REACTIONS OF P-PHENYLAZOBENZOYL CHLORIDE WITH AMINO ACIDS AND DIPEPTIDES

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by

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ABSTRACT

Part of the work of Karrer, Keller and Szonyi (22) has been repeated to determine whether improved yields of p-phenylazobenzoyl glycine and p-phenylazobenzoyl-DL-alanine could be obtained. An attempt was also made to adapt their procedure to the preparation of p-phenylazobenzoyl-DL-phenylalanine previously unreported. A further extension of their method was used to prepare the p-phenylazobenzoyl derivatives of four dipeptides: glycylglycine, glycyl-DL-alanine, DL-alanylglycine and DL-alanyl-DL-alanine; these compounds were not previously reported in the literature.

Unsuccessful attempts were made to prepare p-phenylazobenzoyl glycine using tertiary bases as the reaction medium.

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CHAPTER I

INTRODUCTION

Investigators, in order to separate and identify amino acids and polypeptide compounds obtained from protein hydrolysates, have used numerous procedures in order to accomplish this task.

Many types of derivatives have been used to aid in the identification and separation of amino acids and peptides. Aromatic acid halides have been used quite extensively to form these derivatives, due to (a) relative ease of formation, (b) stability of the derivatives.

Many of the reactions have been carried out using the Schotten-Baumann Reaction (11), or modifications thereof. The reaction is carried out in aqueous medium, in presence of alkali or alkali carbonate. The alkali reagent prevents formation of free hydrochloric acid, (i.e. it acts as "acid binder") and is believed also to function as a catalyst. A variation entails the use of pyridine or quinoline as the basic reagent. The variation has the added advantage of providing an anhydrous medium. This method has not yet proved effective for preparation of amino acid and peptide derivatives.

Emil Fischer (1,2) utilized the Schotten-Baumann (11) reaction in order to prepare acyl derivatives of amino acids and peptides. The derivatives, however, were primarily used

for the syntheses of larger polypeptides. Many of his condensations involved the use of α-halogen acid halides with amino acids and peptides. These α-halogen acyl peptides were then converted to their respective amino compounds, when the desired peptide had been synthesized. He also prepared aromatic sulfonyl derivatives of amino acids to protect the amino group for preparation of polypeptides.

R. Schonheimer (3) also made use of this type of reaction for synthesizing polypeptides. He found that by using p-toluene sulfonyl chloride as the acylating agent, this group could be removed by hydrolysis when the desired polypeptide had been prepared.

Acylamino acids and acylpeptides have also been prepared for other studies, some of which include:

The study of specific linkages involved in the action of certain enzyme systems, and in the hydrolysis by mild alkali (4,5,6,7).

Investigation of the antigenic effect of certain polypeptides (8).

The separation of optically active amino acids: K. Landsteiner and J. Vanderscheer (9,10).

Karrer and his associates (22) used p-phenylazobenzoyl chloride as the acylating agent for the preparation of p-phenylazobenzoyl amino acids because it was readily prepared and produced highly colored derivatives. The work

reported in this thesis was undertaken to extend this procedure to the preparation of p-phenylazobenzoyl derivatives, glycine, DL-alanine, DL-phenylalanine, and of some dipeptides.

The possibility of using an anhydrous base as the condensing agent for the acylation of glycine was also investigated.

CHAPTER II

LITERATURE REVIEW

Schotten and Baumann (11) proposed a method whereby primary alcohols, primary and secondary amines and amino acids could be converted into their respective acyl derivatives. The method consists of reacting an acid chloride with the alcohol or amino compound in the presence of aqueous alkali; in some instances aqueous sodium carbonate, or aqueous magnesium oxide, have been used instead of caustic alkali solution. The Schotten-Baumann Reaction has been used widely in the field of protein chemistry and related studies for the identification and isolation of amino acids and polypeptides.

Even though an aqueous medium is used, the acid chloride generally reacts preferentially with the amino compound. To avoid hydrolysis of the acid chloride to the corresponding acid, which would lead to a decreased yield of the acylated amino acid or polypeptide, the use of pyridine or quinoline has been proposed as the basic reagent. The advantages of such reagents are:

(a) They provide an anhydrous medium.

(b) They function both as "acid binder" and as catalysts. While often effective, this method sometimes suffers from the disadvantage that the crude product has a persistent yellow impurity not encountered in the Schotten-

Baumann procedure (31).

F. A. Menalda (12) studied the acylation of amino acids by the Schotten-Baumann procedure (11), to ascertain the optimum conditions for maximum yield of product. For the cases studied, he concluded that the following criteria must be met:

(1) The reaction temperature must be as low as possible.

(2) Equal moles of acid halide and amino acid must be used along with two moles of aqueous alkali 10%.

In order to identify amino acids and also build up a large series of polypeptides, Emil Fischer (13,14) made much use of the Schotten-Baumann reaction. He used such acyl halides as (a) β-naphthalene sulfonyl chloride, (b) benzoyl chloride, and (c) p-nitrobenzoyl chloride. The use of acyl halides to build up polypeptide compounds proved to be of limited value in that only polypeptides of a certain size could be synthesized. He also prepared p-nitrobenzoyl derivatives in order to separate the optically active forms of serine (32).

E. Abderhalden and Eugene Riesz (5) also used such acylating reagents as p-aminobenzoyl chloride, benzene-sulfonyl chloride, cresoldisulfonyl chloride and o- and p-nitrobenzoyl chloride in order to obtain a method of identifying the constituents of a mixture of amino acids and

polypeptides.

In later years G.W. Muhlemann (15) prepared a series of benzoyl amino acid esters. After mixing them, he separated them using a very tedious series of fractional crystallizations.

E. Cherbuliez and P. Plathner (16) conducted in parallel a series of condensations using aromatic and aliphatic acyl halides. They found that aliphatic acyl derivatives were much more easily separated by fractional crystallization than were the corresponding aromatic acyl derivatives.

Bernard W. Towne (17) attempted to separate mixtures of amino acids by fractional crystallization of their copper salts. This procedure proved to be very tedious and difficult to perform. Still another method of separating amino acids from protein hydrolysates was accomplished by St. J. Przylecki and K. Kaspzyk (18), using as a basis the varying amino acid solubilities in different concentrations of fatty acid solutions to effect separation.

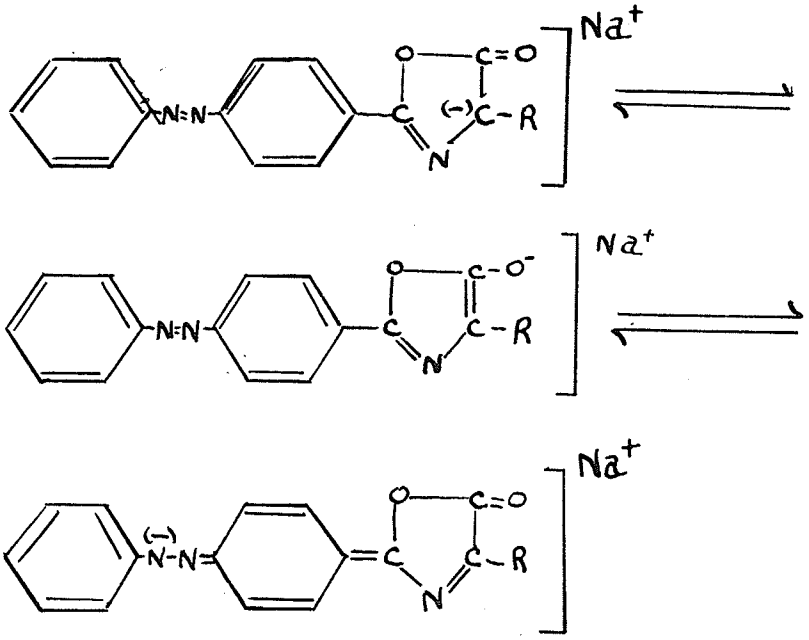
B.C. Saunders (19,20) and B.W. Towne (21), prepared a series of 3,5-dinitrobenzoyl amino acids. B.W. Towne (21) was also able to separate the optically active isomers of the valines and leucines on the basis of the different solubilities of their 3,5-dinitrobenzoyl derivatives in various aqueous organic solvent solutions at different pH

values.

In preparing the p-phenylazobenzoyl derivatives of glycine, L-leucine, L-valine and L-alanine, by the Schotten-Baumann method (11), Karrer and his associates (22) noticed the development of a deep violet color in the aqueous alkali phase; the color eventually disappeared. It is not clear from their paper whether a similar violet color developed during the preparation of p-phenylazobenzoyl L-alanine. p-Phenylazobenzoyl glycine was prepared with good yield, but the L-alanine derivative was obtained only in very low yield. The L-leucine and L-valine derivatives were obtained in small quantities, together with some optically inactive derivatives and some of the corresponding oxazolones. Hydrolysis of the oxazolones of valine and leucine gave p-phenylazobenzoyl DL-valine and p-phenylazobenzoyl DL-leucine respectively. Oxazolones of glycine and L-alanine could not be isolated. Karrer, Keller and Szonyi (22) postulated that oxazolones formed from long chain amino acids were much more stable than those from short chained amino acids.

The violet color observed in the alkaline solution during the preparation of the p-phenylazobenzoyl derivatives of glycine, L-alanine, L-leucine and L-valine was attributed to mesomerism of the anionic form of the oxazolones sodium salt.

The following structural formula represents the mesomeric structure:



In a succeeding paper, Karrer, Keller and Szonyi (23) reported the preparation of the oxazolones resulting from the condensation of p-nitrobenzoyl chloride with L-valine and L-leucine in presence of alkali. This confirmed the views expressed in their previous paper (22) on the grounds that mesomeric forms of the p-nitrobenzoyl lactone would be similar to those of the p-phenylazobenzoyl lactone.

In later years the problem of separating amino acids and polypeptide fractions obtained from protein hydrolysates has been met by newer techniques, such as column and paper chromatography. A. Lollermoser and K. Adelmann (25) separated a large number of these compounds, but stated that this method was not entirely satisfactory,

due to too many amino acids falling into one group.

The separation and identification of proteins and amino acids has recently been greatly simplified by the use of paper electrophoresis (26).

CHAPTER III

EXPERIMENTAL PROCEDURE

(1) Preparation of Starting Materials

(a) p-Phenylazobenzoyl chloride

At the outset of this work, p-phenylazobenzoyl chloride from commercial sources was not yet on hand and its preparation was undertaken. The preparation required p-phenylazobenzoic acid and, pending its arrival, this acid was also prepared. For preparation of p-phenylazobenzoic acid, nitrobenzene was partially reduced to nitrosobenzene, according to the method of Coleman, McCloskey and Stuart (33). Nitrosobenzene was reacted with p-aminobenzoic acid in the presence of glacial acetic acid by the method of H.D. Anspan (34) to give p-phenylazobenzoic acid. This acid was then converted to p-phenylazobenzoyl chloride by a modification of Ladenburg, Fernhalz and Wallis (27).

(b) Dipeptides

Glycyl-glycine, glycyl-DL-alanine, DL-alanyl-glycine and DL-alanyl-DL-alanine from commercial sources were not available in appreciable quantities, making laboratory synthesis necessary.

Using the method of Sheehan and Frank (28) glycine and DL-alanine were converted to phthalyl glycine and phthalyl DL-alanine respectively. These were each converted to the corresponding acid chloride. Condensation of the

latter with the appropriate amino acid gave the phthalyl dipeptide, which was then converted to the free dipeptide by heating with phenylhydrazine and tri-n-butylamine, according to the method of Schumann and Boissonnas (29).

A full account of the preparation of the above starting materials is given later in this section.

(2) Attempted Reaction between Glycine and p-Phenylazobenzoyl chloride in Presence of Tertiary Bases.

Attempts to prepare p-phenylazobenzoyl glycine using tertiary bases, such as pyridine, triethylamine, and tri-n-butylamine, as condensing agents in place of aqueous alkali were unsuccessful.

(3) Repetition of Karrer's Work on p-Phenylazobenzoyl Glycine and p-phenylazobenzoyl-DL-alanine.

The objects in repeating this work were:

(a) To determine the effect of varying the concentration of aqueous alkali on the yield of p-phenylazobenzoyl glycine.

(b) To determine whether isolation and purification of p-phenylazobenzoyl glycine could be simplified.

(c) Karrer and his associates found some unreacted p-phenylazobenzoyl chloride along with some p-phenylazobenzoic acid. It would appear that if these could be eliminated, yields might improve and methods of purification greatly simplified.

(d) To determine whether the low yields of p-phenylazobenzoyl—L-alanine obtained by Karrer could be improved.

(4) Attempted Preparation of p-Phenylazobenzoyl-DL-phenylalanine.

Essentially this was an attempt to use the established methods for the preparation of a derivative not yet reported in the literature.

(5) Preparation of p-Phenylazobenzoyl Peptides

These compounds have not been previously reported in the literature, and their preparation was carried out, once suitable adaptations of Karrer's method were found.

Experimental details of the above preparations, under their appropriate headings, are given in the following pages.

(1) Preparation of Starting Materials

(a) Preparation of p-Phenylazobenzoyl Chloride

Nitrosobenzene

Materials

24 gm. Nitrobenzene (redistilled)

12 gm. Ammonium chloride

18 gm. Zinc dust

13.4 gm. Sodium dichromate dihydrate

60 ml. Sulfuric acid

Method

The nitrobenzene was added slowly and with vigorous stirring, to a solution of ammonium chloride, dissolved in 800 ml. of water, in a 4 litre flask. The zinc dust was added, in small portions, over a period of 5 minutes. After the zinc dust was added, the temperature rose and enough ice was added to keep the temperature at 50-55°C. After a further 20 minutes, the zinc oxide was removed by suction filtration, the filter cake washed with 1 litre of boiling water, and the combined filtrate and washings transferred to a large crock. This solution contained phenylhydroxylamine, which must be oxidized to nitrosobenzene. The solution of phenylhydroxylamine was cooled to 0°-2°C; sufficient ice was added so that 1 kg. remained unmelted after the required temperature was reached.

A cold solution of sulfuric acid (300 ml. and enough ice to lower the temperature to 50°C) was added with stirring, to the cold phenylhydroxylamine solution, followed by rapid

addition of 800 ml. of sodium dichromate solution. The reaction mixture was stirred rapidly. After 2 to 3 minutes the straw colored nitrosobenzene, which had separated, was collected on a buchner funnel and washed with 2 litres of water. The crude nitrosobenzene was steam distilled into an ice cooled receiver. On solidification, the solid was washed with water until the washings were no longer brown in color. The light green solid was then dried between filter papers.

Yield - 12.3 - 14.0 gm. (M.P. 66-67^{0}C)

61.5 - 70% of theory.

p-Phenylazobenzoic Acid

Materials p-aminobenzoic acid - 5.4 gm.

glacial acetic acid -40.0 ml.

nitrosobenzene - 4.2 gm.

The p-aminobenzoic acid was dissolved in 40 ml. warm glacial acetic acid in a 500 ml. erlenmeyer flask, and the solution cooled to room temperature. The nitrosobenzene was added and the mixture stirred until solution and allowed to stand for 12 hours at room temperature. The p-phenylazobenzoic acid separated as a solid and was removed by suction filtration, washed with acetic acid and water. The product was air dried.

Yield - 5.2 - 7.2 gm (M.P. 245^{0}C)

68 - 80%

p-Phenylazobenzoyl chloride

Materials 5 gm. p-phenylazobenzoic acid
25 ml. thionyl chloride (redistilled)
25 gm. sodium carbonate (anhydrous)

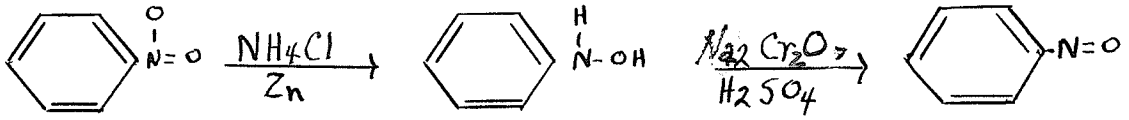
Method

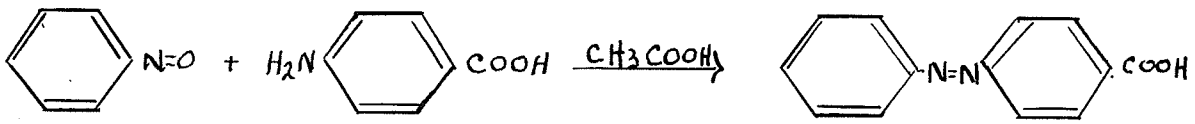
The p-phenylazobenzoic acid and the sodium carbonate were placed in a 600 ml. flask and thoroughly mixed. The thionyl chloride was added and the mixture boiled under reflux for 1½ hours with the condenser fitted with a drying tube to eliminate atmospheric moisture. The condenser was arranged for distillation, and as much thionyl chloride as possible was removed. The acid chloride was collected on a buchner funnel and pressed as dry as possible. After washing the acid chloride twice with petroleum ether, (30-60°C.), it was stored in a vacuum desiccator over phosphorus pentoxide and paraffin shavings.

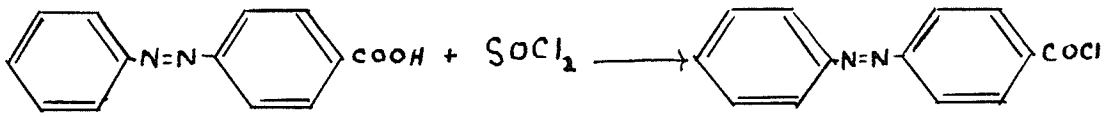
Yield = 3.2 gm. or 61.4% of theory

(M.P. 94.5°C.)

The reactions representing the above preparations are shown below:







(2) Preparation of Dipeptides

Phthalyl glycine

Materials glycine 1.5 gm. (0.02 mole)

phthalic anhydride

2.96gm. (0.02 mole)

Method

The mixture was heated at 150°C on a preheated oil bath. After cooling to room temperature, the solid was refluxed with methanol until solution was complete. The methanol was filtered to remove any solid impurities, concentrated under reduced pressure, and the concentrate allowed to sit overnight in the refrigerator to allow the product to crystallize. The solid, which had crystallized, was filtered by suction and recrystallized from a petroleum ether-benzene mixture.

Yield of Product - 3.2 gm. or 79% of theory

(M.P. - 134°C)

Phthalyl DL-Alanine

Materials DL-alanine 1.78 gm. (0.02 mole)

phthalic anhydride

2.96 gm. (0.02 mole)

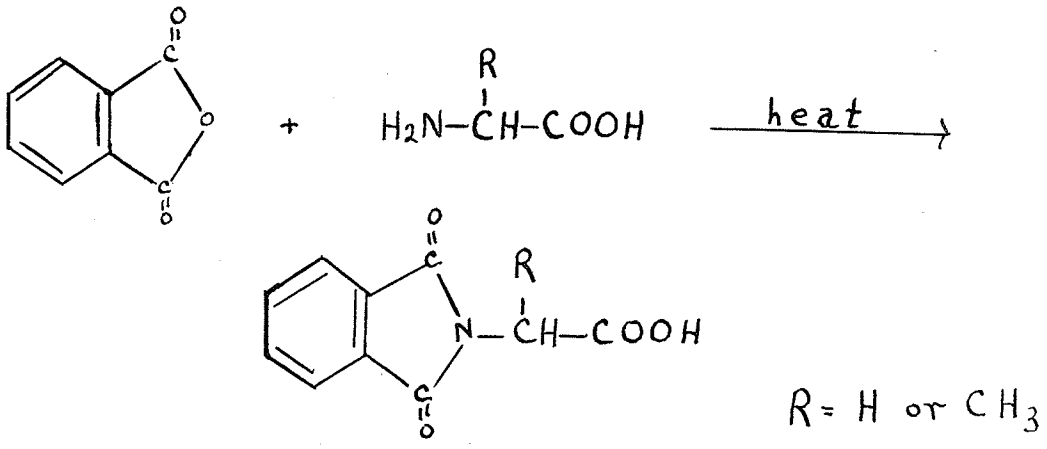
Method

The same procedure was used as for the preparation of phthalyl glycine.

Yield of Product 3.4 gm. or 75% of theory

(M.P. 158°C)

The following equations represent the above reactions.



Phthalyl glycyl chloride

Materials		
	phthalyl glycine	2.05 gm.
	phosphorus pentachloride	2.20 gm.
	benzene	20. ml.

The mixture was heated at 60°C. in a flask fitted with a reflux condenser until solution was complete. The solution was then refluxed for a further two hours, concentrated under reduced pressure and the concentrate set aside in the refrigerator to allow product to crystallize. The solid was filtered off and the resulting filtrate evaporated to dryness. The resulting solid obtained

from evaporation and the previous solid material were washed with petroleum ether. Both solids melted at 81°C. A mixture of the two solids also melted at 81°C.

Yield - 2.05 gm. or 87% of theory

Phthalyl DL-alanyl chloride

Materials	phthalyl DL-alanine	3.4 gm.
	phosphorus pentachloride	3.3 gm.
	benzene	20. ml.

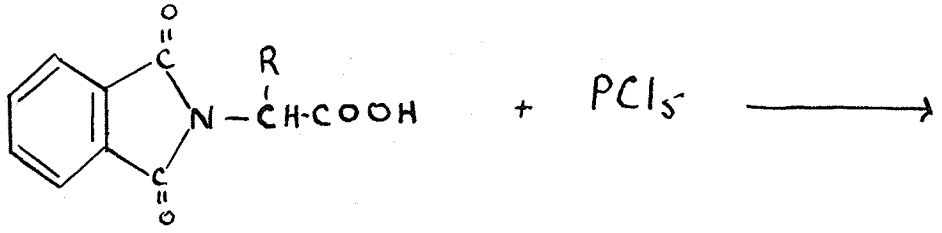
Method

The same procedure was used as for the preparation of phthalyl glycyl chloride.

Yield - 1.8 gm. or 76% of theory

(M.P. - 76°C)

The following equation represents the above reactions:



R = H or CH_3

Phthalyl glycylglycine

Materials	glycine	0.75 gm. (0.01 mole)
	magnesium oxide	0.60 gm. (0.015mole)
	phthalyl glycyl chloride	2.20 gm. (0.01 mole)
	dioxane	25. ml.

Method

A suspension was made from the glycine, magnesium oxide and 10 ml. water, and the phthalyl glycyl chloride dissolved in the dioxane. The dioxane solution was added to the aqueous suspension with stirring over ½ hour, and the reaction temperature kept at 5°C. At the completion of adding the dioxane solution, the reaction mixture was stirred for a further 10 minutes at room temperature. Acidification with dilute hydrochloric acid precipitated a crystalline solid which was recovered by filtration and recrystallized from alcohol. The original filtrate was evaporated under reduced pressure and the solid material recrystallized from alcohol.

Total yield of product - 2.0 gm. or 73% of theory

(M.P. 228°C.)

Phthalyl glycyl-glycine

Materials	glycine	1.5 gm.
	sodium bicarbonate	4.0 gm.
	phthalyl glycyl chloride	4.6 gm. in 40 ml. of benzene

Method

The glycine and sodium bicarbonate were dissolved in 25 ml. water and the benzene solution of phthalyl glycyl chloride added to this, with stirring, over ½ hour. Stirring was continued one hour, the benzene layer removed, and the aqueous layer acidified with dilute hydrochloric acid. The phthalyl glycyl glycine which had separated was filtered by suction. M.P. 218°C. on crystallization from 80% alcohol - 230°C.

Yield - 4.4 gm. or 85% of theory

Phthalyl Glycyl-DL-alanine

Materials	phthalyl glycyl chloride	- 4.6 gm. in 40. ml. benzene
	DL-alanine	- 1.8 gm.
	sodium bicarbonate	- 4.0 gm.

Method

The DL-alanine and sodium bicarbonate were dissolved in 25 ml. water and a similar procedure followed as for the preparation of phthalyl glycyl glycine.

Yield of phthalyl glycyl DL-alanine 2.2 gm or 42% of theory

(M.P. - 220°C)

Phthalyl DL-alanyl glycine

Materials	glycine	0.75gm.
	sodium bicarbonate	2.1 gm.
	phthalyl DL-alanyl chloride	2.37gm. in 40 ml. benzene

Method

Using the same procedure as for the other phthalyl dipeptides, 2.3 gm. of material (43% theory) melting at 221°C. was obtained.

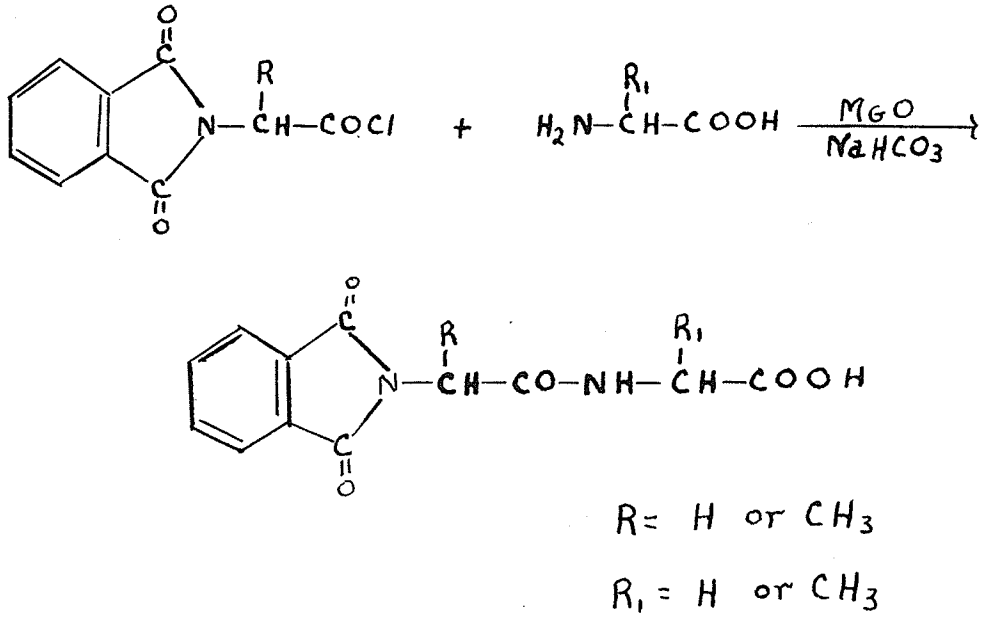
Phthalyl DL-alanyl DL-alanine

Materials	phthalyl DL-alanyl chloride	2.35 gm. in 20 ml. dioxane
	DL-alanine	0.89 gm.
	Magnesium oxide	0.60 gm.

Method

The DL-alanine and the magnesium oxide were suspended in 10 ml. of water. The dioxane solution was added with stirring over a period of ½ hour, with the reaction temperature kept at 5°C. After addition of the dioxane solution, the reaction mixture was stirred at room temperature for a further ½ hour and the solution acidified with dilute hydrochloric acid. The solid which had separated upon acidification was filtered off and recrystallized from

alcohol. The material obtained 1.2 gm. (63% of theory) had a Melting Point 228°C.



Glycylglycine

Materials

phthalyl-glycylglycine	2.4 gm.	(.01 mole)
phenylhydrazine	1.9 gm.	(.02 mole)
tri-n-butylamine	1.65gm.	(.01 mole)
ethyl alcohol	10. ml.	
methyl-ethyl ketone	25 ml.	
glacial acetic acid	0.75ml.	

Method

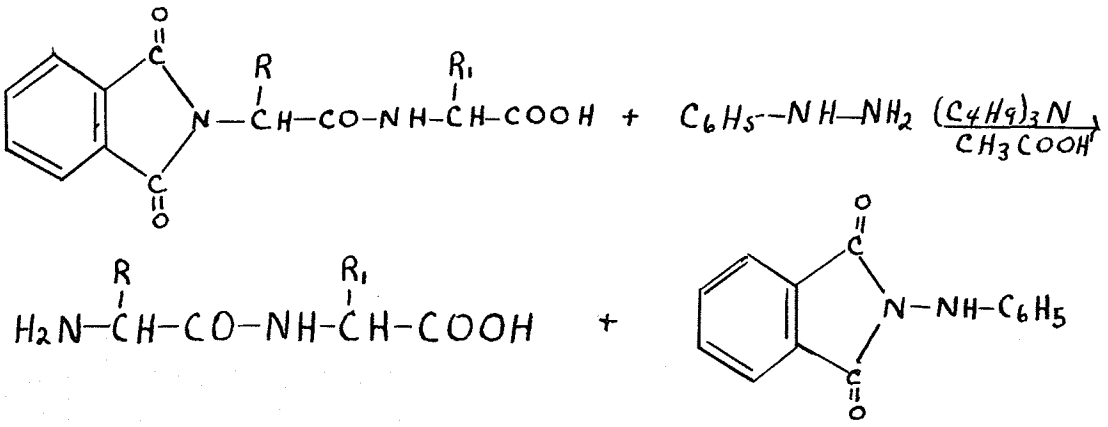
The phthalyl-glycylglycine, phenylhydrazine, tri-n-butylamine and ethanol were placed in a 100 ml. flask and boiled under reflux for two hours. The methyl-ethyl ketone

was then added and solution refluxed for a further 15 minutes. The solution was then cooled to room temperature and the glacial acetic acid added. A white precipitate settled out and was separated by filtration. The white precipitate was washed with methyl-ethyl ketone and dried in air.

Yield of product - 0.40 gm. or 33% of theory

M.P. 215-220°C. decomp.

The other dipeptides were prepared by a similar procedure. The following equations represent the above reactions:



$R = H$ or CH_3

$R_1 = H$ or CH_3

(2) Attempted preparation of p-Phenylazobenzoyl Glycine Using Tertiary Bases

(a) Using Pyridine as a Reaction Medium

Material			
	p-phenylazobenzoyl chloride	0.61	gm.
	pyridine	10.	ml.
	glycine	0.1875	gm.

Method

The acid chloride was dissolved in the pyridine and the glycine was added with constant stirring, in small amounts, over a period of ½ hour. The reaction mixture was stirred for a further 3 hours. The pyridine solution became deep red in color, later changing to an orange color. At the end of the 3 hours, the reaction mixture was poured into a beaker containing 600 ml. of water. After standing 1 hour an orange colored solid had separated, which was filtered, dried on a porous plate, and stored in a vacuum desiccator over phosphorous pentoxide. The solid material was refluxed successively with petroleum ether and benzene, then crystallized from alcohol.

Weight of product = 0.20 gm. or 30% of theory

M.P. = 226°C. (corrected)

Mixed melting point with p-phenylazobenzoic acid = 222 - 228°C.

Mixed M.P. with p-phenylazobenzoyl glycine = 224°C.

After standing one week, the melting point of the product had risen to 227°C. and the mixed melting point with p-phenylazobenzoic acid was 224°C. The melting point data might indicate the possibility of this material being p-phenylazobenzoyl glycine, were it not for the increase in melting point on standing. It would be expected that the mixed melting point of this material with p-phenylazobenzoic acid would be lower than that obtained. Solubility characteristics were generally not those expected for p-phenylazobenzoyl glycine. The possibility of this material being impure p-phenylazobenzoic acid cannot be discounted.

Repetition of this work failed to yield any p-phenylazobenzoyl glycine, but only p-phenylazobenzoic acid. A further study of this procedure was abandoned.

(2) Using Pyridine as a Reaction Medium

Materials	p-phenylazobenzoyl chloride	0.652	gm.
	glycine	0.20	gm.
	pyridine	10.	ml.

Method

The glycine was added to the pyridine solution containing the acid chloride, and the mixture heated under reflux for two hours. Solid material evidently glycine, was always present during the heating. At the end of the two hours, the contents of the flask were poured into a

beaker containing 600 ml. of water. This mixture was set aside, in the refrigerator, for 12-14 hours. After this time, solid material had settled out, which was filtered with the aid of suction, and dried on a porous plate. This material was refluxed with petroleum ether and the undissolved solid removed by filtration. This remaining solid material was then refluxed with benzene. The solid remaining was filtered and dried on a porous plate.

Weight of solid material was 0.30 gm.

Melting point - 260°C.

Melting point values were different from both that of p-phenylazobenzoyl glycine and p-phenylazobenzoic acid. Consistent values for the nitrogen content could not be obtained: sample values being 19.38%, 19,46% and 23.2% nitrogen. The quantity of material was too small to allow further examination. In view of the uncertainty of this reaction, this experiment was not repeated.

(b) Using triethylamine as condensing agent

Materials	glycine	1.5 gm.
	triethylamine	50. ml.
	p-phenylazobenzoyl chloride	5.379 gm.
	benzene	50. ml.

Method

To a suspension of glycine in the triethylamine

was added dropwise, with stirring, 50 ml. of a benzene solution containing the acid halide, and the mixture stirred for 8 hours on a water bath at 35 - 40°C. Although the benzene-triethylamine solution became a deep blue-violet color, resembling the color produced due to azlactone formation, a large quantity of an orange-yellow solid was visible throughout the heating period. The only materials which could be recovered were p-phenylazobenzoic acid and unreacted glycine.

(c) Using tri-n-butylamine as condensing agent

The materials, quantities and procedure were the same as that in (b) above, except that tri-n-butylamine was used in place of triethylamine. Only unreacted glycine and p-phenylazobenzoic acid could be recovered.

Trials were also made using DL-alanine in place of glycine, but the condensations did not take place.

(3) Repetition of Karrer's Preparation of p-Phenylazobenzoyl Glycine and p-Phenylazobenzoyl DL-alanine

These derivatives were prepared using the method proposed by Karrer et al (22) except that minor modifications were used. The modifications consisted of (1) using different concentrations of alkali as the reaction medium, (2) using different reaction times, (3) changes in isolation procedure. Details are given below.

The glycine was dissolved in a measured volume, containing one equivalent of standard alkali, and the p-phenylazo-

benzoyl chloride was dissolved in diethyl ether. The reaction was carried out at 0 - 5°C. with the acid chloride solution being added to the aqueous amino acid solution, in small quantities, over periods of time varying from 1½ to 5 hours. A further equivalent of alkali solution was added to the mixture at 0 - 5°C. over 1-3 hours. As was the case in Karrer's laboratory, a violet color developed in the aqueous layer during the addition of the alkali. Karrer attributed this color to the formation of the sodium salt of the azlactone, although this particular azlactone has never been isolated. Disappearance of the violet color was slow. At the end of the reaction time, the product was precipitated by acidification with hydrochloric acid.

Table I lists in detail quantities of glycine, acid chloride, volumes of alkali, and duration of the various stages of reaction time.

TABLE I

Variations in Quantity of Reacting Materials, Reaction Time and Strength of Alkali

METHOD	I	II	III	IV
Weight in Grams of p-Phenylazobenzoyl Chloride	0.61	0.60	0.652	0.818
Volume in ml. of Ether to Dissolve Acid Chloride	10.0	10.0	10.0	25.0
Weight in Grams of Glycine	0.1875	0.20	0.20	0.25
Volume in ml. of Alkali to Dissolve Glycine	1.2	1.2	1.6	1.2
Strength of Alkali (Normality)	1.593	1.593	1.593	2.82
Duration Time in Hours of Addition and Preliminary Agitation	1½	1½	1½	1½
Volume of Alkali in ml. Added After Preliminary Agitation (1)	1.2	1.2	1.6	1.2
Duration Time in Hours of Second Addition of Alkali (2)	1.0	3.0	3.0	2.0

(1) Reaction mixture allowed to stand 2½ hours at 0-5°C. before adding the extra alkali solution.

(2) Agitation continued a further 4 hours at 0-5°C. on completing addition.

Isolation of Product after Acidification

The methods outlined below are the ones corresponding to Method I, II, III, IV.

METHOD I

The ether layer was separated from the aqueous layer and the solid material in the aqueous layer removed by filtration. It melted at 209°C. The aqueous layer was extracted with ether; the ether layer was separated and added to the original ether layer. The ether solution was evaporated to dryness, which yielded a solid, M.P. 204°C. This solid material was added to the solid obtained from the aqueous layer and the whole heated with benzene under reflux to remove any p-phenylazobenzoic acid. The solid material remaining was separated by suction filtration, then recrystallized from alcohol. The melting point was 198°C. The product was then recrystallized from alcohol and 0.36 gm. of orange colored material melting at 223°C. (corrected) was obtained. Mixed melting point data showed this material to be other than p-phenylazobenzoic acid.

Yield - 63.6% of theory.

METHOD II

The solid material obtained by acidification of the reaction mixture was filtered and extracted with petroleum ether to remove any unreacted p-phenylazobenzoyl chloride. The undissolved material was then extracted with benzene to remove any p-phenylazobenzoic acid. The solid material remaining was then recrystallized from alcohol. An orange colored material 0.36 gm. (67.2% of theory) melting at 224°C (corrected) was obtained, which was proven to be different from p-phenylazobenzoic acid by mixed melting point data.

METHOD III

The crude product obtained by acidification was filtered. The ether layer was separated from the aqueous layer and evaporated to dryness. The solid material obtained by evaporation was added to the main bulk and the whole heated under reflux with petroleum ether, benzene and alcohol successively. On recrystallization from alcohol, 0.43 gm. (68% theory) of an orange colored material which melted at 221°C. was obtained. Mixed melting point data showed it to be other than p-phenylazobenzoic acid.

METHOD IV

The solid material was filtered and the ether layer separated from the aqueous layer, evaporated and material obtained added to the main solid. The combined solid was dried on a porous plate and the dried material

refluxed successively with petroleum ether and benzene. The benzene was filtered off, leaving 0.80 gm. (85.1% theory) of an orange colored solid melting at 220°C. (corrected).

Repeated crystallizations from alcohol yielded 0.75 gm. (80.3% theory) of a product M.P. 223°C. (corrected).

Mixed melting point data showed it to be other than p-phenylazobenzoic acid.

Mixed melting point determinations of the material obtained in I, II, III, and IV, showed it to be the same compound.

ANALYSIS

Calculated for $C_{15}H_{13}N_3O_3$.....N = 14.83%
Nitrogen obtained by micro kjeldahl.....N = 14.48%
Karrer and associates (22).....N = 14.50%

In Table II is summarized the results of these tests. It shows strength of alkali used as reaction medium, total reaction time, yield and melting point, compared with the work of Karrer, Keller and Szonyi.

TABLE II

Effect of Reaction Time and Strength of Alkali on Yield and Melting Point.

METHOD	STRENGTH OF ALKALI (NORMALITY)	TOTAL REACTION TIME (HOURS)	YIELD %	MELTING POINT °C.
Karrer and Associates	2.0	12½	76.9	225°
I	1.593	2½	63.6	223°
II	1.593	7	67.2	224°
III	1.593	7	68	221°
IV	2.82	7½	80	223°

Preparation of p-Phenylazobenzoyl DL-Alanine

Materials	DL-alanine	0.267 gm.
	p-Phenylazobenzoyl Chloride	0.732 gm.
	Ether	3.0 ml.

Procedure

The p-phenylazobenzoyl chloride was dissolved in 3.0 ml. of ether and added slowly over a period of one hour to 0.267 gm. of DL-alanine dissolved in 3.3 ml. of 0.954 N sodium hydroxide solution at 0-5°C. The reaction mixture was stirred another 7 hours. The reaction mixture was violet in color. The aqueous layer was removed from the ether layer and acidified with dilute hydrochloric acid. On acidification, an orange colored solid precipitated and was removed by filtration. The ether solution was evaporated to dryness and the resultant solid material obtained combined with the original material. Extraction of the solid with carbon disulphide removed unreacted p-phenylazobenzoyl chloride. The remaining solid material was recrystallized from alcohol.

Yield - 0.182 or 25% of theory

(M.P. - 219°C.)

Mixed melting point data ruled out the possibility of this material being p-phenylazobenzoic acid.

In a second trial, 1.22 gm. of acid chloride dissolved in 30 ml. of ether was added, in small portions,

to 0.455 gm. DL-alanine dissolved in 5.3 ml. 0.953 N sodium hydroxide. The reaction temperature was maintained at 0-5°C. and the reaction mixture stirred for 3 hours. The ether layer was separated from the aqueous layer and the former washed four times with 10 ml. of 0.4 N sodium hydroxide. These washings were added to the previously separated aqueous layer. The solution was a deep violet color. On acidification, an orange colored solid was precipitated, which was filtered and dried. The melting point of the crude product was 192-196°C. Extraction with carbon disulphide raised the melting point to 202°C. After twice recrystallizing from 95% alcohol and 70% alcohol respectively, 0.47 gm. of an orange solid material M.P. 215°C. (corrected) was obtained. Mixed melting point data showed distinction from p-phenylazobenzoic acid.

A further trial was made, using the same quantities and procedure as shown above, except that after crystallization from alcohol, the solid material was extracted again with carbon disulphide. The yield of orange solid material, M.P. 215°C. (corrected) was 0.5 gm. (30% theory).

Analysis for $C_{16}H_{15}O_3N_3$ - N = 14.17%

Found - N = 13.78%

Karrer et al (22) N = 14.18%

Mixed melting point data showed the three products to be similar.

Table III gives a summary of yields obtained by the above methods and the melting points of the respective products, together with results obtained by Karrer and associates.

TABLE III

Yield and Melting Points of Products obtained by Varying the Methods of Purification as Compared with Karrer's Method.

METHOD	YIELD %	MELTING POINT °C.
I	25	219° (corrected)
II	31.3	215° (corrected)
III	30	215° (corrected)
Karrer	30	215° (corrected)

As improved yields could not be obtained without an extended investigation, requiring much time, work on the preparation of this derivative was abandoned.

4. Preparation of p-Phenylazobenzoyl-DL-Phenylalanine

Materials

DL-phenylalanine	1.65 gm.
p-phenylazobenzoyl chloride	2.44 gm.

Method

To 1.65 gm. DL-phenylalanine dissolved in 4.5 ml. of 2.5 N sodium hydroxide was added with stirring over a period of $\frac{1}{2}$ hour, 50 ml. of an ether solution containing the p-phenylazobenzoyl chloride. The reaction temperature was maintained at 0-5°C. At the end of this addition 4.0 ml. of 2.5 N sodium hydroxide was added over a period of 2 hours. During the latter period, a bulky solid formed at the bottom of the reaction vessel, and the solution was a violet color. The reaction mixture was acidified with 5 N hydrochloric acid and the solid material separated by filtration. This material was extracted successively with ligroin and carbon disulphide. The remaining solid melted at 187°C. On recrystallization from alcohol 0.25 gm. of an orange colored solid, M.P. 191°C. (corrected), was obtained.

Lack of time and supplies of DL-phenylalanine did not permit further investigations into the preparation of this derivative.

5. Preparation of p-Phenylazobenzoyl Dipeptides

p-Phenylazobenzoyl Glycyl Glycine

Two procedures were used for the preparation. The violet color due to azlactone formation during preparation of the amino acid derivatives was not observed in preparing the p-phenylazobenzoyl dipeptides.

Method 1.

Materials Glycyl Glycine - 0.264 gm. (.002 mole)

p-Phenylazobenzoyl chloride

- 0.489 gm. (.002 mole)

To 0.264 gm. of glycylglycine, dissolved in 2.17 ml. of 0.9242 N sodium hydroxide, was added slowly with stirring, 40 ml. of an ether solution containing the acid chlorides. The reaction temperature was maintained at 0-5°C. On completing the addition of the ether solution, the reaction mixture was stirred for another 1½ hours. A further 2.17 ml. of alkali solution was added in small amounts over a period of 3 hours, and the reaction mixture stirred for another 3 hours. Upon acidification with dilute hydrochloric acid, an orange colored material settled out, which was filtered and dried. This solid material was heated under reflux with petroleum ether (30-60°C.) to extract any unreacted acid chloride. The solid material remaining was then removed and refluxed with benzene to remove any p-phenylazobenzoic acid that may have formed. The product

was then repeatedly recrystallized from alcohol until constant melting point was obtained.

A reddish-orange colored material, 0.56 gm. (82.3% theory) M.P. 237°C. (corrected) was obtained.

Analysis

Calculated $C_{17}H_{16}N_4O_4$- N = 16.46%

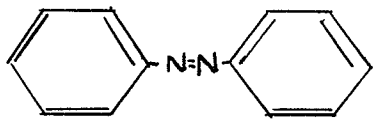
Found N = 16.22%

A small amount of the desired product could also be obtained by evaporating the ether solution and adding the residue obtained to the bulk on the filter paper.

The following equation represents the above reaction:

$$R\text{-}CO\text{-}Cl + H_2N\text{-}CH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH \xrightarrow{NaOH}$$

$$R\text{-}CO\text{-}NH\text{-}CH_2\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH$$

R = 

Method II.

Preparation of p-Phenylazobenzoyl glycyl chloride

Materials p-Phenylazobenzoyl glycine 1.0 gm.

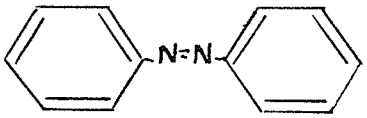
Thionyl chloride (re-distilled) 20.0 ml.

Method

To 1.0 gm. p-phenylazobenzoyl glycine was added 20 ml. thionyl chloride and the mixture heated under reflux for 6 hours. The excess thionyl chloride was removed by distillation. The material remaining which had a tarry consistency was

extracted with ether and the ether solution allowed to evaporate in a vacuum desiccator over phosphorus pentoxide. A deep reddish colored tarry-like material 0.40 gm. was obtained. The following equation represents the above reaction:

$$R\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH + SOCl_2 \longrightarrow R\text{-}CO\text{-}NH\text{-}CH_2\text{-}CO\text{-}Cl$$

R = 

Reaction of p-Phenylazobenzoyl glycyl chloride with glycine

Materials		
	p-Phenylazobenzoyl glycyl chloride	0.3015 gm.
	Glycine	0.075 gm.
	Ether	40.0 ml.

Method

To 0.075 gm. glycine dissolved in 5.0 ml. 0.20 N Sodium Hydroxide was added slowly with constant stirring at 4°C. 20 ml. ether solution containing 0.3015 gm. p-phenylazobenzoyl-glycyl chloride. The reaction mixture was stirred for a total of 4 hours at which time it was acidified with 5 N hydrochloric acid. The solid material was separated by filtration and crystallized from alcohol. An orange colored material 0.11 gm. M.P. 252°C. was obtained. Mixed melting points with previously prepared p-phenylazobenzoyl glycylglycine and p-phenylazobenzoic acid were respectively 218°C. and 200°C.

As the quantity of this material was very small, no further investigation was carried out but it would appear that the above prepared material was not p-phenylazobenzoyl glycylglycine.

p-Phenylazobenzoyl glycyl-DL-alanine

Materials	p-Phenylazobenzoyl chloride	0.48 gm.
	Glycyl-DL-alanine	0.292gm.
	Ether	40. ml.

Method

To 0.292 gm. glycyl-DL-alanine dissolved in 2.17 ml. 0.9242 N sodium hydroxide was added slowly, with constant stirring, 40 ml. ether solution containing 0.48 gm. p-phenylazobenzoyl chloride. The reaction temperature was maintained at 4°C. and the mixture stirred for 1 hour. A further 2.17 ml. 0.9242 N sodium hydroxide was added and the mixture stirred for another 3 hours. The reaction mixture was acidified with 5 N hydrochloric acid and the solid material separated by filtration. The ether phase was separated from the aqueous phase and evaporated to dryness. The resulting solid material was combined with the previous solid material. The resulting solid material was dried on a porous plate, extracted with 30-60°C. ligroin and benzene, then crystallized from alcohol. An orange colored solid

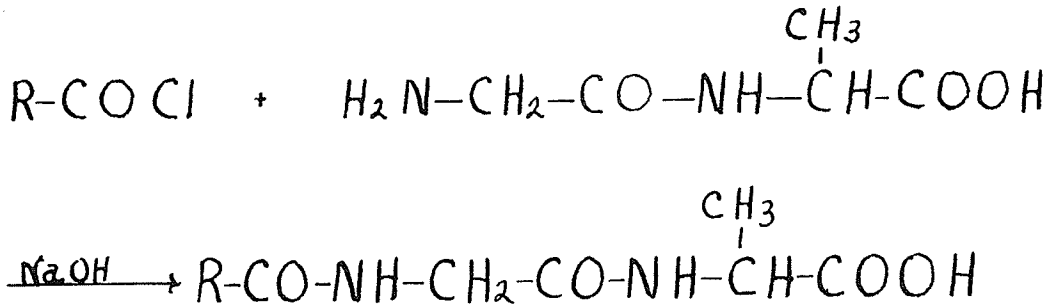
M.P. 236.5 - 237°C. 0.40 gm. (60% theory) was obtained.

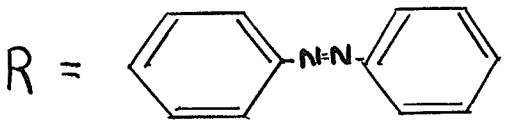
Mixed melting point with p-phenylazobenzoic acid 228°C.

Nitrogen determination.....16.92%

Calculated.....17.17%

The following equations represents the above reactions:





p-Phenylazobenzoyl DL-alanylglycine

Materials		
	p-Phenylazobenzoyl chloride	2.445 gm.
	DL-alanylglycine	1.46 gm.
	Ether	40.0 ml.

Method

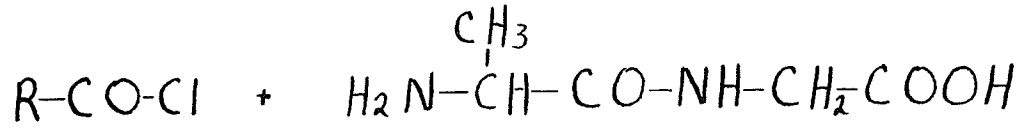
To 1.46 gm. DL-alanylglycine dissolved in 4.0 ml. 2.5 N sodium hydroxide was added slowly with constant stirring at 4°C, 40 ml. ether solution containing 2.445 gm. p-phenylazobenzoyl chloride. The reaction mixture was stirred for 1½ hours, at which time an additional 4.0 ml. 2.5 N sodium hydroxide was added. The mixture was stirred

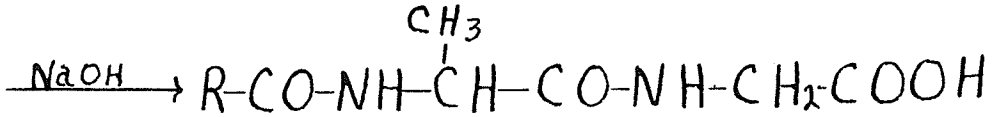
for a total of 7 hours. The reaction mixture was acidified with 5 N hydrochloric acid and the resulting orange colored solid separated by filtration. The ether phase was separated, evaporated to dryness and the solid material obtained combined with the previous solid material. The combined solid material was dried on a porous plate, then extracted successively with ligroin 30-60°C. and benzene. The remaining solid material was crystallized from alcohol after which 0.50 gm. (71% theory) of an orange colored compound M.P. 224.5° - 225.5°C. was obtained.

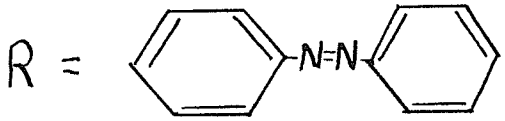
Nitrogen determination.....16.69%

Calculated.....17.17%

The following equations represent the above reactions:







p-Phenylazobenzoyl DL-alanyl-DL-alanine

Materials	p-phenylazobenzoyl chloride	0.489 gm.
	DL-alanyl-DL-alanine	0.320 gm.
	Ether	40.0 ml.

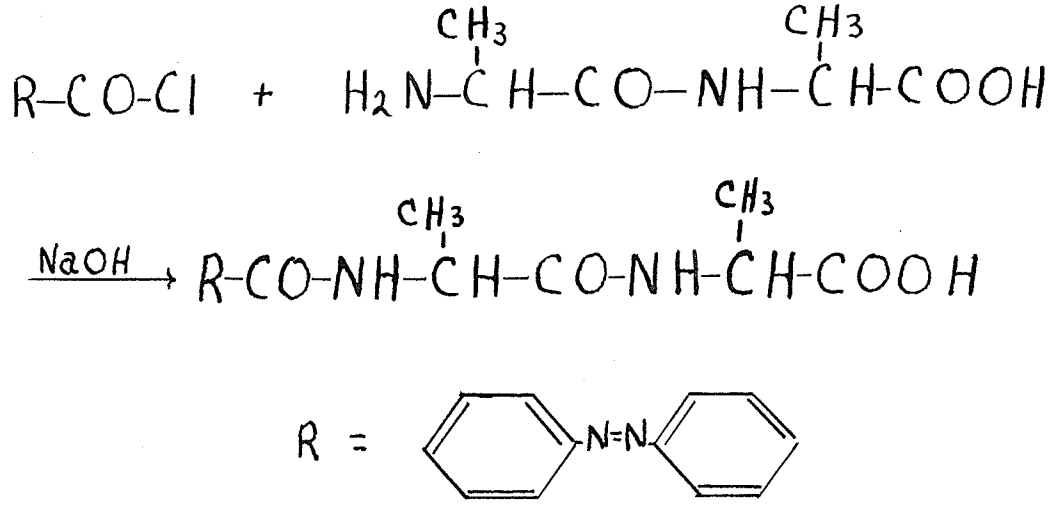
Method

To 0.320 gm. DL-alanyl-DL-alanine dissolved in 2.17 ml. 0.9242 N sodium hydroxide was added slowly, with continuous stirring, 40 ml. of ether solution containing 0.489 gm. p-phenylazobenzoyl chloride. The reaction mixture was maintained at 4°C. and stirred for a period of 2 hours. An additional 2.17 ml. 0.9242 N sodium hydroxide was added and the mixture stirred for a further 2 hours. The mixture was acidified with 5 N hydrochloric acid and the resulting solid material separated by filtration. The ether phase was separated, evaporated to dryness and the solid material obtained, added to the previously obtained material. The solid mixture was extracted with ligroin and benzene and crystallized from alcohol. The orange colored material, yield 0.34 gm. (46% theory), had a M.P. 233° - 234°C. Mixed melting point with p-phenylazobenzoic acid was 220°C.

Nitrogen determination.....15.05%

Calculated.....15.21%

The following equations represent the above reactions:

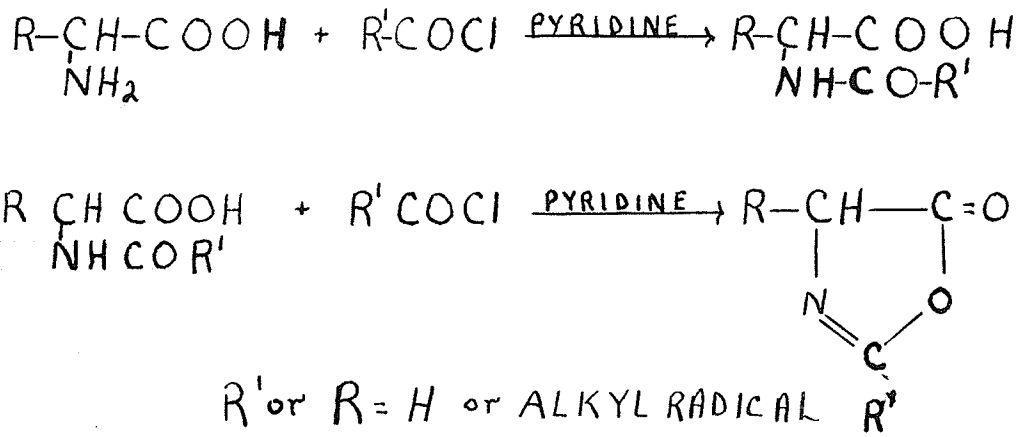

R–CO–Cl + H2N–CH(CH3)–CO–NH–CH(CH3)–COOH
NaOH
R–CO–NH–CH(CH3)–CO–NH–CH(CH3)–COOH
R =
N=N

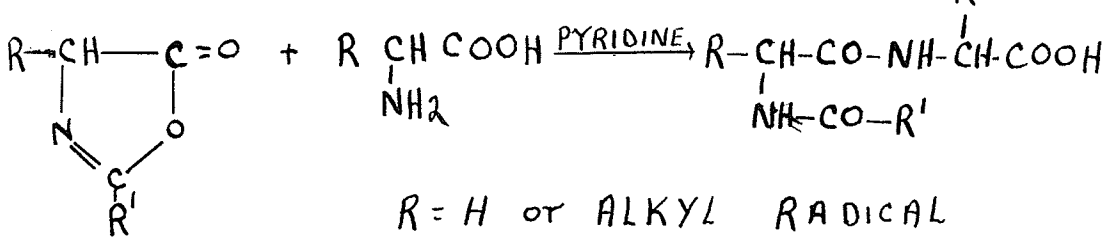
CHAPTER IV

DISCUSSION

Attempted Reaction in the Presence of Tertiary Bases

When the preparation of p-phenylazobenzoyl derivatives of glycine and DL-alanine was attempted at room temperature in the presence of triethylamine, tri-n-butylamine and pyridine, only unreacted amino acid and p-phenylazobenzoic acid could be recovered. Carter, Handler and Stevens (30) found that hippuric acid could not be prepared from benzoyl chloride and glycine in the presence of pyridine. They attributed this to the limited solubility of the amino acids in pyridine. It may be for this reason that no p-phenylazobenzoyl derivatives of glycine and DL-alanine were obtained using tertiary bases. The above authors, in the same article, showed that acylation of amino acids in pyridine, at elevated temperatures, produced a mixture of acylated amino acid and dipeptide as shown below.





This may account for the high melting compound obtained by refluxing p-phenylazobenzoyl chloride with glycine in the presence of pyridine, but, since the quantity of this material obtained was very small, no further identification was possible.

Repetition of Karrer's Work

This work was repeated to determine whether variations in the procedure could lead to improved yield and quality of products. In the case of p-phenylazobenzoyl glycine, it would appear that no significant improvement resulted from slight variations. As for p-phenylazobenzoyl DL-alanine, the results indicate that any improvement in the 24% yield obtained by Karrer and his associates, must be sought in some other direction.

In the preparation of p-phenylazobenzoyl DL-alanine, a violet color was observed in the alkaline solution. Karrer does not mention observing this color in the preparation of p-phenylazobenzoyl L-alanine, but does in the preparation of p-phenylazobenzoyl derivatives of glycine, L-leucine and L-valine. Whether or not this color was

observed by Karrer, or that he just failed to record it, is unknown.

Attempted Preparation of p-Phenylazobenzoyl-DL-phenylalanine

Due to the poor yields obtained in this preparation, of identification methods other than by mixed melting points and solubility characteristics were not permissible. As only one preparation was made of this material, possibly the best reaction conditions and isolation procedures had not yet been found in the initial exploratory trials.

Preparation of p-Phenylazobenzoyl Dipeptides

In the preparation of the p-phenylazobenzoyl dipeptides, no violet color, of the azlactone type, was observed. The structure and identities of these derivatives have been confirmed on the basis of nitrogen analysis and mixed melting point determinations. The procedures used give satisfactory yields, although the reaction time was quite lengthy.

Time and material did not permit repeating the attempted preparation of p-phenylazobenzoyl glycylglycine using p-phenylazobenzoyl glycyl chloride and glycine. The compound obtained had a melting point which was much higher than that obtained by reacting p-phenylazobenzoyl chloride with glycylglycine. It would appear that this method was not very satisfactory.

CHAPTER V

CONCLUSION

1. Dipeptides can be satisfactorily characterized by the use of p-phenylazobenzoyl chloride as the acylating agent, using the Schotten-Baumann procedure. The derivatives can be obtained in fair yields without too much difficulty.
2. Using standard procedures, the yield of p-phenylazobenzoyl DL-alanine cannot be readily improved.
3. Further investigation into the preparation of p-phenylazobenzoyl DL-phenylalanine will be required to determine whether the yields and quality can be improved.
4. The use of tertiary organic bases as reaction media for preparation of p-phenylazobenzoyl amino acids is not practical.

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