-147
DNP-L-val-L-phe	48	0	90-92	199-201	80-84	206-207
DNP-L-leu-gly	24	0	75-80	150-153	72-76	158-159
DNP-L-leu-L-leu	24	0	78-82	135-140	75-78	146-147
DNP-L-leu-L-phe	48	0	80-82	125-140	65-72	147-148
DNP-L-Ileu-gly	24	0	81-84	170-175	72-74	181-182
DNP-L-Ileu-L-leu	4	room	90-94	108-110	86-88	116-117
DNP-L-Ileu-L-phe	8	room	92-98	199-202	86-90	206-207
DNP-L-phe-gly	4	room	72-80	165-168	66-70	170-171
DNP-L-phe-L-leu	4	room	76-80	160-165	70-73	167-168
DNP-L-phe-L-phe	8	room	82-88	165-175	58-64	199-200
DNP-L-try-gly	4	room	80-85	220-225	72-76	227-228
DNP-L-try-L-leu	4	room	82-86	123-130	72-77	127-128
DNP-L-try-L-phe	8	room	86-92	217-220	80-84	223-224

Table 18

Specific rotations and molecular rotations of DNP-dipeptides in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe	+97.6°	+392.7°	+80.6°	+324.3°	+159.5°	+641.8°
DNP-L-val-gly	+101.8°	+346.4°	+40.5°	+137.8°	+167.2°	+569.0°
DNP-L-val-L-leu	+68.9°	+273.1°	+50.2°	+199.0°	+146.8°	+581.9°
DNP-L-val-L-phe	+60.9°	+262.1°	+54.7°	+235.4°	+144.1°	+620.2°
DNP-L-leu-gly	+85.7°	+303.7°	+32.0°	+113.3°	+150.0°	+531.5°
DNP-L-leu-L-leu	+70.4°	+288.9°	+35.7°	+146.5°	+143.0°	+586.9°
DNP-L-leu-L-phe	+55.1°	+244.9°	+38.4°	+170.7°	+127.7°	+567.6°
DNP-L-Ileu-gly	+81.3°	+288.1°	+27.8°	+98.5°	+151.0°	+535.0°
DNP-L-Ileu-L-leu	+64.5°	+264.7°	+35.8°	+146.9°	+139.3°	+571.7°
DNP-L-Ileu-L-phe	+51.4°	+228.4°	+42.2°	+187,6°	+118.5°	+526.7°
DNP-L-phe-gly	-32.0°	-124.3°	-67.7°	-262.9°	+58.0°	+225.2°
DNP-L-phe-L-leu	-22.6°	-100.4°	-52.3°	-232.4°	+70.6°	+313.8°
DNP-L-phe-L-phe	-26.4°	-126.3°	-53.0°	-253.6°	+69.9°	+334.5°
DNP-L-try-gly	-120.3°	-514.1°	-206.9°	-884.3°	+51.4°	+219.7°
DNP-L-try-L-leu	-151.0°	-730.1°	-242.1°	-1170°	+84.3°	+407.6°
DNP-L-try-L-phe	-74.3°	-384.5°	-201.3°	-1042°	+34.9°	+180.6°

$$2,4\text{-}(NO_2)_2C_6H_3NHCH(R)COCl + H_2NCH(R')COO^{\ominus}Na^{\oplus} \xrightarrow{Na_2CO_3} 2,4\text{-}(NO_2)_2C_6H_3NHCH(R)CONHCH(R')COO^{\ominus}Na^{\oplus} + NaCl$$

$$\xrightarrow{HCl} 2,4\text{-}(NO_2)_2C_6H_3NHCH(R)CONHCH(R')COOH + NaCl$$

Materials:

DNP-L-amino acid chloride	0.002 mole
L-Amino acid	0.002 mole
Sodium carbonate	0.02 mole (2.12 gm.)
Water	50 ml.
Benzene	20 ml.

L-Amino acid and sodium carbonate were dissolved in water and to this was slowly added a solution of DNP-L-amino acid chloride in benzene over a two hour period with constant stirring at room temperature of 40°. The solution was stirred for another 6 to 12 hours at the same temperature, 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 ml. of concentrated

hydrochloric acid, which precipitated an orange colored solid or oil. The DNP-dipeptide was extracted with ethyl acetate (3 X 25ml.) and the organic phase washed with ice water. After drying over anhydrous sodium sulfate, the solvent was evaporated at room temperature, the residue washed with 5 ml. of ether and recrystallized from ethanol-water.

The reaction times and temperatures together with the yield and melting point of the products are summarized in Table 19.

Table 19

Reaction time, temperature, yield and melting point of DNP-dipeptides

Compound	Time (hr.)	Temp. (°C)	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP-L-ala-L-phe	12	room	74-80	197-200	60-66	214-215
DNP-L-val-gly	16	room	50-58	185-210	40-44	226-227
DNP-L-val-L-leu	16	room	60-66	115-130	51-54	146-147
DNP-L-val-L-phe	16	room	65-72	160-190	58-62	206-207
DNP-L-leu-gly	20	40	45-52	118-140	20-25	158-159
DNP-L-leu-L-leu	20	40	48-55	90-125	32-36	146-147
DNP-L-leu-L-phe	20	40	50-55	120-140	38-42	148-149
DNP-L-Ileu-gly	14	40	50-56	155-170	35-40	181-182
DNP-L-Ileu-L-leu	14	40	72-78	85-100	48-52	116-117
DNP-L-Ileu-L-phe	14	40	80-85	188-194	55-60	206-207

Specific rotations and molecular rotations of the above DNP-dipeptides in 95% ethanol, dimethylformamide, and glacial acetic acid are summarized in Table 20.

Table 20

Specific rotations and molecular rotations of DNP-dipeptides in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe	+98.0°	+394.3°	+80.6°	+324.3°	+159.8°	+643.0°
DNP-L-val-gly	+102.0°	+347.1°	+41.0°	+139.5°	+167.0°	+568.3°
DNP-L-val-L-leu	+68.5°	+271.5°	+50.4°	+199.8°	+146.3°	+579.9°
DNP-L-val-L-phe	+61.2°	+263.4°	+54.5°	+234.6°	+144.0°	+619.8°
DNP-L-leu-gly	+85.5°	+302.9°	+32.3°	+114.4°	+150.6°	+533.6°
DNP-L-leu-L-leu	+71.1°	+291.8°	+35.6°	+146.1°	+142.5°	+584.9°
DNP-L-leu-L-phe	+55.0°	+244.5°	+39.0°	+173.3°	+127.9°	+568.5°
DNP-L-Ileu-gly	+81.2°	+288.7°	+27.5°	+97.4°	+151.0°	+535.0°
DNP-L-Ileu-L-leu	+64.5°	+264.7°	+36.1°	+148.2°	+140.1°	+575.0°
DNP-L-Ileu-L-phe	+52.0°	+231.1°	+42.2°	+187.6°	+118.8°	+528.0°

Dinitrophenylation method

Due to limited availability of dipeptides, only eight DNP-dipeptides were prepared by this method. The dipeptide was stirred with one equivalent of sodium bicarbonate in 67% ethanol (by volume) for two hours at room temperature. The general equations for the overall

reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}F + H_2N\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOO^{\ominus}Na^{\oplus}$$

$$\downarrow NaHCO_3$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOO^{\ominus}Na^{\oplus} + NaF + CO_2 + H_2O$$

$$\downarrow HCl$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{\overset{|}{C}}HCONH\overset{R'}{\overset{|}{C}}HCOOH + NaCl$$

Materials:

Dipeptide	0.001 mole
FDNB	0.001 mole
Sodium bicarbonate	0.01 mole (0.84 gm.)
Water	20 ml.
Ethanol	30 ml.

Dipeptide and sodium bicarbonate were dissolved in water and to this was added a solution of FDNB in ethanol. The mixture was stirred mechanically for two hours at room temperature in the absence of light, 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

solid. The crystals were filtered with suction, washed with ice water to remove excess hydrochloric acid and dried in a vacuum desiccator.

The crude product was purified by recrystallizing from ethanol-water.

Yield and melting point of the products are summarized in Table 21.

Table 21

Yield and melting point of DNP-dipeptides

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP-L-ala-L-phe	78-82	207-210	59-62	214-215
DNP-L-val-L-leu	88-90	140-143	68-72	146-147
DNP-L-val-L-phe	73-77	201-204	55-60	206-207
DNP-L-leu-gly	80-85	149-151	61-64	158-159
DNP-L-leu-L-leu	85-89	140-145	65-70	146-147
DNP-L-leu-L-phe[1]	73-78	74-82	50-52	147-148
DNP-L-phe-L-leu	62-68	155-165	47-50	167-168
DNP-L-phe-L-phe[2]	78-85	185-190	40-45	199-200

1 = DNP-L-leu-L-phe was purified by first recrystallizing from ethanol-water, then acetic acid-water.

2 = The Dinitrophenylation of L-phe-L-phe requires 24 hours at room temperature and repeated addition of sodium bicarbonate and FDNB.

Specific rotations and molecular rotations of the above DNP-dipeptides in 95% ethanol, dimethylformamide, and glacial acetic acid are summarized in Table 22.

Table 22

Specific rotations and molecular rotations of DNP-dipeptides in 95% ethanol, dimethylformamide and glacial acetic acid

Compound	95% Ethanol		Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe	+97.5°	+392.3°	+80.5°	+323.9°	+160.0°	+643.8°
DNP-L-val-L-leu	+69.0°	+273.5°	+50.0°	+198.2°	+146.5°	+580.7°
DNP-L-val-L-phe	+61.2°	+263.4°	+54.2°	+233.3°	+144.8°	+623.3°
DNP-L-leu-gly	+86.0°	+304.7°	+32.8°	+116.2°	+150.5°	+533.3°
DNP-L-leu-L-leu	+70.6°	+289.8°	+36.1°	+148.2°	+142.9°	+586.5°
DNP-L-leu-L-phe	+55.4°	+246.2°	+38.5°	+171.1°	+127.0°	+564.5°
DNP-L-phe-L-leu	-22.2°	-98.7°	-51.8°	-230.2°	+70.7°	+314.2°
DNP-L-phe-L-phe	-26.5°	-126.8°	-53.2°	-254.5°	+69.2°	+331.3°

Analytical results for the above DNP-dipeptides are summarized in Table 23.

Preparation of DNP-dipeptide hydrazides

A common approach to the synthesis of an N-protected amino acid or peptide hydrazide has been the hydrazinolysis of a suitable ester, methyl and ethyl esters being most preferred (74, 75). In the following preparations, the hydrazinolysis of the DNP-dipeptide ethyl esters was normally carried out in alcoholic solution. In most cases, it was sufficient for the alcoholic solution to stand for a short time with

Table 23

Results of the elemental analyses of DNP-dipeptides

Compound	Formula	Theory (%)			Found (%)		
		C	H	N	C	H	N
DNP-L-ala-L-phe	$C_{18}H_{18}N_4O_7$	53.73	4.51	13.93	53.97	4.74	14.19
DNP-L-val-gly	$C_{13}H_{16}N_4O_7$	45.88	4.74	16.46	46.34	4.74	16.27
DNP-L-val-L-leu	$C_{17}H_{24}N_4O_7$	51.51	6.10	14.13	51.80	6.37	14.24
DNP-L-val-L-phe	$C_{20}H_{22}N_4O_7$	55.81	5.15	13.02	55.72	5.18	13.18
DNP-L-leu-gly	$C_{14}H_{18}N_4O_7$	47.46	5.12	15.81	47.66	5.10	15.52
DNP-L-leu-L-leu	$C_{18}H_{26}N_4O_7$	52.67	6.38	13.65	52.48	6.18	13.54
DNP-L-leu-L-phe	$C_{21}H_{24}N_4O_7$	56.75	5.44	12.61	57.15	5.75	12.80
DNP-L-Ileu-gly	$C_{14}H_{18}N_4O_7$	47.46	5.12	15.81	47.56	5.05	15.45
DNP-L-Ileu-L-leu	$C_{18}H_{26}N_4O_7$	52.67	6.38	13.65	52.18	6.32	13.24
DNP-L-Ileu-L-phe	$C_{21}H_{24}N_4O_7$	56.75	5.44	12.61	56.33	5.35	12.73
DNP-L-phe-gly	$C_{17}H_{16}N_4O_7$	52.58	4.15	14.43	52.29	4.15	14.44
DNP-L-phe-L-leu	$C_{21}H_{24}N_4O_7$	56.75	5.44	12.61	56.84	5.63	12.56
DNP-L-phe-L-phe	$C_{24}H_{22}N_4O_7$	60.25	4.63	11.71	60.11	4.43	11.59
DNP-L-try-gly	$C_{19}H_{17}N_5O_7$	53.40	4.01	16.39	53.54	3.83	16.34
DNP-L-try-L-leu	$C_{23}H_{25}N_5O_7$	57.14	5.21	14.49	57.23	5.09	14.05
DNP-L-try-L-phe	$C_{26}H_{23}N_5O_7$	60.34	4.48	13.53	59.68	4.49	13.33

hydrazine hydrate. A large excess of hydrazine hydrate was required in all cases. The general equation for the overall reaction is shown as below:

$$O_2N-C_6H_3(NO_2)-NH\underset{R}{C}HCONH\underset{R'}{C}HCOOC_2H_5 + H_2NNH_2\cdot H_2O$$

$$\downarrow$$

$$O_2N-C_6H_3(NO_2)-NH\underset{R}{C}HCONH\underset{R'}{C}HCONHNH_2 + C_2H_5OH$$

Materials:

DNP-dipeptide ester	0.001 mole
Hydrazine hydrate	0.02 mole (1.00 gm.)
Absolute alcohol	15 ml.

DNP-dipeptide ester was dissolved in 15 ml. hot absolute alcohol, hydrazine hydrate added, the solution refluxed for 15 minutes and then allowed to stand at room temperature for 5 days in the absence of light. After refrigeration for a few hours, the crystals of the hydrazide were filtered off and washed with a small amount of cold ethanol.

The crude product was purified by recrystallizing with ethanol or ethanol-water.

Yield, melting point and the solvents of recrystallization of the products are summarized in Table 24.

Specific rotations and molecular rotations of the above hydrazides in dimethylformamide and glacial acetic acid are summarized in Table 25.

Analytical results for the above hydrazides are summarized in Table 26.

Table 24

Yield, melting point and recrystallizing solvent of DNP-dipeptide hydrazides

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)	Solvents for recrys.
DNP-gly-gly hydrazide	75-77	212-215	68-71	221-222	E
DNP-gly-L-leu hydrazide	76-80	202-205	72-76	205-206	E-W
DNP-gly-L-phe hydrazide	95-97	219-222	90-92	226-228	E
DNP-L-ala-L-phe hydrazide	95-97	233-235	92-94	234-236	E
DNP-L-val-gly hydrazide	91-93	209-211	85-88	210-211	E
DNP-L-val-L-leu hydrazide	93-95	245-246	89-92	245-246	E
DNP-L-val-L-phe hydrazide	96-98	248-250	93-95	249-250	E
DNP-L-leu-gly hydrazide	84-87	192-195	80-82	195-196	E-W
DNP-L-leu-L-leu hydrazide	93-95	213-216	88-90	218-219	E-W
DNP-L-leu-L-phe hydrazide	94-96	208-210	90-93	210-211	E
DNP-L-Ileu-gly hydrazide	90-92	196-197	87-88	198-199	E-W
DNP-L-Ileu-L-leu hydrazide	94-96	237-239	91-93	239-240	E-W
DNP-L-Ileu-L-phe hydrazide	96-98	226-228	94-96	226-228	E
DNP-L-phe-gly hydrazide	83-86	185-187	81-83	186-187	E-W
DNP-L-phe-L-leu hydrazide	94-96	219-221	90-93	221-222	E
DNP-L-phe-L-phe hydrazide	95-96	245-247	92-94	251-253	E
DNP-L-try-gly hydrazide	90-92	209-211	86-89	211-212	E
DNP-L-try-L-leu hydrazide	92-94	229-231	88-90	237-238	E

Where E = ethanol; E-W = ethanol-water.

Table 25

Specific rotations and molecular rotations of DNP-dipeptide hydrazides in dimethylformamide and glacial acetic acid

Compound	Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-gly-L-leu hydrazide	-26.2°	-96.5°	-30.1°	-110.9°
DNP-gly-L-phe hydrazide	-17.4°	-70.0°	+11.6°	+46.7°
DNP-L-ala-L-phe hydrazide	+76.1°	+316.9°	+207.3°	+863.2°
DNP-L-val-gly hydrazide	+73.5°	+260.4°	+190.6°	+675.4°
DNP-L-val-L-leu hydrazide	+45.0°	+184.7°	+166.4°	+683.0°
DNP-L-val-L-phe hydrazide	+36.7°	+163.1°	+131.8°	+585.8°
DNP-L-leu-gly hydrazide	+19.2°	+70.7°	+128.5°	+473.3°
DNP-L-leu-L-leu hydrazide	+25.1°	+106.5°	+125.6°	+533.1°
DNP-L-leu-L-phe hydrazide	+29.7°	+136.2°	+135.6°	+621.7°
DNP-L-Ileu-gly hydrazide	+36.8°	+135.6°	+186.7°	+688.7°
DNP-L-Ileu-L-leu hydrazide	+18.2°	+77.3°	+149.8°	+635.9°
DNP-L-Ileu-L-phe hydrazide	+28.0°	+128.4°	+158.7°	+727.6°
DNP-L-phe-gly hydrazide	-76.8°	-309.0°	+60.1°	+241.8°
DNP-L-phe-L-leu hydrazide	-67.0°	-307.2°	+105.0°	+481.4°
DNP-L-phe-L-phe hydrazide	-57.9°	-285.2°	+81.5°	+401.4°
DNP-L-try-gly hydrazide	-207.5°	-915.9°	+37.4°	+165.1°
DNP-L-try-L-leu hydrazide	-303.6°	-1510°	+82.2°	+409.0°

Table 26

Results of the elemental analyses of DNP-dipeptide hydrazides

Compound	Formula	Theory (%)			Found (%)		
		C	H	N	C	H	N
DNP-gly-gly hydrazide	$C_{10}H_{12}N_6O_6$	38.46	3.87	26.92	38.83	3.75	26.86
DNP-gly-L-leu hydrazide	$C_{14}H_{20}N_6O_6$	45.65	5.47	22.82	45.69	5.52	22.81
DNP-gly-L-phe hydrazide	$C_{17}H_{18}N_6O_6$	50.74	4.51	20.89	50.78	4.49	21.20
DNP-L-ala-L-phe hydrazide	$C_{18}H_{20}N_6O_6$	51.92	4.84	20.18	52.16	4.97	20.47
DNP-L-val-gly hydrazide	$C_{13}H_{18}N_6O_6$	44.06	5.12	23.71	43.86	4.95	24.03
DNP-L-val-L-leu hydrazide	$C_{17}H_{26}N_6O_6$	49.75	6.39	20.48	49.70	6.31	20.67
DNP-L-val-L-phe hydrazide	$C_{20}H_{24}N_6O_6$	54.05	5.44	18.91	54.15	5.34	18.77
DNP-L-leu-gly hydrazide	$C_{14}H_{20}N_6O_6$	45.65	5.47	22.82	45.91	5.47	23.08
DNP-L-leu-L-leu hydrazide	$C_{18}H_{28}N_6O_6$	50.93	6.65	19.80	50.81	6.85	19.96
DNP-L-leu-L-phe hydrazide	$C_{21}H_{26}N_6O_6$	55.01	5.72	18.33	55.20	5.45	18.18
DNP-L-Ileu-gly hydrazide	$C_{14}H_{20}N_6O_6$	45.65	5.47	22.82	45.70	5.46	22.73
DNP-L-Ileu-L-leu hydrazide	$C_{18}H_{28}N_6O_6$	50.93	6.65	19.80	51.15	6.48	19.64
DNP-L-Ileu-L-phe hydrazide	$C_{21}H_{26}N_6O_6$	55.01	5.72	18.33	54.87	5.62	18.49
DNP-L-phe-gly hydrazide	$C_{17}H_{18}N_6O_6$	50.74	4.51	20.89	50.53	4.31	21.05
DNP-L-phe-L-leu hydrazide	$C_{21}H_{26}N_6O_6$	55.01	5.72	18.33	54.99	5.72	18.26
DNP-L-phe-L-phe hydrazide	$C_{24}H_{24}N_6O_6$	58.53	4.91	17.07	58.23	4.51	16.84
DNP-L-try-gly hydrazide	$C_{19}H_{19}N_7O_6$	51.82	4.12	21.80	51.74	4.18	21.89
DNP-L-try-L-leu hydrazide	$C_{23}H_{27}N_7O_6$	55.53	5.47	19.71	55.69	5.26	19.88

Preparation of DNP-tripeptide esters

Two methods of preparing DNP-tripeptide esters were employed.

(1) By converting DNP-dipeptide hydrazide to azide with nitrous acid, and reacting the azide with the ethyl ester of the pertinent amino acid.

(2) By reacting DNP-dipeptide with the ethyl ester of the pertinent amino acid in the presence of 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate.

From the dipeptide hydrazide

The azide formation was carried in the mixture of acetic acid and hydrochloric acid. Upon addition of sodium nitrite at low temperature, the azide separates as an oil, and was extracted with ethyl acetate. The mixture was dried over anhydrous sodium sulfate prior to the reaction with the amino acid ester. The general equations for the overall reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCONHNH_2 + NaNO_2 + HCl$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCON_3 + 2\,H_2O + NaCl$$

$$\downarrow \; H_2N\overset{R''}{C}HCOOC_2H_5$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCONH\overset{R''}{C}HCOOC_2H_5 + HN_3$$

Materials:

DNP-dipeptide hydrazide	0.001 mole
L-Amino acid ethyl ester hydrochloride	0.001 mole
Triethylamine	0.001 mole (0.101 gm.)
N $NaNO_2$	1 ml.
Glacial acetic acid	20 ml.
6 N hydrochloric acid	5 ml.
Anhydrous ether	100 ml.

DNP-dipeptide hydrazide was dissolved in 20 ml. glacial acetic acid, to this 5 ml. of 6 N hydrochloric acid was added, and the solution was cooled to -5° in an ice-salt bath. with cooling and stirring, the sodium nitrite solution was added slowly and tested constantly with starch-iodide paper, until HNO_2 was present. The mixture was stirred for another 5 minutes at -5°, diluted with ice-water, and extracted with ice-cold ether. The ether layer was quickly washed with ice-water (2 X 25ml.), N $NaHCO_3$ (2 X 25 ml.), ice-water (2 X25ml.) and rapidly dried over anhydrous sodium sulfate. The dry ethyl acetate solution was added to an ethereal solution of the L-amino acid ethyl ester.(prepared by stirring the ester hydrochloride with triethylamine in anhydrous ether for 30 minutes.) After keeping at 5° for 6 days with occasional shaking, the solution was filtered, and the residue washed with 50 ml. of ether. The washed liquid was combined with the filtrate, and evaporated at room temperature to dryness. The crude product was purified by recrystallizing with ethanol-water.

Yield and melting point of the products are summarized in Table 27.

Table 27

Yield and melting point of DNP-tripeptide ethyl esters

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP-L-ala-L-phe-gly ethyl ester	52-58	189-195	45-48	201-202
DNP-L-val-L-leu-L-phe ethyl ester	56-60	216-222	50-55	225-227
DNP-L-Ileu-L-phe-L-leu ethyl ester	65-72	178-181	60-64	184-185

Specific rotations and molecular rotations of the esters in dimethylformamide and glacial acetic acid are summarized in Table 28.

Table 28

Specific rotations and molecular rotations of DNP-tripeptide ethyl esters in dimethylformamide and glacial acetic acid

Compound	Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe-gly ethyl ester	+60.0°	+292.5°	+181.3°	+883.8°
DNP-L-val-L-leu-L-phe ethyl ester	+32.8°	+187.5°	+141.4°	+808.3°
DNP-L-Ileu-L-phe-L-leu ethyl ester	+24.8°	+145.2°	+127.7°	+747.9°

Carbodiimide method

DNP-dipeptides were coupled with amino acid esters in a one step, room temperature reaction using 1-cyclohexyl-3(-2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate as the condensing agent. The desired DNP-tripeptide esters were formed in high yields. The general equations for the overall reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCOOH \; + \; H_2N\overset{R''}{C}HCOOC_2H_5$$

$$\downarrow \; C_6H_{11}\text{-}N{=}C{=}N\text{-}CHCH\text{-}\overset{\oplus}{N}(CH_3)C_4H_8O \; Z^{\ominus}$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCONH\overset{R''}{C}HCOOC_2H_5$$

$$+$$

$$C_6H_{11}\text{-}NHCONHCH_2CH_2\text{-}\overset{\oplus}{N}(H_3C)C_4H_8O \; Z^{\ominus}$$

where Z is $O_3S\text{-}C_6H_4\text{-}CH_3$

Materials:

DNP-dipeptide	0.002 mole
L-amino acid ethyl ester hydrochloride	0.002 mole
Triethylamine	0.002 mole (0.202 gm.)
1-Cyclohexyl-3-(-2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate	0.0024 mole (1.015 gm.)
Tetrahydrofuran	10 ml.
Dichloromethane	40 ml.

DNP-dipeptide was dissolved in 10 ml. tetrahydrofuran, and to this a solution of freshly prepared L-amino acid ethyl ester in dichloromethane (prepared by stirring 1 equivalent of the hydrochloride with 1 equivalent of triethylamine in 20 ml. dichloromethane) was added, followed by a solution of 1-cyclohexyl-3(-2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate in 20 ml. dichloromethane. The mixture was stirred at room temperature for 96 hours in the absence of light. After the reaction was completed, the solvent was evaporated at room temperature and the residue extracted with ethyl acetate (3 X 25ml.). The ethyl acetate solution was washed successively with N HCl (3 X 25ml.), water (3 X 25ml.), saturated $NaHCO_3$ (3 X 25ml.) and water (3 X25ml.). After drying over anhydrous sodium sulfate, the solvent was evaporated at room temperature, and the residue was purified by recrystallizing with appropriate solvents.

Yield, melting point and the solvents of recrystallization of the products are summarized in Table 29.

Table 29

Yield, melting point and recrystallizing solvent of DNP-tripeptide ethyl esters

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)	Solv.
DNP-L-ala-L-phe-gly Et	80-85	195-197	72-76	201-202	DMF-W
DNP-L-val-L-leu-L-phe Et	75-80	220-222	65-70	225-227	E
DNP-L-Ileu-L-phe-L-leu Et	76-82	174-177	69-75	184-185	E-W

Where Et = ethyl ester; DMF-W = dimethylformamide-water; E = ethanol; E-W = ethanol-water.

Specific rotations and molecular rotations of the above esters in dimethylformamide and glacial acetic acid are summarized in Table 30.

Table 30

Specific rotations and molecular rotations of DNP-tripeptide ethyl esters in dimethylformamide and glacial acetic acid

Compound	Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe-gly Et	+60.6°	+295.4°	+181.5°	+884.8°
DNP-L-val-L-leu-L-phe Et	+33.0°	+188.6°	+141.0°	+806.0°
DNP-L-Ileu-L-phe-L-leu Et	+24.7°	+144.7°	+127.6°	+747.3°

Where Et = ethyl ester.

Analytical results for the above DNP-tripeptide ethyl esters are summarized in Table 31.

Table 31

Results of the elemental analyses of DNP-tripeptide ethyl esters

Compound	Formula	Theory (%)			Found (%)		
		C	H	N	C	H	N
I	$C_{22}H_{25}N_5O_8$	54.20	5.17	14.37	54.05	5.13	14.56
II	$C_{28}H_{37}N_5O_8$	58.83	6.53	12.25	58.84	6.78	12.44
III	$C_{29}H_{39}N_5O_8$	59.47	6.71	11.96	59.39	6.72	11.92

Where I = DNP-L-alanyl-L-phenylalanyl-glycine ethyl ester;

II = DNP-L-valyl-L-leucyl-L-phenylalanine ethyl ester;

III = DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester.

Preparation of DNP-tripeptides

Two methods of preparing DNP-tripeptides were employed.

(1) Hydrolyzing the DNP-tripeptide esters with N aqueous sodium hydroxide in 95% ethanol.

(2) Reacting the tripeptides directly with FDNB.

Alkaline hydrolysis method

The hydrolysis was carried out in ethanol with a small amount excess of alkali for 24 hours at room temperature. The general equation for the overall reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\underset{R}{CH}CONH\underset{R'}{CH}CONH\underset{R''}{CH}COOC_2H_5 + NaOH$$

$$\downarrow$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\underset{R}{CH}CONH\underset{R'}{CH}CONH\underset{R''}{CH}COO^{\ominus}Na^{\oplus} + C_2H_5OH$$

$$\downarrow HCl$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\underset{R}{CH}CONH\underset{R'}{CH}CONH\underset{R''}{CH}COOH + NaCl$$

Materials:

DNP-tripeptide ethyl ester	0.001 mole
95% ethanol	20 ml.
N NaOH	1.2 ml.

DNP-tripeptide ester was dissolved in 20 ml. ethanol; to this N NaOH was added in small portions and the solution stirred at room temperature for 24 hours in the absence of light. At the end of the reaction, the solution was diluted with 50 ml. of water, 5 ml. saturated $NaHCO_3$ added, and the solution washed twice with 25 ml. ethyl acetate. The aqueous layer was then acidified with concentrated hydrochloric acid to pH 2, and the DNP-tripeptide was extracted thrice with 25 ml. ethyl acetate. The organic layer was washed twice with 25 ml. of water, and dried over anhydrous sodium sulfate. The solvent was evaporated at room temperature and the residue purified by the following methods.

For DNP-L-alanyl-L-phenylalanyl-glycine, the crude product was dissolved in 10 ml. of acetone, filtered, water added to the filtrate until the solution just turn cloudy, and the mixture refrigerated overnight. The crystals were collected by filtering with suction, dried in a vacuum desiccator and recrystallized from t-butanol-water in the same manner to obtain a pure product.

For DNP-L-valyl-L-leucyl-L-phenylalanine, the crude product was first recrystallized from ethanol-water, then from t-butanol-water to obtain a pure product.

For DNP-L-isoleucyl-L-leucyl-L-phenylalanine, the crude product was first recrystallized from ethanol-water, then from t-butanol-water to obtain a pure product.

Yield and melting point of the products are summarized in Table 32.

Table 32

Yield and melting point of DNP-tripeptides

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP-L-ala-L-phe-gly	52-56	209-212	40-45	224-225
DNP-L-val-L-leu-L-phe	58-66	180-192	48-52	203-204
DNP-L-Ileu-L-phe-L-leu	65-70	191-225	58-62	232-233

Specific rotations and molecular rotations of the above DNP-tripeptides in dimethylformamide and glacial acetic acid are summarized in Table 33.

Table 33

Specific rotations and molecular rotations of DNP-tripeptides in dimethylformamide and glacial acetic acid

Compound	Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-ala-L-phe-gly	+67.2°	+308.7°	+190.1°	+873.4°
DNP-L-val-L-leu-L-phe	+34.1°	+185.4°	+156.0°	+848.0°
DNP-L-Ileu-L-phe-L-leu	+26.6°	+148.3°	+130.6°	+728.2°

Dinitrophenylation method

According to the method employed by Sanger and Thomson (41), the tripeptide was reacted with FDNB in triethylamine. The general equations for the overall reaction are shown as below:

$$O_2N\text{-}C_6H_3(NO_2)\text{-}F + H_2N\underset{|}{\overset{R}{C}}HCONH\overset{R'}{C}HCONH\overset{R''}{C}HCOO^{\ominus}\overset{\oplus}{N}H(C_2H_5)_3$$

$$\downarrow (C_2H_5)_3N$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCONH\overset{R''}{C}HCOO^{\ominus}\overset{\oplus}{N}H(C_2H_5)_3 + (C_2H_5)_3NH^{\oplus}F^{\ominus}$$

$$\downarrow HCl$$

$$O_2N\text{-}C_6H_3(NO_2)\text{-}NH\overset{R}{C}HCONH\overset{R'}{C}HCONH\overset{R''}{C}HCOOH \qquad (C_2H_5)_3\overset{\oplus}{N}HCl^{\ominus}$$

Materials:

Tripeptide	0.001 mole
FDNB	0.001 mole (0.186 gm.)
Triethylamine	0.003 mole (0.303 gm.)
Ethanol	5 ml.

The tripeptide was dissolved in 30 ml. 1% triethylamine (prepared by dissolving 0.3 gm. triethylamine in 30 ml. water) and to this was added a solution of FDNB in 10 ml. ethanol. The mixture was stirred for six hours at room temperature in the absence of light, and the excess triethylamine removed by vacuum distillation from a water bath maintained at 40°, continuing the distillation until the pressure of the system had decrease to 25-30 mm. The residual solution was acidified with concentrated hydrochloric acid to pH 2

and refrigerated overnight. The product was filtered with suction, washed with ice-water to remove excess hydrochloric acid and dried in a vacuum desiccator.

The crude product was purified by recrystallizing from ethanol-water.

Yield and melting of the products are summarized in Table 34.

Table 34

Yield and melting point of DNP-tripeptides

Compound	Crude yield (%)	Crude M.P. (°C)	Pure yield (%)	Pure M.P. (°C)
DNP-L-val-gly-gly	72-78	202-204	66-70	207-208
DNP-L-leu-gly-gly	65-70	185-188	52-56	189-190
DNP-L-phe-gly-gly	55-62	204-206	46-50	213-214

Specific rotations and molecular rotations of the above DNP-tripeptides in dimethylformamide and glacial acetic acid are summarized in Table 35.

Table 35

Specific rotations and molecular rotations of DNP-tripeptides in dimethylformamide and glacial acetic acid

Compound	Dimethyl formamide		Glacial acetic acid	
	$[\alpha]_D^{22}$	$[M]_D^{22}$	$[\alpha]_D^{22}$	$[M]_D^{22}$
DNP-L-val-gly-gly	+50.2°	+199.5°	+174.0°	+691.4°
DNP-L-leu-gly-gly	+24.7°	+101.6°	+142.6°	+588.6°
DNP-L-phe-gly-gly	-54.6°	-243.2°	+43.8°	+195.1°

Analytical results for the above DNP-tripeptides are summarized in Table 36.

Table 36

Results of the elemental analyses of DNP-tripeptides

Compound	Formula	Theory (%) C	Theory (%) H	Theory (%) N	Found (%) C	Found (%) H	Found (%) N
DNP-L-ala-L-phe-gly	$C_{20}H_{21}N_5O_8$	52.29	4.61	15.25	52.64	4.71	15.16
DNP-L-val-L-leu-L-phe	$C_{26}H_{33}N_5O_8$	57.45	6.12	12.88	57.74	5.92	13.02
DNP-L-Ileu-L-phe-L-leu	$C_{27}H_{35}N_5O_8$	58.16	6.33	12.56	58.64	6.74	12.51
DNP-L-val-gly-gly	$C_{15}H_{19}N_5O_8$	45.34	4.82	17.63	45.86	4.88	17.87
DNP-L-leu-gly-gly	$C_{16}H_{21}N_5O_8$	46.72	5.15	17.02	46.97	5.18	16.76
DNP-L-phe-gly-gly	$C_{19}H_{19}N_5O_8$	51.24	4.30	15.73	51.14	4.33	16.17

Thin layer chromatography

Thin layer chromatography was carried out on a Mallinckrodt Chroma-Kit, with Silic AR (TLC-7GF) as adsorbent. Four solvent systems were used for developing, and gave good separations on most of the prepared DNP-peptides and their corresponding esters. Only 0.1 to 0.5 μg of material was required on each individual compound, after developing, as little as 0.1 μg giving a yellow spot that is easily visible by transmitted daylight, therefore no sprayed reagent was required for detection.

Purification of Solvents

(1) Benzene was purified by shaking the organic liquid (1 liter) successively with portions of concentrated sulfuric acid (100 ml.) until free of thiophene, then with water until the washings were neutral to litmus. The water was removed by shaking the benzene with anhydrous calcium chloride, followed by refluxing for six hours over sodium metal. The benzene was distilled, the initial and final 50 ml. of distillate being discarded, and the dry solvent was stored over sodium. It was distilled just prior to use.

(2) Toluene was purified by the method similar to those used for benzene. The toluene (1 liter) was successively shaken with portions of concentrated sulfuric acid (100 ml.) until free of sulfur impurities, then with water until the washings were neutral to litmus. The organic layer was dried over phosphorus pentoxide and then fractionally distilled. The portion distilling between 110° and 111° was collected and stored in a glass stoppered flask.

(3) Glacial acetic acid was purified by the method of Vogel (76).

Approximately 600 gm. of commercial glacial acetic acid was partially frozen and about 300 ml. of liquid removed. The residue was melted and mixed with 6 gm. of potassium permanganate and fractionally distilled. The portion distilling between 115.8° and 116.8° at 758 mm was collected, partially frozen, and about half of the acid discarded as liquid. The solid was melted and fractionally distilled, the portion distilling between 116.2° and 117.2° being collected and stored in a glass stoppered flask. Precautions were taken to prevent the ingress of moisture during the fractional distillation.

(4) Pyridine was purified by standing over freshly fused potassium hydroxide for 24 hours and then fractionally distilled. The portion distilling between 115° and 116° was collected and stored.

(5) Chloroform (contained 0.75% ethanol) was purified by distilling twice through a short column.

(6) Methanol was purified by distilling twice through a short column.

(7) Benzyl alcohol was purified by fractional distillation at reduced pressure with the exclusion of air.

Procedure for thin layer chromatography

A plate with a smooth, uniform layer 0.25 mm thick was prepared, and activated at 80° or 100° in the oven for 2 hours. The samples to be chromatographed were dissolved in acetone and applied on a line parallel to and at least 2 cm from the edge of the coated plate. The samples were applied to the coated plate by means of a 5 μl micro-

pipette. Approximately 2 μl was applied at once, allowing time for solvent evaporation between successive applications, so that the spot size is kept small. After the spots had been applied, the chromatogram was developed vertically in a saturated chamber. The solvent was allowed to move up through the layer to a height of 10 to 15 cm, the position of the solvent marked, and the solvent allowed to evaporated. The distances moved by the samples were measured, and their R_f values calculated.

Thin layer chromatography of DNP-dipeptide esters

DNP-dipeptide esters were chromatographed with chloroform: methanol :glacial acetic (95:5:1) and toluene:pyridine:glacial acetic acid (80:10:1) as solvents. The R_f-values are listed in Table 36a. The distance travelled by the individual DNP-dipeptide esters, DNP-L-amino acids and in the mixture is shown in Fig 1-4.

Table 36a

100 X R_f-values of DNP-dipeptide esters and DNP-L-amino acids with one-dimensional, ascending chromatography on SilicAR TLC-7GF layer. Solvent systems (A) chloroform: methanol:glacial acetic acid (95:5:1) and (B) toluene: pyridine:glacial acetic acid (80:10:1) were used.

Compound	100 X R_f	
	A	B
DNP-gly-gly ethyl ester	25.0	9.5
DNP-glycine	29.5	7.4
DNP-gly-L-leu ethyl ester	30.2	39.8
DNP-L-tryptophan	31.0	5.0
DNP-L-try-gly ethyl ester	31.0	22.2
DNP-gly-L-phe ethyl ester	31.1	36.6
DNP-L-val-gly ethyl ester	32.1	32.5
DNP-L-phe-gly ethyl ester	33.0	33.7
DNP-L-ileu-gly ethyl ester	34.0	40.1
DNP-L-leu-gly ethyl ester	34.1	49.6
DNP-L-try-L-phe ethyl ester	37.3	42.5
DNP-L-try-L-leu ethyl ester	38.2	42.5
DNP-L-leucine	55.0	31.8
DNP-L-val-L-phe ethyl ester	56.3	61.8
DNP-L-val-L-leu ethyl ester	57.2	61.8
DNP-L-leu-L-phe ethyl ester	58.0	62.7
DNP-L-valine	59.3	19.4
DNP-L-leu-L-leu ethyl ester	60.8	62.7
DNP-L-phe-L-leu ethyl ester	63.0	61.5
DNP-L-phe-L-phe ethyl ester	63.0	61.5
DNP-L-Ileu-L-phe ethyl ester	63.5	62.9
DNP-L-phenylalanine	64.3	11.5
DNP-L-Ileu-L-leu ethyl ester	66.2	62.9
DNP-L-isoleucine	67.7	28.0
DNP-L-ala-L-phe ethyl ester	60.8	58.9

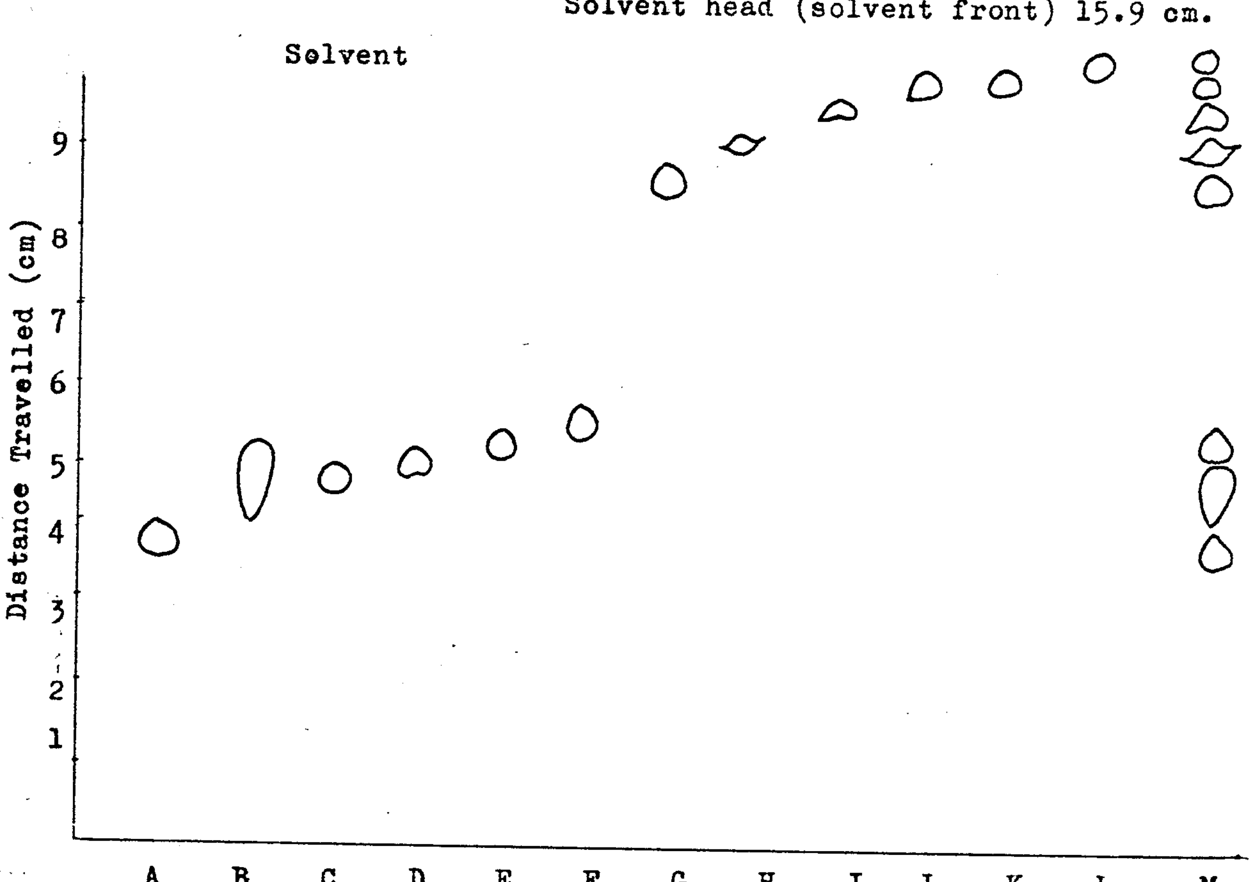


Figure I. Thin layer chromatography of DNP-compounds using chloroform :methanol:glacial acetic acid (95:5:1) as developer. Where A = DNP-glycyl-glycine ethyl ester; B = DNP-glycine; C = DNP-glycyl-L-leucine ethyl ester; D = DNP-glycyl-L-phenylalanine ethyl ester; E = DNP-L-phenylalanyl-glycine ethyl ester; F = DNP-L-leucyl-glycine ethyl ester; G = DNP-L-leucine; H = DNP-L-leucyl-L-phenylalanine ethyl ester; I = DNP-L-leucyl-L-leucine ethyl ester; J = DNP-L-phenylalanyl-L-leucine ethyl ester; K = DNP-L-phenylalanyl-L-phenylalanine ethyl ester; L = DNP-L-phenylalanine; M = a mixture of A,B,C,D,E,F,G,H,I,J,K, and L.

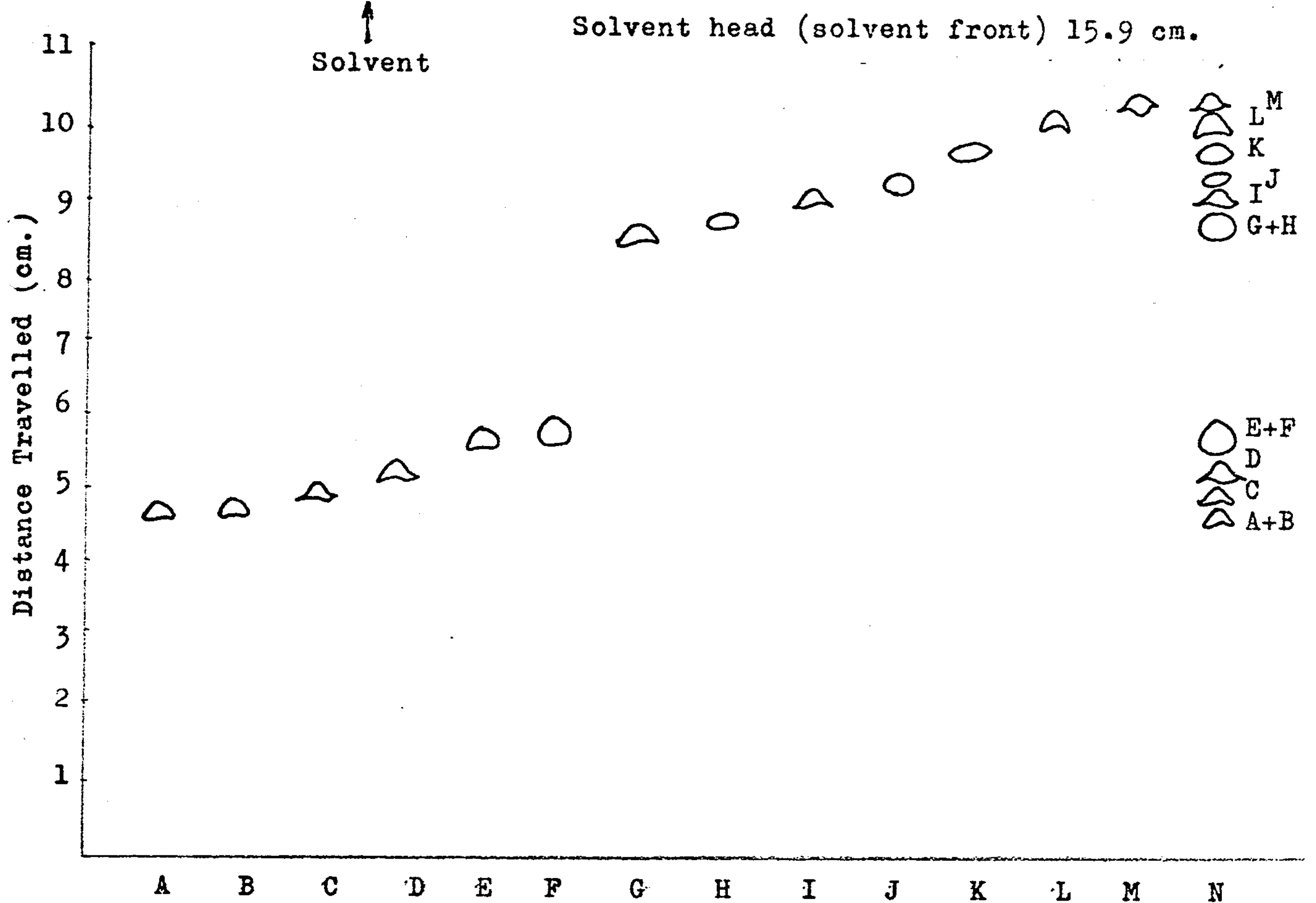


Figure 2. Thin layer chromatography of DNP-compounds using chloroform :methanol:glacial acetic acid (95:5:1) as developer. Where A = DNP-L-tryptophan; B = DNP-L-tryptophyl-glycine ethyl ester; C = DNP-L-valyl-glycine ethyl ester; D = DNP-L-isoleucyl-glycine ethyl ester; E = DNP-L-tryptophyl-L-phenylalanine ethyl ester; F = DNP-L-tryptophyl-L-leucine ethyl ester; G = DNP-L-valyl-L-phenylalanine Et; H = DNP-L-valyl-L-leucine ethyl ester; I = DNP-L-valine; J = DNP-L-alanyl-L-phenylalanine ethyl ester; K = DNP-L-isoleucyl-L-phenylalanine ethyl ester; L = DNP-L-isoleucyl-L-leucine ethyl ester; M = DNP-L-isoleucine; N = a mixture of A,B, C;D,E,F,G,H,I,J,K,L, and M.

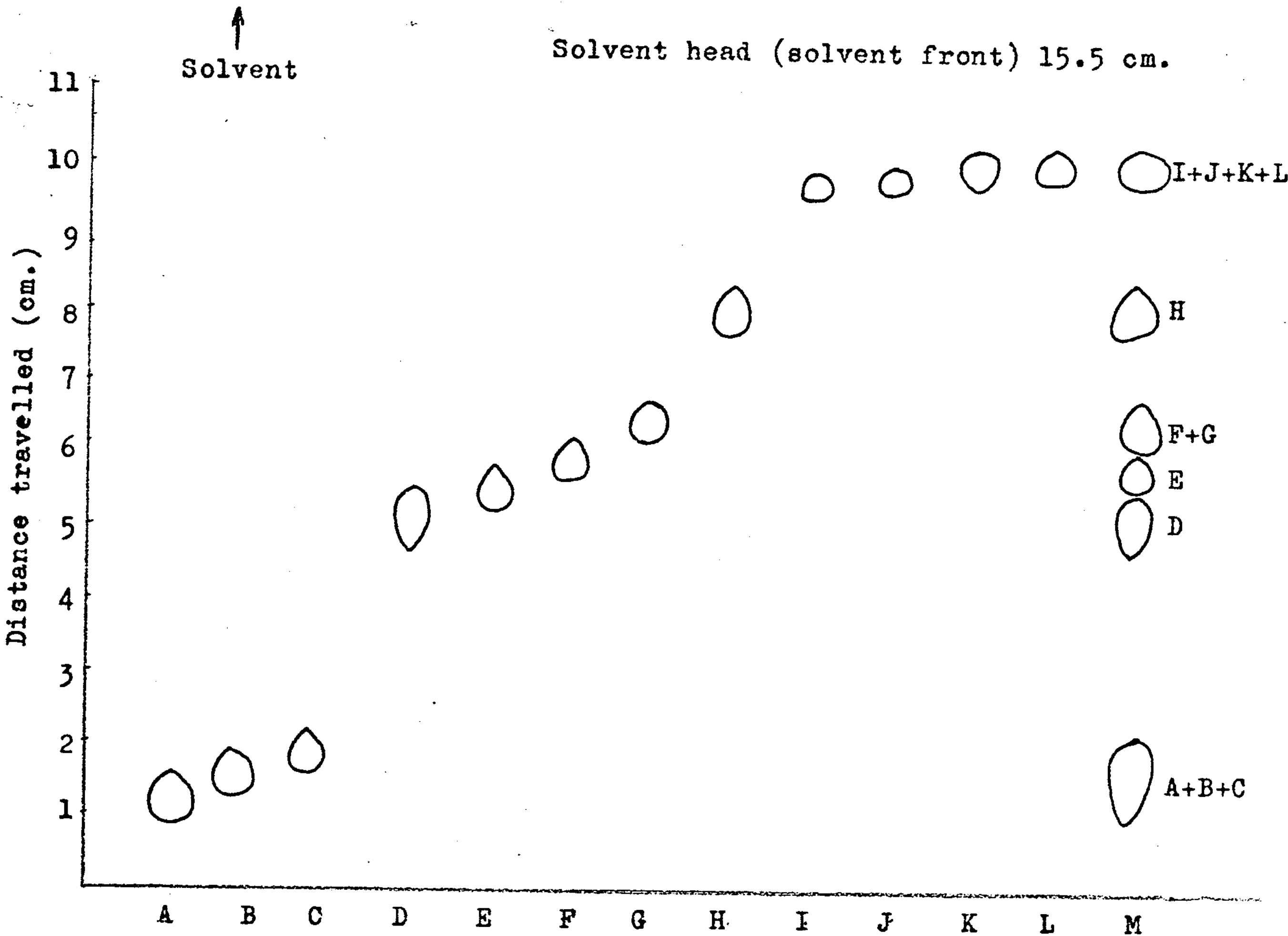


Figure 3. Thin layer chromatography of DNP-compounds using toluene: pyridine:glacial acetic acid (80:10:1) as developer. Where A = DNP-glycine; B = DNP-glycyl-glycine ethyl ester; C = DNP-L-phenylalanine; D = DNP-L-leucine; E = DNP-L-phenyl-alanyl-glycine ethyl ester; F = DNP-glycyl-L-phenylalanine ethyl ester; G = DNP-glycyl-L-leucine ethyl ester; H = DNP-L-leucyl-glycine ethyl ester; I = DNP-L-phenylalanyl-L-phenylalanine ethyl ester; J = DNP-L-phenylalanyl-L-leucine ethyl ester; K = DNP-L-leucyl-L-leucine ethyl ester; L = DNP-L-leucyl-L-phenylalanine ethyl ester; M = a mixture of A,B,C,D,E,F,G,H,I,J,K, and L.

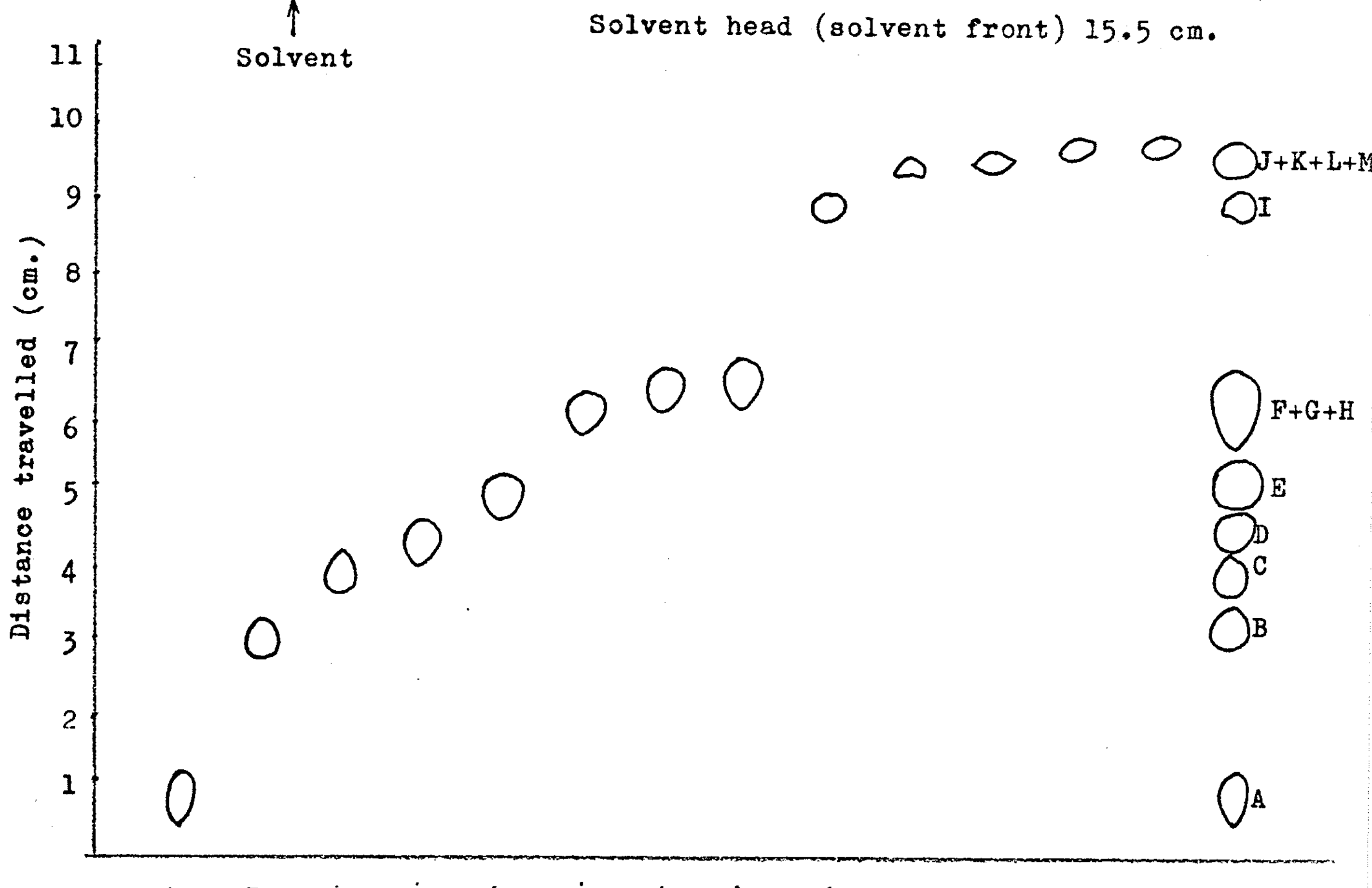


Figure 4. Thin layer chromatography of DNP-compounds using toluene: pyridine:glacial acetic acid (80:10:1) as developer. Where A = DNP-tryptophan; B = DNP-L-valine; C = DNP-L-tryptophyl-glycine ethyl ester; D = DNP-L-isoleucine; E = DNP-L-valyl-glycine ethyl ester; F = DNP-L-isoleucyl-glycine ethyl ester; G = DNP-L-tryptophyl-L-phenylalanine ethyl ester; H = DNP-L-tryptophyl-L-leucine ethyl ester; I = DNP-L-alanyl-L-phenylalanine ethyl ester; J = DNP-L-valyl-L-leucine ethyl ester; K = DNP-L-valyl-L-phenylalanine ethyl ester; L= DNP-L-isoleucyl-L-leucine ethyl ester; M = DNP-L-isoleucyl-L-phenylalanine ethyl ester; N = a mixture of A,B,C,D,E,F,G,H,I,J,K,L and M.

Thin layer chromatography of DNP-dipeptides

DNP-dipeptides were chromatographed with toluene:pyridine:glacial acetic acid (80:10:1), benzene:glacial acetic acid (20:3) and chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as solvents. The R_l-values are listed in Table 37.

Table 37

100 X R_l[1]-values of DNP-dipeptides with one dimensional, ascending chromatography on SilicAR TLC-7GF layer. Solvents (A) toluene:pyridine:glacial acetic acid (80:10:1), (B) benzene:glacial acetic acid (20:3), and (C) chloroform: benzyl alcohol:glacial acetic acid (97:2:1) were used.

Compound	100 X R_l		
	A[2]	B[3]	C[3]
DNP-L-ala-L-phe	31.9	39.4	32.1
DNP-L-val-gly	22.0	16.3	19.5
DNP-L-val-L-leu	92,7	80.0	81.6
DNP-L-val-L-phe	71.7	74.2	64.2
DNP-L-leu-gly	36.6	27.0	28.5
DNP-L-leu-L-leu	100.0	100.0	100.0
DNP-L-leu-L-phe	82.3	94.0	89.7
DNP-L-Ileu-gly	35.0	20.1	26.9
DNP-L-Ileu-L-leu	97.6	100.0	94.8
DNP-L-Ileu-L-phe	84.7	90.5	88.2
DNP-L-phe-gly	24.4	28.6	24.2
DNP-L-phe-L-leu	95.3	94.0	92.7
DNP-L-phe-L-phe	65.2	92.8	68.3
DNP-L-try-gly	4.9	7.2	10.3
DNP-L-try-L-leu	61.0	49.4	42.8
DNP-L-try-L-phe	30.1	62.7	37.3

[1] R_l = migration distance of the sample/migration distance of DNP-L-leucyl-L-leucine.

[2] DNP-L-leucyl-L-leucine travelled 8.2 cm. as standard.

[3] DNP-L-leucyl-L-leucine travelled 8.5 cm. as standard.

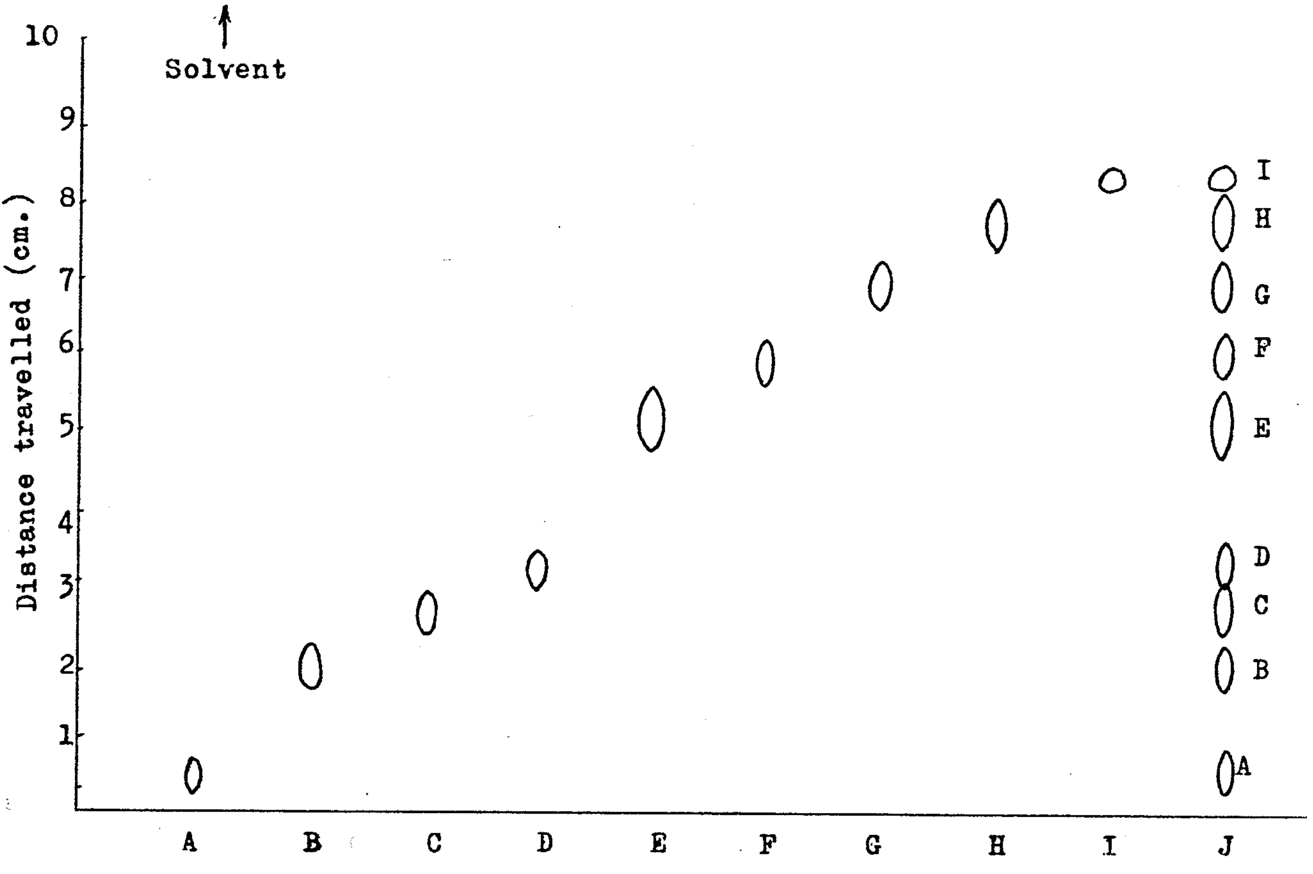


Figure 5. Thin layer chromatography of DNP-dipeptides using toluene: pyridine:glacial acetic acid (80:10:1) as developer. Where A = DNP-L-tryptophyl-glycine; B = DNP-L-valyl-glycine; C = DNP-tryptophyl-L-phenylalanine; D = DNP-L-leucyl-glycine; E = DNP-L-tryptophyl-L-leucine; F = DNP-L-valyl-L-phenylalanine; G = DNP-L-leucyl-L-phenylalanine; H = DNP-L-valyl-L-leucine; I = DNP-L-leucyl-L-leucine; J = A mixture of A,B,C,D,E,F,G,H and I.

DNP-L-leucyl-L-leucine travelled 8.2 cm. as standard.

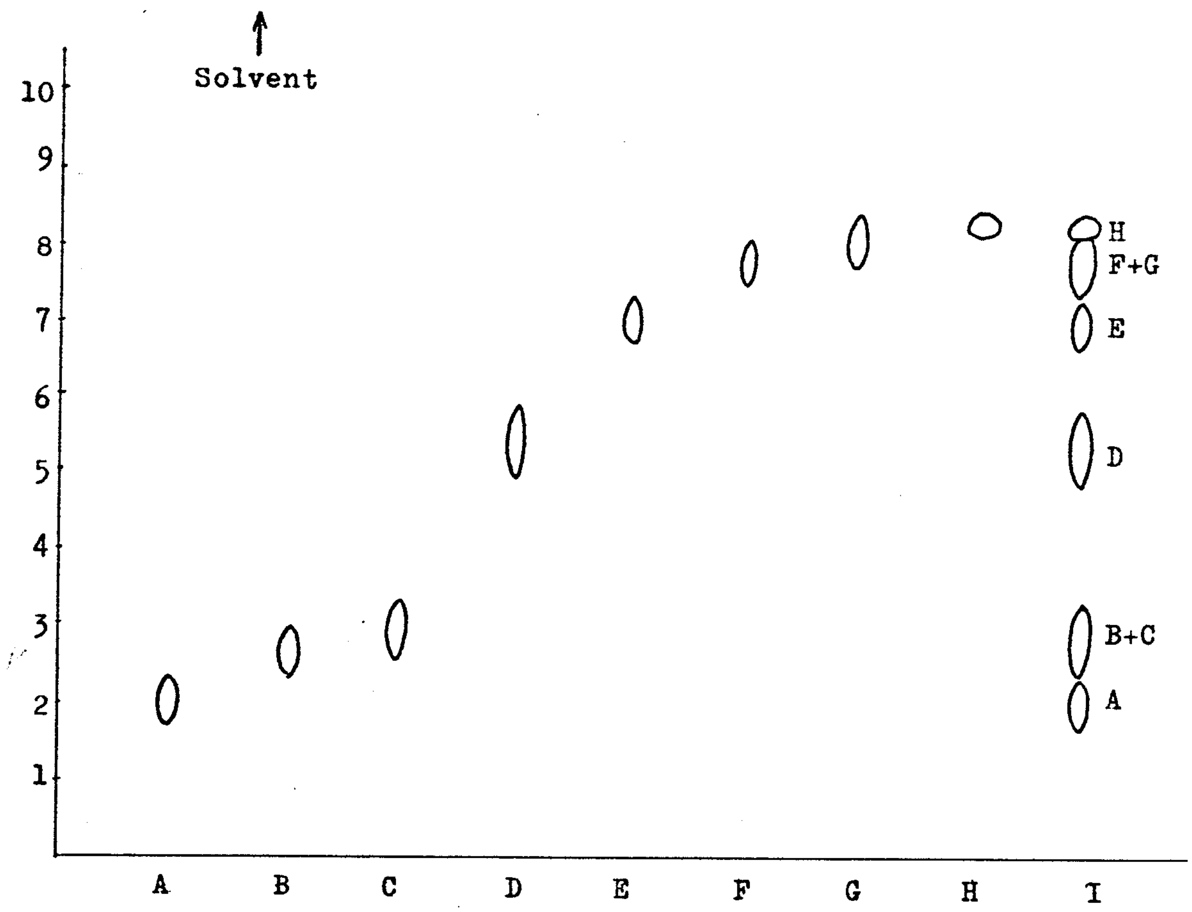


Figure 6. Thin layer chromatography of DNP-dipeptides using toluene:pyridine:glacial acetic acid (80:10:1) as developer. Where A = DNP-L-phenylalanyl-glycine; B = DNP-L-alanyl-L-phenylalanine; C = DNP-L-isoleucyl-glycine; D = DNP-L-phenylalanyl-L-phenylalanine; E = DNP-L-isoleucyl-L-phenylalanine; F = DNP-L-phenylalanyl-L-leucine; G = DNP-L-isoleucyl-L-leucine; H = DNP-L-leucyl-L-leucine; I = a mixture of A,B,C,D,E,F,G, and H.

DNP-L-leucyl-L-leucine travelled 8.2 cm. as standard.

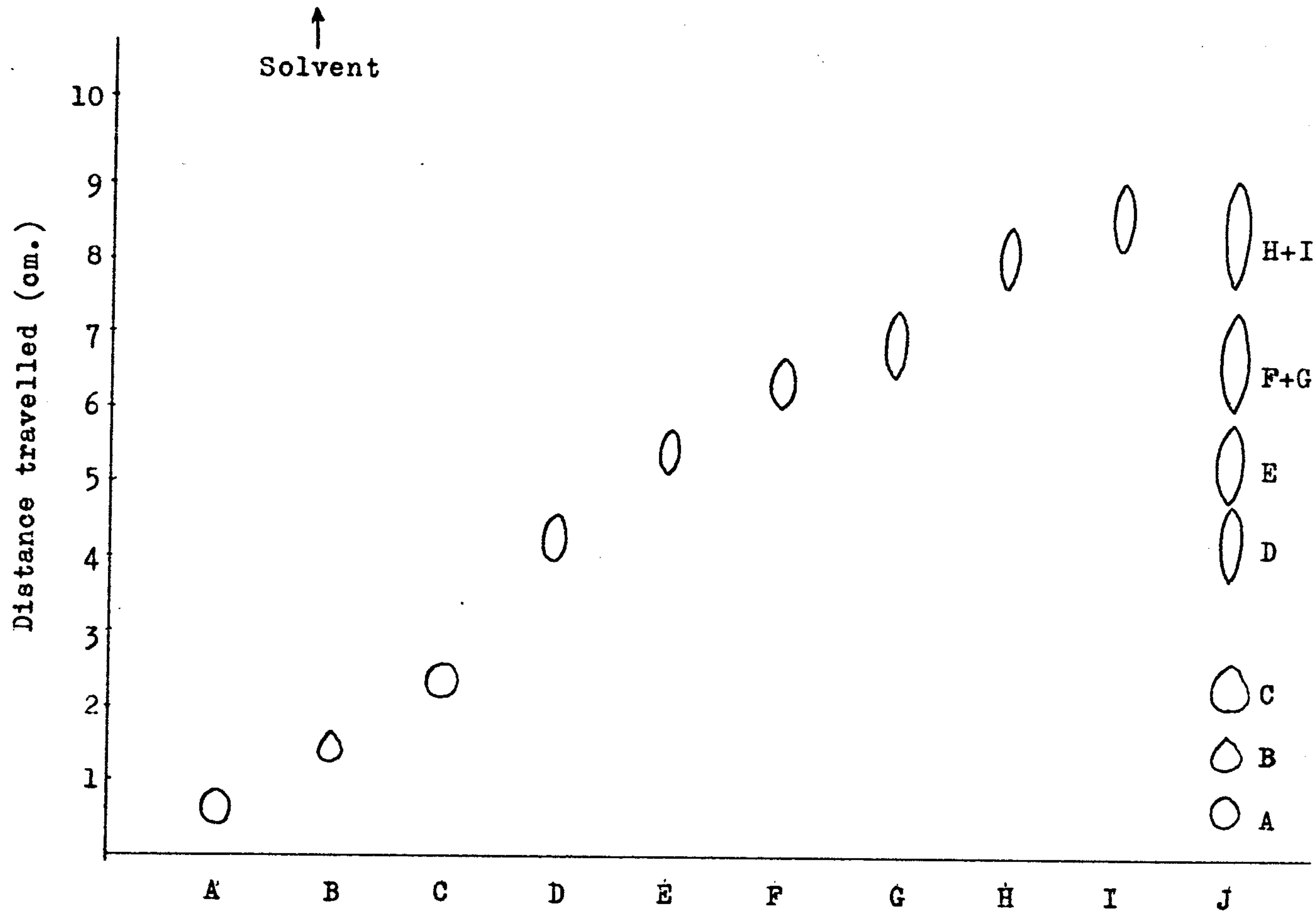


Figure 7. Thin layer chromatography of DNP-dipeptides using benzene:glacial acetic acid (20:3) as developer. Where A = DNP-L-tryptophyl-glycine; B = DNP-L-valyl-glycine; C = DNP-L-leucyl-glycine; D = DNP-L-tryptophyl-L-leucine; E = DNP-L-tryptophyl-L-phenylalanine; F = DNP-L-valyl-L-phenylalanine; G = DNP-L-valyl-L-leucine; H = DNP-L-leucyl-L-phenylalanine; I = DNP-L-leucyl-L-leucine; J = a mixture of A,B,C,D,E,F,G,H and I.

DNP-L-leucyl-L-leucine travelled 8.5 cm. as standard.

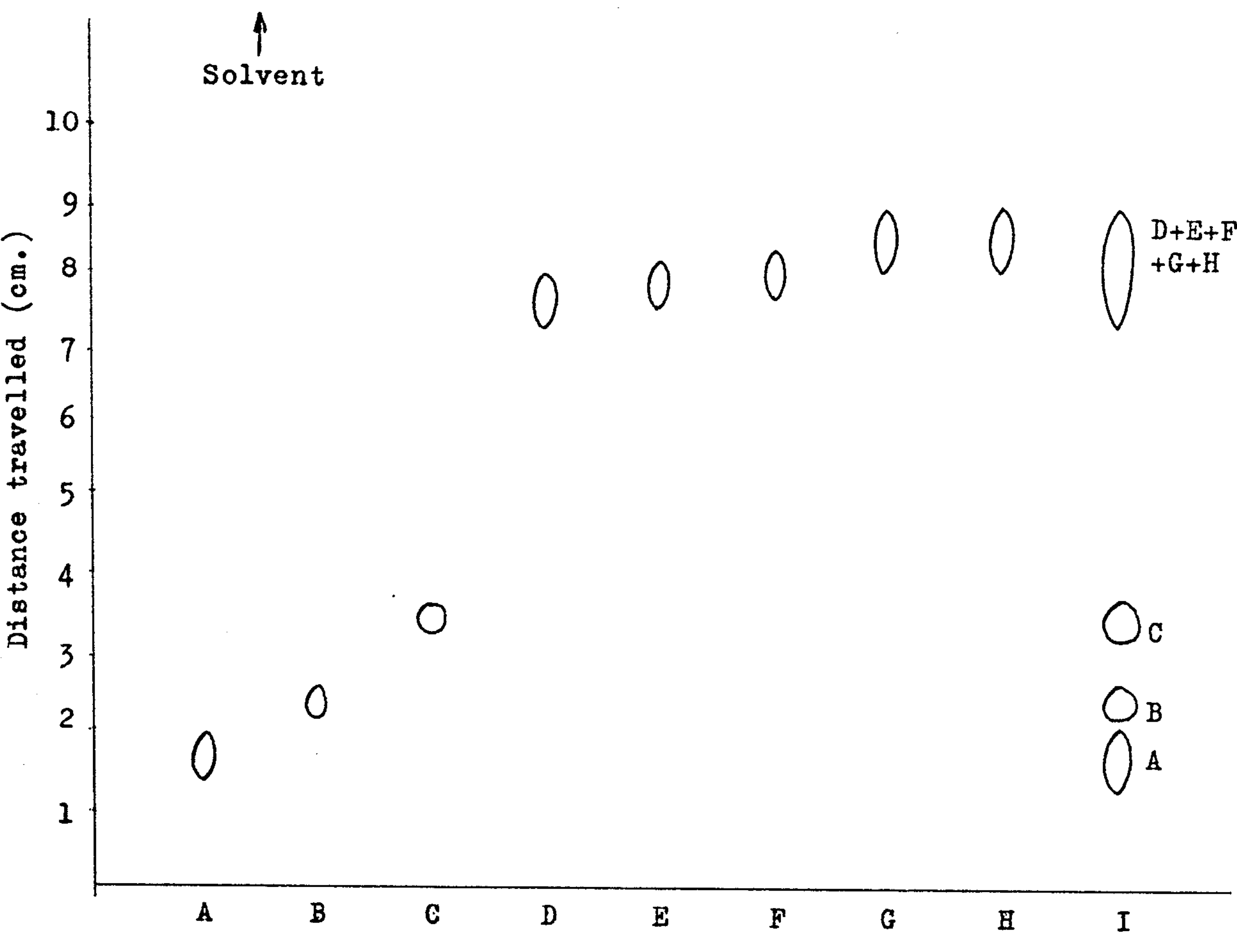


Figure 8. Thin layer chromatography of DNP-dipeptides using benzene:glacial acetic acid (20:3) as developer. Where A = DNP-L-isoleucyl-glycine; B = DNP-L-phenylalanyl-glycine; C = DNP-L-alanyl-L-phenylalanine; D = DNP-L-isoleucyl-L-phenylalanine; E = DNP-L-phenylalanyl-L-phenylalanine; F = DNP-L-phenylalanyl-L-leucine; G = DNP-L-isoleucyl-L-leucine; H = DNP-L-leucyl-L-leucine; I = a mixture of A,B,C,D,E,F,G and H.

DNP-L-leucyl-L-leucine travelled 8.5 cm. as standard.

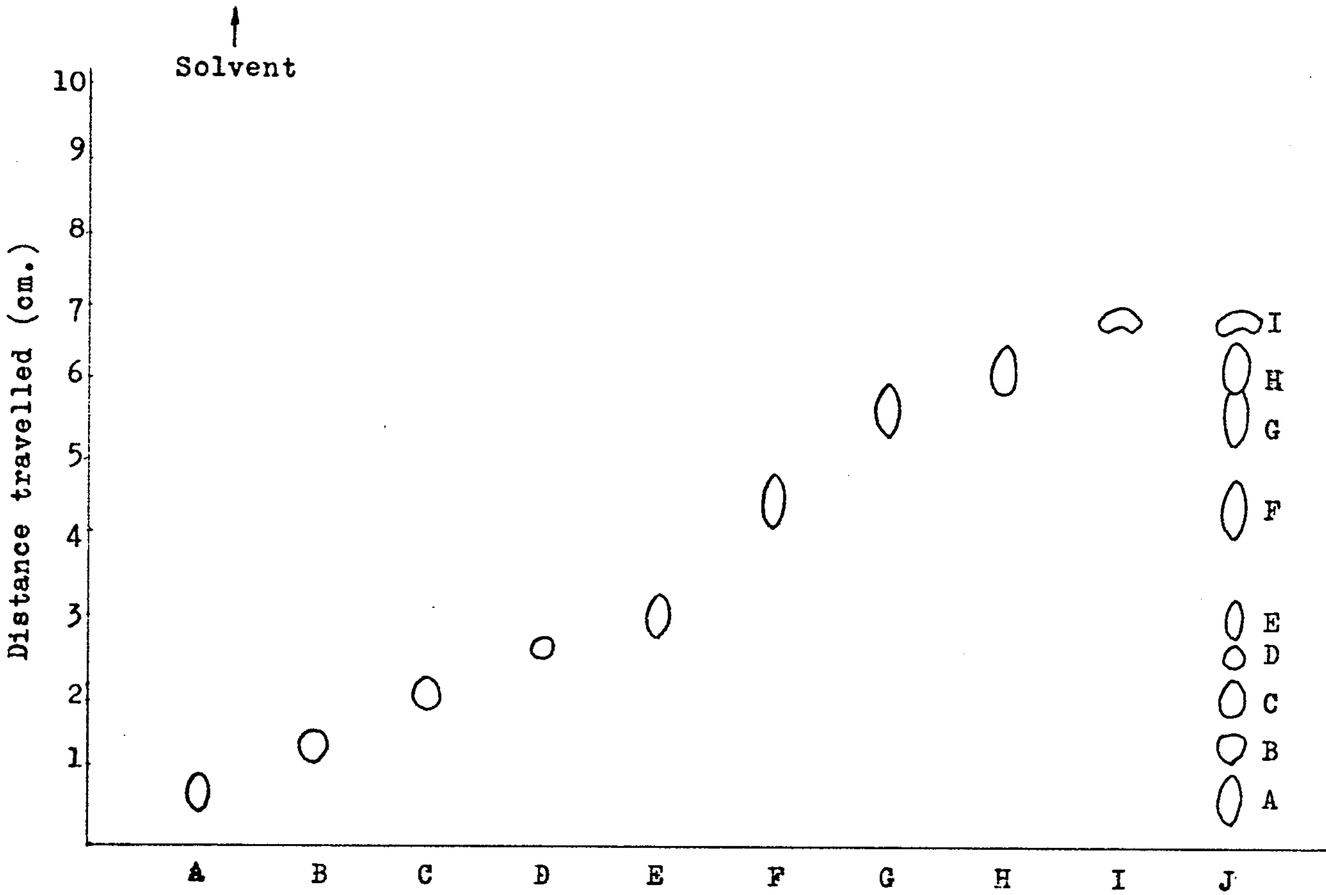


Figure 9. Thin layer chromatography of DNP-dipeptides using chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as developer. Where A = DNP-L-tryptophyl-glycine; B = DNP-L-valyl-glycine; C = DNP-L-leucyl-glycine; D = DNP-L-tryptophyl-L-phenylalanine; E = DNP-L-tryptophyl-L-leucine; F = DNP-L-valyl-L-phenylalanine; G = DNP-L-valyl-L-leucine; H = DNP-L-leucyl-L-phenylalanine; I = DNP-L-leucyl-L-leucine; J = a mixture of A,B,C, D,E,F,G,H and I.

DNP-L-leucyl-L-leucine travelled 8.5 cm. as standard.

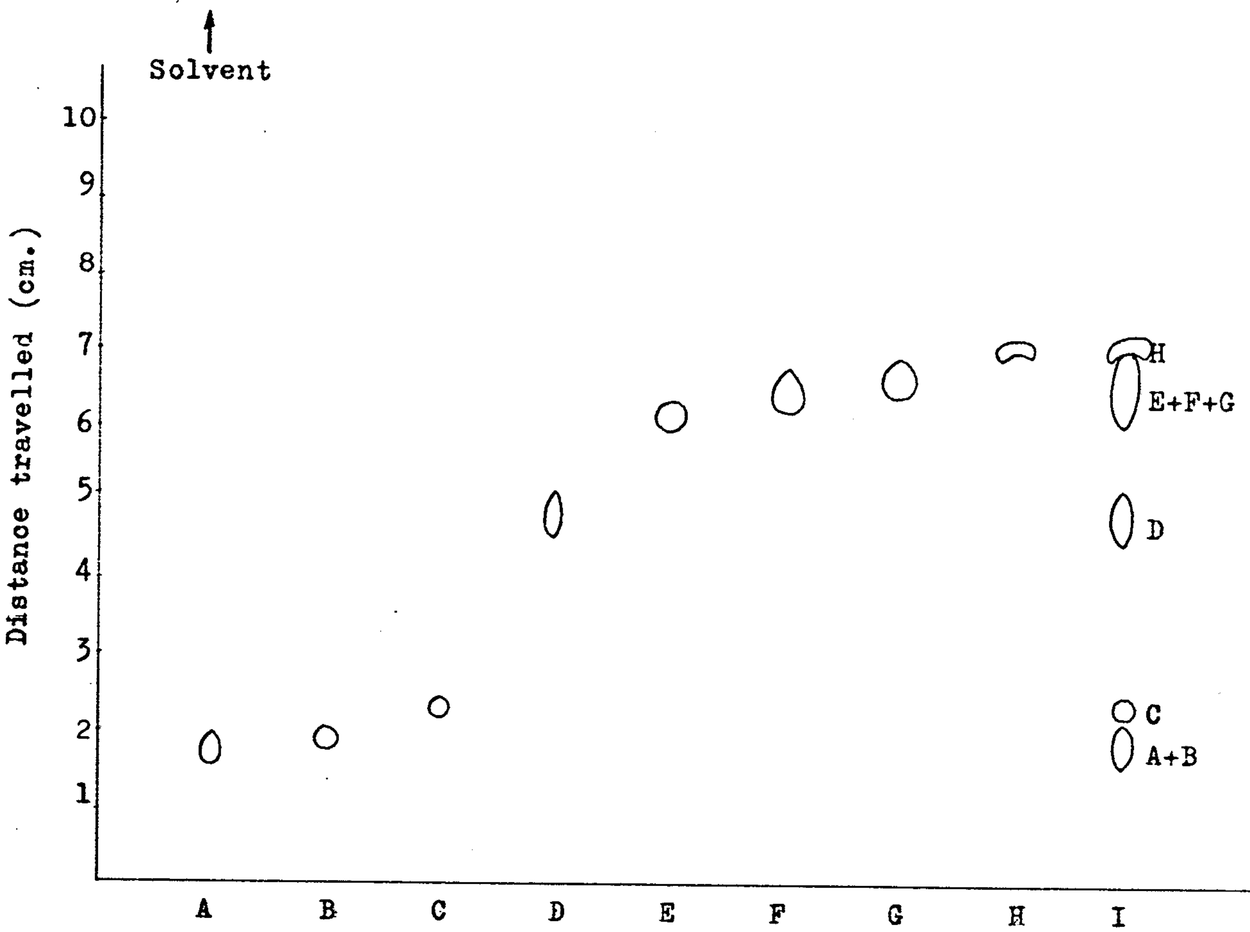


Figure 10. Thin layer chromatography of DNP-dipeptides using chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as developer. Where A = DNP-L-phenylalanyl-glycine; B = DNP-L-isoleucyl-glycine; C = DNP-L-alanyl-L-phenylalanine; D = DNP-L-phenylalanyl-L-phenylalanine; E = DNP-L-isoleucyl-L-phenylalanine; F = DNP-L-phenylalanyl-L-leucine; G = DNP-L-isoleucyl-L-leucine; H = DNP-L-leucyl-L-leucine; I = a mixture of A,B,C,D,E,F,G and H. DNP-L-leucyl-L-leucine travelled 8.5 cm. as standard.

Thin layer chromatography of DNP-tripeptides and their esters

DNP-tripeptides and their esters were chromatographed with toluene :pyridine:glacial acetic acid (80:10:1); chloroform:methanol:glacial acetic acid (95:5:1) and chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as solvents. The R_{ℓ}-values are listed in Table 38. The distance travelled by the individual DNP-tripeptide and their esters and in the mixture is shown in Fig. 11-13.

Table 38

100 X R_{ℓ}^{1}-values of DNP-tripeptide and their esters with one dimensional, ascending chromatography on SilicAR TLC-7GF layer. Solvent systems (A) toluene:pyridine:glacial acetic acid (80:10:1); (B) chloroform:methanol:glacial acetic acid (95:5:1) and (C) chloroform:benzyl alcohol:glacial acetic acid (97:2:1) were used.

Compound	100 X R_{ℓ}		
	A[2]	B[2]	C[3]
DNP-L-val-gly-gly	1.0	17.6	5.0
DNP-L-leu-gly-gly	3.0	24.2	12.0
DNP-L-phe-gly-gly	2.0	21.2	8.0
DNP-L-ala-L-phe-gly	3.5	31.8	22.0
DNP-L-val-L-leu-L-phe	30.0	48.5	58.0
DNP-L-Ileu-L-phe-L-leu	54.0	51.6	72.0
DNP-L-ala-L-phe-gly ethyl ester	61.0	57.6	63.0
DNP-L-val-L-leu-L-phe ethyl ester	96.0	100.0	95.0
DNP-L-Ileu-L-phe-L-leu ethyl ester	100.0	100.0	100.0

[1] R_{ℓ} = migration distance of the sample/migration distance of DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester.

[2] = DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester travelled 6.6cm. as standard.

[3] = DNP-L-Ileu-L-phe-L-leu ethyl ester travelled 10 cm. as standard.

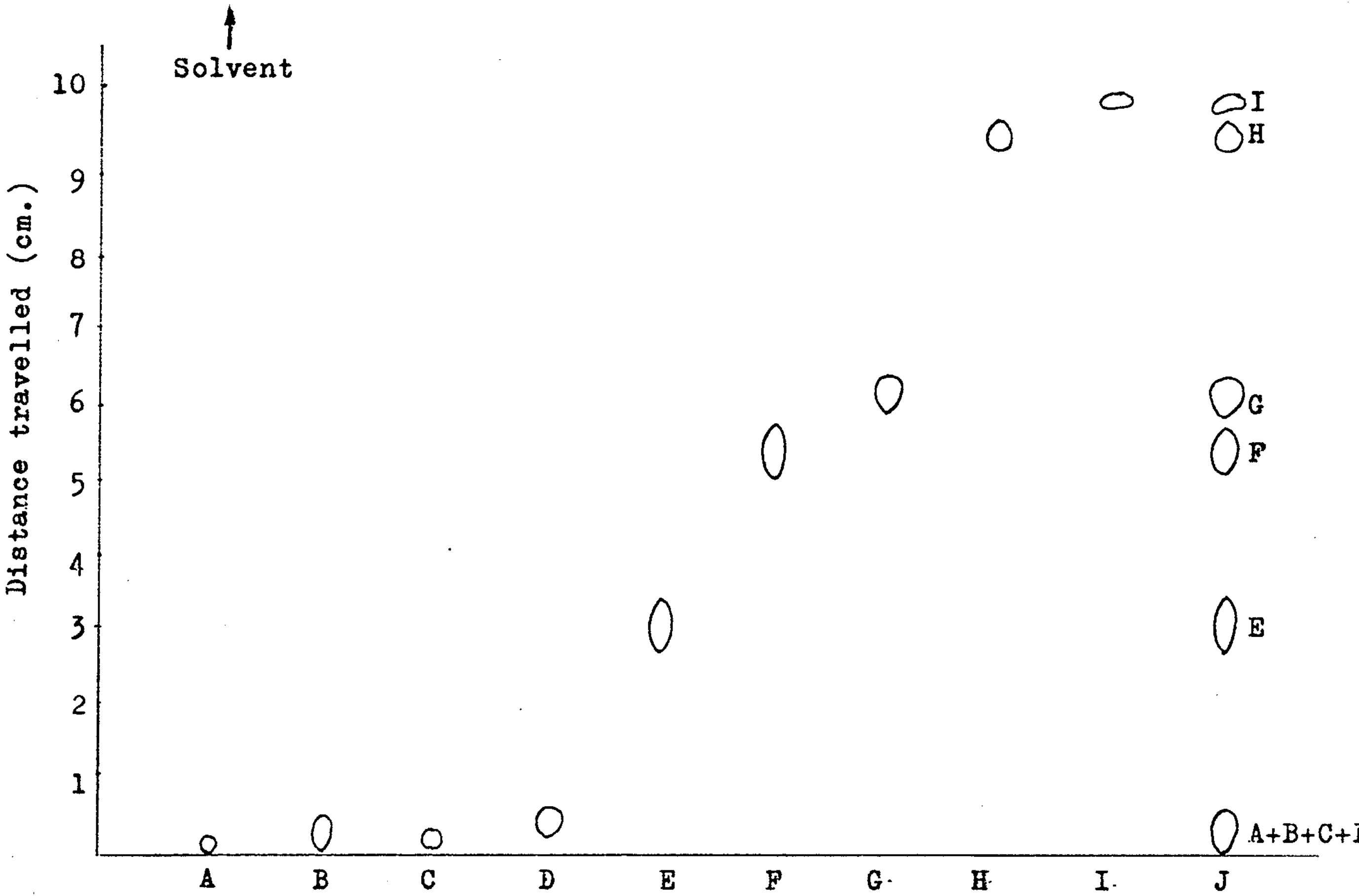


Figure 11. Thin layer chromatography of DNP-tripeptides and their esters using toluene:pyridine:glacial acetic acid (80:10:1) as developer. Where A = DNP-L-valyl-glycyl-glycine; B = DNP-L-phenylalanyl-glycyl-glycine; C = DNP-L-leucyl-glycyl-glycine; D = DNP-L-alanyl-L-phenylalanyl-glycine; E = DNP-L-valyl-L-leucyl-L-phenylalanine; F = DNP-L-isoleucyl-L-phenylalanyl-L-leucine; G = DNP-L-alanyl-L-phenylalanyl-glycine ethyl ester; H = DNP-L-valyl-L-leucyl-L-phenylalanine ethyl ester; I = DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester; J = a mixture of A,B,C,D,E,F,G,H and I.

DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester travelled 6.6 cm. as standard.

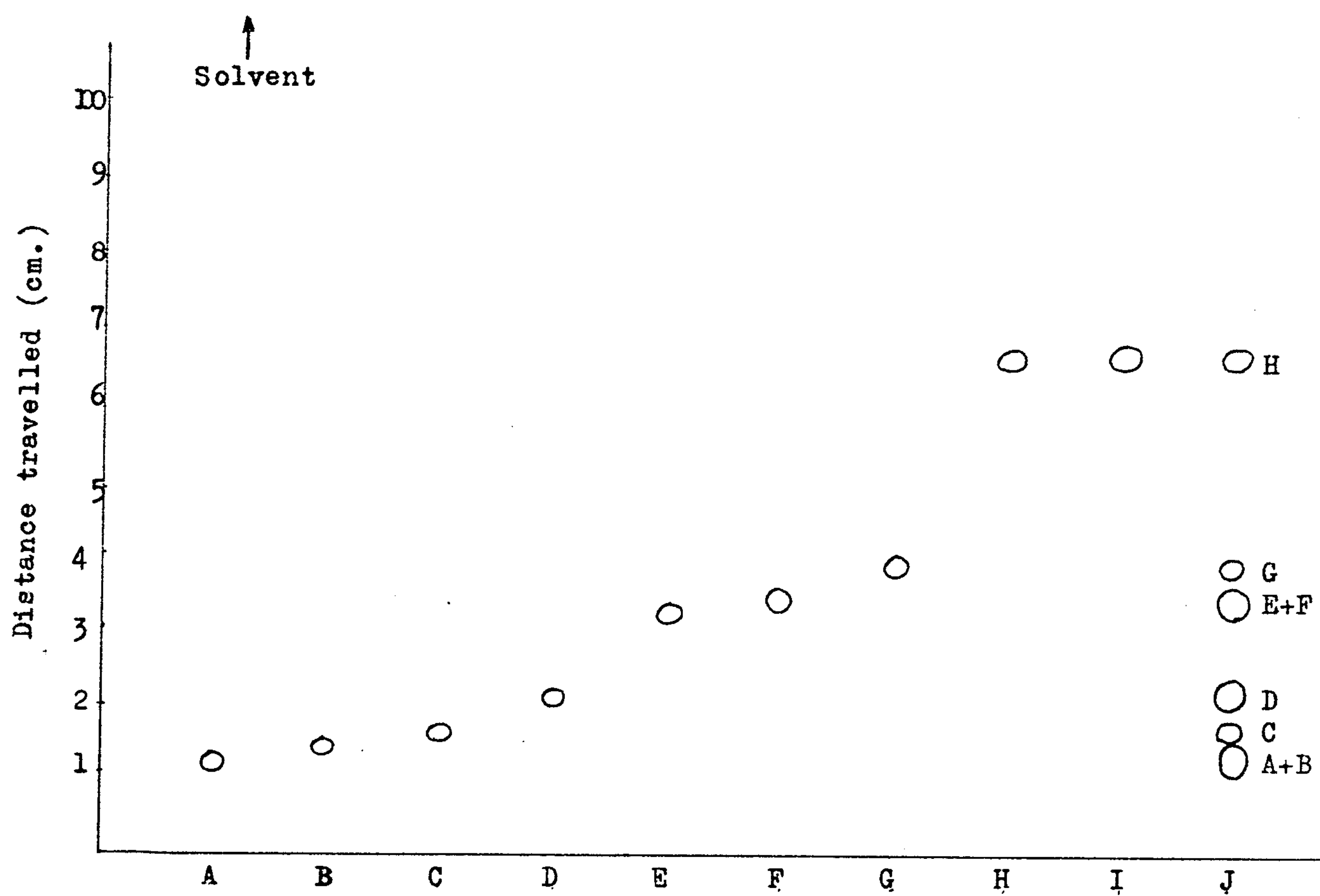


Figure 12. Thin layer chromatography of DNP-tripeptides and their ethyl esters using chloroform:methanol:glacial acetic acid (95:5:1) as developer. Where A = DNP-L-valyl-L-glycyl-glycine; B = DNP-L-phenylalanyl-glycyl-glycine; C = DNP-L-leucyl-glycyl-glycine; D = DNP-L-alanyl-L-phenylalanyl-glycine; E = DNP-L-valyl-L-leucyl-L-phenylalanine; F = DNP-L-isoleucyl-L-phenylalanyl-L-leucine; G = DNP-L-alanyl-L-phenylalanyl-glycine ethyl ester; H = DNP-L-valyl-L-leucyl-L-phenylalanine ethyl ester; I = DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester; J = a mixture of A,B,C,D,E,F,G,H and I.

DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester travelled 6.6 cm. as standard.

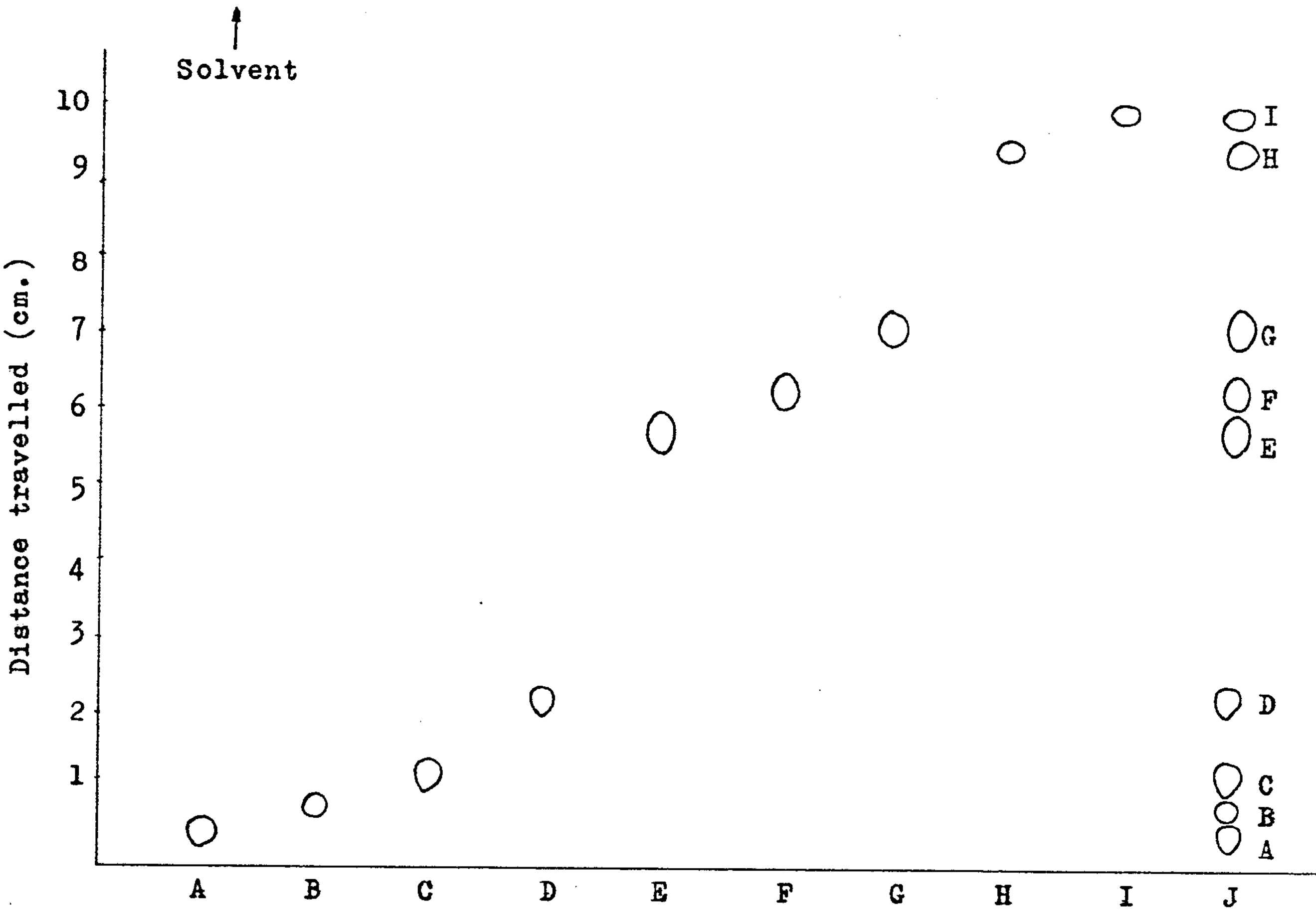


Figure 13. Thin layer chromatography of DNP-tripeptides and their ethyl ester.using chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as developer. Where A = DNP-L-valyl-glycyl-glycine; B = DNP-L-phenylalanyl-glycyl-glycine; C = DNP-L-leucyl-glycyl-glycine; D = DNP-L-alanyl-L-phenylalanyl-glycine; E = DNP-L-valyl-L-leucyl-L-phenylalanine; F = DNP-L-alanyl-L-phenylalanyl-glycine ethyl ester; G = DNP-L-isoleucyl-L-phenylalanyl-L-leucine; H = DNP-L-valyl-L-leucyl-L-phenylalanine ethyl ester; I = DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester; J = a mixture of A,B,C,D,E,F,G,H and I.

DNP-L-isoleucyl-L-phenylalanyl-L-leucine ethyl ester travelled 10 cm. as standard.

Ultraviolet Spectrometry

A PYE UNICAM Model SP 800 spectrophotometer and quartz cells of 1 cm. path length were employed in the ultraviolet studies of all the DNP-compounds prepared. The ultraviolet spectrum of each compound was determined in 95% ethanol solution, glacial acetic acid solution and in 4% sodium bicarbonate solution. A concentration of 7.5 X 10^{-5} molar was used for all samples.

The wavelengths of maximum absorption, λ_{max}, were determined for each compound and the molar absorptivity, ε_M, at those wavelengths was calculated. The results of the determinations are summarized in Table 39-48.

Table 39

Absorption maxima and molar absorbancy of DNP-L-amino acid amides in 95% ethanol solution.

Compound	Molar Absorptivity			
	λ_{max} (mμ)	ε_{max}	λ_{max} (mμ)	ε_{max}
DNP-L-valyl-amide	341	19070	258	10930
DNP-L-valyl-anilide	341	18930	239	24000
DNP-L-valyl-p-toluidide	341	19730	246	26000
DNP-L-leucyl amide	340	17870	257	10930
DNP-L-leucyl anilide	340	18270	240	23500
DNP-L-leucyl-p-toluidide	340	18130	244	26000
DNP-L-isoleucyl amide	341	18400	258	10930
DNP-L-isoleucyl anilide	341	19470	240	24700
DNP-L-isoleucyl-p-toluidide	341	20530	244	26700
DNP-L-phenylalanyl amide	342	17870	258	10930
DNP-L-phenylalanyl anilide	342	19470	240	24000
DNP-L-phenylalanyl-p-toluidide	342	18130	245	24400

Where λ_{max} = wavelength of maximum absorption;

ε_{max} = molecular extinction coefficient (molar absorbancy).

Table 40

Absorption maxima and molar absorbancy of DNP-dipeptide ethyl esters in 95% ethanol solution.

Compound	λmax (mμ)	Molar absorptivity εmax	λmax (mμ)	εmax
DNP-gly-gly ethyl ester	345	17400	258	10000
DNP-gly-L-leu ethyl ester	344	16900	257	10400
DNP-gly-L-phe ethyl ester	345	16400	258	8400
DNP-L-ala-L-phe ethyl ester	341	17100	258	10400
DNP-L-val-gly ethyl ester	344	17500	259	9200
DNP-L-val-L-leu ethyl ester	345	18000	259	9000
DNP-L-val-L-phe ethyl ester	345	16400	259	8800
DNP-L-leu-gly ethyl ester	341	16500	256	10000
DNP-L-leu-L-leu ethyl ester	342	17200	256	10500
DNP-L-leu-L-phe ethyl ester	345	16600	258	8200
DNP-L-Ileu-gly ethyl ester	343	17500	258	9500
DNP-L-Ileu-L-leu ethyl ester	345	16800	258	8600
DNP-L-Ileu-L-phe ethyl ester	345	17000	259	8800
DNP-L-phe-gly ethyl ester	343	16800	258	9800
DNP-L-phe-L-leu ethyl ester	345	16700	258	9100
DNP-L-phe-L-phe ethyl ester	345	18800	258	10400
DNP-L-try-gly ethyl ester	347 290	17000 7400	265	13200
DNP-L-try-L-leu ethyl ester	348 290	17300 7800	265	13500
DNP-L-try-L-phe ethyl ester	346 290	16600 7600	265	13100

Table 41

Absorption maxima and molar absorbancy of DNP-dipeptide ethyl esters in glacial acetic acid solution.

Compound	λmax (mμ)	Molar absorptivity εmax	λmax (mμ)	εmax
DNP-gly-gly ethyl ester	337	18670	261	11470
DNP-gly-L-leu ethyl ester	337	16530	261	10400
DNP-gly-L-phe ethyl ester	337	16000	262	10400
DNP-L-ala-L-phe ethyl ester	337	16800	261	10400
DNP-L-val-gly ethyl ester	337	16530	262	9600
DNP-L-val-L-leu ethyl ester	338	16530	262	9600
DNP-L-val-L-phe ethyl ester	337	16670	262	9870
DNP-L-leu-gly ethyl ester	336	16530	262	9870
DNP-L-leu-L-leu ethyl ester	336	16000	262	9330
DNP-L-leu-L-phe ethyl ester	336	16270	262	9600
DNP-L-Ileu-gly ethyl ester	337	17070	262	10130
DNP-L-Ileu-L-leu ethyl ester	337	18530	262	10670
DNP-L-Ileu-L-phe ethyl ester	338	17070	262	10130
DNP-L-phe-gly ethyl ester	336	16270	262	9600
DNP-L-phe-L-leu ethyl ester	336	16270	262	9600
DNP-L-phe-L-phe ethyl ester	336	17600	262	10130
DNP-L-try-gly ethyl ester	339 288	17070 8800	264	14930
DNP-L-try-L-leu ethyl ester	339 288	17600 9067	264	15330
DNP-L-try-L-phe ethyl ester	339 288	16800 8800	264	14677

Table 42

Absorption maxima and molar absorbancy of DNP-dipeptides in 95% ethanol solution.

Compound	λmax (mμ)	Molar absorptivity εmax	λmax (mμ)	εmax
DNP-L-ala-L-phe	342	18000	259	10270
DNP-L-val-gly	342	17200	260	9600
DNP-L-val-L-leu	342	17600	260	9600
DNP-L-val-L-phe	343	17470	260	9730
DNP-L-leu-gly	340	17200	260	9600
DNP-L-leu-L-leu	340	17330	260	9600
DNP-L-leu-L-phe	341	17470	259	9870
DNP-L-Ileu-gly	342	18400	259	10130
DNP-L-Ileu-L-leu	342	18670	259	10130
DNP-L-Ileu-L-phe	342	18400	259	10130
DNP-L-phe-gly	342	17070	259	9870
DNP-L-phe-L-leu	342	18270	259	10130
DNP-L-phe-L-phe	342	16530	259	9470
DNP-L-try-gly	346 288	17870 8800	265	14400
DNP-L-try-L-leu	346 288	16130 8670	265	13470
DNP-L-try-L-phe	344 288	16800 8800	265	14130

Table 43

Absorption maxima and molar absorbancy of DNP-dipeptides in glacial acetic acid solution.

Compound	Molar absorptivity			
	λmax (mμ)	εmax	λmax (mμ)	εmax
DNP-L-ala-L-phe	337	17470	262	10930
DNP-L-val-gly	337	17330	262	10670
DNP-L-val-L-leu	337	16270	262	10130
DNP-L-val-L-phe	337	16670	262	10130
DNP-L-leu-gly	336	16400	261	10130
DNP-L-leu-L-leu	336	17330	261	10400
DNP-L-leu-L-phe	336	17070	262	10266
DNP-L-Ileu-gly	338	17070	261	10400
DNP-L-Ileu-L-leu	339	16530	261	10130
DNP-L-Ileu-L-phe	338	16400	262	10130
DNP-L-phe-gly	336	16000	261	10130
DNP-L-phe-L-leu	337	16800	261	10400
DNP-L-phe-L-phe	337	16270	261	10130
DNP-L-try-gly	340 287	16400 8800	264	14530
DNP-L-try-L-leu	340 287	15870 8670	264	14670
DNP-L-try-L-phe	338 287	17330 8800	264	15070

Table 44

Absorption maxima and molar absorbancy of DNP-dipeptides in 4% sodium bicarbonate solution.

Compound	Molar absorptivity			
	λ_{max} ($m\mu$)	ε_{max}	λ_{max} ($m\mu$)	ε_{max}
DNP-L-ala-L-phe	349	18130	264	10400
DNP-L-val-gly	350	17330	265	9730
DNP-L-val-L-leu	350	17330	264	9730
DNP-L-val-L-phe	351	17600	265	9330
DNP-L-leu-gly	349	18270	264	10000
DNP-L-leu-L-leu	349	18000	264	10130
DNP-L-leu-L-phe	350	17330	265	9600
DNP-L-Ileu-gly	351	17870	265	9870
DNP-L-Ileu-L-leu	351	17330	265	9600
DNP-L-Ileu-L-phe	351	18400	265	10400
DNP-L-phe-gly	351	17070	264	9470
DNP-L-phe-L-leu	349	18000	264	10130
DNP-L-phe-L-phe	350	16400	264	9600
DNP-L-try-gly	352	18130	268	15200
DNP-L-try-L-leu	352	17600	268	14800
DNP-L-try-L-phe	352	18270	268	15730

Table 45

Absorption maxima and molar absorbancy of DNP-dipeptide hydrazides in glacial acetic acid solution.

Compound	Molar absorptivity			
	λmax (mμ)	εmax	λmax (mμ)	εmax
DNP-gly-gly hydrazide	337	16670	258	12270
DNP-gly-L-leu hydrazide	338	16270	258	12400
DNP-gly-L-phe hydrazide	340	16670	257	16270
DNP-L-ala-L-phe hydrazide	337	16400	258	12270
DNP-L-val-gly hydrazide	338	16800	257	17470
DNP-L-val-L-leu hydrazide	338	16530	258	12000
DNP-L-val-L-phe hydrazide	338	16800	258	12270
DNP-L-Leu-gly hydrazide	336	15470	259	11470
DNP-L-leu-L-leu hydrazide	336	16270	259	12000
DNP-L-leu-L-phe hydrazide	336	16270	258	12130
DNP-L-Ileu-gly hydrazide	338	17600	256	17870
DNP-L-Ileu-L-leu hydrazide	339	16530	256	16000
DNP-L-Ileu-L-phe hydrazide	337	16530	257	12000
DNP-L-phe-gly hydrazide	337	15730	257	12800
DNP-L-phe-L-leu hydrazide	337	16670	256	12270
DNP-L-phe-L-phe hydrazide	338	16530	257	16270
DNP-L-try-gly hydrazide	340 288	16000 8530	261	18800
DNP-L-try-L-leu hydrazide	340 288	17200 8000	262	16000

Table 46

Absorption maxima and molar absorbancy of DNP-tripeptides and their esters in 95% ethanol solution.

Compound	Molar absorptivity			
	λmax (mμ)	ϵmax	λmax (mμ)	ϵmax
DNP-L-ala-L-phe-gly ethyl ester	340	19200	258	11470
DNP-L-Ileu-L-phe-L-leu ethyl ester	342	17730	257	12000
DNP-L-val-L-leu-L-phe ethyl ester	342	17600	258	10670
DNP-L-ala-L-phe-gly	342	18670	259	11070
DNP-L-val-L-leu-L-phe	342	19060	258	11330
DNP-L-Ileu-L-phe-L-leu	341	18800	259	10930
DNP-L-val-gly-gly	340	18930	259	11200
DNP-L-leu-gly-gly	340	19730	259	11870
DNP-L-phe-gly-gly	342	17070	258	10400

Table 47

Absorption maxima and molar absorbancy of

DNP-tripeptides and their esters in glacial acetic acid solution.

Compound	Molar absorptivity			
	λ_{max} (mμ)	ε_{max}	λ_{max} (mμ)	ε_{max}
DNP-L-ala-L-phe-gly ethyl ester	338	16400	261	10270
DNP-L-val-L-leu-L-phe ethyl ester	338	17070	262	10130
DNP-L-Ileu-I-phe-L-leu ethyl ester	338	16930	261	10270
DNP-L-ala-L-phe-gly	337	16400	261	10130
DNP-L-val-L-leu-L-phe	339	16670	261	10270
DNP-L-Ileu-L-phe-L-leu	339	16930	260	10400
DNP-L-val-gly-gly	338	17200	262	10400
DNP-L-leu-gly-gly	337	17330	261	10530
DNP-L-phe-gly-gly	338	17200	261	10400

Table 48

Absorption maxima and molar absorbancy of

DNP-tripeptides in 4% sodium bicarbonate solution.

Compound	Molar absorptivity			
	λ_{max} (mμ)	ε_{max}	λ_{max} (mμ)	ε_{max}
DNP-L-ala-L-phe-gly	350	16930	265	9730
DNP-L-val-L-leu-L-phe	350	18530	265	8930
DNP-L-Ileu-L-phe-L-leu	350	13670	265	10400
DNP-L-val-gly-gly	350	18800	265	10670
DNP-L-leu-gly-gly	349	17600	265	9870
DNP-L-phe-gly-gly	349	16930	264	9200

Infrared spectrometry

The infrared spectra of the above compounds were examined using the mull technique in the region 5000 to 625 cm^{-1}, where the mulls were prepared by grinding about 2 to 5 mg. of the solid with a drop of nujol (a high boiling petroleum fraction). A Perkin-Elmer Model 700 Infrared Spectrophotometer, fitted with sodium chloride windows, was used to obtained all infrared spectra. The spectra are shown in Spectrum 1 to 74.

Discussion

DNP-L-aminoacyl chlorides

All the acid chlorides prepared in this and previous work (9) are liquid except DNP-glycyl chloride. They were obtained in apparently excellent yield of sufficient purity to use directly in the coupling reaction. Attempts to prepare DNP-L-tryptophyl chloride by the same method only resulted in tar formation.

DNP-dipeptide ethyl esters

Acid chloride coupling with L-amino acid esters in tetrahydrofuran or anhydrous ether

Very good yields were obtained in most cases using triethylamine as the condensing agent. The method could not be used for preparing the DNP-L-tryptophyl peptide esters.

Method of Zaoral and Arnold

Failure to obtain DNP-L-tryptophyl chloride by the same means as the other acid chlorides was resolved by using this alternative method, in which thionyl chloride and dimethylformamide combine to form N,N-dimethylchloroformimidium chloride which in turn converts the DNP-L-amino acid to its chloride, the latter coupling with the L-amino acid ester in presence of triethylamine in dimethylformamide. In comparing this method with the previous one, it seen that (i) the DNP-L-tryptophyl dipeptide esters were obtained, and in good yield; (ii) in the few cases where the yield had been low from the previous coupling method, considerable improvement in yield by this modification was achieved; (iii) although all the other DNP-peptide esters obtained in very good yield by the first method were also obtained

in good yield by this modification, nevertheless the yields by this latter method were somewhat lower than by the former.

Carbodiimide method

Dicyclohexyl carbodiimide was satisfactory as condensing agent in preparing the DNP-dipeptide esters, most of the products being obtained in very good yield and quality. Little or no difficulty due to contaminating N,N'-dicyclohexylurea was encountered in purification, although this has frequently been reported in the literature in synthesising other peptide derivatives (15, 16).

In the few instances where this was encountered, the difficulty was resolved by using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate instead of N,N'-dicyclohexyl carbodiimide. Although use of this condensing agent requires a longer reaction time, yields were considerably improved. No advantage in the use of this reagent was gained when used for making those esters prepared in satisfactory yield and quality by means of dicyclohexyl carbodiimide. A DNP-dipeptide ester prepared by any one of those methods had the same melting point and specific rotation as when prepared by the other methods.

DNP-dipeptide hydrazides

Excellent yields were obtained, although the hydrazinolysis is time consuming.

DNP-tripeptide ethyl esters

Coupling a DNP-dipeptide with an amino acid ethyl ester by the

carbodiimide method gave much better yields and much greater ease of purification when 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate was used as condensing agent instead of dicyclohexyl carbodiimide. This adaptation of the carbodiimide method gave much better yields than the azide coupling method which gave only fair yields.

DNP-Peptides

Dinitrophenylation of free peptides gives reasonably good yields, but the free peptides are usually not readily available from commercial sources. Coupling of a DNP-aminoacyl chloride with an amino acid in aqueous solution (basic) was rather unsatisfactory in this study, giving poor yields due to extensive hydrolysis of the acid chloride. Hydrolysis of the DNP-peptide ethyl ester gave much better results in terms of yield of DNP-dipeptides, but substantially lower yields of DNP-tripeptides.

Thin layer chromatography

Free DNP-dipeptides

DNP-L-amino acids in this study were easily removed by extracting the DNP-derivatives with ether. The remaining DNP-peptides were separated by thin layer chromatography. When the chromatogram is developed with toluene:pyridine:glacial acetic acid (80:10:1), DNP-L-try-gly travels a much shorter distance than the other DNP-dipeptides, and DNP-L-leu-L-leu travels further than the rest, hence these two derivatives can be separated from the remainder. On careful examination of the positions of the remaining DNP-dipeptides, two well defined groups can be recognized on the chromatogram.

<u>Group 1</u> DNP-L-phe-gly, DNP-L-val-gly, DNP-L-ala-L-phe, DNP-L-try-L-phe, DNP-L-Ileu-gly, DNP-L-leu-gly.

<u>Group 2</u> DNP-L-try-L-leu, DNP-L-phe-L-phe, DNP-L-val-L-phe, DNP-L-Ileu-L-phe, DNP-L-phe-L-leu, DNP-L-Ileu-L-leu, DNP-L-leu-L-phe, DNP-L-val-L-leu.

<u>Separation of group 1 components</u>

On a chromatogram developed by benzene:glacial acetic acid (20:3), DNP-L-val-gly travels the shortest distance of this group. The longest distance was travelled by DNP-L-try-L-phe, the nearest after that being DNP-L-ala-L-phe. The location of DNP-L-phe-gly is identical with that of DNP-L-leu-gly, but they are separated by toluene:pyridine:glacial acetic acid (80:10:1). The positions of DNP-L-Ileu-gly and DNP-L-val-gly are also so close as to require the toluene:pyridine:glacial acetic acid system for their separation.

<u>Separation of group 2 components</u>

(DNP-L-try-L-leu + DNP-L-phe-L-phe + DNP-L-val-L-phe) and (DNP-L-Ileu-L-phe + DNP-L-phe-L-leu + DNP-L-Ileu-L-leu + DNP-L-Ileu-L-phe + DNP-L-val-L-leu), occupy positions on a chromatogram developed by toluene:pyridine:glacial acetic acid (80:10:1), such that they can be classified in two well defined sub-groups. The locations of DNP-L-try-L-leu, DNP-L-phe-L-phe and DNP-L-val-L-phe on a chromatogram developed by benzene:glacial acetic acid (20:3) are quite distinct from one another. Benzene:glacial acetic acid (20:3) causes the remainder of the second sub-group to move further than DNP-L-val-L-leu. The toluene:pyridine:glacial acetic acid developer causes DNP-L-Ileu-L-phe and DNP-L-leu-L-phe to move the

same distance, so they would form one spot if both present (spot A). DNP-L-phe-L-leu and DNP-L-Ileu-L-leu were found to coincide (spot B) after use of this developer.

If in any study the location of a spot corresponded to spot A, the only way to determine its composition without modifying the C-terminal carboxyl group would be to extract, hydrolyse, and determine whether the hydrolysate contained DNP-L-Ileu or DNP-L-leu (or both). Alternatively, if the DNP-dipeptide carboxyl function could be readily modified, the positions taken up by the new DNP-dipeptide derivatives could be so different as to leave no doubt about the composition of the original spot A. In this regard, it should be noted that the locations of DNP-L-Ileu-L-phe ethyl ester and DNP-L-leu-L-phe ethyl ester on a chromatogram developed by chloroform:methanol:glacial acetic acid(95:5:1) are very far apart.

Similarly, composition of spot B without modifying the C-terminal carboxyl function would require hydrolysis to determine if either or both of DNP-L-phe and DNP-L-Ileu were present. As in the case of spot A, modification of spot B carboxyl function might give a ready means of ascertaining the composition of spot B (DNP-L-phe-L-leu ethyl ester and DNP-L-Ileu-L-leu ethyl ester take up very different positions on a chromatogram developed by the same solvent mixture as above).

Free DNP-tripeptides

DNP-L-val-gly-gly, DNP-L-phe-gly-gly, DNP-L-leu-gly-gly and DNP-L-ala-L-phe-gly hardly travel at all from the base-line when toluene:pyriiine: glacial acetic acid (80:10:1) is used to develop

the chromatogram, not even as far as DNP-L-try-gly, hence at least a partial screening of dipeptide and tripeptide fragments is possible in the form of their DNP-derivatives. These four DNP-tripeptides were later separated from one another by using chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as developer.

With the toluene:pyridine:glacial acetic acid (80:10:1) developer, DNP-L-val-L-leu-L-phe and DNP-L-Ileu-L-phe-L-leu travelled much further, occupying positions on the chromatogram close to those occupied by DNP-L-leu-gly and the DNP-gly-L-phe:DNP-L-try-L-leu: DNP-L-val-L-phe group. Use of chloroform:benzyl alcohol:glacial acetic acid (97:2:1) as developer shifts DNP-L-val-L-leu-L-phe to a position on the chromatogram very different from that occupied by the L-leu-gly and L-Ileu-gly derivatives, and shifts DNP-L-Ileu-L-phe-L-leu to a position very far from any corresponding to any of DNP-L-val-L-phe, DNP-L-try-L-leu or DNP-L-phe-L-phe.

Ultraviolet absorption spectra

As indicated in Part B, molecular weight calculation for DNP-peptides, based upon the usual assumed average value of 16000 for ε_{max} (66), gives a result some 10-20% lower than the correct value. This assumed value for ε_{max} would also introduce error inte any colorimetric determination of the amount of any given DNP-peptide present.

Since every DNP-peptide has been shown to have its own specific value for ε_{max}, it follows that once the DNP-peptides from a given source have been separated and identified, calculation of the amount of each, and of their amounts relative to one another, must utilise the correct value

of ε_{max} for each given compound.

The spectra for the tryptophan-containing DNP-peptides in glacial acetic acid or in ethanol also give valuable additional information concerning any spot on a chromatogram corresponding to a tryptophan-containing peptide, due to the additional peak at 288 $m\mu$. Presence or absence of tryptophan would be shown according to the nature of the curve in this region, and if present the amount could be assessed from the ε_{max} value. If the result were different from the total DNP material, some DNP-peptide material lacking tryptophan would be present.

Infrared absorption spectra

The absorption frequencies found are those which would be assigned as follows:

Carbonyl group

Carbonyl stretching of esters or carboxylic acids at 1740 cm^{-1} region, the latter apparently monomeric.

Ester group

1240 cm^{-1}, strong band, ester C-O-C group asymmetric vibration, and 1040 cm^{-1}, small band, ester C-O-C group symmetric vibration (77, 78).

Secondary amino group attached to a dinitrophenyl group

N-H stretching at 3400 cm^{-1}, C-N stretching at 1330-1305 cm^{-1}.(77)

Amide group

Secondary amide (amide group of peptide linkage) ——

N-H stretching at 3300 cm^{-1}, C-O stretching at 1660 cm^{-1},

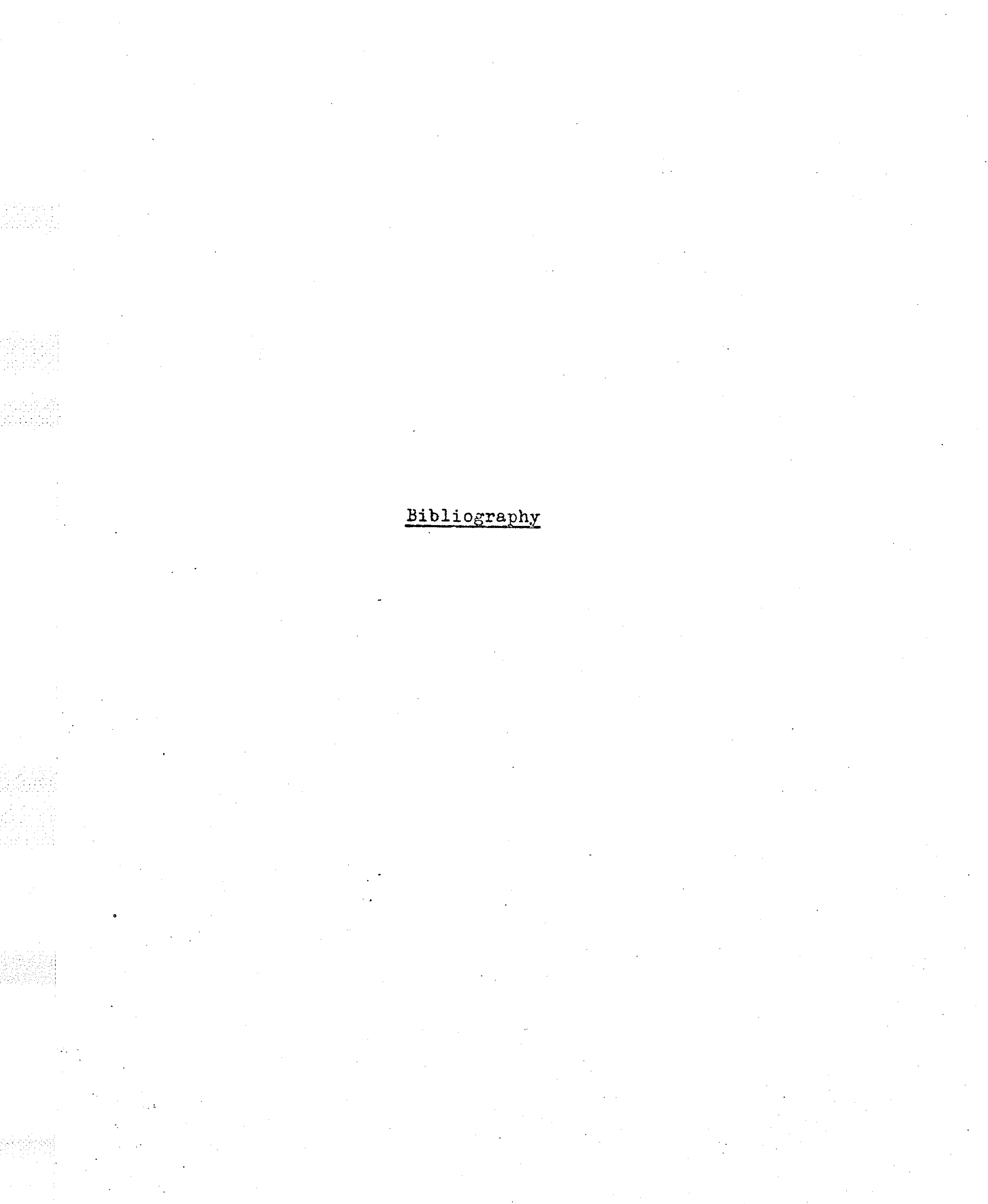
N-H bending at 1550 cm^{-1}, C-N stretching at 1290 cm^{-1}.

Primary amide —— two free N-H stretching bands at 3500 and 3400 cm^{-1}. (77)

Nitro group

Two bands, asymmetric stretching at 1530 cm^{-1}, and the symmetric mode at 1350 cm^{-1}, the latter having greater intensity because of p-amino group. (77-80)

The frequencies and their assignments provide strong additional confirmation of structure of the DNP-peptides, DNP-peptide esters, and the DNP-amino acyl amides, anilides, p-toluidides. Because all the spectra are so very similar they cannot be used to distinguish any one member from another.



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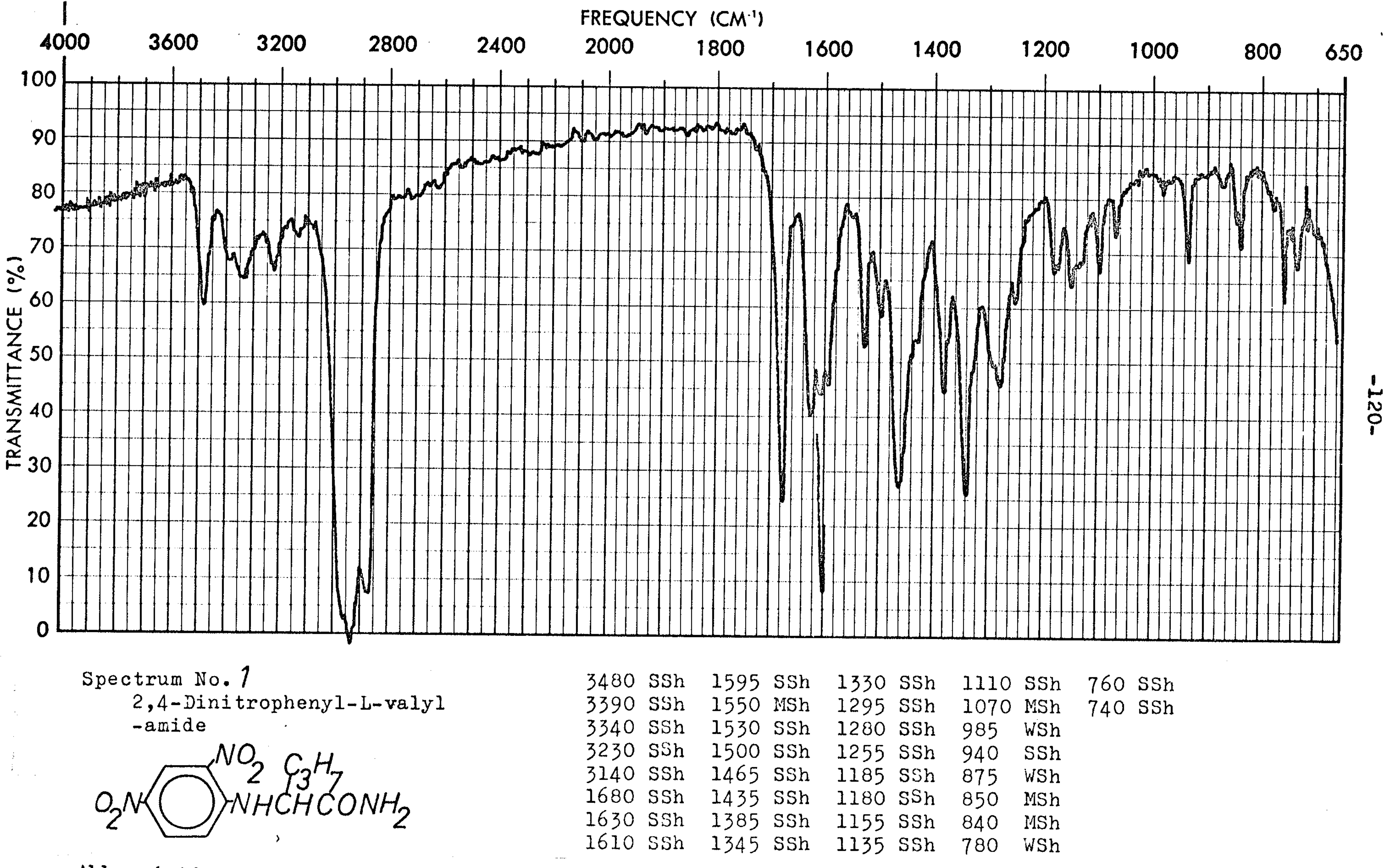
Infrared Spectra

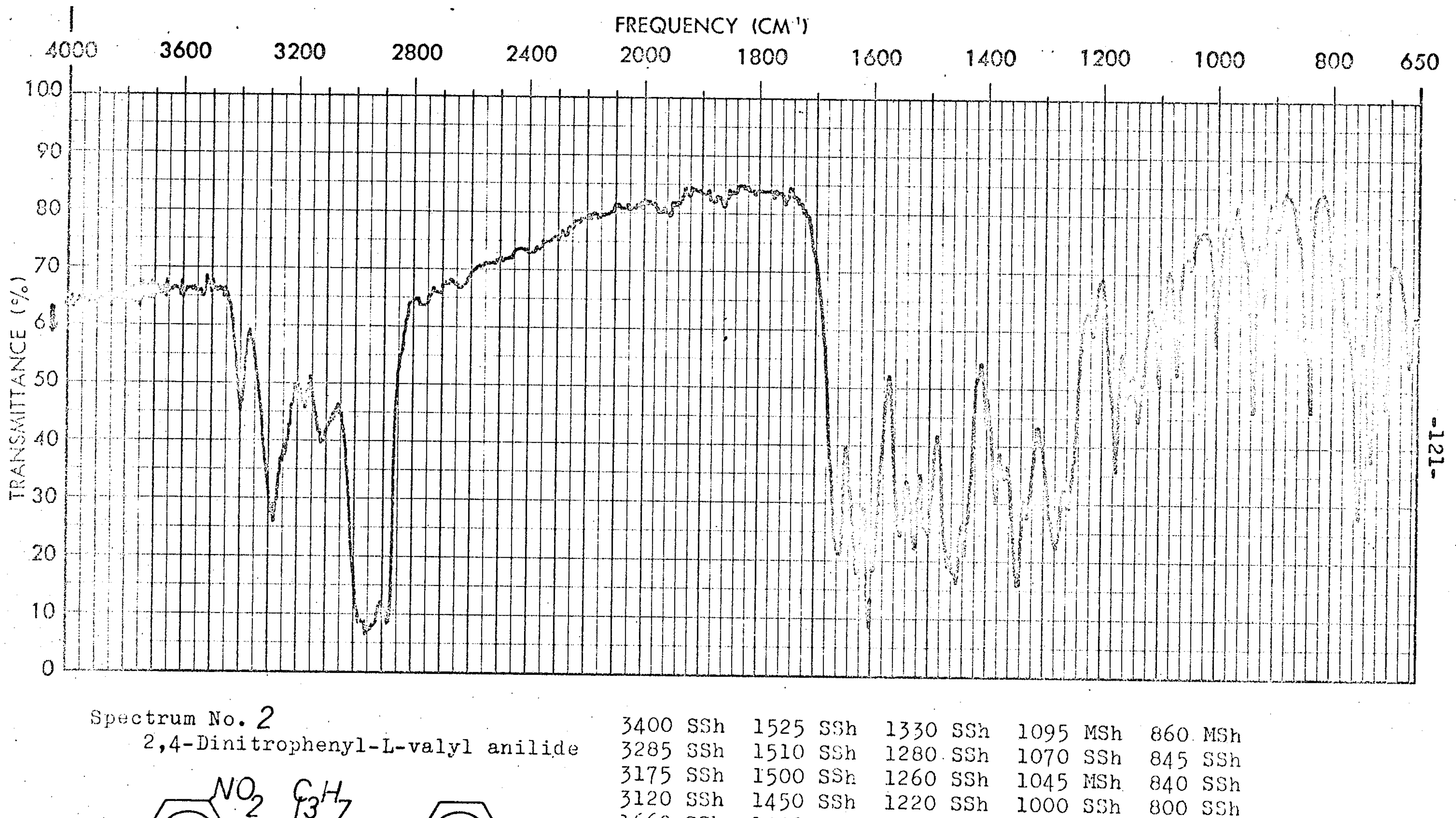


Spectrum No. 1
2,4-Dinitrophenyl-L-valyl -amide

$O_2N-C_6H_3(NO_2)-NHCH(C_3H_7)CONH_2$

3480	SSh	1595	SSh	1330	SSh	1110	SSh	760	SSh
3390	SSh	1550	MSh	1295	SSh	1070	MSh	740	SSh
3340	SSh	1530	SSh	1280	SSh	985	WSh		
3230	SSh	1500	SSh	1255	SSh	940	SSh		
3140	SSh	1465	SSh	1185	SSh	875	WSh		
1680	SSh	1435	SSh	1180	SSh	850	MSh		
1630	SSh	1385	SSh	1155	SSh	840	MSh		
1610	SSh	1345	SSh	1135	SSh	780	WSh		

Abbreviations : S = strong, M = medium, W = weak, Sh = sharp, B = broad.

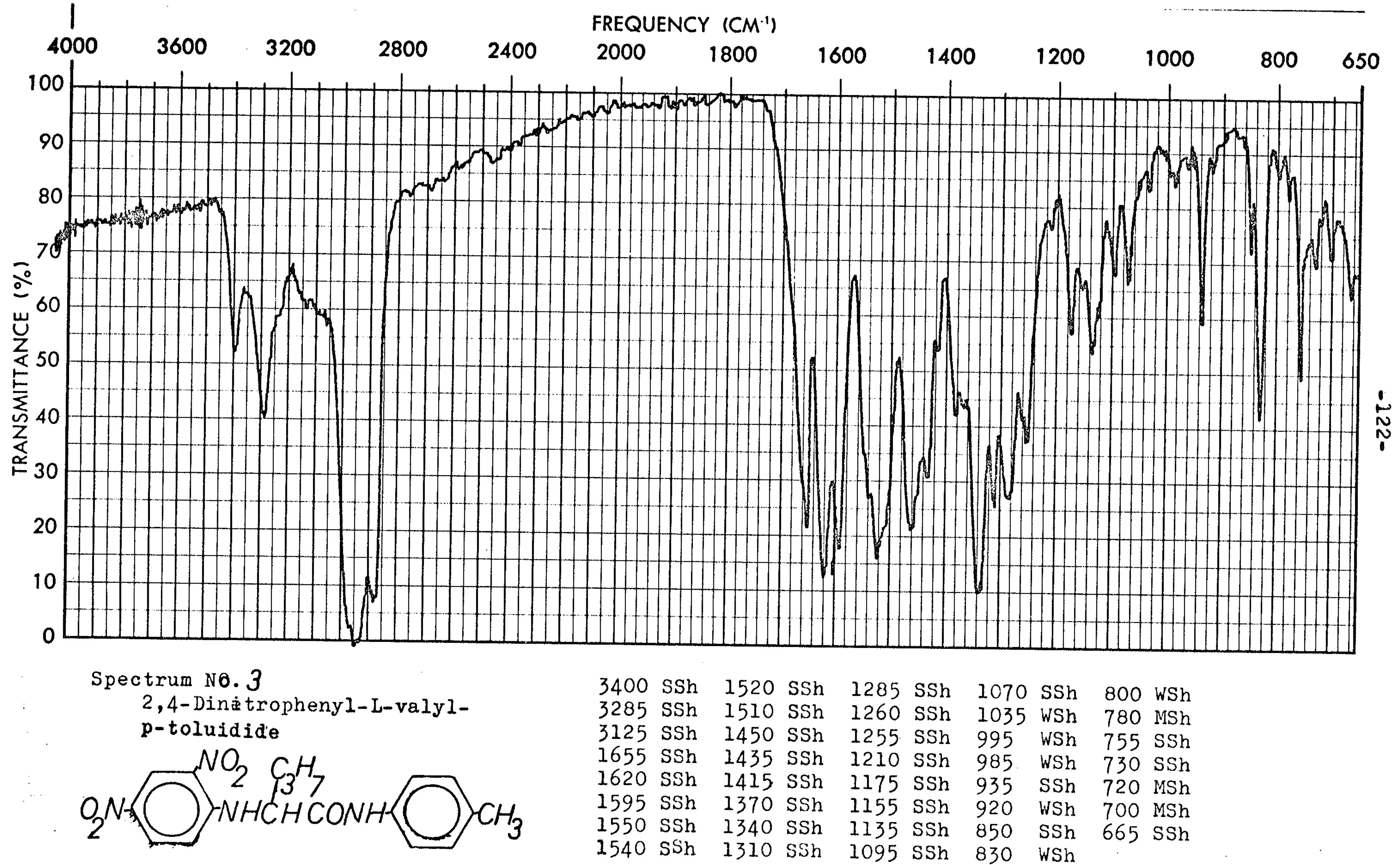


Spectrum No. 2

2,4-Dinitrophenyl-L-valyl anilide

O_2N-C₆H₃(NO_2)-NHCH(C_3H_7)CONH-C₆H₅

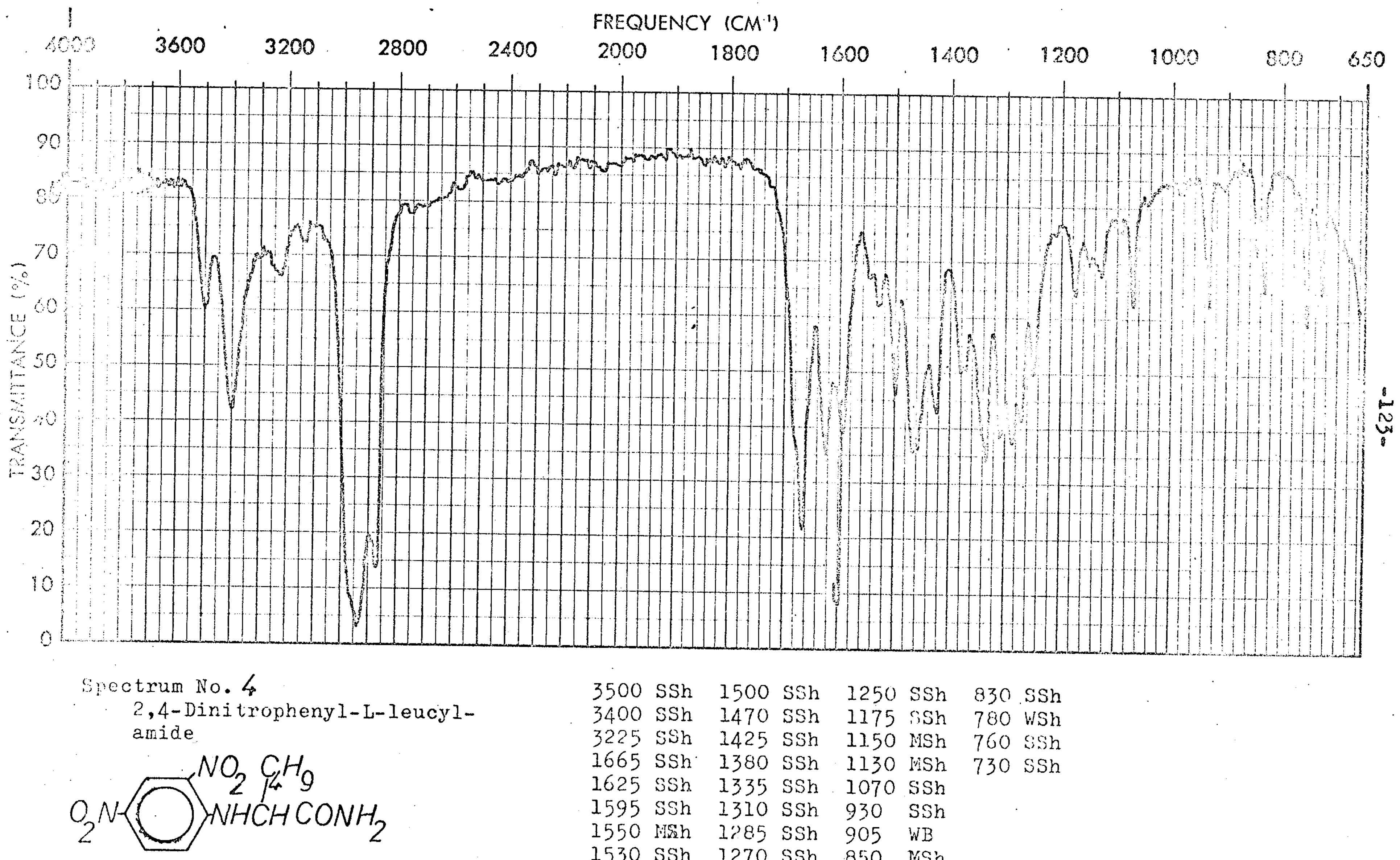
3400	SSh	1525	SSh	1330	SSh	1095	MSh	860	MSh
3285	SSh	1510	SSh	1280	SSh	1070	SSh	845	SSh
3175	SSh	1500	SSh	1260	SSh	1045	MSh	840	SSh
3120	SSh	1450	SSh	1220	SSh	1000	SSh	800	SSh
1660	SSh	1440	SSh	1175	SSh	950	MSh	780	SSh
1625	SSh	1400	SSh	1155	SSh	935	SSh	755	SSh
1595	SSh	1370	SSh	1140	SSh	920	MSh	730	SSh
1550	SSh	1345	SSh	1110	SSh	895	WSh	705	SSh



Spectrum No. 3
2,4-Dinitrophenyl-L-valyl-p-toluidide

O_2N-(2-NO_2-phenyl)-NHCH(C_3H_7)CONH-(phenyl)-CH_3

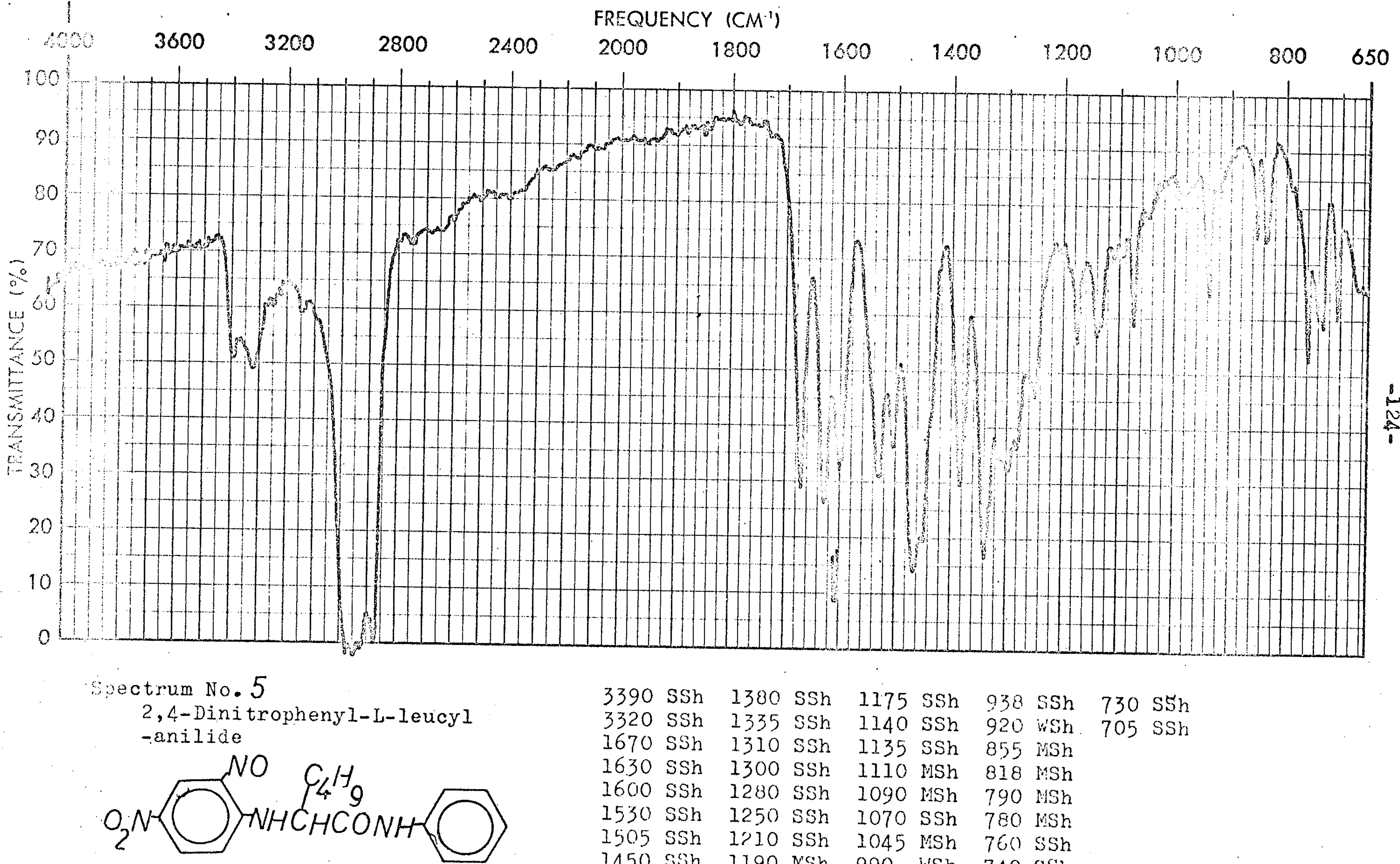
3400	SSh	1520	SSh	1285	SSh	1070	SSh	800	WSh
3285	SSh	1510	SSh	1260	SSh	1035	WSh	780	MSh
3125	SSh	1450	SSh	1255	SSh	995	WSh	755	SSh
1655	SSh	1435	SSh	1210	SSh	985	WSh	730	SSh
1620	SSh	1415	SSh	1175	SSh	935	SSh	720	MSh
1595	SSh	1370	SSh	1155	SSh	920	WSh	700	MSh
1550	SSh	1340	SSh	1135	SSh	850	SSh	665	SSh
1540	SSh	1310	SSh	1095	SSh	830	WSh		



Spectrum No. 4
2,4-Dinitrophenyl-L-leucylamide

$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONH_2$

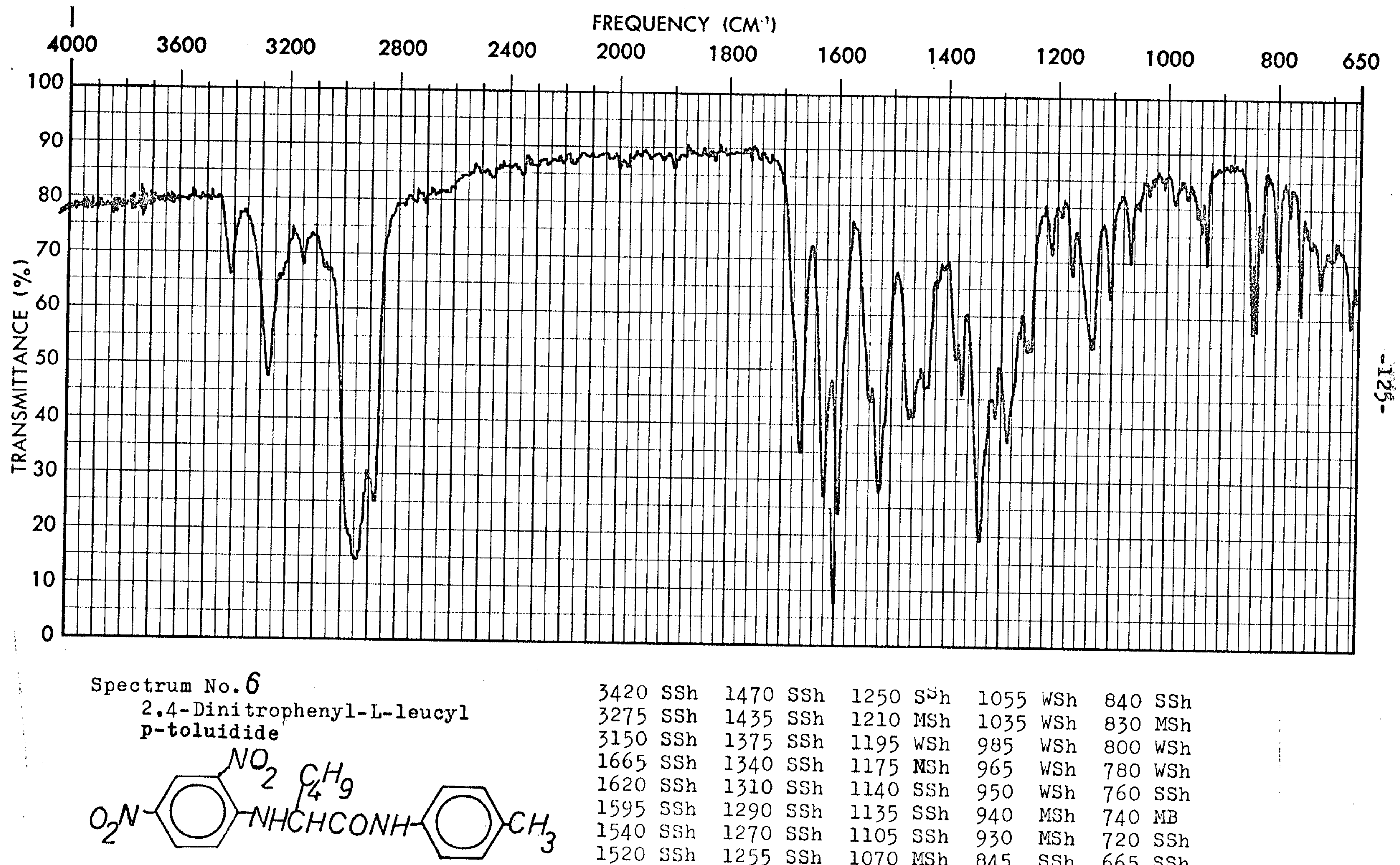
3500	SSh	1500	SSh	1250	SSh	830	SSh
3400	SSh	1470	SSh	1175	SSh	780	WSh
3225	SSh	1425	SSh	1150	MSh	760	SSh
1665	SSh	1380	SSh	1130	MSh	730	SSh
1625	SSh	1335	SSh	1070	SSh		
1595	SSh	1310	SSh	930	SSh		
1550	MSh	1285	SSh	905	WB		
1530	SSh	1270	SSh	850	MSh		



Spectrum No. 5
2,4-Dinitrophenyl-L-leucyl
-anilide

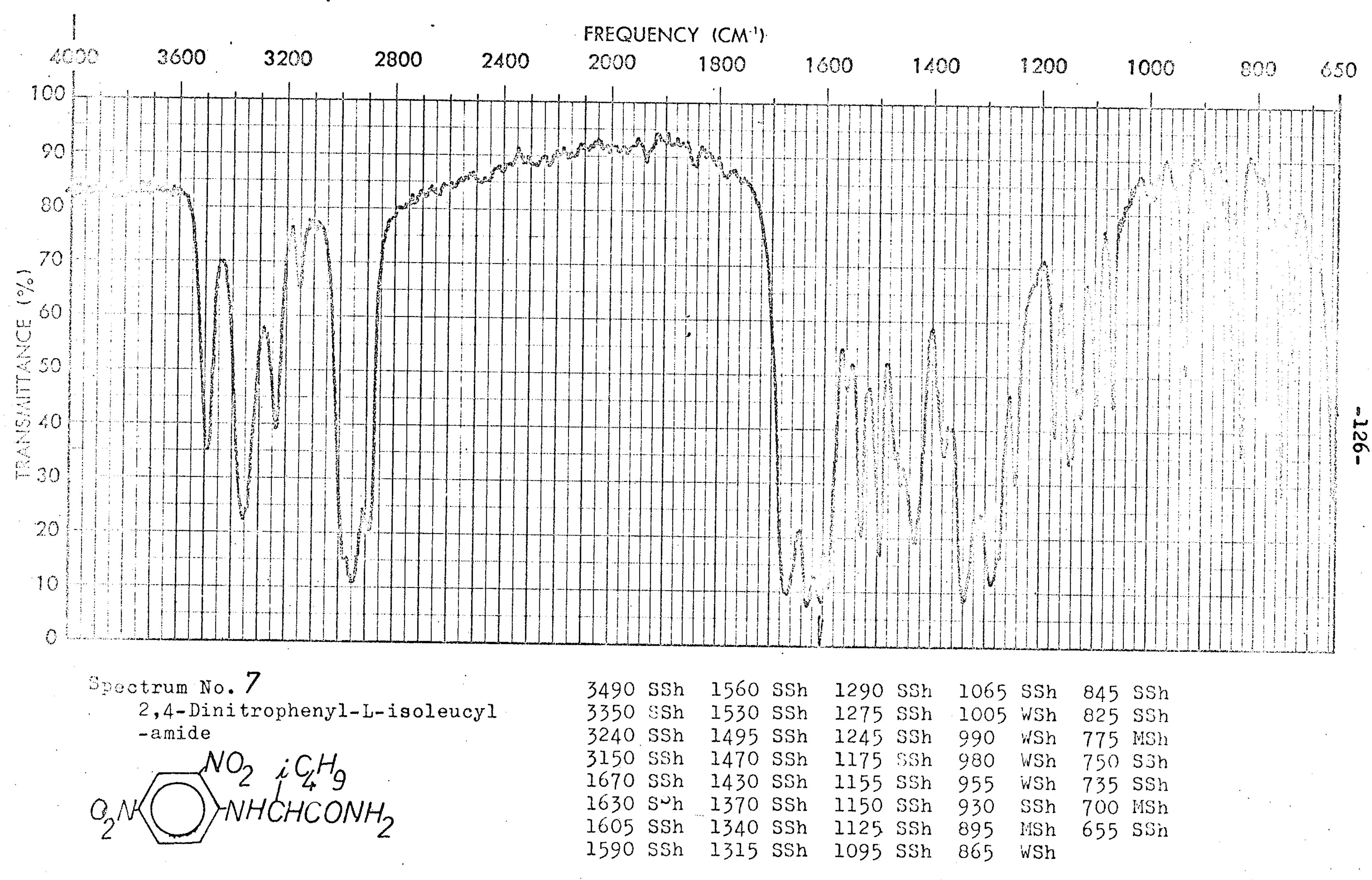
O_2N–(ring)–NO_2; NHCH(C_4H_9)CONH–(phenyl)

3390 SSh	1380 SSh	1175 SSh	938 SSh	730 SSh
3320 SSh	1335 SSh	1140 SSh	920 WSh	705 SSh
1670 SSh	1310 SSh	1135 SSh	855 MSh	
1630 SSh	1300 SSh	1110 MSh	818 MSh	
1600 SSh	1280 SSh	1090 MSh	790 MSh	
1530 SSh	1250 SSh	1070 SSh	780 MSh	
1505 SSh	1210 SSh	1045 MSh	760 SSh	
1450 SSh	1190 MSh	990 WSh	740 SSh	



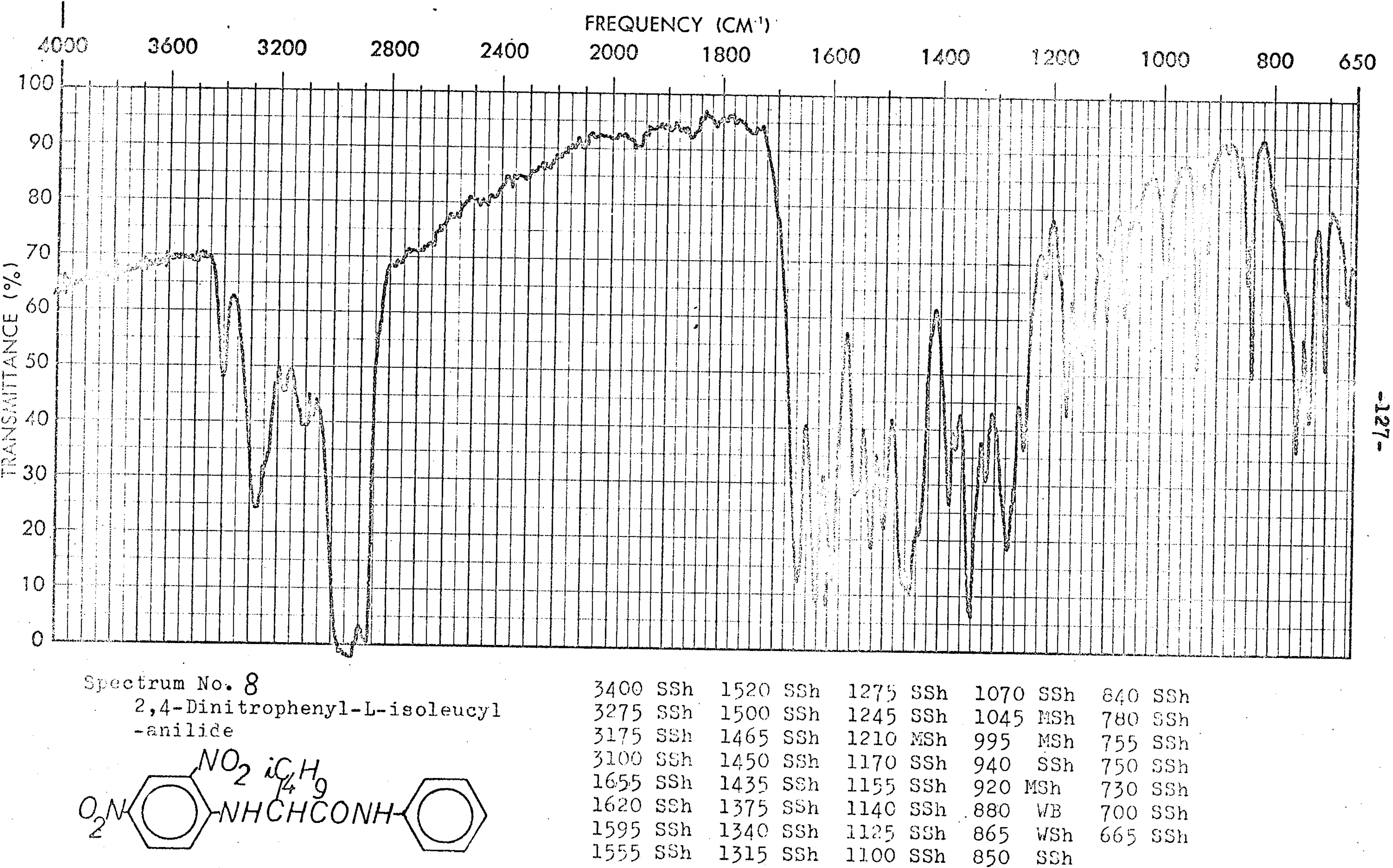
Spectrum No. 6
2.4-Dinitrophenyl-L-leucyl
p-toluidide

3420	SSh	1470	SSh	1250	SSh	1055	WSh	840	SSh
3275	SSh	1435	SSh	1210	MSh	1035	WSh	830	MSh
3150	SSh	1375	SSh	1195	WSh	985	WSh	800	WSh
1665	SSh	1340	SSh	1175	MSh	965	WSh	780	WSh
1620	SSh	1310	SSh	1140	SSh	950	WSh	760	SSh
1595	SSh	1290	SSh	1135	SSh	940	MSh	740	MB
1540	SSh	1270	SSh	1105	SSh	930	MSh	720	SSh
1520	SSh	1255	SSh	1070	MSh	845	SSh	665	SSh



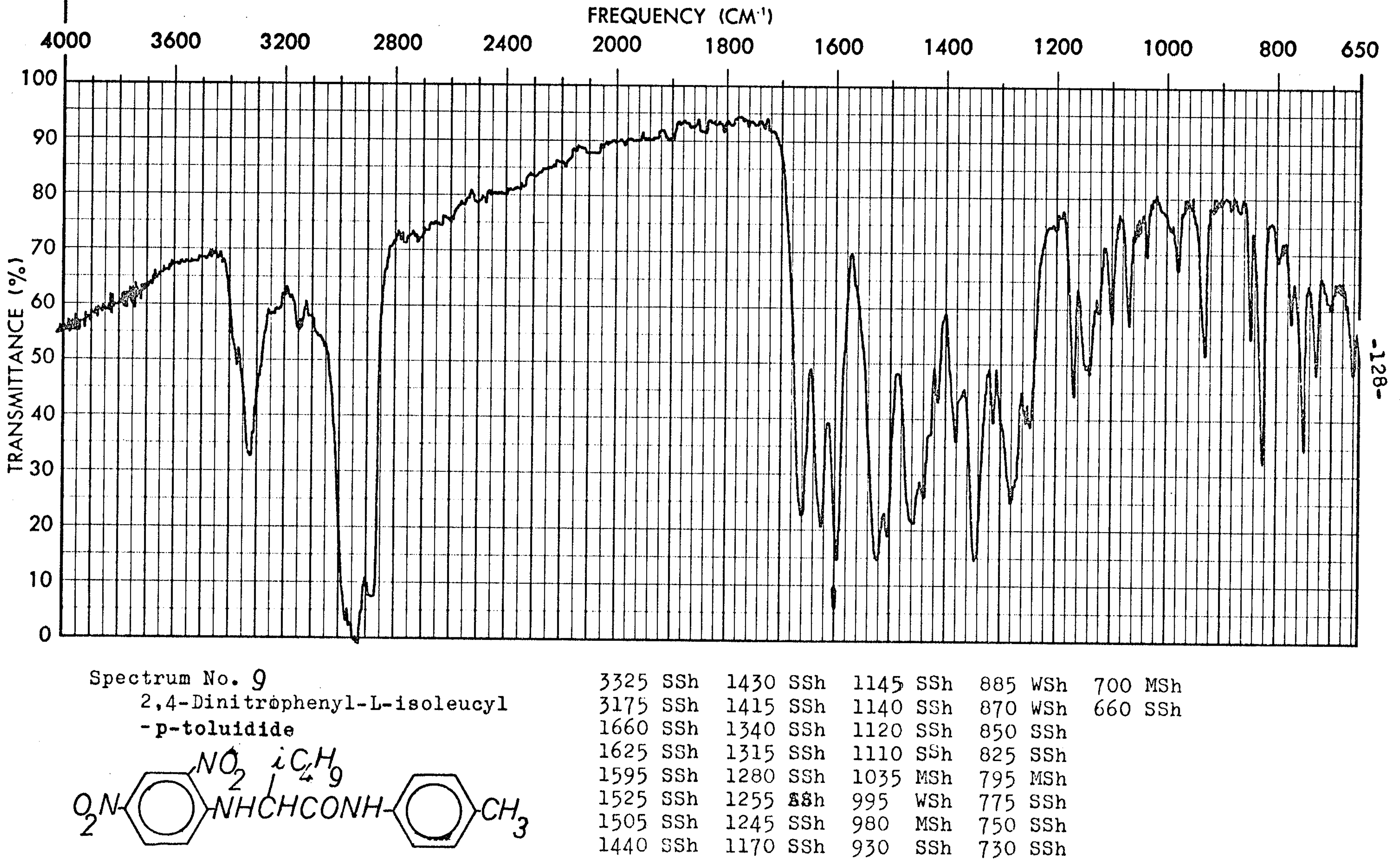
Spectrum No. 7
2,4-Dinitrophenyl-L-isoleucyl
-amide

3490	SSh	1560	SSh	1290	SSh	1065	SSh	845	SSh
3350	SSh	1530	SSh	1275	SSh	1005	WSh	825	SSh
3240	SSh	1495	SSh	1245	SSh	990	WSh	775	MSh
3150	SSh	1470	SSh	1175	SSh	980	WSh	750	SSh
1670	SSh	1430	SSh	1155	SSh	955	WSh	735	SSh
1630	SSh	1370	SSh	1150	SSh	930	SSh	700	MSh
1605	SSh	1340	SSh	1125	SSh	895	MSh	655	SSh
1590	SSh	1315	SSh	1095	SSh	865	WSh		



Spectrum No. 8
2,4-Dinitrophenyl-L-isoleucyl-anilide

3400 SSh	1520 SSh	1275 SSh	1070 SSh	840 SSh
3275 SSh	1500 SSh	1245 SSh	1045 MSh	780 SSh
3175 SSh	1465 SSh	1210 MSh	995 MSh	755 SSh
3100 SSh	1450 SSh	1170 SSh	940 SSh	750 SSh
1655 SSh	1435 SSh	1155 SSh	920 MSh	730 SSh
1620 SSh	1375 SSh	1140 SSh	880 WB	700 SSh
1595 SSh	1340 SSh	1125 SSh	865 WSh	665 SSh
1555 SSh	1315 SSh	1100 SSh	850 SSh	

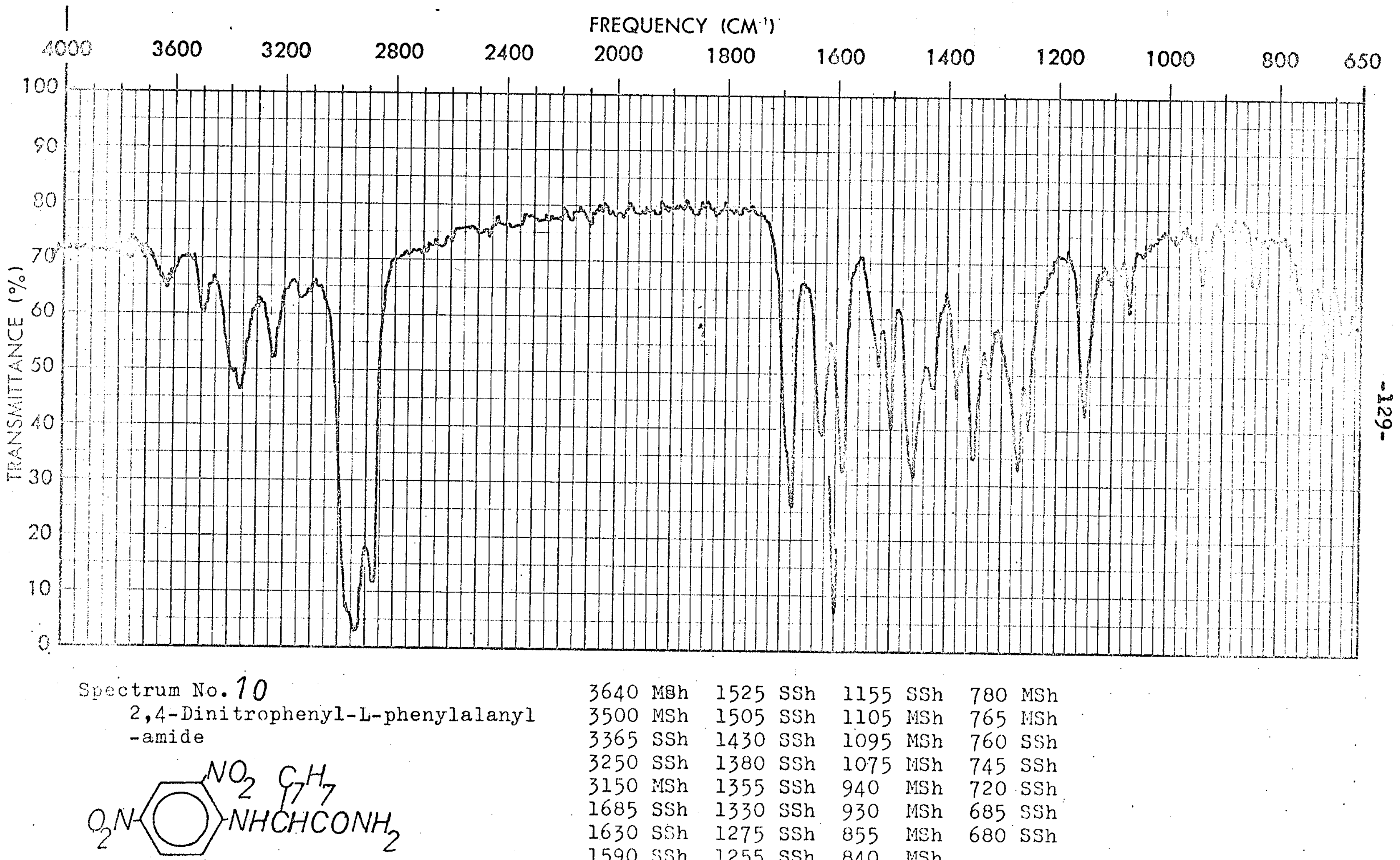


Spectrum No. 9

2,4-Dinitrophenyl-L-isoleucyl-p-toluidide

$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONH-C_6H_4-CH_3$

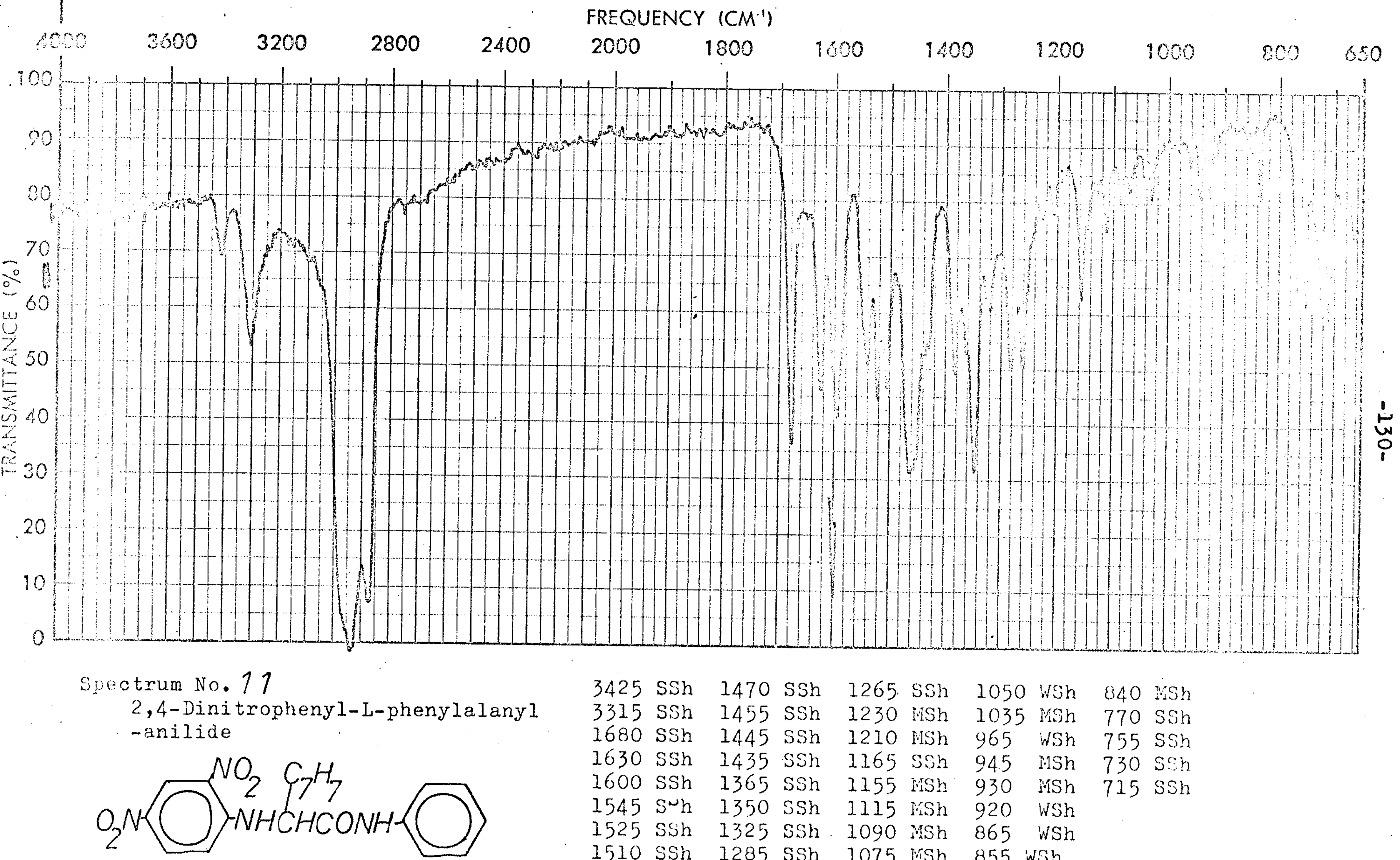
3325 SSh	1430 SSh	1145 SSh	885 WSh	700 MSh
3175 SSh	1415 SSh	1140 SSh	870 WSh	660 SSh
1660 SSh	1340 SSh	1120 SSh	850 SSh	
1625 SSh	1315 SSh	1110 SSh	825 SSh	
1595 SSh	1280 SSh	1035 MSh	795 MSh	
1525 SSh	1255 SSh	995 WSh	775 SSh	
1505 SSh	1245 SSh	980 MSh	750 SSh	
1440 SSh	1170 SSh	930 SSh	730 SSh	



Spectrum No. 10
2,4-Dinitrophenyl-L-phenylalanyl -amide

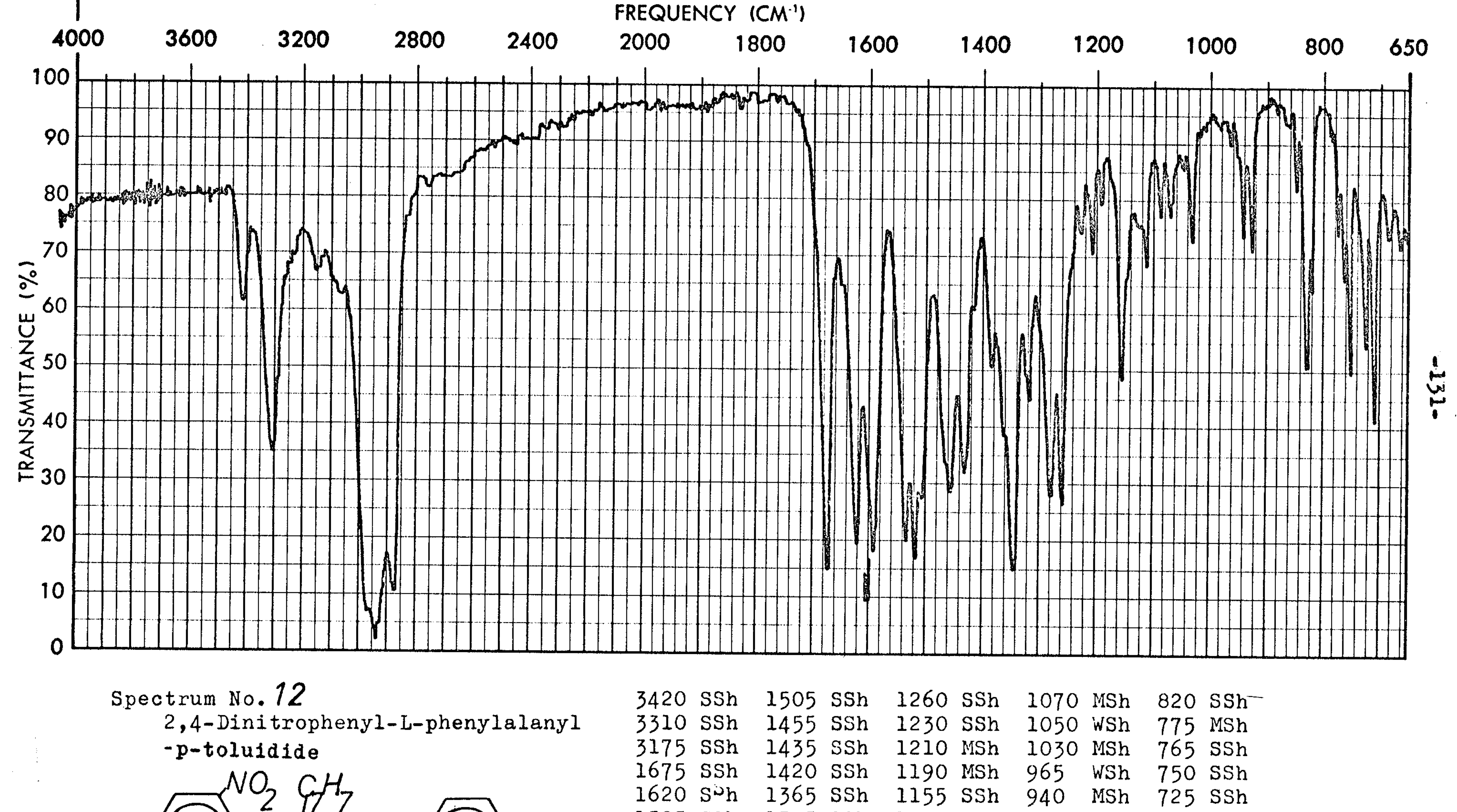
O_2N-C6H3(NO_2)-$NHCH(C_7H_7)CONH_2$

3640	MSh	1525	SSh	1155	SSh	780	MSh
3500	MSh	1505	SSh	1105	MSh	765	MSh
3365	SSh	1430	SSh	1095	MSh	760	SSh
3250	SSh	1380	SSh	1075	MSh	745	SSh
3150	MSh	1355	SSh	940	MSh	720	SSh
1685	SSh	1330	SSh	930	MSh	685	SSh
1630	SSh	1275	SSh	855	MSh	680	SSh
1590	SSh	1255	SSh	840	MSh		



Spectrum No. 11
2,4-Dinitrophenyl-L-phenylalanyl-anilide

3425 SSh	1470 SSh	1265 SSh	1050 WSh	840 MSh
3315 SSh	1455 SSh	1230 MSh	1035 MSh	770 SSh
1680 SSh	1445 SSh	1210 MSh	965 WSh	755 SSh
1630 SSh	1435 SSh	1165 SSh	945 MSh	730 SSh
1600 SSh	1365 SSh	1155 MSh	930 MSh	715 SSh
1545 SSh	1350 SSh	1115 MSh	920 WSh	
1525 SSh	1325 SSh	1090 MSh	865 WSh	
1510 SSh	1285 SSh	1075 MSh	855 WSh	

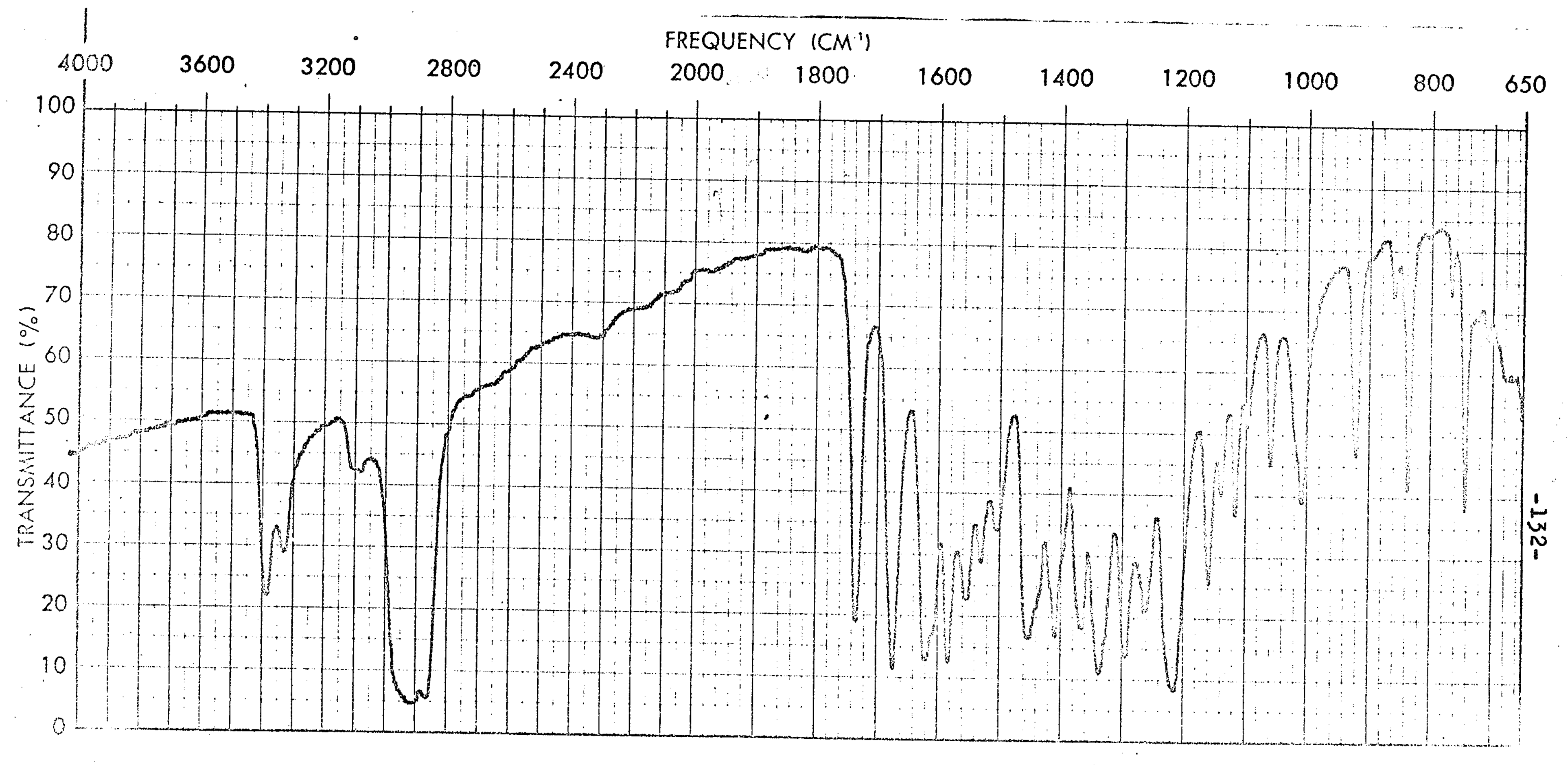


Spectrum No. 12

2,4-Dinitrophenyl-L-phenylalanyl

-p-toluidide

3420 SSh	1505 SSh	1260 SSh	1070 MSh	820 SSh
3310 SSh	1455 SSh	1230 SSh	1050 WSh	775 MSh
3175 SSh	1435 SSh	1210 MSh	1030 MSh	765 SSh
1675 SSh	1420 SSh	1190 MSh	965 WSh	750 SSh
1620 SSh	1365 SSh	1155 SSh	940 MSh	725 SSh
1585 SSh	1345 SSh	1130 MB	925 MSh	710 SSh
1535 SSh	1320 SSh	1110 SSh	850 MSh	685 MSh
1520 SSh	1280 SSh	1085 MSh	830 SSh	665 MSh

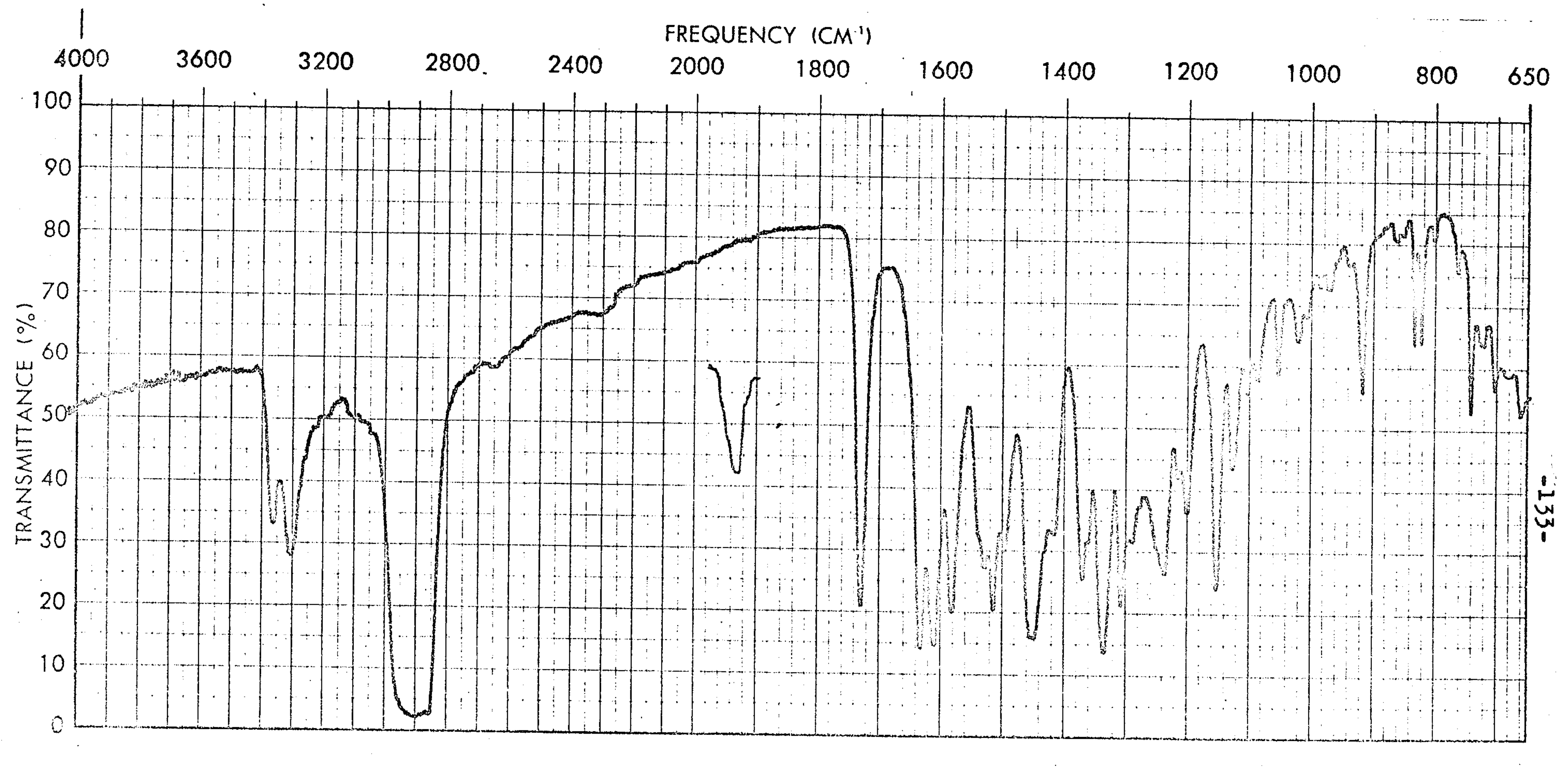


Spectrum No. 13

2,4-Dinitrophenyl glycyl glycine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH_2COCH_2COOC_2H_5$

3400	SSh	1560	SSh	1160	SSh	838	MSh
3340	SSh	1535	SSh	1142	MSh	768	WSh
3120	MSh	1510	SSh	1120	MSh	742	MSh
3080	MSh	1415	SSh	1100	MSh	720	WB
1750	SSh	1345	SSh	1062	MSh	705	WB
1680	SSh	1300	SSh	1010	MSh		
1630	SSh	1260	SSh	920	MSh		
1595	SSh	1220	SSh	860	WSh		

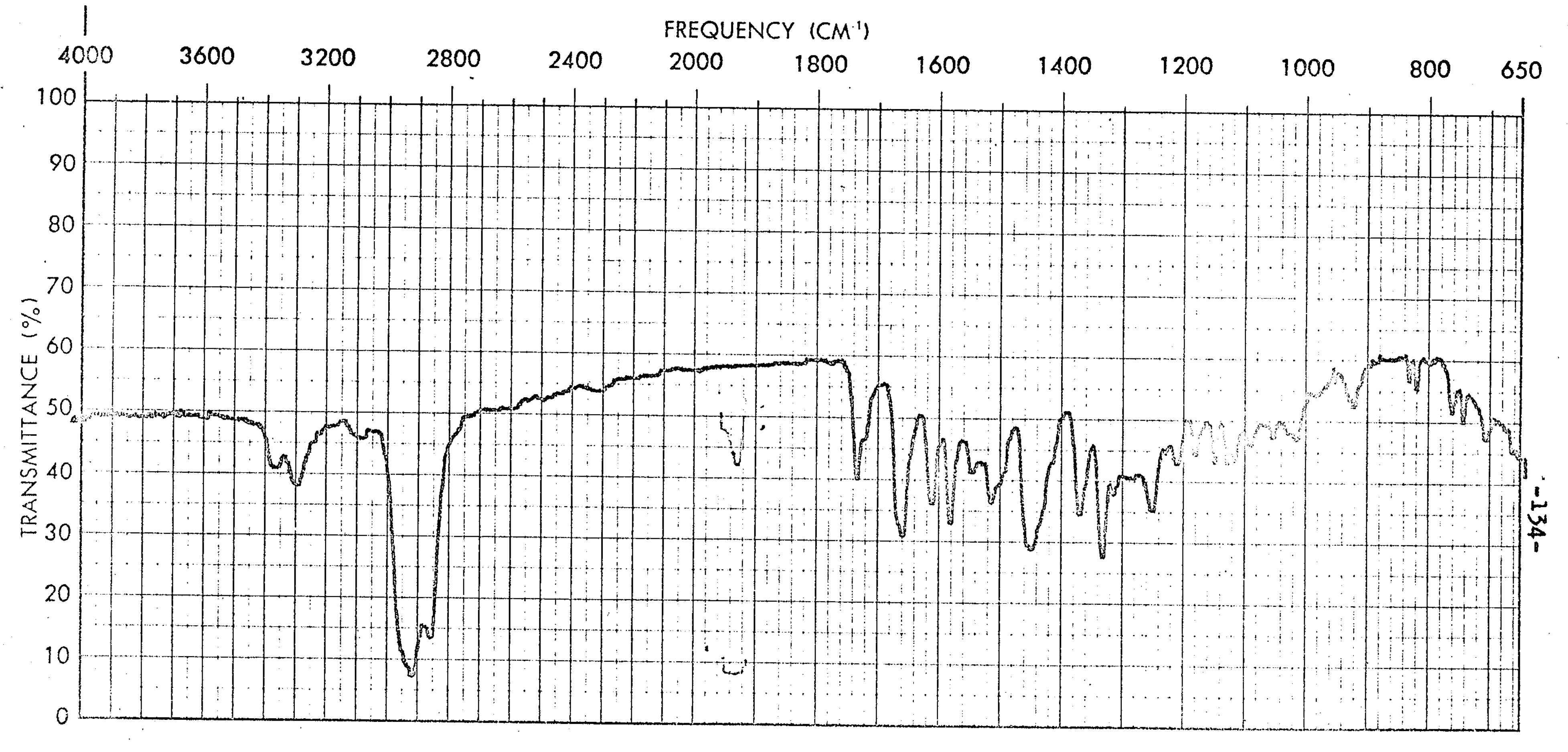


Spectrum No. 14

2,4-Dinitrophenyl glycyl-L-leucine ethyl ester

$2,4\text{-}(O_2N)_2C_6H_3\text{-}NHCH_2CONHCH(C_4H_9)COOC_2H_5$

3370	SSh	1525	SSh	1219	MSh	1010	WB	822	MSh
3330	SSh	1505	SSh	1208	SSh	990	WSh	800	WB
3130	WSh	1420	SSh	1156	SSh	975	WB	762	WSh
1750	SSh	1360	SSh	1132	MSh	941	WB	740	MSh
1650	SSh	1345	SSh	1108	MSh	918	MSh	720	MB
1620	SSh	1310	SSh	1090	MSh	864	WB	700	MSh
1600	SSh	1290	SSh	1055	MSh	850	WB	662	MB
1535	SSh	1240	SSh	1024	MSh	835	MSh	630	MSh

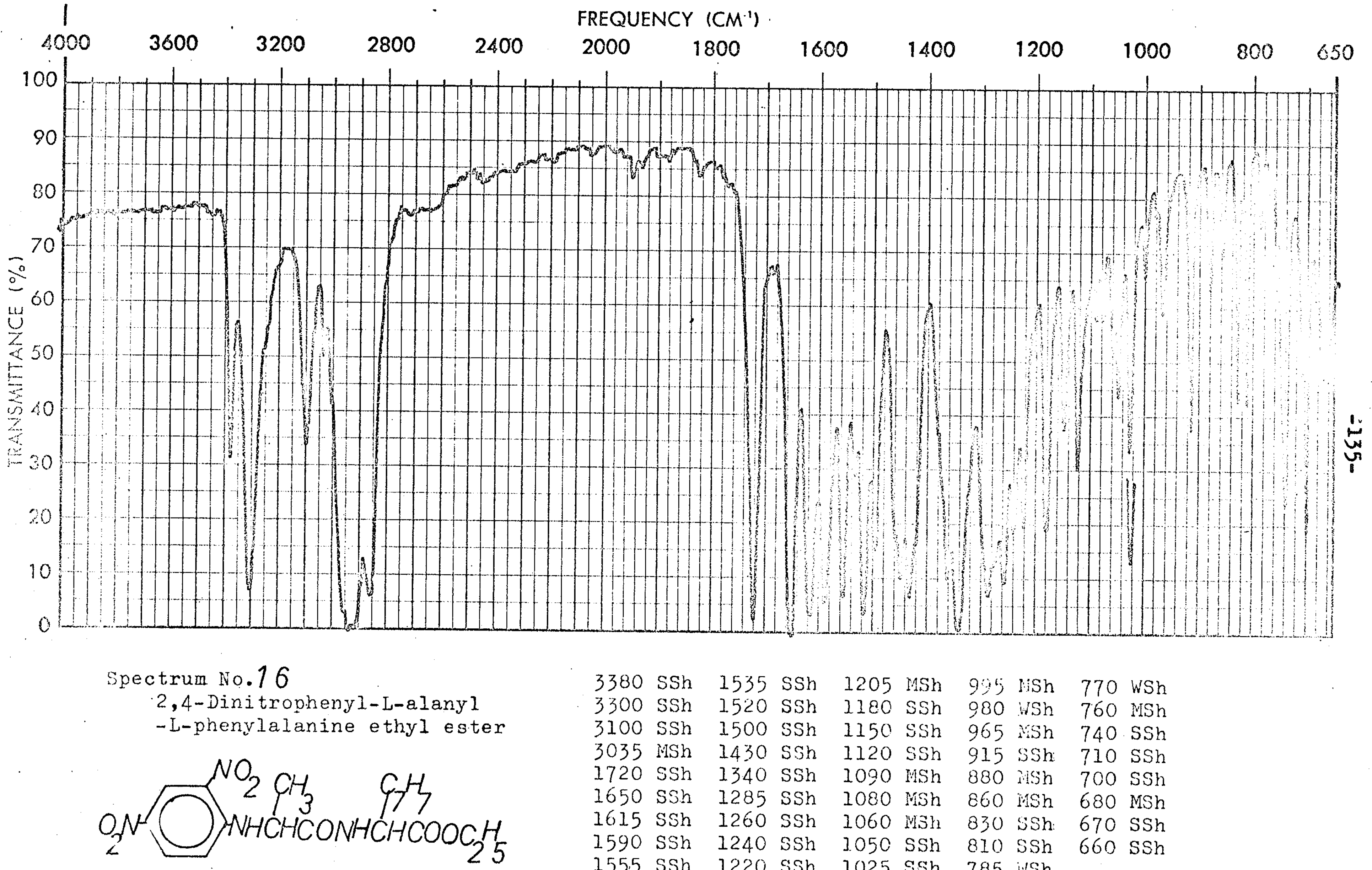


Spectrum No. 15

2,4-Dinitrophenyl glycyl-L-phenlyalanine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH_2CONHCH(C_7H_7)COOC_2H_5$

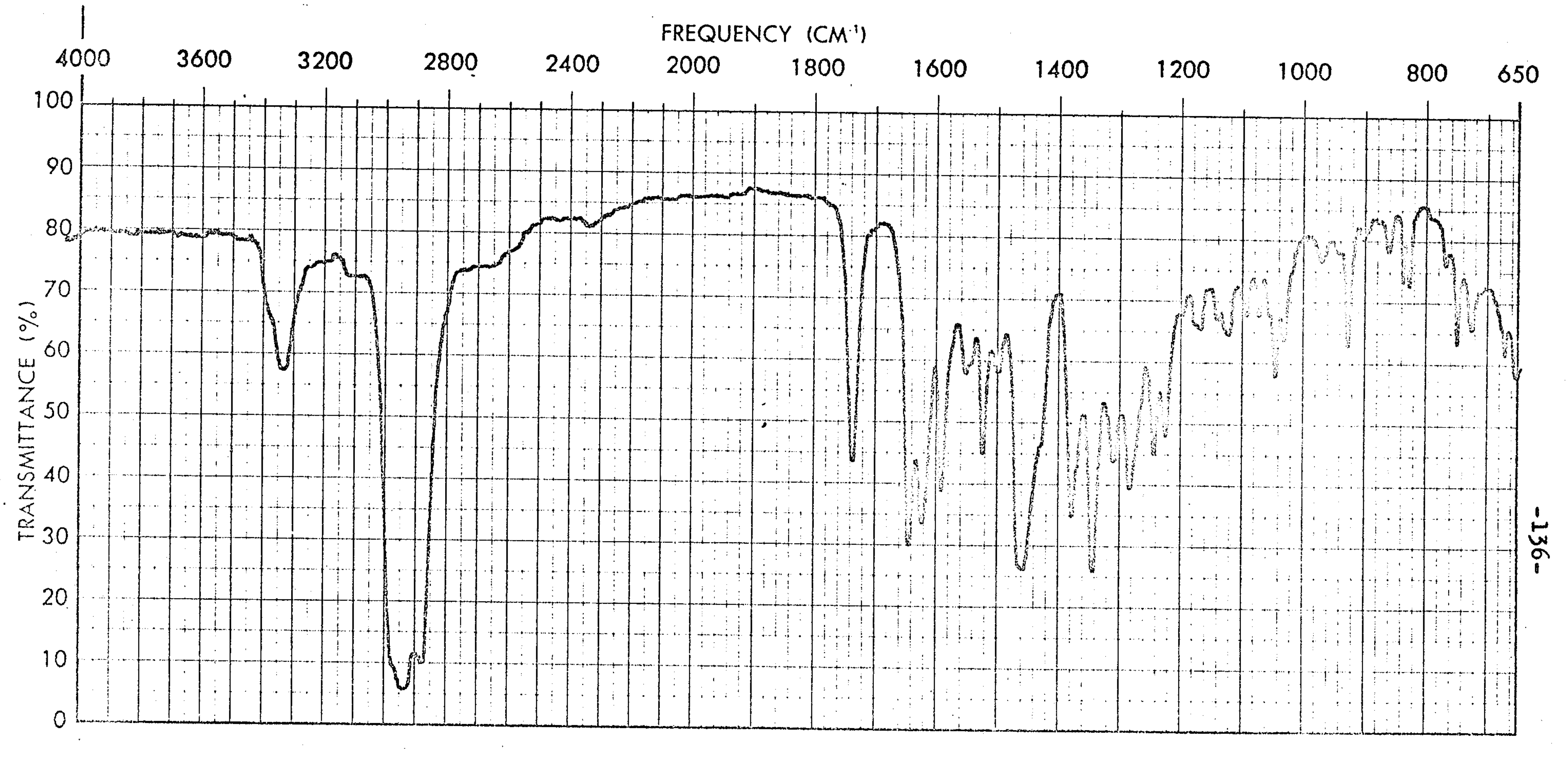
3380 SSh	1545 SSh	1238 SSh	1040 MB	765 MSh
3300 SSh	1520 SSh	1215 SSh	1020 MSh	745 MSh
3090 MB	1500 SSh	1190 SSh	980 WB	710 MSh
1740 SSh	1395 SSh	1158 SSh	935 WSh	665 MSh
1720 MSh	1330 SSh	1140 SSh	920 WSh	
1670 SSh	1315 SSh	1125 SSh	836 WSh	
1615 SSh	1300 SB	1100 MSh	825 WSh	
1590 SSh	1260 SSh	1060 MSh	802 WB	



Spectrum No. 16
2,4-Dinitrophenyl-L-alanyl
-L-phenylalanine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(CH_3)CONHCH(C_7H_7)COOC_2H_5$

3380	SSh	1535	SSh	1205	MSh	995	MSh	770	WSh
3300	SSh	1520	SSh	1180	SSh	980	WSh	760	MSh
3100	SSh	1500	SSh	1150	SSh	965	MSh	740	SSh
3035	MSh	1430	SSh	1120	SSh	915	SSh	710	SSh
1720	SSh	1340	SSh	1090	MSh	880	MSh	700	SSh
1650	SSh	1285	SSh	1080	MSh	860	MSh	680	MSh
1615	SSh	1260	SSh	1060	MSh	830	SSh	670	SSh
1590	SSh	1240	SSh	1050	SSh	810	SSh	660	SSh
1555	SSh	1220	SSh	1025	SSh	785	WSh		



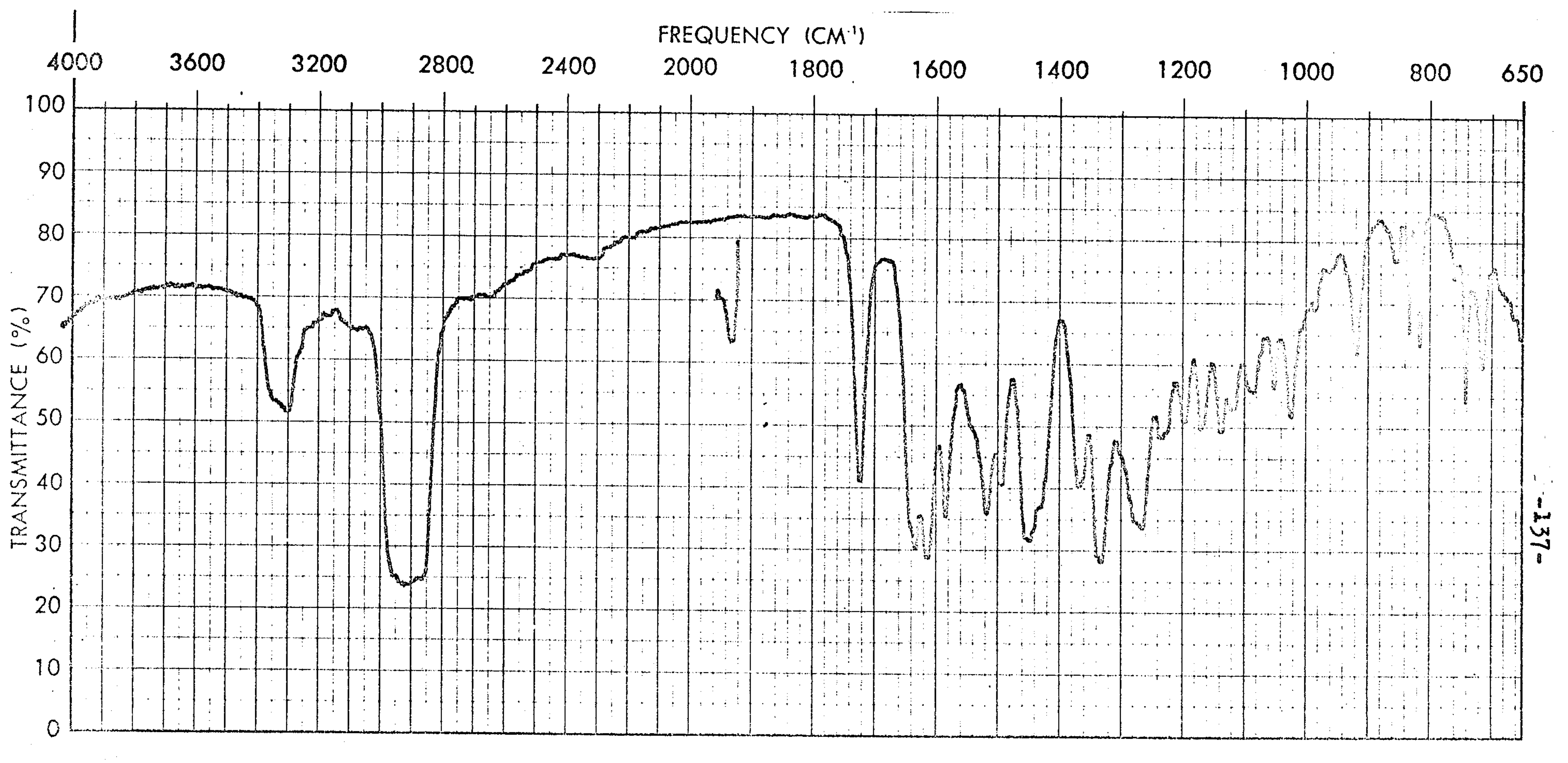
Spectrum No. 17

2,4-Dinitrophenyl-L-valyl-glycine ethyl ester

NO_2, C_3H_7

O_2N–⬡–$NHCHCONHCH_2COOC_2H_5$

3370	SSh	1500	SSh	1220	SSh	1068	MSh	855	WSh
3320	SSh	1425	SSh	1195	MB	1050	MSh	832	WSh
1740	SSh	1360	SSh	1175	MSh	1040	SSh	820	WSh
1645	SSh	1340	SSh	1165	MSh	1026	SSh	762	WSh
1620	SSh	1315	SSh	1135	MSh	966	WB	753	MSh
1590	SSh	1305	SSh	1120	MSh	938	WB	718	MSh
1550	SSh	1280	SSh	1115	MSh	920	MSh	665	MSh
1520	SSh	1240	SSh	1088	MSh	896	WSh	645	MSh

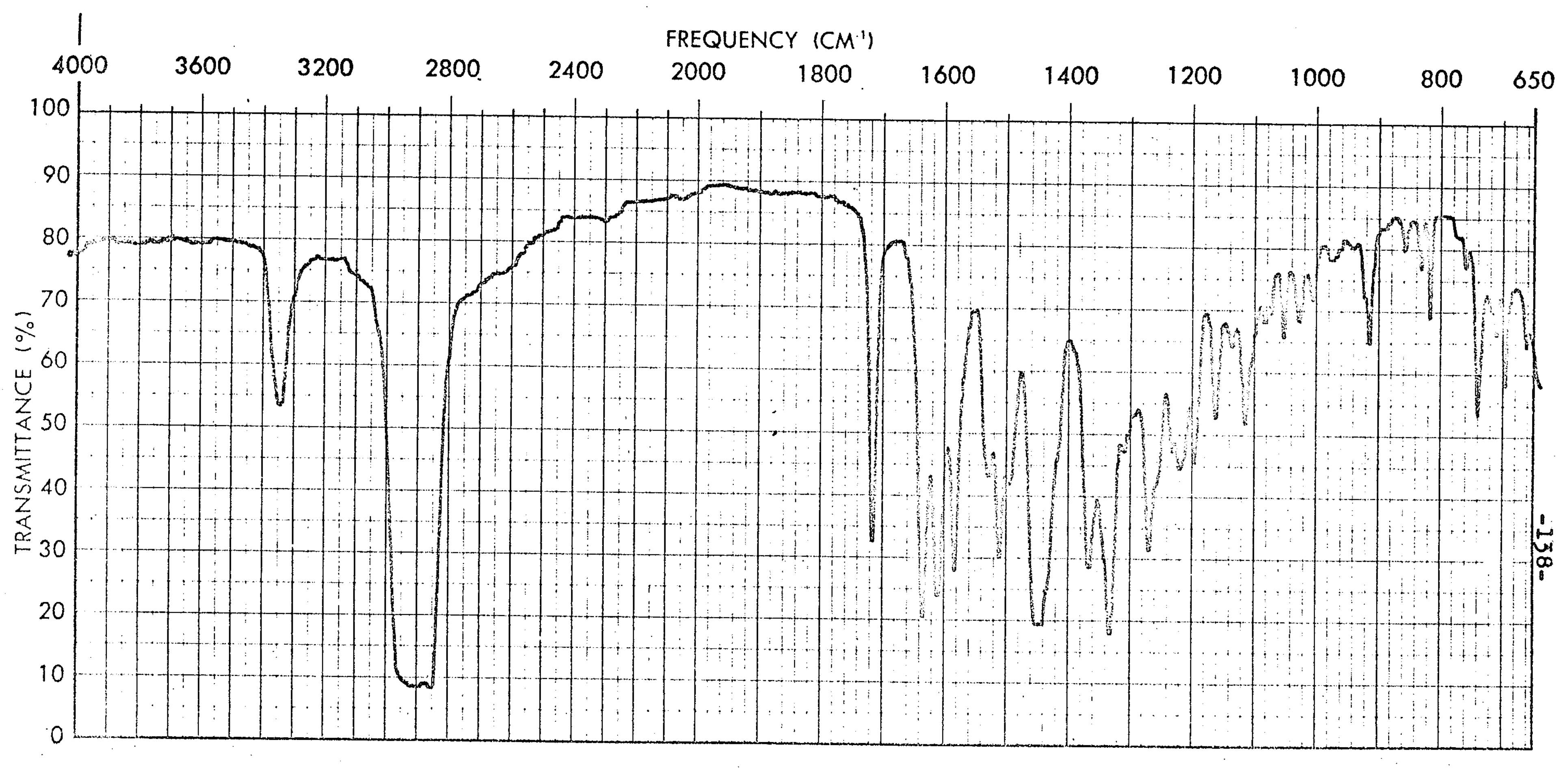


Spectrum No. 18

2,4-Dinitrophenyl-L-Valyl-L-leucine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(C_3H_7)CONHCH(C_4H_9)COOC_2H_5$

3360 SSh	1525 SSh	1225 SSh	988 MSh	665 MSh
3300 SSh	1500 SSh	1200 SSh	920 MSh	650 MSh
1735 SSh	1435 SSh	1175 SSh	855 WB	
1655 SSh	1360 SSh	1140 SSh	835 MSh	
1640 SSh	1340 SSh	1125 SSh	816 MSh	
1620 SSh	1280 SSh	1090 SSh	760 WSh	
1595 SSh	1270 SSh	1055 SSh	742 SSh	
1550 SSh	1240 SSh	1026 SSh	715 SSh	

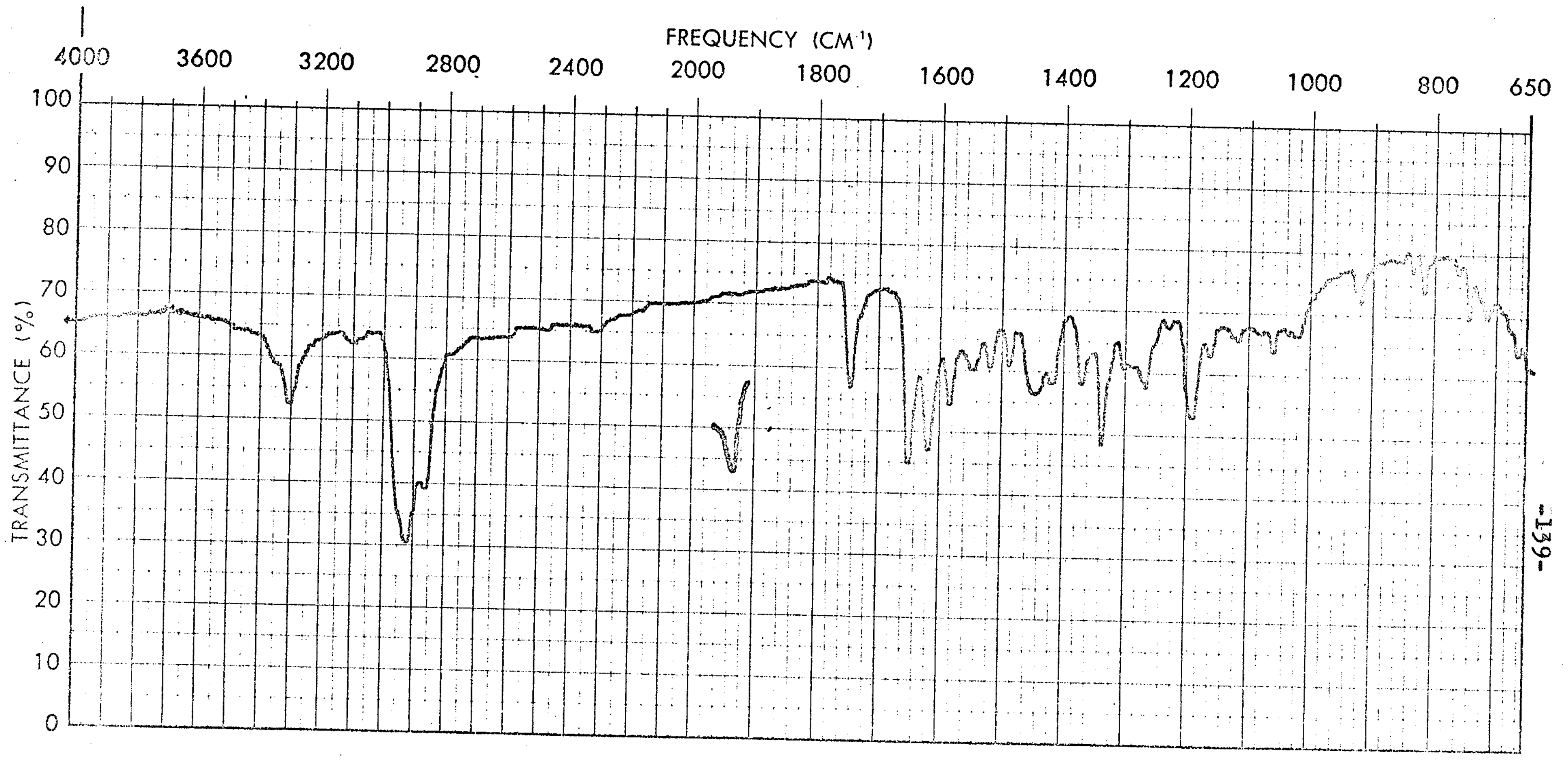


Spectrum No. 19

2,4-Dinitrophenyl-L-valyl-L-phenylalanine ethyl ester

O_2N–(ring, NO_2)–$NHCH(C_3H_7)CONHCH(C_7H_7)COOC_2H_5$

3370	SSh	1500	SSh	1203	SSh	1008	MSh	775	WSh
3350	SSh	1425	SSh	1168	SSh	980	WSh	760	WSh
1730	SSh	1365	SSh	1140	MSh	945	WB	742	SSh
1655	SSh	1340	SSh	1120	SSh	925	MSh	720	MSh
1625	SSh	1315	SSh	1085	MSh	918	MSh	710	MSh
1600	SSh	1275	SSh	1075	MSh	860	WSh	698	SSh
1540	SSh	1240	SSh	1055	MSh	832	WSh	665	MSh
1520	SSh	1225	SSh	1030	MSh	820	MSh		

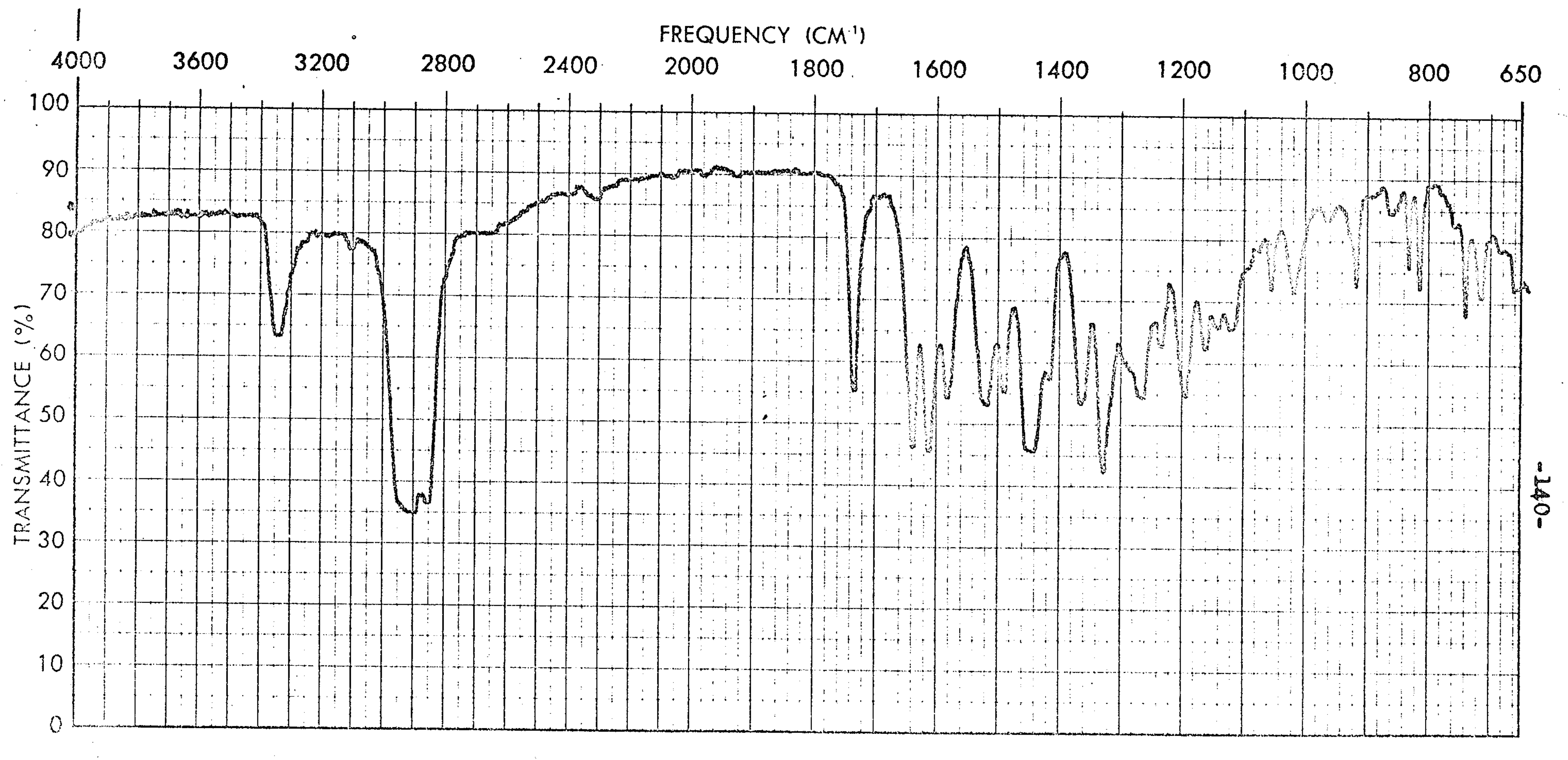


Spectrum No. 20

2,4-Dinitrophenyl-L-leucyl glycine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONHCH_2COOC_2H_5$

3370 SSh	1500 SSh	1160 SSh	918 MSh
3330 SSh	1430 SSh	1125 MSh	832 MSh
1755 SSh	1345 SSh	1115 MSh	815 MSh
1660 SSh	1300 SSh	1060 MSh	760 MSh
1630 SSh	1290 SSh	1033 MSh	752 MSh
1600 SSh	1265 SSh	1012 MSh	740 MSh
1560 SSh	1230 MSh	975 WB	712 MSh
1525 SSh	1190 SSh	925 MSh	662 MSh

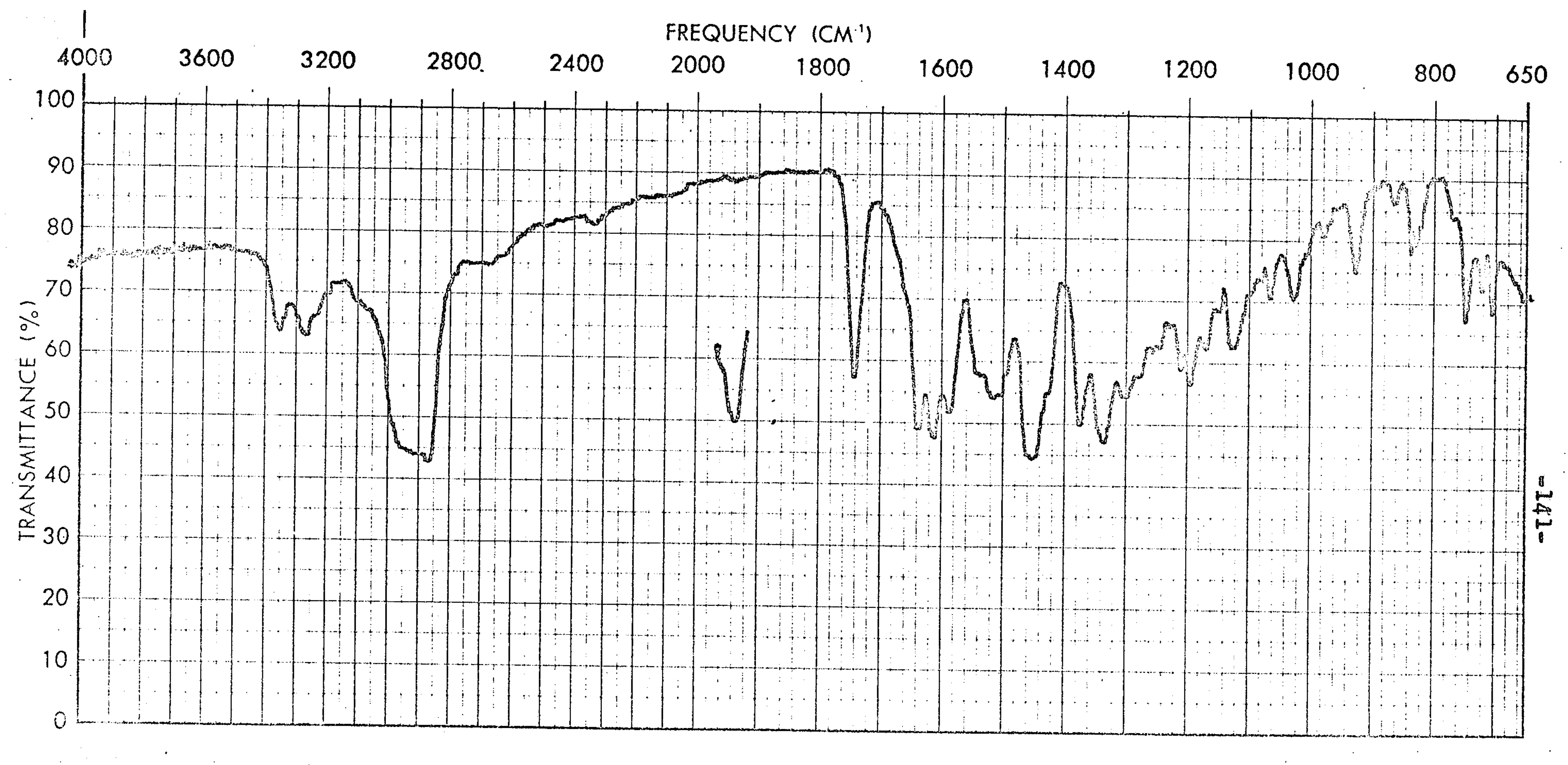


Spectrum No. 21

2,4-Dinitrophenyl -L-leucyl-L-leucine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONHCH(C_4H_9)COOC_2H_5$

3365	SSh	1530	SSh	1205	SSh	976	WSh	665	SSh
3345	SSh	1500	SSh	1170	SSh	920	MSh	655	MB
3115	SSh	1425	SSh	1150	SB	865	WB		
1750	SSh	1340	SSh	1130	SSh	818	MSh		
1650	SSh	1302	SSh	1100	SB	808	MSh		
1625	SSh	1280	SSh	1080	MB	742	MSh		
1595	SSh	1272	SSh	1060	MSh	715	WSh		
1535	SSh	1242	SSh	1022	MSh	687	MSh		

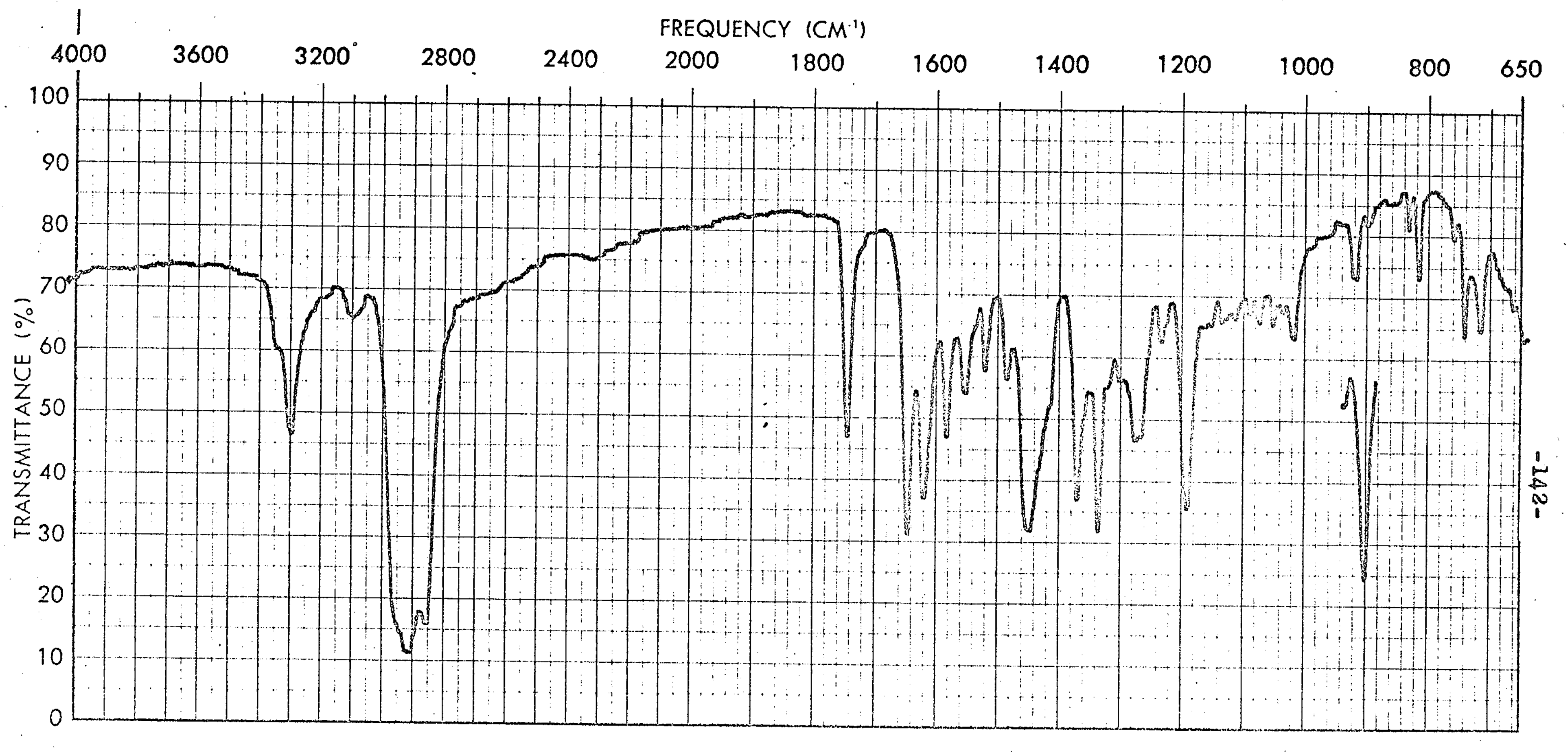


Spectrum No. 22

2,4-Dinitrophenyl-L-leucyl-L-phenylalanine ethyl ester

O_2N–$C_6H_3(NO_2)$–$NHCH(C_4H_9)CONHCH(C_7H_7)COOC_2H_5$

3360	SSh	1500	SB	1192	SSh	1000	MB	715	MSh
3270	SSh	1425	SSh	1168	SSh	972	WB	700	MSh
1750	SSh	1340	SSh	1145	SSh	922	MSh	655	MSh
1645	SSh	1300	SSh	1128	SB	855	WB		
1620	SSh	1280	SB	1118	SB	830	MSh		
1595	SSh	1250	SB	1072	MB	822	MSh		
1545	SSh	1238	SB	1062	MSh	765	WB		
1520	SSh	1210	SSh	1020	MB	740	MSh		

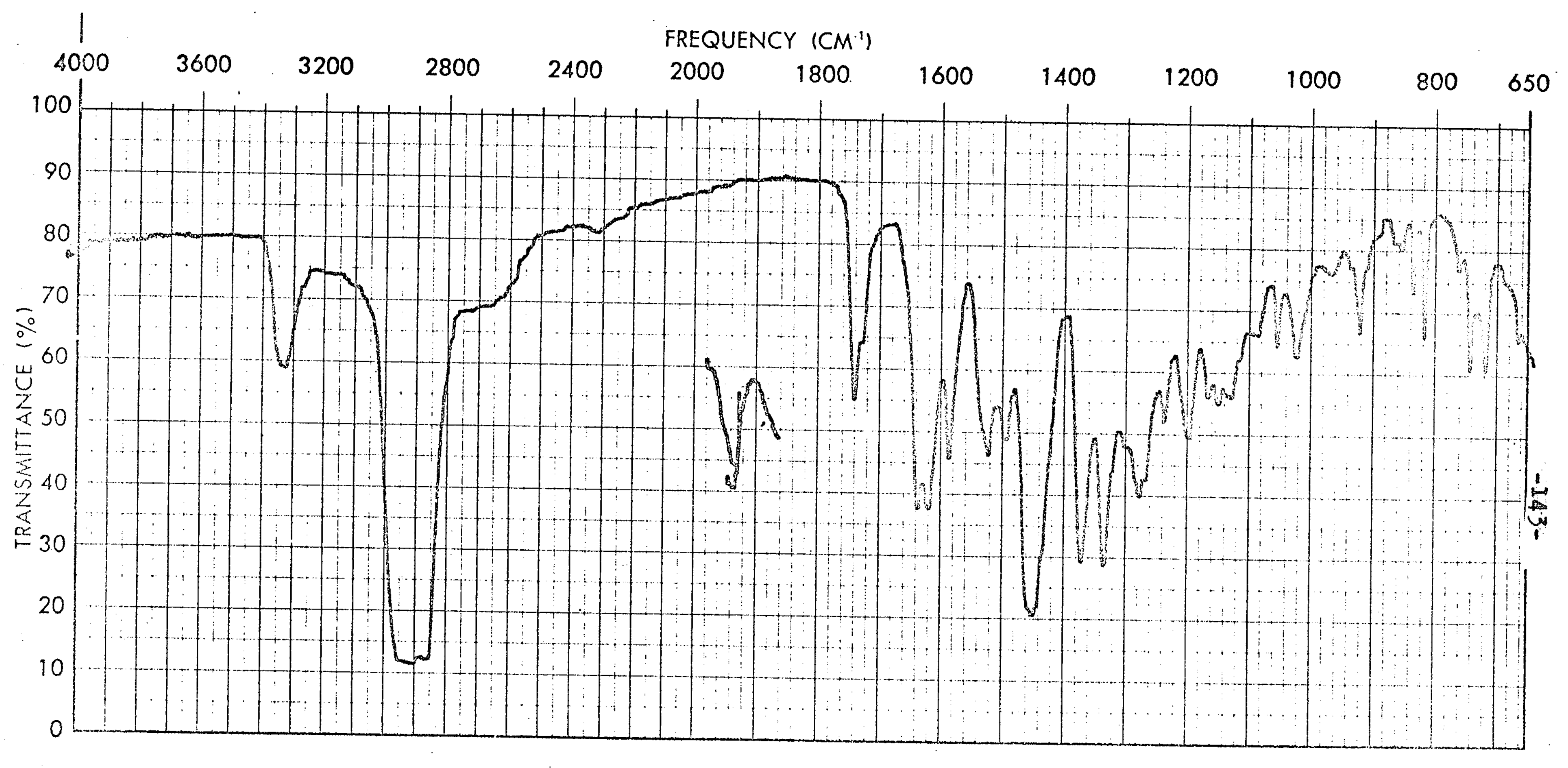


Spectrum No. 23

2,4-Dinitrophenyl-L-isoleucyl-glycine ethly ester

O_2N-$C_6H_3(NO_2)$-$NHCH(iC_4H_9)CONHCH_2COOC_2H_5$

3350	SSh	1530	SSh	1270	SSh	1088	MSh	833	WSh
3310	SSh	1490	SSh	1240	SSh	1055	MSh	815	MSh
3110	SSh	1420	SSh	1200	SSh	1040	MSh	760	WSh
1750	SSh	1355	SSh	1170	MSh	1020	SSh	754	WSh
1660	SSh	1340	SSh	1155	MSh	925	WSh	742	MSh
1625	SSh	1320	SSh	1140	MB	918	MSh	715	MSh
1590	SSh	1300	SSh	1120	MSh	900	WSh	662	MSh
1560	SSh	1280	SSh	1092	MSh	865	WSh	645	MSh

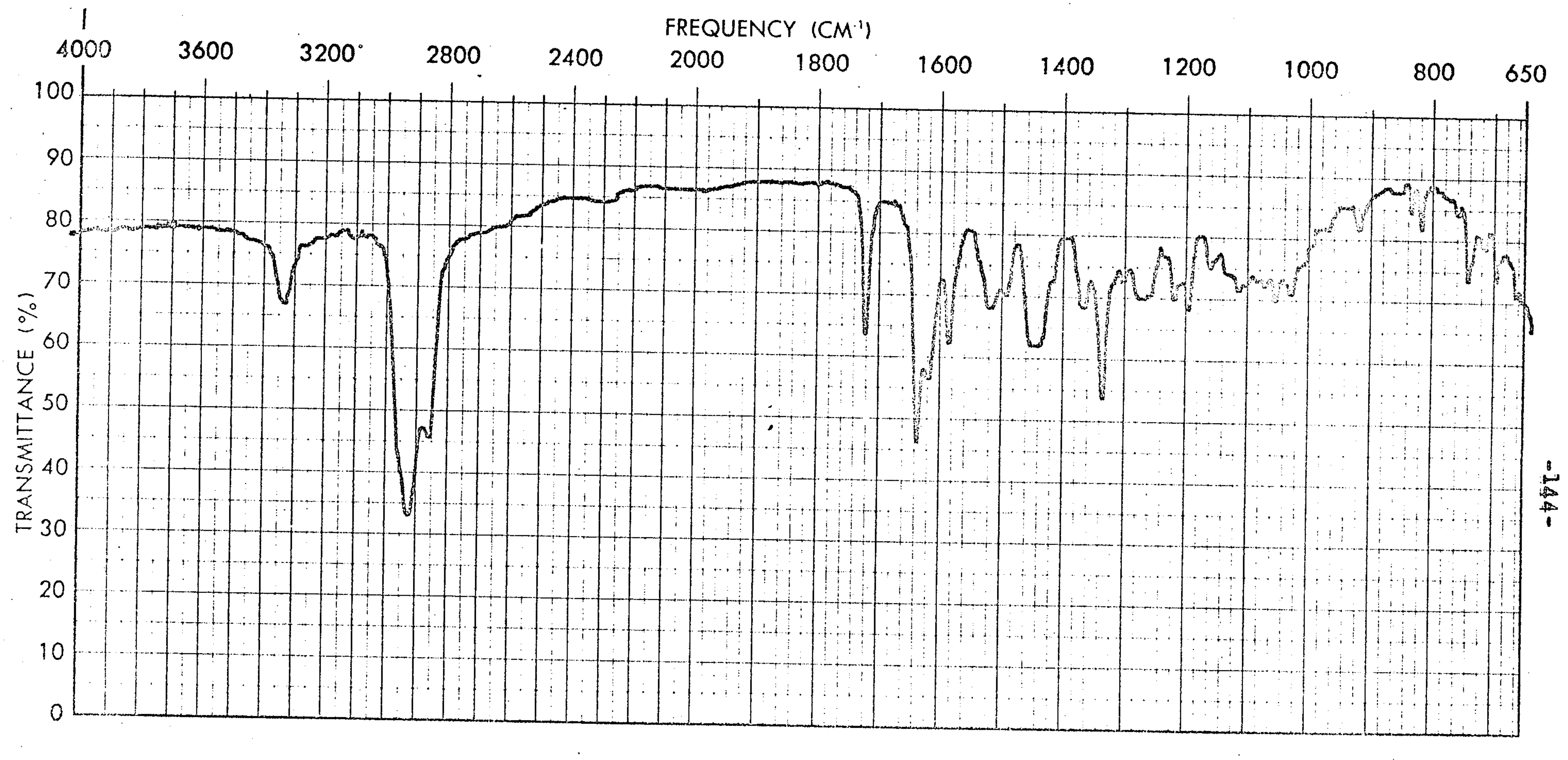


Spectrum No. 24

2,4-Dinitrophenyl-L-isoleucyl-L-leucine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(i\text{-}C_4H_9)CONHCH(C_4H_9)COOC_2H_5$

3350	SSh	1340	SSh	1138	SSh	866	WB
1750	SSh	1305	SSh	1130	SSh	850	WB
1650	SSh	1300	SSh	1116	SSh	835	MSh
1625	SSh	1275	SSh	1088	SSh	818	MSh
1595	SSh	1265	SSh	1055	MSh	760	WSh
1540	SSh	1240	SSh	1020	MSh	742	SSh
1525	SSh	1200	SSh	970	WSh	716	SSh
1500	SSh	1160	SSh	920	MSh	662	MSh

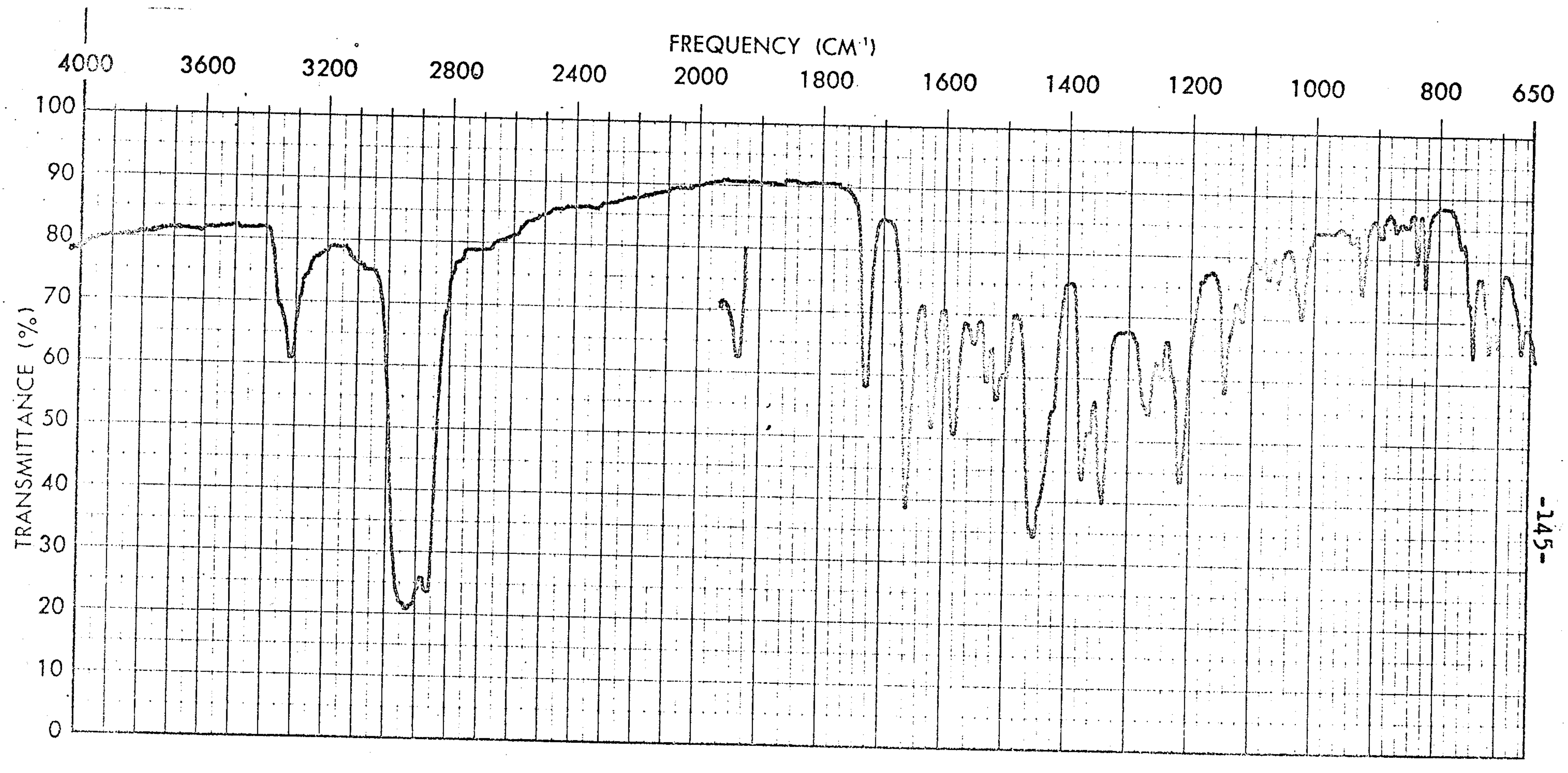


Spectrum No. 25

2,4-Dinitrophenyl-L-isoleucyl-
L-phenylalanine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH(C_7H_7)COOC_2H_5$

3350	SSh	1345	SSh	1120	SSh	922	MSh	698	MSh
1730	SSh	1305	SSh	1105	SSh	870	WSh	665	MSh
1650	SSh	1280	SSh	1088	SSh	835	MSh		
1625	SSh	1260	SSh	1075	SSh	820	MSh		
1595	SSh	1225	SSh	1058	SSh	762	MSh		
1525	SSh	1220	SSh	1032	SSh	755	SSh		
1500	SSh	1168	SSh	1010	SB	743	MSh		
1420	SSh	1140	SB	972	MB	715	SSh		

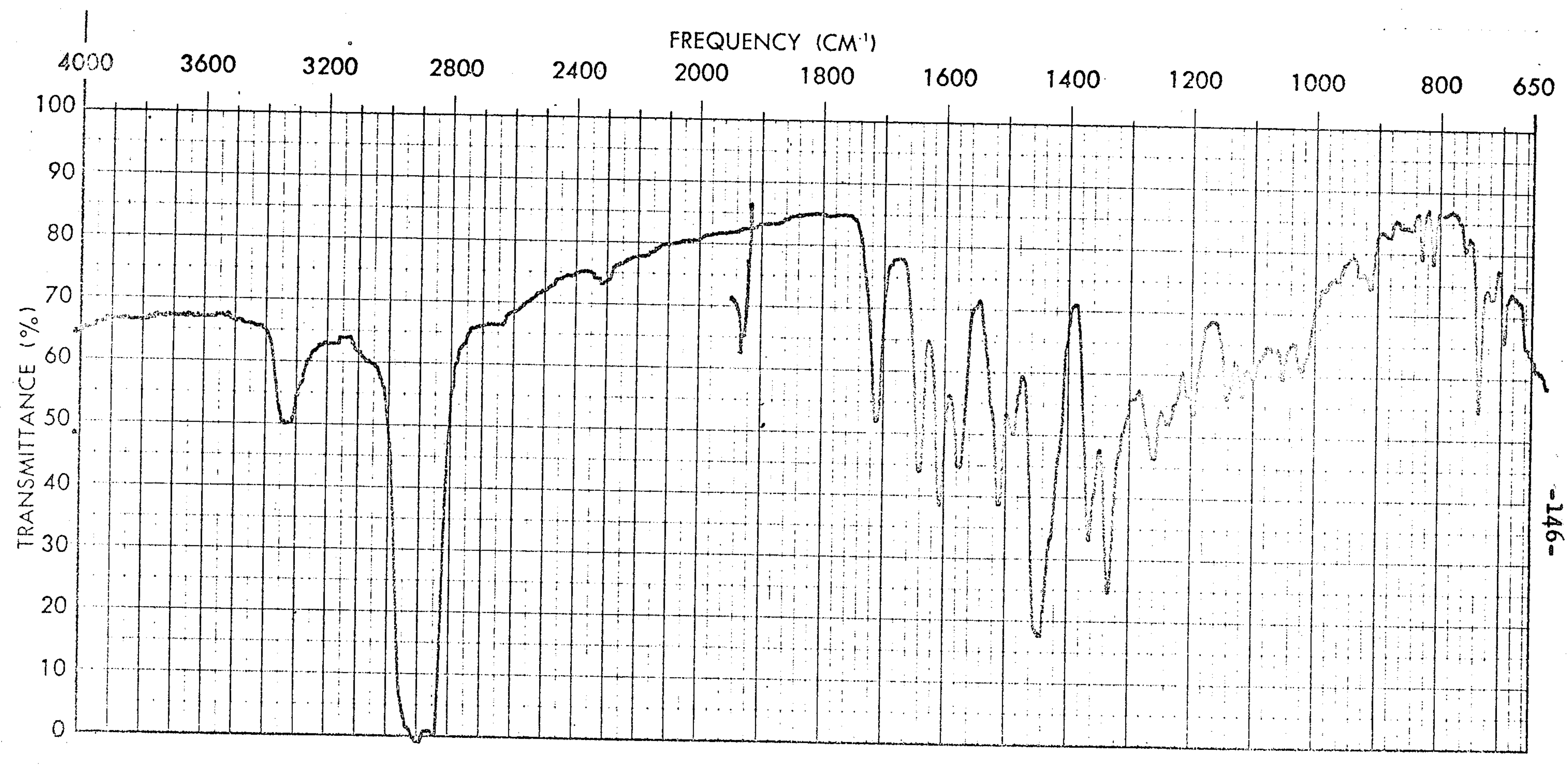


Spectrum No. 26

2,4-Dinitrophenyl-L-phenylalanyl-glycine ethyl ester

O_2N–$C_6H_3(NO_2)$–$NHCH(C_7H_7)CONHCH_2COOC_2H_5$

3350	SSh	1515	SSh	1250	SSh	1055	MSh	830	MSh
3315	SSh	1500	SSh	1230	SSh	1018	MSh	818	MSh
1740	SSh	1440	SSh	1218	SSh	968	WB	760	SSh
1670	SSh	1420	SSh	1143	SSh	940	WSh	740	SSh
1630	SSh	1360	SSh	1132	SSh	920	SSh	712	SSh
1595	SSh	1340	SSh	1116	MSh	890	WB	700	SSh
1555	SSh	1310	SSh	1085	MSh	867	WB	664	SSh
1535	SSh	1265	SSh	1072	MSh	848	WB		

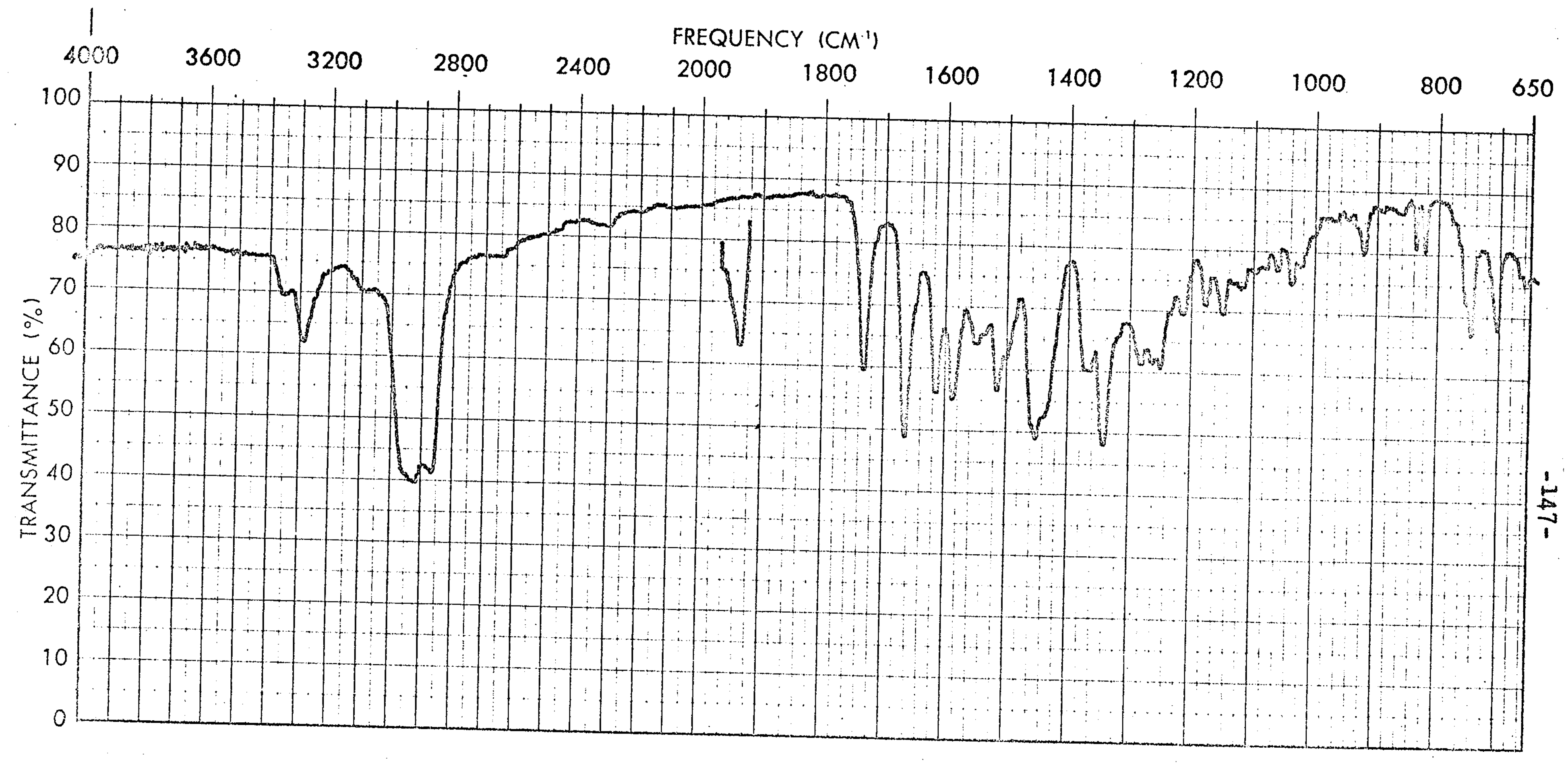


Spectrum No. 27

2,4-Dinitrophenyl-L-phenylalanyl-L-leucine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(C_7H_7)CONHCH(C_4H_9)COOC_2H_5$

3360 SSh	1420 SSh	1150 SSh	925 MB	740 SSh
1725 SSh	1365 SSh	1125 SSh	916 MSh	720 MB
1660 SSh	1345 SSh	1105 SSh	888 WSh	700 MSh
1620 SSh	1268 SSh	1072 SSh	868 WSh	665 MSh
1590 SSh	1245 SSh	1060 SSh	850 WSh	
1535 SSh	1230 SSh	1028 SB	833 MSh	
1520 SSh	1216 SSh	970 MSh	815 MSh	
1500 SSh	1205 SSh	936 MSh	762 WSh	

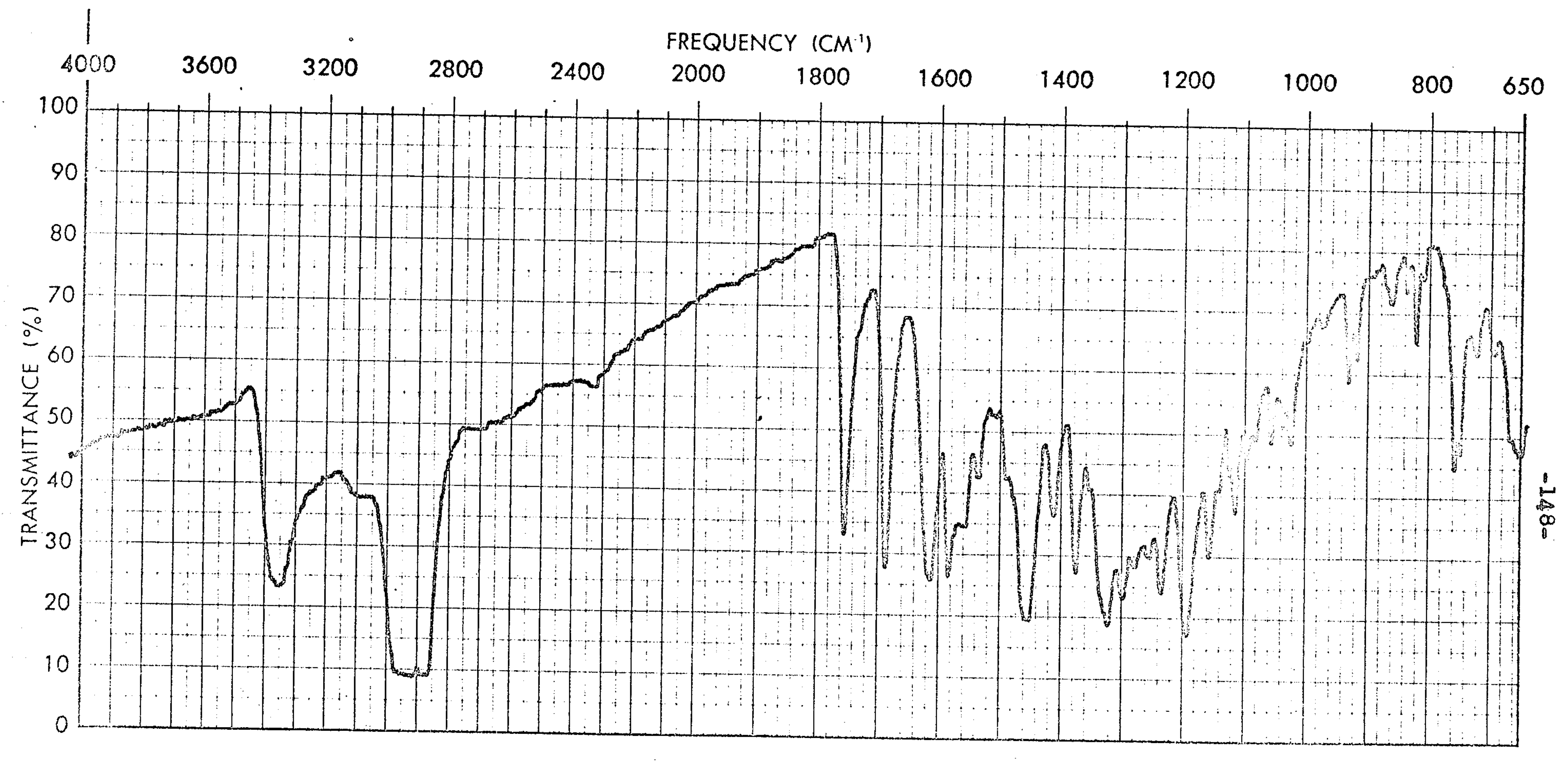


Spectrum NO. 28

2,4-Dinitrophenyl-L-phenylalanyl-L-phenylalanine ethyl ester

O_2N–$C_6H_3(NO_2)$–$NHCH(C_7H_7)CONHCH(C_7H_7)COOC_2H_5$

3360	SSh	1525	SSh	1250	SSh	1075	MSh	833	MSh
3300	SSh	1500	SSh	1230	MB	1060	MSh	818	MSh
1740	SSh	1440	SSh	1214	SSh	1035	MSh	760	SSh
1675	SSh	1365	SSh	1178	SSh	1020	MSh	752	SSh
1625	SSh	1345	SSh	1150	SSh	970	WB	740	MSh
1595	SSh	1320	SSh	1130	SSh	945	WB	700	SSh
1560	SSh	1285	SB	1115	MB	920	MSh	665	MSh
1540	SSh	1265	SSh	1095	MB	860	WSh	652	MSh

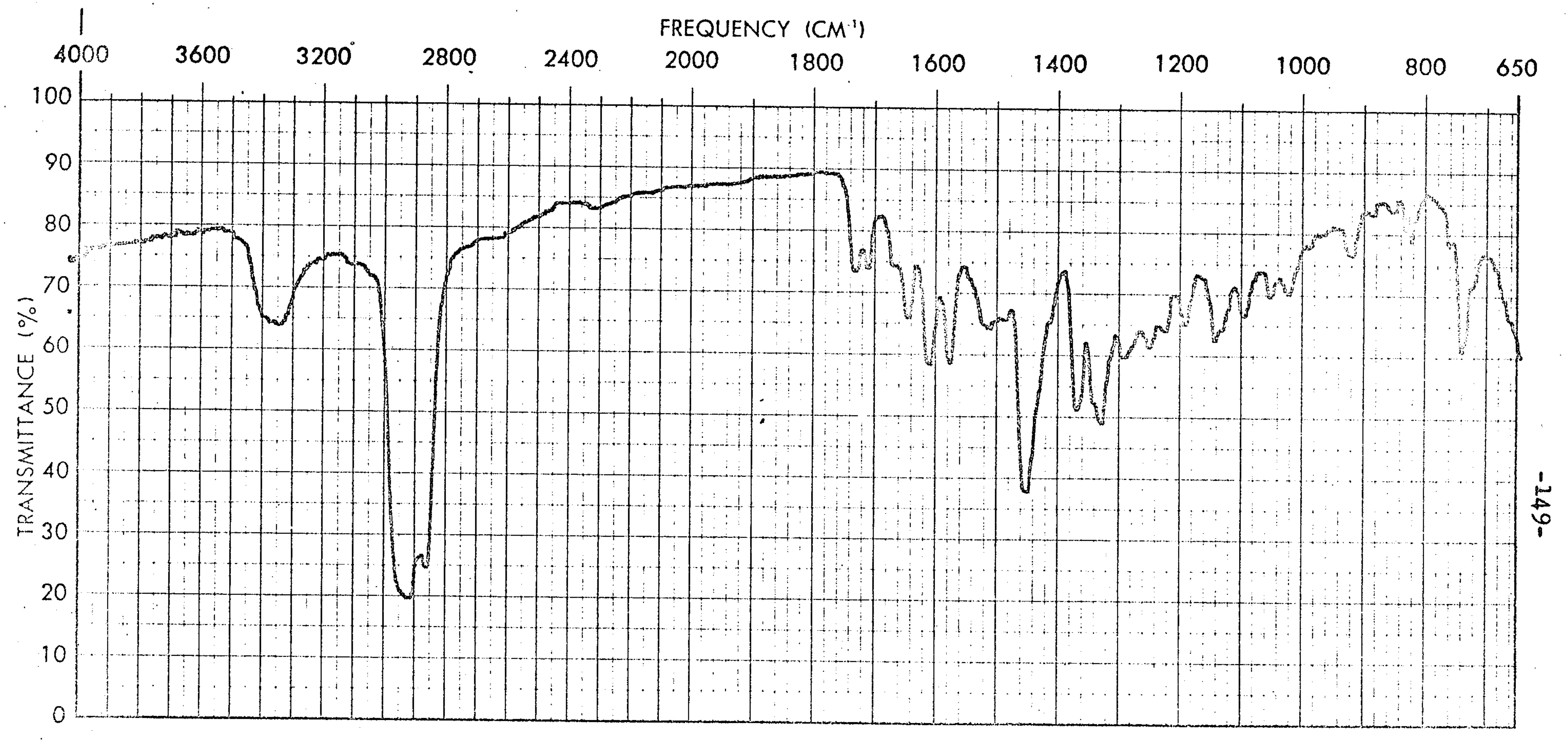


Spectrum No. 29

2,4-Dinitrophenyl-L-tryptophyl-glycine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(C_9H_8N)CONHCH(H)COOC_2H_5$

3360	SSh	1415	SSh	1156	SSh	1020	SSh	755	SSh
1760	SSh	1350	SSh	1138	SSh	968	MB	742	SSh
1690	SSh	1330	SSh	1125	SSh	926	MSh	718	MSh
1625	SSh	1300	SSh	1113	SSh	914	MSh	690	MSh
1590	SSh	1280	SSh	1095	SSh	856	WSh	662	SSh
1560	SSh	1260	SSh	1078	SSh	830	WSh	650	SB
1530	SSh	1235	SSh	1052	SSh	816	MSh		
1495	SSh	1192	SSh	1035	SSh	802	WSh		

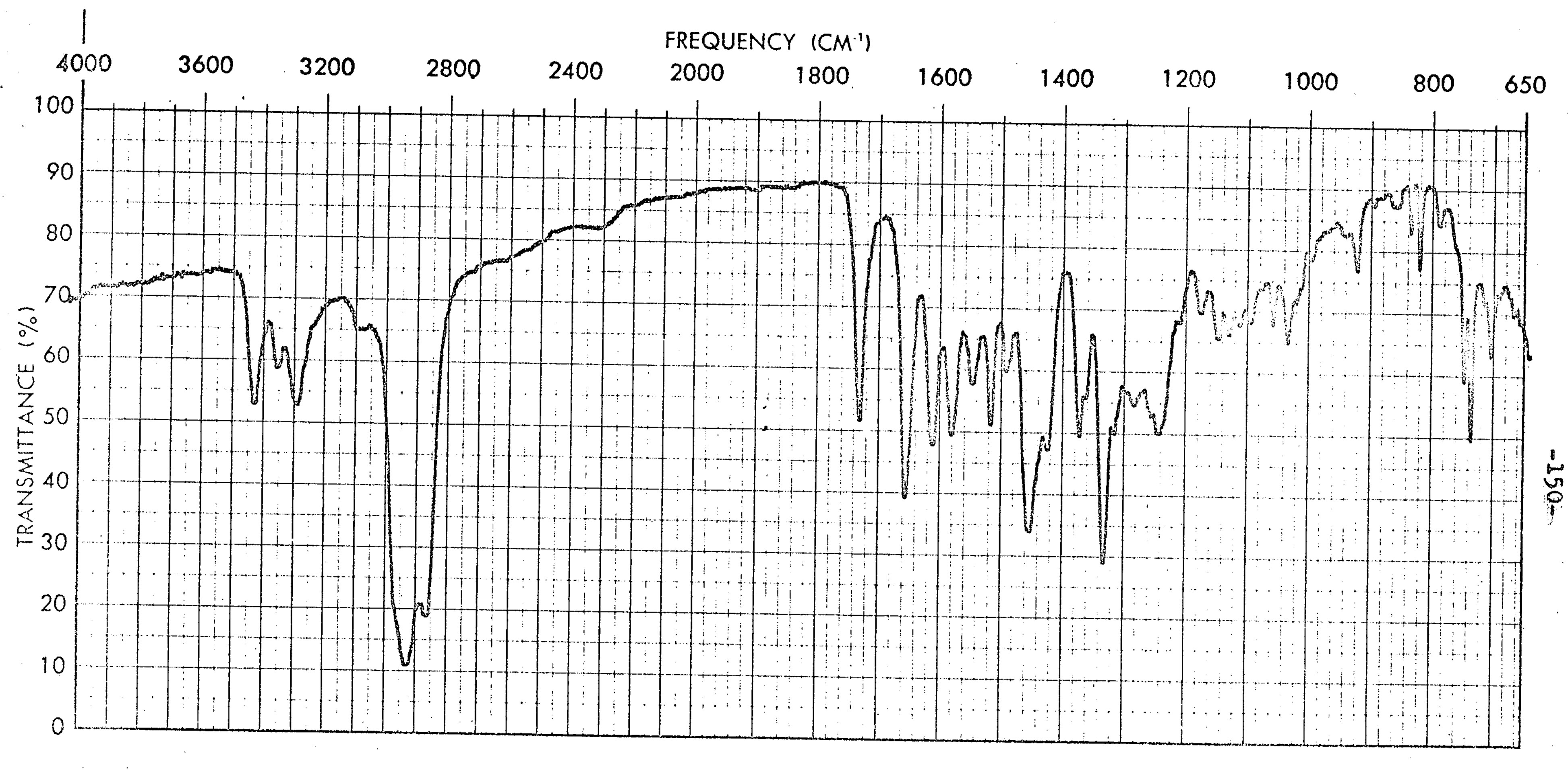


Spectrum No. 30

2,4-Dinitrophenyl-L-tryptophyl-
L-leucine ethyl ester

$$O_2N-C_6H_3(NO_2)-NH\underset{C_9H_8N}{CH}CONH\underset{C_4H_9}{CH}COOC_2H_5$$

3370 SSh	1520 SSh	1200 SB	890 WSh
3330 SSh	1490 SSh	1146 SSh	836 WSh
1745 SSh	1410 SSh	1132 SSh	825 WSh
1720 SSh	1365 SSh	1100 SB	762 WSh
1680 SSh	1340 SSh	1055 SSh	755 SSh
1655 SSh	1300 SSh	1026 SSh	740 SSh
1620 SSh	1250 SSh	930 MSh	
1585 SSh	1225 SB	920 WSh	

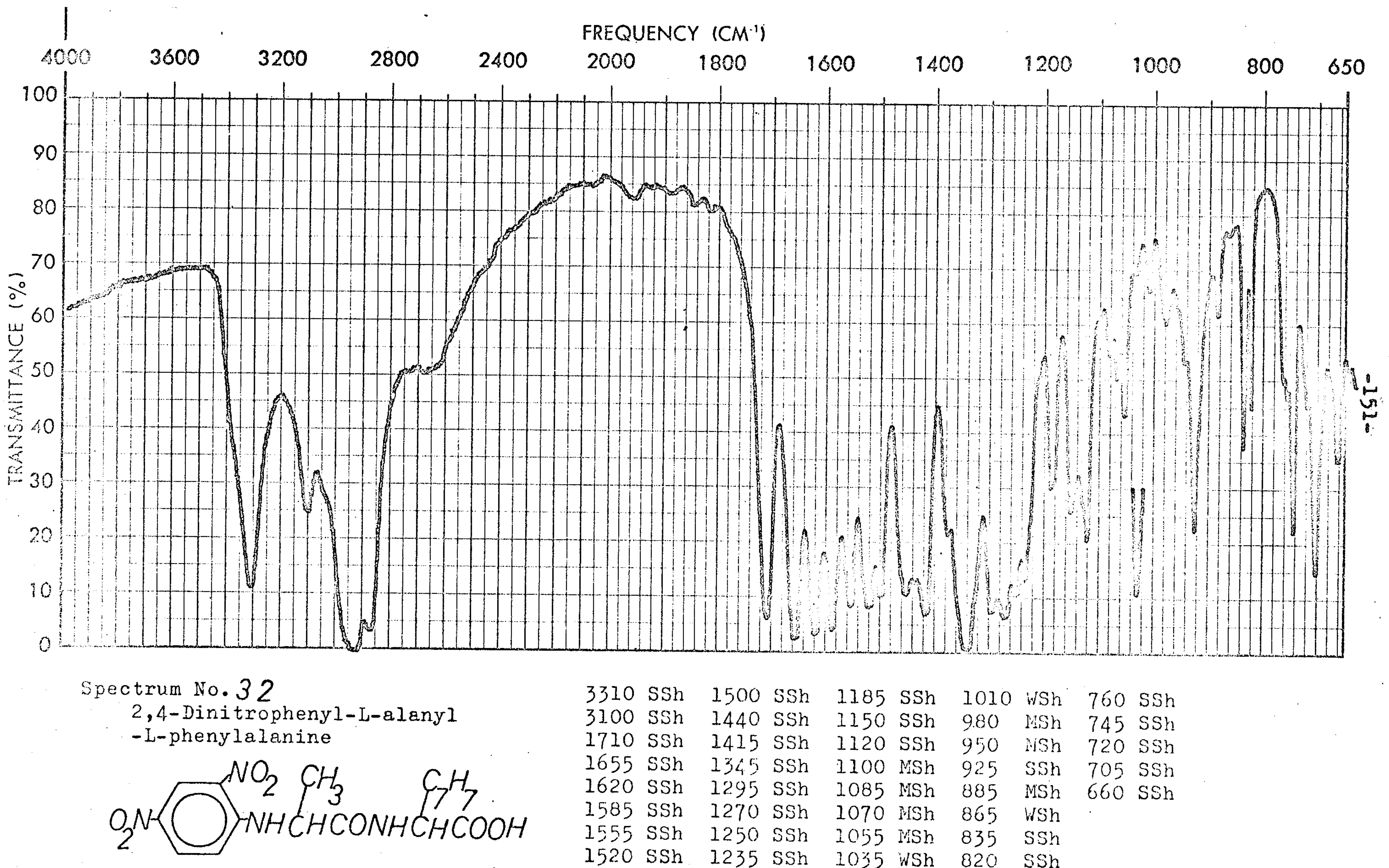


Spectrum No. 31

2,4-Dinitrophenyl-L-tryptophyl-L-phenylalanine ethyl ester

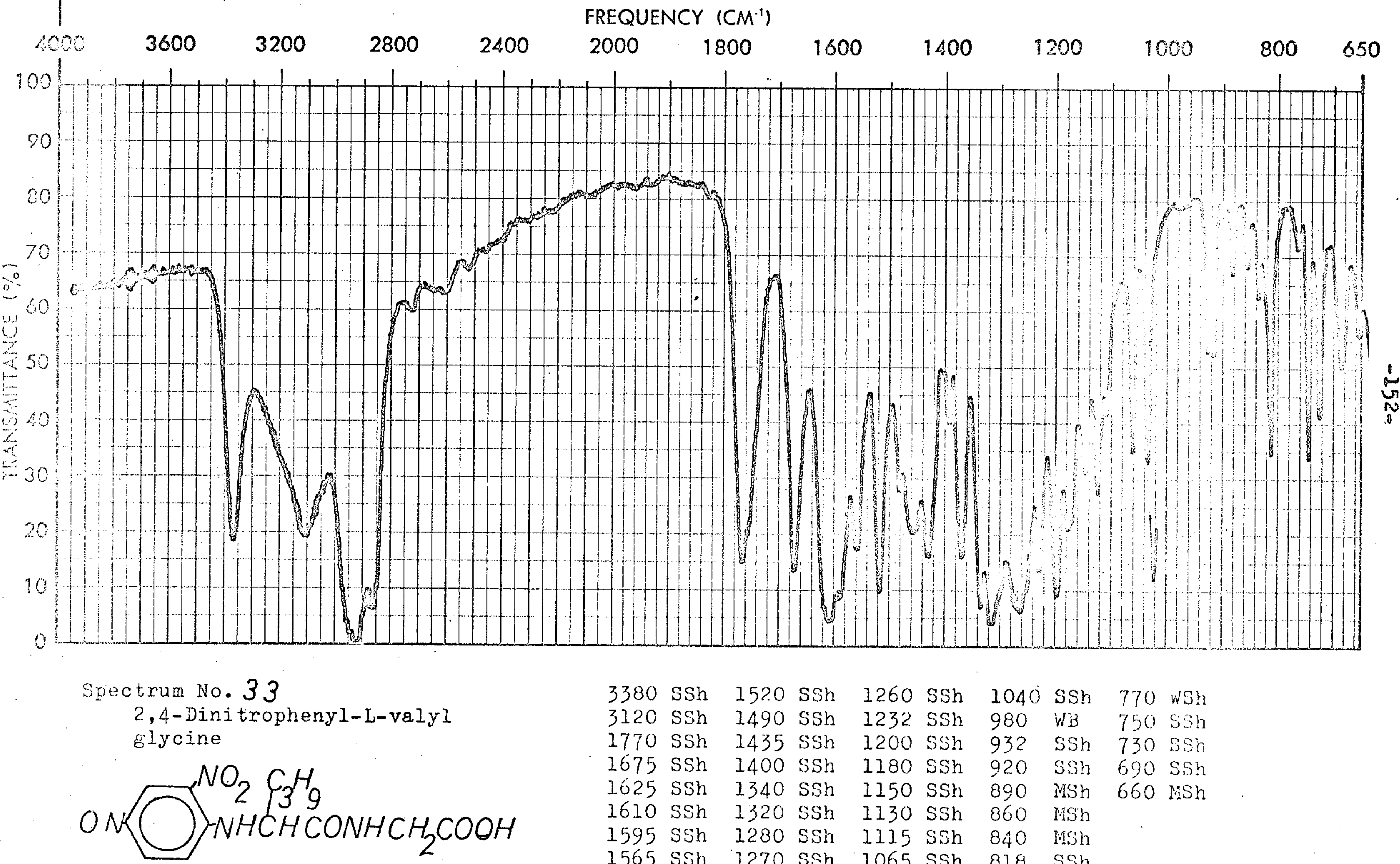
$O_2N-C_6H_3(NO_2)-NHCH(C_9H_8N)CONHCH(C_7H_7)COOC_2H_5$

3430	SSh	1500	SSh	1210	MSh	1020	MSh	786	WSh
3360	SSh	1425	SSh	1178	MSh	1010	MSh	756	SSh
3295	SSh	1360	SSh	1150	SSh	995	MSh	749	SSh
1735	SSh	1335	SSh	1130	SSh	942	MSh	715	MSh
1665	SSh	1320	SSh	1110	SSh	920	MSh	705	SSh
1625	SSh	1300	SSh	1090	SSh	892	MSh	665	MSh
1590	SSh	1285	SSh	1075	MSh	860	WB	660	MSh
1550	SSh	1260	SSh	1060	SSh	835	MSh	650	MSh
1525	SSh	1245	SSh	1032	SSh	820	MSh	640	MSh



Spectrum No. 32
2,4-Dinitrophenyl-L-alanyl
-L-phenylalanine

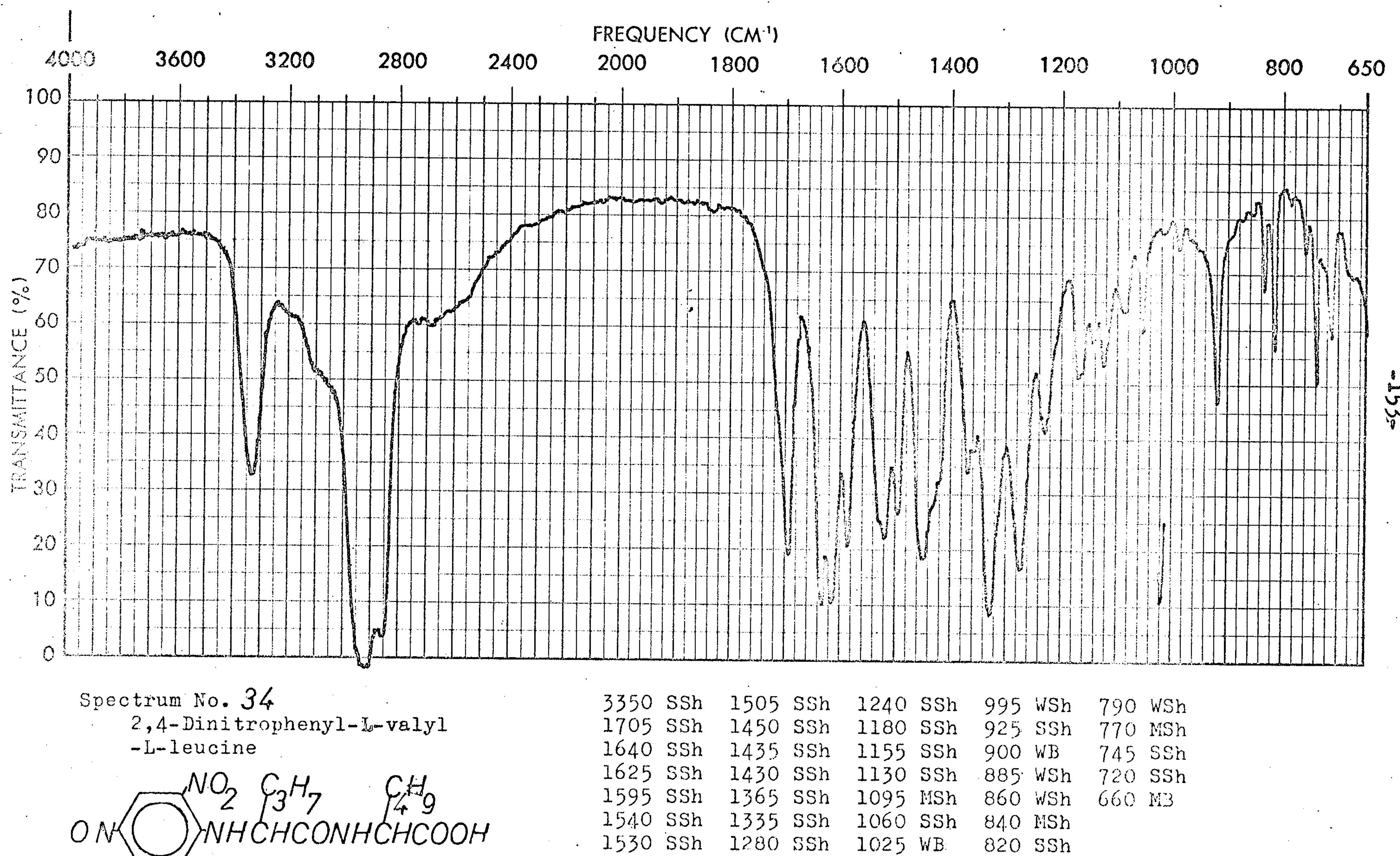
3310	SSh	1500	SSh	1185	SSh	1010	WSh	760	SSh
3100	SSh	1440	SSh	1150	SSh	980	MSh	745	SSh
1710	SSh	1415	SSh	1120	SSh	950	MSh	720	SSh
1655	SSh	1345	SSh	1100	MSh	925	SSh	705	SSh
1620	SSh	1295	SSh	1085	MSh	885	MSh	660	SSh
1585	SSh	1270	SSh	1070	MSh	865	WSh		
1555	SSh	1250	SSh	1055	MSh	835	SSh		
1520	SSh	1235	SSh	1035	WSh	820	SSh		



Spectrum No. 33
2,4-Dinitrophenyl-L-valyl glycine

$O_2N-C_6H_3(NO_2)-NHCH(C_3H_9)CONHCH_2COOH$

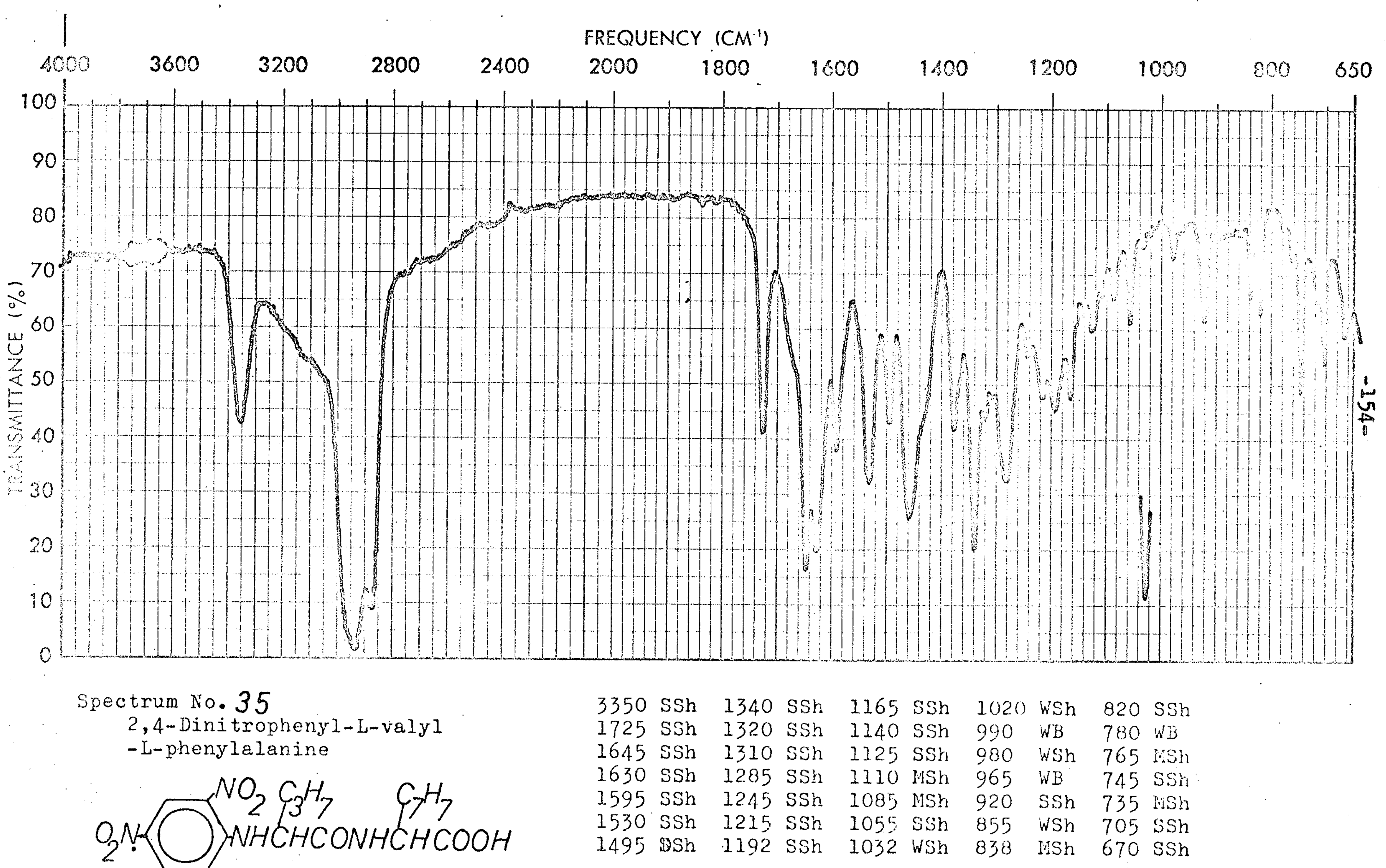
3380	SSh	1520	SSh	1260	SSh	1040	SSh	770	WSh
3120	SSh	1490	SSh	1232	SSh	980	WB	750	SSh
1770	SSh	1435	SSh	1200	SSh	932	SSh	730	SSh
1675	SSh	1400	SSh	1180	SSh	920	SSh	690	SSh
1625	SSh	1340	SSh	1150	SSh	890	MSh	660	MSh
1610	SSh	1320	SSh	1130	SSh	860	MSh		
1595	SSh	1280	SSh	1115	SSh	840	MSh		
1565	SSh	1270	SSh	1065	SSh	818	SSh		



Spectrum No. 34
2,4-Dinitrophenyl-L-valyl
-L-leucine

$O_2N-C_6H_3(NO_2)-NH\,CH(C_3H_7)CONH\,CH(C_4H_9)COOH$

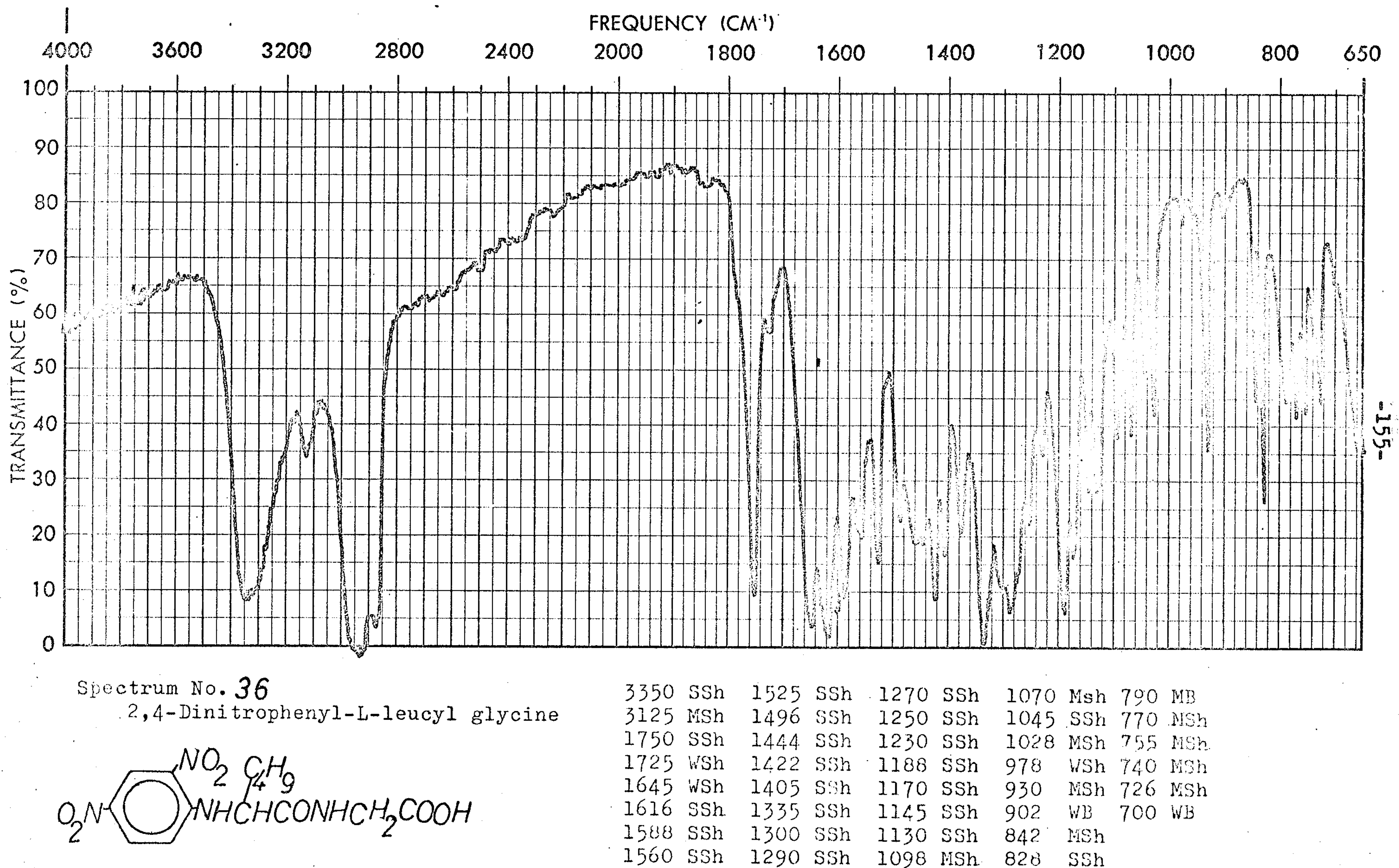
3350 SSh	1505 SSh	1240 SSh	995 WSh	790 WSh
1705 SSh	1450 SSh	1180 SSh	925 SSh	770 MSh
1640 SSh	1435 SSh	1155 SSh	900 WB	745 SSh
1625 SSh	1430 SSh	1130 SSh	885 WSh	720 SSh
1595 SSh	1365 SSh	1095 MSh	860 WSh	660 MB
1540 SSh	1335 SSh	1060 SSh	840 MSh	
1530 SSh	1280 SSh	1025 WB	820 SSh	



Spectrum No. 35
2,4-Dinitrophenyl-L-valyl
-L-phenylalanine

O_2N–C₆H₃(NO_2)–NHCH(C_3H_7)CONHCH(C_7H_7)COOH

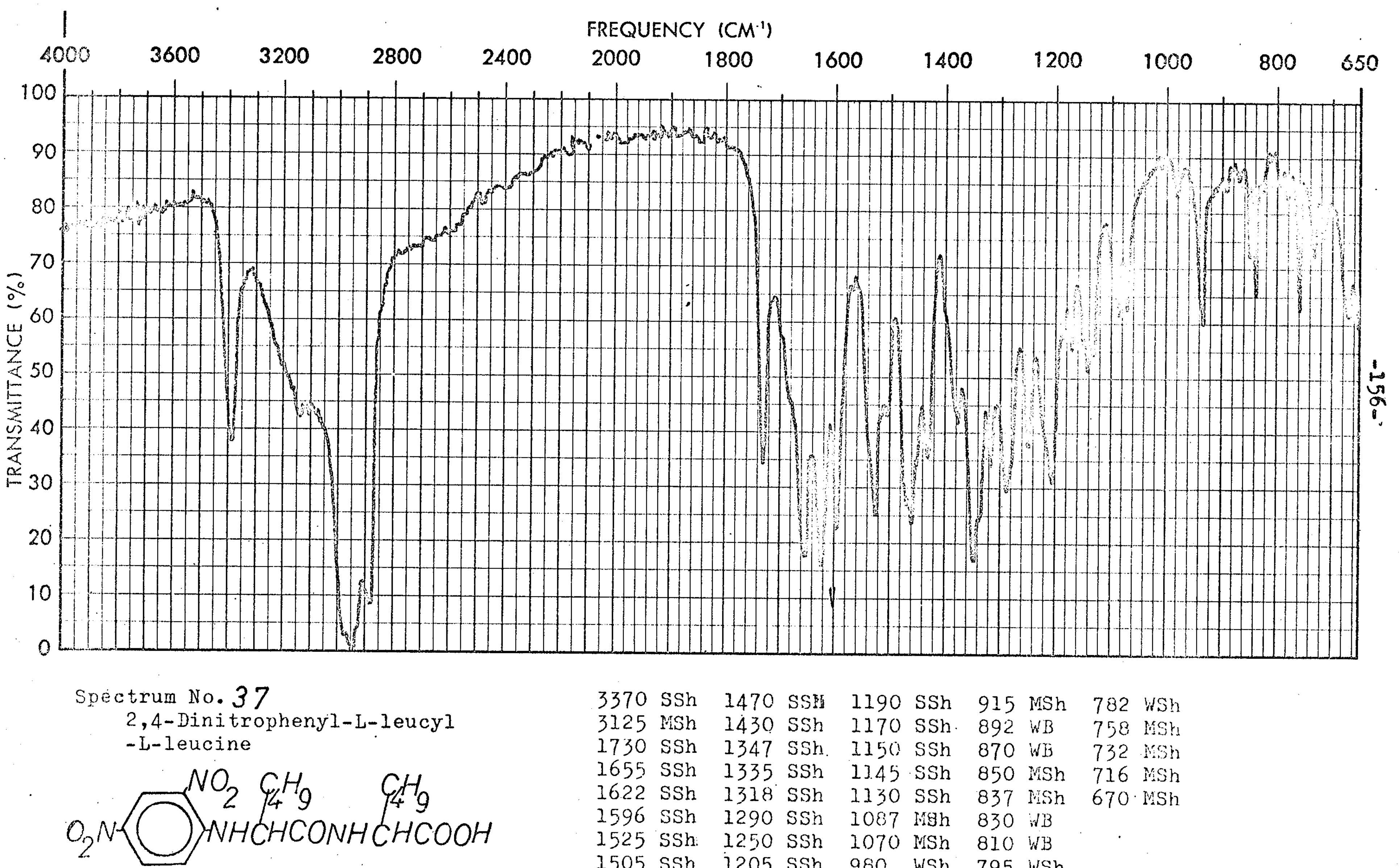
3350	SSh	1340	SSh	1165	SSh	1020	WSh	820	SSh
1725	SSh	1320	SSh	1140	SSh	990	WB	780	WB
1645	SSh	1310	SSh	1125	SSh	980	WSh	765	MSh
1630	SSh	1285	SSh	1110	MSh	965	WB	745	SSh
1595	SSh	1245	SSh	1085	MSh	920	SSh	735	MSh
1530	SSh	1215	SSh	1055	SSh	855	WSh	705	SSh
1495	SSh	1192	SSh	1032	WSh	838	MSh	670	SSh



Spectrum No. 36
2,4-Dinitrophenyl-L-leucyl glycine

O_2N-C_6H_3(NO_2)-$NHCH(C_4H_9)CONHCH_2COOH$

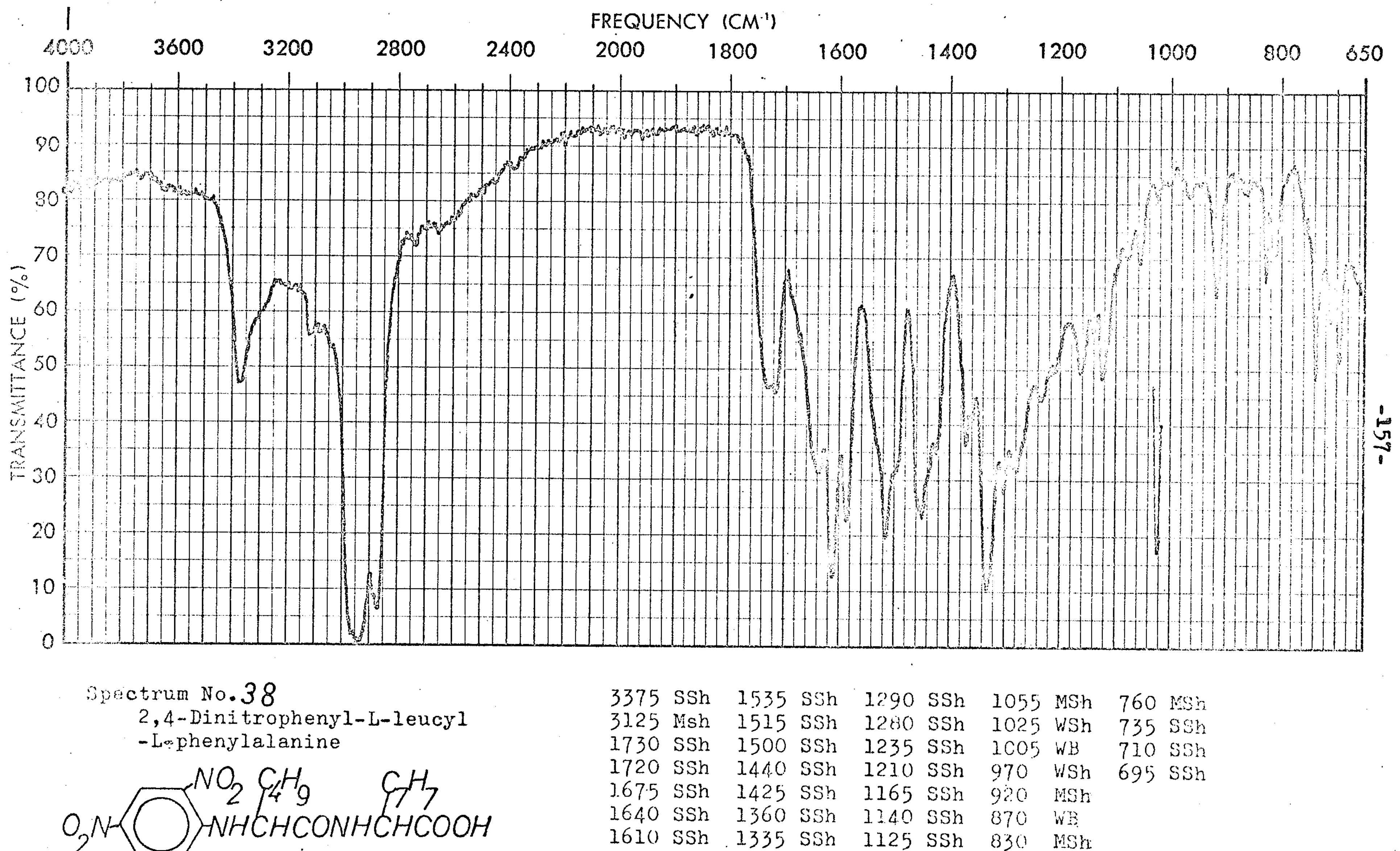
3350	SSh	1525	SSh	1270	SSh	1070	Msh	790	MB
3125	MSh	1496	SSh	1250	SSh	1045	SSh	770	MSh
1750	SSh	1444	SSh	1230	SSh	1028	MSh	755	MSh
1725	WSh	1422	SSh	1188	SSh	978	WSh	740	MSh
1645	WSh	1405	SSh	1170	SSh	930	MSh	726	MSh
1616	SSh	1335	SSh	1145	SSh	902	WB	700	WB
1588	SSh	1300	SSh	1130	SSh	842	MSh		
1560	SSh	1290	SSh	1098	MSh	828	SSh		



Spectrum No. 37
2,4-Dinitrophenyl-L-leucyl
-L-leucine

O_2N-C$_6$H$_3$(NO_2)-NHCH(C_4H_9)CONHCH(C_4H_9)COOH

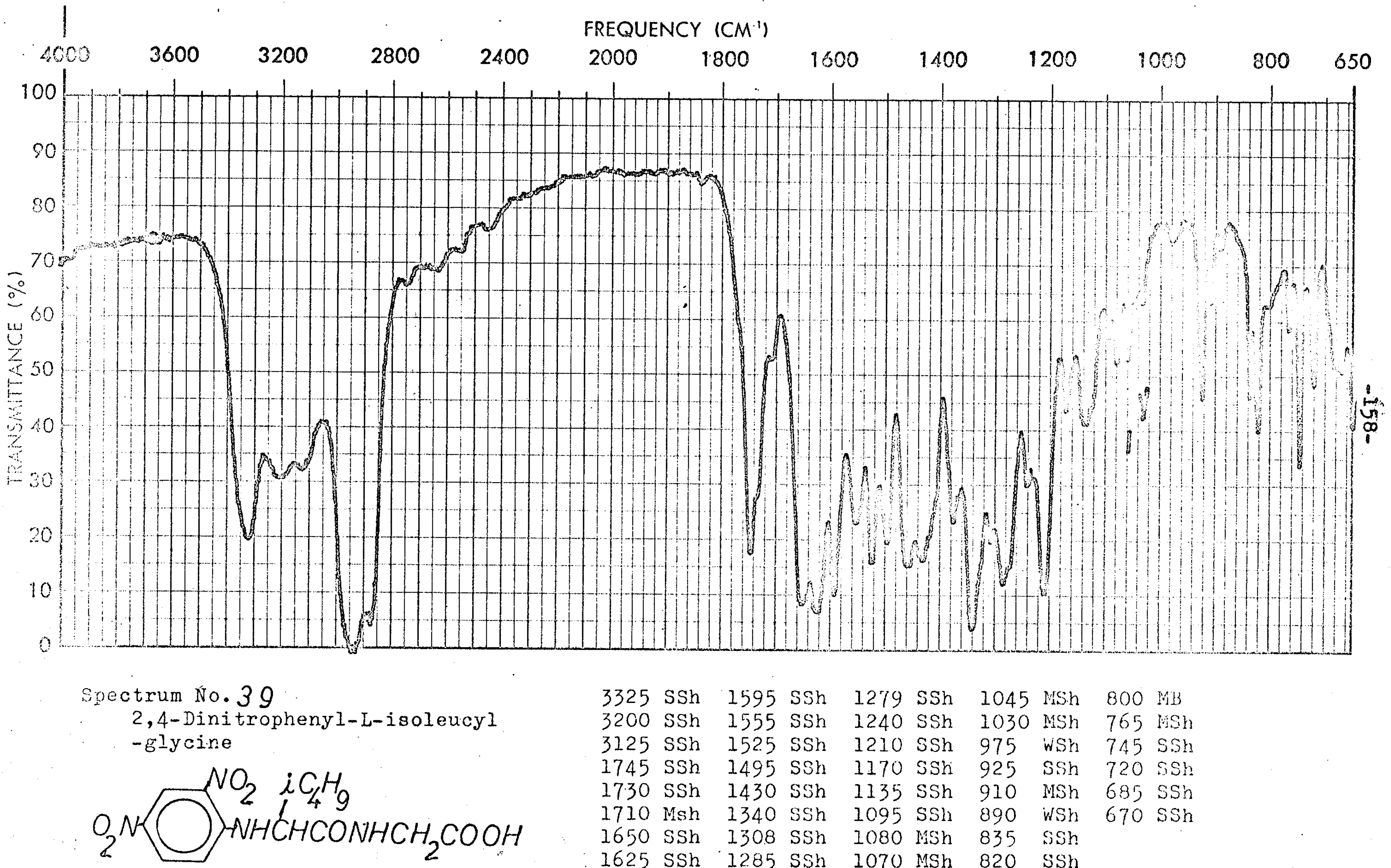
3370 SSh	1470 SSM	1190 SSh	915 MSh	782 WSh
3125 MSh	1430 SSh	1170 SSh	892 WB	758 MSh
1730 SSh	1347 SSh	1150 SSh	870 WB	732 MSh
1655 SSh	1335 SSh	1145 SSh	850 MSh	716 MSh
1622 SSh	1318 SSh	1130 SSh	837 MSh	670 MSh
1596 SSh	1290 SSh	1087 MSh	830 WB	
1525 SSh	1250 SSh	1070 MSh	810 WB	
1505 SSh	1205 SSh	980 WSh	795 WSh	



Spectrum No. 38
2,4-Dinitrophenyl-L-leucyl
-L-phenylalanine

$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONHCH(C_7H_7)COOH$

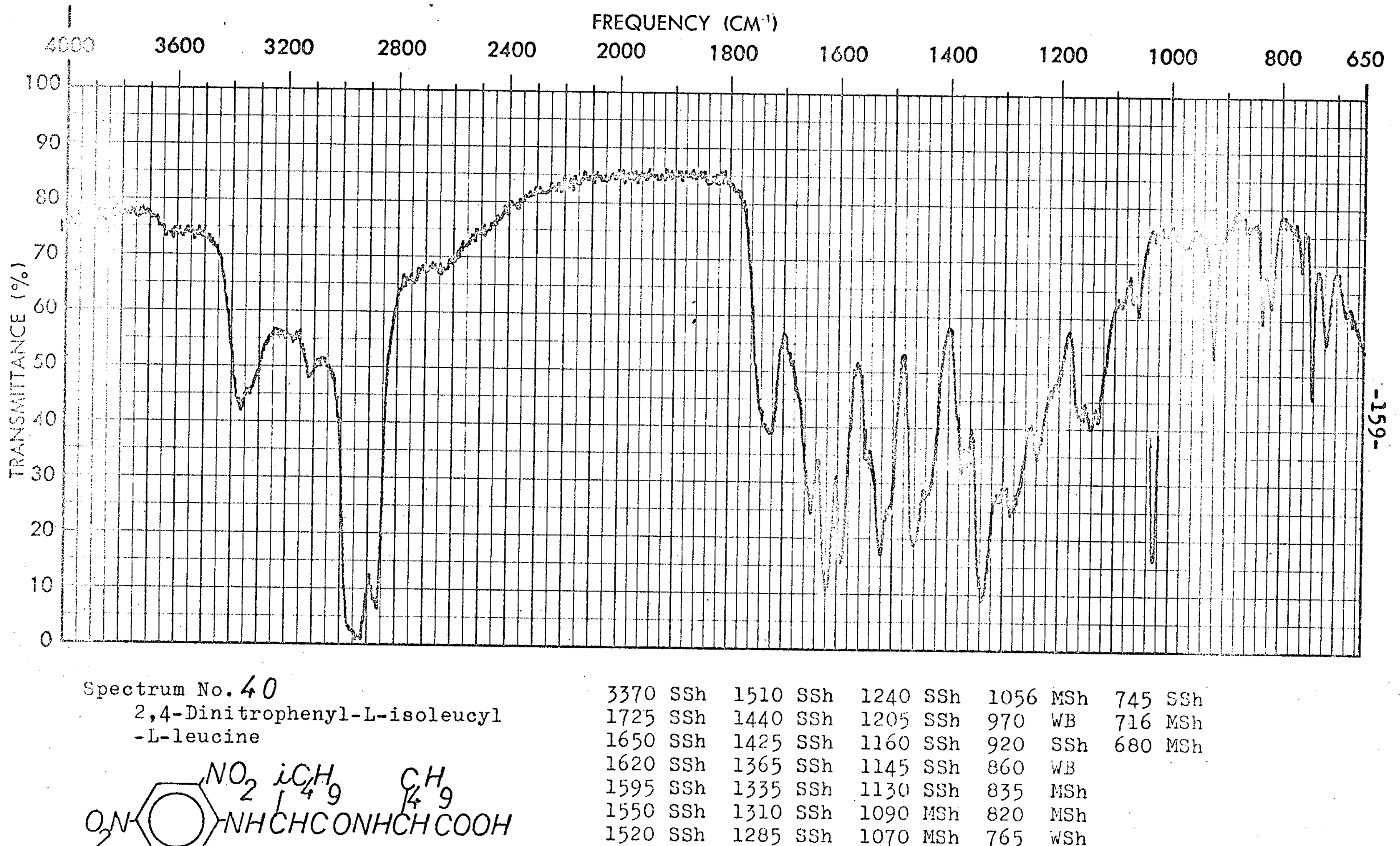
3375 SSh	1535 SSh	1290 SSh	1055 MSh	760 MSh
3125 Msh	1515 SSh	1280 SSh	1025 WSh	735 SSh
1730 SSh	1500 SSh	1235 SSh	1005 WB	710 SSh
1720 SSh	1440 SSh	1210 SSh	970 WSh	695 SSh
1675 SSh	1425 SSh	1165 SSh	920 MSh	
1640 SSh	1360 SSh	1140 SSh	870 WB	
1610 SSh	1335 SSh	1125 SSh	830 MSh	
1585 SSh	1300 SSh	1080 MSh	810 MSh	



Spectrum No. 39
2,4-Dinitrophenyl-L-isoleucyl-glycine

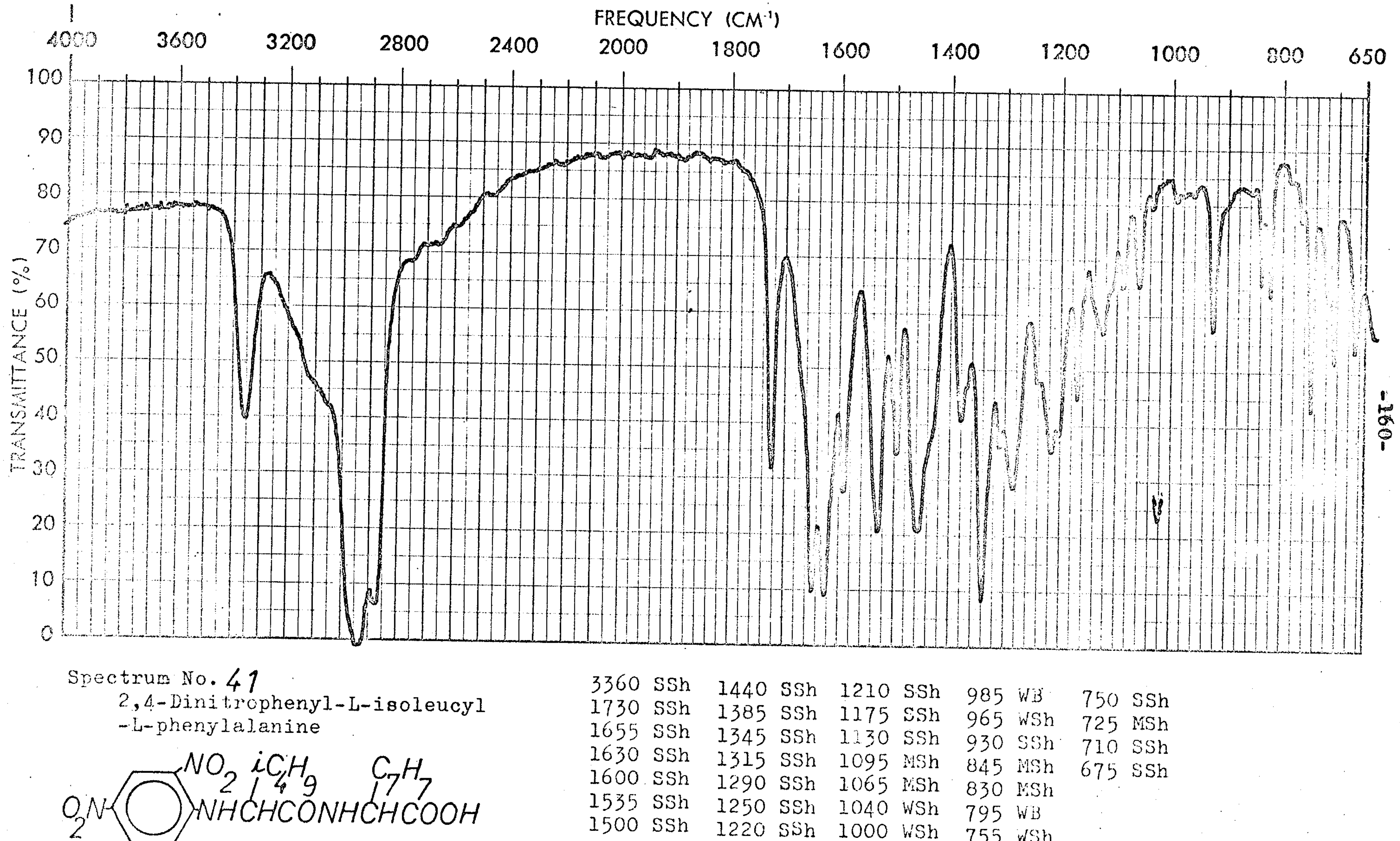
$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH_2COOH$

3325 SSh	1595 SSh	1279 SSh	1045 MSh	800 MB
3200 SSh	1555 SSh	1240 SSh	1030 MSh	765 MSh
3125 SSh	1525 SSh	1210 SSh	975 WSh	745 SSh
1745 SSh	1495 SSh	1170 SSh	925 SSh	720 SSh
1730 SSh	1430 SSh	1135 SSh	910 MSh	685 SSh
1710 Msh	1340 SSh	1095 SSh	890 WSh	670 SSh
1650 SSh	1308 SSh	1080 MSh	835 SSh	
1625 SSh	1285 SSh	1070 MSh	820 SSh	



Spectrum No. 40
2,4-Dinitrophenyl-L-isoleucyl-L-leucine

3370	SSh	1510	SSh	1240	SSh	1056	MSh	745	SSh
1725	SSh	1440	SSh	1205	SSh	970	WB	716	MSh
1650	SSh	1425	SSh	1160	SSh	920	SSh	680	MSh
1620	SSh	1365	SSh	1145	SSh	860	WB		
1595	SSh	1335	SSh	1130	SSh	835	MSh		
1550	SSh	1310	SSh	1090	MSh	820	MSh		
1520	SSh	1285	SSh	1070	MSh	765	WSh		

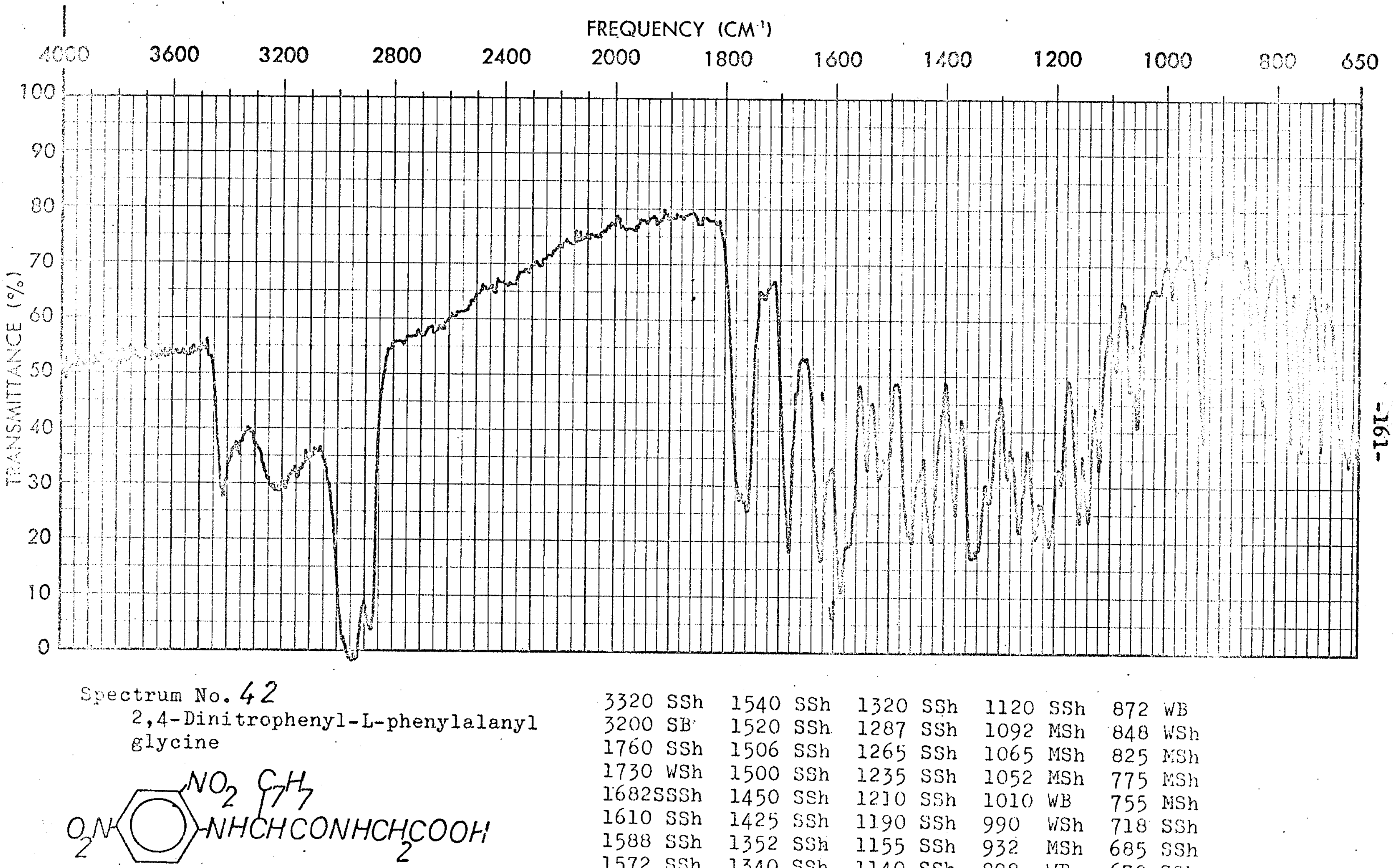


Spectrum No. 41

2,4-Dinitrophenyl-L-isoleucyl
-L-phenylalanine

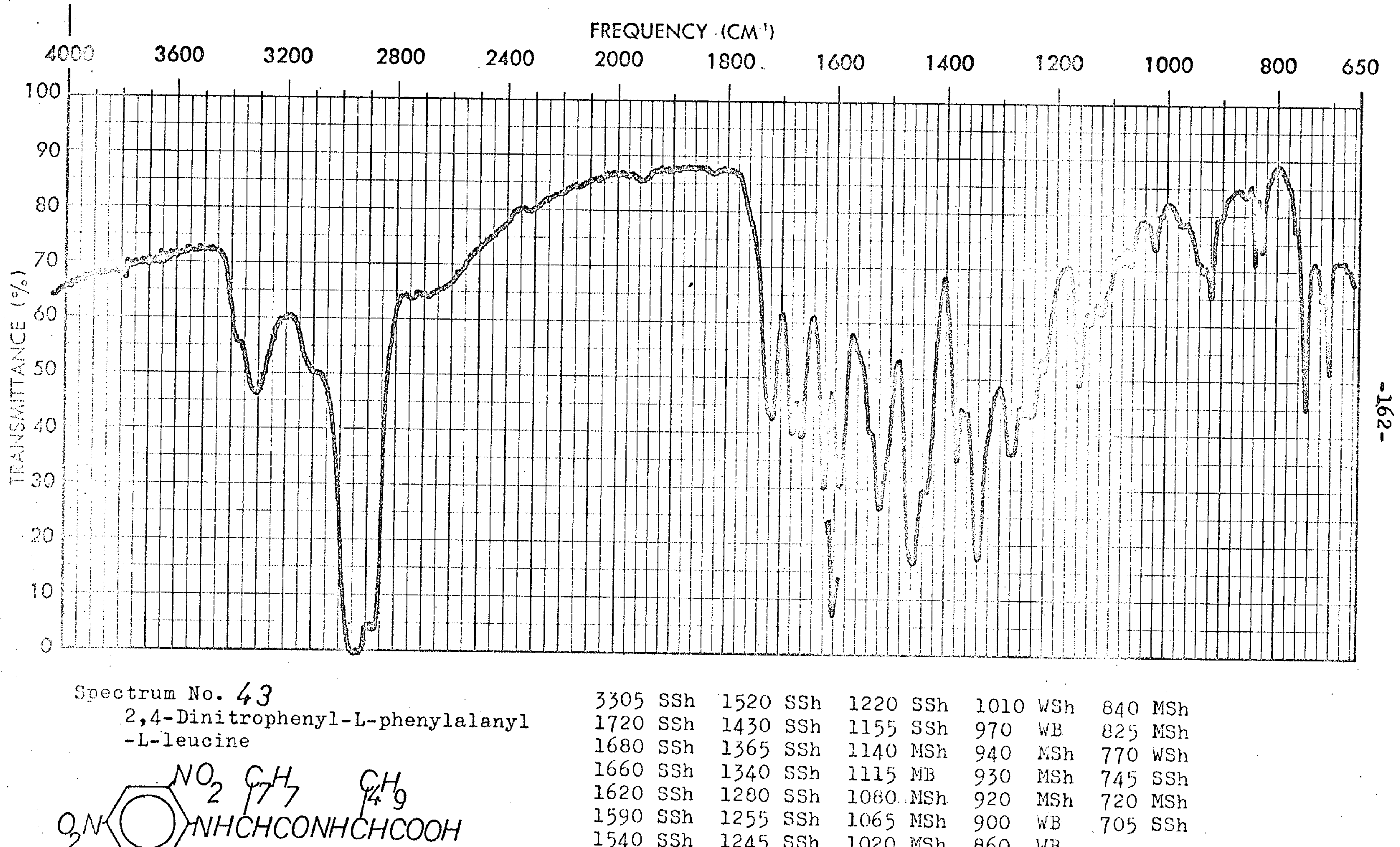
$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH(C_7H_7)COOH$

3360 SSh	1440 SSh	1210 SSh	985 WB	750 SSh
1730 SSh	1385 SSh	1175 SSh	965 WSh	725 MSh
1655 SSh	1345 SSh	1130 SSh	930 SSh	710 SSh
1630 SSh	1315 SSh	1095 MSh	845 MSh	675 SSh
1600 SSh	1290 SSh	1065 MSh	830 MSh	
1535 SSh	1250 SSh	1040 WSh	795 WB	
1500 SSh	1220 SSh	1000 WSh	755 WSh	



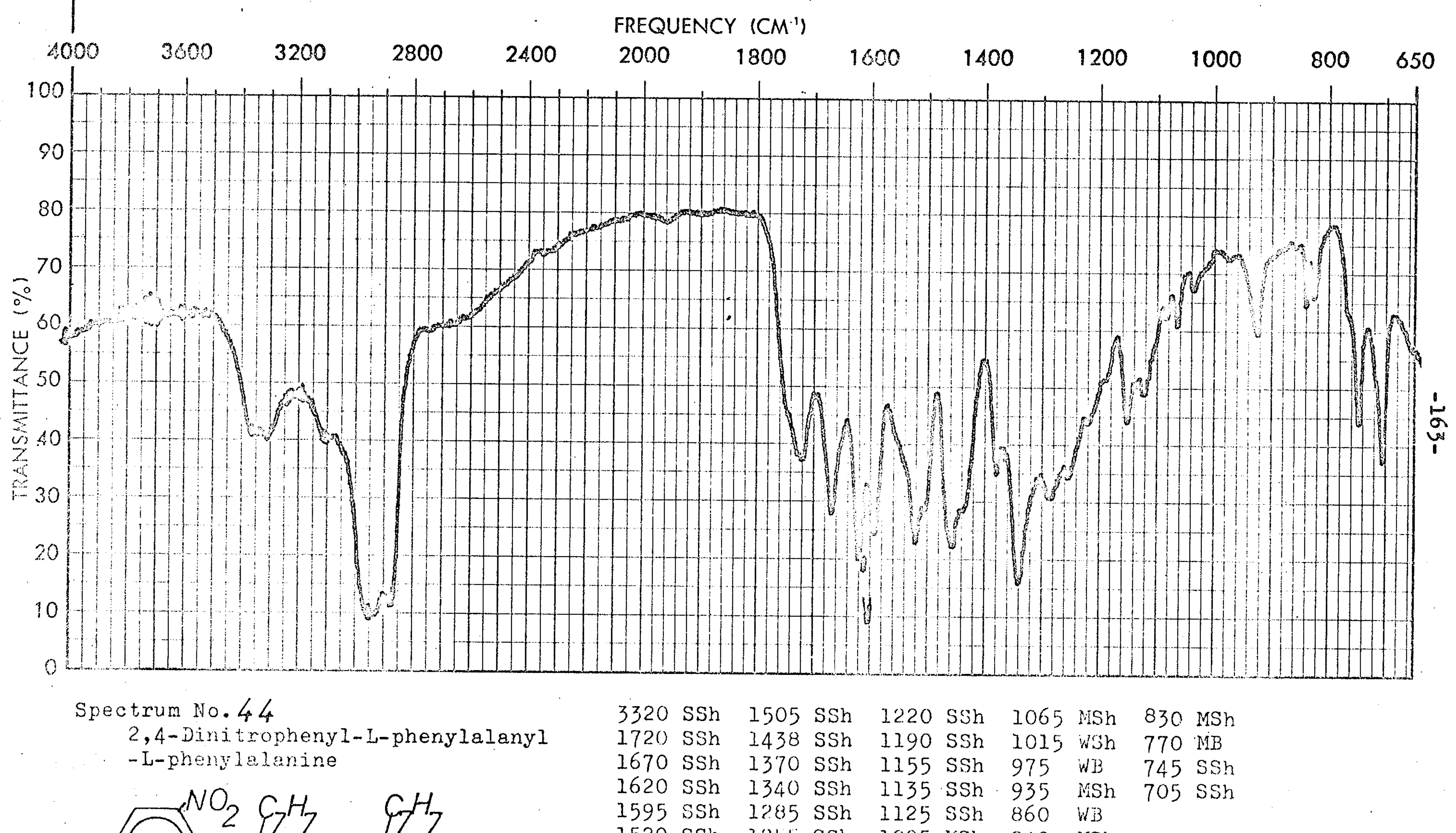
Spectrum No. 42
2,4-Dinitrophenyl-L-phenylalanyl glycine

3320	SSh	1540	SSh	1320	SSh	1120	SSh	872	WB
3200	SB	1520	SSh	1287	SSh	1092	MSh	848	WSh
1760	SSh	1506	SSh	1265	SSh	1065	MSh	825	MSh
1730	WSh	1500	SSh	1235	SSh	1052	MSh	775	MSh
1682	SSSh	1450	SSh	1210	SSh	1010	WB	755	MSh
1610	SSh	1425	SSh	1190	SSh	990	WSh	718	SSh
1588	SSh	1352	SSh	1155	SSh	932	MSh	685	SSh
1572	SSh	1340	SSh	1140	SSh	898	WB	670	SSh



Spectrum No. 43
2,4-Dinitrophenyl-L-phenylalanyl-L-leucine

3305 SSh	1520 SSh	1220 SSh	1010 WSh	840 MSh
1720 SSh	1430 SSh	1155 SSh	970 WB	825 MSh
1680 SSh	1365 SSh	1140 MSh	940 MSh	770 WSh
1660 SSh	1340 SSh	1115 MB	930 MSh	745 SSh
1620 SSh	1280 SSh	1080 MSh	920 MSh	720 MSh
1590 SSh	1255 SSh	1065 MSh	900 WB	705 SSh
1540 SSh	1245 SSh	1020 MSh	860 WB	

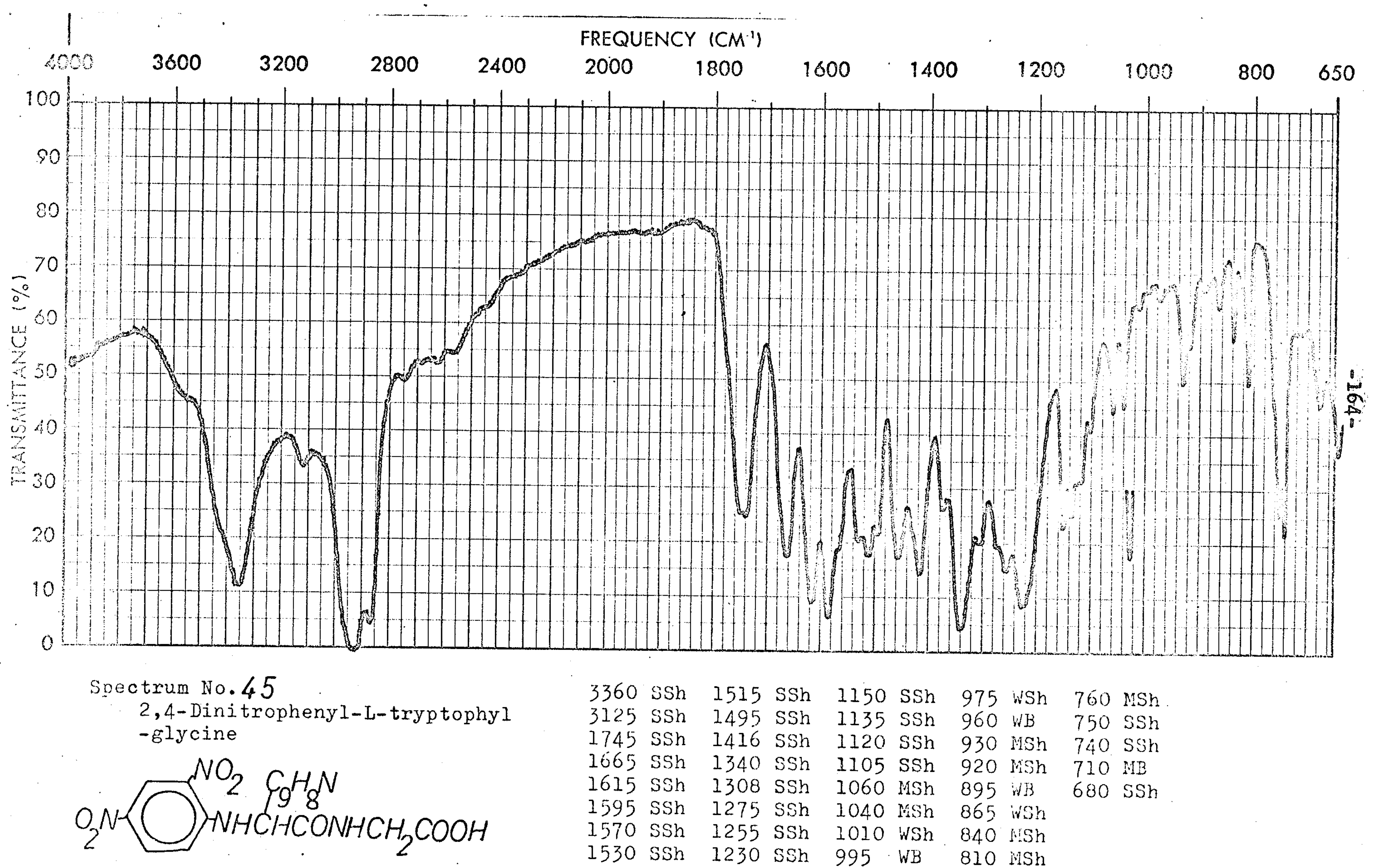


Spectrum No. 44

2,4-Dinitrophenyl-L-phenylalanyl-L-phenylalanine

$O_2N-C_6H_3(NO_2)-NHCH(C_7H_7)CONHCH(C_7H_7)COOH$

3320 SSh	1505 SSh	1220 SSh	1065 MSh	830 MSh
1720 SSh	1438 SSh	1190 SSh	1015 WSh	770 MB
1670 SSh	1370 SSh	1155 SSh	975 WB	745 SSh
1620 SSh	1340 SSh	1135 SSh	935 MSh	705 SSh
1595 SSh	1285 SSh	1125 SSh	860 WB	
1520 SSh	1255 SSh	1085 MSh	840 MSh	

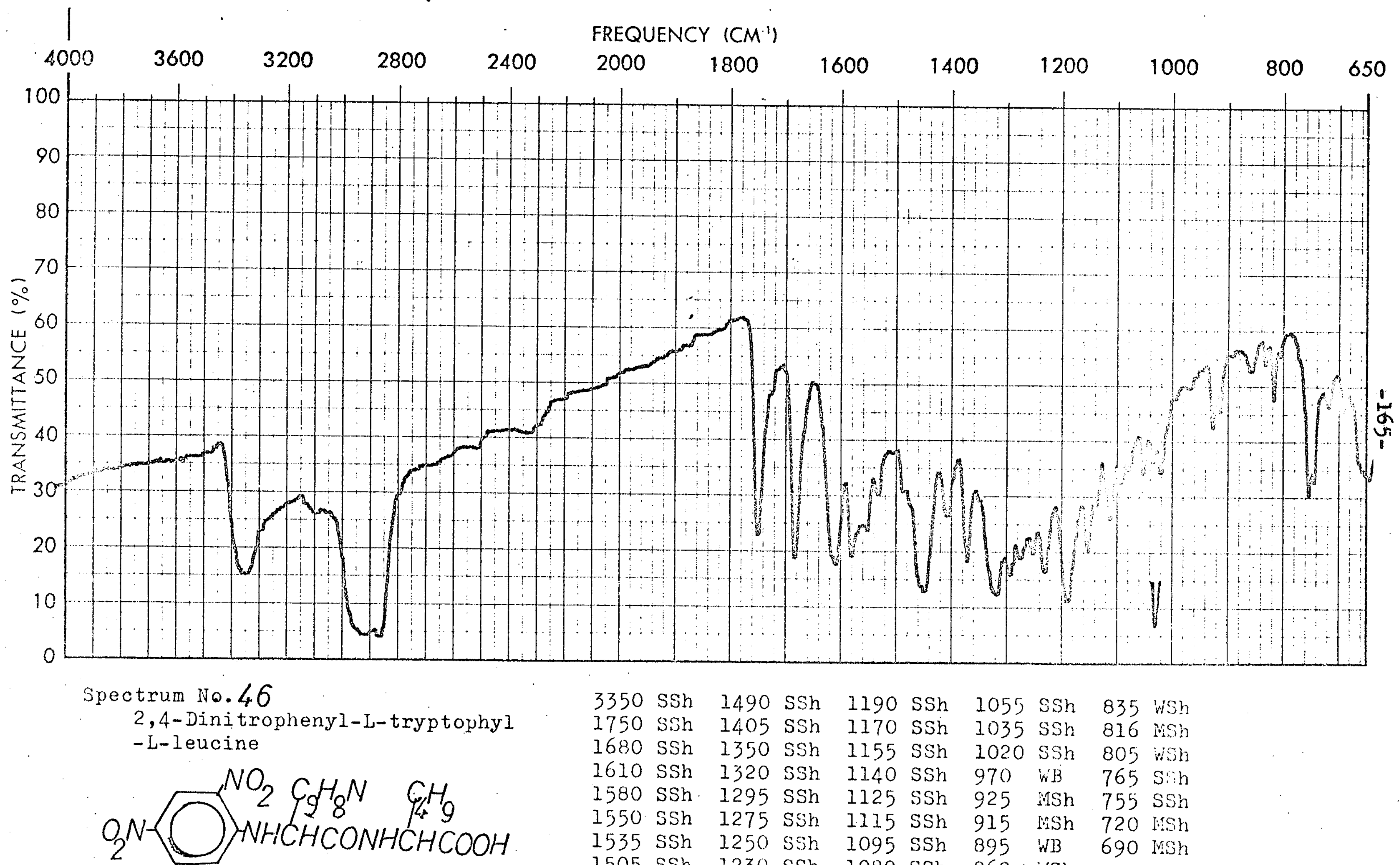


Spectrum No. 45

2,4-Dinitrophenyl-L-tryptophyl-glycine

$O_2N-C_6H_3(NO_2)-NHCH(C_9H_8N)CONHCH_2COOH$

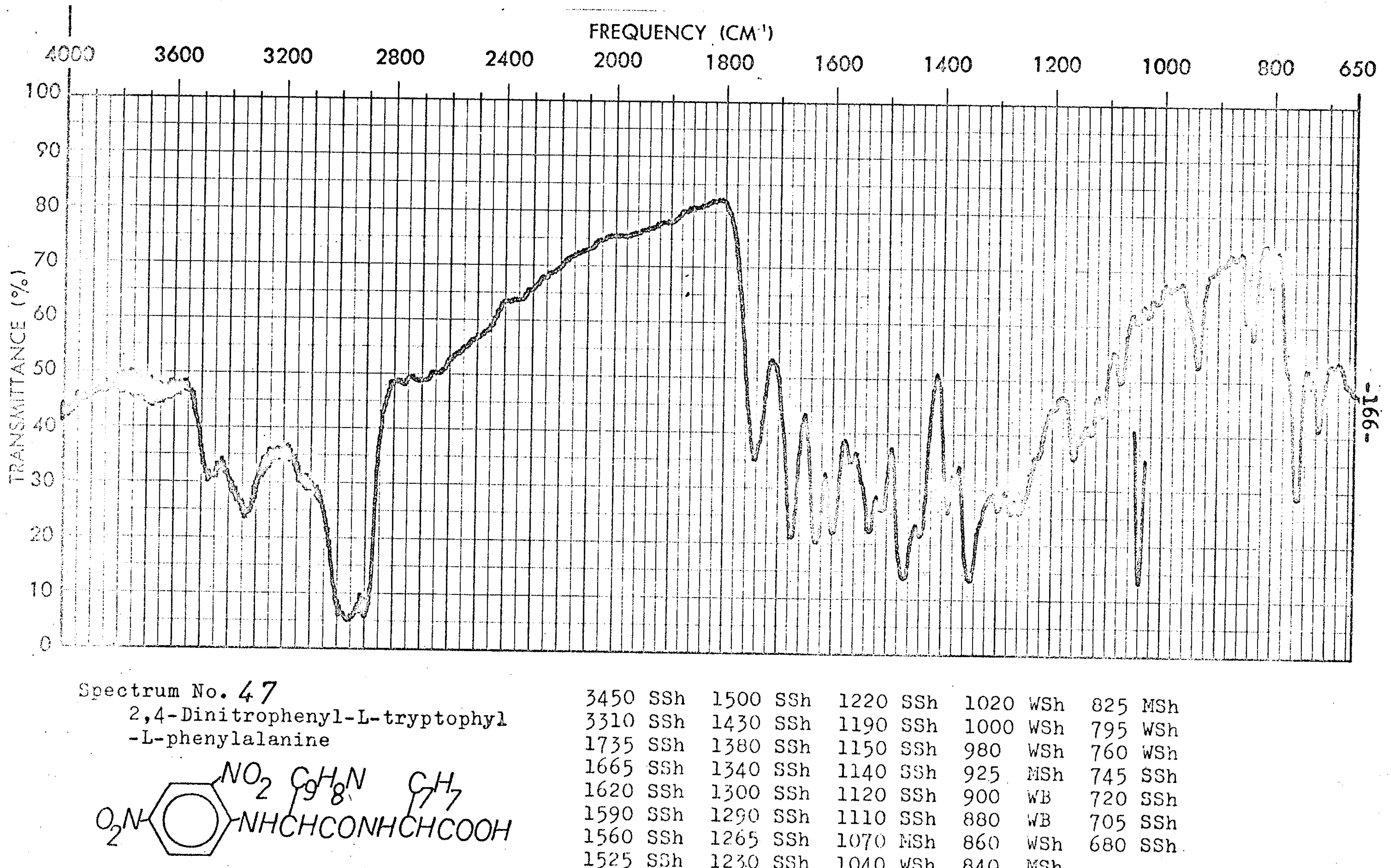
3360	SSh	1515	SSh	1150	SSh	975	WSh	760	MSh
3125	SSh	1495	SSh	1135	SSh	960	WB	750	SSh
1745	SSh	1416	SSh	1120	SSh	930	MSh	740	SSh
1665	SSh	1340	SSh	1105	SSh	920	MSh	710	MB
1615	SSh	1308	SSh	1060	MSh	895	WB	680	SSh
1595	SSh	1275	SSh	1040	MSh	865	WSh		
1570	SSh	1255	SSh	1010	WSh	840	MSh		
1530	SSh	1230	SSh	995	WB	810	MSh		



Spectrum No. 46

2,4-Dinitrophenyl-L-tryptophyl-L-leucine

3350	SSh	1490	SSh	1190	SSh	1055	SSh	835	WSh
1750	SSh	1405	SSh	1170	SSh	1035	SSh	816	MSh
1680	SSh	1350	SSh	1155	SSh	1020	SSh	805	WSh
1610	SSh	1320	SSh	1140	SSh	970	WB	765	SSh
1580	SSh	1295	SSh	1125	SSh	925	MSh	755	SSh
1550	SSh	1275	SSh	1115	SSh	915	MSh	720	MSh
1535	SSh	1250	SSh	1095	SSh	895	WB	690	MSh
1505	SSh	1230	SSh	1080	SSh	860	WSh		

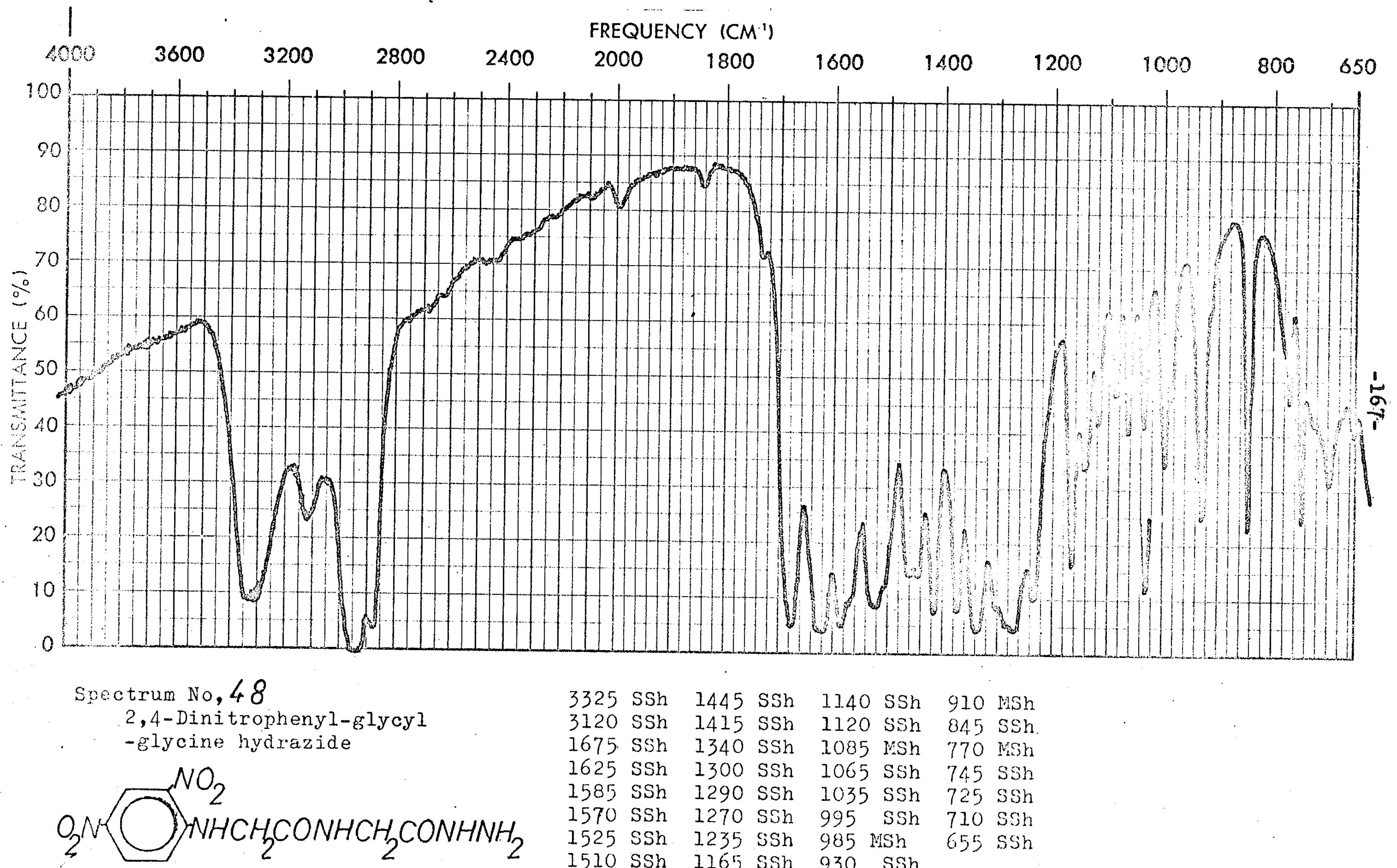


Spectrum No. 47

2,4-Dinitrophenyl-L-tryptophyl -L-phenylalanine

$O_2N-C_6H_3(NO_2)-NHCH(C_9H_8N)CONHCH(C_7H_7)COOH$

3450	SSh	1500	SSh	1220	SSh	1020	WSh	825	MSh
3310	SSh	1430	SSh	1190	SSh	1000	WSh	795	WSh
1735	SSh	1380	SSh	1150	SSh	980	WSh	760	WSh
1665	SSh	1340	SSh	1140	SSh	925	MSh	745	SSh
1620	SSh	1300	SSh	1120	SSh	900	WB	720	SSh
1590	SSh	1290	SSh	1110	SSh	880	WB	705	SSh
1560	SSh	1265	SSh	1070	MSh	860	WSh	680	SSh
1525	SSh	1230	SSh	1040	WSh	840	MSh		

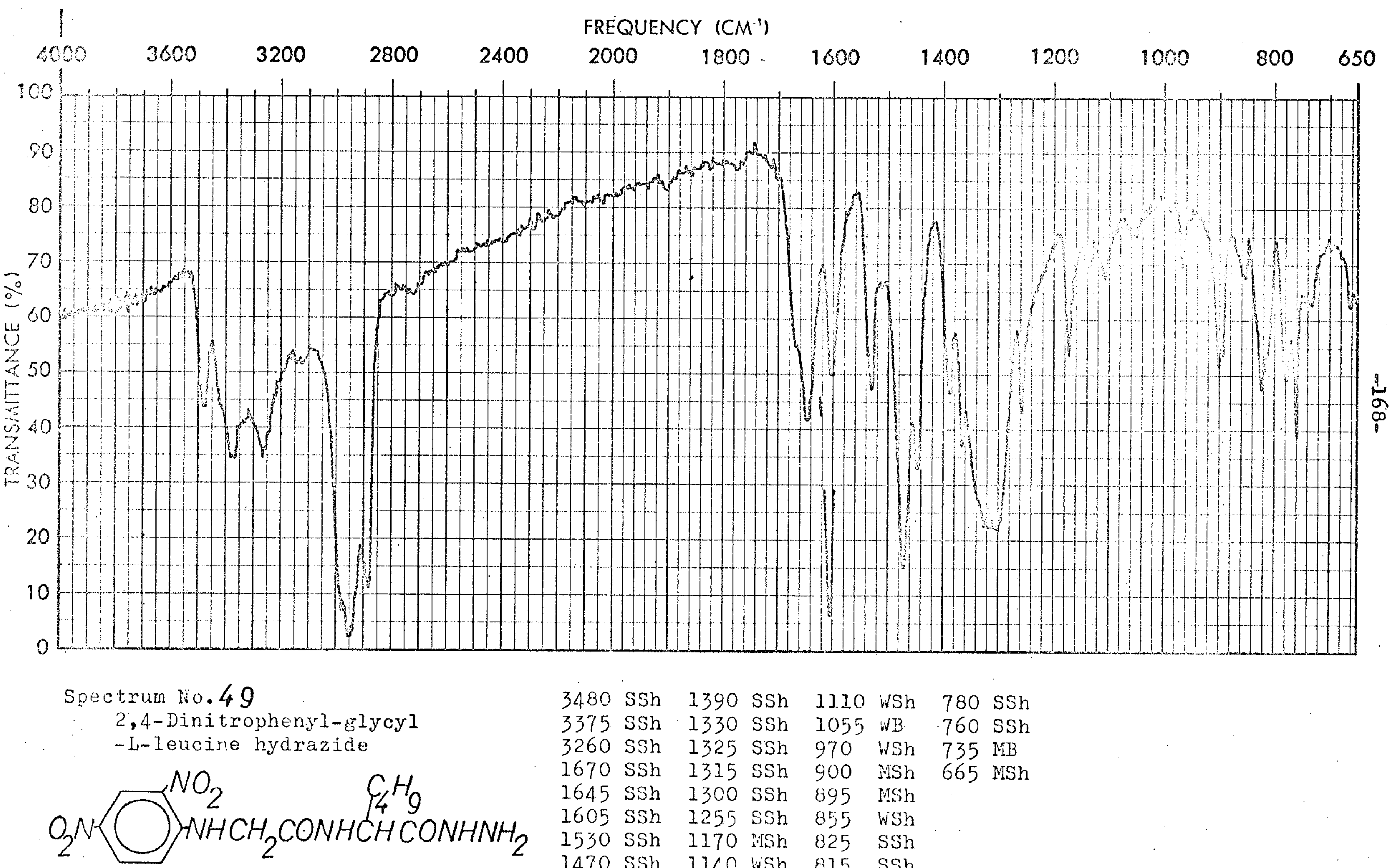


Spectrum No, 48

2,4-Dinitrophenyl-glycyl
-glycine hydrazide

$O_2N-C_6H_3(NO_2)-NHCH_2CONHCH_2CONHNH_2$

3325 SSh	1445 SSh	1140 SSh	910 MSh
3120 SSh	1415 SSh	1120 SSh	845 SSh
1675 SSh	1340 SSh	1085 MSh	770 MSh
1625 SSh	1300 SSh	1065 SSh	745 SSh
1585 SSh	1290 SSh	1035 SSh	725 SSh
1570 SSh	1270 SSh	995 SSh	710 SSh
1525 SSh	1235 SSh	985 MSh	655 SSh
1510 SSh	1165 SSh	930 SSh	

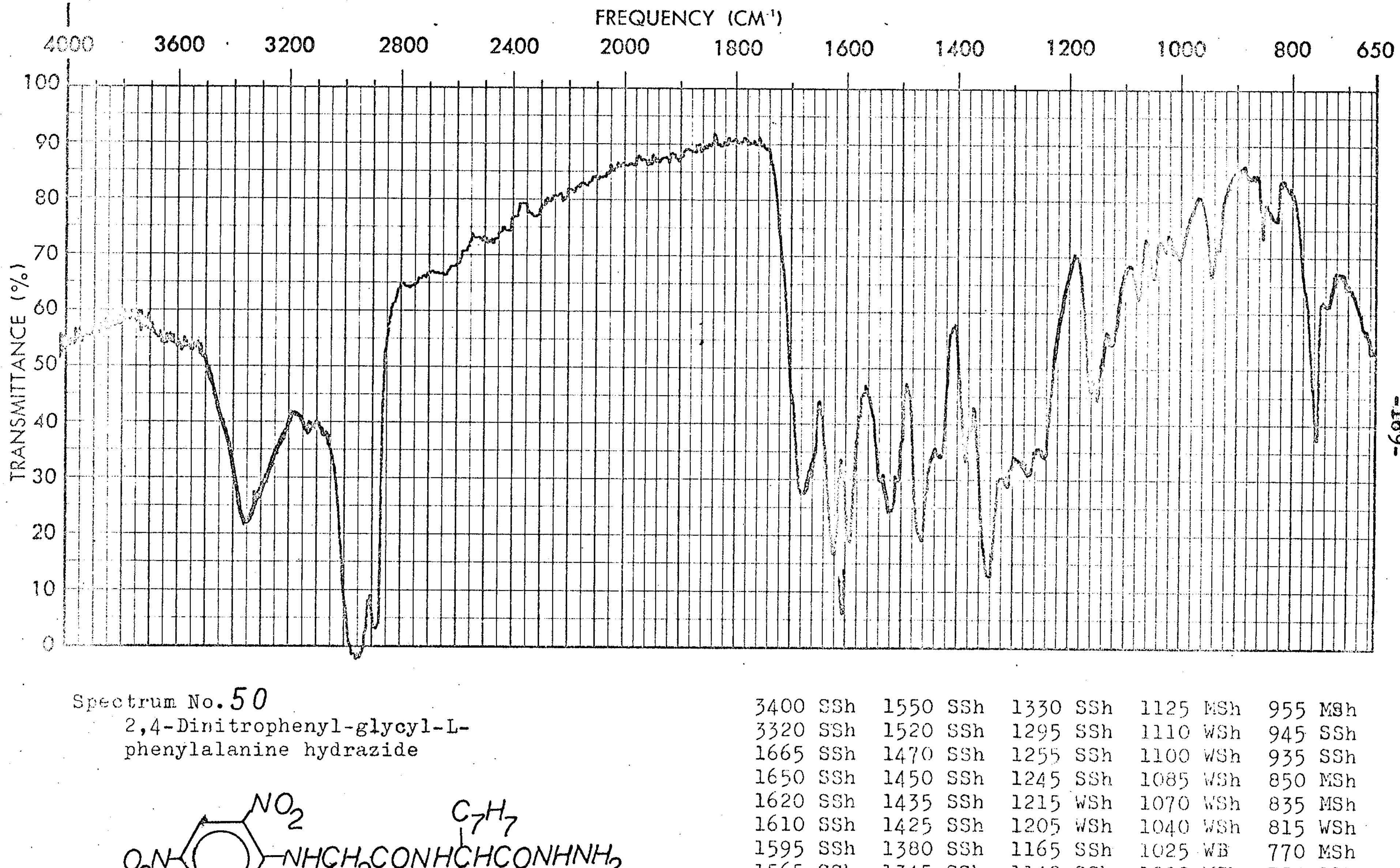


Spectrum No. 49

2,4-Dinitrophenyl-glycyl-L-leucine hydrazide

O_2N–(2-NO_2)C_6H_3–$NHCH_2CONHCH(C_4H_9)CONHNH_2$

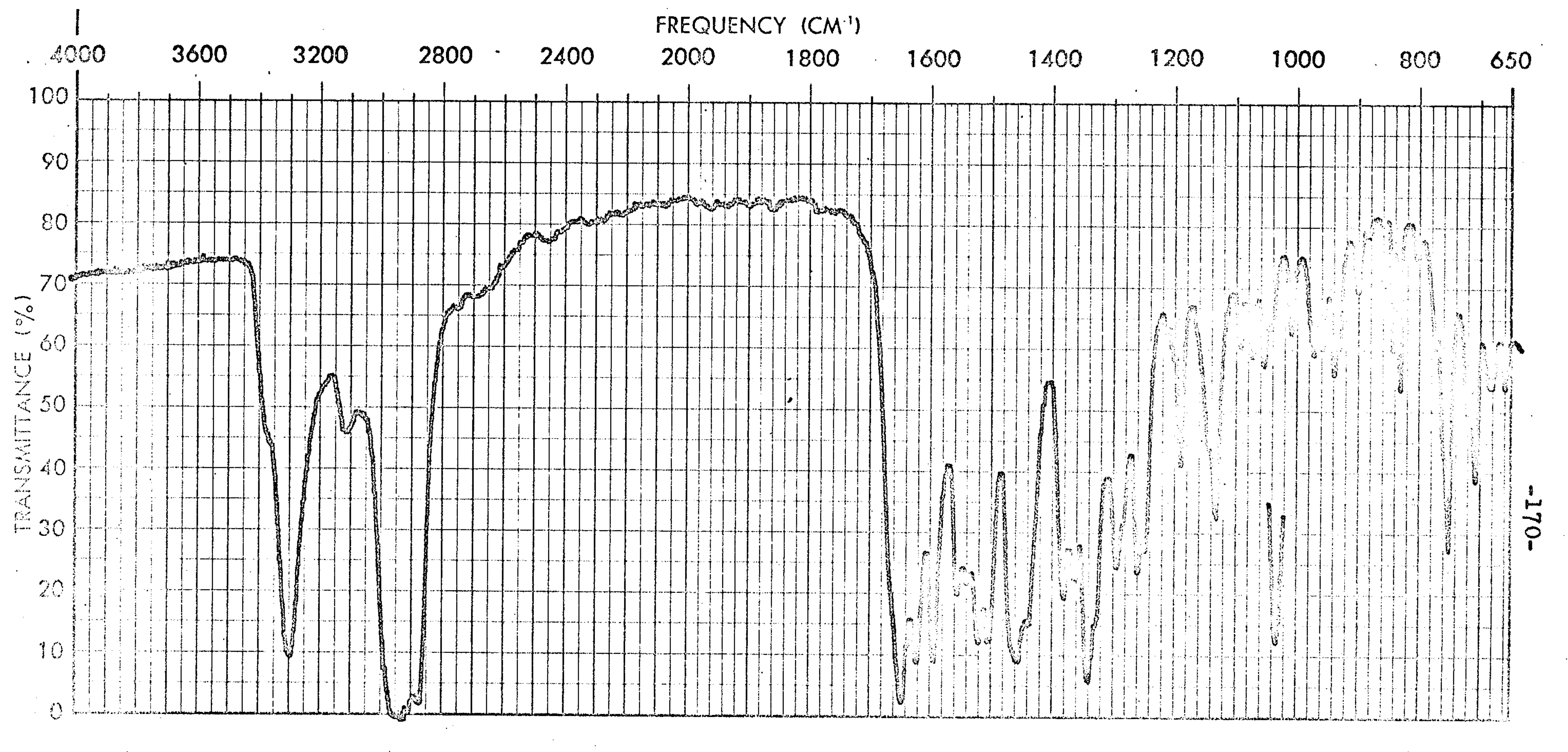
3480	SSh	1390	SSh	1110	WSh	780	SSh
3375	SSh	1330	SSh	1055	WB	760	SSh
3260	SSh	1325	SSh	970	WSh	735	MB
1670	SSh	1315	SSh	900	MSh	665	MSh
1645	SSh	1300	SSh	895	MSh		
1605	SSh	1255	SSh	855	WSh		
1530	SSh	1170	MSh	825	SSh		
1470	SSh	1140	WSh	815	SSh		



Spectrum No. 50

2,4-Dinitrophenyl-glycyl-L-phenylalanine hydrazide

3400 SSh	1550 SSh	1330 SSh	1125 MSh	955 MSh
3320 SSh	1520 SSh	1295 SSh	1110 WSh	945 SSh
1665 SSh	1470 SSh	1255 SSh	1100 WSh	935 SSh
1650 SSh	1450 SSh	1245 SSh	1085 WSh	850 MSh
1620 SSh	1435 SSh	1215 WSh	1070 WSh	835 MSh
1610 SSh	1425 SSh	1205 WSh	1040 WSh	815 WSh
1595 SSh	1380 SSh	1165 SSh	1025 WB	770 MSh
1565 SSh	1345 SSh	1140 SSh	1000 WSh	755 SSh

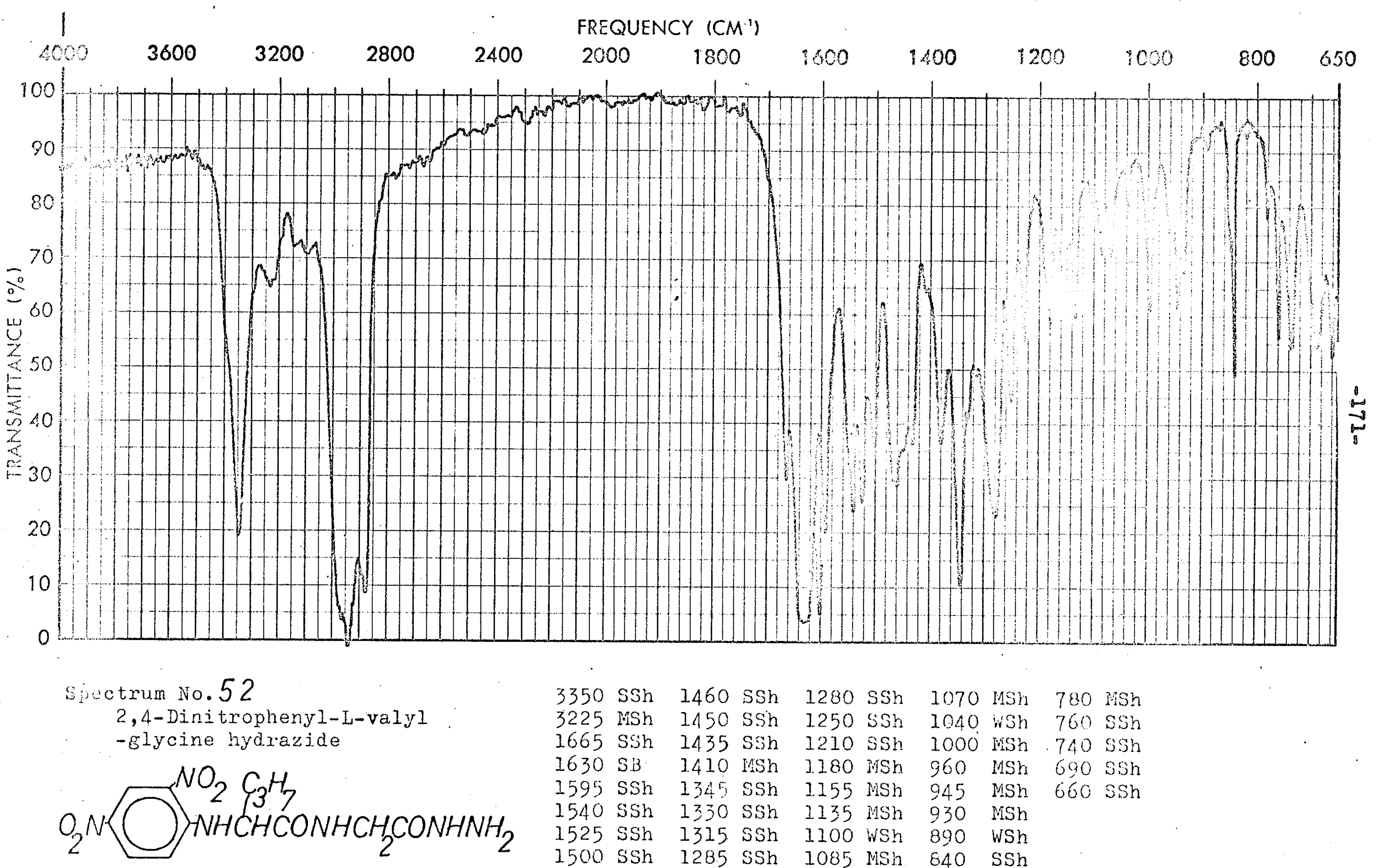


Spectrum No. 51

2,4-Dinitrophenyl-L-alanyl
-L-phenylalanine hydrazide

$O_2N-C_6H_3(NO_2)-NHCH(CH_3)CONHCH(C_7H_7)CONHNH_2$

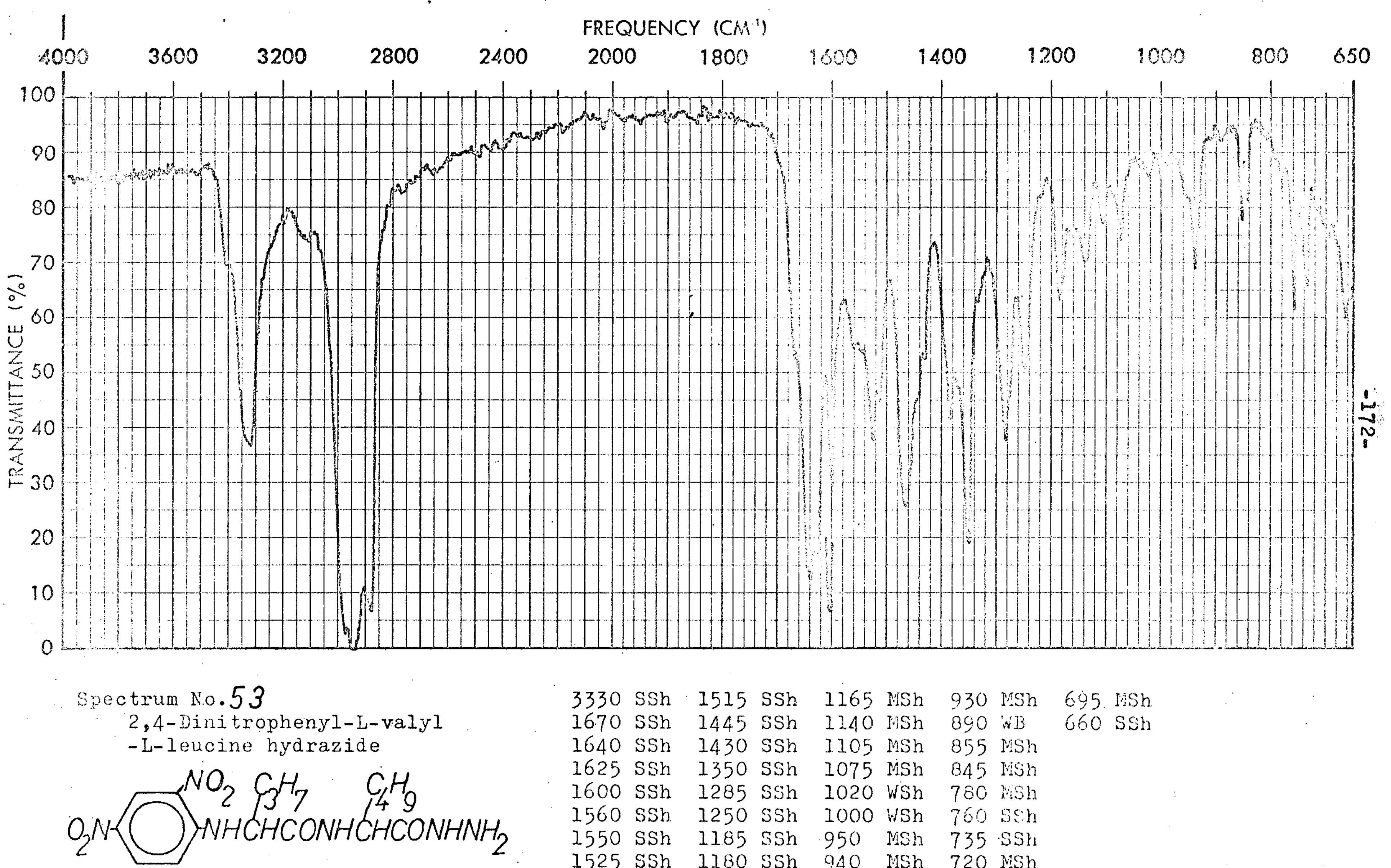
3300 SSh	1440 SSh	1135 SSh	940 MSh	750 SSh
1640 SSh	1360 SSh	1095 MSh	930 MSh	720 SSh
1620 SSh	1340 SSh	1075 MSh	900 WSh	705 SSh
1595 SSh	1295 SSh	1055 MSh	880 WSh	685 MSh
1555 SSh	1260 SSh	1040 MSh	845 MSh	660 MSh
1540 SSh	1250 SSh	1010 MSh	830 MSh	
1520 SSh	1190 SSh	975 MSh	805 WSh	
1505 SSh	1145 SSh	960 MSh	775 MSh	



Spectrum No. 52
2,4-Dinitrophenyl-L-valyl
-glycine hydrazide

$O_2N-C_6H_3(NO_2)-NHCH(C_3H_7)CONHCH_2CONHNH_2$

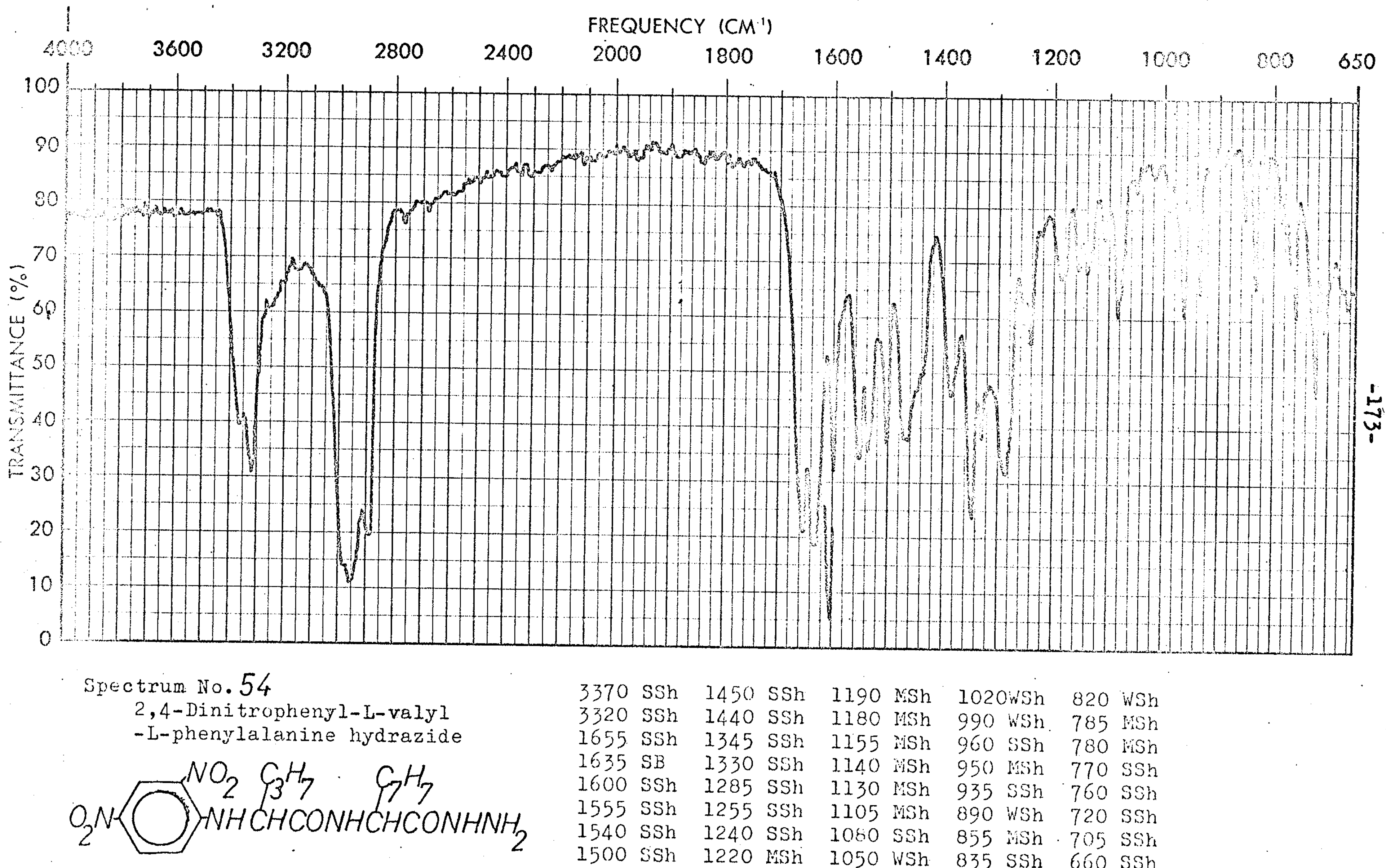
3350	SSh	1460	SSh	1280	SSh	1070	MSh	780	MSh
3225	MSh	1450	SSh	1250	SSh	1040	WSh	760	SSh
1665	SSh	1435	SSh	1210	SSh	1000	MSh	740	SSh
1630	SB	1410	MSh	1180	MSh	960	MSh	690	SSh
1595	SSh	1345	SSh	1155	MSh	945	MSh	660	SSh
1540	SSh	1330	SSh	1135	MSh	930	MSh		
1525	SSh	1315	SSh	1100	WSh	890	WSh		
1500	SSh	1285	SSh	1085	MSh	840	SSh		



Spectrum No. 53
2,4-Dinitrophenyl-L-valyl-L-leucine hydrazide

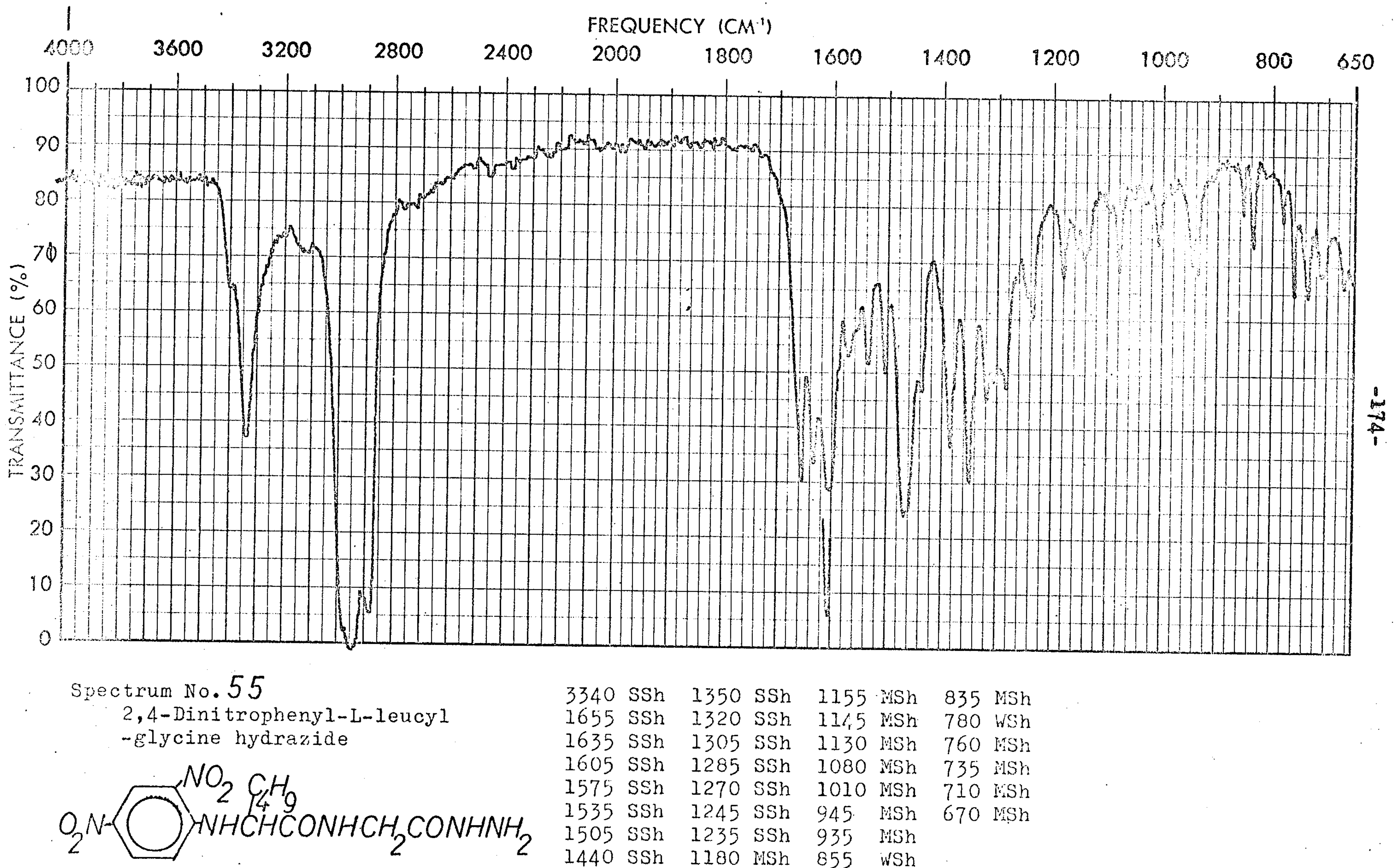
$O_2N-C_6H_3(NO_2)-NHCH(C_3H_7)CONHCH(C_4H_9)CONHNH_2$

3330 SSh	1515 SSh	1165 MSh	930 MSh	695 MSh
1670 SSh	1445 SSh	1140 MSh	890 WB	660 SSh
1640 SSh	1430 SSh	1105 MSh	855 MSh	
1625 SSh	1350 SSh	1075 MSh	845 MSh	
1600 SSh	1285 SSh	1020 WSh	780 MSh	
1560 SSh	1250 SSh	1000 WSh	760 SSh	
1550 SSh	1185 SSh	950 MSh	735 SSh	
1525 SSh	1180 SSh	940 MSh	720 MSh	



Spectrum No. 54
2,4-Dinitrophenyl-L-valyl
-L-phenylalanine hydrazide

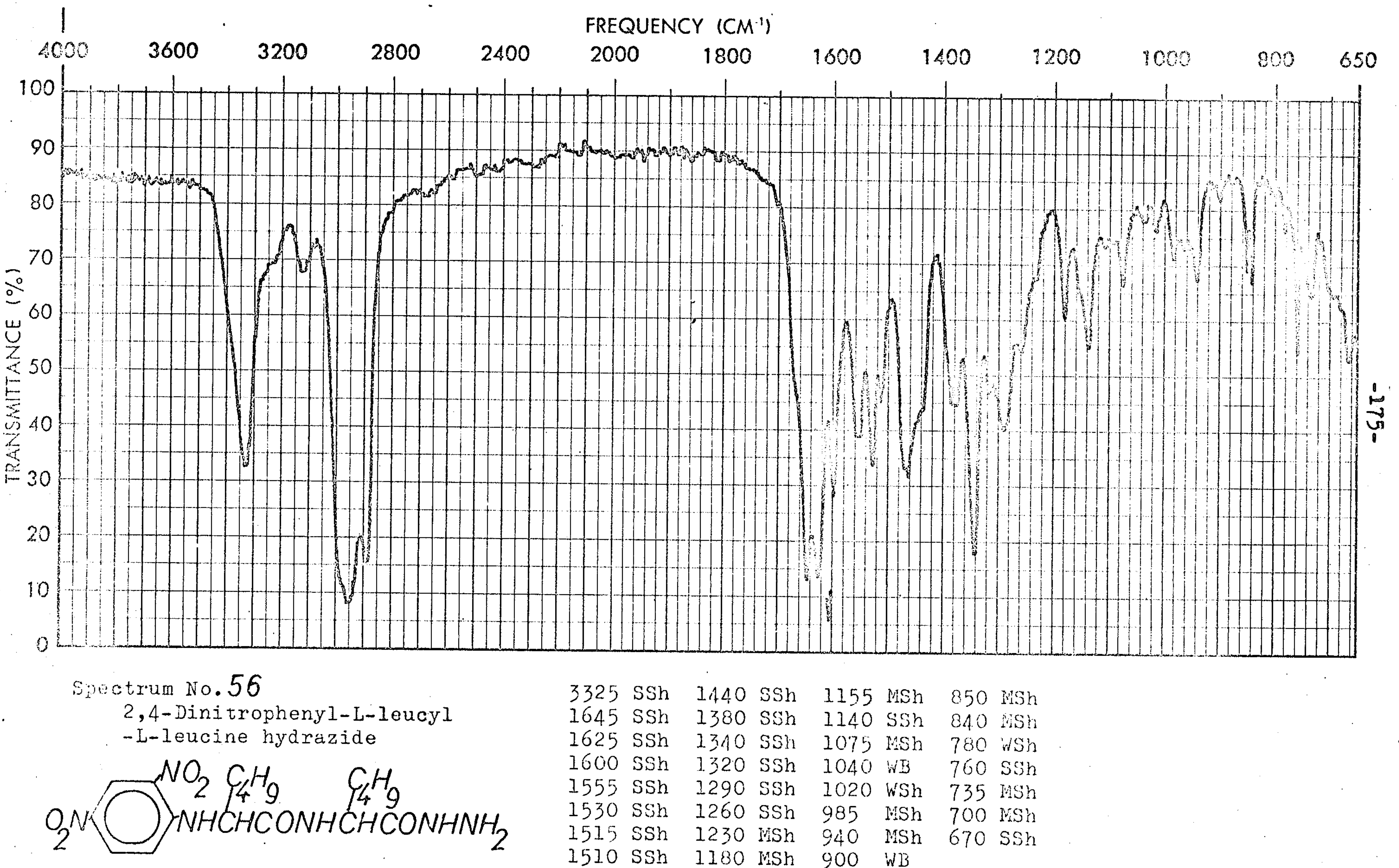
3370 SSh	1450 SSh	1190 MSh	1020 WSh	820 WSh
3320 SSh	1440 SSh	1180 MSh	990 WSh	785 MSh
1655 SSh	1345 SSh	1155 MSh	960 SSh	780 MSh
1635 SB	1330 SSh	1140 MSh	950 MSh	770 SSh
1600 SSh	1285 SSh	1130 MSh	935 SSh	760 SSh
1555 SSh	1255 SSh	1105 MSh	890 WSh	720 SSh
1540 SSh	1240 SSh	1080 SSh	855 MSh	705 SSh
1500 SSh	1220 MSh	1050 WSh	835 SSh	660 SSh



Spectrum No. 55
2,4-Dinitrophenyl-L-leucyl
-glycine hydrazide

$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONHCH_2CONHNH_2$

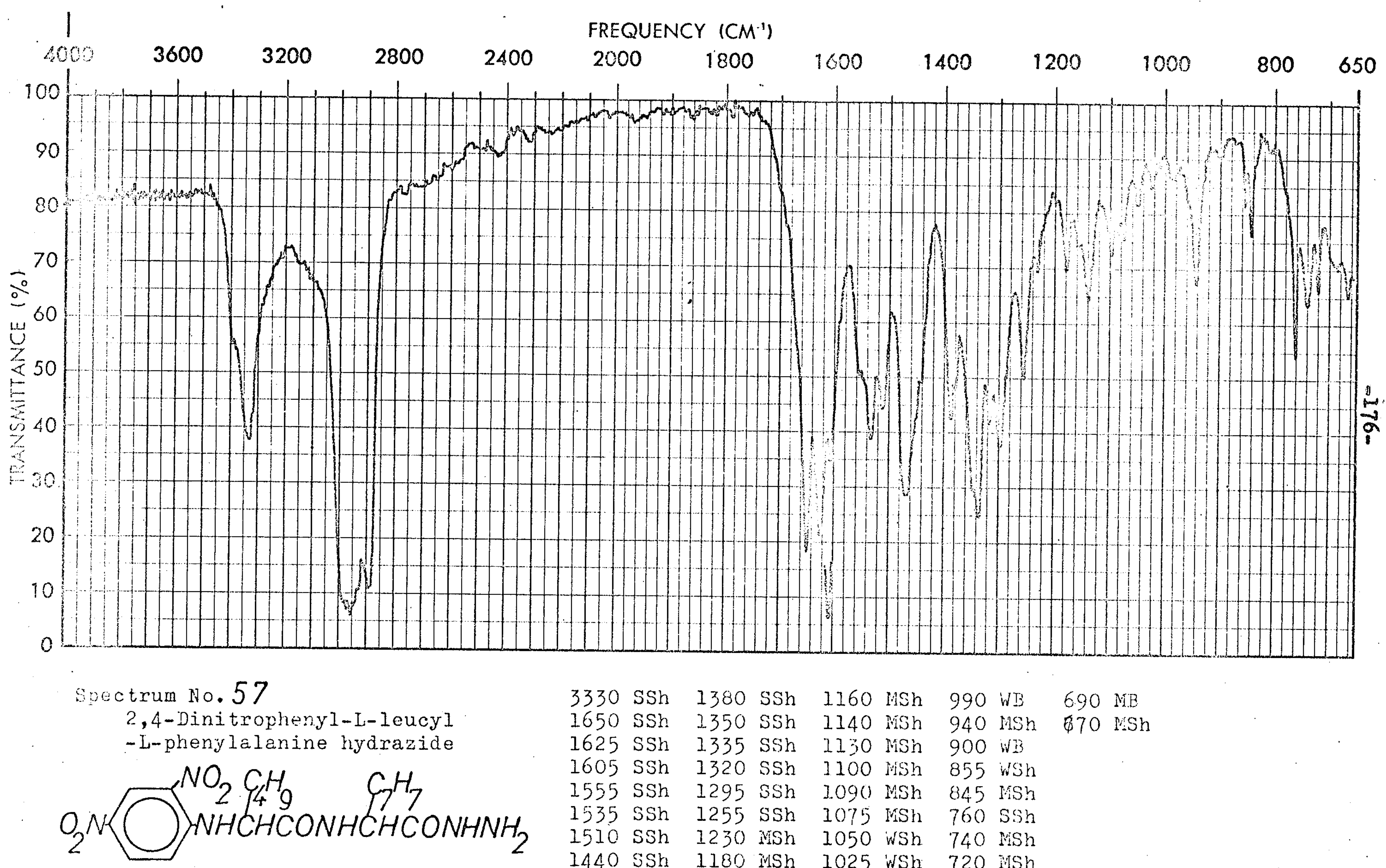
3340 SSh	1350 SSh	1155 MSh	835 MSh
1655 SSh	1320 SSh	1145 MSh	780 WSh
1635 SSh	1305 SSh	1130 MSh	760 MSh
1605 SSh	1285 SSh	1080 MSh	735 MSh
1575 SSh	1270 SSh	1010 MSh	710 MSh
1535 SSh	1245 SSh	945 MSh	670 MSh
1505 SSh	1235 SSh	935 MSh	
1440 SSh	1180 MSh	855 WSh	



Spectrum No. 56
2,4-Dinitrophenyl-L-leucyl -L-leucine hydrazide

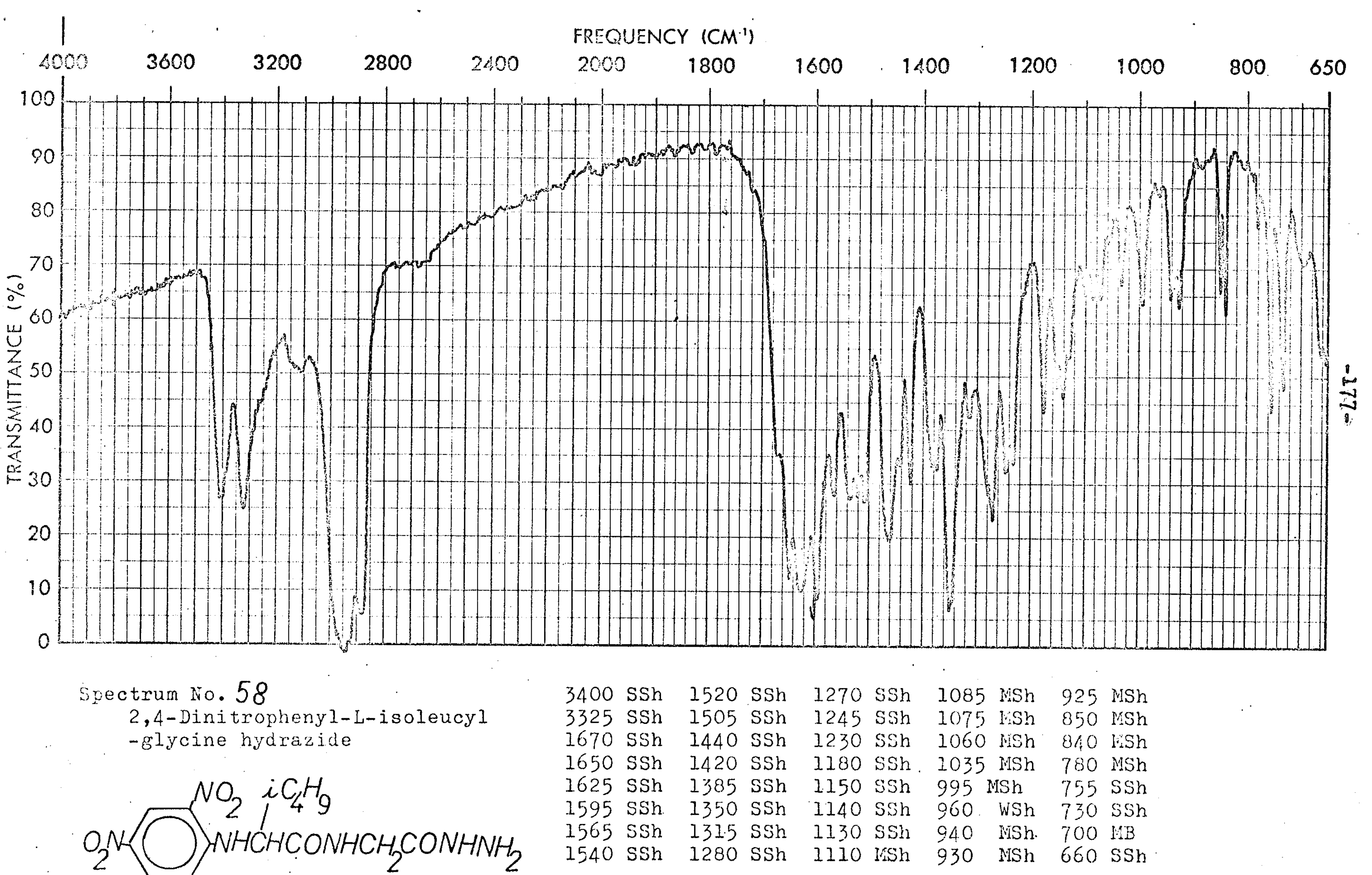
$O_2N-C_6H_3(NO_2)-NHCH(C_4H_9)CONHCH(C_4H_9)CONHNH_2$

3325	SSh	1440	SSh	1155	MSh	850	MSh
1645	SSh	1380	SSh	1140	SSh	840	MSh
1625	SSh	1340	SSh	1075	MSh	780	WSh
1600	SSh	1320	SSh	1040	WB	760	SSh
1555	SSh	1290	SSh	1020	WSh	735	MSh
1530	SSh	1260	SSh	985	MSh	700	MSh
1515	SSh	1230	MSh	940	MSh	670	SSh
1510	SSh	1180	MSh	900	WB		



Spectrum No. 57
2,4-Dinitrophenyl-L-leucyl
-L-phenylalanine hydrazide

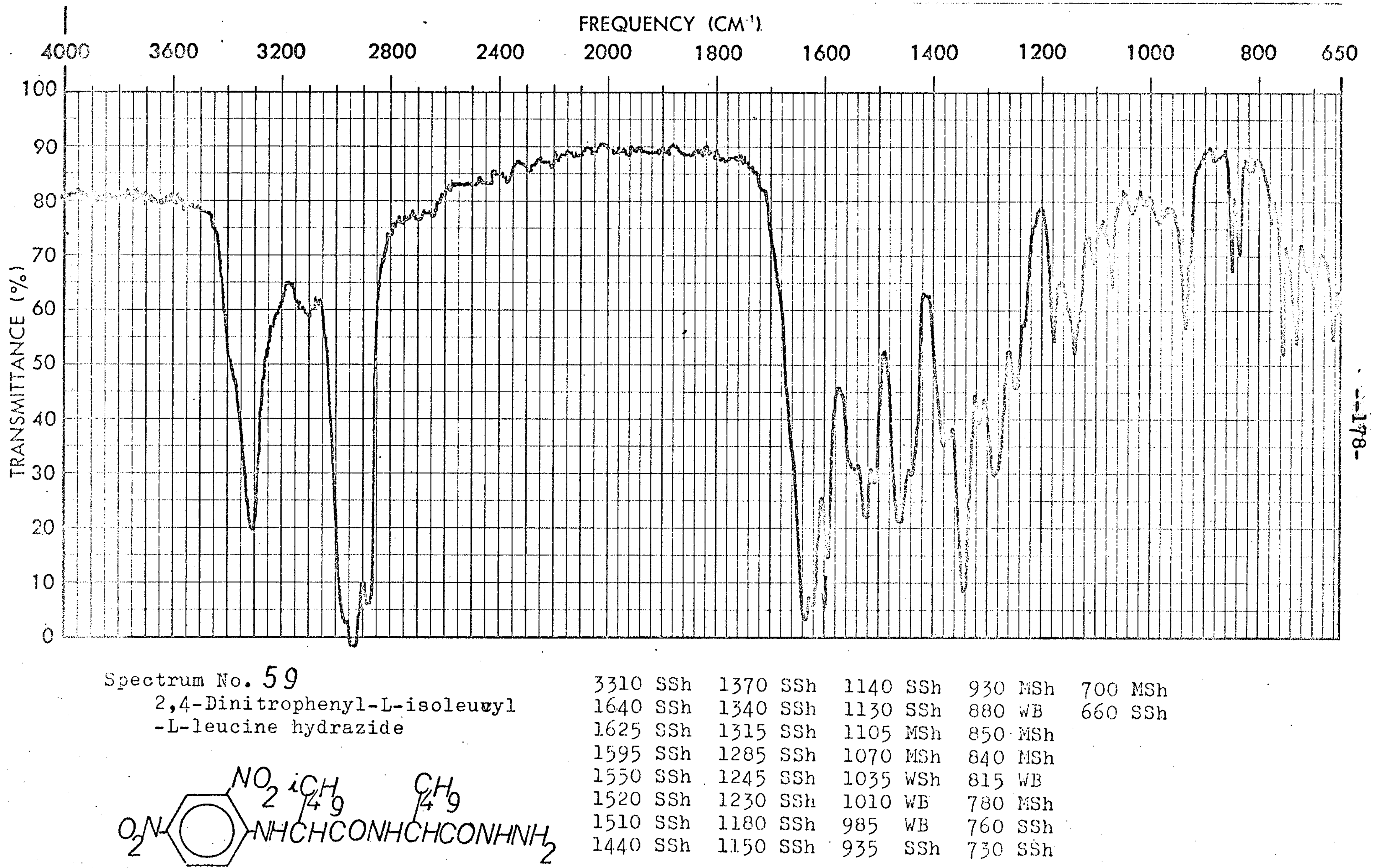
3330 SSh	1380 SSh	1160 MSh	990 WB	690 MB
1650 SSh	1350 SSh	1140 MSh	940 MSh	670 MSh
1625 SSh	1335 SSh	1130 MSh	900 WB	
1605 SSh	1320 SSh	1100 MSh	855 WSh	
1555 SSh	1295 SSh	1090 MSh	845 MSh	
1535 SSh	1255 SSh	1075 MSh	760 SSh	
1510 SSh	1230 MSh	1050 WSh	740 MSh	
1440 SSh	1180 MSh	1025 WSh	720 MSh	



Spectrum No. 58
2,4-Dinitrophenyl-L-isoleucyl
-glycine hydrazide

O_2N-C6H3(NO_2)-NHCH(iC_4H_9)CONHCH$_2$CONHNH$_2$

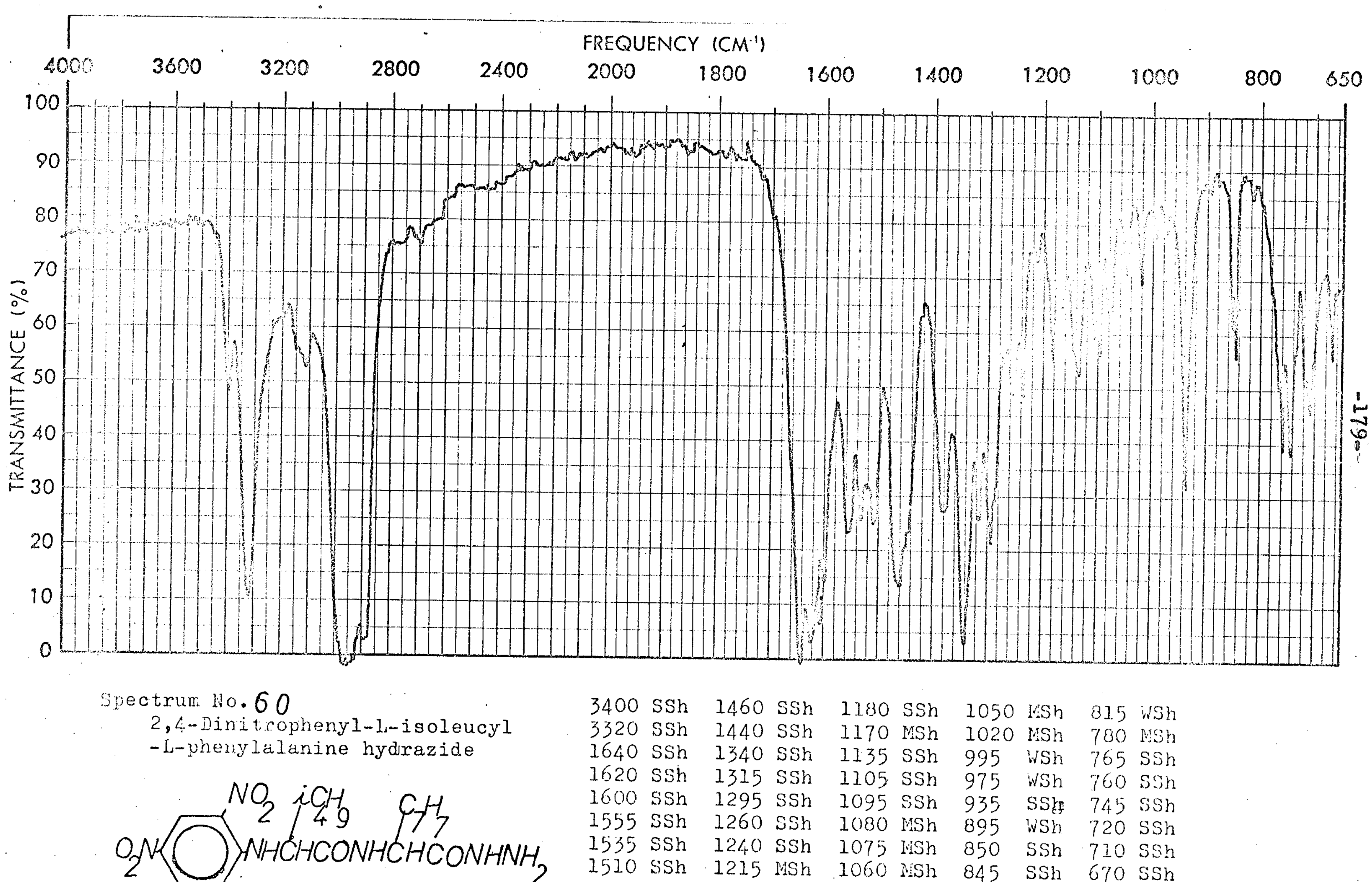
3400 SSh	1520 SSh	1270 SSh	1085 MSh	925 MSh
3325 SSh	1505 SSh	1245 SSh	1075 MSh	850 MSh
1670 SSh	1440 SSh	1230 SSh	1060 MSh	840 MSh
1650 SSh	1420 SSh	1180 SSh	1035 MSh	780 MSh
1625 SSh	1385 SSh	1150 SSh	995 MSh	755 SSh
1595 SSh	1350 SSh	1140 SSh	960 WSh	730 SSh
1565 SSh	1315 SSh	1130 SSh	940 MSh	700 MB
1540 SSh	1280 SSh	1110 MSh	930 MSh	660 SSh



Spectrum No. 59
2,4-Dinitrophenyl-L-isoleucyl
-L-leucine hydrazide

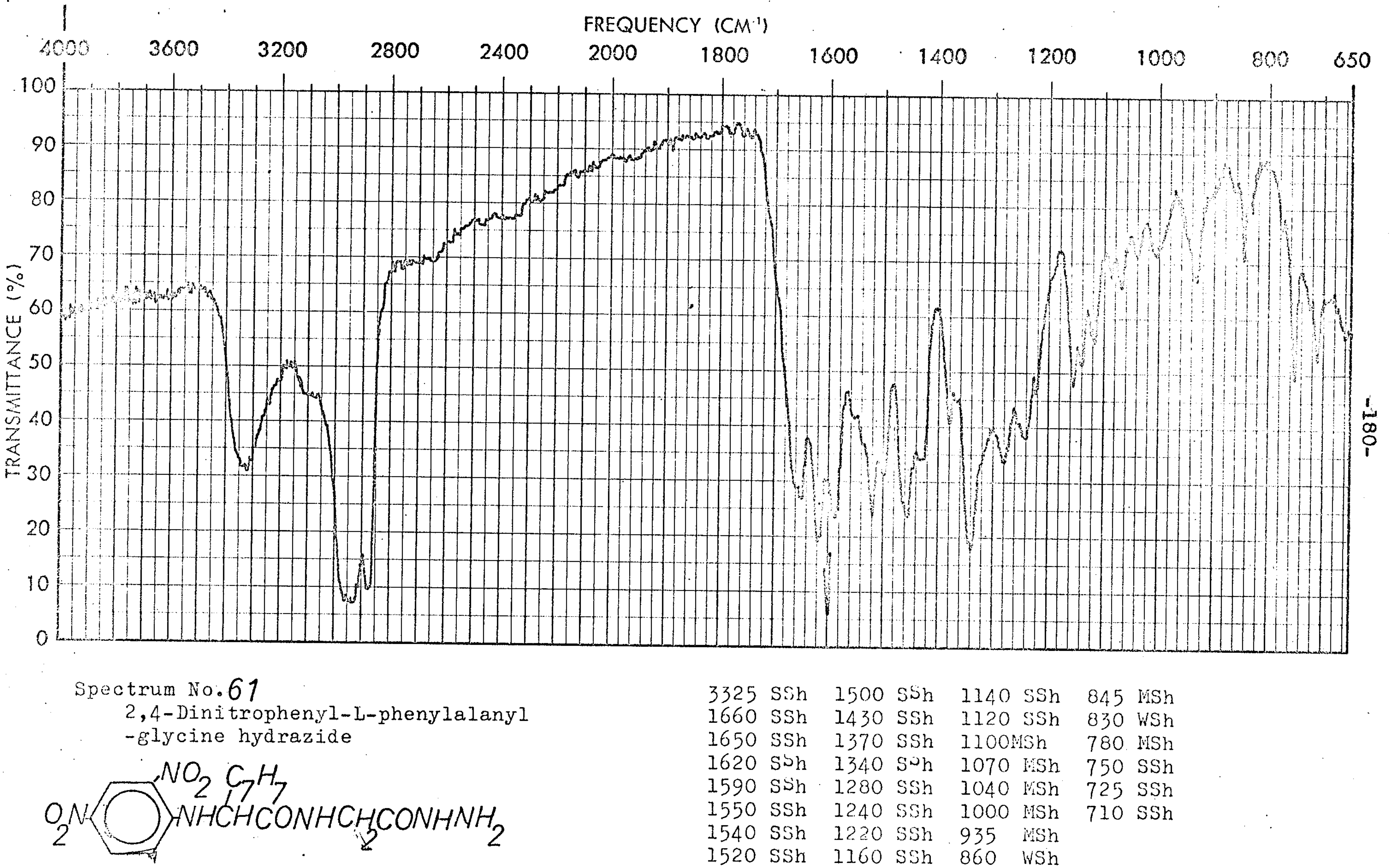
$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH(C_4H_9)CONHNH_2$

3310 SSh	1370 SSh	1140 SSh	930 MSh	700 MSh
1640 SSh	1340 SSh	1130 SSh	880 WB	660 SSh
1625 SSh	1315 SSh	1105 MSh	850 MSh	
1595 SSh	1285 SSh	1070 MSh	840 MSh	
1550 SSh	1245 SSh	1035 WSh	815 WB	
1520 SSh	1230 SSh	1010 WB	780 MSh	
1510 SSh	1180 SSh	985 WB	760 SSh	
1440 SSh	1150 SSh	935 SSh	730 SSh	



Spectrum No. 60
2,4-Dinitrophenyl-L-isoleucyl
-L-phenylalanine hydrazide

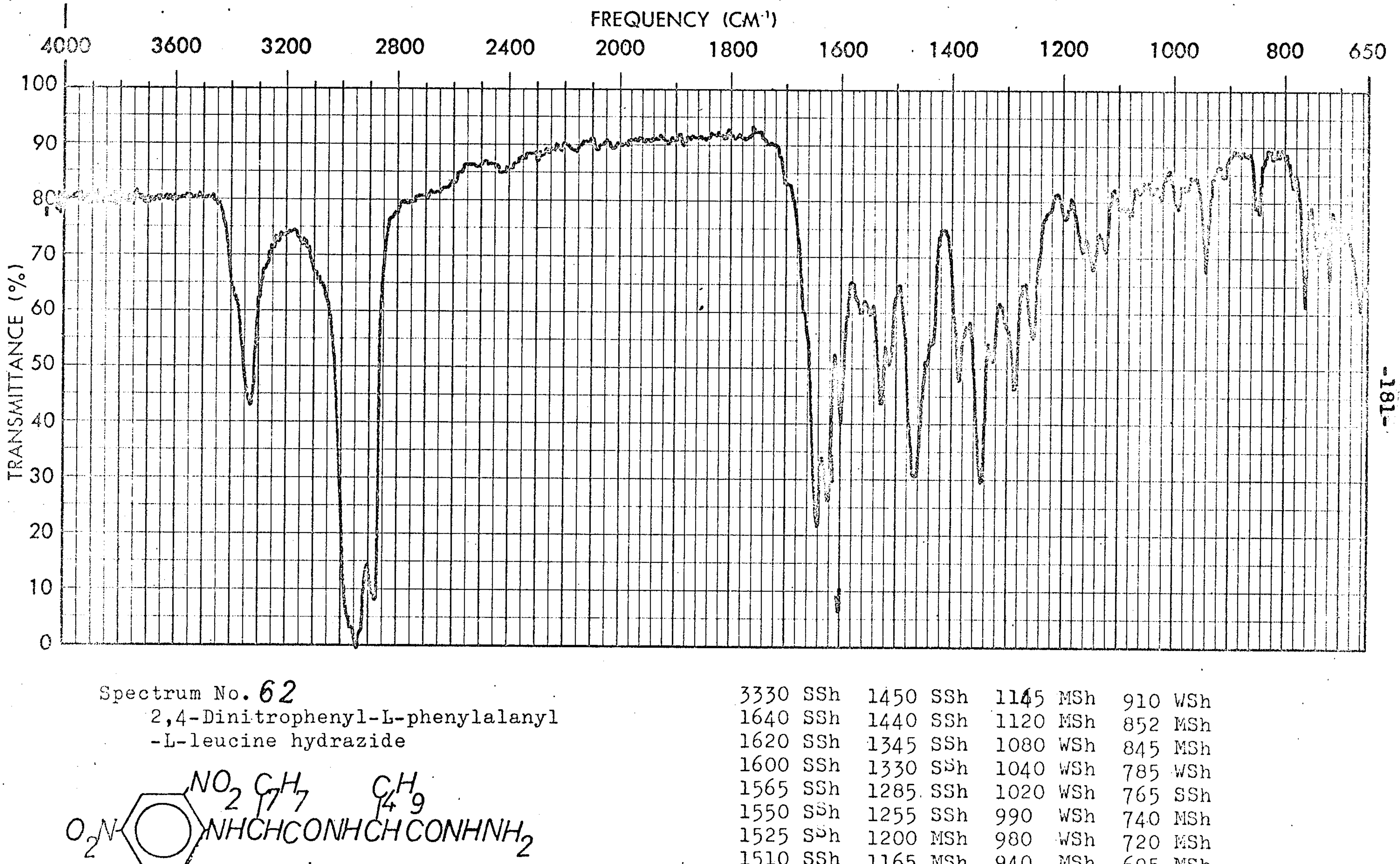
3400	SSh	1460	SSh	1180	SSh	1050	MSh	815	WSh
3320	SSh	1440	SSh	1170	MSh	1020	MSh	780	MSh
1640	SSh	1340	SSh	1135	SSh	995	WSh	765	SSh
1620	SSh	1315	SSh	1105	SSh	975	WSh	760	SSh
1600	SSh	1295	SSh	1095	SSh	935	SSh	745	SSh
1555	SSh	1260	SSh	1080	MSh	895	WSh	720	SSh
1535	SSh	1240	SSh	1075	MSh	850	SSh	710	SSh
1510	SSh	1215	MSh	1060	MSh	845	SSh	670	SSh



Spectrum No. 61
2,4-Dinitrophenyl-L-phenylalanyl
-glycine hydrazide

O_2N–(benzene ring)–$NHCH(C_7H_7)CONHCH_2CONHNH_2$ (with NO_2 at the 2-position)

3325 SSh	1500 SSh	1140 SSh	845 MSh
1660 SSh	1430 SSh	1120 SSh	830 WSh
1650 SSh	1370 SSh	1100 MSh	780 MSh
1620 SSh	1340 SSh	1070 MSh	750 SSh
1590 SSh	1280 SSh	1040 MSh	725 SSh
1550 SSh	1240 SSh	1000 MSh	710 SSh
1540 SSh	1220 SSh	935 MSh	
1520 SSh	1160 SSh	860 WSh	

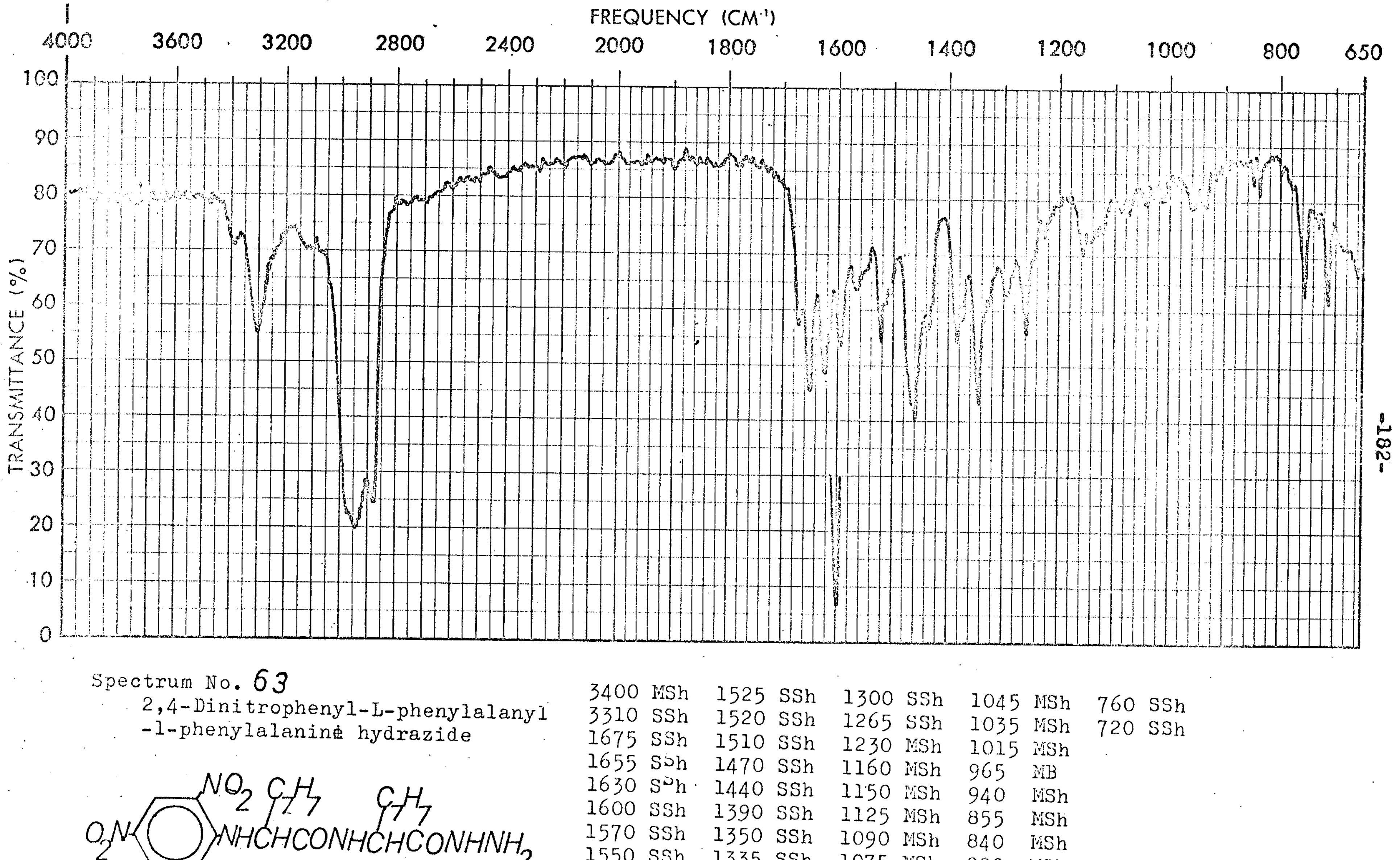


Spectrum No. 62

2,4-Dinitrophenyl-L-phenylalanyl-L-leucine hydrazide

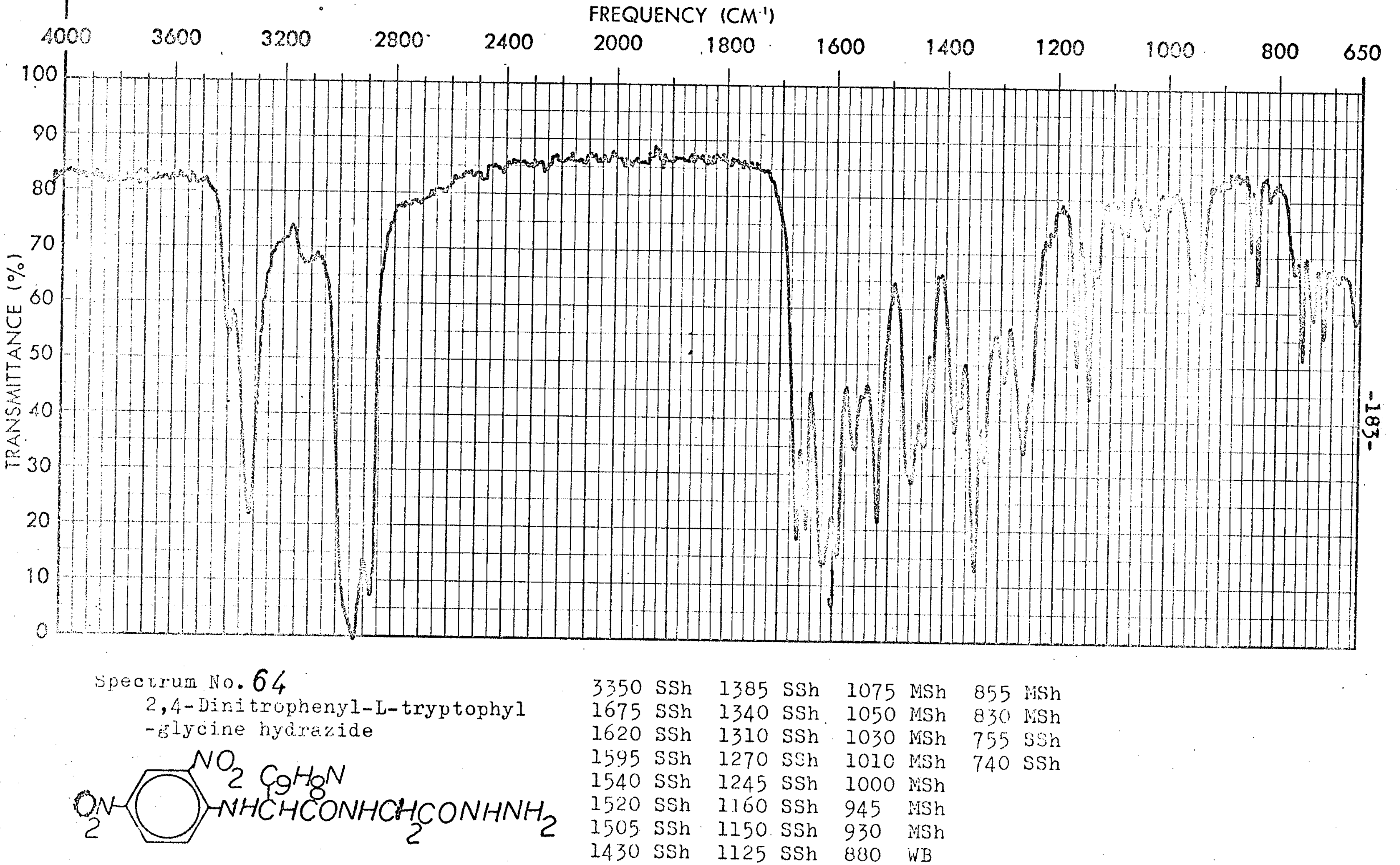
$O_2N-C_6H_3(NO_2)-NHCH(C_7H_7)CONHCH(C_4H_9)CONHNH_2$

3330 SSh	1450 SSh	1145 MSh	910 WSh
1640 SSh	1440 SSh	1120 MSh	852 MSh
1620 SSh	1345 SSh	1080 WSh	845 MSh
1600 SSh	1330 SSh	1040 WSh	785 WSh
1565 SSh	1285 SSh	1020 WSh	765 SSh
1550 SSh	1255 SSh	990 WSh	740 MSh
1525 SSh	1200 MSh	980 WSh	720 MSh
1510 SSh	1165 MSh	940 MSh	695 MSh



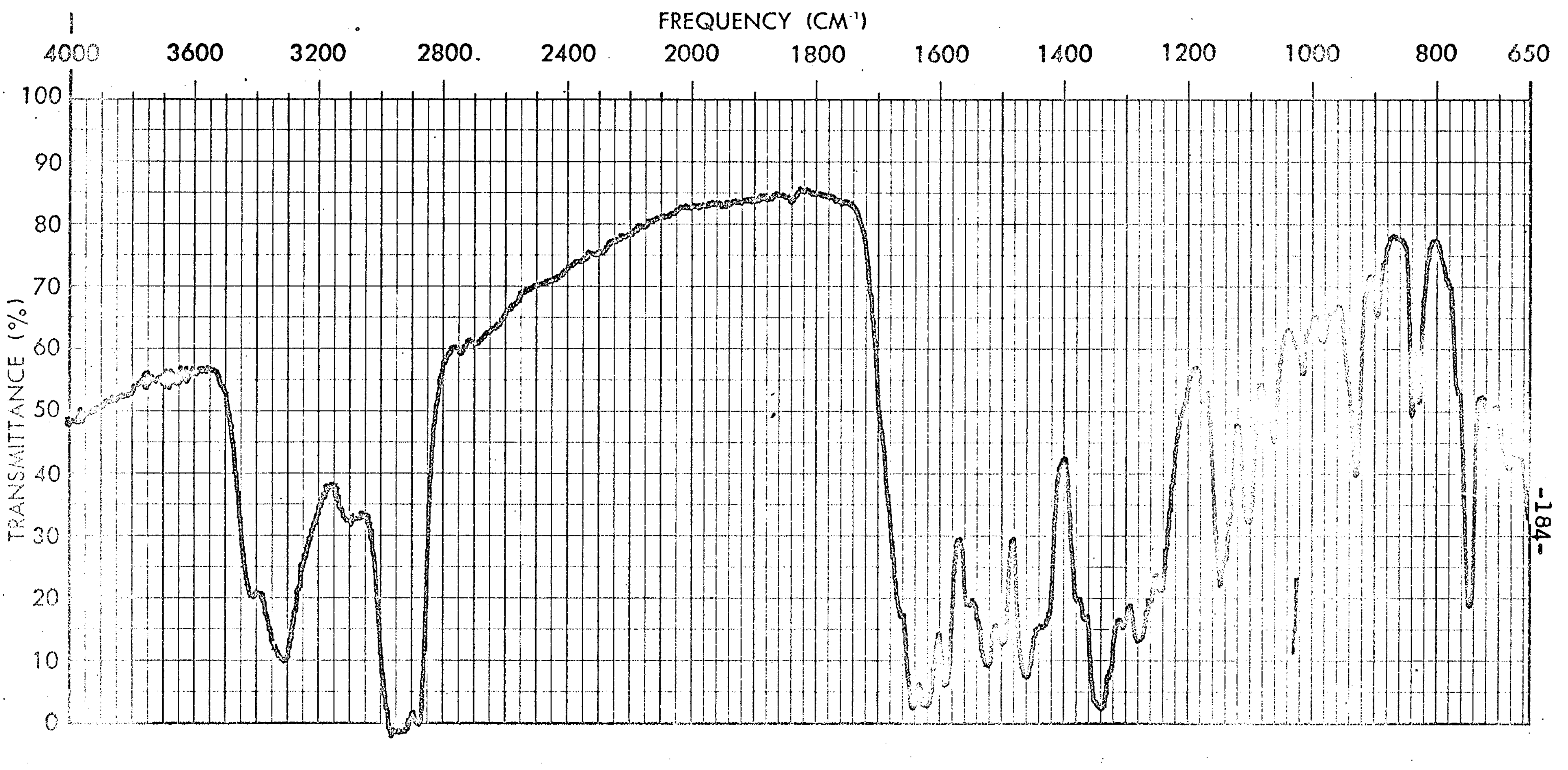
Spectrum No. 63
2,4-Dinitrophenyl-L-phenylalanyl
-l-phenylalanine hydrazide

3400 MSh	1525 SSh	1300 SSh	1045 MSh	760 SSh
3310 SSh	1520 SSh	1265 SSh	1035 MSh	720 SSh
1675 SSh	1510 SSh	1230 MSh	1015 MSh	
1655 SSh	1470 SSh	1160 MSh	965 MB	
1630 SSh	1440 SSh	1150 MSh	940 MSh	
1600 SSh	1390 SSh	1125 MSh	855 MSh	
1570 SSh	1350 SSh	1090 MSh	840 MSh	
1550 SSh	1335 SSh	1075 MSh	800 WSh	



Spectrum No. 64
2,4-Dinitrophenyl-L-tryptophyl
-glycine hydrazide

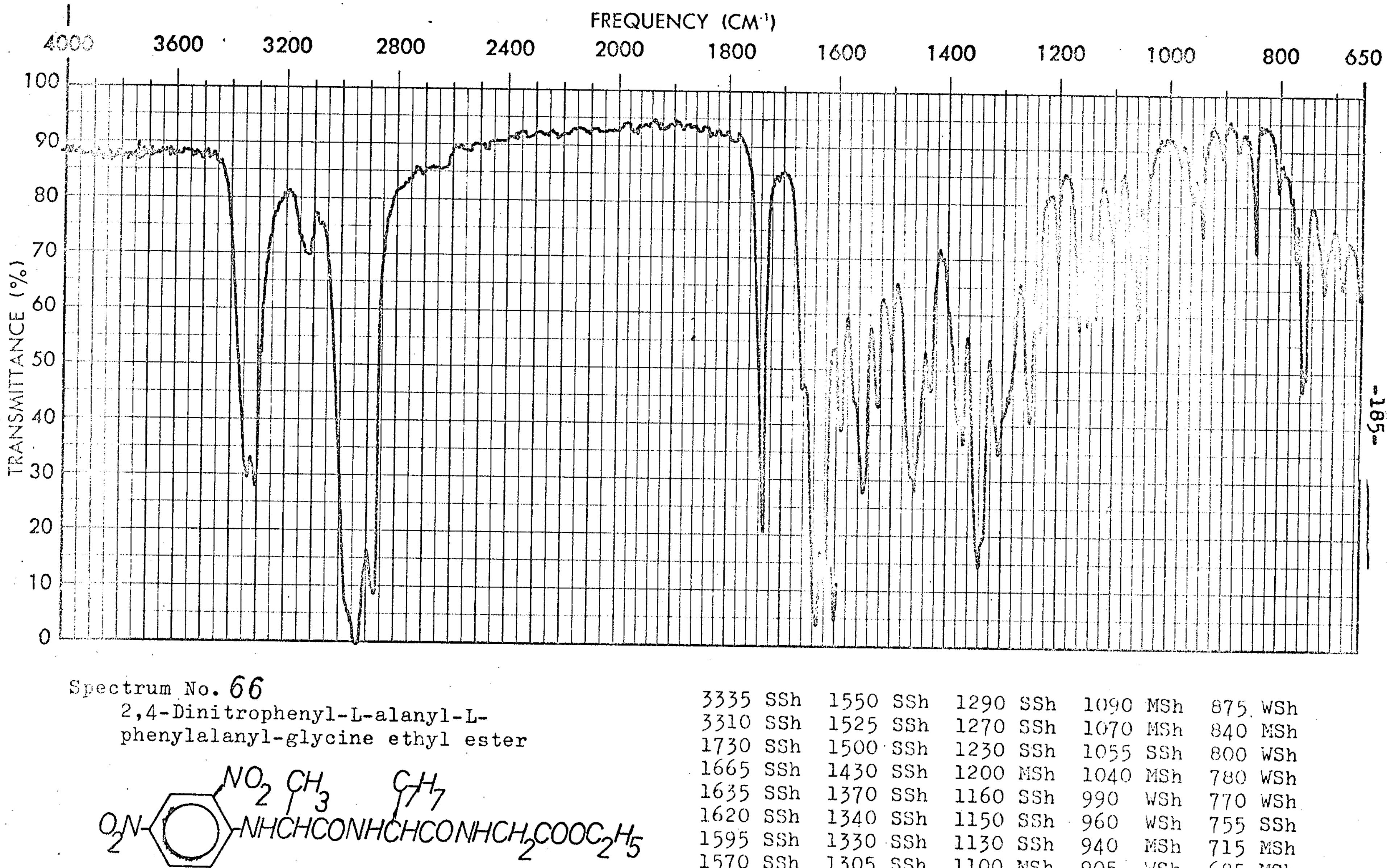
3350	SSh	1385	SSh	1075	MSh	855	MSh
1675	SSh	1340	SSh	1050	MSh	830	MSh
1620	SSh	1310	SSh	1030	MSh	755	SSh
1595	SSh	1270	SSh	1010	MSh	740	SSh
1540	SSh	1245	SSh	1000	MSh		
1520	SSh	1160	SSh	945	MSh		
1505	SSh	1150	SSh	930	MSh		
1430	SSh	1125	SSh	880	WB		



Spectrum No. 65
2,4-Dinitrophenyl-L-tryptophyl
-L-leucine hydrazide

$O_2N-C_6H_3(NO_2)-NHCH(C_9H_8N)CONHCH(C_4H_9)CONHNH_2$

3420	SSh	1430	SSh	1140	SSh	840	MSh
3320	SSh	1380	SSh	1105	SSh	830	MSh
1645	SSh	1340	SSh	1075	SSh	765	MSh
1620	SSh	1305	SSh	1060	SSh	745	SSh
1590	SSh	1275	SSh	1015	MSh	716	SSh
1555	SSh	1245	SSh	985	MSh	680	SSh
1520	SSh	1180	MSh	930	SSh	660	SSh
1500	SSh	1150	SSh	895	MSh		

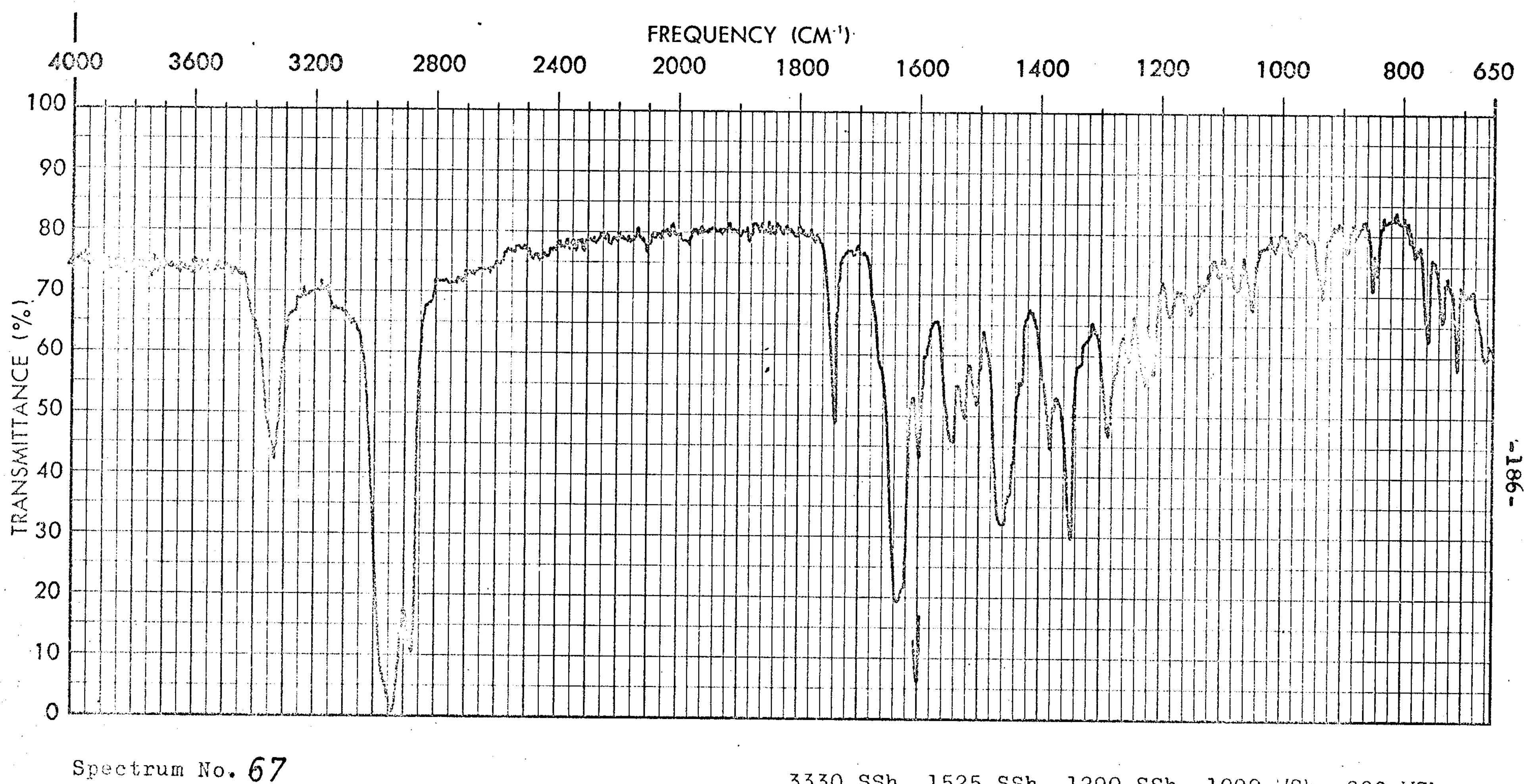


Spectrum No. 66

2,4-Dinitrophenyl-L-alanyl-L-phenylalanyl-glycine ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(CH_3)CONHCH(C_7H_7)CONHCH_2COOC_2H_5$

3335	SSh	1550	SSh	1290	SSh	1090	MSh	875	WSh
3310	SSh	1525	SSh	1270	SSh	1070	MSh	840	MSh
1730	SSh	1500	SSh	1230	SSh	1055	SSh	800	WSh
1665	SSh	1430	SSh	1200	MSh	1040	MSh	780	WSh
1635	SSh	1370	SSh	1160	SSh	990	WSh	770	WSh
1620	SSh	1340	SSh	1150	SSh	960	WSh	755	SSh
1595	SSh	1330	SSh	1130	SSh	940	MSh	715	MSh
1570	SSh	1305	SSh	1100	MSh	905	WSh	685	MSh

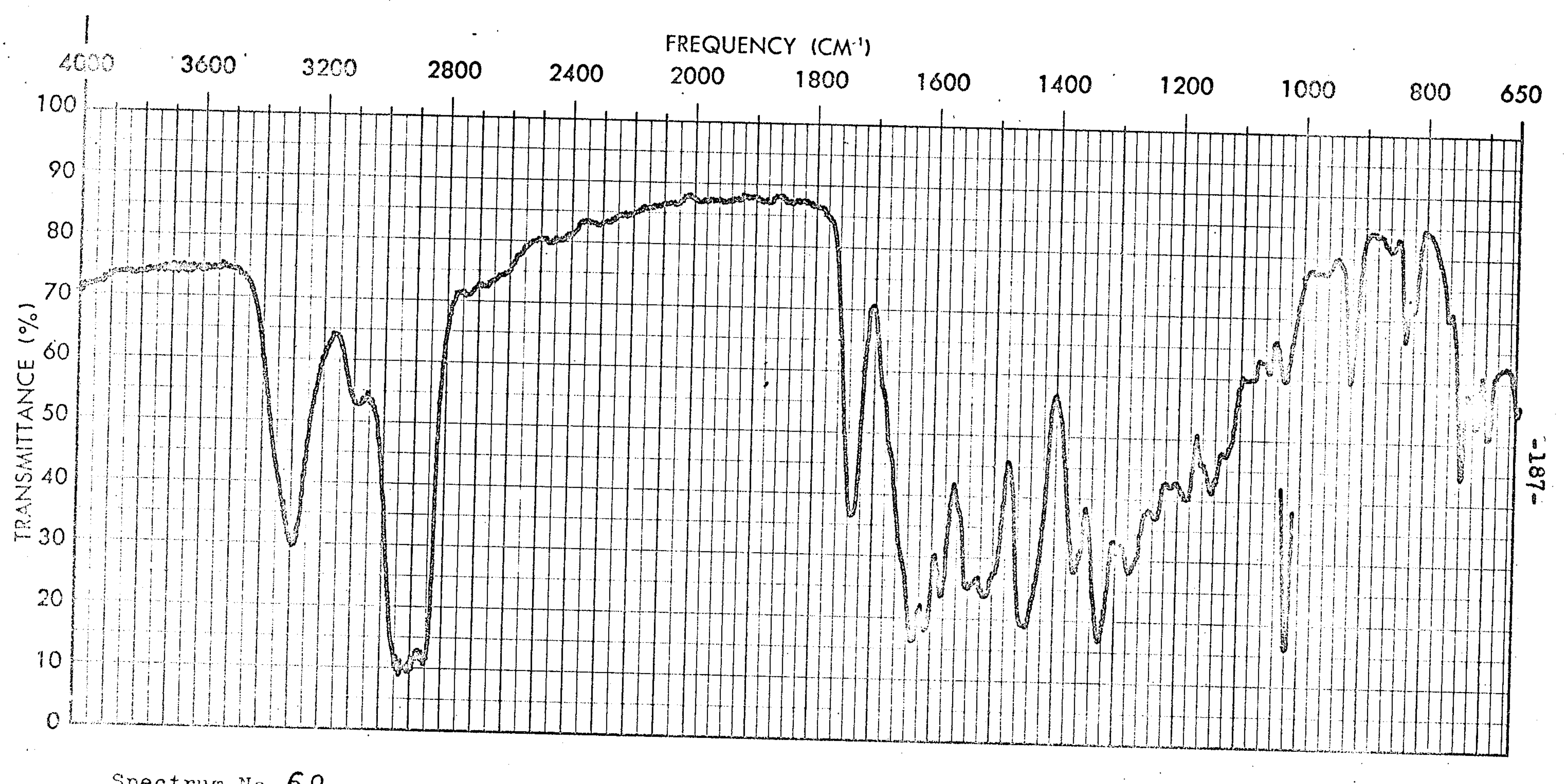


Spectrum No. 67

2,4-Dinitrophenyl-L-valyl-L-leucyl-L-phenylalanine ethyl ester

O_2N–$C_6H_3(NO_2)$–NHCH(C_3H_7)CONHCH(C_4H_9)CONHCH(C_7H_7)$COOC_2H_5$

3330 SSh	1525 SSh	1290 SSh	1090 WSh	880 WSh
1740 SSh	1505 SSh	1250 SSh	1075 WB	850 MSh
1665 SSh	1460 SSh	1230 SSh	1050 MSh	840 MSh
1635 SSh	1450 SSh	1210 SSh	1010 WSh	780 WSh
1625 SSh	1430 SSh	1185 MSh	985 WSh	760 MSh
1600 SSh	1375 SSh	1150 MSh	940 MSh	735 MSh
1590 SSh	1350 SSh	1140 MSh	932 MSh	710 SSh
1545 SSh	1330 SSh	1105 WSh	890 WSh	660 SSh

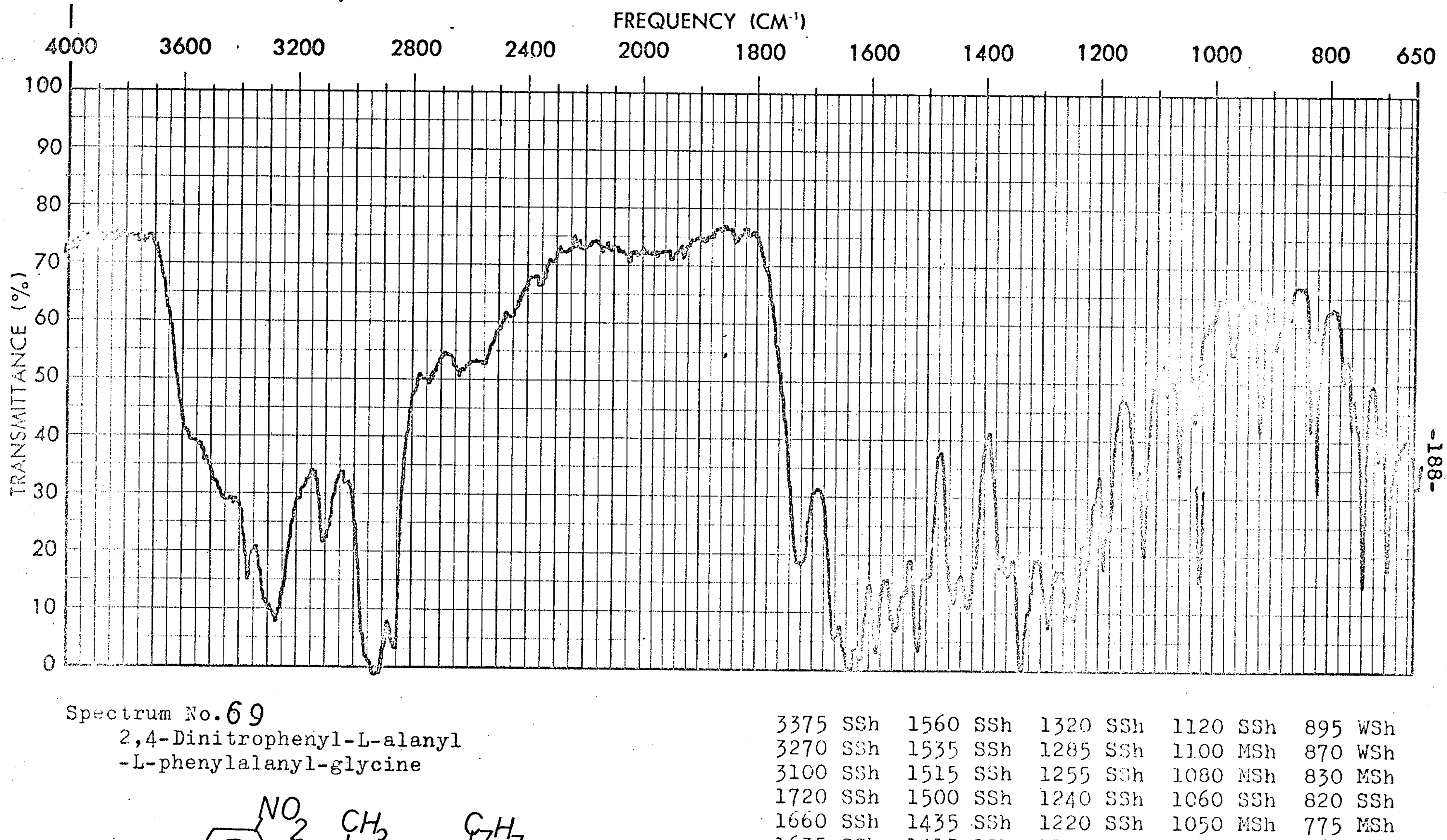


Spectrum No. 68

2,4-Dinitrophenyl-L-isoleucyl
-L-phenylalanyl-L-leucine
ethyl ester

$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH(C_7H_7)CONHCH(C_4H_9)COOC_2H_5$

3320	SSh	1370	SSh	1155	SSh	840	MSh
1745	SSh	1335	SSh	1130	SSh	830	MSh
1640	SSh	1310	SSh	1085	MSh	770	MSh
1620	SSh	1285	SSh	1065	MSh	750	SSh
1595	SSh	1245	SSh	1035	MSh	725	SSh
1550	SSh	1220	SSh	970	WB	705	SSh
1520	SSh	1195	SSh	930	MSh		
1505	SSh	1170	SSh	876	WSh		

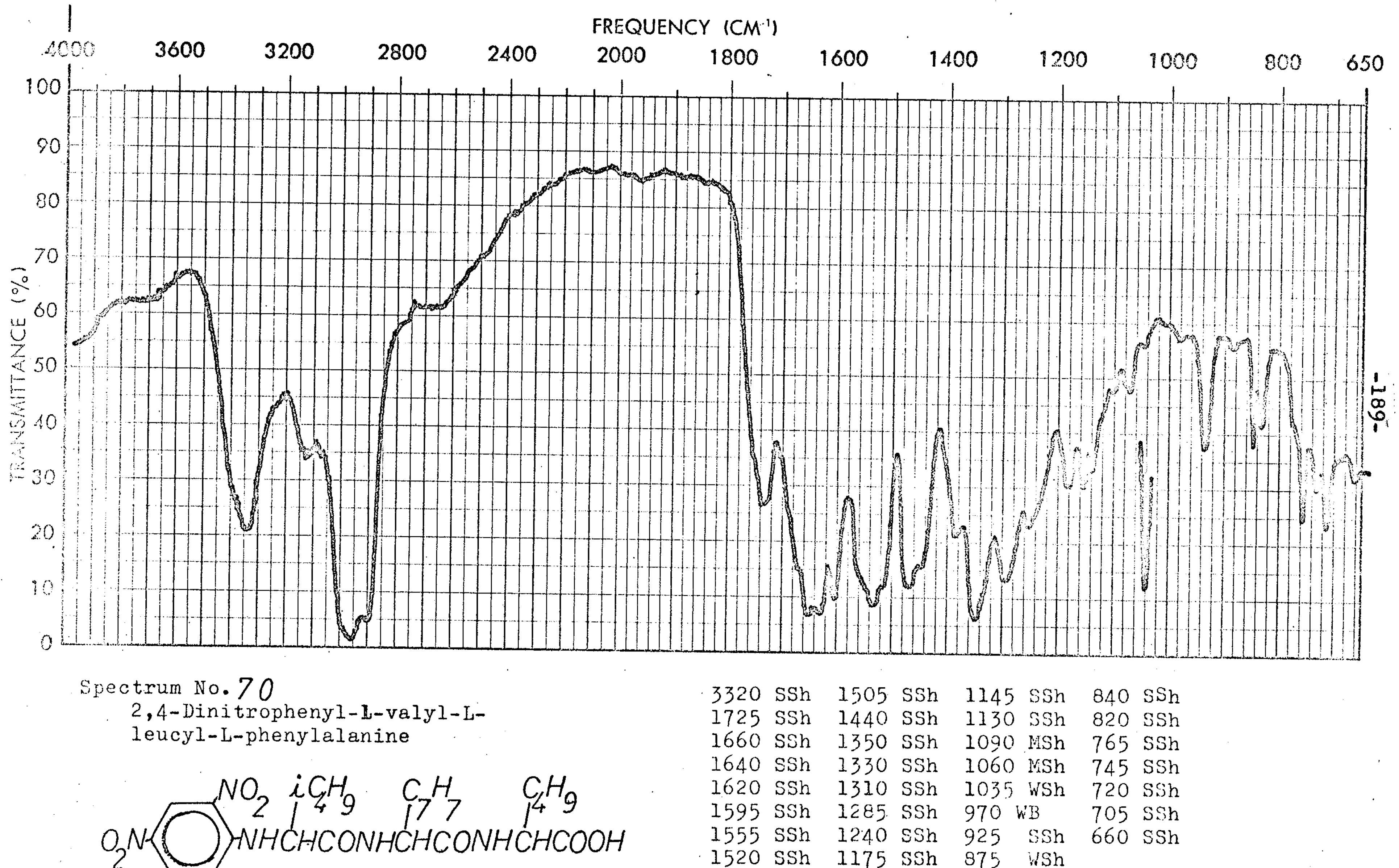


Spectrum No. 69

2,4-Dinitrophenyl-L-alanyl
-L-phenylalanyl-glycine

$O_2N-C_6H_3(NO_2)-NHCH(CH_3)CONHCH(C_7H_7)CONHCH_2COOH$

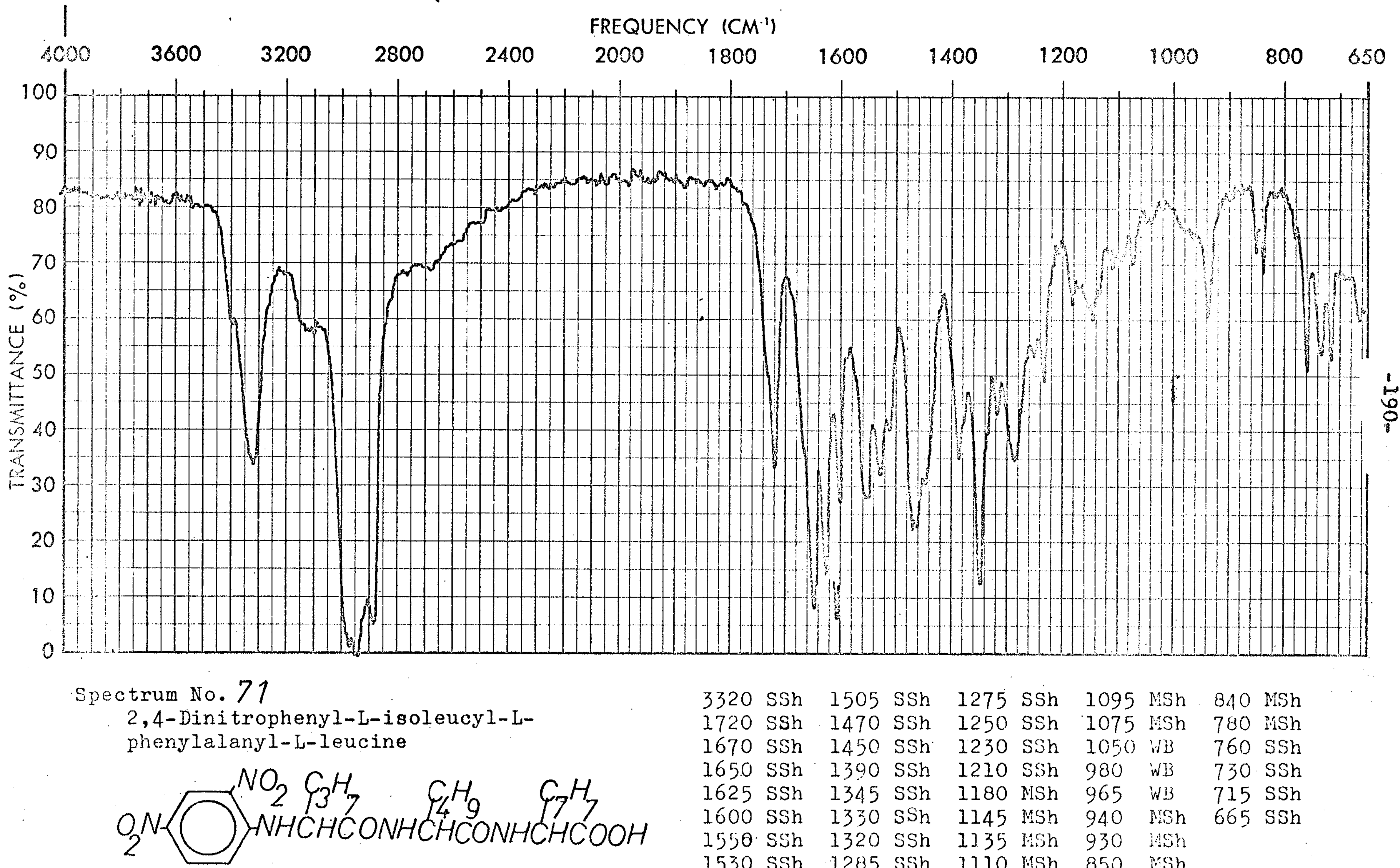
3375	SSh	1560	SSh	1320	SSh	1120	SSh	895	WSh
3270	SSh	1535	SSh	1285	SSh	1100	MSh	870	WSh
3100	SSh	1515	SSh	1255	SSh	1080	MSh	830	MSh
1720	SSh	1500	SSh	1240	SSh	1060	SSh	820	SSh
1660	SSh	1435	SSh	1220	SSh	1050	MSh	775	MSh
1635	SSh	1415	SSh	1195	SSh	1035	MSh	760	MSh
1615	SSh	1360	SSh	1180	SSh	965	WSh	740	SSh
1590	SSh	1335	SSh	1135	SSh	920	MSh	695	SSh



Spectrum No. 70
2,4-Dinitrophenyl-L-valyl-L-leucyl-L-phenylalanine

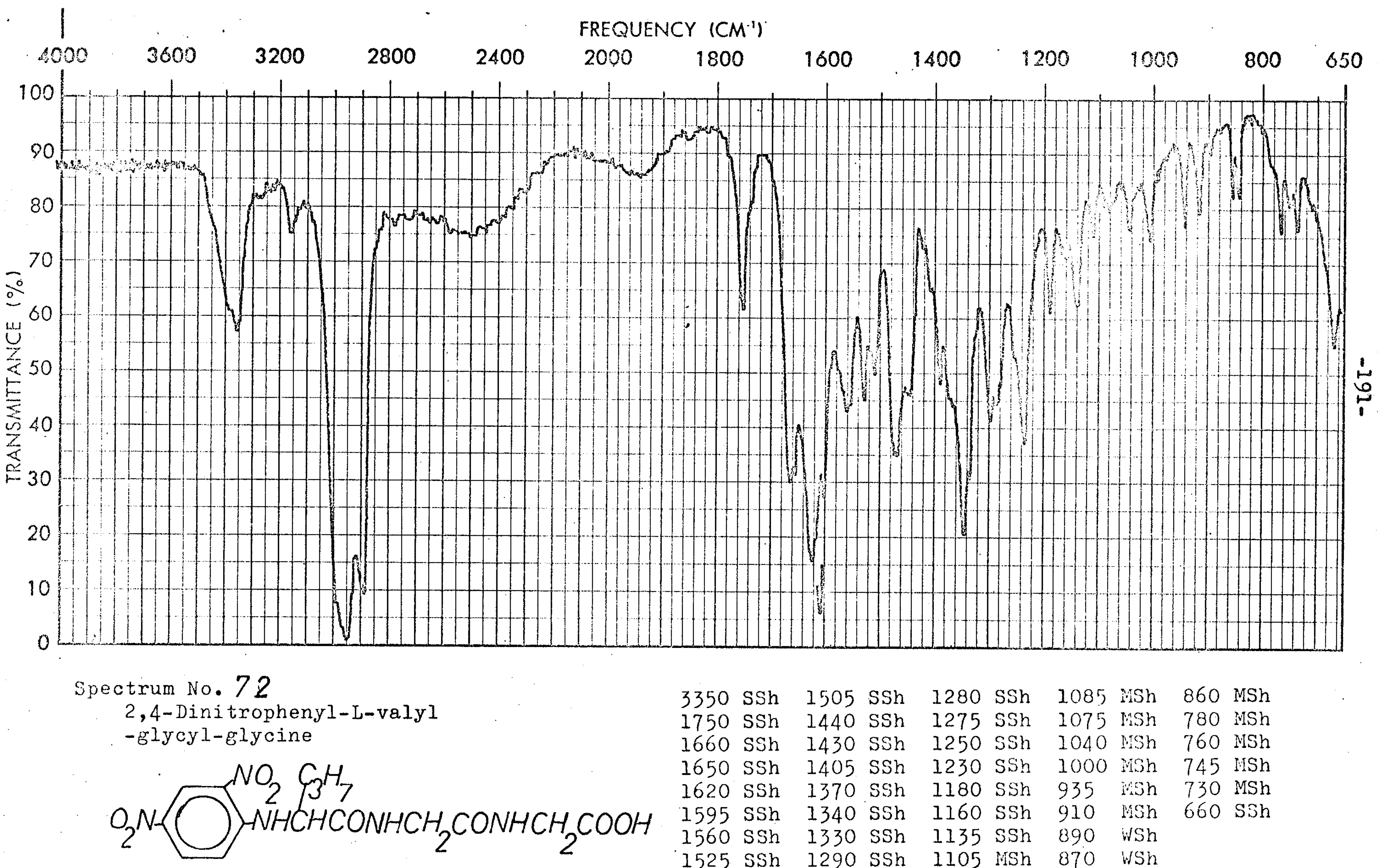
$O_2N-C_6H_3(NO_2)-NHCH(iC_4H_9)CONHCH(C_7H_7)CONHCH(C_4H_9)COOH$

3320	SSh	1505	SSh	1145	SSh	840	SSh
1725	SSh	1440	SSh	1130	SSh	820	SSh
1660	SSh	1350	SSh	1090	MSh	765	SSh
1640	SSh	1330	SSh	1060	MSh	745	SSh
1620	SSh	1310	SSh	1035	WSh	720	SSh
1595	SSh	1285	SSh	970	WB	705	SSh
1555	SSh	1240	SSh	925	SSh	660	SSh
1520	SSh	1175	SSh	875	WSh		



Spectrum No. 71
2,4-Dinitrophenyl-L-isoleucyl-L-phenylalanyl-L-leucine

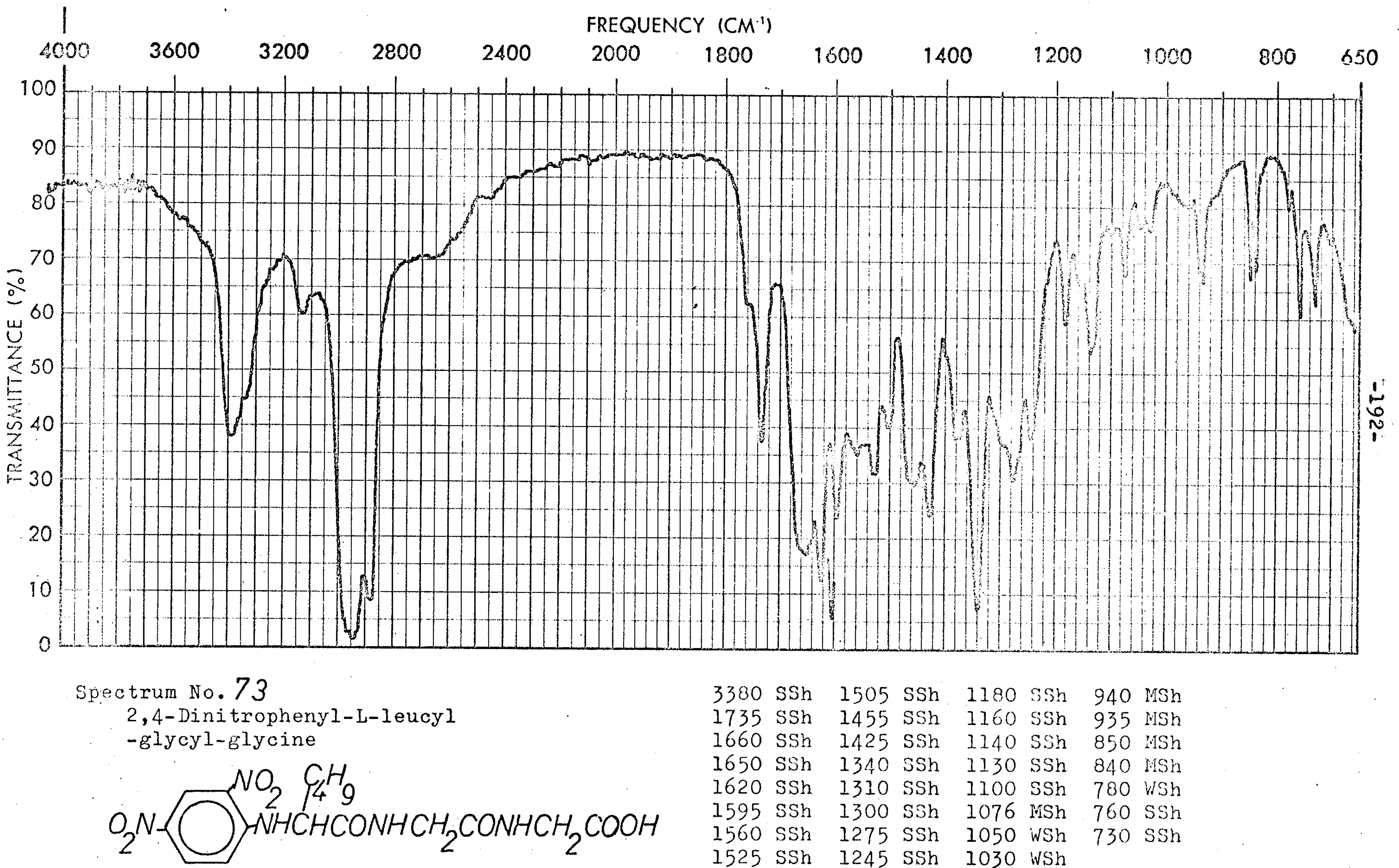
3320	SSh	1505	SSh	1275	SSh	1095	MSh	840	MSh
1720	SSh	1470	SSh	1250	SSh	1075	MSh	780	MSh
1670	SSh	1450	SSh	1230	SSh	1050	WB	760	SSh
1650	SSh	1390	SSh	1210	SSh	980	WB	730	SSh
1625	SSh	1345	SSh	1180	MSh	965	WB	715	SSh
1600	SSh	1330	SSh	1145	MSh	940	MSh	665	SSh
1550	SSh	1320	SSh	1135	MSh	930	MSh		
1530	SSh	1285	SSh	1110	MSh	850	MSh		



Spectrum No. 72
2,4-Dinitrophenyl-L-valyl
-glycyl-glycine

$O_2N-C_6H_3(NO_2)-NHCH(C_3H_7)CONHCH_2CONHCH_2COOH$

3350	SSh	1505	SSh	1280	SSh	1085	MSh	860	MSh
1750	SSh	1440	SSh	1275	SSh	1075	MSh	780	MSh
1660	SSh	1430	SSh	1250	SSh	1040	MSh	760	MSh
1650	SSh	1405	SSh	1230	SSh	1000	MSh	745	MSh
1620	SSh	1370	SSh	1180	SSh	935	MSh	730	MSh
1595	SSh	1340	SSh	1160	SSh	910	MSh	660	SSh
1560	SSh	1330	SSh	1135	SSh	890	WSh		
1525	SSh	1290	SSh	1105	MSh	870	WSh		

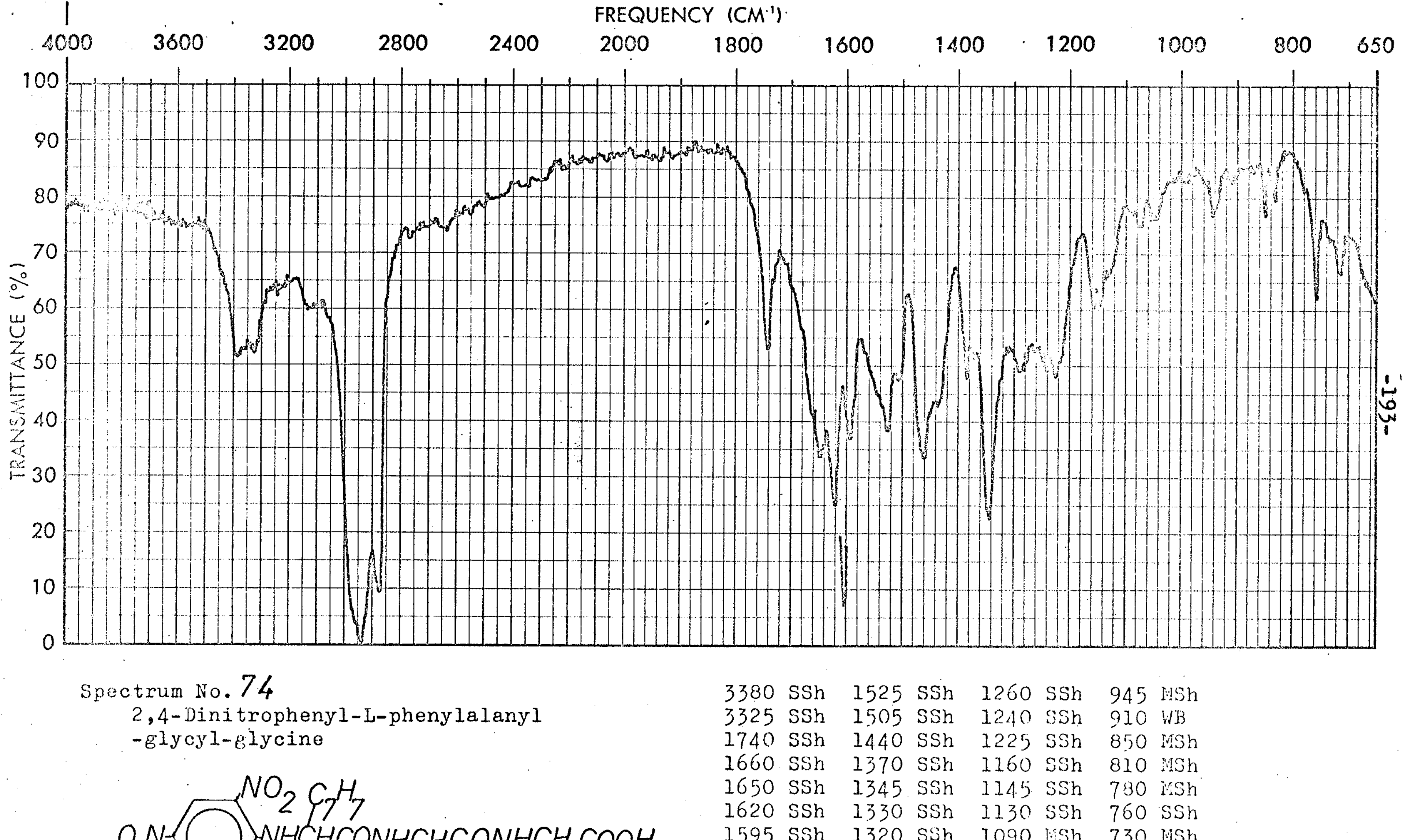


Spectrum No. 73

2,4-Dinitrophenyl-L-leucyl-glycyl-glycine

O_2N–(ring, NO_2)–$NHCH(C_4H_9)CONHCH_2CONHCH_2COOH$

3380 SSh	1505 SSh	1180 SSh	940 MSh
1735 SSh	1455 SSh	1160 SSh	935 MSh
1660 SSh	1425 SSh	1140 SSh	850 MSh
1650 SSh	1340 SSh	1130 SSh	840 MSh
1620 SSh	1310 SSh	1100 SSh	780 WSh
1595 SSh	1300 SSh	1076 MSh	760 SSh
1560 SSh	1275 SSh	1050 WSh	730 SSh
1525 SSh	1245 SSh	1030 WSh	



Spectrum No. 74

2,4-Dinitrophenyl-L-phenylalanyl-glycyl-glycine

$O_2N-C_6H_3(NO_2)-NHCH(C_7H_7)CONHCH_2CONHCH_2COOH$

3380	SSh	1525	SSh	1260	SSh	945	MSh
3325	SSh	1505	SSh	1240	SSh	910	WB
1740	SSh	1440	SSh	1225	SSh	850	MSh
1660	SSh	1370	SSh	1160	SSh	810	MSh
1650	SSh	1345	SSh	1145	SSh	780	MSh
1620	SSh	1330	SSh	1130	SSh	760	SSh
1595	SSh	1320	SSh	1090	MSh	730	MSh
1550	SSh	1290	SSh	1075	MSh	715	SSh

Part B

Introduction

The depression of the freezing point of a solvent has been used with considerable success for determination of the molecular weights of many organic compounds. Consequently, procedures are described in detail in many physical and chemical manuals. (1-5)

Generally, temperature change is measured either by a Beckmann differential thermometer (6), calibrated thermocouple (7), a resistance thermometer (8) or a thermistor (9). The present work used a thermistor to determine the freezing point depression of glacial acetic acid solutions, and hence the molecular weight of the solute (i.e., DNP-peptides).

Freezing point depression and concentration of solution are related in accordance with equation (1)

$$\Delta T_f = \frac{RT_o^2}{\Delta H} \frac{m}{m + 1000/M_A} \qquad \text{----------- \underline{equation} (1)}$$

where ΔT_f is the freezing point depression, T_o is the freezing point of pure solvent, ΔH its molar heat of fusion, M_A its molecular weight and m the molality of the solution. In very dilute solutions, m is negligible compared to $1000/M_A$, and the relation becomes

$$\Delta T_f = \frac{RT_o^2}{\Delta H} \frac{mM_A}{1000} = K_f m \qquad \text{--------- \underline{equation} (2)}$$

where K_f is a constant of the solvent, defined by

$$K_f = \frac{M_A RT_o^2}{1000 \Delta H}$$

When a thermistor of high resistance is used to measure this relatively small temperature change, the change in resistance becomes essentially proportional to temperature change. Over a temperature interval of several degrees the thermistor resistance can be expressed quite accurately by

$$\ln \frac{r}{r_o} = B\left(\frac{1}{T} - \frac{1}{T_o}\right) = B\left(\frac{\Delta T_f}{T_o^2}\right) \text{ -------- } \underline{\text{equation}}\ (3)$$

where r is the resistance of the thermistor at T, r_o the resistance at T_o and B a constant of the thermistor.

Substituting for ΔT_f in terms of the thermistor resistance in equation (2) gives

$$\ln \frac{r}{r_o} = \frac{RBmM_A}{1000\,\Delta H} \text{ ----------------- } \underline{\text{equation}}\ (4)$$

Equation (4) is the resistance-concentration relation found most convenient for use of the thermistor as temperature-sensitive element in the present work.

Greater ease in calculating solute molecular weights from freezing point depression can be achieved by rewriting equation (4) as

$$M_B = \frac{W_A}{W_B} \cdot \frac{RBM_A}{\Delta H} \Big/ \ln \frac{r}{r_o} \text{ ----------- equation (5)}$$

where M_B is the molecular weight of solute, W_A the weight of solute, and W_B the weight of solvent.

The object of this part of the present work was to determine the feasibility of calculating molecular weights of peptide fragments by freezing point depression principles, from their DNP-derivatives, and

to find the equivalent molality of solutions containing mixtures of DNP-peptides as an approximate guide to the number of peptide fragments present. Recognizing the need for adequate solubility of peptide derivative in a solvent of adequate freezing point depression constant, these studies were undertaken, utilising the thermistor as temperature-sensitive element. Recognizing also that a peptide or protein from natural sources may only be available in limited quantity, and that peptide chain length is likely to impose limits on solubility, it was important to ascertain the lowest concentration for which accurate values could be obtained since this would represent the smallest actual weight of material on which these studies could be made.

The thermistor constant B was calculated by two methods, (a) by determining the resistance at certain definite temperatures measured by a Beckmann thermometer and (b) by finding the resistance of glacial acetic acid solutions of benzoic acid of known concentrations (three separate runs on 18 solutions of different concentration); both methods gave the same value for B.

Literature Survey

In addition to the techniques of gel filtration chromatography (10, 11) and gel electrophoresis (12-14) so often used for separating and determining molecular weights of proteins and peptides, calculation from direct measurement of osmotic pressure (15, 16) and calculation from spectroscopic data on DNP-derivatives (17-21) have received attention, and attempts have been made to use mass spectra (22, 23) to calculate peptide molecular weights.

The osmotic pressure method can be used for the determination of molecular weights of less than 1.5×10^6. However, it is difficult to prepare semipermeable membranes which would permit passage of solvent and hold back macromolecules of molecular weight below 30000. Furthermore, it is difficult to estimate accurately the degree of permeability of the membrane to a given protein and the dependence of the rate of permeation of the molecules through the membrane (for molecules of molecular weight below the limit indicated) on the molecular weight (24). Although the equipment required for osmometry is simple, accurate determinations are difficult. In order to avoid bacterial decomposition of the protein, the determinations must be made at low temperatures and over short periods of time. Less than 0.5 ml of solution is required per measurement, and the result extremely accurate.

Insulin had long been regarded as having a molecular weight of approximately 48000 in concentrated solutions, 12000 in dilute solutions and 6000 in non-aqueous solution. Following Sanger's (17) demonstration that insulin contains two peptide chains, with N-terminal glycine and N-terminal phenylalanine as the only N-terminal amino acids in the

molecule, dinitrophenylation and subsequent calculations based on absorption data in the 350 to 360 mμ range with a molecular extinction coefficient in the range of 15000-16000, led to a revised value of 6450 as the correct order of magnitude for the molecular weight, detailed knowledge of the exact amino acid sequence of the two chains leading to a value of 5733 as the actual molecular weight of a single insulin molecule (25). From the value obtained, the spectroscopic method will give the molecular weight of DNP-protein with an error close to ± 10%.

The application of spectroscopic methods to determine the molecular weights of some proteins were reported by Craig (18-21). By using a molecular extinction coefficient of 14500 and measuring the molar absorbance of the DNP-proteins at 350 mμ, a value of 1300, calculated 1142 for gramicidin S (18); a value of 1353, calculated 1270 for tyrocidine A (19); a value of 1470, calculated 1411 for Bacitracin A (20) and a value of 1340, calculated 1420 for Bacitracin B (21) were obtained; these values gave an error close to ± 10%.

Kamerling et al (22) and Aplin et al (23) attempted to use the mass spectra to calculate peptide molecular weights. Important observations were (a) the method appeared satisfactory for dipeptide but not always satisfactory for tripeptides (vapor pressure too low); (b) some peptide derivatives were prepared to obtain compounds of some what higher vapor pressure (i.e. ethoxycarbonyl peptide methyl ester, acetyl-peptide methyl ester, DNP-peptide methyl ester, etc.); (c) the method was only of borderline suitability for tripeptide ester molecular weights and not at all suitable for longer chains when the

amino function was dinitrophenylated, since some DNP-tripeptide esters still have too low a vapor pressure; (d) no difficulty was encountered when the ethoxycarbonyl and acetyl groups were used for tripeptide esters and even longer chains could be used with this as the amino modifying group.

Temperature measured either by Beckmann differential thermometer, calibrated thermocouple or resistance thermometer were widely used, and different devices were described by many workers in order to measure the freezing point depression and determine the molecular weights.

Use of the thermistor to measure the freezing point depression, especially at low temperatures, did not receive too much attention before 1945. In 1949, Zeffert and Hormats (26) reported the results obtained in the application of the thermistor for the accurate determination of freezing points over an extended range at low temperature. This was further investigated by Muller and Stolten (27), who stated that high resistance thermistors are admirably suited for differential temperature measurements of a high order of sensitivity and precision. By using the thermistor to determine the freezing point depression of solvents, they found the magnitude of the signal produced by thermistors permits analytical determinations with a precision exceeding 1%.

Use of a thermistor as the temperature sensitive element in an automatic recording system was first described by Johnson and Kraus (28), who determined the freezing point depressions of aqueous uranyl fluoride solutions.

In 1956, Mc Mullan and Corbett (9) described an apparatus for the adaptation of thermistors to accurate measurement of freezing point

depression without the use of elaborate or expensive equipment. They pointed out that measurement can be made by means of a thermistor with an uncertainty less than that obtainable with the conventional Beckmann thermometer techniques. A few years later, Neumayer (29) employed isothermal distillation with two thermistors, and determined the molecular weights up to 3500 with a relative error of less than 2%.

In 1961, Wilson et al (30) used a simple apparatus for routine molecular weight determinations, they found that a highly sensitive thermistor method for molecular weight determination with water, benzene, 1,4-dioxane, carbon tetrachloride, ethyl acetate and chloroform as solvents gave the average deviation from theory of slightly less than 1%. Letton et al (31) in 1963 described an apparatus for routine determination, with reasonable accuracy, of molecular weights of some known compound in the range of 150-600, using a few milligrams of material in a very dilute solution of benzene, dioxan and nitrobenzene.

Experimental and Results

Apparatus

The apparatus (Fig 1 and 2) is essentially the same as that used by Mc Mullan and Corbett (9). The Wheatstone bridge circuit used in these experiments is shown in Fig. 1. The bridge circuit included three 1.5 volt dry cells in parallel, with a 500 ohm resistor in series as the current source, a Jay-galvanometer for the detector, two 1000-ohm standard resistors for the fixed arms and two decade boxes (1-, 10-, 100-, 1000- ohm steps) in parallel for the variable resistance.

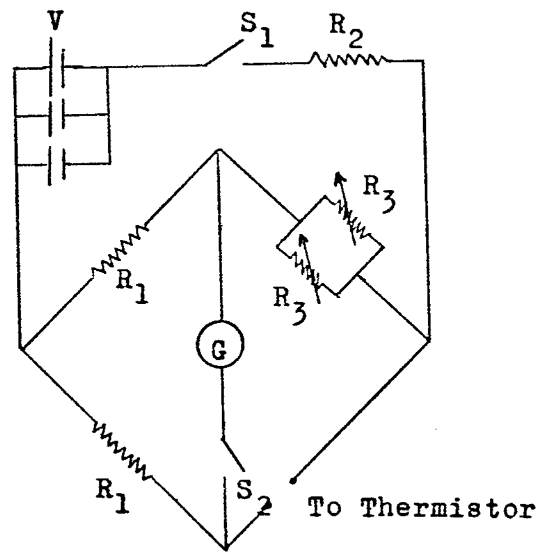


Figure 1. Wheatstone bridge circuit.

Where V: three 1.5 volt dry cells; S_1 and S_2: single-pole, single-throw switch; R_1: 1000 ohm standard resistor; R_2: 500 ohm carbon resistor; R_3: decade resistance box (1-, 10-, 100-, 1000- ohm steps); G: galvanometer, Jay Instruments.

The cryometric cell and thermistor assembly are shown in Fig. 2. The cryometric cell employed was of all-glass construction, an air jacket surrounds the sample chamber and can be evacuated to provide

better insulation. The thermistor, which is attached through a Wheatstone bridge circuit to a galvanometer is placed in a thin wall glass tube, and the bottom inch of this tube was filled with petroleum ether for better thermal contact with the system. The solution in the sample chamber is stirred magnetically with a Teflon-covered stirring bar and the cooling bath is about 3° below the freezing point of the pure solvent.

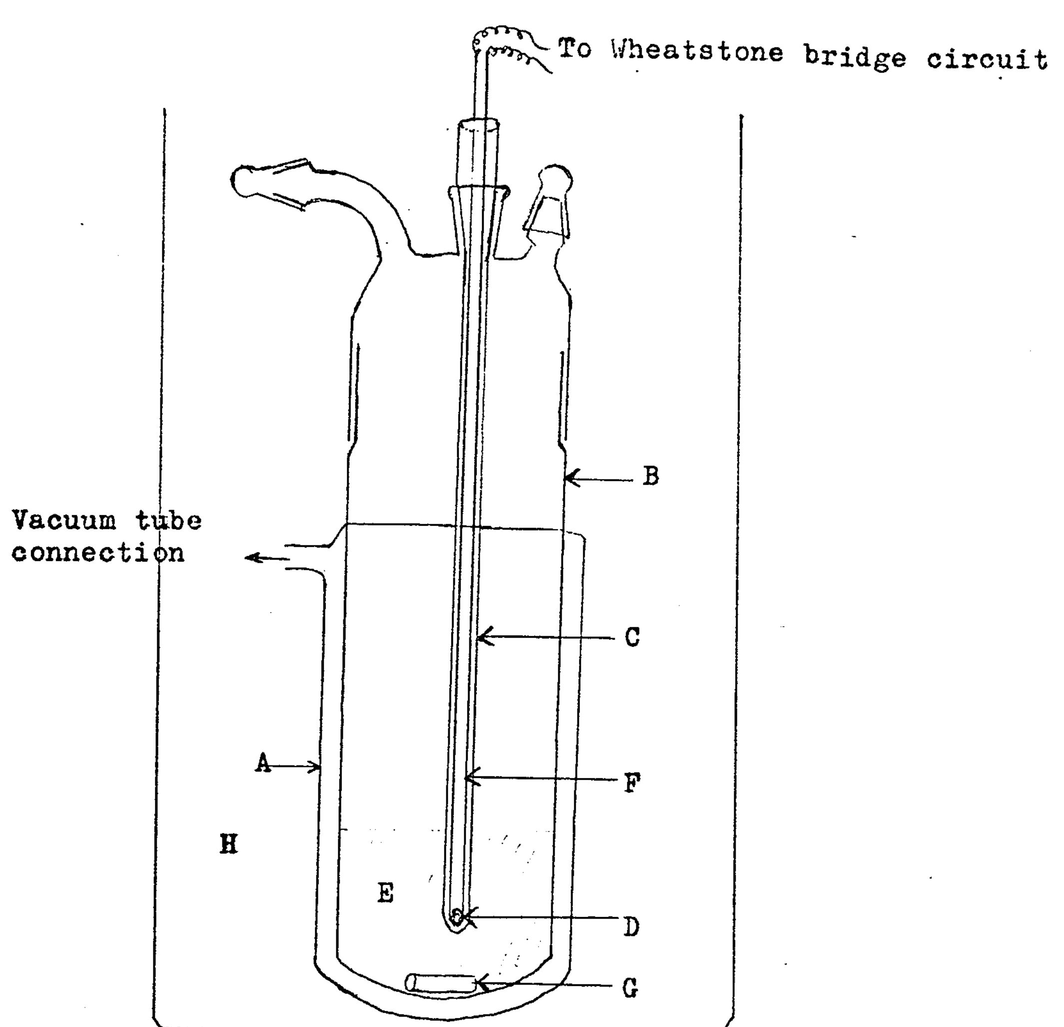


Figure 2. Freezing point cell.
Where A: 8 mm thick air jacket; B: sample chamber with a diameter 35mm; C: 7mm glass; D: Thermistor, carbaloy D-102; E: solvent; F: Petroleum ether; G: Teflon-covered stirring bar; H: constant temperature water bath.

Experimental procedure and results

Calibation of thermistor

A. From Beckmann differential thermometer

This need be done only over a narrow range (1-3°C) below the freezing point of the solvent. A Beckmann differential thermometer was set to give a reading at the freezing point of the solvent. The thermistor, which was placed in a thin glass tube containing petroleum ether, and the Beckmann thermometer were inserted into the sample chamber of the cryometric cell, at the bottom of which is a small Telfon-coated magnetic bar, and a volume of solvent (i.e. glacial acetic acid) sufficient to cover the thermistor and the bulb of the thermometer. The cryometric cell was placed into the cooling bath which was well circulated and already prepared and adjusted to give a temperature of not more than 3°below the freezing point of the solvent. The bath rests just above the surface of a rapid rotatory magnet which is switched on, and after a convenient time, the air jacket of the cell was evacuated, readings on the Beckmann thermometer were taken at 30 seconds intervals simultaneously with the readings of the resistance in ohms, until the solvent was about 0.1° below its normal freezing point. A minute crystal of solvent was added by means of a long handled spoon from the top of the cell to induce crystallization, and the temperature rose to the freezing point, which was then recorded from both the Beckmann thermometer and the thermistor.

The apparatus was carefully removed from the cooling bath, the insulating jacket opened and the solvent warmed at room temperature with stirring until the crystals were melted. About 50 milligrams

of a solid (i.e. benzoic acid) were added by means of the spoon, and the freezing point determined as before. This addition and freezing were repeated a further four times. From the relations between the resistance of the thermistor and the change in temperature, we can calculate B the constant of the thermistor.

In this experiment, glacial acetic acid was used as the solvent, and benzoic acid used as the solute, and the constant B was obtained from the equation (3)

$$B = \ln \frac{r}{r_o} \left(\frac{TT_o}{\Delta T_f} \right) \quad \text{--------- equation (3)}$$

The results are shown in Table 1.

Table 1

Calibration of the thermistor by Beckmann differential thermometer

r_o = 1454.22 ohms. T_o = 289.78 °K.

r (ohms)	T (°K)	$\ln \frac{r}{r_o}$ ($\times 10^{-2}$)	ΔT_f (°K)	TT_o ($\times 10^4$)	B ($\times 10^3$)
1469.03	289.555	1.01326	0.225	8.391	3.779
1482.35	289.355	1.91590	0.425	8.385	3.780
1501.25	289.075	3.18284	0.705	8.377	3.782
1529.10	288.670	5.02097	1.110	8.365	3.784
1556.95	288.270	6.82591	1.510	8.354	3.776
1587.92	287.840	8.79553	1.940	8.341	3.782
					Ave.=3.780

B. From a known concentration of sample.

In the calibration of a thermistor, it is not necessary to use the Beckmann thermometer. From the relations between the concentration and the ratio of the resistance r at the freezing point of a given solution to the resistance r_o for the pure solvent at the start of the measurement, it is easy to calculate the thermistor constant by equation (4).

$$B = \frac{1000\,\Delta H}{RM_A m} \ln \frac{r}{r_o} \qquad \text{--------- equation (4)}$$

For glacial acetic acid, where $\Delta H = 2.803 \times 10^3\,{}^\circ K$ cal $mole^{-1}$(32); R is the ideal gas constant and is equal to 1.987 cal. ${}^\circ K^{-1} mole^{-1}$; M_A is the molecular weight of glacial acetic acid and is equal to 60.052; m is the number of moles of solute per 1000 grams of solvent.

Procedure

A known weight of glacial acetic acid (30 grams) was placed in the sample chamber and a Telfon coated magnetic bar was on the bottom of the chamber. The thermistor was slipped into the thin glass tube, which fits into the cryometric cell. The cryometric cell was placed in the cooling bath and the resistance at the freezing point of the solvent was determined as described above. Then, the apparatus was carefully removed from the cooling bath, the solvent was warmed as described above, 15-30 milligram of pure benzoic acid placed in a spoon and weighed accurately on a microbalance, the spoon carefully introduced via the top of the cell and its content emptied into the solvent by rotating the spoon, which was withdrawn carefully and reweighed. The resistance at the freezing point of the solution

was then determined as before. The addition and freezing were repeated a further four times and the constant B was then calculated from the above equation. The results are shown in Table 2.

Table 2

Calibration of the thermistor by known concentration of sample.

r_o (ohms)	m X10^{-2} (moles)	r (ohms)	ln $\frac{r}{r_o}$ (X10^{-3})	B X10^3
1455.05	0.52	1456.27	0.8381	3.786
	1.06	1457.53	1.7030	3.774
	2.10	1459.96	3.3688	3.768
	3.05	1462.22	4.9156	3.786
	4.22	1464.99	6.8082	3.790
	5.58	1468.13	8.9492	3.767
				Ave.= 3.778
1454.88	0.65	1456.40	1.0442	3.774
	1.17	1457.62	1.8815	3.778
	2.09	1459.80	3.3760	3.795
	3.22	1462.40	5.1555	3.761
	4.13	1464.60	6.6587	3.787
	5.34	1467.37	8.5483	3.760
				Ave.=3.776
1454.99	0.50	1456.17	0.8038	3.776
	1.12	1457.63	1.8128	3.802
	2.08	1459.86	3.3415	3.774
	3.31	1462.75	5.3192	3.775
	4.25	1464.93	6.8084	3.763
	5.18	1467.14	8.3159	3.771
				Ave.=3.777
				Total ave.=3.777

Determination of Molecular weight

A. For DNP-peptides

The molecular weight of eight of those previous prepared DNP-peptides were determined by freezing point depression method. The procedure was carried out exactly as the method B for the calibration of the thermistor, using glacial acetic acid as the solvent and DNP-peptides as the solute. Five consecutive additions of weighed sample were made to the solvent and the above procedure was repeated after each addition. The molecular weight of those DNP-peptides were calculated from equation (5), and a measured value of B of 3.777×10^3 degrees obtained as described above was used.

$$M_B = \frac{W_B}{W_A} \cdot \frac{RBM_A}{\Delta H} \Big/ \ln \frac{r}{r_o} \quad \text{------ equation (5)}$$

The results are shown in Table 3.

Table 3

Molecular weight determinations of DNP-peptides by freezing point depression method.

Compound	W_B(gm.)	r(ohms)	$\ln\frac{r}{r_o} \times 10^{-3}$	Molecular weight Theor.	Found	Error%
DNP-L-leu-L-leu	0.0639	1462.31	0.8073	410.44	414.72	+1.04
r_o = 1461.13 ohms	0.1329	1463.57	1.6685		417.31	+1.67
W_A = 30.6885 gm.	0.2427	1465.62	3.0683		414.43	+0.97
	0.3828	1468.24	4.8543		413.16	+0.66
	0.4986	1470.28	6.2427		418.46	+1.95
	0.6291	1472.66	7.8602		419.33	+2.17
					Ave.=416.23;	+1.41

Table 3 (Continued)

Compound	W_B(gm.)	r(chms)	$\ln\frac{r}{r_o} \times 10^{-3}$	Molecular weight Theor.	Found	Error%
DNP-L-ala-L-phe	0.0576	1453.30	0.7710	402.37	398.38	-0.99
r_o=1452.18 ohms	0.1173	1454.40	1.5276		409.45	+1.76
W_A= 30.1538 gm.	0.2346	1456.59	3.0322		412.55	+2.53
	0.3612	1458.97	4.6648		412.87	+2.61
	0.4755	1461.28	6.2469		405.88	+0.87
	0.6048	1463.61	7.8401		411.34	+2.23
					Ave.=408.41;	+1.50
DNP-L-val-L-leu	0.0714	1460.03	0.9525	396.41	401.46	+1.27
r_o=1458.64 ohms	0.1248	1461.10	1.6851		386.64	+0.06
W_A= 30.0226 gms	0.2355	1463.24	3.1487		400.56	+1.05
	0.3639	1465.67	4.8080		405.34	+2.25
	0.4662	1467.64	6.1512		405.89	+2.39
	0.5910	1470.23	7.9144		399.92	+0.86
					Ave.=401.64;	+1.32
DNP-L-phe-gly	0.0642	1457.70	0.8648	388.35	391.10	+0.71
r_o=1456.44 ohms	0.1305	1458.97	1.7356		395.10	+1.99
W_A= 30.5210 gm.	0.2436	1461.21	3.2698		392.47	+1.06
	0.3685	1463.73	4.9929		385.96	-0.62
	0.4731	1465.72	6.3515		392.40	+1.04
	0.5964	1468.00	7.9058		397.41	+2.33
					Ave.=392.57;	+1.09

Table 3 (Continued)

Compound	W_B(gm.)	r(ohms)	$\ln\frac{r}{r_o} \times 10^{-3}$	Molecular weight Theor.	Found	Error%
DNP-L-Ileu-L-phe	0.0740	1454.10	0.8384	444.46	446.46	+0.45
r_o=1452.88 ohms	0.1374	1455.17	1.5749		441.80	-0.60
W_A= 31.7504 gm.	0.2679	1457.33	3.0582		443.62	-0.19
	0.3972	1459.39	4.4707		449.91	+1.23
	0.5199	1461.50	5.9155		445.07	+0.14
	0.6507	1463.53	7.3035		451.18	+1.51
					Ave.=446.34;	+0.42
DNP-L-ala-L-phe-gly	0.0663	1461.83	0.7596	459.43	460.37	+0.20
	0.1323	1462.96	1.5323		455.40	-0.87
r_o=1460.72 ohms	0.2646	1465.18	3.0486		457.79	-0.36
W_A= 30.4836 gm.	0.4122	1467.70	4.7671		456.08	-0.73
	0.5418	1469.91	6.2717		455.65	-0.82
	0.6879	1472.48	8.0186		452.49	-1.51
					Ave.=456.30;	-0.68
DNP-L-val-L-leu-L-phe	0.0930	1464.60	0.8675	543.59	539.22	-0.80
	0.1695	1465.62	1.5637		545.22	+0.30
r_o=1463.33 ohms	0.3261	1467.76	3.0228		542.63	-0.18
W_A= 31.9665 gm.	0.4874	1470.10	4.6158		542.02	-0.29
	0.6507	1472.28	6.0976		536.76	-1.26
	0.8202	1474.52	7.6179		541.55	-0.37
					Ave.=541.23	-0.43
DNP-L-Ileu-L-phe	0.0819	1456.67	0.7417	557.62	551.19	-1.15
-L-leu	0.1539	1457.58	1.3662		562.29	+0.84
r_o=1455.59 ohms	0.3297	1459.89	2.9498		557.91	+0.05
W_A= 32.2114 gm.	0.5103	1462.28	4.5855		555.49	-0.39
	0.6876	1464.56	6.1435		558.67	+0.19
	0.8766	1466.96	7.7809		562.35	+0.85
					Ave.=557.98;	+0.06

B. For the mixture of DNP-peptides

The mean molecular weight of the mixture of eight DNP-peptides was determined, the procedure was the same as described above. The resistance at the freezing point of pure glacial acetic acid was measured, then 0.005 mole of each DNP-peptides were added together to the glacial acetic acid, the resistance at the freezing point of the solution was measured again, and the molecular weight was calculated. Three separate runs on the mixtures were made, the results of the mean molecular weight determinations are shown in Table 4.

Table 4

Mean molecular weight of DNP-peptides mixture by freezing point method.

Run	r_o(ohms)	W_A(gm.)	W_B(gm.)	r(ohms)	Mean molecular weight Theor.	Found	Error%	m $X10^{-2}$
1	1456.68	32.2805	0.5815	1466.23	450.33	443.24	-1.57	4.06
2	1455.30	30.1947	0.5439	1464.57	450.33	456.13	+1.29	3.95
3	1457.29	30.7532	0.5540	1466.63	450.33	453.37	+0.68	3.97
						Ave.=450.91;	+0.13;	3.99

Where m = the equivalent molality of DNP-peptides in the mixture found by the freezing point depression method.

C. For DNP-poly-L-valine

The molecular weight of poly-L-valine or its DNP-derivative was not known. In the present studies, we attempted to use freezing point depression method to determine this molecular weight. Unfortunately, DNP-poly-L-valine does not dissolve in glacial acetic acid, and a suitable solvent could not be found for the molecular weight determination. An indirect method was used, DNP-poly-L-valine was

hydrolyzed by concentrated hydrochloric acid, and the hydrolysate dinitrophenylated with FDNB. The product was then used for molecular weight determination by freezing point depression method.

(1) Preparation of DNP-poly-L-valine

Crystalline poly-L-valine[1] (0.5 gram) and 1 gram sodium bicarbonate were dissolved in 10 ml. of water, 20 ml. ethanol and 0.5 ml. FDNB added, and the mixture stirred mechanically for two hours at room temperature. The DNP-poly-L-valine which had precipitated as an insoluble yellow powder was centrifuged down and washed with water, ethanol and ether, then dried under vacuum. The yield of the product was 0.48 gram.

With a SP. 800 spectrophotometer, the ultraviolet absorption of DNP-poly-L-valine in 96% sulfuric acid at 338 $m\mu$ was measured, using the Beer-Lambert equation A = abc, where A = absorbance; b = the cell length = 1 cm.; c = the concentration of the solution; a = molecular extinction coefficient[2] = 16000. The molecular weight of DNP-poly-L-valine was obtained as follows:

DNP-poly-L-valine (10 milligram) was dissolved in 100 ml. of 96% sulfuric acid and the ultraviolet absorption of the solution at 338 $m\mu$ was measured, a value of 0.952 for the absorbance was obtained. From the above equation, a value of 5.95×10^{-5} M was obtained for the concentration of the solution, therefore, the molecular weight of DNP-poly-L-valine is equal to $0.1/5.95 \times 10^{-5} \doteq 1680$.

(2) Hydrolysis of the DNP-poly-L-valine

DNP-poly-L-valine (100 milligram) and 10 ml. 12 N hydrochloric acid were heated in a sealed evacuated tube for 16 hours at 105°. After

[1] From Nutritional Biochemicals Corporation, molecular weight 1000-5000.

[2] An average value of DNP-L-val-gly (16530), DNP-L-val-L-leu (15600), DNP-L-val-L-phe (15700) and DNP-L-val-L-leu-L-phe (16130) found in 97% sulfuric acid solution.

cooling, it was extracted three times with 25 ml. ether. The ether extract was evaporated to dryness in vacuo and the DNP-L-valine was purified by preparative thin-layer chromatography. The adsorbent used was SilicAR TLC-7GF, the layer was 2 mm., and the sample was applied in a narrow band, and developed in the solvent (chloroform/methanol/glacetic acetic acid; 95:5:1). After developing, the adsorbent containing the desired component was scraped from the glass plate, and extracted thrice with ether, the solvent evaporated, and the residue used for ultraviolet absorption measurement.

The amount of DNP-L-valine obtained from hydrolysis was estimated spectroscopically. DNP-L-valine was dissolved in 100 ml. glacial acetic acid, and 10 ml. of the resulting solution was diluted to 100 ml. With a spectrophotometer, the ultraviolet absorption at 338 $m\mu$ was measured. By using the above equation and a measured value of 17200 for the molecular extinction coefficient, the concentration of DNP-L-valine in glacial acetic acid solution was calculated, therefore the molecular weight of DNP-poly-L-valine. The results are shown in Table 5.

Due to the instability of DNP-L-valine during hydrolysis, a survey was made of the rates of breakdown of this compound. A standard solution was made of DNP-L-valine in 12 N hydrochloric acid, and some poly-L-valine was added in case the rate of breakdown was influenced by the presence of hydrolytic products of the peptide, and the mixture was heated at 105° in a sealed tube for the required time. The hydrolysate was extracted and purified as previously described. The amount of DNP-L-valine recovered was measured spectroscopically,

and it was found that 88% of DNP-L-valine remained unchanged after the hydrolysis. This correction factor was used to calculate the amount of DNP-L-valine obtained after the hydrolysis of DNP-poly-L-valine.

Table 5

Molecular weight of DNP-poly-L-valine obtained by spectroscopic method.

Run	a	A	c ($\times 10^{-5}$)	m_1 ($\times 10^{-5}$)	m_2 ($\times 10^{-5}$)	Molecular weight DNP-poly-L-valine	Molecular weight poly-L-valine
1	17200	0.93	5.41	5.41	6.14	1627.53	1461.43
22	17200	0.96	5.58	5.58	6.34	1576.66	1410.56
3	17200	0.91	5.29	5.29	6.01	1663.30	1497.20
						Ave.=1622.50;	1456.40

Where a = molecular extinction coefficient; A = absorbance;

c = concentration of DNP-L-valine in glacial acetic acid solution;

m_1 = total amount of DNP-L-valine;

m_2 = total amount of DNP-L-valine after correction = $m_1 \times 100/88$;

molecular weight of poly-L-valine = molecular weight of DNP-poly-L-valine - 166.10

(3) Dinitrophenylation of DNP-poly-L-valine hydrolysate

The hydrolysate after extraction with ether was diluted with 20 ml. of water, the solvent was distilled under reduced pressure at 60°, and to the residue was added 10 ml. of water and 0.8 gram of sodium bicarbonate. The mixture was stirred at room temperature for 5 minutes, and 0.2 gram of fluorodinitrobenzene in 20 ml. ethanol was added. The solution was stirred two hours at room temperature in the absence of light. After the reaction was completed, the alcohol was removed by vacuum distillation

at 40° , and the excess of fluorodinitrobenzene was extracted thrice with 20 ml. of ether. The solution was acidified with concentrated hydrochloric acid to pH 2, and the DNP-L-valine extracted thrice with 25 ml. ether. The ether solution was washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated to dryness under vacuum, and the residue was purified by the preparative thin layer chromatography method used above. The yellow crystals of DNP-L-valine were used for freezing point depression determinations.

(4) Determination of freezing point depression of the dinitrophenylated products.

The resistance of 30 grams (accurately weighed) of glacial acetic acid at the freezing point was measured by the method as described above; to this DNP-L-valine obtained from dinitrophenylation of the hydrolysate was added, and the resistance at the freezing point of the solution was measured again. From the freezing point depression, the number of moles of DNP-L-valine present was calculated from equation (10), and the molecular weight of DNP-poly-L-valine was calculated as follows:

The structural formula of DNP-poly-L-valine is

$$\text{DNP-NH}\underset{|}{\overset{C_3H_7}{\text{CH}}}\text{CO-(NH}\underset{|}{\overset{C_3H_7}{\text{CH}}}\text{CO-)}_y\text{OH}$$

The number of moles of DNP-poly-L-valine in 100 milligram is equal to

$$N_B = (0.1/x)\, y \text{ ----------------------- (a)}$$

Also

$$x = 283.32 + 99.13\, y \text{ ---------------- (b)}$$

Where N_B = number of moles of DNP-L-valine = m • 30/1000;

x = molecular weight of DNP-poly-L-valine;

the molecular weight of DNP-NHCH(C_3H_7)COOH = 283.32

and -NHCH(C_3H_7)CO- = 99.13.

From equation (a) and (b),

$$x = \frac{28.332}{0.1-99.13N_B}$$

The results are shown in Table 6.

Table 6

Molecular weight of poly-L-valine obtained by freezing point depression method.

Run	r_o (ohms)	r (ohms)	B ($X10^3$)	m ($X10^{-2}$) mole	N_B ($X10^{-4}$) mole	Molecular weight DNP-poly-L-valine	poly-L-valine
1	1458.18	1464.80	3.777	2.817	8.45	1745.66	1579.56
2	1460.21	1466.77	3.777	2.788	8.364	1657.81	1491.71
3	1460.33	1466.94	3.777	2.809	8.427	1721.26	1555.16
						Ave.=1708.24;	1542.14

Where ln 1464.80/1458.18 = 4.52963×10^{-3};

ln 1466.77/1460.21 = 4.48244×10^{-3};

ln 1466.94/1460.33 = 4.51616×10^{-3}.

Discussion

A very simple apparatus has been adapted to routine molecular weight determination of peptide fragments (from freezing point data for their DNP-derivatives), giving results of reasonable accuracy. The thermistor is a very simple device to use as the temperature-sensitive element in cryoscopy, requiring very little attention or manipulation. Over a temperature range of approximately 2 °C, the resistance-temperature behavior of the thermistor is described by an equation involving only one constant, and over a period of several months the calibration remained unchanged. Calibration by direct measurement of thermistor resistance and of temperature (measured by a Beckmann thermometer) at the freezing point of glacial acetic acid gave a value of 3.78×10^3 degrees for B, and calibration of resistance with solutions of benzoic acid at different concentrations in glacial acetic acid (three separate runs were made, each involving six different concentrations) gave a weighted average value of 3.777×10^3 degrees for B.

Glacial acetic acid is a good solvent for DNP-dipeptides and DNP-tripeptides, has a fairly large freezing point depression constant, and the solutions appear to obey Raoult's Law in dilute solution. The resistance of pure solvent at its freezing point and of the solutions at their respective freezing points were measured to $\pm$ 0.01 ohm, and the temperature coefficient of resistance of solvent at its freezing point was 54 ohms per °C, indicating the precision of the data to be of the order of 0.0002 °C or better.

The consistency of the results obtained would seem to indicate that the molecular weights of DNP-peptides can be determined by this

method to within ± 1.5% of the correct value, down to a concentration of 5 X 10^{-3} molal with the apparatus described, using only 1.5 X 10^{-4} mole of material. This represents the lowest order of magnitude of concentration for which reliable results were obtained, but construction of a cryometric cell with narrower cross section would permit the handling of smaller volumes of solution, hence the examination of still smaller quantities of solute, and a thermistor of greater resistance-temperature coefficient would be helpful in the study of still more dilute solutions. The availability of a cell capable of handling smaller volumes of solution together with a thermistor of greater resistance-concentration sensitivity would enable very small quantities of material to furnish results of reasonable accuracy.

When compared with the spectroscopic calculation of molecular weights, using the assumed average value of 16000 for $\mathcal{E}_{max}$, the results obtained spectroscopically for the DNP-dipeptides and DNP-tripeptides are in general 10 to 20% lower than the correct value; in general, spectroscopically determined molecular weights reported in the literature for peptides and proteins have been regarded as having ± 10% accuracy, requiring use of other related information to obtain the correct value. If the identity of a peptide is known and the amount below the limit which can be used for cryoscopic study, the correct value of $\mathcal{E}_{max}$ for that compound must be used for its quantitative estimation. If it is unknown (hence $\mathcal{E}_{max}$ is unknown) and the amount is within the range which can be used for cryometric study, its molecular weight could probably be calculated more accurately by this adaptation of freezing point depression principles than from spectroscopic data.

The average of all value of $\mathcal{E}_{max}$ tabulated in Tables 42 to 44 and 46 to 48 for the free DNP-peptides studied are 17849 in 95% ethanol, 16815 in glacial acetic acid and 17751 in 4% sodium bicarbonate solution respectively. Recalculation of DNP-peptide molecular weights after rounding off these coefficients to 18000, 17000 and 18000 respectively gives molecular weights of accuracy ranging from $\pm$ 4% in 95% ethanol, $\pm$ 2.33% in glacial acetic acid and $\pm$ 2.91% in 4% sodium bicarbonate solution. The molecular weight of a DNP-peptide fragment could probably be calculated therefore to $\pm$ 5% of the correct value by using the assumed $\mathcal{E}_{max}$ value for the solvent used, if the identity were unknown and the amount too small for cryoscopic study.

When compared with attempts to determine peptide molecular weights from the mass spectra of the DNP-derivatives (22), results of reasonable accuracy were obtained cryoscopically for the DNP-tripeptides without any difficulty, even though the carboxyl group was free. This compares favourably with the difficulties encountered with DNP-tripeptides in mass spectra studies, even when the carboxyl group was modified.

The DNP-tripeptides examined in this work appeared to dissolve more slowly in glacial acetic acid than did the other DNP-compounds, and all DNP-peptide esters apparently dissolved in this solvent more rapidly than any free DNP-peptide. Although DNP-peptides appear capable of giving relatively more concentrated solutions in tertiary butyl alcohol, it was glacial acetic acid which was found most suitable as solvent for the cryoscopic studies. These observations indicate that molecular weight determinations on longer peptide chains (as DNP-derivatives) would require modification of the carboxyl group, also, if dinitro-

phenylation were chosen to modify α-amino function.

The freezing point depression data obtained from the mixture of DNP-peptides in glacial acetic acid indicate that no matter what interaction may occur between the solutes, no complication exists in calculating the equivalent molality of the mixture in glacial acetic acid solution. This finding, together with the observation that all peptides mentioned in this study can eventually be separated from one another, identified and estimated, indicates that considerable insight into the structure of a large peptide could be gained if a mixture of this type resulted from fragmentation of the large peptide. Further study involving the fragmentation of a large peptide into such a mixture and examining the mixture by combined cryoscopic and thin layer chromatography studies would be necessary to test this deduction to its fullest extent, and substantial confirmation was afforded by the studies involving DNP-poly-L-valine.

The molecular weight of poly-L-valine (as DNP-poly-L-valine) could not be determined directly from freezing point depression data, DNP-poly-L-valine being insoluble in acetic acid (presumably due to peptide chain length), so the direct determination with the chain still intact was made spectroscopically after dinitrophenylation, giving a molecular weight of 1514 for poly-L-valine. The chain would therefore seem to possess some 15 amino acid residues; if the result were accurate to $\pm$ 5%, it would still be correct to less than one amino acid residue. When the DNP-peptide was hydrolysed and the N-terminal DNP-L-valine estimated spectroscopically, the molecular weight of poly-L-valine (after correcting for possible breakdown

of derivative) was calculated to be in the 1400 to 1500 range; the indirect spectroscopic calculation gives 13 to 15 as the number of amino acid residues.

Removal of the DNP-L-valine from the hydrolysate, dinitrophenylation of the remainder, and calculation of the number of DNP-L-valine residues in the latter by the cryoscopic method led to an estimate of 14 amino acids; this together with what had been previously removed gave 15 as the total number of amino acid residues in the poly-L-valine, and a calculated average molecular weight of 1542. Although this cryoscopic calculation was indirect, the result is much more closely consistent with the value of 1514 noted in the previous paragraph. Apparently molecular weight of a larger peptide can be calculated more accurately by the freezing point depression method (even though it has to be done indirectly) than by the indirect spectroscopic calculation.

Conclusion

Peptide fragments can be separated and identified as their DNP-derivatives by thin layer chromatography; their quantitative estimation by spectroscopic means requires an accurate knowledge of their respective values of ε_{max} in the solvent used.

When molecular weight can be determined directly, by cryoscopic means, using the DNP-derivatives, the method is at least as satisfactory as spectroscopic calculation, if not better. It is certainly easier to determine tripeptide molecular weights with reasonable accuracy from the DNP-derivatives by the cryoscopic method than to study their mass spectra, because the vapour pressure problems encountered in the latter study do not present any problem in the cryoscopic study. When cryoscopic calculation of molecular weight has to be made indirectly on a large fragment, the result is more consistent with a direct spectroscopic calculation than is an indirect spectroscopic calculation.

Indirect calculation of molecular weight from cryoscopic study of a mixture of dinitrophenylated fragmentation products, in conjunction with their separation and identification by thin layer chromatography affords a very simple and rapid means of gaining at least a partial insight into the structure of a peptide chain.

Thin layer chromatography also affords at least a partial screening of peptides (as DNP-derivatives) according to chain length.

Separation and identification of peptide fragments (as suitable derivatives) containing side chain alcoholic, side chain phenolic and side chain basic groups should be studied; some preliminary study was made of a peptide containing side chain carboxyl function in earlier

work (9).

The cryometric cell and thermistor should be modified to permit molecular weight determination on much smaller quantities of peptide and this method tested on a polypeptide substantially larger than a tripeptide, followed by selective fragmentation to ascertain its sequence (at least in part) from combined cryoscopic and chromatographic study.

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