THE SYNTHESIS AND PROPERTIES OF SOME PEPTIDES AND THE SPECIFICITY OF PEPSIN

by

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ABSTRACT

A number of peptide intermediates and derivatives have been synthesized from both optically-pure and racemic amino acids. Carbobenzoxy-DL-alanyl chloride was coupled with L-leucine methyl ester and the mixed, crystalline carbobenzoxy-DL-alanyl-L-leucine methyl ester was isolated. Carbobenzoxy-DL phenylalanyl chloride and carbobenzoxy-DL phenylalanyl azide were coupled with L-leucine methyl ester and two products were separated by fractional crystallization. One of the products has been identified as carbobenzoxy-L-phenylalanyl-L-leucine methyl ester.

A series of synthetic, dipeptide derivatives, containing some of those peptide bonds (present in the phenylalanyl chain of insulin) that Sanger and coworkers (31,32) found to be split by pepsin, were subjected to the action of pepsin at pH 2.0 and at pH 4.0. Proteolysis was detected by paper chromatography of the enzyme-substrate solutions. Of the compounds tested, carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester and carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester were found to be hydrolysed by pepsin at pH 2.0 but not at pH 4.0. Synthetic compounds containing the peptide bonds - phenylalanyl-valyl, leucyl-valyl, glutamyl-alanyl, alanyl-leucyl, and glycyl-phenylalanyl - were resistant to pepsin even though it had been found (31,32) that, in insulin, these peptide bonds were pepsin-sensitive.

These results indicate that, in the case of pepsin, observations on the action of pepsin on synthetic, dipeptide-type substrates should not be used to predict, or to explain, the specificity of pepsin on proteins or on high molecular weight peptides.

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DIAGRAMS

INTRODUCTION

The work reported in this thesis consists of two parts; first, a study of peptide synthesis and the resolution of diastereoisomeric peptide derivatives; second, a re-investigation of the specificity of the gastric protease, pepsin. The compounds prepared in the first part of the work were tested, in the second part of the work, as potential pepsin substrates.

HISTORICAL

A. Synthesis of Peptide Derivatives

With the increasing importance of naturally occuring peptides or peptide derivatives containing D-amino acids (23, 36) and with the expense or unavailability of the "unnatural" D-amino acids it would be of great value if inexpensive, effective methods of resolution of the racemic amino acids could be obtained.

Most early workers when preparing peptides containing a D-amino acid or an L-amino acid first resolved a racemic mixture of the amino acid by the use of an optically active base. This method is both expensive and time consuming.

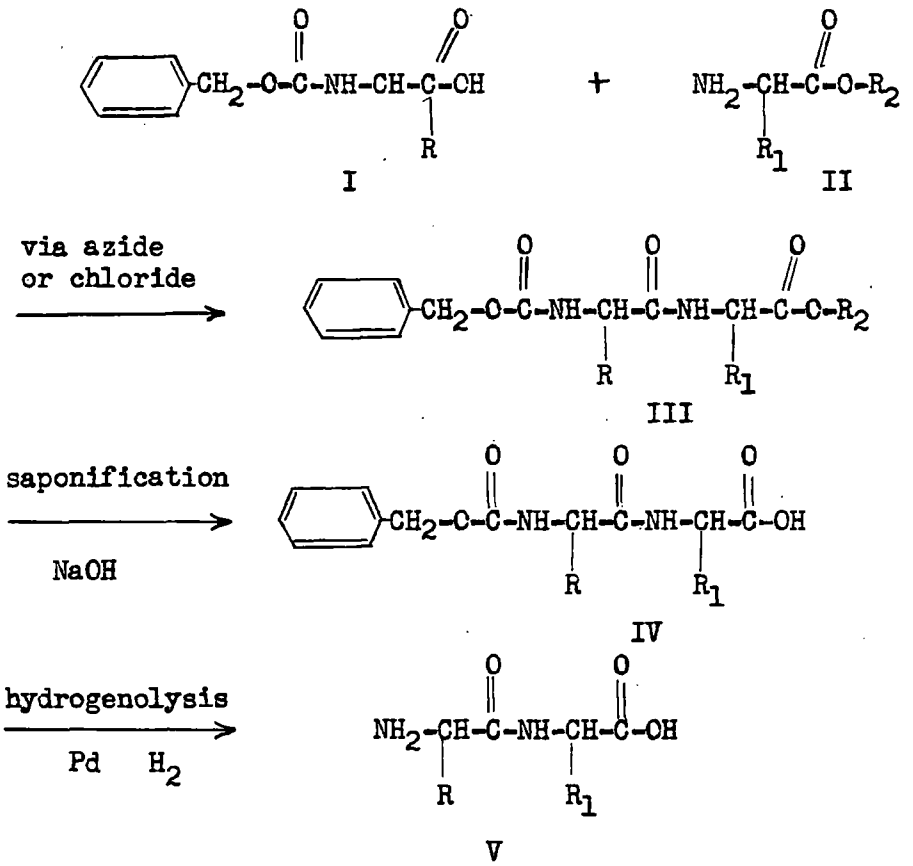
More recently, the stereo specificity of certain enzymes has been used for the preparation of optically pure amino acids. An outstanding example of this is the method used by Greenstein (12, 28, 16) in which an aqueous extract of rat or hog kidney is allowed to act, rapidly and asymmetrically, upon many N-acylated DL-amino acids. The acylase hydrolyses preferentially the N acyl-L-amino acid bond producing the free L-amino acid and the N-acyl D-amino acid. From such a digest, the free L-amino acid is separated by addition of alcohol, leaving the soluble acyl-D-amino acid in the mother liquor. The acyl-D-amino acid is then hydrolyzed by hot mineral acid to yield the free D-amino acid.

This enzymatic method cannot be applied to the resolution of all D, L-amino acids. In the case of arginine and histidine, it is difficult to acylate both basic groups with susceptible, and conveniently removed, acyl radicals, and the N-acyl derivatives of cystine and proline are completely resistant to enzymatic hydrolysis by animal enzyme preparations (22). An alternative method of resolution based upon the asymmetric enzymatic hydrolysis of the racemic amino acid amides by a non-specific amidase has, therefore, been developed (17, 22).

Other enzymatic methods of resolution of racemic amino acids include that of asymmetric synthesis with aniline (11). Such a method makes use of the enzymatic synthesis of the insoluble anilide of the L-isomer which subsequently crystallizes. The diasterioisomer can then be recovered from the mother liquor. The anilide form of the L-isomer is, however, of no further use in coupling unless subjected to hydrolysis which may result in loss by racemization.

Scattered reports of resolution of unsubstituted amino acids have been recorded. However, most of the workers make use of salt formation between an optically active acid or base and a racemic amino acid with subsequent separation of one or both of the diastereoisomers by fractional crystallization (30, 29, 37, 21).

In the preparation of a dipeptide by the carbobenzoxy method (4) it is often possible to obtain one or two crystalline intermediate compounds, as well as the final crystalline dipeptide. The steps of this method are as follows:



I II III IV V

Thus, if a carbobenzoxy DL-amino acid (I) is coupled to an L-amino acid ester (II) there may be as many as three synthetic steps at which to effect a separation of the resulting diastereoisomers. Furthermore, the resulting optically pure carbobenzoxy dipeptides (IV) or dipeptide esters (III) are in such a form that they can be used directly for further couplings to produce longer chain peptides.

This method of resolution has been used in a few cases. Polglase and Smith (27) coupled CARBOBENZOXY L-leucine azide with the methyl ester of DL-alanine. Carbobenzoxy-L-leucyl-D-alanine methyl ester crystallized from the reaction mixture. The diastereoisomer could not be crystallized from the mother liquor, but after saponification with alkali, the resulting carbobenzoxy-L-

leucyl-L-alanine crystallized. Hunt and du Vigneaud (20) prepared carbobenzoxy-L-alanyl-L-histidine from carbobenzoxy-DL-alanine and L-histadine methyl ester. After saponification of the resulting product the carbobenzoxy-L-alanyl-L-histidine crystallized. The combined mother liquors were hydrogenolysed and the dipeptide D-alanyl-L-histidine isolated by means of its copper salt. Behrens, Doherty and Bergmann (3), prepared acetyl-D-phenylalanyl-L-leucine and acetyl-L-phenylalanyl-L-leucine by reduction of acetyldehydrophenylalanyl-L-leucine and fractional crystallization from fifty percent dioxane. Cook, Cox and Farmer (7) coupled L-α-bromoisovaleryl chloride and DL-N-methyl valine. These products were converted to the hydroxy isomeric lactones and then separated chromatographically.

In this thesis, the first part of the experimental section describes the coupling of carbobenzoxy-DL-alanine and of carbobenzoxy-DL-phenylalanine with L-leucine methyl ester, and the separation or attempted separation of the corresponding isomers.

Some of the L-leucine used in the syntheses was prepared by purification of technical leucine as described by DeWitt and Ingersoll (10).

B. Specificity of Pepsin

The proteolytic enzymes of vertebrates fall into two groups, the first of which is mainly concerned with the degradation of the large molecules of the food proteins to yield smaller fragments, the second group completing the process initiated by the first and leading eventually to the liberation of free amino acids. The first group includes pepsin

which arises from the gastric juice, and trypsin and chymotrypsin formed from precursors present in the pancreatic juice. The second group is composed of carboxypeptidase, contributed by the pancreatic juice, aminopeptidase, and dipeptidase, which are present in the intestinal secretions.

Pepsin is secreted by the gastric mucosa in the form of pepsinogen, which is activated, in the first instance, by the hydrochloric acid of the gastric juice to yield pepsin itself. Pepsin, once formed, is capable of activating more pepsinogen so that the activation of pepsinogen is an autocatalytic process. Northrup (24) was the first to isolate the crystalline enzyme from commercial pepsin preparations. This was the second crystalline enzyme to be reported and the first crystalline proteolytic enzyme.

Since 1930, many more enzymes have been isolated in the crystalline state (25). Biologically speaking, it is possible to draw some sort of distinction between pepsin and the trypsins on one hand, formerly called proteinases, and the group of peptidases, carboxypeptidase, aminopeptidase and dipeptidase on the other. The digestion of the food proteins is begun by the "proteinases" and the fragmentary products thus formed are further degraded by the "peptidases" to yield, finally, free amino acids. It was formerly believed that pepsin and the trypsins were able to attack only large molecules of the same order of size as the protein molecule of the food and that the "peptidases" were only able to deal with molecules of the order of size found among polypeptides and perhaps peptones. More recent work, which became possible only when Bergmann's "Carbobenzoxy method of peptide synthesis (4), had made a wide variety of synthetic peptides available, has shown that pepsin, trypsin and chymotrypsin, as

well as the peptidases, are able to act upon comparatively simple peptides, provided that the peptide linkages of the right kind and the appropriate configuration are present. Whereas the substrate specificities of trypsin and chymotrypsin were clearly delineated in Bergmann's first experiments (19, 14), the specificity of pepsin is still incompletely understood. In their original work (15), Bergmann and Fruton outlined the substrate specificity for pepsin as follows:

1) Presence of at least two carboxyl groups in the peptide.

2) Absence of a proximal free amino group.

3) Presence of an aromatic residue (phenylalanyl or tyrosyl) on the amino side of the linkage to be attacked.

A typical "Bergmann substrate" was carbobenzoxy-L-glutamyl-L-tyrosine or carbobenzoxy-L-glutamyl-L-phenylalanine. The pH optima however, for these two substrates were pH 4.0 and pH 4.5, respectively, as compared with an optimum of 1.8 - 2.0 for the peptic hydrolysis of proteins. Moreover, the rate of hydrolysis of the synthetic substrates is extremely slow, compared to the rate of hydrolysis of proteins (25). In 1944, Harrington and Pitt-Rivers (18), studied the action of crystalline pepsin on the N-carbobenzoxy derivatives of cysteinyl- and cystinyl tyrosine and of tyrosyl-cysteine and cystine, and on the corresponding free peptides both at pH 4.0 and pH 1.8 . They found that both cysteinyl (cystinyl)-tyrosine and tyrosyl-cysteine (cystine) peptides were split by pepsin and although the N-carbobenzoxy derivatives were the most readily hydrolysed, the free dipeptides were also attacked. In agreement with Bergmann, Harrington and Pitt Rivers found that the action of pepsin on their synthetic substrates was much more marked at pH 4.0 than at pH 1.8. They

also found that the substrates, whether acylated or not, were more susceptible to the action of pepsin when they were in the reduced form. Although the synthetic substrates tested by Harrington and Pitt-Rivers do not meet the specificity requirements set forth by Bergmann (15) they were split, in some cases, just as rapidly as substrates which satisfied Bergmann's criteria. The splitting of a free dipiptide by pepsin was contrary to all the previously conceived ideas of peptic action. The observation that the substrates are attacked by pepsin much more rapidly in the reduced (-SH) form than in the oxidized (-SS) was interesting in view of the fact that pepsin acts much more vigorously on denatured than on native proteins and that denaturation of proteins is accompanied by the appearance of -SH groups. A few years later Fruton and coworkers (8), tested the action of pepsin on carbobenzoxy-L-methionyl-L-tyrosine and L-methionyl-L-tyrosine. They found substantial hydrolysis (35% and 25% hydrolysis in 24 hr., respectively) of the two compounds, again with an optimum pH of 4.0. The observation of Northrup (26) that tyrosine is liberated from a self-digestion of pepsin and the report of Calvery and Schock (6) that tyrosine is liberated from a peptic hydrolysis of egg albumin can possibly be explained by assuming that at least one point of attack of the protein by pepsin may be a cysteinyl tyrosine, tyrosyl-cysteine or methionyl tyrosine linkage. Using peptide derivatives containing two aromatic amino acids such as acetyl-L-phenylalanyl-L-tyrosine or acetyl-L-phenylalanyl-L-diiodotyrosine, Baker (2) has shown an optimum pH for peptic hydrolysis of 1.8, the pH optimum for hydrolysis of proteins. The experiment was extended to substrates which contained benzyl, hydroxybenzyl or diiodohydroxybenzyl group. All the compounds were hydrolysed quite

rapidly, the rate being faster at pH 2.0 than at pH 3.0 or 4.0. Under the condition of hydrolysis used, Baker also found carbobenzoxy-L-glutamyl-L-tyrosine to be hydrolyzed much more rapidly at pH 2.0 than at pH 4.0. The differences between Baker's and Bergmann's results are difficult to explain unless it is assumed that the enzyme preparations used by the two workers were different.

In their study of the sequence of amino acids in the insulin molecule, Sanger and Tuppy (31), and Sanger and Thompson (32) employed proteolytic enzymes. They found that the specificities of trypsin and chymotrypsin, as delineated by Bergmann and his associates (with synthetic substrates) were almost completely applicable to the large polypeptide fragments obtained from insulin. Sanger and associates suggested (31, 32) that specificity of action of trypsin and chymotrypsin on an intact protein was probably similar to the specificity of these enzymes for synthetic substrates. On the other hand, pepsin appeared to have a considerable lower degree of specificity than had the other two enzymes. In the glycyl chain of insulin, Sanger and Thompson (32), found the following bonds to be split by pepsin: - glutamyl - glutamyl - ; - valyl - seryl - ; - leucyl - tyrosyl - ; tyrosyl - glutamyl - ; -glutamyl - leucyl - ; - leucyl - glutamyl - ; glutamyl - aspartyl - ; in the phenylalanyl chain (31) the following bonds were split by pepsin: phenylalanyl - valyl - ; - glutamyl - histidinyl - ; - leucyl - valyl - ; - glutamyl - alanyl - ; - alanyl - leucyl - ; - leucyl - tyrosyl - ; - tyrosyl - leucyl - glycyl - phenylalanyl - ; - phenylalanyl - phenylalanyl - and - phenylalanyl - tyrosyl - . It does not appear that the specificity

of pepsin is limited to bonds adjacent to aromatic residues as was previously believed (15, 18, 2, 8). Pepsin would appear to have a specificity for the more fat soluble parts of the peptide chain. This finding of a rather wide specificity for the action of pepsin on the insulin molecule is in agreement with the results of Desnuelle, Rovery and Bonjour (9, 35) who studied the N-terminal residues liberated on treatment of ovalbumin and horse globin with pepsin. Generally speaking it was found that peptic hydrolysis proceeded differently with globin than with albumin. In the case of globulin, hydrolysis proceeded in two successive phases. During the first phase, very large peptides and duodeca peptides (on the average) are formed, simultaneously, from the protein chains. The large peptides are themselves rapidly transformed into duodecapeptides which can be considered as typical of this particular stage. The first phase of degradation is rapid. Characterization of terminal amino acids shows that this degradation occurs according to certain rules of specificity. Pepsin, during this phase, breaks up preferentially those bonds in which alanine, phenylalanine, leucine and serine are involved through their amino groups. The characterization of the carboxyl terminal groups liberated by pepsin was not attempted. During a second phase of the hydrolysis, the peptides undergo a further degradation down to the tetra peptide (average) stage. This phase proceeds very slowly and seems non-specific, (at least as far as the amino side of the linkage is concerned).

On the other hand, in the case of albumin, there does not seem to be any general formation of large peptides. A first phase proceeds rapidly

to the hexa- or penta-peptide stage. No specificity can be demonstrated in the hydrolysis of individual linkages. After this rapid initial degradation, very little further hydrolysis occurs, although eventually, again, an average of four amino acids per peptide residue is reached. No specificity can be demonstrated in this second phase (9, 35).

Anfinsen (1) in considering a peptic digestion of ribonuclease suggests that the initial stage of digestion consists of a change in the fine structure of the molecule, involving minimal cleavage of peptide bonds and no change in sedimentation constant. Subsequent digestion by pepsin then results in the rupture of approximately ten peptide bonds, with the resulting peptides containing, on the average, 7 - 8 amino acid residues per molecule.

From the foregoing discussion, it is clear that the substrate specificity of pepsin needs further study. Such a study requires that suitable, chemically-defined, potential substrates be prepared. Usually, in work on enzyme specificity, the synthesis and the isolation and characterization of substrates become the major research tasks, while the work of testing enzyme specificity (the ultimate objective of the research) may be completed in a relatively short time. This has been true for the research reported herein.

All melting points are reported in degrees centigrade and are uncorrected.

EXPERIMENTAL

A. Synthesis of Peptide Derivatives

I. Carbobenzoxy Chloride (4)

One liter of toluene was cooled in an ice bath and phosgene (200 g.) introduced slowly (34). Benzyl alcohol (160 ml.) was then added and the solution allowed to stand for 1 hr. in an ice bath and a further 2 hr. at room temperature. At the end of this time the excess phosgene was flushed out with a stream of dry air and the solution concentrated under reduced pressure to a volume of 120 ml.

The carbobenzoxy chloride was tested by preparation of carbobenzoxy glycine, as follows: glycine (7.5 g.), was dissolved in 25 ml. of 4 N sodium hydroxide. To this solution, carbobenzoxy chloride (20 g.) and 4 N sodium hydroxide (25 ml.) were added in equivalent, small portions over a period of 35 min. After 1 hr. at room temperature, the solution was acidified to congo red with concentrated hydrochloric acid. The precipitate was filtered and recrystallized from water-ethanol; yield: 18.5 g., m.p. 118-119°. This indicated a yield of 88.5% for the carbobenzoxy chloride.

II. Purification of L-Leucine (10)

Technical L-leucine, 131 g., (1 mole, assuming pure leucine) was suspended in 350 ml. of water and treated with 3 moles of

acetic anhydride and a solution of 7 moles of sodium hydroxide in 350 ml. of water. Throughout the addition, the mixture was kept slightly alkaline. The reagents were added over a period of 2 hr., with stirring and cooling in an ice-salt bath. Twenty minutes after the last addition, the mixture was acidified with 7 moles of 37% hydrochloric acid and placed in the refrigerator. The product was collected on a Buchner funnel the next day. The precipitate was washed with 200 ml. of cold water and 200 ml. of acetone; yield: 110 g. For recrystallization, this compound was dissolved in 9 parts of 33% methanol and treated with charcoal. A second recrystallization was from a solution of 2.5 ml. methanol and 5 ml. hot water per gram of material; m.p. 179-180°.

Twice recrystallized, N-acetyl-L-leucine (17.3 g.), was refluxed with 35 ml. of 3 N hydrobromic acid. At the end of 2 hr., the solution was diluted with 100 ml. of hot methanol and brought to pH 6.0 with aqueous ammonia. The solution of L-leucine was cooled overnight and filtered; yield: 10.8 g. $[\alpha]^{25}_{D}$ +15.6 (c 4, 6N HCl). The reported rotation for L-leucine (10) is: $[\alpha]^{25}_{D}$ +15.3 (c 4, 6 N HCl). A paper chromatogram of the purified L-leucine showed only one spot corresponding to a sample of pure L-leucine.

III. Preparation of Amino Acid Esters

The corresponding amino acid ester hydrochlorides (see pages 14, 20, 30, 32) were dissolved in the least amount of water and covered with a layer of ether. The solution was cooled to 0° and an excess of 12 N sodium hydroxide was added slowly with shaking.

Anhydrous potassium carbonate was then added to absorb all the water. The mixture was filtered and the precipitate extracted repeatedly with ether. The combined filtrates were dried over sodium sulfate.

IV. Carbobenzoxy-DL-Alanyl-L-Leucine Methyl Ester

a) Carbobenzoxy-DL-alanine

DL-Alanine (5 g.) was dissolved in 15 ml. of 4 N sodium hydroxide which had been cooled to 0°. Carbobenzoxy chloride (10.5 g., 20% excess) and 4 N sodium hydroxide (15 ml.) were added over a period of thirty minutes with shaking. After standing at room temperature for 1 hr., the solution was acidified to congo red with concentrated hydrochloric acid. The precipitate was filtered and recrystallized from water-acetone.

TABLE I

Preparation of carbobenzoxy-DL-alanine

Preparation No.	Starting amt. of DL-alanine (g.)	Yield (g.)	m.p. (°C)
1	5.0	8.5	109 - 111
2	10.0	14.2	109 - 111
3	5.0	8.0	109 - 111
4	2.5	4.2	110 - 112.5

b) L-Leucine methyl ester hydrochloride

L-Leucine (11.3 g.) was dissolved in ten times its weight of anhydrous methanol and the solution saturated with dry hydrogen

chloride gas at 0°. The solution was allowed to stand at 0° overnight. The reaction mixture was then concentrated under reduced pressure, whereupon crystallization resulted. The concentration was repeated three or four times, after the addition of anhydrous methanol. The resulting amino acid ester hydrochloride was suspended in ether, filtered, and recrystallized from methanol-ether.

TABLE II

Preparation of L-leucine methyl ester hydrochloride

Preparation No.	Starting amt. of L-leucine (g.)	Yield (g.)	m.p. (°C)
1	10.0	2.0	148 - 149.5
2	10.0	4.0	145 - 147
3	5.8	4.5	147 - 148
4	10.0	6.8	147 - 148
5	9.0	3.9	147 - 148.5
6	11.3	9.92	148.5- 149.5
7	20.0	5.1	147 - 148
8	9.0	3.86	147 - 148.5
9	10.0	4.31	146.5- 148.5
10	10.0	5.72	146 - 148

c) Carbobenzoxy-DL-alanyl-L-leucine methyl ester

Carbobenzoxy-DL-alanine (5.45 g.), was dissolved in 20 ml. of anhydrous ether and the solution cooled to 0°. Phosphorous pentachloride

(5.45 g.) was added and the solution shaken at 0° until all but a trace of the latter had dissolved. Light petroleum ether (previously cooled to -50°) was added, whereupon the solution turned cloudy. On stirring at -50° the carbobenzoxy-DL-alanyl chloride crystallized.[1] The supernatant liquid was decanted and the chloride washed by decantation, with light petroleum ether (previously cooled to -50°). The product was then dissolved in 30 ml. of chloroform (previously cooled to -50°) and added to a solution of L-leucine methyl ester (from 8.9 g. of the hydrochloride) in ether. After 30 min. at 0° and 2 hr. at room temperature, 2.24 g. of L-leucine ester hydrochloride was filtered from the reaction mixture. The filtrate was washed with saturated sodium bicarbonate, N hydrochloric acid and fianlly with water. The chloroform-ether solution was dried over sodium sulfate and concentrated to a syrup. The syrup was dissolved in ethyl ether and crystallization occured on slow evaporation. The compound was recrystallized from ether-petroleum ether (30-60°); $[\alpha]^{25}_{D}$ - 26.0 (c 1 ethanol)

TABLE III

Preparation of carbobenzoxy-DL-alanyl-L-leucine methyl ester

Preparation No.	Starting amt. of DL-alanine (g.)	Yield (g.)	m.p. (°C)
1	3.05	1.5	74.5 - 75.5
2	5.45	4.19	74 - 75
3	5.45	1.23	72 - 73

[1] This compound has previously been reported only as an oil (20, 27).

Anal. Calcd. for $C_{18}H_{24}O_5N_2$: N, 8.00; Found, N, 7.81, 7.94. Upon admixture with an authentic sample of carbobenzoxy-D-alanyl-L-leucine methyl ester,[1] the melting point was depressed to 62-67°.

V. Carbobenzoxy-DL-Alanyl-L-Leucine

Carbobenzoxy-DL-alanyl-L-leucine methyl ester (2.18 g.) was dissolved in 20 ml. of acetone and treated with 6.05 ml. of N sodium hydroxide. After 45 min. at room temperature, the solution was acidified, and the acetone evaporated in a stream of air. The resulting syrup was extracted into ethyl acetate and this solution extracted with a saturated solution of sodium bicarbonate. The sodium bicarbonate solution was acidified and extracted with ethyl acetate and this extract dried with sodium sulfate. The DRIED ethyl acetate solution was concentrated to a syrup. Attempts to induce crystallization were unsuccessful.

VI. DL-Alanyl-L-Leucine

Carbobenzoxy-DL-alanyl-L-leucine from above was dissolved in methanol (15 ml.), water (1 ml.) and glacial acetic acid (1 ml.) and hydrogenated over a palladium catalyst. Hydrogenolysis was complete in 28 hrs. The catalyst was filtered off and the filtrate concentrated under reduced pressure to a syrup from which the acetic acid and water were removed by repeated concentration with anhydrous methanol. The syrup could not be induced to crystallize. However, on standing at room

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[1] A sample of carbobenzoxy-D-alanyl-L-leucine methyl ester was kindly supplied by Dr. Emil L. Smith of the University of Utah.

temperature it did solidify; $[\alpha]^{20}_{D}$ - 7.78 (c 5 water). The recorded rotation (27) for L-alanyl-L-leucine is $[\alpha]^{21}_{D}$ - 17.0 (c 5 water)

VII. Carbobenzoxy-DL-Alanyl-L-Leucinamide

Carbobenzoxy-DL-alanyl-L-leucine methyl ester (200 mg.) was dissolved in methanol (15 ml.) previously saturated at 0° with ammonia. After 40 hrs. at room temperature, the solution was concentrated, whereupon crystallization occurred. The product was recrystallized from ethanol-water; m.p. 163-164°; $[\alpha]^{20}_{D}$ - 27.9 (c 1, ethanol).
Anal. Calcd. for $C_{17}H_{23}O_4N_3$: N, 12.54 Found, N 12.43. The constants reported (27) for the two diastereo-isomers are:

Carbobenzoxy-D-alanyl-L-leucinamide; m.p. 187-188°;

$[\alpha]^{30}_{D}$ - 6 (c 1 ethanol)

Carbobenzoxy-L-alanyl-L-leucinamide; m.p. 188-189°;

$[\alpha]^{30}_{D}$ - 41 (c 1 ethanol)

VIII. Carbobenzoxy-L-Alanyl-L-Leucine Methyl Ester

a) Carbobenzoxy-L-alanine

L-Alanine (2.5 g.) was treated in the same way as for the preparation of carbobenzoxy-DL-alanine. The oily product was dissolved in ether and extracted with sodium bicarbonate. The bicarbonate layer was acidified and re-extracted with ether. The ether layer was dried over sodium sulfate and the compound was crystallized by the addition of petroleum ether (30-60°); yield: 3.2 g; m.p. 81-84°. The reported melting point (4) for carbobenzoxy-L-alanine is 84°.

b) Carbobenzoxy-L-alanyl-L-leucine methyl ester

Carbobenzoxy-L-alanine (3.2 g.) was coupled to L-leucine methyl ester (from 5.56 g. of the hydrochloride) through the acid chloride by the usual procedure (see page 15). Carbobenzoxy-L-alanyl chloride was induced to crystallize at -50° although like carbobenzoxy-D,L-alanyl chloride, previous reports (27) have described this substance as an oil. Carbobenzoxy-L-alanyl-L-leucine methyl ester could only be isolated as an oil. It was dried in vacuo; weight, 3.0 g.

IX. Carbobenzoxy-L-alanyl-L-Leucine

Carbobenzoxy-L-alanyl-L-leucine methyl ester (3.0 g.) was dissolved in acetone (20 ml.) and treated with N sodium hydroxide (9.37 ml.) in the same manner as for the preparation of carbobenzoxy-DL-alanyl-L-leucine (page 17). The product could not be induced to crystallize and was isolated as an oil.

X. Carbobenzoxy-?-Phenylalanyl-L-Leucine Methyl Ester

a) Carbobenzoxy-DL-phenylalanine

DL-Phenylalanine (6.6 g.) was dissolved in 2 N sodium hydroxide (20 ml.) which had been cooled to 0°. To this solution, carbobenzoxy chloride (7.0 g.), and 2 N sodium hydroxide (20 ml.) were added gradually, with shaking. After 0.5 hr. at room temperature the solution was acidified to congo red with concentrated hydrochloric acid. An oil separated which solidified on stirring. The compound was re-

crystallized from toluene.

TABLE IV

Preparation of carbobenzoxy-DL-phenylalanine.

Preparation No.	Starting amt. of DL-phenylalanine (g.)	Yield (g.)	m.p. (°C)
1	6.6	5.67	102 - 103
2	6.6	10.1	102 - 103
3	6.6	8.5	100 - 103
4	6.6	3.5	97 - 99

b) DL-Phenylalanine ethyl ester hydrochloride

TABLE V

Preparation of DL-phenylalanine ethyl ester hydrochloride

Preparation No.	Starting amt. of DL-phenylalanine (g.)	Yield (g.)	m.p. (°C)
1	10.0	6.38	124 -125.5
2	10.0	7.85	125 -126
3	25.0	19.0	124 -125.5
4	25.0	21.6	127.5-129.5

DL-Phenylalanine was suspended in ten times its weight of anhydrous ethanol and saturated with dry hydrogen chloride gas at 0°. The reaction mixture was heated under reflux for 2 hr. and concentrated repeatedly with

anhydrous ethanol under diminished pressure. The resulting amino acid ester hydrochloride was crystallized from ethanol-ether.

c) Carbobenzoxy-DL-phenylalanine ethyl ester (30)

DL-Phenylalanine ethyl ester hydrochloride (3.0 g.) was dissolved in 50 ml. of chloroform and treated, portion-wise, under ice cooling and vigorous shaking with carbobenzoxy chloride (2.4 g.) and N sodium hydroxide (27 ml.). After 0.5 hr. at room temperature, the chloroform layer was washed with N hydrochloric acid (30 ml.) and dilute potassium carbonate (10%) and dried over sodium sulfate. On concentration under reduced pressure, an oil remained which crystallized on standing. The product was re-crystallized from ethyl ether-petroleum ether (65-110°).

TABLE VI

Preparation of carbobenzoxy-DL-phenylalanine ethyl ester

Preparation No.	Starting amt. of DL phenylalanine (g.)	Yield (g.)	m.p. (°C)
1	3.0 3.0	5.5	79.5 - 80
2	5.5	5.0	80 - 82
3	6.0	6.4	77.5 - 79.5
4	6.0	5.5	80 - 81

d) Carbobenzoxy-DL-phenylalanyl hydrazide (30)

Carbobenzoxy-DL-phenylalanine ethyl ester (4.0 g.) was

suspended in 12 ml. of absolute ethanol and warmed with 0.91 g. of hydrazine hydrate (2.2 moles of 95%), and left at room temperature for 24 hr. At the end of this time, the reaction mixture had crystallized into a solid mass. The crystals were brought into solution by the addition of more absolute ethanol with warming. The solution was treated with a second portion (0.2 g.) of hydrazine hydrate. At the end of 24 hr., the reaction mixture was diluted with water and the crystalline product collected on a Buchner funnel. The compound was re-crystallized from dilute ethanol.

TABLE VII

Preparation of carbobenzoxy-DL-phenylalanyl hydrazide

Preparation No.	Starting amt. of carbobenzoxy-DL-phenylalanine ethyl ester (g.)	Yield (g.)	m.p (°C)
1	4.0	3.0	135 - 135.5
2.	4.0	3.61	134.5 - 135
3	6.0	5.42	133 - 135
4	4.66	4.04	134.5 - 136

e) Carbobenzoxy ? phenylalanyl - L - leucine methyl ester

1. Coupling through carbobenzoxy-DL-phenylalanyl chloride

Carbobenzoxy-DL-phenylalanine (4.7 g.) was suspended in 15 ml. of anhydrous ether and cooled to 0°. Phosphorous pentachloride (3.3 g.) was added and the mixture shaken until all but a trace of the latter had reacted. Light petroleum ether (200 ml.)

was then added, whereupon the carbobenzoxy-DL-phenylalanyl chloride crystallized. This was filtered off (yield: 4.0 g.) and added to an ether solution of L-leucine methyl ester (from 7.5 g. of the hydrochloride). The solution was kept at 0° for 1 hr., then left at room temperature overnight. At the end of the reaction the precipitated L-leucine methyl ester hydrochloride was filtered off and the filtrate washed with saturated sodium bicarbonate, N hydrochloric acid and water and dried over sodium sulfate. The dried ether solution was concentrated whereupon crystallization occurred; yield: 2.63 g. By fractional crystallization from ethyl ether and then from ethyl ether - petroleum ether (65 - 110°) two different compounds were separated; "Xa" which had: m.p. 123.2 - 124°, $[\alpha]_D$ - 20.8 (c-1, ethanol). Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57, Found: N, 6.15; and "Xb" which had: m.p. 108 - 108.5° $[\alpha]^{17.5}_D$ - 21.64 (c-1, ethanol). Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57, Found: N, 6.17, 6.03.

2. Coupling through carbobenzoxy-DL-phenylalanylazide

Carbobenzoxy-DL-phenylalanyl hydrazide (2.18 g.) was dissolved in a mixture of 17 ml. of glacial acetic acid and 41 ml. of 2N hydrochloric acid and cooled to -3° in an ice-salt bath. The cooled solution was treated, with stirring, with a solution of sodium nitrate (0.59 g.) in 4 ml. of water. The azide, which separated immediately as a thick, stringy precipitate, was extracted into 40 ml. of ice cold ether. The ether solution was washed with water, sodium bicarbonate and again with water (all solutions ice cold) and dried for a short time (10 min.) with sodium sulfate. The dried solution of

carbobenzoxy-DL-phenylalanyl azide was then treated with an absolute ether solution of 0.98 g. of L-leucine methyl ester (prepared from 1.23 g. of the hydrochloride). After standing for 24 hr. at 0°, the reaction mixture was washed with N hydrochloric acid, sodium bicarbonate and water and dried over sodium sulfate. The solvent was removed under reduced pressure; yield (crystals): 1.82 g. By fractional crystallization from ethyl ether and then from ethyl ether - petroleum ether (65 - 110°), the same two compounds were as before (p. 23) when the coupling was performed with carbobenzoxyl - DL - phenylanylchloride; "Xa", m.p. 123.5 - 124° $[\alpha]^{23}_{D}$ - 15.9 (c-1, ethanol). Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57, Found: N, 6.25; "Xb", m.p. 101 - 105° $[\alpha]^{22.5}_{D}$ - 23.5 (c-1, ethanol). Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57, Found: N, 5.84.

XI. Carbobenzoxy-L-Phenylalanyl-L-Leucine Methyl Ester.

a. Carbobenzoxy-L-phenylalanine

L-Phenylalanine (6.6 g.) was treated in the same manner as for the preparation of carbobenzoxy-DL-phenylalanine; yield: 5.3 g., $[\alpha]^{24.5}_{D}$ + 5.53 (c-1.5 glacial acetic acid). The carbobenzoxy-L-phenylalanine was recrystallized from acetone-petroleum ether (65 - 110°); m.p. 128 - 130°. The constants previously reported for carbobenzoxy-L-phenylalanine (5) are $[\alpha]^{21}_{D}$ + 4.9), m.p. 126 - 128°.

b. L-Phenylalanine ethyl ester hydrochloride

L-Phenylalanine (5.0 g.) was treated in the same manner as for the preparation of DL-phenylalanine ethyl ester hydrochloride (p.20); yield: 4.4 g., m.p. 152 - 153° $[\alpha]^{23}_{D}$ - 6.58 (c-2 water).

c. Carbobenzoxy-L-phenylalanine ethyl ester.

L-Phenylalanine ethyl ester hydrochloride (3.0 g.) was treated in the same manner as for the preparation of carbobenzoxy-DL-phenylalanine ethyl ester. The product, after standing about a week at 5° crystallized into an oily mass, and was transformed directly into the hydrazide.

d. Carbobenzoxy-L-phenylalanyl hydrazide.

The carbobenzoxy-L-phenylalanine ethyl ester from the above preparation was dissolved in 15 ml. of absolute ethanol and warmed with 1.0 g. of hydrazine hydrate (98%) until complete solution resulted. At the end of 48 hr. the reaction mixture was treated with water and the precipitate filtered; yield: 3.0 g. On recrystallization from ethanol - water, the carbobenzoxy-L-phenylalanyl hydrazide had the following constants: m.p. 138 - 139°, $[\alpha]^{25}_{D}$ + 19.6 (c 2 ethanol).

e. Carbobenzoxy-L-phenylalanyl-L-leucine methyl ester.

1. Coupling through carbobenzoxy-L-phenylalanyl chloride.

Carbobenzoxy-L-phenylalanine (2.58 g.) was converted to the chloride in the same manner described in the preparation of carbobenzoxy-?-phenylalanyl-L-leucine methyl ester (p. 22). The chloride required about 1 hr. to crystallize; yield: 1.9 g. The solid chloride was then added to an ether solution of L-leucine methyl ester (from 2.15 g. of the hydrochloride) and left at 0° for 0.5 hr. and at room temperature overnight. After washing and drying the reaction mixture in the usual manner, the solvent was removed under

reduced pressure to yield an oil. This was dissolved in ethyl ether and the product was crystallized by the addition of petroleum ether (65 - 110°); m.p. 107 - 108°, $[\alpha]_D^{23}$ - 20.7 (c 1 ethanol). Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57. Found: N, 6.43. Upon admixture with a sample of carbobenzoxy-?-phenylalanyl-L-leucine methyl ester, m.p. 108 - 108.5°, the melting point was 106 - 107.5°. Upon admixture with a sample of carbobenzoxy-?-phenylalanyl-L-leucine methyl ester, m.p. 122.8 - 123.5°, the melting point was depressed to 82 - 88°.

2. Coupling through carbobenzoxy-L-phenylalanyl azide.

Carbobenzoxy-L-phenylalanyl hydrazide (2.1 g.) was coupled to L-leucine methyl ester (from 2.0 g. of the hydrochloride) in the same manner as described for the preparation of carbobenzoxy-DL-phenylalanyl-L-leucine methyl ester (p. 23); yield: 1.26 g., m.p. 105 - 105.5° $[\alpha]_D^{23}$ - 21.7. Anal. Calc'd. for $C_{24}H_{30}O_5N_2$: N, 6.57, Found: N, 6.42. Upon admixture with a sample of carbobenzoxy-?-phenylalanyl-L-leucine methyl ester, m.p. 101 - 105°, the melting point was 101 - 104°. Upon admixture with a sample of carbobenzoxy-?-phenylalanyl-L-leucine methyl ester, m.p. 123.2 - 124°, the melting point was depressed to 88 - 91°.

XII. Carbobenzoxy-DL-Phenylalanyl-DL-Phenylalanine Ethyl Ester. (38)

Carbobenzoxy-DL-phenylalanyl hydrazide (2.4 g.) was dissolved in a mixture of 18 ml. of glacial acetic acid and 42 ml. of 2 N hydrochloric acid and cooled to -3° in an ice-salt bath. The cooled solution was then treated, under stirring, with a solution of

sodium nitrite (0.54 g.) in 4 ml. of water. The azide, which appeared immediately as a thick, stringy precipitate, was dissolved in 40 ml. of ice cold ether and the ether solution washed with water, sodium bicarbonate and again with water (all solutions ice cold) and dried for a short time (10 min.) with sodium sulfate. The dried solution of carbobenzoxy-DL-phenylalanyl azide was then treated with an absolute ether solution of 2.3 g. of DL-phenylalanine ethyl ester (from 2.74 g. of the hydrochloride). After standing 24 hr. at 0°, the reaction mixture was washed with N hydrochloric acid, sodium bicarbonate, and water and dried with sodium sulfate. Upon concentration of the dried filtrate there remained behind a pale yellow glass which crystallized on standing; yield: 1.74 g., m.p. 92 - 98°. A second experiment yielded 2.36 g. of carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester as a white amorphous mass, which was saponified with N sodium hydroxide, directly. An attempt to synthesize carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester by coupling carbobenzoxy-DL-phenylalanyl chloride to DL-phenylalanine ethyl ester was unsuccessful.

XIII. Carbobenzoxy-DL-Phenylalanyl-DL-Phenylalanine.

Carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester (2.36 g.) was dissolved in 30 ml. of ethanol and treated with 15 ml. of N sodium hydroxide (3 mol.) at room temperature for 12 hr. Upon acidification with N hydrochloric acid and removal of the ethyl alcohol in vacuo, an oil remained, which was induced to crystallize from methanol - water. Recrystallization from the same solvents gave crystals: yield: 1.05 g., m.p. 107 - 136°. On drying at 100° and then at 118° in vacuo, the melting range became 132 - 136°. Anal.

Calc'd. for $C_{26}H_{26}O_5N_2$: N, 6.28 Found: N, 6.10, 6.03.
$C_{26}H_{26}O_5N_2H_2O$: N, 6.04

XIV. DL-Phenylalanyl-DL-Phenylalanine

Carbobenzoxy-DL-phenylalanyl-DL-phenylalanine (0.5 g.) was dissolved in 80 ml. of methanol, 6 ml. of glacial acetic acid and 5 ml. of water. Palladium catalyst was added and hydrogen was introduced in a slow stream until no further evolution of carbon-dioxide occurred, as determined by testing the exhaust with a saturated barium hydroxide solution. At the end of the reaction (5 hr.), the catalyst was filtered off and the solution concentrated to dryness. The residue was taken up in anhydrous methanol, and concentrated in vacuo several times to remove excess water and acetic acid; yield: 0.31 g. Recrystallization from hot methyl alcohol gave two fractions. Fraction 1 was insoluble in hot methanol (91 mg.) and gave only one spot in a paper chromatogram with R_f .82 (n-Butanol, Acetic acid, water: 4:1:5). Anal. Calc'd. for $C_{18}H_{20}O_3N_2$: N, 8.97. Found: N, 8.48. Fraction 2 was soluble in hot methanol and gave two spots on a paper chromatogram with R_f values .82 and .86, respectively (same solvent system as above). Anal. Calc'd. for $C_{18}H_{20}O_3N_2$: N, 8.97, Found: N, 10.19.

XV. Carbobenzoxy Glycyl-DL-Phenylalanine Ethyl Ester.

a. Carbobenzoxy glycine (4)

Glycine (7.5 g.) was dissolved in 25 ml. of 4 N sodium hydroxide at 0° and treated alternately and in portions over a period of about 20 min. with 17.0 g. of carbobenzoxy chloride and an additional 25 ml. of 4 N sodium hydroxide. On acidification to congo

red with concentrated hydrochloric acid the compound crystallized; yield: 18.7 g. Recrystallization from chloroform gave m.p. 115 - 117°.

b. Carbobenzoxy glycyl chloride (4)

Carbobenzoxy glycine (6.3 g.) was suspended in 35 ml. of anhydrous ether and shaken with 6.7 g. of finely powdered phosphorous pentachloride for 20 min. under ice cooling. At the end of the reaction, the ether was removed under reduced pressure and the residue washed twice with cold petroleum ether (30 - 60°). Under extreme cooling (acetone and dry ice) the chloride crystallized; yield: 3.6 g. A second similar experiment resulted in an oil which was dissolved in ether and coupled to DL-phenylalanine ethyl ester.

c. Carbobenzoxy glycyl-DL-phenylalanine ethyl ester.

Carbobenzoxy glycyl chloride (syrup from above) was dissolved in ether and added to an ether solution of 3.4 g. of DL-phenylalanine ethyl ester (from 3.8 g. of the hydrochloride). The reaction mixture was kept at 0° for 0.5 hr. and at room temperature overnight, washed with N hydrochloric acid, sodium bicarbonate and water and dried with sodium sulfate. A precipitate which remained after the washing was filtered off and recrystallized from ethyl acetate - petroleum ether; m.p. 87.5 - 89. Crystallization occurred during the drying so that it was necessary to extract the drying agent (sodium sulfate) with hot ethyl acetate. The ethyl acetate extracts were combined and the solvent removed under diminished pressure. The resulting residue was recrystallized from ethyl acetate - petroleum ether; yield: 1.45 g., m.p. 88 - 89.5°. Anal. Calc'd. for

$C_{21}H_{24}O_5N_2$: N, 7.29, Found: N, 7.30, 7.21.

XVI. Carbobenzoxy glycyl-DL-phenylalanine.

Carbobenzoxy glycyl-DL-phenylalanine ethyl ester (0.73 g.) was dissolved in acetone and treated with 57 ml. of N sodium hydroxide at room temperature for 8 hr. On acidification to congo red with N hydrochloric acid and removal of the acetone under reduced pressure an oil remained, which was extracted into ethyl acetate. The ethyl acetate solution was extracted with sodium bicarbonate, the bicarbonate layer acidified and re-extracted with ethyl acetate. After drying the final ethyl acetate extract with sodium sulfate and concentrating it to dryness, a white crystalline precipitate was obtained; yield: 0.63 g. The product was recrystallized from ethyl acetate - ether - methanol; needles, m.p. 159 - 160°. Anal. Calc'd. for $C_{20}H_{22}O_5N_2$: N, 7.86. Found: N, 7.92, 7.95.

XVII. Carbobenzoxy-DL-Phenylalanyl-DL-Valine Methyl Ester

a) DL-Valine methyl ester

DL-Valine (10 g.) was suspended in 125 ml. of anhydrous methanol. The suspension was saturated with dry hydrogen chloride gas and then heated under reflux for 3 hr. Repeated concentration, under reduced pressure with methanol, yielded a syrup which was treated twice more by the same procedure. The final syrupy product was taken up in methanol and crystallization was effected by careful addition of ethyl ether; yield: 8.1 g., m.p. 107 - 108°. Smith, Spackman and Polglase (33) report a melting point of 120 - 122° for a preparation of this compound which gave correct nitrogen and

methoxyl analyses. Fox and Minard (13) report a melting point of 90 - 97° for the same compound.

b) Carbobenzoxy-DL-phenylalanyl-DL-valine methyl ester.

Carbobenzoxy-DL-phenylalanyl azide (from 2.65 g. of the hydrazide) was treated with an absolute ether solution of 1.98 g. of DL-valine methyl ester (from 2.7 g. of the hydrochloride). The reaction mixture was kept at 0° overnight and 1.04 g. of carbobenzoxy-DL-phenylalanyl -DL-valine methyl ester which had crystallized was filtered off. The filtrate was washed and dried in the usual manner. Concentration of the filtrate yielded another 0.41 g. Recrystallization from ethyl ether - ethanol - petroleum ether (65 - 110°) gave crystals which were dried at 100° in vacuo, m.p. 132 - 133.5°. Anal. Calc'd. for $C_{23}H_{28}O_5N_2$: N, 6.68. Found: N, 6.56, 6.64.

XVIII. Carbobenzoxy-DL-Phenylalanyl-DL-Valine.

Carbobenzoxy-DL-phenylalanyl-DL-valine methyl ester (1.22 g.) was dissolved in acetone and treated with 2.96 ml. of N-sodium hydroxide at room temperature for 40 min. On acidification of the mixture and evaporation of the acetone in a stream of air, a crystalline precipitate was left, which was taken up in ethyl acetate and extracted with sodium bicarbonate. The sodium bicarbonate extract was acidified and the resulting crystalline precipitate filtered under suction; yield: 0.1 g. Recrystallization from methanol - water gave a melting range of 142 - 149°. Anal. Calc'd. for $C_{22}H_{26}O_5N_2$: N, 7.04. Found: N, 7.41.

XIX. Carbobenzoxy-L-α-Glutamyl-DL-Alanine Methyl Ester.

a) Carbobenzoxy-L-glutamic acid.

L-glutamic acid (8.8g.) and magnesium oxide (7.4 g.)

were added to 100 ml. of water that had previously been covered with a layer of 30 ml. of ether. To this mixture there was added, over a period of 0.5 hr., under ice cooling and with shaking, 20.4 g. of carbobenzoxy chloride. The mixture was then shaken at room temperature until almost all of the magnesium oxide had gone into solution. The mixture was then acidified to congo red with concentrated hydrochloric acid and extracted four times with ethyl acetate. The ethyl acetate extract was washed with 1 N hydrochloric acid, filtered through a dry filter and concentrated to a syrup in vacuo. The syrup was dissolved in a little ethyl acetate and crystallized by careful addition of petroleum-ether (30 - 60°); yield: 11.0 g., m.p. 114 - 116°. A second experiment gave 11.13 g., m.p. 113 - 116°. It was found in later experiments that a large excess of carbobenzoxy chloride was not necessary and that 1 mole of carbobenzoxy chloride to 1 mole of glutamic acid was satisfactory.

b) Carbobenzoxy-L-glutamic acid anhydride (4)

Carbobenzoxy-L-glutamic acid (10.0 g.) was warmed with 29 ml. of acetic anhydride for 5 min. at 100°. The solution was then concentrated to dryness under reduced pressure. The resulting syrup was dissolved in a little anhydrous chloroform and crystallized by the addition of petroleum-ether (30 - 60°) followed by cooling; yield: 5.5 g., m.p. 90 - 93°.

c) DL-Alanine methyl ester hydrochloride

DL-Alanine (10 g.) was treated in the same manner as for L-leucine methyl ester hydrochloride (p. 14); yield: 7.0 g. The product obtained upon recrystallization from methanol - ethyl acetate

gave m.p. 155 - 156°.

d) Carbobenzoxy-L-α-glutamyl-DL-alanine methyl ester

Carbobenzoxy-L-glutamic anhydride (5.0 g.) was added, at room temperature, to an anhydrous ether solution of DL-alanine methyl ester (from 5.26 g. of the hydrochloride). After 0.5 hr., sodium bicarbonate was added* until the solution was slightly alkaline. After 3 hr. stirring and 2 days at room temperature, the sodium bicarbonate layer was acidified and the resulting oil extracted into ethyl acetate. The ethyl acetate extract was dried with sodium sulfate and concentrated to dryness under reduced pressure. Attempts to crystallize the compound were unsuccessful; weight of dried oil: 5.10 g.

XX. Carbobenzoxy-L-α-Glutamyl-DL-Alanine.

Carbobenzoxy-L-α-glutamyl-DL-alanine methyl ester (3.1 g.) was treated with 17.0 ml. (2 moles) of N sodium hydroxide for 40 min. at room temperature. The acetone was then evaporated in a stream of air and the resulting oil extracted into ethyl acetate. The ethyl acetate layer was extracted with sodium bicarbonate. The bicarbonate solution was acidified and re-extracted with ethyl acetate. The final ethyl acetate extract was dried with sodium sulfate and the solvent removed under reduced pressure. Attempts to crystallize the compound were unsuccessful; weight of syrup: 2.18 g.

XXI. Carbobenzoxy-DL-Phenylalanyl-L-Tyrosine Methyl Ester.

a) L-Tyrosine methyl ester hydrochloride

L-Tyrosine (5 g.) was suspended in 35 ml. of anhydrous

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* Should use a larger excess of the ester.

methanol and saturated with dry hydrogen chloride gas. An additional 35 ml. of methanol was then added and the solution refluxed for 2 hr. Repeated concentration under diminished pressure gave needles; yield: 6.0 g., m.p. 187 - 188°. A second similar experiment yielded 4.1 g., m.p. 184 - 186°.

b) L-Tyrosine methyl ester.

L-Tyrosine methyl ester hydrochloride (4.1 g.) was dissolved in water and treated with 2.0 g. of calcium carbonate. The solution was extracted several times with ethyl acetate. Evaporation of the ethyl acetate solution under reduced pressure yielded crystals which were recrystallized from ethyl acetate; yield: 0.5 g., m.p. 132 - 133.5°.

c) Carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester

Carbobenzoxy-DL-phenylalanyl hydrazide (1.7 g.) was converted to the azide (p. 23) and treated with an absolute ethyl acetate solution of L-tyrosine methyl ester (from 1.3 g. of the hydrochloride). After 24 hr. at 0°, the solution was washed with N hydrochloric acid, sodium bicarbonate and water and dried with sodium sulfate. Concentration of the solution under diminished pressure yielded a glass which would not crystallize; weight: 1.96 g.

XXII. Carbobenzoxy-DL-Phenylalanyl-L-Tyrosine.

Carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester (1.5 g.) was dissolved in 20 ml. of ethanol and treated with 9.45 ml. of N sodium hydroxide (3 mol.) for 1 hr. at room temperature. Evaporation

of the ethanol in a stream of air yielded a syrup which was dissolved in methanol. On evaporation of the methanol a brown crystalline precipitate remained.

XXIII. Carbobenzoxy-L-Leucyl-DL-Valine Methyl Ester.

a) Carbobenzoxy-L-leucyl hydrazide.

L-Leucine methyl ester hydrochloride (5 g.) was dissolved in 30 ml. of water and covered with 55 ml. of chloroform and cooled to 0° in an ice-salt bath. Magnesium oxide (1.8 g.) and carbobenzoxy chloride (7.2 g.) were added alternately, and in three portions, over 0.5 hr. Pyridine was then added to decompose the excess carbobenzoxy chloride and the mixture was acidified with 5 N hydrochloric acid. The chloroform layer was washed twice with water, sodium bicarbonate and N hydrochloric acid, dried with sodium sulfate and evaporated under reduced pressure. The residue was dried by repeated concentration with anhydrous methanol, dissolved in 30 ml. of absolute methanol and treated with 2.0 g. of hydrazine hydrate (98%) for 24 hr. at room temperature. The mixture was then refluxed for 1 hr. and concentrated several times with anhydrous ether. A white crystalline mass resulted which was recrystallized from ethanol - water; yield: 3.0 g., m.p. 117 - 119°.

b) Carbobenzoxy-L-leucyl-DL-valine methyl ester.

Carbobenzoxy-L-leucyl hydrazide (2.25 g.) was dissolved in a mixture of 30 ml. of water, 8.0 ml. of glacial acetic acid and 20 ml. of 2 N hydrochloric acid, cooled to -3° and treated with sodium nitrite (1 g.) in 5 ml. of water. The resulting carbobenzoxy-L-leucyl azide was extracted into ether (previously cooled to 0°), washed with water,

sodium bicarbonate and again with water (all solutions at 0°), and dried with sodium sulfate. The azide was then treated with an absolute ether solution of DL-valine methyl ester (from 1.35 g. of the hydrochloride). After 24 hr. at 0°, the solution was washed and dried in the usual manner, and concentrated in vacuo. The resulting oil could not be crystallized readily; weight: 1.05 g.

B. Specificity of Pepsin.

The seven carbobenzoxy dipeptide esters, one carbobenzoxy dipeptide amide, and numerous other peptide derivatives, that were synthesized as described in Part A, above, were tested for their susceptibility to peptic hydrolysis. These compounds contained peptide bonds which Sanger and Tuppy (31) found to be susceptible to pepsin in the phenylalanyl chain of insulin.

In the experiments to be described below, proteolysis was detected by paper chromatography of the enzyme-substrate solutions. The amino acid(s) liberated by pepsin action were detected by means of the ninhydrin spray reagent, and the identity of the liberated amino acid was established by a parallel chromatogram of the authentic amino acid. The main difficulty in the enzymatic work was the insolubility of many of the substrates at the pH of the hydrolysis. This difficulty was overcome, in most cases, by dissolving the substrate first in methanol and adding buffer solution until the desired pH, substrate concentration and methanol-buffer ratio were reached. In most cases the substrate remained dissolved or as a fine suspension. A control experiment was

done with bovine γ -globulin as substrate. The hydrolysis of bovine γ-globulin by pepsin in the presence of increasing concentration of methanol is given in Table VIII.

TABLE VIII

Pepsin hydrolysis of bovine γ-globulin at increasing concentrations of methanol.

	Ratio of methanol - 0.01N HCl			
	0 - 5	2 - 5	3 - 5	4 - 5
Enzyme action	++++	+++	+++	+

γ -Globulin concentration, 2 per cent (1.0 ml. used)
Enzyme concentration, 2 mg. per ml., (0.5 ml. used)
pH, 2.0 (0.01N HCl) Temperature 36.7°

In the case of the carbobenzoxy dipeptide esters, the substrate was dissolved in 1.43 ml. of methanol and diluted to 5 ml. with 0.01N HCl (pH 2.0) or with 0.1 M acetate buffer, pH 3.5 (pH 4.0). It was found that when 3.57 mls. of 0.1 M acetate buffer (pH 3.5) was diluted to 5 mls. with methanol, the pH changed to 4.0. In all the enzymatic experiments γ -globulin was run as a control substrate. Substrate and enzyme blanks were also run and were negative in all cases. Table IX shows the results of the action of pepsin on a series of carbobenzoxy dipeptide esters and on one carbobenzoxy dipeptide amide.

TABLE IX

Action of pepsin on various synthetic substrates

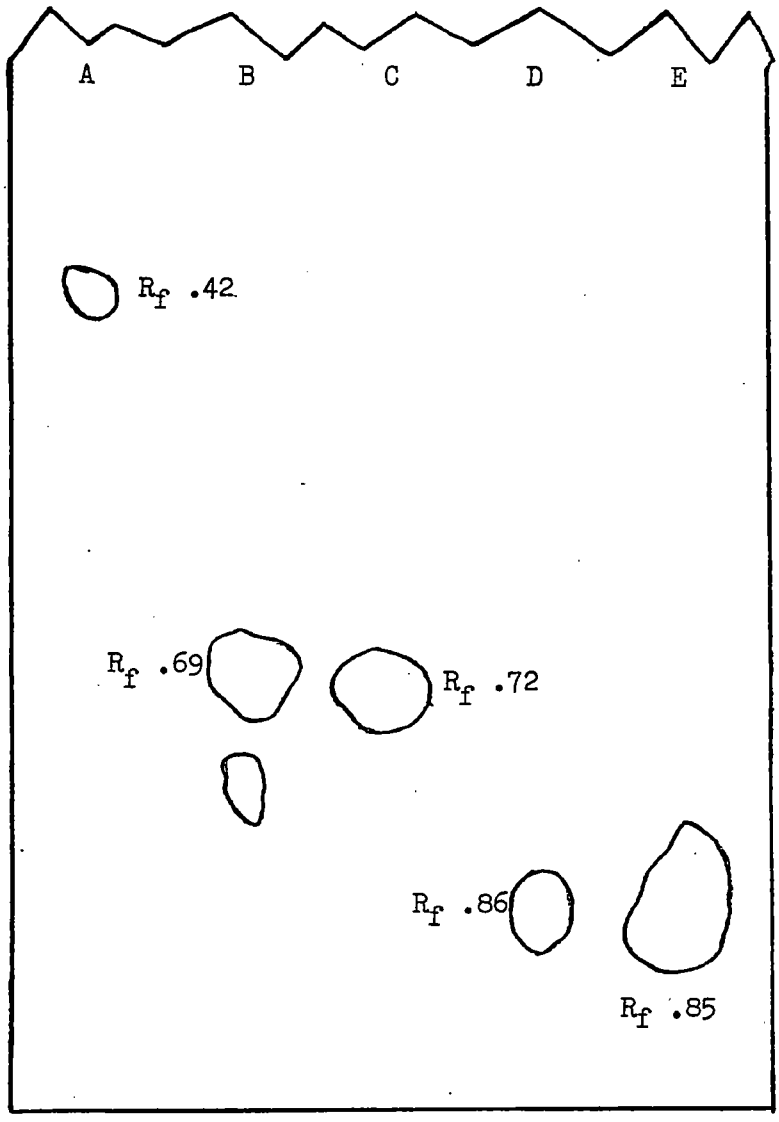
Substrate	Hydrolysis pH 2.0	Hydrolysis pH 4.0
1. Carbobenzoxy-DL-phenylalanyl-DL-valine methyl ester †	-	-
2. Carbobenzoxy-L-phenylalanyl-L-leucine methyl ester	-	-
3. Carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester	+	-
4. Carbobenzoxy-DL-phenylalanyl-DL-phenylalanyl ethyl ester	+	-
5. Carbobenzoxy-DL-alanyl-L-leucine methyl ester	-	-
6. Carbobenzoxy glycyl-DL-phenylalanine ethyl ester	-	-
7. Carbobenzoxy-DL-alanyl-L-leucinamide	-	-
8. Carbobenzoxy-L-leucyl-DL-valine methyl ester*	-	-
9. Enzyme blank (pepsin only)	-	-
10. γ-Globulin control	+	-

Synthetic substrate concentration = 4 mM per ml. (1.0 ml. used)
γ-Globulin concentration = 2 per cent (1.0 ml. used)
Enzyme concentration = 2 mg. per ml. (0.5 ml. used)
Temperature = 36.7°
Time of hydrolysis: pH 2.0, 10 hr.; pH 4.0, 18 hr.
* As an oil, therefore exact concentration not known.
† Some substrate crystallized out during the reactions

Another chromatogram was set up which contained the substrates carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester, carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester, plus pepsin at pH 2.0, as well as L-tyrosine, L-tyrosine methyl ester hydrochloride, and DL-phenylalanine ethyl ester hydrochloride. The results are shown drawn to scale in diagram I.

DIAGRAM I

Chromatogram of pepsin hydrolysis of synthetic substrates



A - Tyrosine
B - Carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester plus pepsin
C - L-Tyrosine methyl ester hydrochloride
D - Carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester plus pepsin
E - DL-Phenylalanine ethyl ester hydrochloride

Table X shows the results of pepsin action on a series of carbobenzoxy dipeptides and on one dipeptide.

TABLE X

Action of pepsin on various synthetic substrates

	Substrate	Hydrolysis pH 2.0	Hydrolysis pH 4.0
1.	Carbobenzoxy-DL-phenylalanyl-DL-valine †	±	-
2.	Carbobenzoxy-DL-phenylalanyl-L-tyrosine	-	-
3.	Carbobenzoxy glycyl-DL-phenylalanine	-	-
4.	Carbobenzoxy-L-α-glutamyl-DL-alanine *	-	-
5.	Carbobenzoxy-DL-phenylalanyl-DL-phenylalanine	-	-
6.	DL-Phenylalanyl-DL-phenylalanine	-	-
7.	γ-Globulin	+	-

Synthetic substrate concentration - 4 mM per ml. (1.0 ml. used)
γ-Globulin concentration, 2 per cent (1.0 ml. used)
Enzyme concentration - 2 mg. per ml. (0.5 ml. used)
Temperature - 36.7°
Time of hydrolysis - pH 2.0, 24 hr.; pH 4.0, 40 hr.
* As an oil, therefore exact concentration not known.
† Some substrate crystallized out during hydrolysis

DISCUSSION

A. Chemical Part.

One of the objectives of this research was to determine if peptides (or peptide derivatives) formed by reaction between an L-amino acid (or derivative) and a DL-amino acid (or derivative) can be separated readily to yield an L,D-peptide and an L,L-peptide (or suitable derivatives thereof). If this type of resolution of optical isomers could be widely used, synthetic peptides could be obtained more readily, at reasonable cost.

In one case, described below, the method was applied unsuccessfully while, in a second example, resolution was, apparently, achieved.

A crystalline product (I) was obtained when carbobenzoxy-DL-alanyl chloride was coupled with L-leucine methyl ester. The melting point of this product was 74 - 75°. Carbobenzoxy-D-alanyl-L-leucine methyl ester (II) had been reported previously (27) to have a melting point of 72 - 73°. When (I) and (II) were mixed, a melting point of 62 - 67° was observed. This depression of the melting point proves that the crystalline compound (I) obtained from the coupling with racemic carbobenzoxy-alanine was not carbobenzoxy-D-alanyl-L-leucine methyl ester.

Carbobenzoxy-L-alanyl-L-leucine methyl ester (III) has been prepared previously but only as a syrup (27). Therefore, it was not possible to compare (I) with (III) by the mixed melting point technique.

On the other hand, both carbobenzoxy-D-alanyl-L-leucinamide (IV) and carbobenzoxy-L-alanyl-L-leucinamide (V) have been characterized previously (27). It was therefore decided that the amide of (I) would allow a decision to be made regarding its identity. The results are summarized:

from ref. 27:

	m.p.	$[\alpha]_D$ (ethanol)
Carbobenzoxy-D-alanyl-L-leucinamide (IV)	187-188°	-6°
Carbobenzoxy-L-alanyl-L-leucinamide (V)	188-189°	-41°

from this work:

The amide obtained (VI) from product (I) from the coupling of carbobenzoxy-DL-alanyl chloride with L-leucine methyl ester	163-164°	-27.9°

The rotation of an equimolar mixture of carbobenzoxy-D-alanyl-L-leucinamide and carbobenzoxy-L-alanyl-L-leucinamide would be expected to be about -24°. This suggests that the crystalline amide (VI) ($[\alpha]_D$ - 27.9°) obtained in this work was an equimolar mixture of carbobenzoxy-D-alanyl-L-leucinamide and carbobenzoxy-L-alanyl-L-leucinamide, which may be called carbobenzoxy-DL-alanyl-L-leucinamide. The original crystalline product from the coupling of carbobenzoxy-DL-alanyl chloride and L-leucine methyl ester is therefore believed to be carbobenzoxy-DL-alanyl-L-leucine methyl ester.

If these deductions are correct, it indicates that diastereoisomers of this type may readily crystallize together to give a [illegible] MIXTURE and that separation of pure diastereoisomers (at this stage of

the synthesis at least) in the peptide series frequently may be difficult. Further work would have to be carried out to determine if separation of diastereoisomers may be practical at a later stage in peptide synthesis.

When carbobenzoxy-DL-phenylalanyl chloride was coupled to L-leucine methyl ester, two crystalline products were separated by fraction crystallization. One of the products (VII) had a melting point of 123.2 - 124° while the other (VIII) had a melting point of 108 - 108.5°. Carbobenzoxy-L-phenyl alanyl-L-leucine methyl ester (IX) was prepared (experimental) and found to have a melting point of 107 - 108°. When (VII) and (IX) were mixed a melting range of 82 - 88° was observed. When (VIII) and (IX) were mixed a melting point of 106 - 107.5° was obtained. When equal portions of (VII) and (VIII) were mixed a melting range of 90 - 92° was observed. Mixed melting point data suggest that (VIII) is probably carbobenzoxy-L-phenylalanyl-L-leucine methyl ester. Carbobenzoxy-D-phenyl-alanyl-L-leucine methyl ester (X) was not prepared and no report of its synthesis has been found in the literature, therefore it was not possible to compare (X) with either (VII) or (VIII) by the mixed melting point technique. Essentially the same compounds were obtained when carbobenzoxy-DL-phenylalanyl azide was coupled to L-leucine methyl ester, although the constants were slightly different. Although the rotations of (VIII) and (IX) were quite similar (-21.6 and -20.7, respectively), the rotations of the corresponding compounds prepared from coupling carbobenzoxy-DL-phenylalanyl azide with L-leucine methyl ester were quite different (-15.9 and -23.5, respectively). The rotation of (VII) (-20.8) was also the same as (VIII) and (IX). This similarity in

rotations of (VII) and (VIII) is difficult to understand if (VII) is assumed to be either carbobenzoxy-D-phenylalanyl-L-leucine methyl ester or carbobenzoxy-DL-phenylalanyl-L-leucine methyl ester (XI). The results are summarized in Table XI.

TABLE XI

Compounds separated from the coupling of carbobenzoxy-phenylalanine with L-leucine methyl ester.

	m.p.		$[\alpha]_D$ (ethanol)	
	A	B	A	B
Carbobenzoxy-?-phenylalanyl-L-leucine methyl ester	123.2 - 124°	123.2 - 124°	-20.8°	-15.9°
Carbobenzoxy-?-phenylalanyl-L-leucine methyl ester	108 - 108.5°	101 - 105°	-21.64°	-23.5°
	C	D	C	D
Carbobenzoxy-L-phenylalanyl-L-leucine methyl ester	107 - 108°	105 - 105.5°	-20.7°	-21.7°

A - Coupling performed with carbobenzoxy-DL-phenylalanyl chloride

B - Coupling performed with carbobenzoxy-DL-phenylalanyl azide

C - Coupling performed with carbobenzoxy-L-phenylalanyl chloride

D - Coupling performed with carbobenzoxy-L-phenylalanyl azide.

In the synthesis of carbobenzoxy-DL-phenylalanyl-L-leucine methyl ester, therefore, it has been possible to separate at least one of diastereoisomers by fractional crystallization.

It was observed, upon paper chromatography of DL-phenylalanyl-DL-phenylalanine, that two spots appeared. Since four isomers are formed

from the coupling, D-phenylalanyl-L-phenylalanine (XII), D-phenylalanyl-D-phenylalanine (XIII), L-phenylalanyl-D-phenylalanine (XIV) and L-phenylalanyl-L-phenylalanine (XV), it is probable that one spot is composed of (XII) and (XIV) while the other spot is made up of (XIII) and (XV).

B. Enzymatic Part.

From the results of the enzymatic work it can be seen that the specificity of pepsin is much higher in the hydrolysis of simple dipeptide derivatives than it is in the splitting of larger polypeptide or protein molecules. The splitting of carbobenzoxy-DL-phenylalanyl-DL-phenylalanine ethyl ester (XVI) and carbobenzoxy-DL-phenylalanyl-L-tyrosine methyl ester (XVII) by pepsin is unique in that no cases of peptic hydrolysis of a carbobenzoxy dipeptide ester has been reported. The phenylalanyl-tyrosine bond has also not previously been reported as being split by pepsin. Baker (2) treated carbobenzoxy-L-phenylalanyl-L-phenylalanine amide with pepsin at pH 2.0 for 6 days without getting any hydrolysis. Baker's substrate, however, was not in solution but was present as a fine suspension. The fact that both (XVI) and (XVII) contain two aromatic residues and are hydrolysed at pH 2.0 is in agreement with Baker's results. The resistance to pepsin of carbobenzoxy-DL-phenylalanyl-DL-phenylalanine was unexpected since Baker (2) found that acetyl-DL-phenylalanyl-DL-phenylalanine was hydrolysed quite rapidly at pH 2.0. Greenstein and co-workers (12), however, have observed that in the treatment of certain N-carbobenzoxy racemic amino acids (aspartic, asparagine, serine, glutamic, glutamine, alanine and arginine) by a crude aqueous extract of hog kidney,

the amide bond was either resistant to hydrolysis, or hydrolysis was much slower than with the corresponding N-acetyl racemic amino acids. For example, the rate of hydrolysis of N-carbobenzoxy-DL-glutamic acid by the above enzyme preparation was 0.8 micromoles per hr. per mg. protein nitrogen, as compared to 140 for the corresponding acetyl derivative. It appears, therefore, that in using synthetic substrate for the determination of pepsin specificity, the type of N-acyl blocking group is critical.

A more precise delineation of enzyme specificity might be obtained if longer chain peptides were used as substrates. In this type of substrate, the blocking group would be a peptide and the structure of a protein molecule would be more closely approximated. It is known that a peptide bond between two amino acids in a protein molecule is weaker than a bond involving the same two amino acids in a simple dipeptide. This fact may explain why certain bonds that Sanger found to be split in the phenylalanine chain of the insulin molecule were not split when present as the carbobenzoxy dipeptide ester. It may also be possible that the folding of the protein molecule may determine whether hydrolysis will take place or not and if this is the case, each protein may have to be considered separately as far as peptic hydrolysis is concerned. It therefore appears that, whereas pepsin has a low specificity toward the hydrolysis of protein molecules, it does have a fairly high degree of specificity towards simple synthetic substrates. In the latter, at least one of the constituent amino acids must be aromatic.

A comparison between the action of pepsin on a large polypeptide and on simple synthetic substrates is given in the following Table.

TABLE XII

A summary of the bonds split by pepsin

Peptide bond	In the insulin molecule (from Sanger)	In synthetic substrates	Reference
Glutamyl-glutamyl	++	Negative	15
Valyl-cysteic acid	++	not tested	
Tyrosyl-glutamyl	++	"	
Glutamyl-leucyl	++	"	
Leucyl-glutamyl	+++	"	
Glutamyl-aspartyl	+++	"	
Phenylalanyl-valyl	++	negative	**
Glutamyl-histidinyl	++	not tested	
Leucyl-valyl	+++	Negative	**
Glutamyl-alanyl	++	"	**
Alanyl-leucyl	++	"	**
Leucyl-tyrosyl	++ ; +++*	not tested	
Tyrosyl-leucyl	+++	" "	
Glycyl-phenylalanyl	++	negative	**
Phenylalanyl-phenylalanyl	+++	positive	**, 2
Phenylalanyl-tyrosyl	+++	"	**
Glutamyl-tyrosyl	bond not present	"	15
Glutamyl-phenylalanyl	" " "	"	"

TABLE XII (cont.)

Peptide bond	In the insulin molecule (from Sanger)	In synthetic substrates	Reference
Glutamyl-glycyl	bond not present	Positive	15
Glycyl-tyrosyl	" " "	Split weakly	"
Tyrosyl-tyrosyl	" " "	Positive	15, 2
Cysteinyl-tyrosyl	" " "	"	18
Cystinyl-tyrosyl	" " "	"	"
Tyrosyl-cysteinyl	" " "	"	"
Tyrosyl-cystinyl	" " "	"	"
Methionyl-tyrosyl	" " "	"	8
Tyrosyl-phenylalanyl	" " "	"	2

+++ indicates major site of action by pepsin
++ indicates weak action by pepsin
* This bond was split strongly by pepsin in the glycyl chain of insulin but much more weakly in the phenylalanyl chain.

** Table IX of this thesis.

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