THE SYNTHESIS OF PEPTIDE BONDS

A Thesis

by

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GENERAL INTRODUCTION

This thesis reports work which is an integral part of a larger project underway in these laboratories under the direction of Dr. R. V. V. Nicholls, the ultimate goal of which is the synthesis of several naturally-occurring oligopeptides with physiological activities. The purpose of the present work is to initiate an investigation of the problems associated with the synthesis of peptide bonds. Two aspects are under consideration here; first, to study the methods by which amino acids can be rendered monofunctional so that their reactions may be strictly controlled; second, to examine amidation reactions from the standpoint of the controlled synthesis of peptide bonds.

Acceptable methods of synthesizing the peptide linkage should be general and controllable; in other words, they should make it possible to combine the alpha-amino acids or other starting materials in any sequence desired. The reactions involved should start with readily accessible materials, proceed with relative ease, and should give high yields.

The immensely important problem of peptide synthesis renders investigations of new and improved methods advisable. This thesis is an endeavour to contribute towards the solution of the problem.

HISTORICAL INTRODUCTION

Peptides are usually defined as substances which contain peptide bonds of the general type, -CHR-CO-NH-CHR'-, yield alpha-amino- or imino-acids on hydrolysis, and differ from proteins in the smaller size of their molecules or in the smaller variety of amino-acid species per molecule. The upper limit for molecular weight of peptides has been arbitrarily placed at 10,000, which represents about 100 amino acid moieties (1).

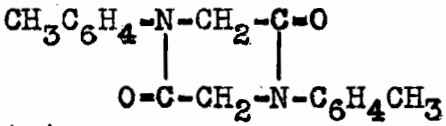
The relationship between the proteins and alpha-amino acids was recognized more than a century ago. It was in 1820 that Braconnot isolated glycine from the acid hydrolyzate of gelatin and called it "sugar of gelatin" because of its sweet taste. In the same year he succeeded in isolating another amino acid, leucine, from a similar hydrolyzate of muscle fibres (cf. e.g. 2, 3). Since that time the number of amino acids obtained from proteins has increased, until we now have 24 amino acids acknowledged to be "building stones" of proteins, and some 10 more amino acids not definitely established as protein "building stones" (3). With the exception of glycine, each one of them has one optically active carbon atom to which the amino-, carboxyl-, and alkyl radicals are attached. The "natural" amino acids have an L configuration, although claims were made for the occurrence of D-amino acids in hydrolysates of several naturally-occurring peptides (1).

It was chiefly the work of two schools, that of Theodor Curtius at Heidelberg University, and of Emil Fischer at the University of Berlin, which helped to clarify the essential importance of the amino acids in the structure of proteins. Investigations instrumental in reaching this conclusion were based on the studies of amino acid esters and diketopiperazines.

Curtius developed a method for preparing methyl or ethyl esters of amino acid hydrochlorides from the amino acid, alcohol and dry hydrogen chloride (4, 5). For investigations of free amino acid esters he removed the hydrogen chloride with silver oxide (5). Fischer improved this method by using for this purpose either a cold solution of potassium carbonate, or sodium alkoxide (6,7) and, therefore, this method is now generally known as Fischer's method of esterifying amino acids (e.g. 8). The free amino acid esters then served Curtius for studies on aliphatic azides, which terminated in the development of the classical Curtius method of coupling the amino acids through a peptide bond (9, 10, 11). During these studies Curtius noted the relative instability of the amino acid esters, which could be stored only in the form of their hydrochlorides. A "biuret base" was obtained from a dry ethereal solution of glycine ethyl ester on standing, while in aqueous solution, diketopiperazine was obtained (4,5). Fischer reported that esters of higher amino acids did not

form diketopiperazines readily in aqueous solutions; however, they did so in the dry state, slowly at room temperature, faster at temperatures above 100°C. (6, 7, 12, 13). Also he noted that methyl esters formed diketopiperazines much more easily than ethyl esters (13).

It seems that the first reported diketopiperazine was that of leucine, which had already been isolated in 1848 by Bopp, who named it "leucinimid" (14). It was prepared later by heating leucine in a stream of carbon dioxide (15) or hydrogen chloride (16). Abenius and Widman observed that a diketopiperazine derived from o-toluylglycine (I) could be split open by a short heating in strong hydrochloric acid (17). By this method, Fischer and Fourneau prepared glycylglycine hydrochloride from its diketopiperazine (II); neutralization of this product yielded the first recorded unsubstituted dipeptide prepared in vitro from amino acids (18).



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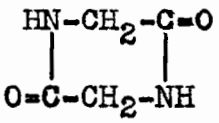


(II)

Another historically important peptide derivative is the "gamma acid" which Curtius prepared by several methods. In 1881, attempting to prepare benzoylglycine from benzoyl chloride and silver glycinate, he obtained mixed products, from which he isolated "alpha acid", identified as benzoylglycine, "beta acid" assumed to be benzoylglycylglycine, and "gamma acid", structure of

which was not determined (19, 20). In 1883 he obtained the "gamma acid" by heating ethyl hippurate with glycine (4). And in 1902 he succeeded in synthesizing by the azide method benzoyl (pentaglycyl) glycine (10), which was found to be identical with the "gamma acid" (21). So it was shown that the first two reactions were essentially polymerization reactions of glycine. Similar polymerization products were obtained by simple heating of amino acids, either alone (in the case of dicarboxylic acids) (22, 23), or in the presence of some alcohol (24, 24). However, as had been noted already by Fischer in 1906 (26), the simple polymerization reactions of the amino acids or their derivatives, however useful they might be from the preparative standpoint, will only play a relatively small role in the clarification of the composition of proteins. Even if some of these polymers happened by chance to be similar to some naturally-occurring substances, their preparation would reveal to the scientist exploring protein structure no more than a fast train journey across some country would disclose to a conscientious traveller wishing to explore its customs and geography. The protein molecules appear to have such complex structures that only painstaking, step-by-step studies can have any chance of helping to clarify their nature. Recognizing this, Fischer attempted to solve the problem of controlled synthesis of peptide bonds. The development of the acyl chloride method was the most important result of this effort (27, 28).

However, even the genius of Emil Fischer combined with the immense resources of the Berlin Institute of Chemistry did not solve the problem of synthesizing even the natural polypeptides, not to speak of proteins. The multitude of articles which resulted shows the size of their effort in this field. It took several more decades of research in several laboratories the world over to bring about the first syntheses of naturally-occurring peptides (viz., the tripeptide, glutathione, in 1935 and the octapeptide, oxytocine, in 1953) (29, 30, 31). Most important advances of the latter period were the introduction of improved protecting groups, development of the azlactone method (32), investigation of mixed carboxylic anhydrides (33) and of acyl derivatives of several mineral acids (34, 35, 36, 37, 38, 39, 40). Of all these recently developed methods, the one which appears most promising involves the use of mixed carbonic-carboxylic acid anhydrides. Parallel advances in the field of polymerization leading to poly-alpha-amides of high molecular weight, however far-reaching they are, are outside the scope of our work. For an authoritative review in this field, Katchalski's article published in 1951 (41) may be consulted.

The considerable improvement in protecting the amino or carboxyl groups lies in the application of radicals removable by non-hydrolytic methods, which do not endanger the peptide bonds. This approach was based on reports that some radicals,

like the benzyl group, when attached to oxygen (or sulphur) atoms, are easily cleaved by hydrogenolysis (cf. e.g. 42, 43, 44). Of these, most important are the carbobenzyloxy (basis of the famous Bergmann's method) (45), carballyloxy (46), p-toluenesulphonyl (47) and benzyl (48) groups. Another important protecting group, the phthaloyl radical (49, 50), can also be cleaved by a non-hydrolytic method, using hydrazine.

Several reviews of methods of forming peptide bonds have been prepared in the past. Most recent are those of Fruton (51), Wieland (52), Nicholls (53) and Shapiro (54). The first two are actually historical reviews, in the sense that the methods are discussed more or less in chronological order. The first author to bring a different arrangement to his review was Nicholls, who divided the reactions leading to the peptide bond synthesis into two groups (condensation and addition reactions) and discussed the protecting groups as a separated, although integral, part of the review. This system was closely followed by Shapiro in his more recent review.

We felt that a more exact system was desirable in view of the complexity of the problem. Arranging all the information at hand into such a system should offer, besides the obvious advantages of having the related data grouped in a logical sequence, some indication of yet uninvestigated

routes of synthesizing the amido bond. The great importance of the natural peptides makes investigation of any further possible method of this synthesis desirable. Therefore we had undertaken to compile a systematic classification of methods for the controlled synthesis of peptide bonds, the result of which is presented in the next chapter.

We should mention here also the important role of enzymes in the peptide chemistry. The study of the interactions between the various enzymes and the vast quantity of substrates ranging from the amino acids up to the proteins and their derivatives had already yielded a monumental amount of literature, which is, however, more a part of biochemistry. From the standpoint of the synthesis of peptide bonds only four aspects of this study are of immediate interest. They are:

i) formation of peptide bonds by the action of endopeptidases on the hydrolyzates from natural proteins;

ii) transamidation (or, in a narrower sense, transpeptidation);

iii) polymerization of amino acid esters;

iv) controlled formation of peptide bonds.

Investigations of the first aspect, the interactions between the proteases and hydrolyzates, started in the past century. It was in 1886 that Danielewski (cf. 55, 58)

observed the formation of a precipitate upon addition of gastric juice to a concentrated solution of peptones. More recent experiments in this field are, among others, those of Virtanen (56, 57, 58), who demonstrated the importance of the degree of depolymerization of the hydrolyzates on the type of products isolated from the precipitates; and of Tauber (59), who was able to gelatinize the natural hydrolyzates (e.g. of egg albumin, bovine albumin, zein) by the action of chymotrypsin at pH about 7.0 and temperature 37° C. He estimated the molecular weight of the products to be 250,000 to 500,000.

Transamidation is another fascinating aspect of the enzymic action. A good review of the transpeptidation theory was compiled by Hanes et al (60), who investigated the transpeptidation reactions of glutathione and its derivatives. Fruton (61) suggested on the basis of his experiments (e.g. with the N_{15}-tagged amino-acid derivatives (62)) that the proteolytic enzymes catalyze reactions in which peptide chains are lengthened not by direct condensation reactions (with elimination of water), but by transpeptidations involving replacement of a short peptide chain, or of an amino acid, by a longer one. He was even able to replace the amido-NH_2 group of benzoyl or carbobenzyloxy amino acid amides by further amino acid moieties (63). Feasibility of this theory was further demonstrated by Waley and Watson (64)

who found that enzymatic degradation of certain tripeptides yielded dipeptides in which the sequence of amino acid residues differed from that of the original tripeptide.

The polymerization of the amino acid esters catalyzed by the enzymes is outside the scope of this discussion. It should suffice here to refer to the most recent articles dealing with this type of polymerization, which were written by Brenner and co-workers (65) and by Wieland and Schäfer (66).

Many investigations on the possibility of controlled synthesis of peptide bonds catalyzed by enzymes were undertaken in order to clarify the functioning of enzymes and to devise methods which would enable us to form, under very mild conditions, peptide bonds of sterically "natural" configuration, regardless of the optical activity of the available starting material. Invariably the products were insoluble and apparently it was their removal by precipitation which allowed further condensation reaction to proceed. The carboxyl group was always in the form of an amide or a substituted amide (anilide, phenylhydrazide, etc.). A considerable amount of work was done by the group of Bergmann et al (67, 68, 69) who wrote in 1944 a short but authoritative review on this field (70; cf. also 71). Even the most recent publications (e.g. 72, 73, 74) show that this method of forming peptide bonds is still at the stage of exploratory experiments and, for the controlled synthesis of longer peptide

chains cannot yet be compared in usefulness with methods which use energy-rich intermediates.

SYSTEMATIZATION OF SYNTHETIC METHODS

Introduction

The alpha-amido bonds can be formed either by polymerization, or by a controlled combination of two chosen moieties in a desired sequence. There is also a theoretical possibility of making such bonds by rearrangements of suitable compounds, viz. the Beckmann rearrangement of polymeric oximes. If such a method of synthesizing the poly-alpha-amino acids should prove feasible, this would become a third type of method of forming the alpha-amido bonds, in addition to the polymerization and controlled combination mentioned above. For our purposes, we are concerned with the controlled synthesis of peptide bonds only.

Study of the pertinent literature brought to light a surprising fact, that, although several reviews of methods for synthesizing the peptide bonds exist, no true systematization of these methods has yet been compiled (cf. p. 7). We felt that such a system is indispensable for the previously-mentioned long-term project (cf. "General Introduction" p. 1) and accordingly proceeded immediately to survey the pertinent literature and to construct a systematic review of the methods available. Also included were methods which appeared to have severe disadvantages from the standpoint of laboratory technique. The resulting systematic review, which is presented in Table I (p. 14) soon indicated several possible paths for the synthesis of peptide bonds as yet untested, which were also tentatively included in this table.

Methods of Amidation

From the standpoint of controlled formation of the alpha amido (peptidic) bond, the direct condensation of the amino acids or peptides which were rendered monofunctional (by attaching the protecting groups to the amino and carboxyl radicals respectively) is not an easy task because of the high amount of energy it requires (75, 76). Therefore, attempts were made to prepare and use amino acid derivatives containing "energy-rich bonds", capable of forming the amido linkage with greater facility. Since the direct formation of amido linkage is accompanied by a loss of one mole of water, most of the intermediary compounds are mixed anhydrides of the amino acids with some suitable compound. The actual condensation is then accompanied by a loss of the elements of this compound instead of water. The intermediates may be either linear, or cyclic anhydrides. Some of the latter type of anhydrides form peptide bonds by addition reactions with no by-products.

A special case is the synthesis of the amido linkage by a condensation reaction catalyzed by an enzyme. A general report on this type of reactions is summarized on pp. 8-11.

The chemically controlled reactions can be generally classified into three groups, according to which bond is apparently formed, i.e. whether the RCO-NHR, RCONH-R, or R-CONHR bond. Traditionally, the RCO-NHR bond was the only one to be considered, though one should not lose sight of the theoretical possibilities of the other two approaches.

Table I

Synthesis of an Amido-Linkage

* in specific cases can be catalyzed by enzymes

A.1. Direct Condensation of Carboxyl and Amino Groups

$$RCOOH + NH_2R' \rightarrow RCONHR' + H_2O$$

The direct condensation of two amino acid (or peptide) moieties, rendered monofunctional by the use of suitable protecting groups so that one has a free carboxyl and the other a free amino group, is essentially an endothermic and reversible reaction. The necessary energy can be conveniently provided in the form of heat. Since the position of the equilibrium is unfavourable to the production of the amide and water, the removal of the products from the reaction is a necessity. Thus, for example, the amide might be removed by precipitation or the water be removed by evaporation.

Even simple heating of free amino acids sometimes yields polymerization products. Several examples are here included, because their general importance in peptide chemistry as the oldest methods of formation of peptide bonds. Schiff (23) condensed aspartic acid by heating it to 190-200° C. He isolated from the reaction mixture an octa- and tetra-aspartic acid and Schaal's "aspartides". (Schaal (22) obtained in 1871 "tetra-" and"octaaspartides", exact constitutions of which were not elucidated, by condensing aspartic acid and asparagine in a stream of hydrochloric acid.) The situation here is complicated by the possibility of the amino group reacting either with the alpha or the beta carboxyl groups. Balbiano and Trasciatti described the preparation of

polyglycine by heating glycine in glycerol (24, 25). They obtained a water-insoluble polymer, resembling a horny protein, which yielded on hydrolysis glycine in quantitative yield. Mailard (77, 78) found that triglycylglycine and diketopiperazine were also formed, their amounts being dependent on conditions used. He found also that aqueous solutions of triglycylglycine or glycine anhydride deposit an insoluble material on standing, which was apparently a hexapeptide. This observation would indicate that diketopiperazine may be an intermediate in the polymerization process. Shibata (79) obtained polymers by heating glycine anhydride and tyrosine anhydride in glycerol. On the other hand, in view of the relative ease of polymerization of esters of alpha-amino acid (cf. section A.2.a.1. below), Carothers (80) suggested the possibility of glycerol esters being the intermediate. Since esters are universally used as protecting groups for the amino-acid or peptide carboxyl groups, attempts at a controlled formation of peptide bonds by direct condensation of carboxyl and amino groups, facilitated by addition of energy, create a need for a more stable protecting group for the carboxyls. As will be seen in the chapter on the protecting groups, this problem is not yet satisfactorily solved.

Kahn's work with the photochemically induced reactions of diketopiperazine indicates that adding the energy in the form of irradiation may also facilitate the formation of

peptide bonds (81).

Some polymerization reactions based on the direct condensation of the amino groups with carboxyl (or ester) groups were apparently brought almost to completion by the removal of the product from the reaction mixture by its precipitation. Most of the enzymatically-catalyzed syntheses of the peptide bonds have as their significant characteristic the insolubility of their products (cf. e.g. 70, 71). The idea of forcing the formation of the peptide bond by removing the amide from the reaction mixture leads logically to the conclusion that a similar effect should be achieved by forcible removal of the by-product of this reaction, water.

A.2.a. Condensation of the Amino and Thiocarboxyl Groups

$$RCOSH + NH_2R' \longrightarrow RCONHR' + H_2S$$

In 1898 Pawlewski reported a new method for forming an amido bond by reacting the methanecarbothiolic acid with various aromatic amines. Since the by-product is gaseous hydrogen sulphide, instead of water, the reaction is faster and gives better yields than with the analogous acetic acid (82, 83). However, application of this method to the formation of peptides in hindered by the fact that the thioamino acids (alpha-amino thiolic acids) or their N-acylated derivatives have not yet been prepared. During attempts to

synthesize these compounds, Wieland (84) succeeded in preparing at least their S-phenyl esters, the reactions of which are described below (cf. A.2.b.2.).

A.2.b.1. Amino-Acid Esters and Their Derivatives

$$RCOOR'' + NH_2R' \longrightarrow RCONHR' + R''OH$$

Considered from the standpoint of synthesis of the peptide bonds, the amino-acid esters can be regarded as mixed "anhydrides", since we can obtain them as condensation products of the amino-acid carboxyl group and alcohols, with the elements of water as a by-product. Compared with the thioesters and mixed carboxylic anhydrides, the esters are least reactive towards the amino groups. As a matter of fact, they are often used as protecting groups for blocking the carboxyl radical in the alpha-amino acids and thus freeing the amino group from the zwitter-ion formation. However, under certain conditions the esters also react with the amino groups. (For example, the famous "biuret base" obtained by Curtius from ethyl glycinate when its ethereal solution was left standing at room temperature (4, 5) and which was later identified as tetraglycylglycine ethyl ester (85).) Fischer noted that methyl esters polymerize more easily than the corresponding ethyl esters (13). A number of other workers have followed Fischer's lead and have used amino acid esters for the synthesis of peptides (86, 87, 88).

A good review of this type of polymerization can be found on p. 129 of Katchalski's article on poly-alpha-amino acids (41).

Recently, Brenner, et al had shown that the incubation of esters of various amino acids with several alcohols in the presence of chymotrypsin or pepsin leads to the synthesis of peptides (65). Wieland concluded that the esters seem to be the energy-rich compounds of these reactions, in which the splitting of ester bonds supplies the energy necessary for the synthesis of peptides (66).

A.2.b.2. Amino-Acid Thioesters and Their Derivatives

$$RCOSR'' + NH_2R' \longrightarrow RCONHR' + R''SH$$

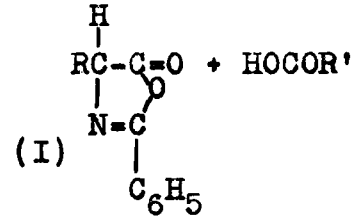
An indication exists that the ability of the esters of carbothiolic acids (for the sake of brevity called here thioesters) to transfer their acyl groups on the amino group might explain at least partly the bio-synthesis of peptides (66). So, for example, Lynen et al found in 1951 that the so-called "active acetic acid" from the cellular matter is a Coenzyme A methanecarbothiolate, CH_3COS-CoA (where HS-CoA is Coenzyme A) (89). Generally speaking, the behaviour of the thioesters (at least where R'' in the equation above represents a phenyl group) towards the amino group lies between that of ordinary esters and of mixed carboxylic anhydrides (66). On the other hand, the sensitivity of the thioesters towards hydroxyl groups seems to be less than that of ordinary

esters (84). This relative insensitivity towards hydrolysis made it possible to form peptide bonds even in aqueous solutions, e.g. by reacting N-carbobenzyloxy amino acyl thiophenol with a free amino acid in alkaline solution (i.e. amino acid salt), as was demonstrated by Wieland et al (84). Some amino acid thioesters with an unprotected amino group have a tendency to polymerize even at room temperature (66, 90).

A.2.b.3. Mixed Carboxylic Acid Anhydrides

$$RCOOCOR'' + NH_2R' \longrightarrow RCONHR' + R''COOH$$

When Wieland et al attempted unsuccessfully to improve the azlactone method of forming the peptide bond (cf. A.4.a.4.) by preparing an azlactone with a benzyloxy instead of phenyl radical attached to its ring, they noticed a theoretical analogy between the azlactones (I) and the mixed anhydrides (II) with the carboxylic acids (33):



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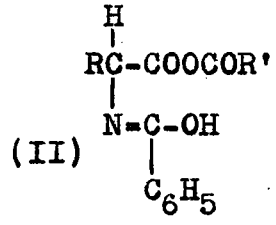


(II)

Consequently, instead of continuing the attempts to prepare benzyloxy-azlactones as intermediates for the synthesis of N-carbobenzyloxy-dipeptides, they turned their attention to the mixed anhydrides of N-carbobenzyloxy amino acids with

other carboxylic acids. Anhydrides of benzoic or acetic acid with N-substituted amino acids (prepared via benzoyl- or acetyl chloride and N-carbobenzoxy amino acid salt) were treated with aniline (33) and then with a salt, or ester of another amino acid (91) in alkaline aqueous solution. The benzoyl anhydrides were found to be superior to the acetyl anhydrides. However, Wessely et al (92) could not repeat the above results with N-carbobenzyloxy phenylalanine and benzoylchloride. Only symmetric anhydrides were obtained which, when reacted with the sodium salt of phenylalanine, gave only a small yield of dipeptide (detected chromatographically).

Vaughan and Osato (93) experimented with this reaction under anhydrous conditions. They tried 25 carboxylic acid chlorides (aliphatic or aromatic) for the preparation of mixed N-carbobenzoxy-glycine anhydrides and reacted these anhydrides with aniline in an anhydrous medium (toluene). Best results were obtained with alpha- and beta-branched chain aliphatic acids. The aromatic acids gave lower yields and a less pure product, while the straight chain aliphatic acids were least satisfactory. Since the isovaleric anhydrides (R = Me_2CHCH_2-), gave best results, isovaleryl chloride was then used in the preparation of a series of ethyl esters of N-carbobenzoxy- or phthaloyl-dipeptides. The yields varied from 29 to 86 per cent. Wieland and Stimming (94) prepared in 1953 several mixed anhydrides of hippuric

acid and various carboxylic acids in order to determine the preference of the amino group reacting with one or the other of the two acyl groups under anhydrous conditions. The reactions with hydroxylamine had shown the following order of preference (decreasing from first to last): chloracetyl, formyl, hippuryl, acetyl, propionyl, butytyl, phenacetyl, benzoyl. Compared with the above mentioned experiments of Vaughan and Osato, we see that in both cases the reactivity of amines with the acetyl, phenacetyl, and benzoyl groups decreased in this order (i.e. from acetyl down to benzoyl).

A.2.b.4.a. Amino Acyl Halides

$$RCOX + NH_2R' \longrightarrow RCONHR' + XH$$

(where X represents Cl; Br was also used, although no experimental data were described)(92)

This equation represents the classical method developed by Emil Fischer at the beginning of this century. In 1903 he decided to apply Meyer's method (95) of preparing the acyl chloride of pyridine carboxylic acid (by means of thionylchloride) to the conversion of the acylamino acids to the corresponding acylamino acyl chlorides (96). These compounds were then used for the condensation reactions with the amino acid or peptide esters (usually in ethereal or chloroform solutions). Phosphorus pentachloride was also used instead of thionylchloride. In 1905 Fischer (27) succeeded in

converting free amino acids into crystalline hydrochlorides of the aminoacyl chlorides by shaking the finely pulverized monoamino-monocarboxylic acids with phosphorus pentachloride in acetyl chloride. The products were condensed with amino acid esters (28) yielding dipeptide esters (after the hydrochloric acid was removed by neutralizing it with sodium methylate); however, the yields were not too satisfactory. On the other hand, the conversion of the amino acids with a protected amino group to the acyl chlorides via phosphorus pentachloride was used until recent times (30, 50, 92, 97, 98, 99).

This method was used generally for simpler amino acids, like glycine and alanine. In other cases, bothersome side-reactions were observed. So, for example, hydroxyl-containing amino acids may have replaced the hydroxyl group by chlorine (100). Dicarboxylic amino acids cannot be coupled by this method if peptides are desired, since disubstitution products would be obtained. Carbobenzyloxy amino acyl chlorides have a tendency to form oxazolidinediones (cf. A.4.a.1.). For the prolongation of peptide chains it is better to condense their free amino group with the aminoacyl chloride, instead of halogenating their free carboxyl, because this procedure will be accompanied by side reactions of the halogenating agent with peptide bonds (88).

The application of phosphorus trichloride for synthesizing the peptide bond was studied by Süs and Hoffmann (101),

who treated phenacetylglycine with this compound in the presence of ethyl glycinate, obtaining ethyl ester of phenacetylglycylglycine. However, on the basis of Goldschmidt's experiments with the phosphor-azo compounds (102) Süs and Hoffmann formulated a theory of phosphorus trichloride activating the amino group instead of the carboxyl group. This will be discussed later (cf. A.3.b.).

A.2.b.4.b. Amino Acyl Azides

$$RCON_3 + NH_2R' \longrightarrow RCONHR' + HN_3$$

This classical method was developed by Th. Curtius. During the period 1890-1900 he studied the reactions of hydrazides and azides. He discovered that azides of carboxylic acids can replace acyl chlorides in the Schotten-Baumann reaction. In 1902 Curtius described (10) the application of this reaction to the synthesis of benzoyl (pentaglycyl) glycine by condensing benzoylamino acyl azide with the free amino acid in alkaline aqueous solution (i.e. with the salt of the amino acid). Incidentally, he proved later (21) that this polypeptide is identical with the "gamma acid" he obtained in 1881 from benzoylchloride and silver glycinate (19, 20) and in 1883 from ethyl hippurate and glycine (4). Coupling the acyl azides with amino acid (or peptide) ethyl esters, Curtius and his collaborators prepared ethyl esters of benzoyl peptides of several amino acids (103). The azides

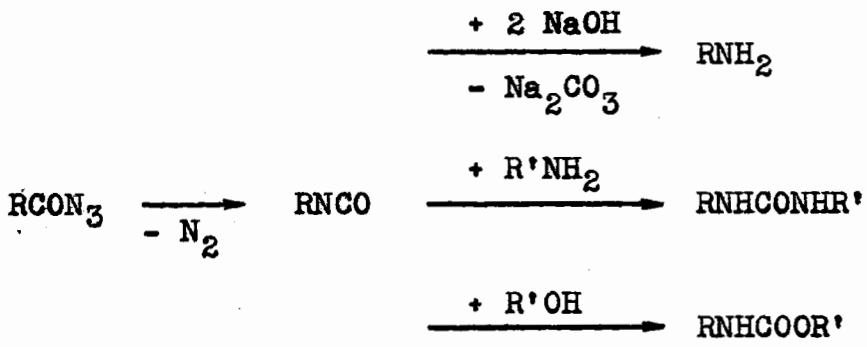
were prepared by treating the corresponding esters with hydrazine hydrate and converting the hydrazides thus obtained to the azides by means of nitrous acid:

$$-COOR \xrightarrow{NH_2NH_2 \cdot H_2O} -CONHNH_2 \xrightarrow{HNO_2} -CON_3$$

Hydrazine can be substituted by carbobenzyloxyhydrazine (104), which has the advantage of introducing a valuable protecting group to the carboxyl (cf. section on protecting groups below) which, under proper conditions can easily be converted into the reactive acyl azide.

Compared with the reaction of acyl chlorides with amino acid esters, which proceeds quite rapidly (it is usually completed in less than one hour), the azides react with amino acid esters much more slowly. For this reason it is customary to allow the reaction mixture to stand overnight at room temperature. On the other hand, the azide method seems to be less plagued by unwelcome side reactions. For example, as was mentioned above, the peptidic carboxyl groups cannot be well chlorinated, because of the possible side reactions of the halogenating agent with the peptide bonds (88). Therefore the prolongation of peptide chains is better accomplished by the azide route. Hydroxyl-containing amino acids, which cannot be halogenated (100), were successfully converted into azides (105). While the by-product of the acyl chloride method is hydrochloric acid, which requires prompt neut-

ralization, the acyl azide method produces hydrazoic acid, which quickly decomposes to nitrogen and hydrogen. On the other hand, the azides are not completely devoid of unwelcome side reactions; they may undergo a Curtius Rearrangement to isocyanate derivatives, which then react with amino groups to form ureido compounds, or with hydroxy groups to form urethanes (100, 106):



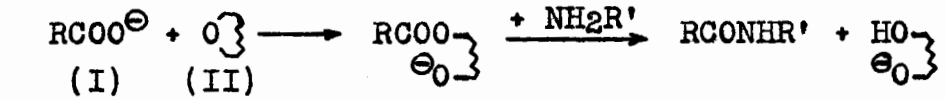
Another disadvantage is the need to convert the carboxyl radical either into an ester or into an acyl halide before it can be transformed into the hydrazide and then into the azide. However, since the amino acids are usually employed in the form of an ester, this disadvantage is not so serious.

A.2.b.4.c. Anhydrides with Sulphuric Acid & its Derivatives

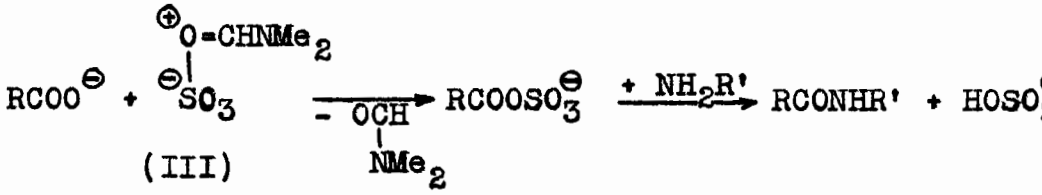
$$RCOO(SO_2)OR'' + NH_2R' \rightarrow RCONHR' + HO(SO_2)OR''$$

While mixed anhydrides were usually prepared by reacting a salt of one acid with a chloride of the other acid in non-aqueous medium, Kenner and Stedman (37, 107) experimented

with reacting the NH_2-protected amino acid salt (I) with a cyclic anhydride of some dibasic acid (II) This idea can be presented schematically as follows:



They obtained poor yields with various cyclic sulphonic anhydrides (e.g. o-sulphobenzoic anhydride, beta-sulphopropionic anhydride, etc.). Substituting sulphur trioxide for the cyclic anhydrides above they obtained much better yields. Sulphur trioxide was added in crystalline form as its complex with dimethylformamide (III):



By this method they condensed, e.g., carbobenzyloxy glycyl-L-phenylalanine with glycine without affecting the steric configuration.

Wieland and Bernhard (39) reported that, when benzene- or p-toluenesulphonyl chloride were used, the preparation of the amido bond was not successful. However, a reactive intermediate was obtained from sulphuryl chloride and N-carbobenzyloxy glycine (in the presence of a tertiary base

in an inert solvent at about 0° C.) which condensed with glycine in alkaline aqueous solution to yield N-carbobenzyloxy glycylglycine. Since the yields did not exceed 50 per cent, the authors believe there is a probability of the symmetric amino acid anhydride (IV) being the reactive form:

$$2\ XNHCH_2COOH \xrightarrow{+\ SO_2Cl_2} (XNHCH_2CO)_2SO_4 \xrightarrow[-SO_3]{} (XNHCH_2CO)_2O$$

(IV)

(X = carbobenzyloxy group)

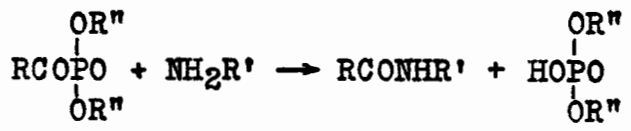
A.2.b.4.d. Anhydrides with Sulphurous Acid & its Derivatives

$$RCOO(SO)OR'' + NH_2R' \longrightarrow RCONHR' + HO(SO)OR''$$

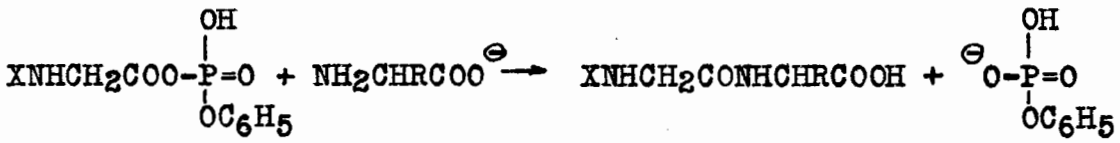
By analogy with their previous experiments with sulphuryl chloride, Wieland and Bernhard reacted thionyl chloride with N-carbobenzyloxy glycine (39) in an inert solvent in the presence of a tertiary base at about 0° C. The reactive intermediate was then condensed with glycine in alkaline aqueous solution. Although the yields were somewhat higher here than in the previous case, again they did not exceed 50 per cent. Consequently the authors believe that disporportionation of the unsymmetrical anhydride took place also in this case and that the amino group actually reacted with the symmetric anhydride of the carbobenzyloxy glycine.

A.2.b.4.e.

Anhydrides with Phosphoric Acid and its Derivatives



The importance of organic phosphorus compounds, especially the energy-rich acyl phosphates, for peptide synthesis in living cells has been known for some time (108). However, application of this theory to the synthesis of peptide bonds in vitro was possible only after the development of feasible phosphorylation techniques (109, 110, 111). After several preliminary experiments (112) Chantrenne reported (34) that phenyl carbobenzoxyglycyl phosphate (prepared by reacting carbobenzoxyglycylchloride with di-silver phenylphosphate) reacts rapidly with amino acid salts forming the corresponding carbobenzoxyglycyl peptides. The yields were quite high even at M/100 concentrations of both reacting substances.



(X carbobenzyloxy group)

Sheehan and Frank (113) isolated the anhydride obtained by shaking silver dibenzylphosphate suspended in a solution of phthaloylglycyl chloride in dry benzene at room temperature. This crystalline anhydride readily disproportionated

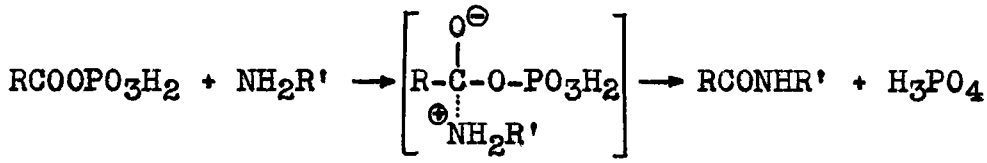
into symmetric anhydrides on attempted recrystallization from benzene, or in the presence of a small amount of triethylamine, or, more slowly, when left standing at room temperature. Reaction of this anhydride with aniline was very rapid and exothermic, yielding almost quantitative amounts of phthaloylglycyl anilide and of anilinum salt of dibenzylphosphate. No evidence of N-(dibenzylphosphoryl) aniline could be found. Similar results were obtained with excess of benzylamine. Reaction with glycine or phenylalanine in alkaline aqueous solution (pH 7.4) gave about 80 per cent yield of the phthaloyl dipeptide. Similar experiments with carbobenzoxyglycine gave only 50 per cent yield of anhydride.

Preparations of these anhydrides by other methods were not successful. Reaction of phthaloylglycyl chloride with triethanolammonium salt of dibenzylphosphate gave chiefly symmetrical anhydrides, and silver phthaloylglycinate gave with dibenzylphosphoryl chloride only a tarry product, from which the desired compound could not be isolated (113).

Using diphenylphosphoryl chloride and a solution of carbobenzoxyglycine and ethylpiperidine in anhydrous tetrahydrofuran, Wieland and Bernhard (39) reported only very small yields of dipeptides when the reaction mixture was shaken with alkaline aqueous solution of glycine. They ascribed this to the precipitated diphenylphosphoric acid, which was extremely difficult to remove. Similar experiments

with phenylphosphoric acid dichloride resulted in a 20 to 40 per cent yield of carbobenzoxyglycylglycine. Since they were not able to increase the yield above 50 per cent, the authors believe that disproportionation to carbobenzoxyglycine anhydride and phenyl metaphosphate took place. Only traces of carbobenzoxyglycylglycine were obtained when the synthesis was started with the reaction of phosphor oxychloride ($POCl_3$) and three moles of carbobenzoxyglycine under the conditions described above.

Koshland (114) used the acetylation of amines and amino acids by acetylphosphate in aqueous solution to study the kinetics of peptide-bond formation. He found the reaction to be first order with respect to acetyl phosphate and to amino compound and concluded that it involves a nucleophilic attack by the nitrogen of the amino compound on the carbonyl compound of the acyl phosphate.



A.2.b.4.f. Anhydrides with Phosphorous Acid & its Derivatives

$$RCOOP(OR'')OR'' + NH_2R' \rightarrow RCONHR' + HOP(OR'')OR''$$

Anderson et al (115) applied diethyl chlorophosphite as a reagent for peptide synthesis. They found that this reactant can be used either for activating the amino group, which will be discussed in section A.3.a.2., or the carboxyl group by forming an anhydride:

$$RCOOH + ClP(OEt)_2 \xrightarrow[-(R')_3NHCl]{+(R')_3N} RCOOP(OEt)_2 \xrightarrow[-HPO_3Et_2]{+R''NH_2} RCONHR''$$

Other diesterchlorophosphites were also used for this purpose (35). The structures of the intermediate anhydride or of the phosphite by-products have not been rigorously established. However, the observation that a quantitative yield of trialkylammonium chloride was obtained in the above reaction indicates that the mixed anhydride is formed, at least as a transitory intermediate. The intermediate was then allowed to react with an amino acid (or peptide) ester under anhydrous conditions. N-acyl dipeptide esters were prepared by this method in 80-90 per cent yields, while similar tripeptide derivatives were obtained in 25-50 per cent yields. The authors reported no apparent racemization of optically active derivatives. Similar results were obtained when tetraethyl

pyrophospite replaced the diethylchlorophosphite (116, 117). This procedure obviated the need for the presence of trialkyl amine, because here the by-product, diethylphosphite, is a weak acid and a liquid, which can be easily separated.

$$RCOOH + (EtO)_2P\text{-}O\text{-}P(OEt)_2 \rightarrow RCOOP(OEt)_2 + (EtO)_2POH$$

A.2.b.4.g. Anhydrides with Arsenous Acid & its Derivatives

$$RCOO\underset{\underset{OR''}{|}}{As}OR'' + NH_2R' \rightarrow RCONHR' + HO\underset{\underset{OR''}{|}}{As}OR''$$

The application of diethyl chloroarsenite as a reagent for peptide synthesis was studied by Vaughan (36). Again it was found that, as with chlorophosphites described above, either the amino group (an application of which will be discussed later), or the carboxyl group can be activated. The technique of forming the mixed anhydride and reacting it with the amino acid ester is the same as described above for chlorophosphites. While the chloroarsenite is easier to prepare and is more stable than chlorophosphite, the yields with diethyl chloroarsenite were somewhat lower.

A.2.b.4.h. Anhydrides with Carbonic Acid & its Derivatives

$$RCOOCOOR'' + NH_2R' \rightarrow RCONHR' + CO_2 + R''OH$$

The possibility of applying mixed anhydrides of an acyl

amino acid with carbonic acid was studied recently by several authors. Wieland and Bernhard (39) added a solution of phosgene in toluene to a cold solution of acyl amino acid and tertiary amine in benzene, hoping to obtain the following type of anhydride:

$$2\ RCOOH + COCl_2 \longrightarrow RCOOCOOCOR + 2\ HCl$$

However, this anhydride, if formed, is very unstable. Carbon dioxide was soon evolved and a symmetrical anhydride of the amino acid was obtained. This anhydride, when allowed to react with glycine, gave a dipeptide in less than 50 per cent yield.

More successful were the experiments with chloroformates, which were reported independently from three different laboratories in 1951 (38, 39, 40). The reactive form is the mixed anhydride of the carboxyl group with a carbonic acid monoester. The first step is the addition of an alkyl or aryl chlorocarbonate (chloroformate) to a solution of trialkylammonium salt of an NH_2-protected amino acid (or peptide) in an inert solvent at low temperature (about 0°C.) in order to obtain the mixed carbonic-carboxylic acid anhydride. This anhydride can then react either with an amino acid (or peptide) ester in an anhydrous medium, or with a salt of an amino acid (or peptide) in an aqueous solution, producing the amide bond. The yields are generally in the vicinity of 60-70 per cent. Carbon

dioxide and alcohol are the by-products:

$$RCOOH + R'_3N + ClCOOR'' \xrightarrow[-\,R'_3NHCl]{} RCOOCOOR'' \xrightarrow{+\,R''NH_2} RCONHR'' + CO_2 + R''OH$$

Boissonnas (38) used chloroform as anhydrous medium and prepared phthaloyl (or carbobenzoxy) dipeptides and their esters and one tetrapeptide (phthaloylglycyl-DL-phenylalanylglycyl-L-leucine). Wieland and Bernhard (39) made carbobenzoxy derivatives of di-, tri-, and tetra-glycine in this manner, using anhydrous tetrahydrofuran as a medium in which the anhydride was formed, and adding alkaline aqueous solution of glycine to it. They compared eight various alkyl (from methyl up to octyl) and two aryl (benzyl and phenyl) chloroformates. Of these esters, methyl and phenyl chloroformates did not yield good results, while ethyl and isopropyl esters gave very pure dipeptides, and esters of higher alcohols gave better yields in cases of higher peptides. In all cases they noted that the preparation of mixed anhydrides must be carried out at low temperature (about 0°C.), or partial disproportionation will occur (formation of symmetrical amino acid anhydride and of symmetrical carbonic acid ester anhydride, which immediately loses carbon dioxide). Wieland's group then proceeded to synthesize by this method naturally-occurring beta-amido compounds (118, 119). Vaughan used either toluene or chloroform as the anhydrous medium (40). He recommended branched-chain alkyl chlorocarbonates. Best

results were obtained with secondary or with isobutyl esters, the secondary butyl chlorocarbonate giving slightly higher yields than the isobutyl isomer (70, 120). No racemization occurred in the preparation of simple carbobenzoxy dipeptide ethyl esters (120), while some racemization was observed when similar derivatives of tripeptides were prepared.(121). The presence of chloroform was found to increase considerably the tendency towards racemization. Also, when an aqueous solution of the sodium salt of the amino acid was used, partial racemization was observed in prepared tripeptides, while no racemization occurred when a carbobenzoxy dipeptide was prepared (122). Applying these results, a tetrapeptide (dicarbobenzoxy-L-lysyl-L-valyl-L-phenylalanylglycine ethyl ester) was prepared with little, if any, racemization (122).

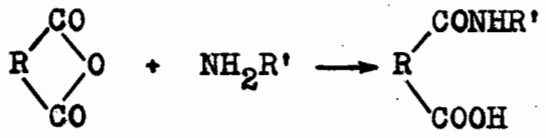
A.2.b.4.i. Anhydrides with Silicic Acid & its Derivatives

$$RCOOSi- + NH_2R' \longrightarrow RCONHR' + HOSi-$$

Schuyten et al reported in 1947 a preparation of acetoxysilanes from sodium acetate and silicon tetrachloride, or trimethyl chlorosilane, in anhydrous medium (123). Malatesta prepared acetoxysilanes from silicon disulphide (124, 125), while McKenzie used ethyltrichlorosilane (126) and silicon tetrachloride (127) to obtain silicone derivatives of several carboxylic acids. Malatesta had shown (125) that acylsilanes

can acylate amines, but did not report the yields. These anhydrides, sometimes called "esters", have not been applied to peptide syntheses.

A.2.c.1. Internal Dicarboxylic Anhydrides



The dicarboxylic amino acids can be treated like other amino acids only if the condensation of both carboxyl groups is desired. However, in natural peptides, the dicarboxylic amino acids are supposed to be linked by their alpha carboxyl groups only, the second one being either free, or in the form of a carboxyl amide ($CONH_2$). Consequently, similar bonding was desired in preparation of synthetic peptides. For this purpose, these amino acids are converted to cyclic dicarboxylic anhydrides. Such anhydrides can be prepared upon treatment with acetic anhydride (32, 128, 129). This reaction is usually not accompanied by racemization if carbobenzyloxy- (45) or phthaloyl-derivatives (130) are used, while acetyl and benzoyl amino acids are easily racemized by acetic anhydride (45).

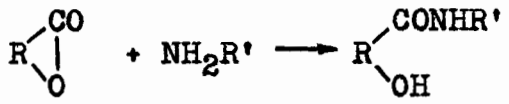
It is usually alpha-carboxyl to which the new radical becomes attached when such an anhydride reacts with amines (amino acid esters) (45, 131, 132, 133) or with alcohols (29, 134). However, the anhydride ring may not always open

exclusively in this direction (135, 136, 137). Generally, glutamic acid anhydride appears to have a stronger tendency towards alpha-carboxyl attachment of the radical than the aspartic acid anhydride. So, for example, carbobenzyloxy-L-aspartic acid anhydride yields with L-tyrosine ethyl ester a beta peptide bond, while a similar derivative of L-glutamic acid forms an alpha peptide (137, 138). On the other hand, phthaloyl glutamic anhydride gave gamma-amides, peptides, or esters (130, 139). It is of interest that hydrazine reacts preferentially with the anhydride ring of phthaloyl glutamic anhydride (instead of with the phthaloyl-imide ring, removing the phthaloyl group) forming the gamma hydrazide (139). With carbobenzyloxy glutamic anhydride the hydrazine forms a mixture of alpha and gamma hydrazides (140, 141).

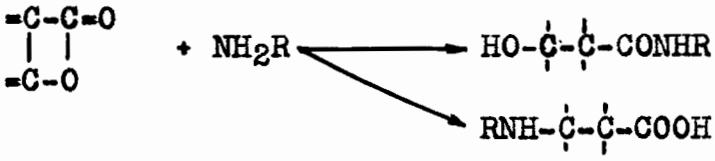
Very often, the product contains both derivatives. In that case we can utilize the fact that the alpha carboxyl group has higher acidity than the beta or the gamma carboxyl group, respectively, to separate these derivatives by fractional extraction of their solutions in organic solvents with aqueous solution of sodium carbonate (141). Sometimes the difficulty of isolating one of the two possible peptides in pure form can be circumvented by preparing a mono-ester of known constitution from the internal dicarboxylic anhydride and an alcohol (or alkoxide) and then either converting the remaining free carboxyl into some reactive form, or transforming

the esterified carboxyl into the azide. An example of the first type of synthesis is the reaction of carbobenzyloxy-L-aspartic anhydride with benzyl alcohol (134). Alpha-benzyl ester was obtained and the beta carboxyl was then converted into an acyl chloride with phosphorus pentachloride. The other type of synthesis was demonstrated by Le Quesne and Young (141) who subjected the mono-ester to fractional extraction and reacted the purified form with hydrazide at room temperature. Such a product could then be applied to the preparation of alpha or gamma (beta) peptides by an unambiguous path.

A.2.c.2. Lactones



Since several naturally-occurring amino acids contain a hydroxyl group, a knowledge of the reactivity of lactone rings with aliphatic (primary or secondary) and aromatic amines (142) is also of interest in peptide chemistry. The amine can react in two ways, forming either an amide, or a N-substituted amino acid.



A.3.a.1. Amides of Phosphoric Acid & its Derivatives

$$RCOOH + (R''O)_2(PO)NHR' \rightarrow RCONHR' + (R''O)_2(PO)OH$$

Crystalline amido phosphonates can be obtained from amines and dialkoxyphosphoryl chlorides. This reaction was used by Saunders et al to identify dialkoxy phosphoryl chlorides (or fluorides) during his studies on esters containing phosphorus (143, 144).

Si-Oh-Li applied this reaction to amino acid esters (145). Out of ten amino acid ethyl esters, only four (methyl esters of glycine, Dl-alanine, DL-phenylalanine, and DL-tryptophan) gave products which could be isolated with satisfactory yields. The products were found to be quite stable in acidic medium. No application of this intermediate to peptide chemistry has yet been reported.

A.3.a.2. Amides of Phosphorous Acid & its Derivatives

$$RCOOH + (R''O)_2P\text{-}NHR' \rightarrow RCONHR' + (R''O)_2POH$$

Diethyl chlorophosphite forms with amino acid esters, in the presence of an equivalent of triethyl amine (acid acceptor) in an inert solvent, oils, at least some of which are distillable (115). They react with carbobenzoxy amino

acids in inert solvents at elevated temperatures to form carbobenzoxy peptide esters (115, 146). The by-product is presumably diethylphosphate $(EtO)_2POH$. Tetraethylpyrophosphite can also be used for this purpose (116). Using this reagent, anilides were prepared in good yield (117). While no racemization took place when dipeptide derivatives were prepared, some racemization was observed with tripeptide derivatives (116).

A.3.a.3. Amides of Arsenous Acid & its Derivatives

$$RCOOH + (R''O)_2As\text{-}NHR' \rightarrow RCONHR' + (R''O)_2AsOH$$

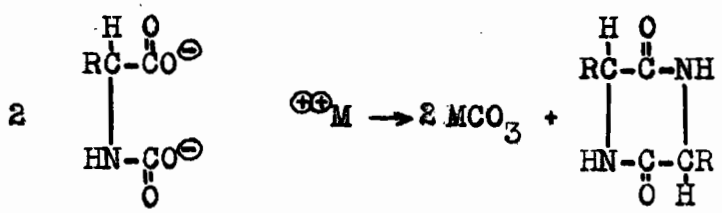
Diethyl chloroarsenite forms with amino acid esters (under conditions described above for chlorophosphites) non-distillable oils. After removal of the precipitated triethylammonium chloride, the solution of diethyl arsenite amide in toluene is refluxed for one hour with an equivalent of a second amino acid which has a protected amino group. The by-product, presumably diethyl arsenite, is precipitated quantitatively as arsenic trioxide by addition of water (36).

A.3.a.4. Amides of Carbonic Acid (Carbamates)

$$RCOOH + {}^{-}OCONHR' \rightarrow RCONHR' + CO_2 + {}^{-}OH$$

N-Carboxyl amino acids have been prepared in the form of alkaline earth metal salts by passing a stream of carbon dioxide through an ice-cold suspension of barium (or calcium)

hydroxide in a water solution of an amino acid (156, 157). These salts, sometimes termed Siegfried's salts, were also prepared by adding barium (or calcium) hydroxide to an ice-cold aqueous solution of oxazolidinediones (41). Heating of these salts produces diketopiperazines and metal carbonate (157):



Similar sodium salts were prepared from the aqueous solution of the amino acid and sodium carbonate by adding methanol, which precipitates the product in crystalline state (4). Farthing suggested that this product could be used for purification of the amino acids, since elevated temperature releases carbon dioxide, or for polymerization of these amino acids by reacting these salts with phosgene, or with thionyl chloride. However, the latter seems to work with glycine only (158).

A.3.b. Phosphazo Compounds

$$2\ RCOOH + R'N{=}P{-}NHR' \longrightarrow 2\ RCONHR' + HPO_2$$

N-Monosubstituted carboxylic amides can be prepared by a one-step reaction of a carboxylic acid, phosphorus trichloride,

and a primary amine. Investigations carried out by Grimmel et al (147) indicate that the previous belief, that the acyl chloride is the intermediate in this reaction, was wrong. It was concluded that it is a phosphazo compound which is the intermediate:

$$5\ RNH_2 + PCl_3 \longrightarrow RN{=}P{-}NHR + 3\ RNH_2.HCl \quad *$$

When heated, the phosphazo compound then reacts with the carboxylic acid:

$$RN{=}P{-}NHR + 2\ R'COOH \longrightarrow 2\ R'CONHR + HPO_2$$

Grimmel et al tried unsuccessfully to react these phosphazo compounds with alpha-amino acids. The explanation of their failure probably lies in the unfortunate fact that they used free amino acids, not realizing the need of first breaking up the zwitter-ion structure of the alpha-amino acids by protecting the amino group.

Free amino acid ethyl esters (not hydrochlorides; hydrochloric acid was removed before by Hillmann's method (cf. 148))

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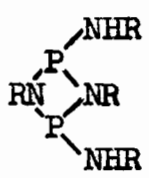
* Since the molecular weight determinations show that these compounds exist as dimers in solutions, the structure of the phosphazo compound should, perhaps, be written:

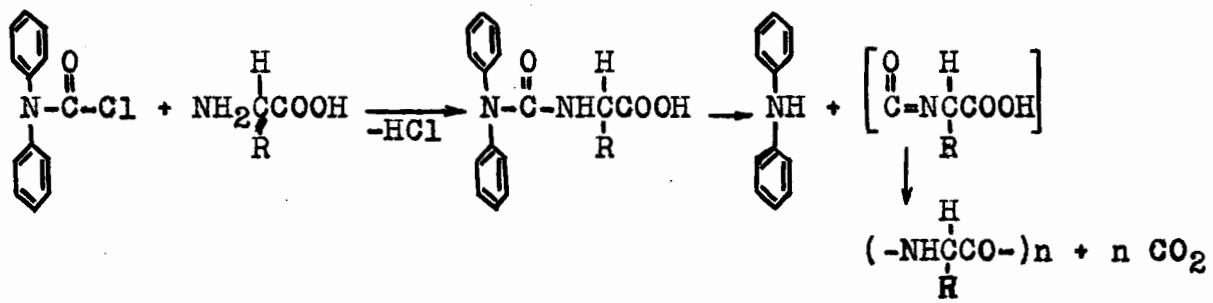


were used for the reaction with phosphorus trichloride and acyl (phenacetyl, benzoyl, carbobenzyloxy) glycine in an inert solvent by Süs and Hoffmann (101). The results were only fair. Slightly better results were obtained by Goldschmidt (102, 149). Anderson et al (146) applied this method to the preparation of carbobenzyloxy- and phthaloyl amino acid anilides and to the preparation of a (presumed) phosphazo derivative of ethyl DL-phenyl-alaninate. The latter was successfully combined with carbobenzyloxy glycine to form the dipeptide derivative, but in poor yield.

A.3.c. Isocyanates

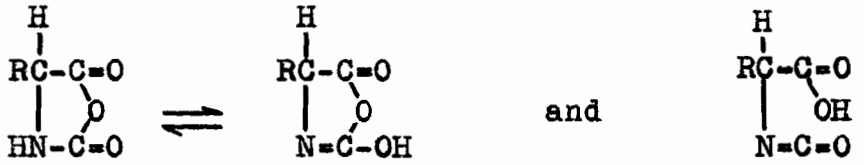
$$RCOOH + OCNR' \longrightarrow CO_2 + RCONHR'$$

Isocyanates prepared from the free alpha-amino acids are very labile compounds and polymerize easily to polypeptides (150, 151):



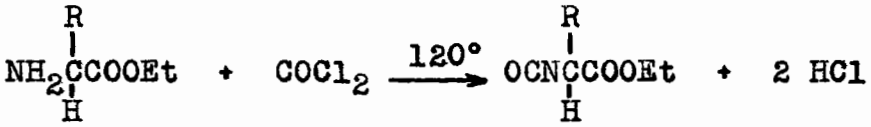
The considerable similarity of the conditions and results of this polymerization as compared with the polymerization of oxazolidinediones suggests a possibility that isocyanates

may be the intermediates in the polymerization reactions of oxazolidinediones, , or vice versa (see A.2.a.1.). The possible relationship of both types of compounds is best apparent when their structural formulas are compared:



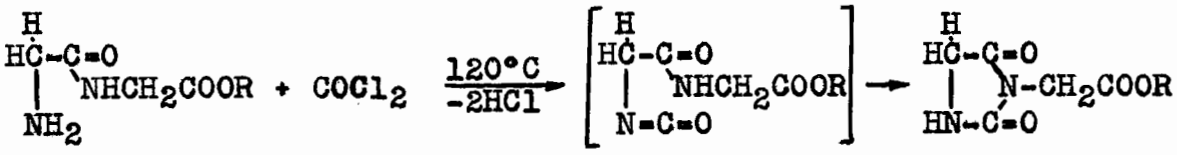
oxazolidinedione-2,5 isocyanate

The application of isocyanates to the controlled synthesis of peptides was pioneered by Goldschmidt in 1950 (102, 152). He prepared isocyanates of amino acid ethyl esters by Gattermann's method (153):



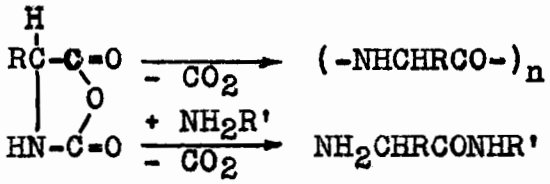
The yields were above 90 per cent in cases where the esterification of the amino acids used was complete (sometimes it was found advantageous to re-esterify the amino acid esters before reacting them with phosgene). The N-carbonyl amino acid esters are colourless, lachrymatory liquids, which can be distilled in vacuo without decomposition. A similar preparation of carbonyl dipeptide esters was unsuccessful. The N-carbonyl group closed a ring by the transfer of the hydrogen atom from the amido group, forming a hydantoin in almost

quantitative yield:



Addition of alcohol to N-carbonylamino acid esters produced N-carbalkoxyamino acid esters (152). A similar reaction of carboxylic acids with isocyanic acid esters yielding N-substituted carboxyl-amides had been described already in 1854 by Wurtz (154). Inconvenient side reactions were observed with aromatic isocyanates only (155). Accordingly, the application of Wurtz's method to the acyl-amino acids (which were either melted with the N-carbonyl amino acid esters at 110°C., or a mixture of both reactants in some inert, anhydrous solvent was heated to 110°C. until no more carbon dioxide was evolved) resulted in good yields of peptide derivatives (152).

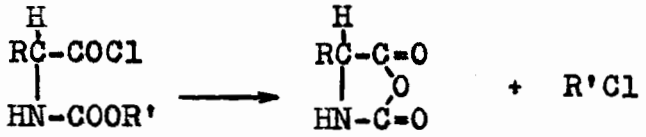
A.4.a.1. <u>Oxazolidinediones-2,5</u>



Oxazolidinediones-2,5 are known under several names, viz., N-carboxy anhydrides, N-carbonic acid anhydrides,

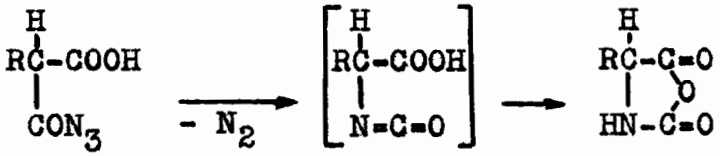
anhydro-N-carboxy amino acids, oxazolid-2:5-diones, Leuchs' anhydrides, isatoic acid anhydrides (159), azasuccinic anhydrides (160).

There are several methods of preparing this type of azole. Heating of an N-carbalkoxy amino acid chloride yields cyclic anhydride of an amino acid-N-carbamic acid with simultaneous formation of alkyl chloride:



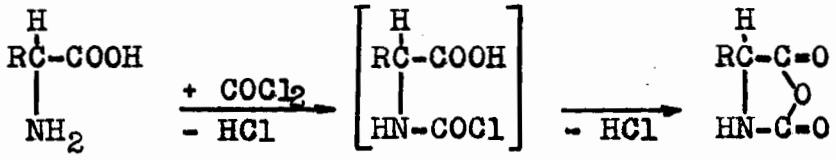
This method was developed by Leuchs (161, 162, 163) when he investigated in 1903 the decomposition of N-carbethoxy glycyl chloride. Higher yields were obtained with methyl derivatives. Bergmann and Zervas (45) reported that carbobenzyloxy amino acyl chlorides will also undergo similar reaction.

Another method originated by Curtius and Sieber (159). When attempting to prepare isocyanates from substituted malonic acid azides, they obtained oxazolidinediones (and some diketopiperazines and polypeptides):



Hurd and Buess (160) reported similar results with alpha-substituted alpha-carboxy hydroxamic acids, which were easily converted into oxazolidinediones (azasuccinic anhydrides).

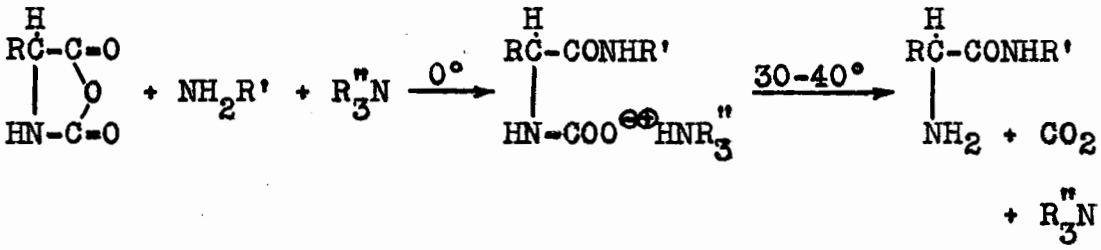
The reaction of phosgene with amino acids also yields these cyclic anhydrides. This reaction was reported first by Fuchs, who used N-phenylglycine (164), and was later applied to unsubstituted amino acids by Farthing and Reynolds (165):



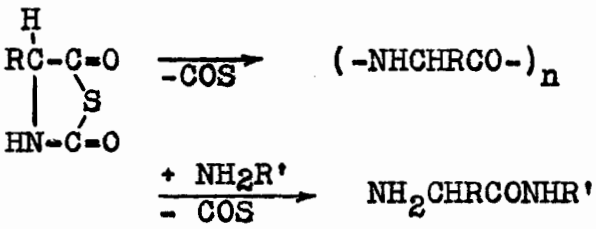
The presence of the intermediate carbamic acid chloride was proved by obtaining N-phenyl hydantoic acid when aniline was added to the reaction mixture (165).

The ability of oxazolidinediones to polymerize was noted many years ago by Leuchs (161) when he obtained a compound similar to the polyglycine of Balbiano and Trasciatti (24). Since that time, these anhydrides have been developed into important starting materials for preparing simple poly- and co-poly-alpha-amino acids. On the other hand, there are only a few available reports on the aminolysis of oxazolidinediones, which are of greater interest from the standpoint of the controlled synthesis of peptides. Leuchs and Mannase (162) obtained from N-phenylglycine-N-carbonic anhydride with alcoholic ammonium an amide of N-phenylglycine. Fuchs (164) reacted the same anhydride with aniline, obtaining the anilide of phenyl glycine. Wessely et al (166, 167, 168) treated phenylalanine-N-carbonic anhydride with various primary amines

obtaining fair yields of N-substituted amides of phenylalanine. Replacing the amines with an excess of glycine ester, they obtained phenylalanylglycine ester in moderate yield. Hunt and du Vigneaud (169) used this method to prepare alanyl histidine. Bailey (170, 171) found that if a tertiary amine is present, the yields are improved, apparently because the triethylammonium salts of the intermediate carbamates are less stable than the salts of the carbamates with the amino group of the amino acid ester as the cation:

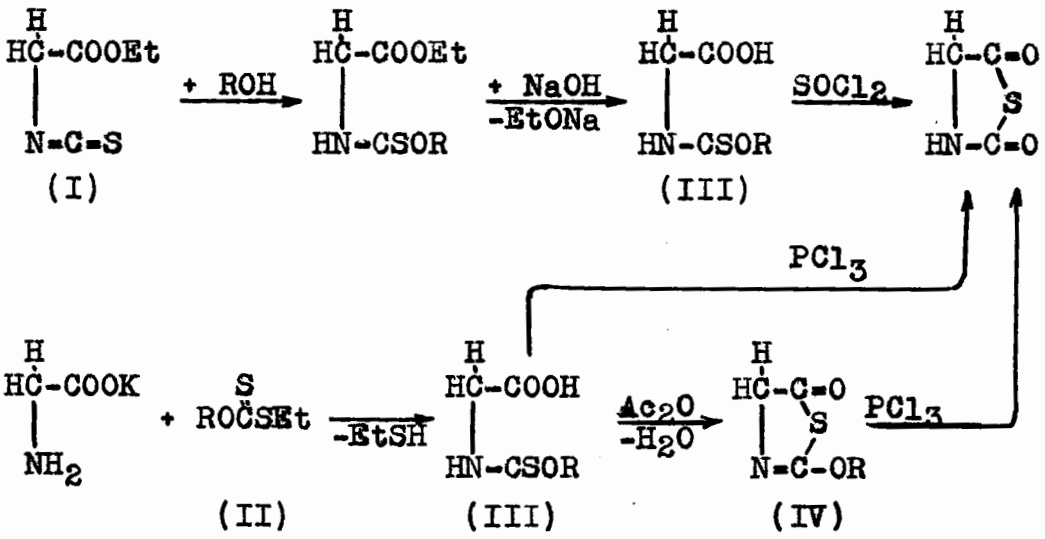


A.4.a.2. Thiazolidinediones-2,5



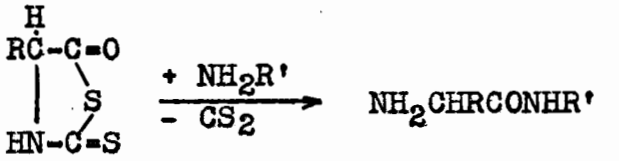
Thiazolidinediones can be prepared from N-thiocarbalkoxy amino acids by the action of either thionyl chloride (171) or phosphorus trichloride (172). The former reaction was studied by Bailey (171) who assumed that an acyl chloride was formed, which cyclized into a 2-thiooxazolidone-5 (cf. the Leuchs method of preparing oxazolidinediones above).

However, Aubert et al, who used phosphorus trichloride (or tribromide) prepared an identical compound also from phosphorus trichloride and 2-alkoxythiazolone-5 (172). Both methods are schematically shown below; Bailey started with carbethoxymethyl isothiocyanate (I) and used thionyl chloride, while Aubert et al started with ethyl alkylxanthate (II) and used phosphorus trichloride on both N-thiocarbalkoxyglycine (III) and 2-alkoxythiazolone-5 (IV):

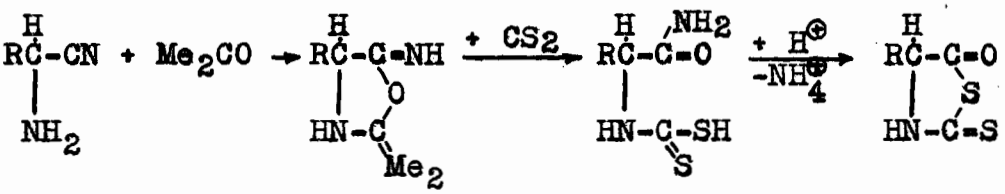


This cyclic anhydride readily polymerizes on heating in aqueous solution to give what is believed to be a polyglycine (173). Bailey used this anhydride to prepare glycylglycine ethyl ester, but did not report the yield (171).

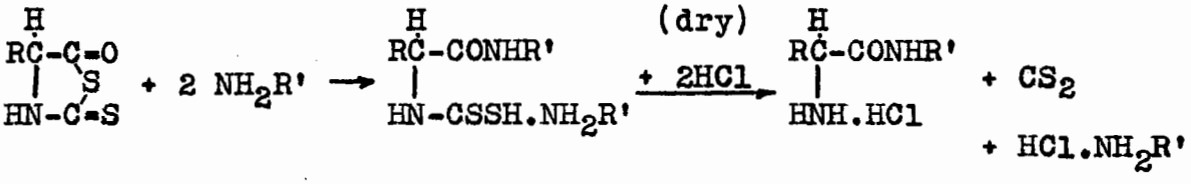
A.4.a.3. 2-Thiothiazolidinones-5



These compounds can be prepared from alpha-amino nitriles as follows:



While their analogues, oxazolidinediones-2,5 are generally very unstable and undergo polymerization readily, 2-thiothiazolidinones-5 are quite stable. However, they are still readily attacked by primary or secondary amines to give amides in the form of dithiocarbamate salts (175, 176):

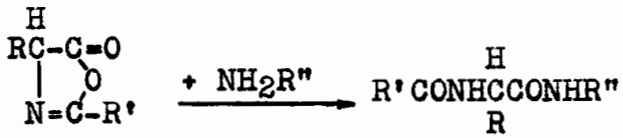


Equimolecular amounts of the amino acid ester and trialkyl amine can be used, the tertiary amine serving to form salts with the dithiocarbamate of the dipeptide (177, 178). Thiazolidinones can react also with the amino acids in alkaline aqueous solution. Acidification separates the dipeptide,

which is invariably mixed with its component amino acid. The subsequent purification, e.g. by a cation exchange resin, decreases considerably the yield (178, 179). Moreover, 4-substituted-2-thiothiazolidinenes-5 (i.e. in the above equations: R ≠ H) give still lower yields, because the acidification of the intermediate dithiocarbamate, even in non-aqueous media, tends to regenerate the original thiazolidinone (180, 181).

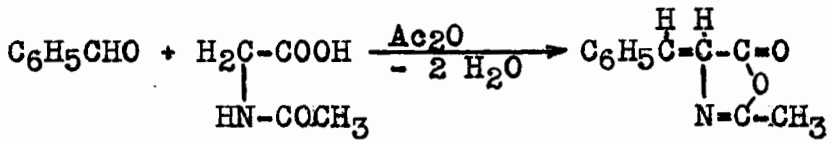
This method suffers from the disadvantage that alpha-amino nitriles are needed as a starting material, and these are not easily obtained. The direct addition product of alpha-amino acids and carbon disulphide were found to be unsuitable for the synthesis of peptides (181).

A.4.a.4. 2-Substituted Oxazolinones-5



2,4-Substituted oxazolinones, better known as azlactones, may be considered anhydrides of N-acyl-alpha-amino acids. They can be prepared by several methods, which may be classified into two types: azlactonization of N-acyl-alpha-amino acids, and Erlenmeyer's (182) azlactone synthesis. In methods of the first type, azlactonization is achieved by removal of water by the action of acyl (usually acetyl) anhydride, or

chloride, the latter either in pyridine solution, or in aqueous solution of the acyl amino acid sodium salt. The second type consists of a reaction of an aromatic aldehyde with an N-acyl glycine in the presence of acetic anhydride:



The aromatic aldehydes may be replaced by alpha-beta-unsaturated aliphatic aldehydes, phthalic anhydride, and pyruvic acid. Saturated aliphatic aldehydes generally give low yields (183). In general, the azlactones of saturated acylamino acids are more difficult to purify than those of alpha-beta-unsaturated analogues (184).

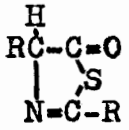
Mohr and Stroscheim prepared azlactones of benzoylalanine and of benzoylphenylalanine by methods of the first type in 95 per cent yields and reacted them with aniline and alpha-methyl alanine ethyl ester to obtain anilides and a benzoyl "dipeptide" ethyl ester (185). Azlactone of alpha-acetaminocinnamic acid (cf. the equation above) was prepared by Bergmann et al by a method of the second type and used to introduce the acetylphenylalanyl unit into the peptides (32).

After this azlactone was allowed to react with the free amino group of an amino acid ester (or peptide). The catalytic hydrogenation turned this moiety into the acetylphenylalanyl unit. However, it was found simpler to directly

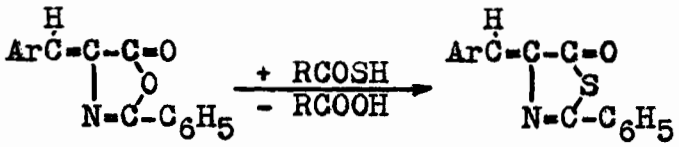
convert acetylphenylalanine into the azlactone by a first-type method (32). In a similar manner Bergmann et al introduced a benzoylphenylalanyl and benzoyltyrosyl unit into the dipeptides (69). Later Bergmann returned to Erlenmeyer's method, after reports about the existence of a dehydropeptidase in swine kidney (which hydrolyses the unsaturated peptide glycyldehydrophenylalanine) indicated that dehydrogenated peptides might occur as metabolites (186). Benzaldehyde was used to obtain unsaturated phenylalanine unit, and p-hydroxybenzaledhyde to obtain an unsaturated tyrosine unit. In this way, unsaturated di, tri, tetra, and penta peptides were prepared (186).

Generally, azlactones give good yields of dipeptides (higher even than with oxazolidinediones), but low yields in the preparation of tripeptides (92). The attempts to introduce a better NH_2-protecting group (e.g. carbobenzyloxy group) were unsuccessful (33).

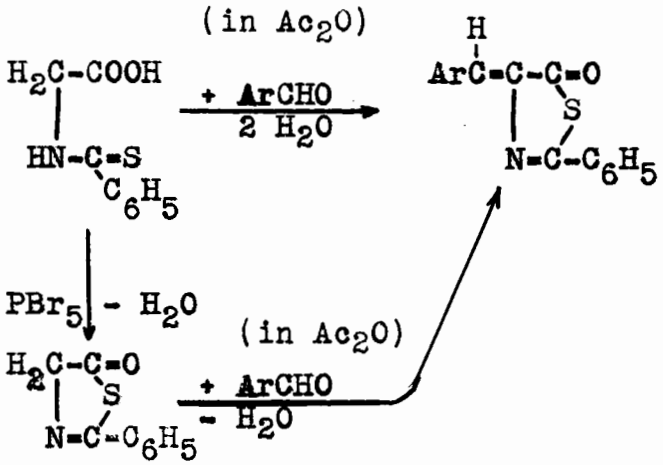
A.4.a.5. 2-Substituted Thiazolidinones-5



Behringer found that reaction of carbothiolic acids with 2-phenyl-5-alkylidene azlactones yields analogous thioazlactones or 2-substituted thiazolidinones-5 (187):

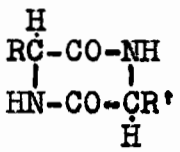


The structure of this compound was identical to that prepared by Robinson and Jepson (cf. 188):



The above reactions are described here because if more suitable derivatives of the above products could be prepared, a new valuable method of peptide synthesis could be developed.

A.4.b. Diketopiperazines



Diketopiperazines can be prepared from esters (cf. page 3 above), Siegfrieds salts (cf. page 42 above) or amides (189). They can be split open by hydrolysis into dipeptides

(17, 18, 65, 190), more effectively under alkaline conditions than in acidic medium (191, 27). An interesting variation is Kahn's reported condensation of glycine anhydride with glycylglycine under ultraviolet light (81).

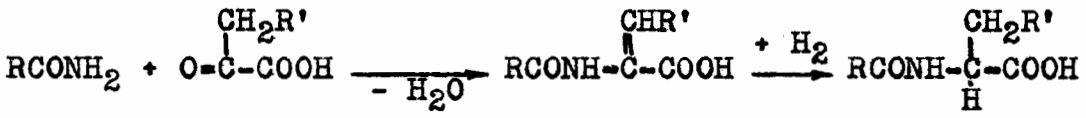
Wider application of the diketopiperazine method is hindered by partial racemization (192) which is more pronounced when alkaline hydrolysis is employed (193, 194). In a few cases careful acid hydrolysis yielded optically-active dipeptides in good yield, e.g. L-cysteinyl-L-cysteine (195) or L-methionyl-L-methionine (65), the latter being obtained in better yield by this method than by the azide method. Even mixed dipeptides were secured in quite pure state (196) although, more often, a mixture of two possible dipeptides was obtained, from which the two isomers were difficult to separate (197).

B.1.a. Condensation of Amides with Hydroxy Acids

$$RCONH_2 + NaOR' \longrightarrow RCONHR' + NaOH$$

Bresler and Selezneva (198, 199) succeeded in preparing N-ethyl acetamide from acetamide and sodium ethoxide in almost quantitative yield. Similarly, polyalanine was prepared from alpha-hydroxypropanamide (lactamide) and sodium in dioxane and heating the isolated sodium alkoxide in vacuo at 80° C. for three to four weeks. A clear, resinous mass was obtained, which yielded alanine upon either acidic or pancreatic hydrolysis.

B.1.b. Condensation of Amides with Keto-Acids



This reaction is presently of interest in peptide synthesis more because of its possible relationship to the biological synthesis of peptide bonds (70) than because of its preparative value. Several preparations of acylaminoacids (from acylamide and ketoacid) and of dehydropeptides were reported (200, 201, 202); the yields were not high (cf. preparation of similar compounds by the azlactone method (page 52 above).

B.1.c. Condensation of Amides with Amines (Transamidation)

a) $RCONH_2 + NH_2R' \longrightarrow RCONHR' + NH_3$

b) $RCONHR'' + NH_2R' \rightleftharpoons RCONHR' + NH_2R''$

Transamidation reactions do not perhaps belong properly in a section dealing with the formation of the RCONH-R bond, since the mechanism of these reactions involves in all probability an exchange of radicals at the RCO-NHR bond. The reason for placing them here is that these reactions, like all other reactions in this section, are those of amides, i.e. of compounds which already have an amido bond.

At room temperature the transamidation reactions are extremely slow, almost non-existent, and require the catalytic effect of enzymes in order to proceed with any apparent

results (the enzymatically catalyzed transamidations and transpeptidations are discussed on page 9). On the other hand, transamidations at elevated temperatures have been known since the 19th century (203, 204, 205, 206). However, these occurred only when aromatic amines were heated with unsubstituted amides and the yields were only fair. The transamidation reactions of substituted amides with aromatic amines at elevated temperatures were studied only recently by Jaunin (207) who heated acetanilide with p-toluidine or with p-anisidine. He found them to be reversible reactions, catalyzed by acids (e.g. benzoic acids).

No transamidation reactions were observed with aliphatic amines (208, 209) until Galat and Elion (210) applied Hoffman's discovery (211) that acetamide reacted satisfactorily with the hydrochloride of an amine (in this case hydroxylamine). Thus, aliphatic amines, when in the form of their hydrochlorides or sulphates, can also undergo transamidation reactions with unsubstituted amides at elevated temperatures (210). Ammonia then separates in the form of salts with these acids. Boron trifluoride was also found to be an effective catalyst (212). McGregor and Ward reported that if the alkyl radicals contain at least eight carbon atoms, monoalkylamides of aliphatic monobasic acids can be prepared by simple heating of aliphatic monoamide and a long chain alkyl amine to 150-200° C. until ammonia ceases to be evolved(213). Non-enzymatic transamidation has yet to be applied to the synthesis of peptides.

B.2.a. Metalo-amides

$$RCONHM + XR' \longrightarrow RCONHR' + MX$$

(where M represents an alkali metal and X a reactive radical, e.g. a halogen atom)

Sodamide is capable of forming sodium derivatives of acyl amides in liquid ammonia (214):

$$RCONH_2 + NaNH_2 \xrightarrow{\text{liquid } NH_3} RCONHNa + NH_3$$

Such a derivative may be able to form an amide bond by condensation reaction with compounds containing some reactive group, such as a halogen atom.

C.1. Formyl-amides

$$RX + HCONHR' \longrightarrow RCONHR' + HX$$

(where X represents a reactive radical, e.g. a halogen atom)

The formyl radical has been recently introduced as a NH_2-protecting group to peptide synthesis (215, 216). The possibility of reacting N-formyl amino acids or their derivatives with amines which have in the alpha position a radical capable of condensing with the active hydrogen atom of the N-formyl group (probably alpha-chloro amines) is worthy of consideration.

Protecting Groups

The roles of protecting (masking, blocking) groups in peptide synthesis are manifold. The most important role is to render the amino acid temporarily monofunctional. The formation of the amido-linkage can then be controlled, because the protecting groups make uncontrolled polymerization impossible. Moreover, by breaking up the zwitter-ion configuration, the immobilization of one group activates the other group of the amino-acid (or peptide) molecule. Still another role may be to render the product insoluble in the reaction medium. A generalized review of the types of protecting groups is summarized in Table II:

Table II

It is essential that such a protecting groups can be introduced with ease and in good yield and subsequently, at any stage, removed with ease and in good yield, without destroying the amido-linkages and without altering the steric configuration. In some cases it is advantageous to use a protecting group of such a kind that after its removal it will leave the respective carboxyl or amino group in a reactive form, and thus make it possible for one to pass directly to the next condensation step.

Protection of the Carboxyl Group

1. Esters RCO-OR'

For the purposes of peptide syntheses the carboxyl group of amino acids has been most frequently protected in the form of an ester group. This type of protection is not ideal, since the ester group may react under certain conditions with the amino group either of the same or of another compound, forming an amido bond (cf. the spontaneous formation of polyamides and diketopiperazines from the amino acid esters described above on page 3). However, if precautions are taken, such as storing the esters of amino acids in the form of salts (most often as hydrochlorides) and avoiding elevated temperature whenever possible, the esters do serve their purpose of protecting the carboxyl group from unwanted reactions and enhancing the reactivity of the alpha-amino group by breaking up the zwitterion configuration.

From the standpoint of subsequent cleavage, we recognize two types of esters. Simple esters, like methyl or ethyl esters, require hydrolysis for this purpose, while those containing beta-gamma unsaturated radicals, like benzyl esters, can be cleaved by non-hydrolytic methods. Although it has been shown (93, 101, 217) that in certain cases the conditions required for the hydrolysis of methyl or ethyl esters can be so mild that the peptide bonds are scarcely, if at all, affected, a reaction which is certain not to break any peptide bonds is always more desirable. In the past, the reason for not using the benzyl esters more often has rested in the difficulty of preparing pure amino acid benzyl esters in reasonable yields. They have been prepared either from the amino acyl chloride hydrochloride and benzyl alcohol (48, 218, 219) or by heating the suspension of amino acid in benzyl alcohol saturated with dry hydrogen chloride (Fischer's method; cf. 8, 220). Bergmann even considered the coupling of N-carbonic acid anhydrides of amino acids (oxazolidinediones) with benzyl alcohol (221). Considerable improvement of yields obtained by Fischer's method was reported when the hydrogen chloride was replaced by p-toluene-sulphonic acid (222, 223). The benzyl group can be removed either by catalytic hydrogenation (224), by phosphonium iodide (48, 225), or by sodium in liquid ammonia. No tendency for the benzyl ester to undergo ammonolysis in liquid ammonia has been observed (48).

2. Amides RCO-NHR' (where R' represents hydrogen, alkyl, aryl, or their derivatives)

Amides have sometimes been used in enzymatic studies, most often in the form of anilides (cf. e.g. 67, 69). They are removable by mild hydrolysis.

Substituted acid hydrazides have recently been introduced into peptide chemistry as a means of protection for the carboxyl groups. Waldschmidt-Leitz and Kühn (74) worked with the phenylhydrazide (where the R' symbol above represents the $-NHC_6H_5$ group) and the p-nitrophenylhydrazide groups, which can be removed by a somewhat involved procedure using a warm aqueous solution of cupric salts (e.g. acetate) and filtration of the cupric oxide formed. Hofman et al (104) reported good yields of crystalline carbobenzyloxyhydrazides (where the R' symbol above represents the $-NHCOOCH_2C_6H_5$ group) prepared by an interaction of 4-substituted 2-thiothiazolidones-5 (174) with carbobenzyloxyhydrazine. Hydrogenolysis conveniently liberates a free hydrazide group ($R' = -NH_2$) which may then be converted to an azide (by the action of nitrous acid) for further coupling reactions.

3. Salts $RCO-O^{\ominus}$

Conversion of the amino acid into metallic salts releases the alpha-amino group from the zwitter-ion structure.

Therefore, alkaline aqueous solutions of amino acids are sometimes used for coupling reactions with the compounds which contain an activated carboxyl group (cf. e.g. 38, 40). The disadvantage of this method lies in the fact that activated carboxyl groups are invariably reactive also towards hydroxylic solvents. Consequently the presence of water decreases the yield of the amide by introducing unwelcome side reactions. If it were possible to find some cation which would form a carboxylate soluble in some inert solvent, the salts of amino acids might become much more valuable intermediates than either esters or amides, because of the unsurpassable ease of liberating the carboxyl group by a simple neutralization reaction.

B. Protection of Amino Group

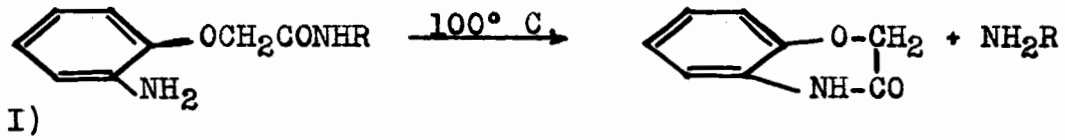
1.a. Amides of Carboxylic Acids

RNH-COR' (where R' represents hydrogen, alkyl, aryl, or their derivatives)

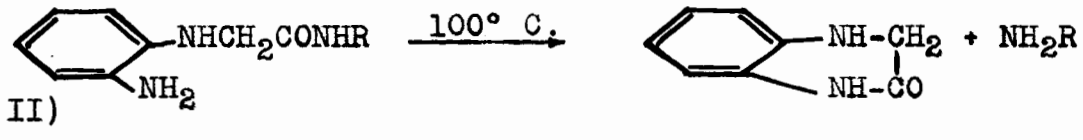
The N-acylation of amino acids or peptides is one of the oldest methods of rendering them monofunctional. Acetyl and benzoyl groups (10, 32, 226) are typical of earlier representatives of this class of protecting groups. Their disadvantage lies in the reactions necessary for their removal after they have served their purpose. Hydrolysis must be

used, which is a reaction capable of cleaving the newly formed peptide bond. Although Bergmann et al (32) have shown that some acetylated dipeptides can be deacetylated with dilute acid, selective hydrolysis of even the acetyl group cannot be relied upon in all cases.

Recently, several variations of this method of protecting the amino group have been reported. Holley and Holley (227) announced that two modifications of the acetyl group, namely, the o-aminophenoxyacetyl (I) and o-aminophenylglycyl (II) groups, can be removed simply by boiling in water. The explanation of this ready decomposition lies in the ability of these groups to form lactam rings at elevated temperatures.



(I)



(II)

The first case found practical application when o-nitrophenoxyacetylamino acids were used in the peptide synthesis, and the nitro group was then reduced by a catalytic hydrogenation to amino group. In the other case, the reaction of chloroacetylamino acids or peptides with o-phenylenediamine made it also possible to remove the acyl group, which separated

in the form indicated by the second equation above. The reported yields of these deacylation reactions were in the range of 30-75 per cent.

Another interesting variation is the N-trifluoroacetyl group, which can be introduced by treating the amino acid with trifluoroacetic anhydride. Derivatives of several amino acids were purified by sublimation in vacuo. They were stated to be easier to hydrolyze than the N-acetylamino acids (228).

The formyl group was used by Sheehan and Armstrong (215) to protect the amino group in cysteine, which in turn made it possible to protect the thiol group by treatment with acetone and acetic anhydride. The resultant N-formyl thiazolidine ring was subsequently removed by treatment with one per cent aqueous hydrochloric acid. The authors noted that the N-formyl group can be removed almost quantitatively by treatment at 0° C. with methanol containing one per cent hydrochloric acid. The ready hydrolysis of N-formyl derivatives in aqueous solution was noted earlier by Fischer and Warborg (229), but the conditions they used are likely to racemize peptides. In a study of the alcoholysis of various acylamino-acids, Hillmann and Hillmann (230) mention that formyl groups are easily removed, but state that unsatisfactory yields were obtained in peptide syntheses. Good yields were obtained by Waley (216) who removed the formyl group in cold dilute alcoholic solution of hydrochloric acid. He

prepared the N-formyl derivatives by heating the amino acid, acetic anhydride, and formic acid at 35°C. for one-half hour. It was noted that the high solubility of some derivatives, like formylglycylglycine methyl ester, in water may be inconvenient.

1.b. Amides of Carbodithioic Acids

RNH-CSR' (where R' represents hydrogen, alkyl, aryl or their derivatives)

A representative of this type of protection was prepared by Kjaer (231) who thiobenzoylated esters or amides of several alpha-amino acids by treatment with carboxymethyldithiobenzoate ($C_6H_5CSSCH_2COOH$) in alkaline solution. The technique of removing the thioacyl group was not described.

1.c. Amides of Carbonic Acid Derivatives

1. Carbamic Acid Esters RNH-COOR'

N-Carbethoxyamino acids were first prepared by Fischer (232, 233). However, his hope of being able to remove the carbethoxy group under mild conditions was not fulfilled. He reported the removal of the ethyl group by saponification and was surprised at the high stability of the remaining N-carboxyl group, which did not evolve carbon dioxide (96). Actually, as was later proved by Wessely (234) he was dealing with secondary rearrangement products. Leuchs' experiments

with the attempted removal of the carbethoxy group in carbethoxyglycine led to the development of the well-known method of preparing polymers from the alpha-amino acids via oxazolidinediones (161).

A much more important protecting group is the carbobenzyloxy group, first employed by Bergmann and Zervas (45). A great advantage of this radical lies in its easy removal by hydrogenolysis, with the formation of toluene, carbon dioxide, and the amino acid or peptide. The hydrogenolysis may be accomplished by catalytic hydrogenation (225), sodium in liquid ammonia (236), phosphonium iodide in glacial acetic acid at 40°C. (29), and hydrogen iodide also in glacial acetic acid (74). The presence of amino acids containing sulphur sometimes prohibits the use of the catalytic method (237), since the metallic catalysts are readily inactivated by sulphur compounds. In some cases, carbobenzyloxy groups may be removed by treatment with absolute ethanol and dry hydrogen chloride at 0°C. (238). This ease of removal, combined with the facile preparation of the carbobenzyloxy amino acid or peptides (from benzyl chloroformate and the amino compound in a Schotten-Baumann reaction) has resulted in a wide application of this group in the preparation of peptides. However, there are several disadvantages inherent in its use. First of all, some workers have reported difficulty in storing benzyl chloroformate and they felt impelled to prepare it periodically from phosgene and benzyl

alcohol. Carbobenzyloxyamino acid chlorides have a troublesome tendency of forming oxazolidinediones (161). Therefore, if the carbobenzyloxyamino acids are employed in the acyl chloride method, rapid operation under anhydrous conditions and strong chilling of the reaction mixture during the halogenation and subsequent coupling is required in order to minimize this tendency. Carbobenzyloxy derivatives of some amino acids are not readily available (if at all) in crystalline form, and are therefore not easily purified.

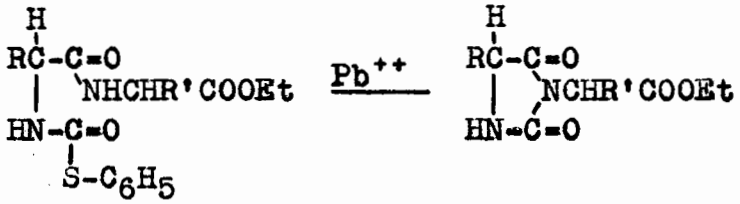
Several substituted carbobenzyloxy derivatives were recently introduced in order to improve the crystallizing tendencies. Young et al (239) found that p-bromobenzylchloroformate (which is a stable, low-melting solid) gave good yields of more easily crystallizable derivatives, while p-methylbenzyl and naphthylmethyl-chloroformates gave no better results than the unsubstituted benzyl chloroformates. Carpenter and Gish (240, 241) reported better results with p-nitrobenzyl chloroformates.

Recently Stevens et al (46) have advocated allyl chloroformate ($ClCOOCH{=}CH_2$), which is stable and commercially available, as a reagent for protecting the amino group. The structural analogy of the carballyloxy group with the carbobenzyloxy group (the presence of the unsaturated bond in the position alpha to the oxygen atom) explains the reason why this group can also be removed by hydrogenolysis. Sodium in liquid ammonia or phosphonium iodide is usually used for

this purpose. Catalytic hydrogenation cannot be applied here owing to the competitive hydrogenation of the olefinic double bond, which converts the carballyloxy group into the stable carbethoxy group (cf. 96).

2. Thiocarbamic Acid Esters RNH-COSR'

Phenyl chlorocarbothiolate, which can be prepared from thiophenol and phosgene, reacts with amino acid esters in ether at room temperature. The acylated amino acid ester can then be converted into an acylated amino acid by an acid hydrolysis (242). These phenyl-thiocarboxylamino acids are more crystalline than the corresponding carbobenzyloxy compounds. However, their removal, which is easily accomplished either by a five-minute heating with lead acetate in 20 per cent ethanol, or by a cold reaction with lead thiophenolate in 0.1 N alkali, was found to lead, in some cases, to the formation of hydantoins instead of dipeptides (234; cf. also 244).



3. Carbamide Derivatives RNH-CONHR'

The phenyl ureido group was used by Fischer and Suzuki (245) for masking the alpha-amino group. Mild hydrolysis was required to remove this group.

An interesting carbamide derivative was reported by Fischer (233), who prepared it by the treatment of the dipeptide ester with phosgene in toluene:

$$COCl_2 + 2\ NH_2CH_2CONHCH_2COOEt \rightarrow OC\begin{matrix}\diagup NHCH_2CONHCH_2COOEt \\ \diagdown NHCH_2CONHCH_2COOEt\end{matrix} + 2\ HCl$$

It was later shown by Wessely (234) that this compound also appeared during Fischer's unsuccessful attempts to remove the carbethoxy group.

1.d. Amides of Sulphonic Acids

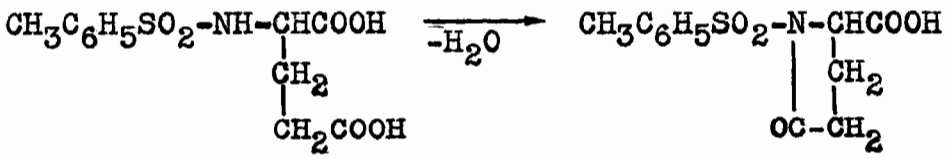
$RNH\text{-}SO_2R'$

Sulphonyl chlorides have been used to introduce methanesulphonyl (246), benzenesulphonyl (247), beta-naphthalenesulphonyl (233, 248), or p-toluenesulphonyl (47) groups into alpha-amino acids. Generally the yields were not too good. In 1926 Schönheimer took advantage of Fischer's discovery (249) that p-toluenesulphonylamino acids can be converted to the parent amino acid by hydrogenolysis with a mixture of hydriodic acid and phosphonium iodide, and proposed a novel method by

which N-substituents could be removed from amino acids and peptides by a procedure not involving hydrolysis with strong acids. Another reagent which has been used for the same purpose is sodium in liquid ammonia (250). The reaction is:

$$RSO_2NHR' + H_2 \longrightarrow RSO_2H + NH_2R'$$

One sees that the p-toluenesulphonyl group has been somewhat neglected, since in the few cases when it has been used the authors reported complete satisfaction (31, 105, 250, 251). The only unwelcome side reaction was reported by Harrington and Moggridge (252) who observed that p-toluenesulphonylglutamic acid could not be converted to its cyclic anhydride, owing to its dehydration to a derivative of 5-oxopyrrolidine-2-carboxylic acid:

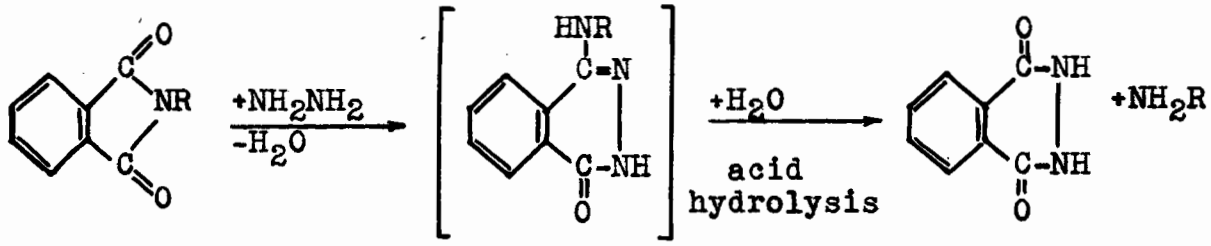


However, since this ring can be easily opened by the action of aqueous ammonia (which cleaves the carbonyl group from the nitrogen, leaving it in the form of an amide), this side reaction actually assures an unambiguous alpha-amide bond formation with di-carboxylic amino acids (253, 254).

2. Imides $RN{=}(CO)_2R'$

The phthaloyl group is the only one of the imido type which has been used in peptide synthesis. Succinyl and maleic derivatives of amino acids have received passing attention (255). Phthaloylglycine was first prepared in 1883 by adding glycine to the molten anhydride of phthalic acid (256). Billman and Hartung used this method for preparing a series of phthaloyl derivatives of amino acids intended to be used for purposes of identification (255).

An elegant way of removing the phthaloyl group from its imide was discovered by Radenhausen (257) when he attempted to prepare phthaloylglycine hydrazide from the ethyl ester of phthaloylglycine and hydrazine hydrate. He obtained instead a precipitate, which he identified as phthaloylhydrazide, and ethyl glycinate. Ing and Manske (258) applied this reaction to the preparation of amines from the corresponding alkyl halides. (They modified the classical Gabriel synthesis of amines by which the N-substituted phthalimide is boiled with potassium hydroxide yielding dipotassium phthalate and the amine.) Through the use of hydrazine they subjected the intermediate to an acid hydrolysis:



(probable structure)

Since the phthaloyl hydrazide was found to be a moderately strong acid, Barber and Wragg (259) concluded that the intermediate in the above reaction is actually a salt of the amine with the phthaloyl hydrazide. Accordingly they suggested that in cases where the acid hydrolysis would be detrimental to the product, thermal dissociation, solvent extraction, or basification of the intermediate salt should be employed for precipitating phthaloyl hydrazide. Hydrazine sulphate (260) and phenylhydrazine (261, 262, 263) have also been used to remove the phthaloyl group.

It was chiefly the work of King's group in England (49, 244) and of Sheehan and Frank in the United States (50) which directed attention to the phthaloyl group as an important factor in peptide synthesis. Phthaloyl derivatives of oligopeptides were prepared by these workers via various intermediates, viz. acyl chlorides (50, 99, 264), carbonic-carboxylic mixed anhydrides (38, 40), internal dicarboxylic anhydrides (130, 139) and so on. Preparation of phthaloyl

peptides by the azide method was also reported. However, the phthalimido-acid azides must be prepared via the acyl chlorides (244) owing to the preferential attack of hydrazine on the phthaloyl group, which was discussed above. The imido method of protecting the amino group is sometimes advantageous, as several instances have been reported in which the remaining amino-hydrogen atom participates in undesirable side reactions (cf. e.g. 252).

3. Alkylidine Derivatives $RN{=}\dot{C}-$

Benzalamino acids ($C_6H_5CH{=}N{-}CHRCOOH$) were used by Wieland and Schafer (90) in their experiments. They were prepared from the potassium salts of the amino acids and benzaldehyde in dimethylformamide in good yields. The technique of removing this group was not reported.

4. Salts of the Amino Group $RNH_3^{\oplus}\ X^{\ominus}$ (where $X^{\ominus}$ represents a strongly electronegative anion)

Strong acids are able to break up at least partially the zwitter-ion configuration of the alpha-amino acids and thus to render the carboxyl group more susceptible to the desired reactions. For example, esterification of the amino acids is feasible only in the presence of a strong acid (e.g. 4, 5, 223). Peptide synthesis via amino acyl

chlorides, which have their amino groups protected by the hydrochloric acid, was described in 1905 by Fischer (27, 28). This method takes advantage of the fact that several amino acids, when shaken with phosphorus pentachloride and acetyl chloride, are readily converted into crystalline hydrochlorides of the amino acyl chlorides. These were then condensed with amino acid esters. The yields were not too high, one of the side reactions being the formation of diketopiperazine rings from the dipeptide esters. Only isolated cases of successful application of this method have been reported (265, 266).

The proton can be removed either by a calculated amount of sodium alkoxide (7) or a tertiary amine (which forms a tertiary ammonium salt with the anion; cf. e.g. 38) in anhydrous medium, or, in aqueous solution, by a simple neutralization with concentrated alkali in the presence of solid potassium carbonate and immediate extraction of the free base with ether or ethyl acetate (18). In the case of peptide ester hydrochlorides, the concentrated alkali is usually omitted, and only carbonate is used. Ion-exchange resins also offer attractive possibilities.

5. Amino Group Precursors

The problem of protecting the amino group by some radical, which can not only be easily attached to the amino group, but also removed without endangering the peptide bonds, can be

neatly circumvented by simply using some other group, which can be conveniently transformed into the amino group. This idea was first realized by Fischer, when he used chloroacetyl chloride for making the glycyl peptides; he replaced the alpha-chlorine atom by the amino group simply by treating the chloroacetyl peptide with 25 per cent ammonia at 100°C. or by allowing the reaction mixture to stand at room temperature for two to three days (96). In similar manner, several other alpha-haloacids were used to synthesize the peptides of corresponding DL-alpha-amino acids (27, 267, 268). The extension of this method to the optically-active derivatives, however, entails several difficulties. Preparation of the optically-active acids in pure state is a difficult and time-consuming procedure (D-alpha-halo acids have to be used if L-alpha-amino acids are desired in the peptide, because of the Walden inversion which accompanies the amination step) (269, 270). Anomalous reactions often occur at the amination step (271). Moreover, the optical purity of many of the peptides prepared by the halogen acyl halide method is open to doubt (cf. e.g. 190).

The azido radical was used to replace the alpha-amino group in an interesting modification of the halogen acyl halide method (272, 273). The alpha-azido group can be converted either by aluminium amalgam (274), or by catalytic hydrogenation in the presence of platinum or palladium (272) into the alpha-amino group. However, since the alpha-azido

acyl halides are prepared from alpha-halo acyl halides, this modification does not appear to offer any decided advantages.

Still another modification of the alpha-halogen acyl chloride method entails the use of alpha-benzyloximino acids (275, 276):

$$C_6H_5CH_2ON{=}\overset{R}{\dot{C}}COOH \xrightarrow{SOCl_2} C_6H_5CH_2ON{=}\overset{R}{\dot{C}}COCl \quad \text{etc.}$$

The benzyloximino group is reduced to the amino group and benzyl alcohol by a Raney-nickel hydrogenation. Note that this is another example of the cleavage of the benzyl radical by hydrogenation. The preparation of the alpha-benzyloximino acids is a rather involved procedure, starting with an alpha-dicarboxylic acid and an alkyl nitrate, and benzylating the intermediate product (alpha-oximino acid) with benzyl chloride. The reports did not make clear how this method can be applied to the preparation of peptides of desired spatial arrangement where optically active moieties are involved.

The cyano group can be reduced to a $-CH_2NH_2$ radical. Wessely reported (277) that he "protected" the delta-amino group by using gamma-cyano-alpha-amino acid (for polymerization via oxazolidinediones).

BENZYL ESTERS OF AMINO ACIDS

Introduction

While the literature offers a wealth of methods for protecting the amino group, not many practical ones are available for the carboxyl, as can be seen from the review of protecting groups on page 60. Methyl or ethyl esters are almost universally used, although the removal of these groups involves hydrolysis, which is certain to affect the peptide bonds, at least in some degree. The benzyl-ester type of protection appears to be more advantageous, because the benzyl group can be removed by hydrogenolysis, which does not affect the peptidic bonds. The benzyl esters of at least some of the amino acids should be relatively easy to prepare from the commercially available reactants. They have been prepared by treating benzyl alcohol with an amino acyl chloride (the amino group being blocked by a hydrogen halide) (48, 218, 219), with an N-carbonic acid anhydride (221), or with an amino acid, which had the amino group protected by a hydrogen halide or a sulphonic acid (8, 223).

Discussion of Results

We have prepared benzyl esters of glycine, DL-phenylalanine, DL-leucine, and L-leucine by a method recently described by Miller and Waelsch (223). This method is based on

dissolving equimolecular amounts of the respective amino acid and benzenesulphonic acid in a large excess of benzyl alcohol and removing the excess of benzyl alcohol by vacuum distillation. The role of the sulphonic acid is apparently threefold; first, to render the amino acid soluble in benzyl alcohol; second, to eliminate the zwitter-ion form of the amino acid; and third, to catalyze the removal of water formed by the condensation reaction, which is separated from the reaction mixture when the excess of benzyl alcohol is removed by vacuum distillation.

The products, benzyl esters of the respective amino acid benzenesulphonate, were found to be rather unstable, apparently due chiefly to the strong hygroscopicity introduced by the sulphonate group. Moreover, the melting points of products obtained in several preparations differed from case to case, an effect which was probably caused by the polymorphism typical of sulphonates (cf. 278).

Free benzyl esters were obtained by mixing equimolecular amounts of the above salts with triethyl amine in chloroform at low temperature (below 5°C.) and precipitating the triethylammonium benzenesulphonate by addition of excess of dry ether. Vacuum evaporation of the ethereal solutions yielded free benzyl esters of the respective amino acids, which were all viscous oils. Therefore, it was found advisable to convert these esters into their hydrochlorides, which are crystalline compounds with definite melting points and quite stable (Expt's l.a.l.a.).

A variation of the above method by replacing the benzenesulphonic acid by equimolecular amounts of p-toluenesulphonic acid was tested (Exp'ts 1.a.1.b.). These experiments have proved the suitability of this new reagent for the preparation of the benzyl esters of amino acids, although no significant improvements in the yields were observed in comparison with the benzenesulphonates.

Recognizing the obvious advantages of having crystalline benzyl esters of amino acids which might not need to be converted to their salts, we decided to experiment with the preparation of analogues of benzyl esters of higher molecular weight. At first, the syntheses of 4-nitrobenzyl esters were attempted, following the well-known method of identifying carboxylic acids by condensing them with 4-nitrobenzyl chloride (279, 280). Esterification of hippuric acid (benzoylglycine) by this method yielded a crystalline product (Exp. 1.b.1.). However, no ester could be isolated with glycine. No further attempts with other amino acids were made, because preliminary experiments in freeing the carboxyl by hydrogenating the 4-nitrobenzyl ester of hippuric acid by sodium in liquid ammonia indicated its instability; the solution of 4-nitrobenzyl ester in liquid ammonia became reddish-brown after sodium was added and it turned darker with time.

The potentialities of chlorine-substituted benzyl esters were then investigated. For the formation of these esters, 2-, 4-chloro-, and 2,4-dichlorobenzyl chlorides (Heyden

Chemical Corp., New York) were utilized. No information about the esters of this type was found in the literature. Therefore it was decided to follow generally the method of preparation of 4-nitrobenzyl esters (279). Since none of the benzyl chlorides described above is soluble in water, a solution of the particular benzyl chloride in ethanol was mixed with a water solution of equivalent amounts of the amino acid (or its derivative) and potassium carbonate. Then, as much alcohol was added as was necessary to make the solution homogeneous at the refluxing temperature. After being refluxed, usually for three hours, the solution was left standing at room temperature to cool slowly. If the reaction occurred, the ester should crystallize out of the solution. On the other hand, unreacted chlorobenzyl chloride should separate out as a liquid layer. 4-Chloro- and 2,4-dichlorobenzyl hippurates were obtained (Exp'ts l.c.), which were found to be crystalline compounds of sharp melting points (139°C. and 134.5°C. respectively). Since these esters were not reported in the literature before, their identity was confirmed by an elementary analysis. No crystals were obtained when 2-chlorobenzyl chloride and hippuric acid were subjected to the same procedure. Neither was any product obtained by subjecting free amino acids, like glycine, DL-phenylalanine, and DL-leucine to the same procedure, regardless of which of the above chloro derivatives of benzyl chloride was used. Even when the preformed salts of these amino acids (prepared in

liquid ammonia) were shaken for several hours with an excess of 2,4-dichlorobenzyl chloride (or 4-benzyl chloride), no product was obtained.

Since the derivatives of hippuric acid had shown, as expected, that chlorobenzyl esters have appreciably higher melting points (m.p. of benzyl hippurate is 91-92°C. (218)), successful preparation of chlorobenzyl esters of amino acids was desired. The possibility of preparing chlorobenzyl esters from the corresponding alcohol was considered, but its application postponed, since no chlorobenzyl alcohols were found to be commercially available to date. Further search for these alcohols seems desirable.

It is well known that several commercially available esters are manufactured from the corresponding acids and alcohols by methods based on the removal of the condensation by-product, water, by azeotropic distillation. However, this procedure has not been applied as yet to the preparation of the esters of the amino acids. Its chief advantages in the preparation of benzyl esters should be elimination of the need for a very large excess of the expensive benzyl alcohol used in the above-mentioned method of Miller and Waelsch and a decrease in the required reaction temperature. Preliminary experiments, where the same amounts of reactants as in the method of Miller and Waelsch (223) were used and water was removed by distillation with carbon tetrachloride (the boiling point of the water-carbon tetrachloride azeotropic

mixture is 76.75°C.; it contains 4.1 per cent water(281)), had shown the feasibility of this method. However, because of the insolubility of benzenesulphonic salts in carbon tetrachloride, it was necessary to dissolve the amino and benzenesulphonic acids in benzyl alcohol first, and the distillation had to be carefully conducted in order to keep the reaction mixture in emulsion-like form. For this purpose, some excess of benzyl alcohol was still required (3.9 equivalents as contrasted with 9.7 equivalents used in the old method). Further improvement was introduced by removing the necessity of using anhydrous ether for precipitating the benzyl ester of glycine benzenesulphonate. Simple cooling of the anhydrous reaction mixture (addition of more dry carbon tetrachloride was sometimes beneficial) gave the same result (Exp'ts 1.a.2.a. 3 and 4).

Simple addition of the reactants to carbon tetrachloride and azeotropic distillation was never conducive to esterification, as glycine remained undissolved throughout the whole procedure, and its zwitter-ion structure prevented its participation in the esterification reaction. This difficulty might be removed if a sulphonic acid with a long alkyl chain could be used, since its salts with the amino acids ought to be soluble in organic solvents. Such an acid was found in Nacconol ZSA (manufactured by the National Aniline Division, Allied Chemical & Dye Corporation), which is essentially a p-laurylbenzenesulphonic acid, $C_{12}H_{25}C_6H_4SO_3H$. It allowed

glycine to dissolve in the presence of only one equivalent of benzyl alcohol. However, a difficulty arose since it was discovered that triethyl ammonium p-laurylbenzenesulphonate is also soluble in dry carbon tetrachloride or in dry diethyl ether (other amines were tried with similar results) which made its separation from benzyl glycinate impossible. Several solvents were tried. Finally it was found that when petroleum ether was used as a solvent, triethyl ammonium p-laurylbenzenesulphonate separated in the form of an oily layer (Exp. 1.a.2.b.). The improved yield which was obtained justified our expectations.

Experimental Procedures

Experiments 1.a.1.

Preparation of Benzyl Esters by Vacuum Distillation

a) Using Benzenesulphonic Acid:

The method described by Miller and Waelsch (223) was employed in the preparation of the benzyl esters of glycine, DL-phenylalanine, DL-leucine, and L-leucine hydrochlorides. It was found that the temperature of the oil bath (which must remain below 130°C.) and the careful exclusion of moisture throughout the experiments are critical factors, which influence considerably the amounts and qualities of the products obtained.

Saturation of the solution of benzyl ester of either

glycine, or DL-phenylalanine in diethyl ether with dry hydrogen chloride resulted in an abundant precipitation. On the other hand, benzyl esters of DL- or L-leucine hydrochlorides remained in the solution. It was necessary to take the ethereal solution saturated with hydrogen chloride to dryness in vacuo, to dissolve the residue in the mixture of fourteen parts of cyclohexane and one part of chloroform at elevated temperature, and to leave the solution standing for sixteen hours at 0 - 5°C. The precipitated hydrochloride was then isolated by filtration. Yields and melting points of the products are listed in Table III.

Table III

Product	Experimental		Literature	
	yield	m.p.°C.	yield	m.p.°C.
Benzyl glycinate hydrochloride	69%	131-2	70%	131-2
Benzyl DL-phenylalaninate hydrochloride	81%	191-1.5	75%	196
Benzyl DL-leucinate hydrochloride	79%	145-5.5	--	--
Benzyl L-leucinate hydrochloride	67%	126-7	88%	129

Since no data on benzyl-DL-leucinate hydrochloride were available, it was identified by elementary analysis:

Calculated for $C_{13}H_{20}O_2NCl$: C 60.6 ; H 7.83

Found : C 60.51; H 7.90

b) Using p-Toluenesulphonic Acid:

Experiments 1.a.1.a. were repeated, the benzenesulphonic acid being replaced by equimolecular amounts of p-toluenesulphonic acid. The results were not significantly different from those above.

Experiments 1.a.2.

Preparation of Benzyl Esters by Azeotropic Distillation

a) Using Benzenesulphonic Acid:

1.

Glycine (3.75 g., 50 millimoles) and benzenesulphonic acid (9.0 g., 51 millimoles) were suspended in 25 ml. (26.25 g., 242 millimoles) benzyl alcohol and dissolved by heating the suspension on the steam bath with occasional stirring. (It is imperative to dissolve both the amino and sulphonic acids first, in order to bring about the formation of a benzenesulphonate salt. Preliminary experiments had shown that if glycine, benzenesulphonic acid, and benzyl alcohol are simply added to carbon tetrachloride, glycine remains undissolved throughout the whole operation and does not seem to participate in the esterification reaction.) After 300 ml. of carbon tetrachloride was added (which solidified the benzyl alcohol solution by cooling it), the reaction mixture was heated to the relux temperature. As the temperature increased, a layer of benzyl alcohol solution of glycine benzenesulphonate formed on top of the carbon tetrachloride phase. When

boiling started, both layers were churned into a milky suspension. Refluxing was continued for two hours, after which slow distillation was started and continued for six hours (after first three hours, 100 ml. carbon tetrachloride was added in order to keep the level of the reaction mixture above the top of the heating mantle) and then continued quickly on the steam bath until no more liquid distilled over. The residue was poured, while still hot, into an evaporating dish, cooled somewhat, and 50 ml. of anhydrous ether was poured over it. A white precipitate began to form immediately. The coarse grains were broken up, separated by filtration and suspended in 30 ml. of anhydrous ether. Filtration and drying in vacuum desiccator yielded 15.05 g. (46.6 millimoles) of benzyl ester of glycine benzenesulphonate; (93.2 per cent yield), m.p. 137-138°C.

Part of this product (13.0 g.) was converted to 6.97 g. of the benzyl ester of glycine hydrochloride by the triethylamine method (223); recrystallization from ethyl cellosolve acetate gave 5.65 g. (28.1 millimoles; 72.5 per cent yield); m.p. 130.5-132°C.

2.

The same amounts of glycine and of benzenesulphonic acid as in Exp. 3.a.2.a.1. were suspended in 5.5 ml. (5.72 g., 53.5 millimoles) benzyl alcohol. Heating of this suspension on a steam bath did not dissolve all the suspended material. Therefore, benzyl alcohol was slowly added in 0.5 ml. quanti-

ties, the mixture being heated all the time on the steam cone and occasionally stirred. After 1 ml. was added, a homogeneous solution was obtained; i.e. 6.5 ml. of benzyl alcohol was needed to dissolve all of the glycine and benzenesulphonic acid. Further procedure was the same as described in Exp. 3.a.2.a.2. It was noted that addition of carbon tetrachloride precipitated glycine benzenesulphonate into coarse lumps, so that the fine suspension of the previous experiment could not be obtained. The product (12.6 g., m.p. 123-6 + 137 + 147-153°C.) was apparently a mixture of several compounds. The inability to prepare from it benzyl glycinate hydrochloride (addition of triethylamine did not clear the suspension of this product in chloroform and saturating the ethereal-chloroform mixture with dry hydrogen chloride caused only clouding of the liquid -- no appreciable amount of precipitate was obtained) shows that the esterification was not complete.

3.

Experiment 1.a.2.a.1. was repeated throughout, except that 20 ml. (21 g., 194 millimoles) benzyl alcohol was used and carbon tetrachloride was not removed after the azeotropic distillation was stopped. Instead, the reaction mixture was left standing overnight to cool slowly to room temperature. White microcrystalline precipitate separated, which was isolated by filtration and washed with anhydrous ether. There was 13.75 g. (42.6 millimoles) of benzyl ester of glycine benzenesulphonate; (85.2 per cent yield) m.p. 120-4°C.

The conversion of 4.35 g. (13.5 millimoles) of this product to the hydrochloride by the above-mentioned (Exp. 1.a.2. a.1.) triethyl-amine method yielded 1.63 g. (8.1 millimoles; 60 per cent yield) of benzyl glycinate hydrochloride, by recrystallization of which from ethyl cellosolve acetate, 1.3 g., m.p. 131-2°C., was obtained.

4.

In this repetition of Exp. 3.a.2.a.3., hot carbon tetrachloride was slowly added to the warm solution of glycine benzenesulphonate in benzyl alcohol under constant stirring. After approximately 50 ml. of carbon tetrachloride was added, the solution began to be slightly cloudy. After some refluxing, however, it turned clear again. The reaction mixture was very "bumpy" and it was necessary to continue stirring even during the periods of refluxing and of slow distillation. The solution was poured into 200 ml. carbon tetrachloride and put into the refrigerator overnight. The white precipitate was isolated by filtration, washed with ether and dried in a vacuum desiccator; 11.38 g. (35.3 millimoles of benzyl ester of glycine benzenesulphonate; 70.6 per cent yield), m.p. 135.5-137°C.

A sample of this product (4.35 g., 13.5 millimoles) was converted by the above-described method (cf. Exp. 1.a.2.a.1.) to 1.84 g. (9.15 millimole; 67.7 per cent yield) of benzyl ester of glycine hydrochloride, which, after being recrystallized from ethyl cellosolve acetate, gave m.p. 130.5-132°C. (1.6 g.).

b) Using p-Lauryl-benzenesulphonic Acid:

Glycine (7.5 g., 0.1 mole), p-lauryl-benzenesulphonic acid* (32.5 g., 0.1 mole) and 300 ml. of carbon tetrachloride (previously dried over calcium hydride) were refluxed briefly in a 500 ml. round-bottomed flask. While the sulphonic acid dissolved completely, about half the glycine remained undissolved. Benzyl alcohol (11 g., 0.1 mole) was added and refluxing continued in a Soxhlet extractor, the paper thumble of which contained lump calcium hydride. During the first hour or so of refluxing almost all the remaining glycine dissolved. However, the reaction mixture remained somewhat cloudy. Refluxing was continued for eighteen hours. The orange solution, which contained a few fragments of white solid material, was filtered by suction as rapidly as possible, its volume made up to 300 ml. with dry carbon tetrachloride, and the resultant solution was used as a stock solution for the following experiments.

One gram (1.4 ml., 10 millimoles) of triethylamine (previously dried over calcium hydride) was added to a 30-ml. aliquot of the stock solution. A precipitate (solid or liquid) failed to separate even on long standing in the refrigerator.

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* Nacconol ZSA, manufactured by the National Aniline Division, Allied Chemical & Dye Corp, Buffalo, N.Y., and containing up to 7 per cent sulphuric acid as received by us, was dried initially by azeotropic distillation with toluene followed by holding the material in a vacuum at 50°C. overnight.

To another 30-ml. aliquot, 1.4 g., (10 millimoles) of hexamethylenetetramine was added. It dissolved slowly and a gelatinous precipitate separated, apparently a hexamine sulphonate. Attempts to separate this precipitate by suction filtration were abandoned because the gelatinous character of the precipitate made its isolation by filtration a time-consuming process.

Triethanolamine (1.5 g., 1.2 ml., 10 millimoles) was added to a third 30-ml. aliquot. An oily phase appeared on standing in a refrigerator, but it was so intimately distributed through the carbon tetrachloride solution and in such a small amount, that its separation was not attempted.

A 90-ml. aliquot of the stock solution was now evaporated on a steam bath to remove the carbon tetrachloride. The oily residue was heated for about an hour on the steam bath to get rid of the last traces of solvent which were held tenaciously. The residue was triturated with a small volume of anhydrous diethyl ether in which it dissolved freely; no precipitate separated. The volume was increased to 45 ml. by adding more anhydrous ether. To 15 ml. of this solution, 1 g. (1.4 ml., 10 millimoles) of dry triethylamine was added; no second phase (solid or liquid) separated. To another 15 ml. of the ethereal solution 2.25 g. (10 millimoles) triamylamine was added. Again, no second phase separated.

Another 90 ml. aliquot of the stock solution was freed of carbon tetrachloride as before. The residue was triturated

with a small volume of dry petroleum ether (b.p. range 36-42°C.) in which it dissolved freely. The volume was increased to 45 ml. by adding more petroleum ether. To 15 ml. of this solution, one gram (1.4 ml., 10 millimoles) of dry triethyl amine was added. After agitating the solution for a few minutes, a second liquid phase separated. On standing in a refrigerator, two distinct layers appeared, an upper, pale-yellow ethereal layer (considered to be a solution of benzyl glycinate in petroleum ether) and a lower, dark-orange oily layer (considered to be the triethyl ammonium sulphonate). The upper layer was decanted and the lower layer was washed twice with an equal volume of dry petroleum ether. The upper layer and washings were combined, cooled in an ice-salt bath, and dry hydrogen chloride was bubbled through the solution. An abundant precipitate appeared, which, after filtration, washing with petroleum ether and drying in vacuum dessicator, weighed 1.46 g. (72.6 millimoles of benzyl glycinate hydrochloride; 72.5 per cent yield), m.p. 129-130°C.

Experiments 1.b.

Preparation of 4-Nitrobenzyl Esters:

1.

The potassium salt of hippuric acid was prepared by suspending 1.8 g. (10 millimoles) hippuric acid and 0.69 g. (5 millimoles) potassium carbonate in 5 ml. distilled water and stirring until the evolution of carbon dioxide stopped.

The mixture was heated on a steam bath in order to bring all the reactants into the solution and then boiled briefly to remove carbon dioxide. A solution of 1.71 g. (10 millimoles) of 4-nitrobenzyl chloride in 6 ml. 95 per cent ethanol (this amount of ethanol was found to be sufficient to keep all the reactants dissolved at the reflux temperature) was added, the reaction mixture was refluxed for two hours, and then cooled in a refrigerator. A white precipitate separated, which was isolated by filtration and washed with water: 1.25 g. (4.0 millimoles of 4-nitrobenzyl hippurate; 40 per cent yield) m.p. 129-135°C. Addition of water to the filtrate yielded a second crop: 0.16 g., m.p. 65-72°C. (intensely yellow; discarded). Recrystallization of the first crop from 15 ml. of 63 per cent ethanol (two volumes of 95 per cent ethanol and one volume of water) yielded 1.10 g., m.p. 135-136°C. (lit. 136°C. (279)).

2.

Attempts to prepare 4-nitrobenzyl esters of glycine by the method described in Experiment 1.b.1. were unsuccessful.

Experiments 1.c.

Preparation of Chlorobenzyl Esters

1.

An aqueous solution of potassium hippurate was prepared as described in Exp. 1.b.1. A solution of 1.5 ml. (approximately 10 millimoles) 4-chlorobenzyl chloride in 5 ml. of

95 per cent ethanol was added and the mixture was heated to the reflux temperature. Since the reaction mixture was milky (apparently because excess of water precipitated some chlorobenzyl chloride) another 5 ml. of ethanol was added to make the solution homogeneous at the reflux temperature. Refluxing was then continued for three hours and the solution was left standing at room temperature overnight. White crystals which separated during the night were isolated by filtration: 1.18 g., m.p. 138-9°C. (39 millimoles of 4-chlorobenzyl ester of hippuric acid; 39 per cent yield). The filtrate was diluted with excess of water. The white precipitate was found to have an m.p. 135-6°C. Because of its minute amount, its weight was not determined.

Recrystallization of a part of the first crop from 63 per cent ethanol gave crystals of m.p. 139°C.

Calculated for $C_{16}H_{14}O_3NCl$: C 63.3 ; H 4.65 ; N 4.62

Found : C 63.25; H 4.84 ; N 4.50

2.

Similar experiments with 2-chlorobenzyl chloride and hippuric acid did not yield any crystals even after the reaction mixture was left standing in the refrigerator for two days, nor after the solution was diluted with a large excess of water.

3.

Similar treatment of 2,4-dichlorobenzyl chloride and hippuric acid (the proportion of water and ethanol had to be

changed to 5:15 in order to keep the refluxing mixture clear) yielded 1.42 g. of white needles (42 millimoles of 2,4-dichlorobenzyl hippurate; 42 per cent yield), m.p. 133-4°C. No second crop was obtained as the addition of water to the filtrate resulted in a milky solution from which no solid precipitate could be isolated.

Recrystallization of a part of the first crop from 63 per cent ethanol gave crystals of m.p. 134.5°C.

Calculated for $C_{16}H_{13}O_3NCl_2$: C 56.8 ; H 3.88 ; N 4.14

Found : C 57.06 ; H 4.16 ; N 3.90

4.

Similar experiments with glycine, DL-leucine and DL-phenylalanine did not give any crystalline products.

5.

The sodium salts of glycine, DL-leucine, and DL-phenylalanine were prepared by adding sodium to the solution of the particular amino acid in liquid ammonia until the solution started to become blue. (Solutions of metallic sodium in liquid ammonia have an intensely blue colouration.) Mechanical shaking of a suspension of such a preformed sodium salt of amino acid in an excess of 2,4-dichlorobenzyl chloride (or of p-chlorobenzyl chloride) for several hours did not give any products.

PHTHALOYL AMINO ACIDS

Introduction

Although the phthaloyl derivatives of some amino acids have been known for a long time (256) they have only recently become accepted as valuable intermediates in the synthesis of peptide bonds. Keenest advocates of their use are King and Kidd in England (49, 130) and Sheehan and Frank in the United States (50). Both these groups published independently papers in which each claimed to be the first to use the phthaloyl derivatives for the synthesis of peptides. Several advantages were gained by this method of protecting the amino group. Phthalic anhydride is a commercially available, stable compound, and it reacts easily with amino acids to give stable derivatives in good yields, most of which are readily crystallized and easily purified. In these derivatives, no hydrogen atom, which could possibly complicate later reactions, remains on the amino group. Eventual removal of the phthaloyl group can be effected easily by the action of hydrazine (or its derivatives). This reaction proceeds essentially to completion because of the extreme insolubility of the phthaloylhydrazide produced.

The points outlined above led us to the conclusion that the phthaloyl group offers most advantages as a protecting device. Therefore, phthaloylation of several amino acids under various conditions was investigated.

Discussion of Results

The traditional technique for preparing phthalimides from amines may be designated "the hot-melt method". According to this technique an intimate mixture of equimolecular amounts of phthalic anhydride and the amine (or its derivative) is held at a temperature just above the melting point of phthalic anhydride for about thirty minutes. After cooling, the glassy product is recrystallized from a suitable solvent.

While the preparation of phthaloylglycine in this way offered no difficulty, phthaloyl-DL-leucine was not easily purified by recrystallization. Apparently it has a very low temperature quotient of solubility. Evaporation of the solvent in order to obtain a supersaturated solution also did not bring about any crystallization. It only increased the viscosity of the solution, until a solid gel was obtained. After being dried completely in a vacuum dessicator, the gel passed over into an adherent, glassy crust but remained, of course, in impure form. Addition of a non-solvent to a solution of phthaloyl-DL-leucine, e.g. petroleum ether to an ethereal solution, or cold water to an acetic acid solution, merely caused its separation into two layers, although in both cases the two liquids in pure state are freely miscible. This observation indicates some form of association between phthaloyl-DL-leucine and the solvent.

Further support for this view is offered by the observation that, when recrystallization from water was attempted, and dilution of a concentrated viscous solution was desired, addition of water to the aqueous solution resulted in the appearance of two layers, a clear colourless layer above an oily yellow layer containing the solute. Crystallization was finally indiced by seeding an ethereal solution with a microcrystal of the compound.

Similar difficulties in an exaggerated form were encountered with phthaloyl-DL-isoleucine, which could not be separated from the reaction mixture in the crystalline form at all; only an oil was obtained. Accordingly, attention was given to the possibility of carrying out the reaction in glacial acetic acid, which was expected to yield a product of higher purity and thus easier to recrystallize. Vanags (282) had reported that this solvent was highly satisfactory for the preparation of N-phenylphthalimide from phthalic anhydride and aniline. After some preliminary experimentation with glycine, a technique was devised which, when applied to DL-leucine, proved to be a distinct improvement over the hot-melt method. However, it failed when applied to DL-isoleucine; only oils were obtained from which no crystals could be separated.

It is well known that phthalimides, substituted on the benzene ring, have higher melting points than the corresponding unsubstituted phthalimides. Thus Sah and Mah (283)

and Allen and Nicholls (284) have advocated the use of 3-nitro- and of tetrachlorophthalimides, respectively, in qualitative organic analysis. Accordingly, we felt that the analogous 3-nitro- and tetrachlorophthaloyl derivatives of amino acids, having a higher melting point, would prove to be more easily crystallizable. Exploratory experiments with glycine, using the acetic acid method, served to confirm this prediction.. Of even greater importance was the further observation that 3-nitrophthaloyl-DL-isoleucine could be isolated with ease in crystalline form. A similar derivative of norleucine could be obtained in crystalline form only with difficulty. However, a phthaloyl derivative of even this amino acid was easily isolated by using tetrachlorophthalic anhydride.

Even bearing in mind the sensitivity of the phthaloyl-amino group to alkaline conditions (cf. page 159 and ref. 216) we may conclude that 3-nitro- and tetrachloro-phthaloyl amino acids, prepared in glacial acetic acid, have a real, if limited, value as intermediates in the synthesis of peptides.

The results of the experiments discussed above are summarized in Table IV:

Table IV

Product	Method	Experiment No.	Yield %	M.P. °C.
Phthaloyl-				
-glycine	hot melt	2.a.1.a	84.5	191
	AcOH	2.a.1.b	92.5	191
-DL-phenylalanine	AcOH	2.a.2	89.5	177
-DL-leucine	hot melt	2.a.3.a	45.0	140-142
	AcOH	2.a.3.b	89.0	141.5-142
-DL-isoleucine	hot melt	2.a.4.a	0.0	-
	AcOH	2.a.4.b	0.0	-
3-Nitrophthaloyl-				
-glycine	AcOH	2.b.1	70.4	212
-DL-isoleucine	AcOH	2.b.2	59.3	166
-DL-norleucine	AcOH	2.b.3	76.5	116-118
Tetrachlorophthaloyl-				
-glycine	AcOH	2.c.1	97.5	310
-DL-norleucine	AcOH	2.c.2	95.2	212.5

Experimental Procedures

Experiments 2.a.1.

(a) Phthaloylglycine by the "hot-melt method":

Phthalic anhydride (3.7 g., 25.0 millimoles) and glycine 1.9 g., 25.3 millimoles) were mixed thoroughly in a large Pyrex test-tube and heated in an oil-bath at 175-180°C. Frothing started after about two minutes. To reduce this frothing

the contents were stirred for about fifteen minutes. There was no apparent sublimation. After another fifteen minutes the hot melt was quickly poured into a 100 ml. beaker, where it was dissolved in ether. As the ether evaporated, a crystalline precipitate separated. Yield, 4.34 g. (84.5 per cent); m.p. 189-191°C. Recrystallization of 2.91 g. of this material from water gave 2.55 g., m.p. 191-2°C.(lit.191-2°C)(255).

(b) Phthaloylglycine by the "acetic-acid method":

Phthalic anhydride (3.7 g., 25.0 millimoles) and glycine (1.9 g., 25.3 millimoles) were dissolved in 25 ml. glacial acetic acid. The solution was refluxed for three hours. Upon cooling, a white crystalline precipitate appeared, which was isolated by suction filtration and washed twice with diisopropyl ether. Yield, 3.05 g.; m.p. 190-192°C. The combined filtrate and washings were evaporated to one-third volume and cooled, whereupon a second crop of crystals appeared and was treated as described above; 1.7 g., m.p. 189-190°C. Further evaporation of the mother liquor yielded 0.42 g. of crystals, m.p. 159-164°C., which were discarded. The total yield of air-dried material was then 4.75 g. (92.5 per cent).

Experiment 2.a.2.

Phthaloyl-DL-phenylalanine by the "acetic-acid method":

Phthalic anhydride (3.7 g., 25.0 millimoles) and DL-phenylalanine (4.1 g., 24.8 millimoles) were dissolved in 3 ml.

glacial acetic acid and further treated as described in Exp. 2.a.1.b. Freezing of the solvent was necessary to initiate the crystallization of the first crop; 4.9 g., m.p. 177-8°C. (lit. 174-5°C.) (255). The second crop, 1.7 g., had an m.p. 176.5-8°C. The third crop, 0.2 g., m.p. 173-6°C., was discarded. The first two crops represented a yield of 89.5 per cent.

Experiments 2.a.3.

(a) Phthaloyl-DL-leucine by the "hot-melt method":

Phthalic anhydride (7.4 g., 50.0 millimoles) and DL-leucine (6.55 g., 49.9 millimoles) were reacted under conditions described in Exp. 2.a.1.a. Crystallization from an ethereal solution was induced only after the addition of a "seed", obtained by evaporating a few drops of the solution to dryness. The first crop weighed 5.85 g., m.p. 140-2°C. The second crop was obtained from the filtrate only after all the ether was evaporated; 6.19 g., m.p. undeterminable, the material started to soften in the vicinity of 120°C. Recrystallization of the second crop from 20.0 ml. ethanol, to which 50 ml. of hot water was added, was attempted. However, after cooling only an oily layer separated, which remained at the bottom. Therefore the mixture was heated again, until a homogeneous solution was obtained and this was stirred vigorously while cooling. A white precipitate was obtained, 5.75 g., softening above 135°C. Another recrystallization was attempted from an ethereal solution, by

adding petroleum ether. It caused a slight cloudiness, which did not increase after more petroleum ether was added. After a while, an oil began to separate at the bottom. When the container was placed in an ice/sodium chloride cooling mixture and its contents stirred for about three hours, some of the solvent evaporated and a microcrystalline crust formed on the walls of the beaker. The crystals (5.10 g.) softened at 136°C. and melted at 140-1°C. Recrystallization of a small sample from water was unsuccessful; an oil was obtained. Recrystallization of another small sample from glacial acetic acid (the solution was frozen into a crystalline mass and then allowed to melt at room temperature) gave crystals (0.60 g.) of m.p. 140-1°C. (lit. 140-1°C.) (255).

(b) Phthaloyl-DL-leucine by the "acetic-acid method":

Phthalic anhydride (1.5 g., 10.1 millimoles) and DL-leucine (1.32 g., 10.1 millimoles) was dissolved in 5.0 ml. glacial acetic acid and refluxed for one hour. The reaction mixture was then frozen into a crystalline mass and allowed to melt at room temperature. The white precipitate which appeared was separated by suction filtration; 1.71 g., m.p. 141.5-142°C. On slow evaporation of the filtrate to one-quarter volume, 0.64 g. of precipitate separated, m.p. 139-141°C. The residue obtained by evaporating the filtrate to dryness (0.1 g., m.p. 135-9°C.) was discarded. The combined weight of the first two crops (2.35 g.) was 89.0 per cent of the theoretical yield.

Experiments 2.a.4.

Phthaloyl-DL-isoleucine, prepared either by a "hot-melt method" (cf. Exp. 2.a.1.a.), or by an "acetic-acid method" (cf. Exp. 2.a.1.b.), could not be induced to separate in crystalline form. Only a yellowish oil was obtained.

Experiments 2.b.

(1) 3-Nitrophthaloylglycine

3-Nitrophthalic anhydride (4.8 g., 24.8 millimoles) was stirred into 25.0 ml. of hot glacial acetic acid; only about 80 per cent of the anhydride dissolved. Glycine (1.9 g., 25.3 millimoles) dissolved easily in this solution and simultaneously brought the remaining undissolved anhydride into the solution. The dark green-brown solution was refluxed for one hour and allowed to stand overnight. A mass of rosettes of fine needles separated. The precipitate was separated by suction filtration, washed twice with a little water, and air-dried; 3.7 g., m.p. 206-212°C. (first crop). Cooling of the filtrate in the refrigerator yielded 0.6 g. of microcrystalline material, which softened and frothed at 178°C. (second crop). Evaporation of the filtrate to about 5 ml. yielded 0.6 g. of crystals, m.p. 206-212°C. (third crop). The combined weight of the first and third crops (4.3 g.) represents 70.4 per cent of the theoretical yield. Recrystallization of the product from glacial acetic acid gave greenish crystals of m.p. 213-213.5°C.

Calculated for $C_{10}H_6O_6N_2$: C 48.0 ; H 2.42 ; N 11.20

Found : C 47.8 ; H 2.49 ; N 11.27

(2) 3-Nitrophthaloyl-DL-isoleucine

A solution of 3-nitrophthalic anhydride (4.8 g., 24.8 millimoles) and DL-isoleucine (3.3 g., 25.1 millimoles) in 25 cc. glacial acetic acid was refluxed for one hour. Cooling to room temperature, or evaporation to about one-third of the original volume failed to initiate crystallization. The solution was then made up to its original volume while refluxing by the slow addition of water. Two liquid phases appeared; the upper was colourless and mobile, the lower was grey-green and viscous. The usual devices failed to produce crystallization. The mixture was reheated to its boiling point and, on addition of 2.3 cc. glacial acetic acid, became homogeneous. Two phases again appeared when the solution was cooled to room temperature. On standing overnight the lower phase solidified to a mass of microcrystalline material. The mass was broken up, filtered by suction, washed with a small volume of water, and dried: weight, 4.5 g., m.p. 163-5°C. (59.3 per cent yield).

A small amount of material was retained for seeding purposes. The remainder was suspended in 20 cc. boiling water and the larger portion dissolved. Complete solution was achieved by adding about 5 cc. glacial acetic acid to the boiling mixture. On careful cooling and seeding, micro-

crystalline material (not a viscous liquid) separated. After slow cooling to room temperature and standing in a refrigerator overnight, the crystals were removed by filtration, washed with water and dried: weight, 3.7 g., m.p. 166-7°C.

Calculated for $C_{14}H_{14}O_6N_2$: C 54.84; H 4.61; N 9.15

Found : C 55.2 ; H 4.6 ; N 10.4

Water-methanol, rather than water-acetic acid, mixtures were tested as possible recrystallizing media but they repeatedly produced "an oil".

(3) 3-Nitrophthaloyl-DL-norleucine

A solution (4.8 g., 24.8 millimoles) of 3-nitrophthalic anhydride and DL-norleucine (3.3 g., 25.1 millimoles) in 25 cc. glacial acetic acid was refluxed for one hour. The method of isolation of a crystalline product, which had proved successful in the case of 3-nitrophthaloyl-DL-isoleucine (i.e. evaporation of the reaction mixture to about one-third of its volume, addition of hot water to make up the loss by evaporation, addition of 2-3 cc. glacial acetic acid to render the mixture homogeneous at its boiling point, cooling and standing overnight) failed to initiate crystallization in the lower, viscous layer. The only technique which was eventually successful in producing crystals involved the addition of sufficient 3-5 cc.) methanol to render the mixture homogeneous even at room temperature, seeding with material

obtained by the evaporation of a few drops of the reaction mixture, and cooling in a refrigerator. The conglomerate mass of microcrystals was broken up, filtered, washed with acetic acid, and dried: weight, 5.0 g., m.p. 116-8°C. Yield: 66.0 per cent.

Calculated for $C_{14}H_{14}O_6N_2$: C 54.84; H 4.61; N 9.15
Found : C 55.1 ; H 4.7 ; N 10.6

Experiments 2.c.

(1) Tetrachlorophthaloylglycine

A solution of tetrachlorophthalic anhydride (7.0 g., 24.5 millimoles) and glycine (1.9 g., 25.3 millimoles) in 25 cc. boiling glacial acetic acid was prepared. After a brief period of refluxing (two to three minutes) an abundant crop of crystals separated, causing the suspension to solidify. The suspension was cooled to room temperature and the precipitate filtered, washed with a small volume of acetic acid, and dried: weight, 7.3 g., m.p. (by dip method) 310-311°C. (with slight discolouration). On further refluxing of the combined filtrate and washing, additional material separated: weight, 0.9 g., m.p. (on slow heating) 308-311°C. (with some discolouration); (by dip method), 310-311°C. (with slight discolouration). Combined weight of product, 8.2 g.; yield, 97.5 per cent.

Calculated for $C_{10}H_3O_4NCl_4$: C 35.05; H 0.88; N 4.08
Found : C 35.0 ; H 1.1 ; N 4.5

(2) Tetrachlorophthaloyl-DL-norleucine

A solution of tetrachlorophthalic anhydride (7.0 g., 24.5 millimoles) and DL-norleucine (3.3 g., 25.1 millimoles) in 25 cc. boiling glacial acetic acid was prepared. After refluxing for five minutes, no spontaneous separation of a crystalline product having occurred, the slightly cloudy solution was filtered hot. The filtrate was cooled slowly and carefully seeded with material obtained by the evaporation of a few drops of the reaction mixture. The crystalline precipitate was filtered, washed with acetic acid, and dried: weight, 6.7 g., m.p. 211.5-213.5°C. The combined filtrate and washings were evaporated to a small volume to produce a second crop of crystals; weight, 2.3 g.; m.p. 212.5-213.5°C. The combined filtrate and washings were heated and diluted with water to incipient cloudiness to produce a third crop: weight, 0.3 g., m.p. 212-213°C. The combined filtrate and washings were evaporated to dryness to yield 0.2 g. of obviously very impure material.

Combined weight of product, 9.3 g.; yield, 95.2 per cent.

Calculated for $C_{14}H_{11}O_4NCl_4$: C 42.2; H 2.77; N 3.53

Found : C 42.5; H 2.9 ; N 3.9

P-TOLUENESULPHONYL AMINO ACIDS

Introduction

Recognizing the single important limitation of the phthaloyl group as a means of protecting the amino group, namely its relative instability in alkaline medium, we considered alternative means for NH_2-protection. One group, which seemed to be worthy of further investigation, was the p-toluenesulphonyl radical. Known for a long time as a useful N-substituent for alpha-amino acids (285) its value for peptide synthesis increased considerably after Schönheimer's discovery (47) that this radical can be cleaved by hydrogenolysis. This possibility of being removed by a non-hydrolytic method, combined with the ready availability of p-toluenesulphonyl chloride, by means of which the sulphonation is universally effected, makes the p-toluenesulphonyl group very attractive for our purpose. Several authors have reported complete satisfaction with this group when using it in peptide synthesis (31, 105, 250, 251, 252).

A factor which has apparently caused the p-toluenesulphonyl group to be somewhat neglected seems to be the relatively low yields obtained on sulphonation of alpha-amino acids. As most of the sulphonation reactions reported were undertaken for the purpose of identification of the amino acids, the yields were of secondary importance and very often were not mentioned at all. The most authoritative work in this field seems to be

that of McChesney and Swann (286), who reported that the yields are generally in the vicinity of 50 per cent of the theoretical amount.

Because of the insolubility of p-toluenesulphonyl chloride in water, it is usually dissolved in ether and this solution is shaken with the aqueous solution of some alkali and the amino acid (47, 251, 285, 286, 287, 288, etc.). Several cases were reported, where p-toluenesulphonyl chloride was simply suspended in the aqueous solution of the amino acid and sodium hydroxide and shaken, often at elevated temperatures (105, 247, 250, 289). The products precipitated after the aqueous layer was acidified. Sometimes extraction of the aqueous solution by an organic solvent was advised (252), although in other cases no further product could be isolated in this way (105). The crude precipitate was invariably found to be of sufficient purity for further experiments (105, 251), although recrystallization usually increases its melting point considerably.

Discussion of Results

Our initial sulphonation experiments with p-toluenesulphonyl chloride dissolved in diethyl ether, using equimolecular amounts of the reagents, resulted in disappointingly low yields (Exp'ts 3.a.1.a; 3.b.) and high recoveries of unreacted p-toluenesulphonyl chloride. Evidently the poor yields were due to incomplete sulphonation and not to side

reactions. Therefore we studied the effect of changing the amino acid/alkali, and amino acid/p-toluenesulphonyl chloride ratios with a view to forcing the reaction more nearly to completion (Exp'ts 3.a.l.b - d.). It was found that an increased amount of p-toluenesulphonyl chloride did not seem to have any effect on the amount of the product, while doubling the amount of alkali increased the yield. An attempt to improve the intermixing of the aqueous and ethereal layers by adding an emulsifier (Nacconol NR, sodium salt of a long-chain-alkyl benzenesulphonic acid) resulted only in a further decrease of the yield (Exp. 3.a.l.e.). One may theorize at this point that poor yields arise from conditions (high alkalinity, relatively high concentration of the sulphonyl chloride in the water phase) which are conducive to disulphonation, as it is known that the second sulpho group in a disulphonamide is very loosely bound (290). In harmony with this theory it was found that the yield increased appreciably when the alkali was added to the reaction mixture over a longer period instead of adding the full amount at the beginning of the reaction (Exp. 3.a.l.f.). However, even here we have a disquietingly high recovery of p-toluenesulphonyl chloride from the ethereal solution.

Another attempt in the direction of forcing sulphonation to completion was to operate at higher temperatures. Di-n-butyl ether was used because it has a higher boiling point (122.5°C.) than water. Thus heating the aqueous solution of

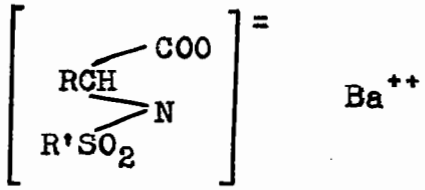
the amino acid to a vigorous reflux churned the aqueous lower layer through the ethereal upper layer, allowing it to be thoroughly mixed with p-toluenesulphonyl chloride in di-n-butyl ether, while the potassium hydroxide solution was slowly added (Exp. 3.a.l.g.). The product,obtained in about the same yield as in the original experiments, was of lower purity. Surprisingly, the amount of residue obtained by evaporation of the ethereal layer was much less than in the previous experiments. Apparently higher temperature was more conducive to the hydrolysis of the sulphonyl chloride (forming a water soluble potassium salt of p-toluenesulphonic acid) than to the sulphonation.

Realizing that the relatively high solubility of diethyl ether in water (7.5 per cent at 20°C.) might be the factor which supports the disulphonation (cf. above), we decided to substitute this ether by one which is less soluble in water and thus permits a lower concentration of the sulphonyl chloride in the aqueous phase. Diisopropyl ether (0.2 per cent solubility in water at room temperature) was our choice (Exp. 3.a.l.h.). The increase of the yield to the 70 - 80 per cent range was in accordance with our deductions.

A completely different approach to the problem of improving the yield of sulphonation reaction was taken when we replaced potassium hydroxide by barium hydroxide. This approach was based upon a report of McChesney and Swann (286) that yields increased from the usual 50 per cent to almost 100 per

cent in the case of phenylalanine and tyrosine. They ascribed these high yields to the fact that the sodium salts of these sulphonated acids happen to precipitate during the reaction. Accordingly means were sought to cause sulphonated amino acids to separate in all cases. The most promising possibility seemed to be to replace potassium hydroxide by barium hydroxide, for it is known that, whereas barium salts of carboxylic acids are generally freely soluble in water, the barium salts of sulphonic acids are not.

The prediction was confirmed by a preliminary experiment with glycine (Exp. 3.a.2.a.) when we observed rapid precipitation of barium p-toluenesulphonyl glycinate from the solution of barium glycinate. It was further observed that a full mole of barium hydroxide must be added to assure maximum yield. Consequently it seems likely that the water-insoluble barium salts of sulphonated amino acids (which have not been described previously) have the form :



Fair yield (48.1 per cent) of a very pure product was obtained by this procedure.

Unfortunately, the voluminous, curdy precipitate of the barium salt imbibes large amounts of liquids. The chief consequence of this tendency appears to be that the sulphonation

is incomplete and that the precipitate is contaminated with water-insoluble p-toluenesulphonyl chloride. Accordingly, in Exp. 3.a.2.b., the barium hydroxide was added slowly in four equal lots and each crop of barium salt was isolated by filtration, washed with ether, and the ether washings returned to the reaction mixture before proceeding. A modest improvement in yield resulted. However, the full advantage was not gained until the diethyl ether was replaced by diisopropyl ether and the amount of water was doubled (Exp. 3.a.2.c.). By their use good yields (61.2 per cent) of product were obtained for the first time, although still not as good as with the best run with potassium hydroxide. We ascribe this improvement to the following factors: the lower solubility of diisopropyl ether in water (see above) reduced the adsorption of p-toluenesulphonyl chloride on the precipitated salt, which is more dispersed because of the larger amount of water, and the lower volatility of this ether reduces evaporation and deposition of the chloride during filtration.

In summary, we suggest that where maximum yields of product are important and the time consumed in reaction is relatively insignificant, for example, when the sulphonated amino acid is to be used in peptide syntheses, the potassium-hydroxide method (Exp. 3.a.1.h.) may be followed; whereas if conditions are reversed, for example, when the product is to be used for purposes of identification, the barium hydroxide

method (Exp. 3.a.2.c.) may be followed.

During the earlier part of this work, p-toluenesulphonyl-DL-leucine was also prepared. Since no data on this compound were found in the literature (Schöhnheimer mentioned this compound as an intermediate in his reactions without describing it (47)), the description of its preparation is included in the experimental section below (Exp. 3.b.).

Experimental Procedures

Experiments 3.a.

1) p-Toluenesulphonylglycine by the "Potassium Hydroxide Method":

a.

A solution of 7.5 g. glycine (0.10 mole) and 6.0 g. potassium hydroxide (0.107 mole) in 100 ml. water was hand-shaken with a solution of 21.0 g. (0.11 mole) p-toluenesulphonyl chloride in 100 ml. diethyl ether at frequent intervals for several hours. The two layers were then separated and the ethereal layer was evaporated to dryness. The remaining residue, apparently unreacted p-toluenesulphonyl chloride, weighed 14.0 g. The light orange aqueous solution was acidified with 10 ml. of concentrated hydrochloric acid (representing 0.121 mole of the acid). White, microcrystalline material precipitated immediately and was removed by vacuum filtration. The weight of the precipitate was 8.5 g., (38.2 millimoles of p-toluenesulphonyl glycine) m.p. 138-9°C., softening at 136°C. Recrystallization from water yielded 7.6 g. of crystals, m.p.

147.5-148°C. (lit. 147°C.) (286).

The filtrate was made neutral to litmus with potassium hydroxide solution and subjected to evaporation until it became a solid, damp paste. This paste was then triturated with about 50 cc. hot ethyl alcohol and, while still hot, filtered on a heated funnel. Insoluble material (apparently containing potassium chloride, unreacted glycine, and a small amount of potassium p-toluenesulphonate) weighed 8.5 g. when air dried. Allowing 7.5 g. (0.1 mole) for the potassium chloride which was probably present, this observation suggests that one gram of original glycine was recovered.

The volume of the ethanolic filtrate was reduced to about 25 ml. and the remaining solution was allowed to stand in the refrigerator overnight. Crystals were obtained which were separated by filtration and dried: 2.5 g., no melting up to 160°C.

b.

A repetition of Exp. 3.a.1.a., using 12.0 g. (0.214 mole) of potassium hydroxide and 20 ml. of concentrated hydrochloric acid, resulted in a yield of 4.5 g. of white crystals (19.7 millimoles), m.p. 136-8°C., softening at 134°C. The aqueous phase remained almost colourless throughout. Evaporation of the ethereal solution left a residue weighing 15.0 g., which would indicate that only 6.0 g. of p-toluenesulphonyl chloride participated in the reaction.

c.

The same amounts of reactants were used as in Exp. 3.a. 1.a., except that the p-toluenesulphonyl chloride was increased to 31.5 g. (0.1655 mole). The aqueous phase became darker orange than in the first two experiments. The ethereal layer yielded 22.5 g. of residue. By acidification of the aqueous layer with 10 ml. of concentrated hydrochloric acid, 8.5 g. of white microcrystalline precipitate (38.2 millimoles) was obtained, m.p. 139-140°C., softening at 134°C.

d.

Glycine (7.5 g., 0.10 mole) was treated with 12.0 g. (0.214 mole) of potassium hydroxide and 31.5 g. (0.1655 mole) of p-toluenesulphonyl chloride in the manner described in Exp. 3.a.1.a. The aqueous layer remained colourless, and its acidification with 20 ml. of concentrated hydrochloric acid precipitated only 6.0 g. of white microcrystalline material (26.2 millimoles), m.p. 140-142.5°C., softening at 134°C. The amount of residue, which remained after evaporating the ethereal solution to dryness, was 25.0 g.

e.

The addition of 1 g. of sodium "lauryl" benzenesulphonate (Nacconol NR, manufactured by the National Aniline Division, Allied Chemical & Dye Corp, Buffalo, N.Y.) to the aqueous solution of potassium glycinate (the amounts of all the reactants and the procedure was the same as described in

Exp. 3.a.1.a.) did not improve the yield. On the contrary, the amount of the product was smaller (estimated at 1.5 g.), while the amount of the residue from the ethereal solution increased to 17.5 g.

f.

A solution of 7.5 g. (0.10 mole) glycine in 50 ml. water was shaken mechanically with 100 ml. ethereal solution of 20.0 g. (0.11 mole) p-toluenesulphonyl chloride, while 5.6 g. (0.10 mole) potassium hydroxide dissolved in 50 ml. water was added drop-wise and continuously to the reaction mixture over a period of two hours. The mixture was then mechanically shaken for another two hours, by which time the aqueous layer had become orange in colour. The two layers were separated in a separatory funnel and the aqueous layer was washed twice with ether in order to remove any unreacted p-toluenesulphonyl chloride. The aqueous solution was then reduced to a volume of about 50 ml. and, while still hot, acidified to Congo Red with approximately 8 ml. of concentrated hydrochloric acid. The orange colour tended to lighten somewhat. No oily droplets or layer separated. On cooling slowly a slight tendency to "oil out" was noted. However, on continued cooling massive amounts of crystals separated which, after being isolated by filtration, washed with a little water and dried at 50°C., weighed 11.5 g. (50.3 millimoles), m.p. 134-7°C. (softened at 126°C.). Evaporation of the ethereal layer left 10 g. of residue.

g.

In this experiment, diethyl ether was replaced by an equal amount of di-n-butyl ether (b.p. 122.5°C.), amounts of all the other reactants being the same as in Exp. 3.a.l.f. Vigorous refluxing, which churned the aqueous lower layer through the ethereal upper layer, was used instead of shaking. The solution of alkali was added drop-wise during a period of one hour (the ethereal layer started to become pale-yellow after half an hour), refluxing was then stopped and the reaction mixture was allowed to cool to room temperature while being vigorously shaken mechanically for two hours. Both layers were cloudy, but after standing overnight they became clear (water layer very light yellow, the ethereal layer pale-yellow). About one gram of viscous sticky liquid had settled to the bottom of the flask. The ethereal and aqueous layers were decanted away from the viscous layer, which was found to be reasonably soluble in deithyl ether (this ethereal solution was washed with a little water and the washings were added to the aqueous layer above, while the diethyl ether solution was discarded). The aqueous solution was extracted twice with di-n-butyl ether and the extracts were discarded. The aqueous material, after addition of 3 ml. concentrated hydrochloric acid, was evaporated to about 50 ml. volume and 5 ml. more of concentrated hydrochloric acid was added. On cooling, a crystalline precipitate separated slowly (unlike in previous experiments, where this precipitation was

prompt) from the somewhat oily, orange solution. The suspension was then placed in the refrigerator. Solid precipitate was isolated by filtration, washed with ether, and dried. Weight, 7.5 g. (32.7 millimoles), m.p. 130-7°C. (softens at 127°C.).

The di-n-butyl ethereal layer was distilled to dryness. The weight of somewhat charred residue was 2.5 g.

h.

One-tenth of a mole of glycine (7.5 g.) dissolved in 50 ml. of water was shaken mechanically with 19.0 g. (0.10 mole) of p-toluenesulphonyl chloride dissolved in 100 ml. diisopropyl ether for four hours. The solution of 11.2 g. (0.20 mole) potassium hydroxide in 40 ml. water was added in four 10-ml. batches. The first one was added just before the shaking of the reaction mixture was started and the remaining three at one-hour intervals, so that the last batch was added after three hours of shaking. This was followed by one more hour of shaking and by separation of the two layers in the separatory funnel. The aqueous layer, pale yellow in colour, was washed twice with isopropyl ether and acidified with concentrated hydrochloric acid on Congo Red (about 16 ml. of the acid was required). The resulting precipitation yielded 17.5 g. of white microcrystals (76.4 millimoles), m.p. 139.5-142°C., softening at 137°C. This melting point was increased by recrystallization from water to 147.5-148°C. (lit. 147°C.) (286), the final yield being 16.1 g.

(70.5 per cent yield). By evaporating the combined ether layer and ether washings (from washing the crude product) a residue weighing 2 g. was obtained.

2) p-Toluenesulphonylglycine by the "Barium Hydroxide Method":

a.

Water (100 ml.) was freed from dissolved carbon dioxide by addition of about 0.5 g. of anhydrous barium hydroxide and filtration. To the filtrate, 7.5 g. (0.10 mole) glycine and 8.55 g. (0.05 mole) anhydrous barium hydroxide was added. On warming, these substances passed into solution and left a slightly cloudy solution. A solution of 21 g. (0.11 mole) p-toluenesulphonyl chloride in 100 ml. diethyl ether was added and the suspension was vigorously shaken by hand. After about fifteen minutes a voluminous, curdy precipitate of white colour separated. Some of the liquid phase appeared to be locked in the stiff curds. The precipitate was separated by filtration on a Büchner funnel and pressed down with a stopper to force the liquid phase completely out of the curds. The precipitate was then washed with ether and suspended in 100 ml. hot water. Acidification to Congo Red (by about 10 ml. of concentrated hydrochloric acid) cleared the suspension as most of the precipitate passed into solution. However, a small amount of an oil remained on the bottom of the beaker. This was considered to be unreacted p-toluenesulphonyl chloride (it has a density greater than one, and its melting point is

reported as 69°C.). Accordingly the hot aqueous solution was decanted into another beaker and left standing to cool to room temperature. On cooling, white crystals separated, which, washed with a little water, and air-dried, weighed 8.5 g. m.p. 142-145°C., softens at 137°C. Evaporation of the filtrate (which was neutralized by potassium hydroxide before heating and reacidified to Congo Red afterwards) until its volume was reduced to about 30 ml. yielded another 2.5 g. of white precipitate, which begins to soften at 129°C. and to melt at 134°C. (melting incomplete at 160°C.).

It was observed that when the filtrate, obtained by separating the barium salt of p-toluenesulphonylglycine from the reaction mixture which contained both the aqueous and ethereal layers,was allowed to stand for some hours with occasional shaking, another crop of white precipitate was obtained. It was separated by filtration and to the filtrate, 8.5 g. of anhydrous barium hydroxide was added. The mixture was shaken mechanically overnight. Another crop of white precipitate was obtained. Both crops were combined and p-toluenesulphonylglycine isolated as above. Weight 2.5 g., m.p. 137-140°C., softens at 132°C. Thus the combined yield was 11.0 g. (48.1 millimoles).

b.

A solution of 7.5 g. (0.10 mole) glycine and 4.3 g. (0.025 mole) anhydrous barium hydroxide in 100 ml. water was vigorously hand-shaken with a solution of 20 g. p-toluenesulphonyl

chloride (0.11 mole) in 100 ml. of diethyl ether. After a few minutes a precipitate began to separate, which stiffened after another fifteen minutes. The curdy precipitate was separated by filtration or a Büchner funnel, pressed down with the bottom of a snug-fitting beaker, and then suspended in about 50 ml. diethyl ether. The suspension was warmed and agitated for a few minutes and poured again through the Büchner funnel. The filtrate was added to the previous one, 4.3 g. anhydrous barium hydroxide was stirred in, and the resultant reaction mixture was treated as described above. The whole procedure was then repeated a third and a fourth time. On the fourth occasion the reaction was shaken not for fifteen minutes only, but overnight.

The four crops of barium p-toluenesulphonylglycinate were combined, suspended in 50 ml. hot water (to form a thin, homogeneous paste), and acidified to Congo Red (about 8 cc. of concentrated hydrochloric acid was needed). While warm, the upper aqueous layer was decanted from the lower oily layer, and the upper layer was poured through a filter paper in a heated funnel while hot. On cooling white crystals separated. After filtration, washing with a little water, and air-drying at 50°C. they weighed 9.0 g. (39.3 millimoles), m.p. 137-140°C., softening at 129°C.

The filtrate was evaporated to about 20 ml. and allowed to stand in the refrigerator. A second crop separated, 1.5 g., which softened at 130°C., largely melted over range 136-140°C.

(melting incomplete at 160°C.).

c.

Heating of a suspension of 7.5 g. (0.10 mole) glycine and 8.6 g. (0.05 mole) anhydrous barium hydroxide in 200 ml. of water on a steam bath turned it into a slightly cloudy solution. It was shaken mechanically with 19.0 g. (0.10 mole) of p-toluenesulphonyl chloride dissolved in 100 ml. diisopropyl ether. After about fifteen minutes, a curdy precipitate began to separate and it increased in quantity with time. Since larger volumes of water and of ether were used this time than in the previous experiments, the white suspension remained sufficiently fluid to permit efficient mixing for a total time of one hour. Then the suspension was filtered and the precipitate pressed down with a beaker. Slightly damp filter-cake was suspended in about 50 ml. of boiling diisopropyl ether for a few minutes and isolated again by filtration. The approximate weight of the air-dried barium salt was 16 g.

The ether-washings were added to the reaction mixture, 8.5 g. of anhydrous barium hydroxide added (it passed rapidly into solution in the aqueous layer) and the mixture subjected again to mechanical shaking, this time for two hours. Again, barium salt separated as a curdy precipitate, but this time more slowly. This second crop, after being treated as described above for the first crop, weighed about 8 g.

Both crops of the barium salt were combined and suspended

in 50 ml. of hot water, acidified with concentrated hydrochloric acid to Congo Red (solution was slightly cloudy at this point, but no oily layer separated), filtered while hot and allowed to cool. White precipitate which crystallized out of the solution, was isolated by filtration and washed with a little water. Air-dried weight 14.0 g. (61.2 millimoles of p-toluenesulphonylglycine), m.p. 140-1°C., softens at 136°C.

Experiment 3.b.

Preparation of p-Toluenesulphonyl-DL-leucine

A solution of 13.12 g. (0.10 mole) DL-leucine in 115 ml. N sodium hydroxide was shaken mechanically with a solution of 38.0 g. (0.20 mole) p-toluenesulphonyl chloride in 205 ml. diethyl ether for one hour. Another 100 ml. of N sodium hydroxide was added and shaking continued for a further three hours. The water layer was then separated in the separatory funnel, acidified with concentrated hydrochloric acid to Congo Red (abundant white, microcrystalline precipitate separated) and filtered. The precipitate was then dried in vacuum. It melted between 117-128°C. Recrystallization from 20 per cent ethanol yielded three crops: 4.85 g. (m.p. 140-140.5°C.), 5.69 g. (m.p. 139-140°C.), and 1.70 g. (m.p. 136-8°C.); total 12.24 g. (43 millimoles; 43 per cent yield).

Calculated for $C_{13}H_{19}O_4SN$: C 54.80 ; H 6.72 ; N 4.90 ;

Found : C 55.01 ; H 6.81 ; N 4.70

ENZYMATIC AMIDATION

Introduction

Several enzymes are known which catalyze the hydrolysis of naturally-occurring polyamides. Like all catalysts, these enzymes generally do not change the equilibrium point of the reaction, or, in other words, they accelerate the rate of reaction in both directions. Consequently, they should prove to be capable of catalyzing also the reaction proceeding in the reverse direction, i.e. the condensation reaction. If some means of upsetting the equilibrium could be found, e.g. by separation of the product as an insoluble precipitate, these enzymes might be put to practical use. The proteinase-catalyzed formation of the peptidic bonds, being stereochemically specific, should offer the advantage of obtaining peptides of "natural" configuration. Thus it should be possible to prepare optically-active peptides even from racemic mixtures of amino acids, which should lower the cost of starting materials.

No enzymatic syntheses of peptides from the free amino acids have yet been described. Apparently they are not feasible, because of the considerable amount of energy required for the transformation of the amino-acid zwitter-ions into a reactive form by a proton transfer (58, 75, 291). On the other hand, enzymatic synthesis of peptide derivatives from substituted amino acids has been observed. So, for example,

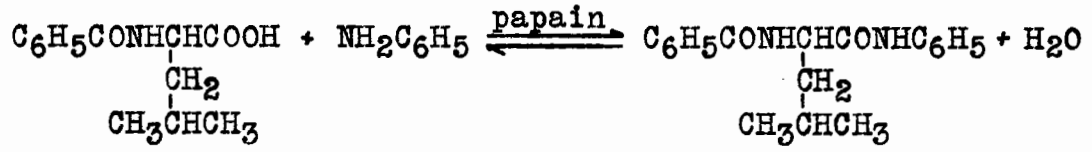
Bergmann and Fraenkel-Conrat used papain to condense benzoyl-L-leucine with aniline (67) and Waldschmidt-Leitz and Kühn to combine carbobenzoxyglycine with glycine phenylhydrazide (74), while Bergmann and Fruton reacted benzoyl-L-tyrosine with glycine anilide in the presence of chymotrypsin (69). In all cases the removal of the product by precipitation from the reaction mixture upset the equilibrium and thus was the driving force behind the reaction.

Discussion of Results

The experiments with enzyme catalyzed formation of the peptide bonds were undertaken in order to demonstrate whether the phthaloyl derivatives, which have recently come into vogue, are suitable for this type of condensation or not. Later, when it was decided to replace the phthaloyl by the p-toluenesulphonyl group for protecting the amino groups, the suitability of the latter for the enzymatic synthesis was also tested. No report on the use of phthaloyl or p-toluenesulphonyl derivatives in the enzymatic peptidation has been found in the literature.

Papain was the enzyme chosen for these experiments because of the wide range of its proteolytic activity.

At first, benzoyl-L-leucyl anilide was prepared by condensation of benzoyl-DL-leucine and aniline, catalyzed by



papain (67) in order to provide experience with some successful example of enzymatic synthesis (Exp. 4.a.1.). The same method was then applied to the attempted syntheses of phthaloyl-L-leucyl anilide (Exp. 4.a.2.), phthaloylglycine anilide (Exp. 4.a.3.), benzyl ester of phthaloylglycylglycine (Exp. 4.a.4.), and of benzyl ester of benzoylglycylglycine (Exp. 4.a.5.). While the preparation of benzoyl-L-leucine anilide was successful, no precipitates were obtained in the remaining 4.a. experiments. This would indicate that the phthaloylamino acids are not susceptible to the catalytic effect of papain. On the other hand, Experiment 4.a.5., where benzoyl-DL-leucine and benzylglycinate were incubated with papain without any precipitation, shows that the benzyl esters are apparently also not too suitable for this type of synthesis. Fruton (51) had also reported difficulties in this direction.

In order to extend the investigation of whether the phthaloyl amino acids can undergo enzymatic synthesis or not, it was resolved to try various pH's. Since no recipes for citrate buffers below pH 5.0 were found in the literature, other types of buffers were considered. It was decided to try the phthalate type of buffers (292). Benzoyl,

phthaloyl, and p-toluenesulphonylglycines were reacted with aniline at pH's 4.4, 5.0, and 5.6, as described in experiments 4.b.1., 4.b.2., and 4.b.3. Again, no results were obtained with the phthaloyl amino acid, while benzolglycine anilide and p-toluenesulphonylglycine anilide were obtained at pH 5.0 and, to a lesser degree, at pH 5.6.

Recently Fox has reported (73) that higher concentration of the citrate buffer improves the yields in the enzymatic synthesis of anilides. He found that various amino acid derivatives have different optimum pH's, although they are invariably in the neighbourhood of pH 5.0. Therefore the experiments were repeated to determine the suitability of the phthaloyl amino acids for the enzymatic anilide synthesis, using higher citrate buffer concentration and at various hydrogen ion concentrations.

The first difficulty encountered was absence in the available literature of any tables or recipes for citrate buffers below pH 5.0. Fox does not mention the composition of the buffers in his articles. According to a private communication he simply mixed the citric acid and sodium hydroxide stock solutions with control by a pH meter.

After preparing the approximately one molar solutions of the citrate buffer of pH 4.4, 5.0, and 5.6 by a method of trial and error, it was observed that the solutions were somewhat viscous. The solubilities of the amino-acid derivatives were much less in these concentrated citrate buffer

solutions. This observation suggests that the increase of the yields with the increased molarity of the citrate buffers, described by Fox (73), is due, not so much to the possibly higher stability of the reaction mixture to the changes in pH, as to the lower solubility of the compounds, which enhances the precipitation of the product.

In none of the above mentioned experiments did the phthaloyl amino acids participate in the enzymatic synthesis of the peptide bonds. Apparently, papain activity requires the presence of the alpha-amino hydrogen atoms, which are not present in phthaloyl derivatives. This suggestion is in line with the explanation of the activity of the endopeptidases by the possible hydrogen bonding between the substrate and the enzyme, resulting in a stress on the bond between the carbonyl and amino groups (cf. 293).

On the other hand, the preparation of p-toluenesulphonylglycine anilide, which is apparently the first case of successful application of tosylated amino acid to the enzymatic synthesis of the peptide bonds, indicates that this type of amino acid or peptide derivatives should be suitable for enzymatic syntheses.

Experimental Procedures

Experiments 4.a.1.

Benzoyl-DL-leucine (1.20 g., 5.10 millimoles) previously prepared from sodium leucinate (DL-leucine dissolved in 10 per cent sodium hydroxide) and benzoyl chloride, was added to a 1/3 M. water solution of sodium acetate which was buffered by a pH 5.0 citrate buffer (15 ml. distilled water + 6 ml. 2 M sodium acetate + 15 ml. citrate buffer). Not all of the benzoyl-DL-leucine dissolved, even after bringing the suspension to boiling point. Therefore it was filtered at 40°C; the undissolved residue weighed 0.45 g. An excess of aniline (1 ml.) and 10 ml. of papain solution, activated by 0.05 g. cysteine hydrochloride, was added to the filtrate. Placing the flask in a 40°C. water bath for about one minute and vigorous shaking brought all the reactants into solution. The flask was then placed in an incubator at 40°C. for four days. A brownish precipitate was formed, which was separated by filtration, washed with distilled water and air dried. Its weight was 0.42 g., m.p. 202-7°C. A control run with the unactivated papain (no cysteine hydrochloride was added) produced only a very small amount of a dark brown precipitate. Repetition of the above experiment, using 0.75 g. (3.19 millimoles) of benzoyl-DL-leucine, yielded 0.38 g. (1.22 millimoles of benzoyl-L-leucine anilide) of brownish precipitate, m.p. 198-203°C.

The products were combined and recrystallized from hot 95 per cent ethanol. Slightly brownish crystals were obtained: 0.30 g. of the first crop, m.p. 211-2°C., and 0.38 g. of the second crop, m.p. 205-8°C. (lit. 213°C.) (67).

The solutions which were used above were prepared as follows:

1. N sodium hydroxide: 4 g. sodium hydroxide dissolved in sufficient distilled water to make 100 ml. solution.
2. Citrate buffer pH 5.0: 2.1 g. citric acid dissolved in 20 ml. N sodium hydroxide in a 100-ml. volumetric flask and made up to 100 ml. by addition of distilled water.
3. Papain solution: 0.36 g. papain suspended in a mixture of 40 ml. distilled water and 40 ml. citrate buffer; filtered after thirty minutes.
4. 2 M sodium acetate: 16.41 g. sodium acetate dissolved in sufficient distilled water to make 100 ml. solution.

Experiments 4.a.2 - 5.

No precipitates were obtained even after four weeks under the conditions of Experiments 4.a.1. with:

4.a.2: Phthaloyl-DL-leucine and aniline;

4.a.3: Phthaloylglycine and aniline;

4.a.4: Phthaloylglycine and benzyl glycinate;

4.a.5: Benzoyl-DL-leucine and benzyl glycinate.

Experiments 4.b.

Benzoyl-, phthaloyl-, and p-toluenesulphonylglycine were reacted with aniline at pH's 4.4, 5.0, and 5.6, using potassium acid phthalate buffers. Two and a half millimoles of the particular amino acid derivative was dissolved in approximately 45 ml. of the buffer solution by careful heating on a steam cone and cooling to room temperature. Then, 0.23 ml. (2.53 millimoles) of aniline and 2 ml. of the papain solution were added. After measuring the pH by means of a glass-electrode pH-meter and, if necessary, adjusting it by 10 M sodium hydroxide solution, the reaction mixture was made up to 50 ml. by adding buffer solution. The mixture was then poured into a stoppered test-tube and incubated at 40°C. with occasional shaking.

It was noted that the addition of aniline to the phthaloylglycine solutions turned them yellow, while the solutions of other glycine derivatives remained colourless until the second day of incubation, when all the solutions became brownish yellow. The intensity of this colouration was practically the same in all reaction mixtures, while the intensity of the yellow colour which the solutions of phthaloylglycine acquired after addition of aniline, was highest at pH 5.6 and lowest at pH 4.4.

In the incubator, a brown precipitate began to appear in very small quantities in all test-tubes the second day after starting the incubation. In addition a white

precipitate appeared in pH 5.0 solutions containing benzoylglycine and p-toluenesulphonylglycine on the fifth day and slowly increased in amount on the following days. On the seventh day, white precipitates started to form in the pH 5.6 solution containing benzoylglycine and on the eighth day in pH 5.6 solution containing p-toluenesulphonylglycine. The amounts of the white precipitates increased until the second week of incubation. No further change was then observed (except for a slight darkening of the solutions) until the end of the third week, when the incubation was stopped. Then the brownish white precipitates were observed only in the four test-tubes containing pH 5.0 and pH 5.6 solutions of benzoylglycine and pH 5.0 and pH 5.6 solutions of p-toluenesulphonylglycine. All the other test-tubes contained only small amounts of dark brown sediment.

The four precipitates were separated by filtration and extracted by hot 95 per cent ethanol. Cooling of the ethanolic solutions brought about crystallization only in the pH 5.0 cases. The crystals were isolated by suction filtration and washed successively with small amounts of 0.1 N sodium bicarbonate, 0.1 N hydrochloric acid, and with distilled water. The other two ethanolic solutions yielded on evaporation to dryness only small amounts of brownish crystals.

The crystals obtained from benzoylglycine (pH 5.0) were white with a yellowish brown tinge and weighed 0.21 g., m.p.

207-212°C. The second recrystallization from 95 per cent ethanol yielded 0.11 g. of white crystals, m.p. 215-215.5°C. The mixed melting point determination with this compound prepared by the carbonic anhydride method had shown no depression.

p-Toluenesulphonylglycine yielded 0.135 g. of brownish crystals, m.p. 154-157.5°C. The second recrystallization from 95 per cent ethanol gave 0.07 g. of white crystals, m.p. 159-159.5°C. No depression in the mixed melting point with this compound prepared by the carbonic anhydride method was observed.

The solutions used above were prepared as follows:

1. pH 4.4 buffer solution: 7.35 ml. of 0.2 M sodium hydroxide solution was added to 0.2 M solution of potassium acid phthalate and made up to 200 ml. with distilled water.
2. pH 5.0 buffer solution: 23.65 ml. of M/5 sodium hydroxide processed as described above.
3. pH 5.6 buffer solution: 39.70 ml. of M/5 sodium hydroxide processed as described above.
4. Papain solution: 0.5 g. of papain suspended in 20 ml. of the particular buffer and filtered after about thirty minutes; 0.5 g. of cysteine hydrochloride (Merck) was added.

Experiments 4.c.

Benzoyl-, phthaloyl-, and p-toluenesulphonylglycine were reacted with aniline at pH's 4.4, 5.0, and 5.6, using higher concentrations of citrate buffer. The technique was the same as described in Experiments 4.b., with the phthalate buffers being replaced by the concentrated citrate buffers. After preparing approximately one molar solutions of the citrate buffer of pH's 4.4, 5.0, and 5.6 by a method of trial and error (mixing the 1 M aqueous solution of citric acid with the 10 M solution of sodium hydroxide and measuring the pH by means of the glass-electrode meter), it was observed that the solutions were somewhat viscous. The amino-acid derivatives did not dissolve well in the concentrated buffers. In all cases, the solubilities decreased with decreasing pH of the buffer. The undissolved parts were removed by filtration. Therefore when Experiments 4.c. were repeated, only half amounts of amino acid derivatives were used. Even so, small amounts of phthaloylglycine remained still undissolved in pH 4.4 solution, and had to be removed by filtration.

The preparation of the papain solutions by dissolving 0.5 g. of papain in 20 ml. of the particular buffer solution was not easy; papain itself dissolved fairly well, but the filtration(by suction using ordinary filter paper) was very slow and it was possible to finish it only by exchanging a fresh filter paper for the old one two or three times during the filtration. However, the small amount of cysteine

dissolved quite well. Because these papain solutions did not cause browning of the reaction mixtures in the incubator, as was observed in previous experiments, it was decided to repeat Experiments 4.c. with the papain solutions filtered through a cloth. Although some degree of the brown colouration of the reaction mixtures was observed in this series, again no yield was obtained even after three weeks in the incubator.

A Z E O T R O P I C A M I D A T I O N

I n t r o d u c t i o n

Since the formation of a peptide (amido) bond is essentially a condensation of a carboxyl with an amino group, the elements of a molecule of water being the by-product, the continuous removal of water from the reaction mixture should force the reaction to proceed towards completion. The following equation summarizes the reactions involved:

$$X\text{-}COOH + NH_2Y \rightleftharpoons [X\text{-}COO]^{\ominus} + [NH_3Y]^{\oplus} \rightleftharpoons X\text{-}CO\text{-}NH\text{-}Y + H_2O$$

Temperatures of the order of 200°C. are normally required to drive this reaction to completion in the desired direction. Since such conditions might prove too severe when applied to the more unstable amino acid (or peptide) derivatives, a milder method of displacing the equilibrium was sought. It is well known that water can be removed from reaction mixture continuously and quantitatively at temperatures below its boiling point by azeotropic distillation, as has been demonstrated by using this technique to advantage in esterification reactions (294). Therefore it was decided to examine the effect of heating equimolecular amounts of a reactant containing a free carboxyl group and of a reactant containing a free amino group in a medium which is able to form an azeotropic mixture with the water produced.

Discussion of Results

As sample reactants for the synthesis of an amido bond by azeotropic distillation, phenylacetic acid and p-toluidine were chosen. At first, the traditional "hot-melt method" was applied (Exp. 5.a.1.). Simple melting of an equimolecular mixture of phenylacetic acid (m.p. 76.7°C.) and p-toluidine (m.p. 45.0°C.) failed to produce the desired p-toluidide. However, if the temperature of the melt was raised to and held at 180°C., the appearance of effervescence and water vapour gave evidence of reaction. From the reaction mixture phenylaceto-p-toluidide was isolated in an 72.2 per cent yield.

Next, an equimolecular mixture of phenylacetic acid and p-toluidine was subjected to prolonged boiling in toluene, the toluene and its water-azeotrope being slowly removed by distillation (Exp. 5.a.2.). Toluene boils at 110.5°C., while its water-azeotrope, which contains 19.6 per cent water, boils at 84.1°C. (281). On cooling the solution remaining in the distilling flask, the p-toluidide separated in remarkably pure form. On repeating twice the addition of fresh toluene to the filtrate and subsequent azeotropic distillation, a total yield of 85.6 per cent was obtained.

Having demonstrated that azeotropic distillation can be applied to the synthesis of the amido bond, we applied it to a NH_2-protected amino acid. Hippuric acid (benzoylglycine)

was chosen for the purpose. The literature describes but one method of preparing hippuro-p-toluidide, which starts with hippuramide and p-toluidine (295). Since no melting point of the hippuro-p-toluidide is mentioned in the literature, it was deemed necessary to prepare some of it by the hot-melt method (Exp. 5.b.1.). Heating an equimolecular mixture of hippuric acid and p-toluidine at 190°C. for one hour gave a fair yield (54.1 per cent) of hippuro-p-toluidide. Its melting point was 210.5-211°C. Prolonged azeotropic distillation of an identical mixture (it was necessary to use a much larger volume of toluene than in the previously-described preparation and even then some of the hippuric acid remained undissolved) also produced the toluidide, although in somewhat lower yield (Exp. 5.b.2.).

Hippuric acid is not an attractive type of amino acid derivative for the synthesis of peptides, since the protecting benzoyl group cannot be removed subsequently by any other method than hydrolysis. Phthaloylglycine, however, has been recently advocated for this purpose and it was decided to use this derivative. The opportunity was seized to carry out the reaction in a continuous azeotropic-distillation apparatus, adapted from a design recommended by Clarke and Davis for the esterification of oxalic acid with ethanol (294). An equimolecular mixture of phthaloylglycine and aniline was dissolved in toluene. Again, a relatively large amount of toluene was used, due to the relatively low

solubility of phthaloylglycine. In this instance sufficient toluene was employed, so that a solution, rather than a suspension, was obtained. After prolonged distillation the sole product obtained (other than unreacted phthaloylglycine) was a compound which melted at 117-8°C. Phthaloylglycine anilide melts at 230-1°C.

Further variations were tried. At first, the relative amount of aniline was increased in the hope of forcing the reaction more nearly to completion. Then the amount was decreased to zero in order to ascertain whether it actually did participate in the reaction leading to the formation of "the 117-8°C. compound". The results of these experiments are summarized in Table V.

Table V

Exp. No.	Reactants used		Crude Product		Recrystallized Product
	Phthaloyl-glycine g.	Aniline ml.	Weight g.	M.P. °C.	M.P. °C.
5.c.1	1.25	0.5	1.09 0.26	117-118 mixture	
5.c.2	1.25	2.0	1.40	113-118	117-119
5.c.3	1.25	2.0	1.30	115-120+ 172-173	
5.c.4	1.25	2.0	1.02	179-187	
5.c.5	2.0	0.0	1.84	189.5-191	190-191

The divergence of the results is a disappointing feature of these experiments. Moreover, none of them indicates that the formation of the anilide did indeed take place during the azeotropic distillation. The highest melting points observed were in the range of the melting point of phthaloylglycine, 190-1°C. This observation suggests that in all runs some starting material remained and in several no reaction whatsoever had occurred. This conclusion was confirmed by a mixed melting point with an authentic sample of phthaloylglycine. The identity of the product with a melting point in the 115-120°C. range, though interesting, was not studied in detail. For comparison, the melting points of compounds, which were considered to be possibilities, are listed below:

Phthalic acid	190-200°C. (with decomp.)
Phthalic anhydride	131
Phthalic acid monoanilide*	164
Phthalic acid dianilide	231 (with decomp.)
Phthalimide	233.5
N-phenylphthalimide	203
Glycine	232.6
Glycine anhydride	275 (with decomp.); sublimate 26 oz.

* Phthalic acid monoanilide is easily transformed on heating to N-phenylphthalimide.

The possibility that p-toluenesulphonic acid might have a catalytic effect on the formation of an amido bond was investigated in Experiment 5.c.6. This attempt was motivated by a report of the catalytic effect of sulphonic acids on the hydrolysis of this bond (296). However, no crystalline product was obtained but only a black, highly viscous, sticky sediment, further manipulation of which did not produce a crystalline product. One may suppose that p-toluenesulphonic acid catalyzes the oxidation of aniline to such an extent that its application to the synthesis of anilides does not seem feasible.

A fair and conservative opinion, based on this work, would appear to be that the amido bond can be created at moderate temperatures by the direct interaction of an amino group and a carboxyl group through the technique of azeotropic distillation but that application of the method to the stepwise synthesis of peptides must await the development of a more suitable method for protecting the carboxyl groups of amino acids. Metal salts of amino acids are unsuitable as intermediates, because of their general insolubility in organic liquids; even if an organic solvent could be found, no doubt an undesired metathetical reaction would take place between the two components, acid and salt, with transfer of the metallic ion. Esters of the amino acids have an inconvenient tendency either to polymerize or to form diketopiperazine rings at even moderately elevated

temperatures. And amides are not suitable if a controlled synthesis is desired because of the possibility of transamidation. In this connection strongly basic resins, such as those containing quarternary ammonium groups, are worthy of consideration for the protection of the carboxyl groups of amino acids. On the other hand, the creation of a peptide bond through azeotropic distillation ought to lead to a method for the synthesis of polypeptides by "block polymerization" involving the use of tri- or higher peptides as starting material.

Experimental Procedures

Experiments 5.a.

1) Phenaceto-p-toluidide by the "hot-melt method":

Simple melting of a mixture of phenylacetic (alpha-toluic) acid and p-toluidine did not result in condensation reaction; the phenylacetic acid was recovered.

Heating of phenylacetic acid (6.8 g., 50 millimoles) and p-toluidine (5.5 g., 56.5 millimoles) to 180°C. resulted in a slight effervescence and water vapour condensed on the walls of the test-tube. After two hours of heating and occasional stirring, no more water vapour evolved. The melt was dark brownish-green, but transparent. It was poured into a beaker, left to cool and then dissolved in hot ethanol. The greenish-white crystals which formed on

standing were separated by suction filtration, then washed with dilute sodium carbonate solution (to remove unreacted p-toluidine) and with water, then dried in a vacuum oven (temperature 80°C.). White crystals (thick leaflets) were obtained: 8.36 g., (74.2 per cent yield), m.p. 129-131°C. Recrystallization from hot toluene gave thick crystalline leaflets, m.p., 133.5°C. Slow recrystallization from hot toluene yielded white needles of the same melting point. (Lit. 130-2°C.(297) 135-6°C (298).)

2) Phenaceto-p-toluidide by the "azeotropic method":

Both phenylacetic acid and p-toluidine dissolved very easily in hot toluene. Phenylacetic acid (13.6 g., 100 millimoles) and p-toluidine (11 g., 113 millimoles) were dissolved in 30 ml. toluene, placed in a distilling apparatus equipped with a 10-inch column and a moisture trap and subjected to slow distillation. Fresh toluene was added several times so as to keep the level of the boiling solution above the top level of the heating mantle. After five hours of this distillation, the level of the solution was allowed to decrease just below the top of the heating mantle and the reaction mixture was left standing overnight at room temperature. White crystals separated, which were isolated by suction filtration, and dried in vacuo: 1.5 g., m.p. 132-132.5°C. (softening started at 128°C.). Fresh toluene was added to the filtrate and the solution was then subjected

to another twelve hours of azeotropic distillation. On standing, 6.35 g. of white crystals of the same melting point as the first crop was obtained. A third period of azeotropic distillation lasting twelve hours yielded 2.82 g. of white crystals, m.p. 130.5-132°C. (softening starting at 125°C.). The reaction mixture, which turned darker and darker throughout the whole distillation, was at the end almost black. It was evaporated to a volume of about 5 ml. Brownish crystals were isolated which, upon recrystallization from toluene, gave 0.52 g. of white crystals, m.p. 132.5-133.5°C. No further crystals could be obtained from the filtrate.

Table VI

Crop	Crude Product	Recryst. from Toluene
1	1.5 g., 132-132.5°C.(soft.128°)	0.98 g. 132.5-133.5°C.
2	6.35 g, 132-132.5°C.(soft.128°)	4.15 g. 133.0-133.5°C.
3	2.82 g, 132-132.5°C.(soft.125°)	2.20 g. 132.5-133.5°C.
4	(from filtrates 1 + 2 + 3)	1.76 g. 131.5-132.5°C.
5	(from the reaction mixture)	0.52 g. 132.5-133.5°C.
		9.61 g. 85.6% yield

The mixed melting point of the products obtained by the "hot-melt method" and by the "azeotropic distillation method" indicates that both are identical compounds.

Experiments 5.b.

1) Hippuro-p-toluidide by the "hot-melt method":

The procedure described in Exp. 5.a.1. was employed, using hippuric acid (4.5 g., 25.1 millimoles) and p-toluidine (3.0 g., 28.0 millimoles). The oil bath was heated to 190°C., because hippuric acid melts at 187.5°C. The mixture melted at first, effervescing slightly, then began to form white dot-like particles, which grew until they formed a mixture of solid particles joined by a viscous mass. While these particles remained whitish-yellow to orangeish, the viscous mass turned red and after about one hour of heating very dark pink. Since such a dark colouration might indicate a danger of decomposition, and since no further effervescence was apparent, the heating was discontinued at this stage and the mass was cooled and extracted with hot ethanol. The cooling of the ethanolic extracts brought on heavy precipitation. The precipitate was white, m.p. 207-8°C. (started to soften at 200°C.). One-third of the product was recrystallized from ethanol; m.p. 208-9°C., 1.35 g. The other part (one-half of the first crop) was subjected to an attempted recrystallization from toluene, but was found to be almost insoluble in hot toluene. Therefore it was separated by filtration (1.83 g., m.p. 210.5-211°C.). No further crystals were obtained after cooling the filtrate on dry ice, which caused only slight cloudiness; evaporation to one-third

volume slightly increased the cloudiness, but no crystals appeared. The last crop, which represented a 54.5 per cent yield, was analyzed:

Calculated for $C_{16}H_{16}O_2N_2$: C 71.6 ; H 6.02 ; N 10.45
Found : C 71.84; H 6.32 ; N 10.30

2) Hippuro-p-toluidide by the "azeotropic method":

The same amounts of reactants as above (Exp. 5.1.b.) were added to hot toluene. While p-toluidine dissolved easily, hippuric acid dissolved only partly, even after the amount of hot toluene was increased to 250 cc. The mixture was placed in a 500 ml. three-necked flask equipped with a stirrer and a 35-inch glass column, and was subjected to slow distillation for forty hours (spread over three and a half days), after which time the volume of the reaction mixture was decreased to about 100 ml. The cooled mixture was filtered. The white crystalline mass on the filter paper was dried and recrystallized from ethanol, yielding 4.7 g., m.p. 170-185°C. This product was then recrystallized from hot water in order to remove the unreacted hippuric acid (washing with alkaline water solution for this purpose might considerably decrease the yield, as the previous experiments indicate that hippuro-t-toluidide is unstable in either alkaline or acidic medium); the undissolved part was removed by filtration in a heated funnel and recrystallized from ethanol: 2.45 g., m.p. 209.5-211°C.

Mixed melting point with the product from Experiment 5.b.1. was 209-211°C. The aqueous solution gave, after cooling, 0.87 g. of white crystals, m.p. 170-180°C., and after evaporation to one-third volume, 1.06 g. of white crystals, m.p. 187-8°C. (slight browning above 184°C.).

The toluene solution (filtrate from the reaction mixture above) yielded after decreasing its volume to one-third 0.13 g. of white crystals, m.p. 209.5-210.5°C. Both crops together (2.45 g. and 0.13 g.) make 2.58 g., 38.4 per cent of the theoretical yield.

Experiments 5.c.

Attempted synthesis of phthaloylglycine by "azeotropic method": 1.

Aniline (0.5 ml., 0.511 g., 5.50 millimoles) and phthaloylglycine (1.125 g., 5.50 millimoles) were dissolved in toluene. Whereas aniline is infinitely soluble in toluene, this amount of phthaloylglycine required 200 ml. of hot toluene. The reaction solution was placed in a continuous azeotropic-distillation apparatus, adapted from the design recommended by Clarke and Davis for the esterification of oxalic acid with ethanol (294). The distillate was run through an automatic liquid-liquid separator and through a drying bottle containing anhydrous calcium chloride in order to remove water, and then returned to the reaction vessel. At no time during the reaction was water present in the distillate in

sufficiently large amount to appear as a distinct phase in the automatic separator. Toluene was circulated for twenty-four hours (spread over two and a half days). The reaction solution was left to cool overnight and white needles separated; weight 1.09 g., m.p. 117-8°C. Evaporation of the filtrate to about one-quarter of its original volume produced on cooling a second crop of a slightly yellowish precipitate; weight, 0.26 g., m.p.: softening at 116-8°C. slight change at 138-140°C., final melting at 162-205°C. Further evaporation of the filtrate did not give another crop of crystals.

2.

An increased amount of aniline was employed in this run (2 ml., 2.05 g., 22.0 millimoles). After twenty-four hours of distillation the distillate was allowed to accumulate until about 50 ml. of solution remained in the reaction vessel. This solution yielded on cooling 1.4 g. of crystals which melted at 113-8°C. No second crop of crystals was obtained, even when the filtrate was evaporated to dryness. The first crop was recrystallized from 70 ml. toluene to yield 0.72 g. of white crystals, m.p. 117-9°C.

3.

This run was a repetition of Experiment 5.c.2. The first crop, consisting of white crystals, weighed 1.3 g. and melted at 115-120 + 172-3°C. No second crop was obtained.

4.

This run was a second repetition of Experiment 5.c.2. The first crop weighed 1.02 g. and melted at 179-187°C. No further crystals were obtained.

5.

In order to ascertain whether aniline did participate in the formation of the product obtained, a run was performed in its absence. The amount of phthaloylglycine was 2.0 g. and the technique of Experiment 5.c.1. was followed. White crystals were obtained. They weighed 1.84 g. and melted at 189.5-191.0°C. Evaporation of the filtrate yielded a second crop, long white needles, weight 0.23 g., m.p. 190-1°C. By further evaporation a trace of a yellow residue was obtained, m.p. about 200°C., with decomposition starting at 188°C.

6.

The possible catalytic effect of p-toluenesulphonic acid was investigated. The opportunity was taken to improve the azeotropic-distillation apparatus by replacing the drying bottle by a drying U-tube.

Aniline (2 ml., 2.044 g., 22.0 millimoles), phthaloylglycine (1.5 g., 7.325 millimoles), p-toluenesulphonic acid (0.2 g., 1.16 millimoles), and 200 ml. toluene were placed in the reaction flask. On heating, the solution soon turned dark brown and after twenty-four hours it became almost black. Filtration did not yield any crystals. A black

sticky mass remained adhering to the flask. It was not investigated further. The filtrate was evaporated to dryness. A black, sticky residue remained. Repeated attempts to obtain crystalline material from it were unsuccessful.

C A R B O N I C - C A R B O X Y L I C A N H Y D R I D E S

I n t r o d u c t i o n

A new method for synthesizing the peptide bond was reported in 1951 independently by Boissonnas (38), Vaughan and Osato (40, 120), and Wieland and Bernhard (39). The overall reaction can be outlined in the following way:

$$RCOOH \xrightarrow{NR'_3} RCOOH.NR'_3 \xrightarrow[-HCl.NR'_3]{ClCOOR''} RCOOCOOR'' \xrightarrow[-CO_2]{NH_2R'''} RCONHR'''$$

This method has several conspicuous advantages. It uses commercially available reagents (e.g. ethyl chloroformate, tri-n-butylamine, etc.) and amino acid derivatives which are easily prepared (e.g. N-phthaloyl derivatives, esters, salts). The reaction proceeds rapidly under mild conditions and without racemization. The product obtained is in a pure state, since the by-products are either soluble in the organic medium (ethanol, tri-n-butylammonium chloride) or are in gaseous form (carbon dioxide).

D i s c u s s i o n o f R e s u l t s

Preliminary experiments with mixed carbonic-carboxylic anhydrides, designed to familiarize us with this method, have shown that the reactions of these anhydrides with

aniline, amino acid salts, or benzyl esters yielded easily products of remarkable purity. The benzyl esters of the amino acids gave higher yields than the amino acid salts (cf. Exp'ts 6.a.). We prepared several benzyl esters of the phthaloyldipeptides, which have not been reported previously in the literature.

One difficulty which plagued us throughout these experiments was the apparent instability of the anhydrides. Quite often, the solution of the mixed anhydride evolved carbon dioxide, indicating a decomposition (disproportionation) of the anhydride, before the compound containing the free amino group could be added. Such a disproportionation of these anhydrides had been noted by Wieland and Bernhard (39) who suggested that symmetric anhydride of the acyl-amino acid and carbonic acid diester are obtained:

$$2\ RCOOCOOR' \longrightarrow (RCO)_2O + (R'O)_2CO + CO_2$$

The tendency to disproportionate was more pronounced when triethylamine was used instead of tri-n-butylamine. Private communication with Vaughan and co-workers of the American Cyanamide Co. revealed that this difficulty might be diminished if ethyl chloroformate were replaced by isobutyl chloroformate. Our experiments confirmed this. No appreciable effect of the change of chloroformates on the yield of anilides was observed.

The effect of replacing the chloroformate by one of

its thio-analogues on the formation of the peptide bond was also tested. The decision to study the replacement of oxygen by sulphur in these compounds stemmed from the known increase in the stability of analogous N-carbonic anhydrides (oxazolidinediones) when the oxygen atoms were replaced by sulphur atoms. While oxazolidinediones-2,5 are very thermolabile and sensitive to the presence of hydroxylic compounds, thiazolidinediones-2,5 and 2-thiothiazolidinones-5 (mentioned above on pp. 46, 49 and 51) are less so. The experiments showed that thiochloroformates which contain the carbothionic (C=S) group are too unstable to be of any practical value for our purposes. A representative thio-analogue which contained the carbothiolic (C-S-) group, viz. n-pentyl chlorocarbothiolate (a compound not previously mentioned in the literature), was found to be quite stable and was subjected to a comparative testing with the chloroformate. Phthaloylglycine anilide was prepared in two runs, one using n-pentyl chlorocarbothiolate, the other isobutyl chloroformate. The yields were lower in the first case (54 per cent, as compared with 71 per cent in the latter).

In the above-mentioned reactions of the carbonic-carboxylic anhydrides with the alkali salts of the amino acids, water was used as a solvent for the amino acid salts. This had the obvious disadvantage of introducing large quantities of a compound which contains a hydroxyl group capable of

reacting with the anhydride. This side reaction, which competed with the amidation reaction, could explain the lower yields generally obtained when salts of the amino acids in water were used, as compared with those of the amino acid esters in inert solvents. On the other hand, using a base as a protecting group for the carboxyl enhances greatly the ease of synthesizing the peptides, since its removal requires only a simple neutralization reaction. Accordingly, if an inert solvent capable of dissolving such salts could be found, or, conversely, a base capable of forming amino acid salts soluble in organic solvents, a method of far-reaching merits might be introduced to peptide synthesis.

At first, our attention was centred on dimethyl formamide, because it is unaffected by acid anhydrides and is known to be a good solvent for a number of metallic salts of both inorganic and organic acids (299). (It has a high dielectric constant in comparison with other organic solvents.) Sodium glycinate, however, was found to be insoluble in dimethyl formamide. Accordingly, attention was given to other alternatives. At first, amino acid salts of metals of Groups I.B., VI.B., and VIII (viz. Cu, Cr, Ni) looked attractive, because their chlorides and acetates are reported to be highly soluble (299), but they were rejected when it was realized that they might have a tendency to exist as chelate compounds (cf. e.g. 300). Tin and zinc salts remained as possible solutes (299). Accordingly,

stannic and zinc salts of glycine were prepared by well known methods (from zinc or stannic chloride respectively, potassium hydroxide and glycine). Unfortunately, they showed only limited solubility in dimethyl formamide.

Another alternative would be to find an appropriate organic cation. Tertiary amines, which serve so effectively with N-substituted amino acids, are not applicable to free amino acids, because of their apparent inability to break up the zwitter-ion constitution of the amino acids. Exploratory test-tube experiments on the solubility of glycine plus tri-n-butylamine in twelve organic solvents or their combinations (sixty-six combinations of equal volumes of two solvents) had shown that no significant dissolving took place. Neither did glycine dissolve in tri-n-butylamine, triethylamine, diethylethanolamine, or triethanolamine. It was apparent that a much stronger organic base was required. Therefore, experiments with quaternary ammonium salts were undertaken.

The above considerations were confirmed by the successful experiments with benzyltrimethylammonium glycinate. This cation was chosen because of its reasonably simple structure and ready availability (as chloride) from commercial sources. The glycinate was found to be readily soluble in anhydrous chloroform and in this solvent was subjected to reaction with the mixed anhydride derived from phthaloylglycine and isobutyl chloroformate. A good yield (74 per cent) of phthaloylglycylglycine was obtained. The

principle of using a quaternary ammonium cation for the protection of the carboxyl group of amino acids for amidation reactions carried out in non-hydroxylic solvents appears to be established.

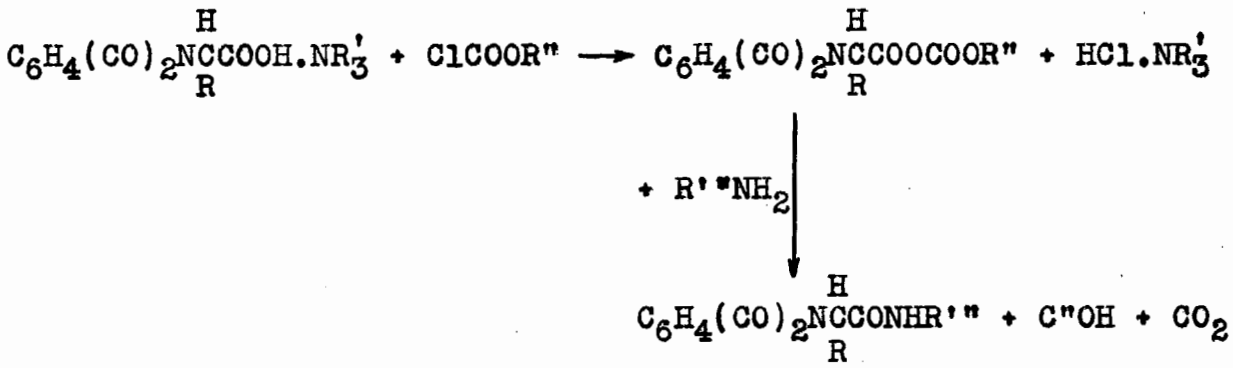
The experimental work reported in this chapter has provided us with firsthand experience in the application of phthaloylamino acids to peptide syntheses. These derivatives have proved themselves to be reasonably satisfactory, with the exception of difficulties encountered in their purification when they had been previously subjected to the effect of high pH media (our observations were confirmed by a private communication with Vaughan and co-workers of the American Cyanamide Co.). The possible type of this effect is indicated by the transformation of phthaloylglycine into o-carboxybenzoylglycine in alkaline solution (301, 302).

Hydrochlorides of the benzyl esters of amino acids proved to be superior to the benzenesulphonates of the benzyl esters of amino acids as starting materials for the synthesis of the peptide bond. For example, benzyl phthaloylglycylglycinate was prepared in pure form in 76 per cent yield, if hydrochloride was employed, while only 44 per cent of impure product was obtained from benzenesulphonate.

Experimental Procedures

Experiments 6.a.

Mixed Carbonic-Carboxylic Anhydride Method:



The phthaloylamino acid (usually 4.2 millimoles) was suspended in an anhydrous solvent (e.g. 2.5 ml. chloroform, or 5 ml. toluene), cooled on ice and an equivalent amount of the tertiary amine (e.g. 0.98 ml. of tri-n-butylamine) was slowly added with constant stirring. In several cases the suspension did not clear completely and a careful addition of a small amount of the solvent was required to bring the amino acid derivative completely into solution. The mixture was then cooled in the freezing compartment of the refrigerator to around -10°C. and an equivalent amount of the chloroformate (e.g. 0.40 ml. ethyl chloroformate) was added. The mixture was shaken briefly and left in the refrigerator with occasional shaking for a pre-determined period (usually ten minutes), after which the solution of equivalent amount of the compound containing the free amino group was added.

Three procedures were used, according to which type of the "amine" was added:

a) Aniline was added dissolved in the same anhydrous solvent as that in which the carbonic-carboxylic anhydride was prepared, and the mixture was vigorously shaken.

b) Amino acid dissolved in alkaline aqueous solution* was added, the mixture stirred on the magnetic stirrer for about one hour.

c) Hydrochloride (or benzenesulphonate) of the amino acid benzyl ester together with the equivalent amount of a tertiary amine (which releases the amino group of the amino acid ester from the salt formation), dissolved in the same solvent as that in which the carbonic-carboxylic anhydride was dissolved, was added and the mixture vigorously shaken.

In all cases gas evolution occurred immediately after the reactive amine was added. The reaction mixture was allowed to warm to room temperature and to stand for a longer time (usually overnight), though the apparent gas evolution rarely lasted for more than a few minutes, in

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* An exact amount of alkali carbonate was used, because excessive alkalinity was found to be detrimental to the phthaloyl derivatives.

order to enable the reaction to proceed as far as possible to completion.

1. Phthaloylglycine anilide separated immediately as a microcrystalline precipitate. It was isolated by suction filtration, washed consecutively with small amounts of water, normal hydrochloric acid, normal solution of sodium bicarbonate, and again with water. Yield was 72 per cent; m.p. 229-230°C. (lit. 230-231.5°C.) (38).

2. Phthaloyl-DL-phenylalanine anilide precipitated also soon after aniline was added and the treatment of the precipitate was the same as in Exp. 6.a.1. Yield was 68 per cent; m.p. 213-4°C. (lit.213-213.8°C.) (50).

3. Phthaloylglycylglycine started to precipitate soon after the aqueous phase was acidified to Congo Red. It was separated by suction filtration and recrystallized from 60 per cent ethanol. Yield was 55 per cent; m.p. 230.5-232°C. (lit. 229-231°C.) (50).

4. Phthaloylglycyl-DL-phenylalanine separated also after the aqueous phase was acidified. It was recrystallized from iso-amyl alcohol. Yield was 59 per cent; m.p. 195-6°C. (lit. 197-198.5°C.) (50).

5.

Phthaloyl-DL-leucylglycine separated after acidification in the form of an oily layer, which later solidified into a crystalline structure. Recrystallization from methanol resulted in a total yield of 46 per cent; m.p. 117-9°C. (lit. 119-120°C.) (303).

6.

Benzyl phthaloylglycylglycinate separated beautifully in the form of a crystalline precipitate, which melted sharply without recrystallization at 180-2°C. The yield was 76 per cent. (Hydrochloride of the benzyl glycinate was used as a starting material in this experiment; if benzenesulphonate was used instead, only 44 per cent of crude product, m.p. 176-180.5°C., was obtained.)

Calculated for $C_{19}H_{16}O_5N_2$: C 64.75; H 4.58

Found: C 64.42; H 4.33

7.

Benzyl phthaloylglycyl-L-leucinate separated more slowly, but again it was a microcrystalline precipitate, m.p. 129.5-131°C. The yield was 62 per cent. (Hydrochloride of the benzyl-L-leucinate was the starting material.)

Calculated for $C_{23}H_{24}O_5N_2$: C 67.6 ; H 5.93

Found: C 67.20; H 5.59

Experiments 6.b.

1) Preparation of n-Pentyl Chlorocarbothiolate

a.

Phosgene gas was condensed in a large test-tube immersed in an acetone/dry ice bath. Approximately 90 ml. (125 g., 1.26 moles) of liquid phosgene was collected. From a burette, 50 ml. (42.8 g., 0.41 mole) n-pentanethiol was added drop-wise with stirring over a period of twenty minutes. The stirring was then continued for two hours more, the container was taken out of the cold bath and left standing at room temperature until most of the phosgene evaporated. The residual phosgene was removed by vacuum distillation at room temperature; 42.6 g. of clear liquid remained, which was divided into two parts. The first part, 15.9 g., was subjected to ordinary distillation. Approximately 1 ml. went over at 91-112°C. No further distillation occurred even after the oil bath temperature was increased to above 200°C., when the material began to decompose (darken). The liquid was then quickly cooled to prevent further decomposition, and subjected to vacuum distillation. During this distillation, the boiling liquid foamed considerably. When the second part (26.7 g.) was subjected to vacuum distillation, no foaming occurred. In both cases, the fractions boiling at 65-90°C. (12 mm. vacuum) were singled out for further purification described below (Exp. 6.b.1.b.). Their weights were 5.1 g. and 13.8 g. respectively. Green colouration in the

Beilstein test indicated the presence of chlorine in both samples. No chlorine, or only traces of it, were detected in lighter and heavier fractions in both distillations. Small amounts of tarry sediments remained after each distillation in the distilling flask.

b.

The previous experiment (6.b.1.a.) was repeated, using approximately 100 ml. of liquid phosgene (139 g., 1.40 moles) and 50 ml. of n-pentanethiol (42.8 g., 0.41 mole). After removing unreacted phosgene by vacuum distillation at room temperature, the clear, colourless liquid was subjected immediately to vacuum distillation on the oil bath. The fraction boiling at 65-90°C. (12 mm. vacuum) was found to colour intensively the copper wire in the flame, while only traces of chlorine were detected in the lower fraction, and no chlorine in the higher fraction. About one ml. of a tarry sediment remained in the distilling flask.

The amount of the middle fraction was 15.7 g. The two fractions from the previous experiment (5.1 g. and 13.8 g.) were added and the mixture was subjected to repeated vacuum distillation on an oil bath heated to 100-125°C. The boiling started at 60°C. (12 mm. vacuum). The temperature of the vapour rose quickly to 84°C., where it remained for a while, and then increased very slowly to 86°C. Rapid rise to above 90°C. followed. The 84-86°C. fraction was collected. It was a colourless liquid with a characteristic odour

mildly reminiscent of onions, weighing 32.63 g. (197 millimoles of n-pentyl chlorocarbothiolate). Since the volume of this product was 30.5 ml. its density is 1.07. As this compound was not described in the literature, it was identified by elementary analysis:

Calculated for $C_6H_{11}OSCl$: C 43.25; H 6.65; S 19.2 ; Cl 21.3

Found: C 43.65; H 6.65; S 19.09; Cl 19.09

2) Preparation of Ethyl Chlorocarbothionate:

a.

The method of Autenrieth and Hefner (304) was generally followed. A known amount of sodium (4.3 g., 187 millimoles) was slowly added to a large excess (50 ml.) of absolute ethanol. After formation of sodium ethoxide was completed, the container was placed in an acetone/dry ice bath and stirred vigorously. Thiophosgene (21.4 g., 186 millimoles), previously cooled to a semifrozen state, was added slowly so that the temperature of the solution did not rise markedly (the reaction was found to be strongly exothermic). The addition took about fifteen minutes. Stirring was then continued for one more hour in the cooling mixture, and then for half an hour at room temperature. Since it was observed that white fumes formed on the surface of the reaction mixture in the light, the container was wrapped in tin foil. The brownish precipitate, which started to form immediately after the first drops of thiophosgene were added to the

solution of sodium ethoxide in ethanol, was now removed by rapid filtration. Washing the precipitate with anhydrous diethyl ether turned it white (evaporation of the ethereal solution yielded some more product, which was added to the filtrate). This white powder melted without decomposition in a strong flame and was easily soluble in water; it weighed 10.23 g., which could represent 176 millimoles of sodium chloride.

The dark orange filtrate, which was found to be strongly lachrymatory, was subjected to distillation. All fractions boiled at temperatures below 100°C., while the boiling point of ethyl chlorocarbothiolate is reported to be 126-7°C. (304) or about 136°C. (305).

Vacuum distillation yielded fractions coloured yellow to orange, which gave positive tests on chlorine(Beilstein test), but negative aniline test (no precipitate formed).

b.

In this repetition of the previous experiment (6.b.2.a.) the precipitated sodium chloride was not removed by filtration. Instead, the observation made during the previous experiment (that addition of water to a small aliquot of the reaction mixture resulted in a separation of clear oily droplets of a brownish tinge) was applied. Two volumes of diethyl ether were added to one volume of the reaction mixture and the resultant ethereal solution was shaken with one and a half volume (i.e. half volume of the ethereal

solution) of water saturated with diethyl ether. The aqueous layer was separated, and the ethereal solution was shaken with another one and a half volume of water. (Note: while the aqueous layers were colourless, the ethereal layer remained yellowish all the time.) The ethereal solution was dried with calcium chloride and evaporated in vacuo in a stream of carbon dioxide (in order to prevent the possible oxidation of the thioester). The residue was an orangeish-yellow viscous liquid with an ozone-like smell. Distillation of a small aliquot (7.0 ml.) of this product had shown that most of it distilled between 137-155°C. This product gave a positive test for chlorine and reacted vigorously with aniline (exothermic reaction) yielding a large amount of white precipitate which solidified the mixture. This would indicate the following reaction:

$$2\ C_6H_5NH_2 + ClCSOC_2H_5 \longrightarrow C_6H_5NHCSOC_2H_5 + C_6H_5NH_2.HCl$$

The melting point of the precipitate was found to be 193-5°C., subl. above 110°C. (lit. m.p. of aniline hydrochloride is 198°C.)

It was established that distillation of the product obtained by the above described procedure, whether done at ordinary pressure (all distilling apparatus being previously filled with carbon dioxide in order to minimize the contact of the vapours with oxygen), by vacuum distillation, or with various types of columns, resulted in extremely small yields of the final product.

Experiment 6.c.

The solubility of 0.15 g. glycine (2 millimoles) and 0.5 ml. tri-n-butylamine (0.389 g., 2.1 millimoles) in 10 ml. of the following solvents was tested: chloroform, carbon tetrachloride, dimethylformamide, pyridine, toluene, cyclohexane, dioxane, tetrahydrofuran, isopropyl ether, dimethyl glycol ether, cellosolve acetate, acetone, ethanol, and glycerol. With the exception of glycerol, no dissolving was observed even after two drops of water were added to each test-tube. Glycerol dissolved glycine, but tri-n-butylamine formed a separate layer.

All possible combinations of two 5-ml. aliquots of the above solvents (excepting ethanol and glycerol), 66 in all, were prepared and tested. No dissolving of glycine and tri-n-butylamine was observed.

Experiment 6.d.

Application of the Quaternary Ammonium Salt of Glycine:

At first, benzyltrimethyl ammonium hydroxide had to be prepared from its chloride by the action of silver oxide. To the solution of 34.0 g. (0.2 mole) silver nitrate in 100 ml. distilled water, 11.2 g. (0.2 mole) of potassium hydroxide in 50 ml. distilled water was added slowly with vigorous stirring. The precipitated silver oxide was isolated by filtration and thoroughly washed with distilled water. The moist silver

oxide was then suspended in 61.5 g. of the 60 per cent aqueous solution of benzyltrimethyl ammonium chloride (Commercial Solvents Corporation, New York), i.e. with 37.0 g. (0.2 mole) of this compound. The dark green-black colour of the silver oxide gave way to a light chocolate brown colour of impure silver chloride. After standing for two hours in a stoppered flask, the silver chloride was separated by a rapid filtration and washed with small volumes of water.

In the clear, viscous solution of benzyltrimethyl ammonium hydroxide in water, glycine (15.0 g., 0.2 mole) was dissolved with ease. Removal of water from the resultant aqueous solution of benzyltrimethylammonium glycinate proved to be a difficult task, due to the highly hygroscopic character of this salt. We were reluctant to heat this solution because of the well-known thermal instability of quaternary ammonium salts. Prolonged passage of a stream of dry air through the solution under vacuum of the water aspirator (about 15 mm. vacuum) failed to reduce appreciably its volume. Increasing the vacuum by using an efficient mechanical vacuum pump (about 1 mm. vacuum) with repeated additions and evaporations of small volumes of chloroform dried over calcium hydride, we obtained a small volume of a highly viscous oil. It was placed in a highly evacuated desiccator over calcium hydride. The final form of the material was a waxy solid in which a small amount of crystalline material was suspended. A small sample of this material was triturated with dry

chloroform. The waxy solid dissolved freely; the crystalline solid remained undissolved. Since the crystals dissolved in water, it was assumed that they are particles of unreacted glycine. Another sample of the waxy solid was tested for its properties. It was found to be extremely hygroscopic (a small piece of it left in air soon turned into a liquid droplet), freely soluble in water, anhydrous ethanol, anhydrous chloroform, and dimethyl formamide, fairly soluble in anhydrous tetrahydrofuran, and insoluble in anhydrous toluene, diethyl ether, and carbon tetrachloride.

The remaining crude product (38 g.) was purified by the above described procedure (suspended in chloroform, filtered and the crystalline solid which weighed 4.5 g. was discarded after being washed several times with dry chloroform). The combined volume of the filtrate and chloroform washings containing the purified product (33.5 g.) was adjusted to 70 ml. by further addition of chloroform. A 5-ml. portion of this solution (containing 2.39 g., i.e. 10.6 millimoles of benzyltrimethylammonium glycinate) was added to the chloroformic solution of the mixed anhydride previously prepared from 2.1 g. (10 millimoles) phthaloylglycine, 2.4 ml. (1.9 g., 10 millimoles) tri-n-butylamine, and 1.4 g. (10 millimoles) isobutyl chloroforminate in 10 ml. chloroform by the procedure described above (Exp. 6.a.). Instantaneous evolution of carbon dioxide occurred, which diminished after a while but continued on shaking of the solution for about

one-half hour. The solution was allowed to stand at room temperature overnight, washed with dilute hydrochloric acid several times, then with water, and heated carefully in order to remove excess chloroform. The yellow oily residue was triturated with a small volume of water. A solid slowly separated. It was isolated by filtration and dried. Weight: 1.91 g., which represents 7.30 millimoles of phthaloylglycylglycine (73.0 per cent yield); m.p. 228°C. (lit. 229-231°C.) (50).

A C Y L - S I L I C O N C O M P O U N D S

I n t r o d u c t i o n

Silicon compounds are known in many cases to be considerably more stable thermally than their carbon analogues. Consequently, the relative instability of the carbonic-carboxylic anhydrides, the detrimental effect of which was discussed in the previous chapter (cf. page 155), led us to consider the potentialities of mixed silicic-carboxylic anhydrides. A survey of the literature revealed a few recent reports on the preparation of acetoxysilanes either from anhydrous sodium acetate and chlorosilanes, like silicon tetrachloride (123, 127), ethyltrichlorosilane (126), or trimethylchlorosilane (123), or from acetic acid and silicon disulphide (124, 125). An isolated report of the ability of these anhydrides (sometimes called "esters") to acylate amines has also appeared (125), although no percentage yields were included.

D i s c u s s i o n o f R e s u l t s

As sample amidation reactions designed to test the feasibility of this method, the formation of phenaceto-p-toluidide and of hippuroanilide were chosen. Preliminary experiments showed that in both cases amidation occurred when silicon tetrachloride was used, while application of trimethylchloro-

silane resulted only in the recovery of the phenacetic or hippuric acid, respectively.

The overall scheme of this new method can be outlined in the following way:

$$4\ RCOOH \xrightarrow{+\ 4\ NHR'_3} 4\ RCOOH.NR'_3 \xrightarrow[-\ 4\ HCl.NR'_3]{+\ SiCl_4}$$

$$\longrightarrow (RCOO)_4Si \xrightarrow[-\ Si(OH)_4]{+\ 4\ NH_2R''} 4\ RCONHR''$$

For example, when phenaceto-p-toluidide is to be prepared, R in the above scheme represents the benzyl group, while R" is the p-toluyl group. The first two steps, formation of triethylammonium phenylacetate and its reaction with silicon tetrachloride in anhydrous benzene, were found to be exothermic reactions which resulted in formation of a heavy precipitate. (It is advisable to add silicon tetrachloride to the carboxylic salt, and not vice versa, because of the possibility of the silicic-carboxylic anhydride being converted to acyl chlorides plus silicic acid in the presence of excess of silicon tetrachloride: $(RCOO)_4Si + SiCl_4 \longrightarrow 4\ RCOCl + H_4SiO_4$ (cf. 306).) On the other hand, when p-toluidine was added to the reaction mixture, no visible changes were apparent. Therefore at this stage the reaction mixture was left shaking on the vibrator for a very long time (e.g. twelve hours) in order to allow the reaction

to proceed as far as possible. Similar observations were made when hippuric acid and aniline were subjected to this procedure. However, while the isolation of the precipitate by filtration in the former case was a relatively easy task, the filtration of the reaction mixture in the second case was rather time-consuming, because of the gelatinous character of the precipitate. Recrystallizations of these precipitates from ethyl alcohol yielded phenaceto-p-toluidide, or hippuroanilide, respectively.

Since the yields were below 50 per cent (cf. Exp'ts 7.a.l. and 7.b.) some doubt existed as to the correct interpretation of the course of the acylation; i.e whether disproportionation had perhaps occurred which would result in the symmetric carboxylic anhydride becoming the acylating agent. In such case, the yield of the amidation reaction could never be above the 50 per cent value of the theoretical yield. Of course, another hindrance might be that, as the number of chlorine atoms in the silane molecule decreases, the tendency of the chlorine atoms to be replaced by the acyloxy group decreases also. Indeed, as was noted above, trimethylchlorosilane did not seem to react in the manner observed with silicon tetrachloride.

A series of experiments was started in order to determine the optimal procedure for isolating the desired product from the reaction mixture (Exp'ts 7.a.l.). Surprisingly, the best yield was obtained by shaking the reaction mixture with

an aqueous solution of sodium hydroxide. Apparently the removal of the silicic acid by dissolving it in alkaline medium resulted in less reduction of yield than when it was removed by filtration. Application of this procedure to the larger amounts of reactants resulted in a 57.5 per cent yield of phenaceto-p-toluidide.

Thus, the feasibility of the new route of amidation, schematically described in the equation on page 174 is proved. A new method has been developed, which has the advantage of having a thermally stable intermediate.

Experimental Procedures

Experiments 7.a.

Preparation of p-Phenacetotoluidide

1)

Phenacetic acid (13.6 g., 100 millimoles) and 14.0 ml. of triethylamine (11.9 g., 100 millimoles) were dissolved in 25 ml. dry benzene. While the reactants dissolved, evolution of heat was observed; therefore the mixture was cooled until no further heat evolution took place. The solution was then cooled on ice and 4.18 g. (24.7 millimoles) of silicon tetrachloride dissolved in 5 ml. of dry benzene was slowly added under constant stirring. Because of the great sensitivity of silicon tetrachloride to moisture, the container was stoppered and both the stirrer and the stem

of the separatory funnel (which contained the benzene solution of silicon tetrachloride) were snugly fitted in the cork stopper. No glass joints were used because they could be sealed by the effect of silicon derivatives. After all the silicon tetrachloride was added to the reaction mixture, a further 10 ml. of dry benzene was added (because the abundant precipitate solidified the reaction mixture) and the contents were shaken on the vibrator for three hours. At the end of this period, a fine, white precipitate settled to the bottom, while the supernatant liquid had a canary-yellow colour. A solution of 10.72 g. (0.10 mole) p-toluidine in 15 ml. dry benzene was added and the mixture was shaken for another twelve hours on the vibrator. The reaction mixture became honey-coloured, homogeneous, viscous liquid. The suction filtration separated the finely divided (almost colloidal) precipitate, which turned out to be a yellowish powder weighing 25.7 g. (after being dried in the dessicator). It did not have any definite melting point (softening started at 30°C.). Evaporation of the filtrate to approximately 5 ml. yielded 1.70 g. (7.55 millimoles of phenaceto-p-toluidide) of white precipitate, m.p. 130-1°C. (lit. 130-2°C.(297), 135-6°C.(298)).

The first crop was divided into five 5-g. parts. Each part was subjected to different treatment in order to determine the best procedure for recovering the product:

a) A 5-g. aliquot was suspended in 40 ml. benzene, the suspension boiled for one minute, and filtered while hot. The undissolved part weighed 2.52 g. A melting point determination showed that this material softened above 100°C., remained unchanged to about 200°C., when further softening was observed; at 235°C. resolidification occurred (discolouration at 230°C.), and no further change was observed up to 270°C. The filtrate was concentrated to approximately 5 ml. It yielded 0.84 g. of white microcrystalline precipitate, which softened at 70°C. and melted at 77-82°C. Washing this precipitate with normal sodium hydroxide yielded 0.66 g. (2.93 millimoles of phenacetop-toluidide), m.p. 130-131.5°C.

b) Another aliquot was suspended in 40 ml. ethyl alcohol and further treated in the manner described above for the first aliquot. The undissolved part weighed 0.57 g., softened at 120-140°C., discolourized above 200°C. and did not change further up to 270°C. The filtrate (after being evaporated to approximately 5 ml.) yielded 1.26 g. of white precipitate, which softened at 75°C., and melted at 79-86°C. Washing this precipitate with normal sodium hydroxide yielded 0.84 g. (3.63 millimoles), m.p. 131-2°C.

c) An aliquot was suspended in 200 ml. hot water and further treated in the manner described above for the first aliquot. The undissolved part weighed 1.38 g., m.p. 125°C. (approximately; softened at 90°C.). Recrystallization from ethanol yielded 0.54 g. (2.40 millimoles), m.p. 130-1°C. The part which did not dissolve in ethanol weighed 0.30 g., no m.p. up to 270°C.

Evaporation of the filtrate yielded a brownish-grey sediment, m.p. about 40°C.

d) An aliquot was suspended in 40 ml. benzene, the suspension was boiled for one minute, 100 ml. of 5 per cent aqueous solution of sodium hydroxide was added, and the mixture was stirred vigorously for one hour. The benzene layer was then separated, reduced to about 5 ml., and allowed to cool. The crystals weighed 1.02 g. (4.53 millimoles), m.p. 131°C.

Multiplying the amounts of the isolated phenaceto-p-toluidide by five and adding the amount obtained by evaporation of the first filtrate (1.70 g., 7.55 millimoles), the following yields were calculated for the four procedures above:

a) 22.25%; b) 26.25%; c) 19.55%; d) 30.25%.

2)

Half amounts of the reactants used in Exp. 7.a.1. were treated in the same manner up to the point where the prolonged shaking of the reaction mixture on the vibrator (for twelve hours) was completed. Instead of isolating the precipitate as was done previously, 100 ml. of normal sodium hydroxide solution was added and the mixture shaken vigorously for one hour. The benzene layer was then separated, heated,and filtered while hot in order to free it from a few semi-solid particles. The filtrate was concentrated to approximately 20 ml. volume. Crystallization upon cooling produced 5.53 g. of white leaflets, m.p. 133-133.5°C. Further evaporation yielded 0.94 g. of the second crop, m.p. 130-3°C. (softening at 124°C.). The total yield, 6.47 g., represents 28.8 millimoles of p-phenacetotoluidide (57.5 per cent yield).

Experiment 7.b.

Preparation of Hippuroanilide:

Hippuric acid (3.58 g., 20 millimoles) and 2.8 ml. of triethylamine (2.02 g., 20 millimoles) were dissolved in 30 ml. toluene with cooling (prolonged shaking was required to bring these reactants into solution). A solution of 0.84 g. (4.97 millimoles) silicon tetrachloride in 5 ml. toluene was added with constant cooling and vigorous stirring. The reaction mixture was shaken on the vibrator for

three hours. Then 1.82 ml. of aniline (1.86 g., 20 millimoles) was added and the mixture was shaken for another twelve hours on the vibrator. The reaction mixture was yellowish and contained a large amount of brownish-yellow precipitate, which was separated by suction filtration. It consisted partly of a microcrystalline yellow matter and partly of a brownish gelatinous jelly, which made filtration very difficult. The filtrate was then evaporated to one-third of its original volume. A small amount of white precipitate separated, m.p. 213-4°C. The original precipitate was dissolved in hot ethyl alcohol. The undissolved colloidal particles were isolated by filtration through a heated funnel. Weight: 0.10 g., no m.p. up to 280°C. (browning observed above 240°C.). The filtrate gave, upon cooling, a yellow precipitate, which had a very wide m.p. (from 170°C. up). Therefore it was recrystallized from 20 per cent ethanol. The product had a brownish tinge; weight 1.57 g., m.p. 206-9°C. (6.19 millimoles of hippuroanilide; 31.0 per cent yield). Second recrystallization of a part of the product from 20 per cent ethanol gave white crystals of m.p. 212-4°C. (lit 213-5°C.) (307).

S U M M A R Y

As was explained in the General Introduction (cf. Page 1), we have concentrated our attention on two aspects of the general problem of peptide synthesis. Therefore, this Summary consists of two groups of statements.*

Results of the study of methods by which amino acids can be rendered monofunctional may be summarized as follows:

A. Means for the protection of the carboxyl group:

a) The technique of azeotropic distillation has been applied with success to the direct esterification of amino acids with benzyl alcohol. For this purpose the amino acid was temporarily converted to an arylsulphonate. Benzenesulphonic acid may be used, but p-laurylbenzenesulphonic acid appears to be preferable, since its amino acid salts are soluble in anhydrous organic solvents.

(cf. Pages 83-85 and Experiments 1.b.)

b) During the course of this work, p-toluenesulphonic acid was found to be satisfactory as a variant for the benzenesulphonic acid in the Miller-Waelsch Method for the

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* In this Summary, each statement is followed by a reference to the relevant pages of discussion and the number of experiments. For the convenience of the reader, the numbers and locations of each experiment (or group of experiments) are listed on Page 192.

synthesis of benzyl esters of amino acids and a compound previously unreported, viz. benzyl ester of DL-leucine hydrochloride, has been prepared.

(cf. Pages 79-81 and Experiments 1.a.)

c) The possibility of preparing crystalline (and stable) esters of amino acids containing substituted benzyl groups has been examined. 4-Chloro- and 2,4-dichlorobenzyl esters of an NH_2-protected amino acid (benzoylglycine) have been prepared from the corresponding benzyl chlorides and shown to have significantly higher melting points than the corresponding simple benzyl esters. However, benzyl chlorides were found to be unsuitable reagents for preparing benzyl esters of unprotected amino acids.

(cf. Pages 81-83 and Experiments 1.d.)

B. Means for the protection of the amino group:

d) Phthaloylamino acids have been prepared from amino acids and phthalic anhydride by an improved method involving glacial acetic acid as a reaction medium. This technique results in easier separation of crystalline products of high purity.

(cf. Pages 98-99 and Experiments 2.a.)

e) 3-Nitro- and tetrachlorophthalic anhydrides have been shown to be more satisfactory than phthalic anhydride for the phthaloylation of several amino acids. The products tend to have higher melting points and to crystallize

with greater facility. In the course of this work the following new compounds have been prepared and characterized: 3-nitrophthaloylglycine, 3-nitrophthaloyl-DL-isoleucine, 3-nitrophthaloyl-DL-norleucine, tetrachlorophthaloylglycine, and tetrachlorophthaloyl-DL-norleucine.

(cf. Pages 99-101 and Experiments 2.b. and c.)

f) Sulphonation, which has recently become a favoured method for the NH_2-protection of amino acids and which commonly involves a solution of the p-toluenesulphonyl chloride in an organic solvent and potassium (or sodium) hydroxide as a base, has been modified to give improved yields. Important factors appear to be the choice of the organic solvent and the mode of additions of sulphonyl chloride and of alkali to the amino acid. In the course of this work, a compound previously mentioned (as an intermediate) but not described in the literature, viz. p-toluenesulphonyl-DL-leucine, has been characterized.

(cf. Pages 111-113 and Experiments 3.a.1. and 3.b.)

g) A "rapid method" for the synthesis of sulphonated amino acids in which potassium hydroxide is replaced by barium hydroxide was developed. A water-insoluble barium salt of the sulphonated amino acid is formed as an intermediate. Compounds of this type have not been previously described.

(cf. Pages 113-116 and Experiments 3.a.2.)

Results of the study of amidation reactions from the standpoint of controlled peptide synthesis may be summarized as follows:

a) p-Toluenesulphonylamino acids have been found to be suitable as starting materials for the enzymatic synthesis of amides and peptides, while phthaloylamino acids have been found to be not susceptible to the effect of papain. (cf. Pages 121-131 and Experiments 4.)

b) A successful attempt has been made to apply the technique of azeotropic distillation to the direct condensation of two compounds, one with a free carboxyl, the other with a free amino group. Several amidations were performed by this technique, which offers promise as a new method for the synthesis of peptides. During the course of this investigation, benzoylglycine p-toluidide was characterized. (cf. Pages 140-145 and Experiments 5.)

c) Applicability of the benzyl esters of amino acids to the Boissonnas-Vaughan-Wieland method of peptide synthesis was proved. In these experiments carbonic-carboxylic mixed anhydrides of phthaloylamino acids were the acylating agents. Benzyl esters of amino acid hydrochlorides have been found to be superior to the benzyl esters of the amino acid benzenesulphonates. Two new compounds were prepared and characterized: benzyl phthaloylglycylglycinate, and benzyl phthaloylglycyl-DL-leucinate.

(cf. Pages 154-155 and 159 and Experiments 6.a.)

d) The problem of using carboxylic salts without simultaneously adding a reactive solvent (e.g. water), which competes with the amino group for the reaction with the mixed anhydride, was solved by employing the quaternary ammonium hydroxide as a base. The resultant salt was found to be fairly soluble in a series of inert organic solvents. (cf. Pages 156-159 and Experiments 6.c. and d.)

e) With a view to overcoming the instability of mixed carbonic-carboxylic anhydrides, thioanalogues of the chloroformates were prepared and evaluated from the standpoint of their applicability to peptide synthesis. Thiochloroformates of the type ClCSOR are unsuitable, while ClCOSR type of thiochloroformates, although applicable does not offer any marked advantages. A new compound, viz. n-pentyl chlorocarbothiolate, was prepared.

(cf. Pages 155-156 and Experiments 6.b.)

f) A new path to the formation of peptide bonds has been explored by studying the reactions of silicon tetrachloride and of trimethylchlorosilane. In both cases attempts were made to prepare mixed silicic-carboxylic type anhydrides and to apply them as intermediates for acylating amino compounds. While trimethylchlorosilane did not seem to give the desired reactions, the application of silicon tetrachloride resulted in development of a new method of amidation. (cf. Pages 173-176 and Experiments 7.)

g) A systematic classification has been compiled of methods reported for the controlled synthesis of an amido-linkage, on the basis of which suggestions have been offered for fruitful lines of investigation. It is hoped that this classification will be of service for further work in the project of which this work is the initiating part. (cf. Pages 12-59 and Table I.)

CLAIMS TO ORIGINAL RESEARCH

1. The technique of azeotropic distillation has been successfully applied for the first time to the esterification of amino acids.

2. The problem of dissolving amino acids, which have their amino group protected in the form of sulphonates, in inert solvents has been solved by employing benzenesulphonic acid having a long aliphatic side chain.

3. During the course of investigating the possibility of preparing crystalline benzyl esters of amino acids, monochloro- and dichlorobenzyl esters of a substituted amino acid have been prepared for the first time and characterized.

4. An improved method for the preparation of phthaloylamino acids has been developed which is marked by the facility with which pure products can be isolated in crystalline form.

5. Substituted phthaloyl groups, 3-nitro- and tetrachloro-, have been shown to offer advantages for the NH_2-protection of amino acids. Such amino-acid derivatives, prepared for the first time, are marked by higher melting points and more facile crystallization than the unsubstituted phthaloylamino acids which have been used heretofore.

6. A variation of an existing method and a new method have been developed for the synthesis of sulphonated amino acids. The former posses the advantage of leading to higher yields, while the latter provides a more rapid synthesis of the products.

7. Sulphonated amino acids have been employed for the first time in enzymatically-catalyzed amidation reactions.

8. Direct condensation of the carboxyl with an amino group by the technique of azeotropic distillation with the resultant formation of the amido bond has been accomplished for the first time.

9. Benzyl esters of the amino acids have been found to be suitable for amido-bond synthesis by the mixed carbonic-carboxylic anhydride method.

10. Use of the salt-type of protection for carboxyl groups in inert solvents has been made possible for the first time by employing a quaternary ammonium hydroxide as the base. Up until now, water has been the only possible, and not very suitable, solvent.

11. The applicability of mixed thiocarbonic-carboxylic anhydrides as intermediates in peptide synthesis has been investigated for the first time.

12. A new method of amidation has been developed, based on the mixed orthosilicic-carboxylic anhydrides.

13. Systematic classification of amidation methods and of protecting groups applicable for peptide synthesis has been arranged for the first time.

14. The following compounds, previously unreported, or mentioned without conclusive data, have been prepared and characterized: benzyl DL-leucinate hydrochloride (p.86), 4-chlorobenzyl benzoylglycinate (p.95), 2,4-dichlorobenzyl benzoylglycinate (p.96), 3-nitrophthaloylglycine (p.105), 3-nitrophthaloyl-DL-isoleucine (p. 106), 3-nitrophthaloyl-DL-norleucine (p. 107), tetrachlorophthaloylglycine (p. 108), tetrachlorophthaloyl-DL-norleucine (p. 109), p-toluenesulphonyl-DL-leucine (p. 126), benzoylglycine p-toluidide (p. 148), benzyl phthaloylglycylglycinate (p. 163), benzyl phthaloylglycyl-L-leucinate (p. 163), n-pentyl chlorocarbothiolate (p. 166), benzyltrimethylammonium glycinate (p. 169).

15. No previous reports were found on the following compounds which figured as intermediates in the above-described work: p-toluenesulphonic acid salts of amino acids (p. 87), glycine p-laurylbenzenesulphonate (p. 91), barium salts of glycine and of p-toluenesulphonylglycine (p. 122-126 and 114), mixed anhydride from phthaloylglycine and

n-pentyl chlorocarbothiolate (p. 156), benzyltrimethylammonium phthaloylglycylglycinate (p. 171), mixed anhydrides of the orthosilicic acid with phenacetic acid (p. 176 and 180) and of the orthosilicic acid with benzoylglycine (p. 180).

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L I S T O F T A B L E S

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