THE SYNTHESIS OF $\gamma$-ALANYL PEPTIDES

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**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>2</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>19</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>30</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>32</td>
</tr>
</tbody>
</table>

**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE I</td>
<td>6</td>
</tr>
<tr>
<td>TABLE II</td>
<td>12</td>
</tr>
<tr>
<td>TABLE III</td>
<td>16a</td>
</tr>
</tbody>
</table>
THE SYNTHESIS OF $\beta$-ALANYL PEPTIDES

INTRODUCTION

Since the discovery of pantothenic acid and coenzyme A, much work has been done on their chemistry and physiology. However, many gaps remain in our knowledge of the chemotherapy of this vitamin, as well as the behavior of the various intermediates in the biosynthesis of the coenzyme, and of analogs of these intermediates. This work was undertaken with the dual purpose in mind (a) to prepare $\beta$-alanine derivatives of biological activity (either inhibitory or stimulatory) and (b) to give some idea of the biological action of these compounds.

Although the several peptides prepared herein were without biological activity, some interesting observations have been made concerning the synthesis of peptides and the utilization of carbodiimides in peptide formation. An unexpected reaction encountered in the use of dialkyl phosphites has been investigated which offers promise of many applications.
RESULTS AND DISCUSSION

Of the many methods available for amide or peptide formation, the majority of these are a form of carboxyl-activated type such as the acid halides, symmetrical and unsymmetrical anhydrides, esters, isourea esters, and vinyl ether esters (from alkoxy-acetylene (3)). One of the few practical methods for amine activation (14) is that of Anderson (1), (2) in which the amine or amino acid ester is reacted with either tetraethyl pyrophosphite or diethyl chlorophosphite to give the desired diethyl phosphite amide. Following Anderson's general procedure both reagents were used to form the phosphite amide of bis-2-aminoethanethiol and S-benzyl-2-aminoethanethiol. It was observed that the disulfide was quickly reduced to the mercaptan, while the S-benzyl compound remained intact at the sulfur linkage. In order to avoid possible complex thiol-ester formation from the disulfide, subsequent efforts were directed toward the S-benzyl-2-aminoethanethiol. Two advantages of the latter compound in place of the disulfide are the reduced possibility of obtaining mixed products such as the mixed disulfides of pantethine and
2-amino-ethanethiol, and the comparative ease of isolation and separation of S-benzyl pantetheine from the reactants in the case of pantothenic acid.

In spite of the apparent formation of the desired phosphite amide, as estimated by the amount of the hydrochloride salt of triethyl amine formed, an insignificant amount or none of the expected product was obtained.

There have been earlier reports to the effect that glutamic acid, \( \beta \) -alanine, and peptides of these had a pronounced growth promoting properties in some microbiological systems used for studying the requirements of pantothenic acid in some microorganisms. Recent work with transpeptidation by Janssen (11), Fruton (7), and Hanes (8) led to the question of the possible functioning of glutamyl-\( \beta \)-alanine and similar compounds in the biosynthesis of coenzyme A. If \( \gamma \) -glutamyl-\( \beta \)-alanine or its amide were to transfer \( \beta \) -alanine to pantoic acid or to a precursor of pantoic acid, then one would expect improved response in a system that required an external source of pantothenic acid. However, the peptides tested were inactive, either as growth promoters or as inhibitors.

For the synthesis of the \( \alpha \) -peptides of glutamic acid and of glutamine the method used by du Vigneaud (26) was found to be quite satisfactory, since the tosylglutamic
acid that results from incomplete reaction is easily removed with water. \( \alpha \)-glutamyl- and glutaminyl-\&alpha;alanine were easily prepared and isolated in good yields; the tripeptide \( \alpha \)-glutamyl-\&beta;alanyl-cysteine prepared in this manner was isolated as its mercury mercaptide in lower yields due to the greater difficulty of purifying the intermediates.

The method of Rudinger (19) in which tosyl-pyroglutamic acid was used to form \( \gamma \)-glutamyl peptides was found to be satisfactory only when the benzyl ester of \( \beta \)-alanine was used but failed to yield workable material when the dipeptide ester was used.

Sheehan (22) reported that good yields of peptides resulted when suitably protected amino acids were reacted in the presence of dicyclohexyl carbodiimide in anhydrous or aqueous solution. Although the carbodiimide reagent has proved to be satisfactory for the synthesis of peptides of \( \alpha \)-amino acids, it became necessary to develop a method to determine when a particular carboxylic acid would form the desired stable ester of the isourea I. It appears that this ester is necessary in order to have peptide bonds formed by an attack of the amine, giving II and the symmetrical dicyclohexylurea as the other product. An attack by another molecule of the acid would result in the formation of the anhydride
III. If the isourea ester is not stable, several possibilities remain for the course of the reaction. First, in the presence of water, a simple hydrolysis to give the urea and the original acid would readily occur. A second possibility is that of internal reaction with another functional group such as a hydroxyl, to give a lactone V. In the event that the intramolecular distances are such that internal reaction is not possible, polymerization VI would then occur.

It is well known (12) that many of the isourea esters of this type are subject to rearrangement to their corresponding isomeric N-acyl ureas IV. If the rearrangement does not occur or does not compete to any great extent, then the stable isourea ester may undergo attack by acids to give an anhydride, by amines to give amides or by amino acid esters to give peptides.

The method used to study the stability of the isourea ester was to react the particular carboxylic acid derivative in question with the dicyclohexyl carbodiimide in anhydrous dimethyl formamide or in anhydrous dioxane. The carbodiimide was usually present in five to ten percent excess. As expected, when glutamic, malic, and acetic acids were used, an immediate precipitate of the N,N'-dicyclohexylurea was observed. Solutions of carbobenzoxy-S-benzyl cysteine, carbobenzoxy-S-alanine,
TABLE I
REATIONS OF CARBODIIMIDE

DCC + R-C=O → R-C-O-C=N-C\textsubscript{6}H\textsubscript{11} → HO-C\textsubscript{6}H\textsubscript{11}N-C\textsubscript{6}H\textsubscript{11} → HO-C\textsubscript{6}H\textsubscript{11}N-C\textsubscript{6}H\textsubscript{11}

DCC + R'-NH\textsubscript{2} → R-C-NHR' + (R-C=O)\textsubscript{2}

DCC + H\textsubscript{2}O → DCU + O=O→R-C-(OR-C)xOR-C-OH

DCC + R-OH → R-C-O-C=N-C\textsubscript{6}H\textsubscript{11}

(C\textsubscript{6}H\textsubscript{11}-NH)\textsubscript{2}C=O (DCU)

DICYCLOHEXYL UREA

(C\textsubscript{6}H\textsubscript{11}-N=)\textsubscript{2}C (DCC)

DICYCLOHEXYL CARBODIIMIDE
and carbobenzoxy-S-benzyl-cysteine in dimethyl formamide gave no precipitate of the urea even after standing at room temperature for four weeks. The precipitation of the urea did not appear until the third day with tosyl-glutamic acid; although it was not isolated the probable product would be N-tosylpyro-glutamic acid (based on the results obtained with the $\alpha$-benzyl ester of tosylglutamic acid).

The unpredictable nature of the carbodiimide on similar structures is borne out in the reaction with tosylisoglutamine and tosylglutamyl-$\alpha$-benzyl ester. The tosyl-isoglutamine was assumed to have formed a stable isourea ester with the carbodiimide which then coupled with benzyl-S-benzyl-$\alpha$-alanyl-cysteine to give the tripeptide derivative in thirty percent yield. On the other hand, the $\alpha$-benzyl ester of tosylglutamic acid immediately cyclized to give the tosylpyroglutamyl benzyl ester in eighty-five percent yield.

When phthaloyl-$\alpha$-alanine was reacted with dicyclohexylcarbodiimide in dimethyl formamide a precipitate was formed; this was removed by filtration, washed with cold dimethylformamide, then with cold chloroform. The precipitate obtained from the chloroform washings by the addition of several volumes of petroleum ether proved to be the anhydride of phthaloyl-$\alpha$-alanine.
This anhydride, obtained in better than fifty percent yield, was reacted with S-benzyl-2-aminoethanethiol to give almost ninety percent yields of phthaloyl-γ-alanyl-2-aminoethanethiol.

Evidence that the solvent participates in the reaction was noted in comparing the reaction of carbobenzoxy-γ-alanine with the carbodiimide in dimethylformamide and in dioxane. When the reaction was carried out in dioxane, about fifty percent of the calculated amount of the urea was produced; this indicated that roughly half of the carbobenzoxy-γ-alanine was converted to the anhydride. The remainder of the reactants were present as the N-acyl urea. However, when the same reaction was carried out in dimethylformamide, the only products obtained were the N-acyl urea (in a sixty percent yield) and unreacted carbodiimide and carbobenzoxy-γ-alanine.

Proof of the existence of the anhydride of carbobenzoxy-γ-alanine in the dioxane solution lies in the ninety percent yield (based on the amount of anhydride presumed to be present as measured by the amount of dicyclohexylurea formed) of bis-carbobenzoxy-γ-alanyl-2-aminoethanethiol from the addition of the disulfide of 2-aminoethanethiol to the clear dioxane solution.

Since the discovery of the chemical nature of panteth(e)ine there have been several different methods
developed for the synthesis of this interesting, potentially useful compound. The method of Wieland (30) is probably the most direct that has been developed. He obtained approximately fifty percent yields of pantethine by means of the mixed anhydride of pantothenic acid and ethyl carbonate reacted with the disulfide of 2-aminoethanethiol. Snell (23) condensed methyl pantothenate with 2-aminoethanethiol to obtain pantethine, but the isolation and purification presented some difficulties. Other methods (13) (4) (23) (28) (29) gave satisfactory yields of easily purified reaction mixtures, but required the preparation of the peptide \( \beta \)-alanyl-2-aminoethanethiol (\( \beta \)-aletheine) or a derivative of it, followed by fusion with pantolactone.

With the introduction of dicyclohexylcarbodiimide as a peptide forming reagent, it was considered feasible to attempt to prepare pantethine by reacting pantothenic acid with 2-aminoethanethiol or its disulfide in a suitable solvent using the carbodiimide as a coupling agent. Sheehan (22) reported that the reagent gave good yields of peptides when the suitably protected amino acids were treated with the reagent in tetrahydrofuran-water mixtures, although better yields were obtained in non-aqueous solvents.

When pantothenic acid was treated with the carbodiimide and the disulfide of 2-aminoethanethiol
in various combinations of solvents (tetrahydrofuran, tetrahydrofuran-water, aqueous dioxane, and a heterogenous mixture of tetrahydrofuran, water, alcohol, and petroleum ether) no pantethine could be isolated. When anhydrous pantothenic acid, prepared by removing the last traces of water by azeotropic distillation with ethyl acetate or chloroform, was reacted with the carbodiimide in either anhydrous dioxane or anhydrous dimethylformamide, heat was evolved and almost quantitative amounts of the symmetrical urea were formed within one hour. Although this indicated the possible presence of the anhydride of pantotenic acid, subsequent addition of the disulfide of 2-aminoethanethiol and of S-benzyl-2-aminoethanethiol failed to yield pantethine or S-benzyl pantetheine. An attempt was made to test for the presence of the anhydride of pantothenic acid by means of the ferric complex with the hydroxamic acid; there was a slow increase in the color formed and this decreased after about forty minutes. However this was hardly proof for the existence of the anhydride since the same positive hydroxamic acid test could be obtained from the solution of carbobenzoxy-β-alanine and the carbodiimide in dimethyl formamide which does not give the anhydride but produces the N-acyl urea predominately and possibly some of the isourea ester. Since the isourea esters have the same fundamental electron
distribution as acid chlorides, anhydrides, or the vinyl-ether esters, it is not surprising that a hydroxamic acid is formed. From the failure to isolate the desired pante-thine by means of the preceding efforts, and from the properties of material obtained, it appears that the principal reaction of the carbodiimide with pantothenic acid is a polymerization to a polyester under these conditions.
TABLE II
STRUCTURE OF REACTIVE INTERMEDIATES IN PEPTIDE SYNTHESIS

\[
\begin{align*}
R-C-Cl & \quad (R-C-)_2O \\
O & \\
R-C-O-P & \\
O-R' & \\
O & \\
R-C-O-C & \\
HN-R' & \\
O & \\
R-C-O-C-OEt & \\
O & \\
R-C-O-C=CH_2 & \\
O-CH_3 & \\
(RO-)_2P-N-R & \\
(HO-)_2P-N-R & 
\end{align*}
\]
The Behavior of Dialkyl Phosphites toward Alkyl Disulfides

Coincident with an unsuccessful attempt to prepare pantethine by means of the reaction of the bis-(2-aminoethanethiol disulfide) diethylphosphite amide with anhydrous pantothenic acid, it was observed that a large amount of the mercaptan was formed at room temperature. Similar results were obtained when tetraethyl pyrophosphite was used as the condensing agent. That the actual reducing agent was diethylphosphite (generated by hydrolysis of the phosphite amide, tetraethyl pyrophosphite, or diethyl-chlorophosphite) was proved by reacting pure diethyl phosphite with an aqueous solution of the disulfide of 2-aminoethanethiol to obtain a significant amount of the mercaptan quickly at room temperature.

Qualitative tests were run on the available alkyl disulfides (cystine, its dibenzyl ester, glutathione, and ethyl disulfide) and on the S-benzyl derivatives of 2-aminoethanethiol and cysteine. As expected, the S-benzyl compounds were not affected by the reagent, while the disulfides were reduced to a considerable extent. The effect of dibenzyl phosphite and of phosphorous acid was then investigated on these same compounds; preliminary experiments demonstrated that water was essential to the reaction and that the mixture must be alkaline. Phosphorous
acid did not reduce the disulfides at any pH value. Dibenzyl phosphite was found to act more slowly than diethyl phosphite, but had the advantage that both it and its oxidation product, dibenzylphosphoric acid, were easily extracted with ether.

It was desirable to test the quantitative aspects of this reaction in an unambiguous manner. Because the phosphites are reducing agents that will react with the usual oxidants for determining sulfhydryl groups, it was necessary to have some system that afforded an easy separation of the mercaptan from the other materials present. Because of the low boiling point of ethyl mercaptan, the reaction was tested by treating a weighed amount of ethyl disulfide dissolved in aqueous alcohol with dilute sodium hydroxide and the theoretical quantity of diethyl phosphite. The reaction was carried out in an ice bath to insure retention of the ethyl mercaptan formed. The pH was maintained above 8 by the dropwise addition of dilute sodium hydroxide. The reaction mixture was transferred to a three-necked flask fitted with a dropping funnel, an inlet for nitrogen, and an outlet for the gases produced in the reaction. The latter were fed into a cold alkaline solution and swept with nitrogen for one hour. The alkaline reaction mixture was acidified with excess dilute sulfuric acid and the mercaptan formed swept into
excess standard iodine in sodium bicarbonate. The excess iodine was then back titrated with standard thiosulfate. The yield of ethyl mercaptan from this procedure varied from sixty percent to one hundred percent after one hour or longer. Dibenzyl phosphite was used in similar experiments, but the dibenzyl phosphite and phosphate were extracted with ether from an acidic solution of cysteine and cystine. The reaction appeared to be somewhat slower but gave slightly over ninety percent yields within one hour.

Of the published material on alkyl phosphites, the few papers in the literature (17) are concerned only with the kinetics of reaction with halogens. Only recently have investigations (18) been reported on the reducing ability of phosphites with regard to organic sulfur compounds. Morrison (15, p.181) studied the reaction of trialkyl phosphites on sulfenyl chlorides. Excellent yields of thiophosphonic esters resulted from the addition of the two reagents, followed by elimination of alkyl chloride. The reaction of triethyl phosphite with diethyl sulfide (10, p.6064) was reported to give equimolar amounts of ethyl mercaptan and ethyldisulfide.

The probable mechanism of mercaptan formation from disulfides and dialkyl phosphites appears to be as follows. The postulated addition complex "A" eliminates
a proton to give the mercaptan and a dialkyl thio-
phosphonate ester which is quickly hydrolyzed in the
presence of a base. It was established that the reaction
involving the elimination of the proton from the complex
"A" did not occur to any measurable extent in acidic or
neutral solution by sweeping the mixture of reactants
with nitrogen at different pH values and collecting the
effluent vapors in a standard iodine solution; no mer-
captan was formed, as determined by titration of the
iodine with thiosulfate.

Since this reaction occurs readily at moderate
temperatures and at an apparently reasonable rate, its
kinetics might easily be studied. The reaction itself
could be useful for reduction of disulfides in biological
systems wherever other methods such as a sodium-liquid
ammonia reduction, hydrogen sulfide, thioglycolic acid,
or cysteine need to be avoided. Another potential appli-
cation would be as an anti-oxidant in systems requiring
the continued presence of sulfhydryl groups. Although
many of the halo-alkyl phosphites and phosphates are
toxic, the dialkyl phosphites are relatively innocuous.
**TABLE III**

**REACTIONS OF ALKYL PHOSPHITES**

\[
\begin{align*}
R'\text{S-Cl} & \; \xrightarrow{\delta^+ \; \delta^-} \; :P(-OR)_3 \\
\text{(EtO-)}_3P & \; \xrightarrow{\delta^-} \; S-R \\
(RO-)_2P-OH & \; \xrightarrow{\delta^-} \; S-R'
\end{align*}
\]

\[
\begin{align*}
R'S-P(-OR)_3 & \xrightarrow{\text{Cl}^-} R'S-P(-OR)_2 + RCl \\
RS-P(-OEt)_3 & \xrightarrow{\text{EtSR}} RS-P(-OEt)_2 + \text{EtSR} \\
R'S-P(-OR)_2 & \xrightarrow{\text{H}^+} R'S-P(-OR)_2 + R'S^- \\
\text{HO-P(-OR)}_2 & \xrightarrow{\text{OH}^-} R'S^- + \text{R'S-}
\end{align*}
\]
Preparation of Peptides from the Various Intermediates.

In order to test the effect of the various peptides obtainable from the intermediates, the slightly purified reaction mixtures were prepared from reductions with sodium in liquid ammonia. The method of du Vigneaud (26) was followed. This affords the selective removal of benzyl and tosyl groups with little or no racemization and practically no cleavage of the peptides or amides. The reduction is essentially a quantitative one provided that a slight excess of sodium is used. The principal products formed, other than the peptides, are toluene, sodium sulfite and sodium chloride or sulfate. Separation of peptides from salt solutions is often a long and arduous task. Since these experiments were intended for an initial survey of the possible function of the several L-alanyl peptides as well as for an evaluation of peptide condensing methods, no elaborate attempts were made to obtain the analytically pure peptides.

Small amounts of the required intermediates were reduced, treated with weak anion and cation exchange resins, and evaporated in vacuo. Generally the dried, salt-containing peptide preparations were chromatographically pure, and these were used in microbiological tests.
The microbiological tests using two yeasts were conducted as described by Sarett and Cheldelin (20). However, none of the peptide preparations contained any biologically active principles, either for growth or inhibition. The preparations were tested at various concentrations ranging from 0.1 μg. to 900 μg., either alone or in the presence of suboptimal quantities of β-alanine.
In the following syntheses and studies, several known compounds had to be prepared for use as intermediates or reagents. This was done, as follows:

1. carbobenzoxy chloride prepared according to the directions of Bergmann and Zervas (5)
2. 2-aminoethanethiol as described by Mills and Bogert (14)
3. dicyclohexylcarbodiimide by the method of Schmidt et al. (20)
4. N-tosyl-L-glutamic acid and N-tosyl-L-pyroglutamic acid as prepared by Harington and Moggridge (8)
5. tosylpyroglutamyl chloride by the method of Swan and du Vigneaud (23)
6. 8-benzyl-L-alanyl-cysteine by the method of Baddiley and Mathias (4)
7. phthaloyl-L-alanine by the method of Turner (24)

N-carbobenzoxy-8-alanyl-N,N'-dicyclohexylurea

A solution of carbobenzoxy-8-alanine in dimethylformamide was reacted with 2.1 grams of dicyclohexylcarbodiimide at room temperature for one hour; the reaction was exothermic and a small amount of dicyclohexyl urea precipitated.
To the clear solution was added 1.67 (0.01 mole) grams of 8-benzyl-2-aminoethanethiol. This was heated on the steam bath for one hour and the reaction left at room temperature overnight. The precipitated compound resulting from the addition of several volumes of water was extracted with ethyl acetate. The ethyl acetate solution was washed with dilute acid and with dilute base, and the solvent removed in vacuo. The product was then recrystallized from fifty percent alcohol to give 2.8 grams of the N-acetyl urea (59.9%). Melting point 146-147°C. Analytical data; Calcd. for C₂₂H₃₅N₃O₄: C, 67.3%; H, 8.15%; N, 9.79%. Found, C, 67.5%; H, 8.35%; N, 9.66%.

**N-phthaloyl-8-alanyl anhydride.** Phthaloyl-8-alanine (4.38 grams) (25) in 20 milliliters of dimethylformamide treated with 4.12 grams of dicyclohexylcarbodiimide in thirty milliliters of dimethylformamide was allowed to react for thirty minutes. During this time moderate heat was evolved and a precipitate formed quickly. The precipitate was removed, washed with three ten-milliliter portions of dimethylformamide, and then extracted with three portions of cold chloroform. The anhydride was precipitated from the chloroform by the addition of several volumes of petroleum ether; recrystallization was accomplished from five parts petroleum ether—one part chloroform. To a chloroform solution of 0.235 grams
of the anhydride was added a four-fold excess (0.18 grams) of 8-benzyl-2-aminoethanethiol. After refluxing for one hour on a steam bath, the reaction was cooled, diluted with three volumes of ether and four volumes of water. The ether layer was evaporated and the solid remaining (including the ether insoluble solids) were dissolved in ethyl acetate. The ethylacetate solution was washed with dilute sodium carbonate solution, then dilute hydrochloric acid, and finally with water. Concentration of the ethyl acetate solution to a small volume and cooling gave N(N-phthaloyl-β-alanyl-)β-benzyl-2-aminoethanethiol. Yield 0.135 grams (87.5%). Melting point 117-125°C. Reported (29) melting point 113-123°C. Yield of anhydride, 2.2 grams (52.5%). Melting point 171-172°C. Analytical data: Calcd. for C₃₂H₂₆N₂O₇  C, 62.8%; H, 3.83%. Found; C, 62.3%; H, 4.03%.

**Benzyl-(β-alanine) hydrochloride.** To a suspension of 8.9 grams of β-alanine in 100 ml. of benzyl alcohol, a rapid stream of dry hydrogen chloride was introduced with vigorous stirring. When the solution began to cool to room temperature, the flask was placed in a hot water bath, and the water and some of the benzyl chloride was removed in vacuo. This was repeated twice, until the β-alanine was completely dissolved. Two hundred and fifty ml. of petroleum ether added to the
benzyl alcohol solution gave an oil which crystallized upon standing. This was recrystallized from absolute ethanol by dissolving small portions in boiling ethanol and chilling rapidly. Yield, 13.6 grams (86%). Melting point, 66.5-67.5°C. Analytical data: Calcd. for C_{10}H_{14}NO_2Cl O, 55.6%; H, 6.5%. Found: O, 55.5%; H, 6.7%.  

**Benzyl-S-benzyl-S-alanyl cysteine hydrochloride.**

To a suspension of 2.8 grams of S-benzyl-S-alanyl cysteine (5, p.2808) in 50 milliliters of benzyl alcohol was introduced a rapid stream of dry hydrogen chloride. The water and some of the benzyl chloride produced were removed in vacuo, and this was repeated twice. The resulting clear yellow benzyl alcohol solution was cooled and diluted with three times its volume of anhydrous ether. The resulting oil slowly crystallized with vigorous shaking. The solution was then placed in the deep-freeze overnight. The ether was decanted off and the crystals washed quickly on a coarse sintered-glass filter with several portions of dry ether. The product was then placed in a vacuum desiccator over phosphorus pentoxide for several days. Analytical data: Calcd. for C_{20}H_{25}N_{2}O_3SCl O, 58.7%; H, 6.16%. Found: O, 58.7%; H, 6.21%. The free base was prepared by treating the hydrochloride in chloroform with excess triethylamine and filtering off the triethylamine.
hydrochloride. The chloroform was evaporated at low pressure and the slurry remaining was extracted with ethyl acetate. The free base was deposited as short needles upon the addition of cold petroleum ether to the ethyl acetate solution. Melting point 72-74°C.

**Preparation of L-Tosylpyroglutamyl-β-alanine.**

Three grams of L-tosylpyroglutamyl chloride (1, pp. 3111-3112) was added in one portion to an ice-cooled suspension of 0.9 gram of β-alanine and 1.0 gram of magnesium oxide in thirty milliliters of water. This was stirred for one hour at 0°C., acidified with dilute hydrochloric acid, and then placed in the refrigerator overnight. The colorless needles were filtered and air-dried. Yield, 3.1 (79.6%). Melting point, 193-195°C. The material was recrystallized from ethanol and dried in air. Melting point, 195-196°C. Analytical data: Calcd. for C₁₅H₁₈N₂O₆S: C, 50.84%; H, 5.12%. Found C, 50.7%; H, 5.2%.

**L-Tosylglutaminyl-β-alanine.** Six grams of tosylpyroglutamyl-β-alanine were dissolved in 28% ammonium hydroxide and kept at room temperature for about fifteen minutes. The reaction mixture was then placed in a vacuum desiccator over concentrated sulfuric acid overnight at ten mm. Hg pressure. The solution was filtered, hydrochloric acid was added to pH 3, and the mixture chilled in ice for four hours. The colorless
crystals (melting point 196-197°C.) were filtered off, washed with cold water, dried and weighed. Yield, 4.7 grams (75%). A sample for analysis was recrystallized from ethanol; melting point, 197-198°C. Analytical data: Calcd. for C₁₅H₂₁N₃O₆S: C, 48.50%; H, 5.70%. Found C, 48.5%; H, 5.76%.

1-Tosylglutamyl-β-alanine. Tosylpyroglutamyl-β-alanine (6.3 grams) was added to eighty milliliters of ice-cold dilute sodium hydroxide solution. This was acidified to the Congo Red end point with hydrochloric acid after standing at room temperature for thirty minutes. When the solution was cooled for several hours, the precipitate was filtered and dried. Recrystallization from 50% ethanol failed to raise the melting point of 175-177°C. Yield of 4.8 grams (72.5%). Analytical data: Calcd. for C₁₅H₂₀N₂O₇S: C, 48.37%; H, 5.42%. Found: C, 48.2%; H, 5.54%.

1-Tosylpyroglutamyl-benzyl ester. To a solution of benzyl alcohol (10 grams) in 50 milliliters of chloroform containing 10 ml. of triethyl amine, a solution of 10.0 grams (0.33 moles) of tosylpyroglutamyl-chloride in 20 ml. of chloroform was added in a steady stream. The reaction gave off much heat, causing the solvent to boil. After standing one hour at room temperature, the chloroform was removed in vacuo and the red oil extracted with
water. The residual crystals, melting point 100.5-101.5°C., were then washed with ether three times and recrystallized from 50% ethanol. Yield 6.3 grams (50.8%). Analytical data: Calcd. for C_{19}H_{19}N_{0.5}S C, 61.1%; H, 5.13%. Found: C, 60.9%; H, 5.7%.

1-Tosylglutamyl-benzyl ester. Ten grams of \textit{l}-tosylpyroglutamylchloride dissolved in chloroform were added to a solution of excess benzyl alcohol and excess triethylamine in chloroform. After the reaction had subsided and had remained at room temperature for one hour, the chloroform was removed \textit{in vacuo}; about ninety milliliters of dilute sodium hydroxide were added to the oil. The mixture was kept at room temperature for thirty minutes, extracted twice with equal portions of ether, and the aqueous phase acidified with hydrochloric acid. The oily precipitate crystallized upon standing overnight in the refrigerator. This was recrystallized from ethanol-water to give long colorless needles with a melting point of 126.5-128°C. Yield, 8.6 grams (66%). Analytical data: Calcd. for C_{19}H_{21}N_{0.5}S C, 58.3%; H, 5.41%; N.E. 391 Calcd. for C_{19}H_{21}N_{0.5}S\cdot H_{2}O C, 55.7%; H, 5.67%; N.E. 409. Found: C, 55.65%; H, 5.55%; N.E. 394.

If the purified crystalline 1-tosylpyroglutamyl benzyl ester is used to prepare this, it was found that the cyclic compound is extremely stable in base, even
failing to react in boiling alkali within ten minutes. However, when approximately an equal amount of benzyl alcohol was added to the reaction mixture, solution occurred within a few minutes at room temperature. The yield in this case was 61% or 800 milligrams from one gram of pure tosylpyroglutamylbenzyl ester.

**Benzyl (β-Benzyl-N-Tosyl-κ-glutamyl)β-alanine.**

To 2.0 grams of tosylpyroglutamyl-benzyl ester (0.0054 moles) in twenty milliliters of chloroform was added 1.0 gram of benzylβ-alanine. The solution was refluxed for one hour; the chloroform was removed by distillation in vacuo. The residual oil was dissolved in 10 milliliters of chloroform and 80 milliliters of ether. This was washed with 100 milliliters of dilute hydrochloric acid, followed by three portions of water. Upon evaporation of the ether, a mixture of crystals and oil remained. The mixture was triturated with absolute alcohol. The crystals (1.1 grams) were pure tosylpyroglutamyl-benzyl ester. The ethanol solution was diluted with water and the oil formed solidified upon standing. The solid was then recrystallized from twenty milliliters of thirty percent methanol. Yield, 0.95 gram (71% based on the tosylpyroglutamyl benzyl ester). Analytical data:

Calcd. for C_{29}H_{32}N_{2}O_{7}S: C, 63.15%; H, 5.83%; N, 5.08%.
Found: C, 61.7%; H, 5.92%; N, 5.13%. Melting point, 54-60°C, with some small needles in the melt.

Two attempts to obtain this compound by other methods were not successful. One method attempted the formation of the mixed anhydride with \( \alpha \)-benzyl-tosyl-glutamic acid and ethylchloroformate followed by the amino acid ester. This yielded only an unworkable mixture. The other method tried was the reaction of \( \alpha \)-benzyl-tosyl-glutamic acid with dicyclohexylcarbodiimide. This gave nearly quantitative cyclization of the tosylglutamyl ester to tosylpyroglutamylbenzyl ester, as indicated by a mixed melting point with an authentic sample.

**Benzyl (N-Tosyl-isoglutaminyl)-\( \alpha \)-alanine.**

To 3.0 grams of tosylpyroglutamamide (0.0106 moles) in chloroform, a solution of excess benzyl-\( \alpha \)-alanine (2.0 grams) was added and the mixture refluxed for one hour. The chloroform solution was filtered, washed with dilute hydrochloric acid, dilute sodium carbonate, and then water. The chloroform solution was dried with magnesium sulfate, and evaporated to dryness. The solid residue was extracted with hot ethyl acetate and the product precipitated by the slow addition of petroleum ether. After washing the crystals with petroleum ether and drying, they were recrystallized from ethanol-water. Yield,
2.05 grams (42%). Melting point, 126-129°C. Analytical data: Calcd. for C_{22}H_{27}N_{3}O_{8} S C, 57.25%; H, 5.9%; N, 8.91%. Found: C, 57.1%; H, 6.93%; N, 9.12%.

**Benzyl-(N-tosyl-γ-glutamyl)-γ-alanine.** To a solution of 2.83 grams (0.01 mole) of tosylpyroglutamic acid in dichloroethane was added excess benzyl-γ-alanine (2.0 grams, 110%) and 1.4 grams of triethyl amine; this was refluxed for one hour. The solvent was removed in vacuo and the oil triturated with dilute acid. The oil was dissolved in dilute sodium bicarbonate solution and extracted with ether. The aqueous phase was acidified to pH 3 and the turbid solution extracted with ethyl acetate. The ethyl acetate solution was dried with magnesium sulfate. Evaporation of the solvent to a small volume yielded 4.0 grams (85%).

**N-Tosyl-isoglutaminyl-γ-alanyl-S-benzyl-cysteine.** To a solution of 2.35 grams of tosylglutamine in fifty milliliters of dimethylformamide containing 1.7 grams of dicyclohexylcarbodiimide was added 3.0 grams of benzyl-S-benzyl-γ-alanyl-cysteine. The urea formed after letting stand for six hours was almost quantitative. The chloroform was replaced with ethyl acetate, and the solution washed with dilute acid and dilute base, then dried over magnesium sulfate. Upon addition of petroleum ether, an oil separates which crystallized on standing overnight. Melting point,
130-140°C. Recrystallization from ethyl acetate gave 1.65 grams of material (32%), melting point, 149-150°C. Analytical data: Calcd. for C_{32}H_{38}N_{4}O_{7}S_{2} C, 58.75%; H, 5.85%; N, 8.57%. Found: C, 58.5%; H, 6.22%; N, 8.89%.

N-Tosyl-α-glutamyl-γ-alanyl-γ-benzyl-cysteine.

To a suspension of 1.4 grams (0.005 mole) of S-benzyl-alanyl-cysteine in 100 milliliters of cold water containing 0.5 gram of magnesium oxide was added with vigorous stirring 1.5 grams (0.0052 mole) of tosylpyroglutamyl chloride. Stirring was continued in an ice-salt bath for ten hours. The mixture was acidified to pH 4 with dilute hydrochloric acid, and the oil formed was extracted with ethyl acetate. The ethyl acetate solution was washed twice with water, dried over magnesium sulfate, and evaporated in vacuo at room temperature. The viscous oil was treated with dilute sodium hydroxide at room temperature for six hours, filtered from the small amount of undissolved material, acidified to Congo Red with dilute acid and extracted with ethyl acetate. Evaporation of the solvent gave a glass that was recrystallized from one part ethyl acetate—three parts petroleum ether yielding 2.25 grams (82%) of slightly yellow powder. Melting point; at 65-70°C the material softens to a glass which decomposes at 120-125°C. Analytical data: Calcd. for C_{25}H_{31}N_{3}O_{8}S_{2} C, 53.1%; H, 5.5%. Found: C, 53.7%; H, 5.5%.
SUMMARY

1. A new method for reducing alkyl disulfides has been described.

2. Several limitations in the use of carbodiimides have been revealed which may be used as a basis for further investigations into the optimum use of the reagent for amide or peptide formation.

3. Some new peptides of glutamic acid and \(\beta\)-alanine have been prepared by methods known to yield isomerically pure \(\alpha\)- and \(\beta\)-glutamyl and glutaminyl peptides.

4. The peptides prepared were tested for biological activity with two yeasts and found to have no stimulatory or inhibitory activity; this may indicate merely that the yeasts used were lacking a specific transpeptidase or peptidase necessary for utilization of the peptides or components of the peptides.

5. The following new compounds were prepared:
   a. N-tosylpyroglutamyl-\(\alpha\)-alanine
   b. \(\alpha\)-(N-tosylglutamyl)-\(\alpha\)-alanine
   c. \(\alpha\)-(N-tosylglutaminyl)-\(\alpha\)-alanine
   d. Benzyl-N-tosylpyroglutamic acid
   e. \(\alpha\)-Benzyl-N-tosylglutamic acid
f. Benzyl-S-benzyl-S-alanyl-cysteine hydrochloride

h. Benzyl-(α-benzyl-N-tosylglutamyl)-β-alanine

i. Benzyl-(N-tosyl-isoglutaminyl)-β-alanine

j. N-Tosyl-γ-glutamyl-β-alanine

K. α-(N-Tosylglutamyl)-β-alanyl-S-benzyl-cysteine

l. γ-(N-Tosyl-isoglutaminyl)-β-alanyl-S-benzyl-cysteine

m. N-Phthaloyl-β-alanyl anhydride

n. N-Carbobenzoxy-β-alanyl-N,N'-dicyclohexyl urea
BIBLIOGRAPHY


