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# Reactions of Diacylamines Containing N-Protected Aminoacyl Groups<sup>\*</sup>

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ABSTRACT: Diacylamines of the type  $R_1CON(R_2)$ -COC<sub>6</sub>H<sub>5</sub>, where  $R_1CO$  is derived from a N-protected  $\alpha$ -amino acid or peptide, have been synthesized by the reaction of imidoyl chlorides with appropriate carboxylic acids. Evidence for the diacylamine structure assigned to the products is offered by (a) the formation

he reactivity of the amide group has received recurrent attention in relation to the chemistry of peptides and proteins. Several communications (McConnan and Titherley, 1906; Wieland et al., 1955; Battersby and Robinson, 1955, 1956; Clayton et al., 1956; Brenner et al., 1957; Schofield, 1964; Shemyakin et al., 1965) describe the base-catalyzed reactions of the amide group with other functional groups, in particular when such reactions are favored by stereochemical factors. The intermediates postulated or shown to be formed belong to the general class of acylated amides (diacylamines).1 Diacylamines have been proposed as intermediates in the intramolecular participation of the CONH group in the solvolysis of esters (Bernhard et al., 1962; Shafer and Morawetz, 1963; Behme and Cordes, 1964) and have been considered as possible intermediates in the catalytic action of pepsin (Neumann et al., 1959; Fruton et al., 1961; Bender and Kézdy, 1965; Delpierre and Fruton, 1965). Relatively

2468

of the substituted imidazolone-5 after removal of the N-protecting group, and (b) their reactivity with hydroxylamine. Hydroxylaminolysis yields preferentially the hydroxamic acid derived from the carboxylic acid of higher apparent  $pK_a$ ; the mechanism of this reaction is discussed.

few diacylamines containing N-protected aminoacyl groups have been described (Bergmann *et al.*, 1929; Bergmann and Tietzman, 1944; Wieland and Urbach, 1958; Kopple and Renick, 1958; Schellenberg and Ullrich, 1959; Cramer and Baer, 1960), and the current interest in the reactivity of such compounds led us to prepare several new diacylamines containing amino acid units, and to examine their properties.

### Experimental Section<sup>2</sup>

Preparation of Imidoyl Chlorides. Powdered benzani-

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<sup>&</sup>lt;sup>1</sup> Various terms have been used in the recent English and German literature to designate the class of compounds RCON-(**R**')COR''. Among these is the term "imide," which appears to be intended for cyclic compounds [*Chem. Abstr. 56, 52N* (1962)]. Other terms are "diacylimide," "diacylamide," and "diacylamine." Pending a definitive recommendation by an appropriate body, we propose to use "diacylamine," since it appears to define this general class of compounds without ambiguity. In the special case where  $\mathbf{R}' = \mathbf{H}$ , however, the term "imide" has become well established through usage.

<sup>&</sup>lt;sup>2</sup> All melting points were uncorrected. Microanalyses were performed by Dr. S. M. Nagy, Massachusetts Institute of Technology. Infrared spectra were recorded with a Beckman 1R-5 spectrophotometer, and ultraviolet spectra were determined with a Beckman DB spectrophotometer.

lide (mp 162°, 0.01 mole) or benzoylbenzylamine (mp  $105^{\circ}$ , 0.01 mole) was mixed with PCl<sub>5</sub> (0.01 mole) in the absence of moisture. Dry benzene (20 ml) was added, and the reaction mixture was heated at 50-75° until the evolution of HCl had stopped (1-2 hr). The solvent and POCl<sub>3</sub> were removed by distillation under reduced pressure. N-Phenylbenzimidoyl chloride was obtained (92%) by trituration of the oily residue with petroleum ether (bp 30-60°), mp 38-40° [Wallach and Hoffmann (1877) reported mp 38-40°]. N-Benzylbenzimidoyl chloride was purified by distillation, bp 104-106° (55-60 mm) [Pechmann and Heinze (1897) reported bp 104° (60 mm)]. A strong infrared band was exhibited by the N-phenyl compound at 5.98  $\mu$ , and by the Nbenzvl compound at 5.92  $\mu$ , attributable to the C=N vibration (Bosshard and Zollinger, 1959; Arnold, 1959).

Preparation of Diacylamines. To a solution of the imidoyl chloride (0.01 mole) in dry benzene or chloroform (15 ml) was added a solution of the acid (0.01 mole) and triethylamine (0.01 mole) in benzene or chloroform (15 ml). The reaction mixtures were kept at room temperature for 2 hr or, if necessary, heated at  $55-60^{\circ}$  for 2 hr. When benzene was used as the solvent, the triethylammonium chloride was removed by filtration; with chloroform as the solvent, the amine salt was extracted with water, and the organic phase was dried over MgSO<sub>4</sub>. The diacylamines were usually recrystallized from benzene, chloroform-petroleum ether, or benzene-*n*-heptane.

*N*-Benzoyl(*N*-acetyl)aniline (Ia) was obtained in 95% yield, mp 63° [Cramer and Baer (1960) reported mp 63°]; *N*,*N*-dibenzoylaniline (Ib) was obtained in 98% yield, mp 160° [Mumm (1910) reported mp 161–162°]; *N*,*N*-dibenzoylbenzylamine (Id) was obtained in 93% yield, mp 107–108° [Beckmann (1893) reported mp 107–108°].

*N*-Benzoyl(*N*-benzyloxycarbonylglycyl)aniline (Ic) was obtained in 98% yield, mp 159–161°. *Anal.* Calcd for  $C_{23}H_{20}N_2O_4$  (388.4): C, 71.1; H, 5.2; N, 7.2. Found: C, 71.2; H, 5.2; N, 7.2.

*N*-Benzoyl(*N*-benzyloxycarbonylglycyl)benzylamine (Ie) was obtained in 95% yield, mp 122–124°. *Anal.* Calcd for  $C_{24}H_{22}N_2O_4$  (402.4): C, 71.6; H, 5.4; N, 6.9. Found: C, 71.3; H, 5.4; N, 6.8.

*N*-Benzoyl(*N*-benzyloxycarbonylglycylglycyl)benzylamine (If) was obtained in 80% yield. After recrystallization from acetone it melted at 138–140°. *Anal.* Calcd for  $C_{26}H_{25}N_3O_5$  (459.5): C, 67.9; H, 5.4; N, 9.1, Found; C, 67.6; H, 5.4; N, 9.1.

*N*-Benzoyl(*N*-tritylglycyl)benzylamine (Ig) was obtained in 75% yield, mp 140°, upon addition of petroleum ether to the benzene solution (after removal of the amine salt). Recrystallization from benzene did not change the melting point. *Anal*. Calcd for  $C_{35}H_{30}N_2O_2$ (510.6): C, 82.4; H, 5.9; N, 5.4. Found: C, 82.5; H, 6.0; N, 5.4. The product reacted at room temperature with hydroxylamine to form a hydroxamic acid, and exhibited infrared bands at 5.85 and 5.98  $\mu$  (KBr) associated with the imide CO groups (Abramovitch, 1957).

The filtrate obtained after removal of Ig with pe-

troleum ether yielded a by-product (10%) which melted at 195-198° after recrystallization from benzene; it exhibited infrared bands at 5.89 and 6.01  $\mu$  (KBr), and did not react with hydroxylamine at room temperature. *Anal.* Found: C, 82.0; H, 5.9; N, 5.3. A solution of this material (0.25 g) in acetone (5 ml) and concentrated hydrochloric acid (0.2 ml) was heated for 3 min on the steam bath. After removal of the solvent *in vacuo*, tritylcarbinol (0.11 g) was extracted with ether, and treatment of the residue with hot acetone gave a crystalline product (0.13 g) that melted at 208-210°; it exhibited an absorption maximum in the ultraviolet at 242 m $\mu$  (ethanol), and infrared bands at 5.93 and 6.10  $\mu$  (KBr). *Anal.* Found: C, 72.0; H, 5.0; N, 10.4. The structure of this by-product has not been established.

*N-Benzoyl*(*N-trityl*)glycinanilide. This compound was obtained in 90% yield from the reaction of *N*-phenylbenzimidoyl chloride with tritylglycine, instead of the expected *N*-benzoyl(*N*-tritylglycyl)aniline. After recrystallization from benzene–*n*-heptane, it melted at 188–189°. Anal. Calcd for  $C_{34}H_{28}N_2O_2$  (496.6): C, 82.3; H, 5.7; N, 5.6. Found: C, 82.0; H, 5.7; N, 5.7. The product did not react with hydroxylamine at room temperature.

A solution of this product (0.5 g, 1 mmole) in acetone (10 ml) and concentrated hydrochloric acid (0.3 ml) was heated on the steam bath for 3 min. Upon chilling the solution, hippurylanilide (0.22 g, 90%, mp 210°) crystallized. Recrystallization from ethanol did not change the melting point, and no depression of melting point was observed upon admixture with an authentic sample of hippurylanilide. From the filtrate, tritylcarbinol (0.24 g, mp 160°) was isolated.

*1-Benzyl-2-phenylimidazolone-5. N*-Benzoyl(*N*-tritylglycyl)benzylamine (Ig, 1.0 g) was dissolved in acetoneacetic acid (1:1) (10 ml) and heated for 4 min on the steam bath. After removal of the solvent *in vacuo* at 15°, the residue was dissolved in acetone (5 ml), 70% perchloric acid (0.2 ml) was added, and the imidazolone perchlorate was crystallized by the addition of ether; yield, 0.6 g (85%); mp 218-220°. *Anal.* Calcd for. C<sub>16</sub>H<sub>15</sub>ClN<sub>2</sub>O<sub>5</sub> (350.8): C, 54.8; H, 4.3; N, 8.0. Found: C, 55.2; H, 4.7; N, 7.9. Titration with sodium methoxide gave a neutralization equivalent of 365. The compound exhibited infrared bands at 5.79 and 6.08  $\mu$  (KBr), and in methanol it gave an ultraviolet absorption spectrum with peaks at 252 m $\mu$  ( $\epsilon$  14,700) and at 320 m $\mu$ ( $\epsilon$  7700).

Detritylation of Ig with acetone-HCl gave the imidazolone hydrochloride, mp 148–150°. Anal. Calcd for C<sub>16</sub>H<sub>16</sub>ClN<sub>2</sub>O (286.7): N, 9.8. Found: N, 9.6. Its ultraviolet absorption spectrum (in methanol) showed peaks at 252 m $\mu$  ( $\epsilon$  12,500) and at 320 m $\mu$  ( $\epsilon$  8800). The product is extremely hygroscopic and turns pink on standing (*cf.* Wieland and Biener, 1961).

Catalytic hydrogenolysis of *N*-benzoyl(*N*-benzyloxycarbonyl)benzylamine (Ie, 0.8 g, 2 mmoles) in methanol (30 ml) and 70% perchloric acid (1 ml), with palladium black as the catalyst, gave the imidazolone perchlorate (0.65 g, 94%, mp 218–220°). Its melting point was not depressed by admixture with a sample of the product obtained upon detritylation of Ig, and the infrared and ultraviolet absorption spectra of the two materials were identical.

The free base was obtained by dissolving the imidazolone perchlorate (0.35 g, 1 mmole) in 60% acetonewater, and adjusting the pH to 7.5 by the addition of 0.1 N NaOH. Dilution with water and cooling gave 0.2 g (80%) of crystalline product, which was recrystallized from methanol-water (mp 69-71°). *Anal*. Calcd for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>O (250.3): C, 76.8; H, 5.6; N, 11.2. Found: C, 76.9; H, 5.5; N, 11.0. Its ultraviolet absorption spectrum (in methanol) showed peaks at 266 m $\mu$  ( $\epsilon$  10,000) and at 315 m $\mu$  ( $\epsilon$  10,500), and the compound exhibited infrared bands at 5.92 and 6.1  $\mu$  (KBr). The shifts in the ultraviolet absorption spectrum upon addition of acid to the free base are in accord with those described for imidazolones (Kjaer, 1953).

Reaction of Diacylamines with Hydroxylamine. For the analytical studies, a solution of hydroxylamine was prepared by mixing equal volumes of a methanolic solution of 1.8 M hydroxylamine hydrochloride with a methanolic solution of 3.1 M NaOH, and removal of NaCl by filtration (Goddu et al., 1955). This solution was used to prepare the reaction mixtures containing a diacylamine (0.05 M) and hydroxylamine (0.25 M) in methanol. After 20 min at room temperature, samples (corresponding to 1-5  $\mu$ moles of diacylamine) were subjected to thin layer chromatography (silica gel) with 2-butanol-formic acid-water (75:15:10) as the solvent (Wieland and Stimming, 1953), and the chromatograms were developed by spraying them with an ethanolic solution of 5.7 mм FeCl<sub>3</sub> and 0.16 м perchloric acid. The areas of gel showing violet spots were removed and eluted with 1% trichloroacetic acid (3 ml); the eluates were centrifuged, and to the supernatant fluids was added 3 ml of the above FeCl<sub>3</sub> solution. The absorbance of the ferric hydroxamates was measured at 540 mµ, and the amounts of the hydroxamates were estimated by reference to the absorbance of the ferric complexes of appropriate standard compounds. The following compounds were used as standards: acetohydroxamic acid,  $R_F$  0.55, mp 89–91° [Hurd and Cochran (1923) reported mp 86-88°]; benzohydroxamic acid,  $R_F$  0.82, mp 130° [Blatt (1943) reported mp 128-130°]; benzyloxycarbonylglycylhydroxamic acid,  $R_F$  0.90, mp 115-118° [Hoffmann and Faiferman (1964) reported mp 118-120°] [Anal. Calcd for  $C_{10}H_{12}N_2O_4$  (224.2): N, 12.5. Found: N, 12.7]; benzyloxycarbonylglycylglycylhydroxamic acid, R<sub>F</sub> 0.65, mp 153-154° [Hoffmann and Faiferman (1964) reported mp 153-154°] [Anal. Calcd for C<sub>13</sub>H<sub>15</sub>N<sub>3</sub>O<sub>5</sub> (281.2): N, 14.9. Found: N, 15.0]. For the preparation of tritylglycylhydroxamic acid, tritylglycine ethyl ester and hydroxylamine were kept in methanol for 35 hr at room temperature. The solution was acidified to pH 2, the ethanol was removed in vacuo, and the residue was extracted with chloroform. The chloroform extract yielded an oil that could not be crystallized. Anal. Calcd for C<sub>21</sub>H<sub>20</sub>N<sub>2</sub>O<sub>2</sub> (332.4): N, 8.4. Found: N, 7.9. The substance has  $R_F$  0.45, and the absorbance of its ferric complex is 90% that of the acetohydroxamic acid complex and 40% of that of the

2470

benzohydroxamic acid complex. In acid solution, tritylglycylhydroxamic acid is unstable; on extended storage it decomposes to yield triphenylmethane (95%, mp 90–91°) and an unidentified oily product.

For the isolation of the products in the reaction of diacylamines with hydroxylamine, N-benzoyl(N-benzyloxycarbonylglycyl)aniline (Ic, 0.39 g, 1 mmole) was dissolved in methanol (5 ml), and methanolic hydroxylamine (3 ml, 5 mmole) was added. After 30 min at room temperature, the solvent was removed in vacuo, and the residue was triturated with water to yield benzyloxycarbonylglycinanilide (0.27 g, 96%, mp 144-146°). Admixture with an authentic sample (mp 146-147°; Vaughan and Osalo, 1951) did not lower the melting point, and the infrared spectra of the isolated and authentic samples were identical. The aqueous filtrate was acidified to pH 3, the water was removed in vacuo, and the residue was extracted with ethanol. Addition of ether gave benzohydroxamic acid (0.09 g, 70%, mp 128-130°).

From the reaction of *N*-benzoyl(*N*-benzyloxycarbonylglycyl)benzylamine (Ie) with hydroxylamine under the above conditions, there were isolated benzyloxycarbonylglycine benzylamide (92%), mp 114° [Schwyzer *et al.* (1955) reported mp 114°], and benzohydroxamic acid (73%). The infrared spectra of the isolated and authentic samples of the benzylamide were identical.

From the reaction of *N*-benzoyl(*N*-acetyl)aniline (Ia, 1.2 g, 5 mmoles) with hydroxylamine (25 mmoles) in methanol (13 ml) there was isolated benzanilide (0.8 g, 77%, mp 162°). Chromatographic analysis of the aqueous filtrate indicated the presence of aceto-hydroxamic acid and of benzohydroxamic acid in a molar ratio of 5:1.

Determination of Ionization Constants. Solutions of the acids (0.05 M) in 60% methanol-water (v/v) were titrated with 0.1 N NaOH, using a Radiometer TTT1 automatic titrator. The apparent  $pK_a$  values were calculated by means of the Henderson-Hasselbalch equation. The glass electrode and calomel electrode were soaked in 60% methanol for 2 days before they were used.

### Results

In the present study, use was made of the rearrangement of isoimides (formed by the reaction of imidoyl chlorides with salts of carboxylic acids) to diacylamines, discovered by Mumm *et al.* (1915). The mechanism of the four-center rearrangement of isoimides to diacylamines has been the subject of recent studies (Stevens and Munk, 1958; Curtin and Miller, 1965). In our work, use was made of *N*-phenyl- and *N*-benzylbenzimidoyl chloride, prepared by treatment of the appropriate arr ide with PCl<sub>5</sub>. Efforts to prepare the corresponding irridoyl chlorides from N-protected glycinanilides or glycine benzylar ides were unsuccessful (*cf.* Braun and Rudolph, 1934).

When equimolar quantities of *N*-phenylbenzimidoyl chloride, triethylamine, and a carboxylic acid (acetic acid, benzoic acid, or benzyloxycarbonyl<u>e</u>lycine) were

allowed to react in benzene at room temperature, the corresponding diacylamines (Ia-c) were obtained in satisfactory yield. Similarly, with *N*-benzylbenzimidoyl chloride, the diacylamines Id-g were obtained by reaction with benzoic acid, benzyloxycarbonylglycine, benzyloxycarbonylglycylglycine, and tritylglycine, repectively. The course of the reaction was followed by treatment of samples of the reaction mixture with hydroxylamine at room temperature, and determination of the extent of hydroxamate formation.

Ia,  $R = C_6H_5$ ;  $R' = CH_3$ b,  $R = C_6H_5$ ;  $R' = C_6H_5$ c,  $R = C_6H_5$ ;  $R' = C_6H_5CH_2OCONHCH_2$ d,  $R = C_6H_5CH_2$ ;  $R' = C_6H_5$ e,  $R = C_6H_5CH_2$ ;  $R' = C_6H_5CH_2OCONHCH_2$ f,  $R = C_6H_5CH_2$ ;  $R' = C_6H_5CH_2OCONHCH_2CO-NHCH_2$ g,  $R = C_6H_5CH_2$ ;  $R' = (C_6H_5)_3CNHCH_2$ 

The reaction of tritylglycine with *N*-phenylbenzimidoyl chloride did not yield the expected diacylamine; instead, an isomeric compound was obtained in nearly quantitative yield. The product did not react with hydroxylamine at room temperature and, upon detritylation, yielded hippurylanilide. The available evidence indicates that the product was *N*-trityl(*N*-benzoyl)glycinanilide (III), probably formed by an intramolecular transacylation reaction of the diacylamine II. An analogous acyl migration has been observed in the conversion of *N*-benzoyl(*N*-phenyl)ethylenediamine to



*N*-benzoyl(*N'*-phenyl)ethylenediamine (Stirling, 1958), and in the interconversion of  $N^{\alpha}$ -acyl- and  $N^{\gamma}$ -acyl- $\alpha$ , $\gamma$ -diaminobutyric acids (Poduska *et al.*, 1965).

Evidence for the diacylamine structure of compounds Ie and g was provided by the isolation of 1-benzyl-2phenylimidazolone-5 (IV) as the perchlorate, upon removal of the benzyloxycarbonyl group of Ie by catalytic hydrogenolysis or upon acidolytic detritylation of Ig. The formation of the imidazolone IV from the open-chain diacylamines Ie and g, of 2-phenylimidazolone-4 (5) from glycyl(benzoyl)imide (Wieland and Biener, 1961), and of imidazolones from *N*-phthaloylglycyl(*N*-glycyl)amino acid esters (Shemyakin *et al.*, 1965) support the hypothesis that the "amino acid inser-



tion" reaction (Brenner et al., 1957) may proceed via diacylamines.

Further evidence for the diacylamine structure of compounds Ia, c, and e-g was obtained from the study of their reaction with hydroxylamine in methanol, and thin layer chromatography permitted the separation, identification, and estimation of the resulting hydroxamic acids (as their ferric complexes). The following values were obtained for the molar percentage of benzohydroxamic acid (formed from all the diacylamines tested) in relation to the total hydroxamic acid formed: Ia, 18%; Ic, ca. 100%; Ie, ca. 100%; If, 63%; Ig, 33% (the last value is subject to greater error than the others because of the instability of tritylglycylhydroxamic acid). These results may be considered in relation to the apparent  $pK_a$  values (determined in 60% methanol-water at 25°) of the following acids: acetic acid, 5.22; benzoic acid, 4.73; benzyloxycarbonylglycine, 4.24; benzyloxycarbonylglycylglycine, 4.58; tritylglycine, 6.35. It will be noted that, in the reaction of the diacylamines with hydroxylamine, the hydroxamic acid that is formed preferentially is derived from the acyl group corresponding to the carboxylic acid of higher  $pK_a$ . In the cases of the diacylamines Ic and e, only traces of benzyloxycarbonylglycylhydroxamic acid were detected by chromatography; when the reactions were run on a preparative scale, the benzyloxycarbonylglycinamides were isolated in nearly quantitative vield.

The reaction of diacylamines with methanolic hydroxylamine in a neutral or alkaline medium may be expected to proceed by nucleophilic attack of the base

2471

on either of the two carbonyl groups to form a tetrahedral intermediate analogous to that proposed for the hydroxylaminolysis of simple amides (Jencks and Gilchrist, 1964). Although the possibility can be considered that, as "activated" acyl compounds, the diacylamines may initially acylate the oxygen of hydroxylamine, the presence of high concentrations of the base would lead to further reaction to yield the hydroxamic acid (Jencks, 1958). If, under our experimental conditions, the rate-controlling step in the hydroxylaminolysis of diacylamines were the formation of a tetrahedral intermediate, and the more electrophilic carbonyl group is the one derived from the acid of lower  $pK_a$ , that group would have been expected to react preferentially. Since the reverse was found, alternative mechanisms must be considered. One possibility is that the observed molar ratio of hydroxamic acids is an expression of the relative ability of the anilides (or benzylamides) and the hydroxamic acids to leave the intermediate. The rate-determining step would be the decomposition of the intermediate to products, a process that would be influenced both by steric factors and by the composition of the solvent.

It should be noted that the results for the reaction of diacylamines with hydroxylamine are in contrast to those obtained for the alkaline hydrolysis of unsymmetrical diacylamines, where the removal of the stronger acid is favored (Lamberton and Standage, 1960). In the case of the hydroxylaminolysis of mixed carboxylic acid anhydrides, the carbonyl group derived from the acid of lower  $pK_a$  was found to react preferentially, with acetic benzoic anhydride an exception (Wieland and Stimming, 1953). The role of the solvent in such reactions is indicated, however, by studies on the chloroacetylation of aniline by chloroacetic acetic anhydride (Emery and Gold, 1950; Tedder, 1955). Clearly, further kinetic studies on the reaction of diacylamines with nucleophiles are needed for an understanding of the mechanism of this process.

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# New Synthetic Substrates for Pepsin\*

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ABSTRACT: The synthesis of several new peptide substrates for crystalline swine pepsin is described. They include benzyloxycarbonyl-L-histidyl-L-phenylalanyl-Lphenylalanine ethyl ester and related peptide derivatives in which one or both phenylalanyl residues have been replaced by L-tyrosyl or L-tryptophyl residues. These compounds, as well as glycylglycyl-L-phenylalanyl-L-phenylalanine ethyl ester, are cleaved rapidly at the peptide bond between the two aromatic amino acid residues, the pH optimum being near 4. Benzyl-

Tince the discovery of the first synthetic substrates (e.g., Z-Glu-Tyr<sup>1</sup>) for crystalline swine pepsin (Fruton and Bergmann, 1939), several investigators, notably Baker (1951), have added peptide derivatives that have proved to be valuable for the study of the kinetics of pepsin action. In such studies (Silver et al., 1965; Cornish-Bowden and Knowles, 1965; Jackson et al., 1965), the preferred substrates have been acetyl dipeptides such as Ac-Phe-Tyr and Ac-Phe-Dityr, which are hydrolyzed optimally near pH 2. In connection with our studies on the effect of pepsin substrates on the inactivation of the enzyme by diphenyldiazomethane (Delpierre and Fruton, 1965), it was necessary to prepare synthetic substrates that do not have a carboxyl group, as the reagent readily attacks such groups to form benzhydryl esters (Hiskey and Adams, 1965; Aboderin et al., 1965). Because the conversion of the carboxyl group of an acetyl dipeptide to an ester or

oxycarbonyl-L-histidyl-L - phenylalanyl-L - phenylalanine is hydrolyzed by pepsin more slowly than the corresponding ethyl ester, with a pH optimum near 3. These differences in pH optima, and the behavior of previously known synthetic substrates such as acetyl-Lphenylalanyl-L-tyrosine, are discussed in terms of the hypothesis that an  $\alpha$ -carboxylate group adjacent to the sensitive peptide bond is inhibitory to pepsin action. Several of the new synthetic substrates enhance the rate of inactivation of pepsin by diphenyldiazomethane.

amide usually tends to make the resulting compound even less soluble in water than the parent compound, we decided to replace the acetyl group by either benzyloxycarbonylhistidyl or glycylglycyl, thus providing a site of protonation of the acylpeptide ester. Accordingly, the following compounds were prepared: Z-His-Phe-Phe-OEt, Z-His-Phe-Tyr-OEt, Z-His-Phe-Trp-OEt, Z-His-Tyr-Tyr-OEt, and Gly-Gly-Phe-Phe-OEt. All these compounds were found to be good substrates for pepsin, the cleavage occurring between the two aromatic amino acid residues. In the present communication, we report the synthesis of the new substrates and of several compounds related to them, some features of the kinetics of their hydrolysis by pepsin, and their effect on the inactivation of pepsin by DDM.

#### **Experimental Section**

*Enzymic Studies.* All experiments (except when otherwise noted) were performed with a single preparation of twice-crystallized swine pepsin (Worthington Biochemical Corp. Lot No. PM 708), whose specific activity was found to be  $2595 \pm 115$  units/mg (mean of five determinations) when assayed with denatured hemoglobin (1.67%) as substrate at pH 1.8 and 30°. After an incubation period of 10 min, 1.67 volumes of 5% TCA was added to the assay mixture, and the absorbance of the filtrate was determined at 280 m $\mu$  (Anson, 1938). One pepsin unit is defined as the amount of enzyme that produces in this assay an increase in absorbance of 0.01 above the blank, using

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<sup>&</sup>lt;sup>1</sup> Abbreviations used: Gly, glycyl; Phe, L-phenylalanyl; Tyr, L-tyrosyl; Trp, L-tryptophyl; His, L-histidyl; Glu, L-glutamyl; Dityr, diiodo-L-tyrosyl; Ac, acetyl; Bz, benzoyl; Z, benzyloxycarbonyl; OEt, ethoxy; OBu', *t*-butoxy; Onp, *p*-nitrophenoxy; DCC, dicyclohexylcarbodiimide; DDM, diphenyldiazomethane; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; DMF, dimethylformamide; THF, tetrahydrofuran. The abbreviated designation of derivatives of amino acids and peptides accords with the proposals of the Joint IUPAC-IUB Commission on Biochemical Nomenclature.