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Peptide Synthesis from N-Carboxy-α-amino Acid Anhydrides in Water. Aminoacylpenicillanic Acids

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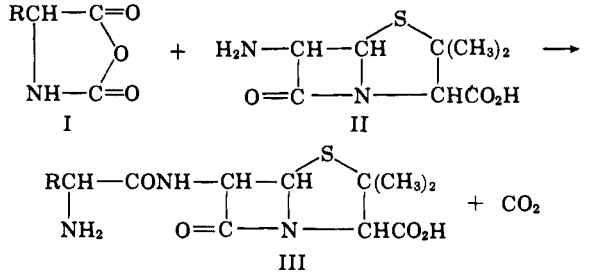
The coupling in aqueous systems between N-carboxyanhydrides of amino acids (NCA's) and amino acids such as 6-aminopenicillanic acid (6-APA) can be controlled to give high yields of dipeptide. Minimization of rearrangement, polymerization, and hydrolysis reactions depends upon selection of a pH level and ratio of reactant concentrations capable of maintaining the total base strength of attacking 6-APA, established by both the pK_2 and the concentration of the anionic species, above the total base strength of the dipeptide and of the NCA hydrolysis product. With N-carboxy-D-phenylglycine anhydride, the pH optimum ranges from 5.0 (anhydride:amino acid ratio = 1.5) to 6.0 (ratio = 0.1). The optimum pH is about 5.0 with 6-APA and equal concentrations of the NCA's of glycine, L-alanine, D-tryptophan, and 1-aminocyclobutanecarboxylic acid. Higher dipeptide yields result from increasing the relative amount of amino acid. Replacement at fixed pH of part or all of the excess 6-APA by strongly or weakly basic amines is ineffective. Because of the low solubility of the NCA's, the reaction is heterogeneous over much of its course. However, solubilizing the NCA's by the addition of water-miscible solvents inhibits rather than promotes the reaction.

Introduction

When an N-carboxy-α-amino acid anhydride condenses with an amino acid or ester, carbon dioxide is split off and the amino group is liberated. Activation of the carboxyl group and blockage of the amino group in one step, followed by carboxyl coupling and removal of the blocking agent in a second step, would appear to make this an attractive synthetic method. There has, however, been general pessimism regarding the use of these anhydrides for controlled, stepwise peptide synthesis because of extremely facile polymerization.[1-4] In addition to simple dipeptides and large polypeptide polymers, reaction products in organic solvents have been shown to include the amino acid from which the anhydride is derived, the N-carboxyamino acid, various carbamic acids, ureido derivatives, and hydantoins.

The present study examines the reaction in water between several N-carboxy-α-amino acid anhydrides (4-substituted 2,5-oxazolidinediones, I) and a rather complex amino acid, 6-aminopenicillanic acid (6-APA, II). The amino acid carries a free carboxyl on a carbon remote from that to which the amino group is attached and a second carboxyl, bound in a lactam, on the same carbon as the amino group. The problem of forming



I II

III

aminoacylpenicillanic acids underscores two of the recurrent difficulties in peptide synthesis: retention of steric configuration and avoidance of conditions promoting rearrangement or hydrolysis. The D-epimer of α-aminobenzylpenicillin (III, R = phenyl) is superior to the L-epimer as a broad-spectrum antibiotic.[5] In addition, although this compound is unusually stable in acid solutions, its β-lactam has the characteristic penicillin lability to bases. Synthesis from D-(−)-N-carbobenzoxy-α-amino-α-phenylacetic acid[5] successfully avoids racemization, but an exceptionally large amount of catalyst is needed in the hydrogenolysis step, probably stemming from poisoning by the penicillin sulfur. Moreover, we have recently found that in the presence of bases the β-lactam of 6-APA can be ruptured to form poly-6-aminopenicillanic acid[6] as well as the penicilloic acid.

Experimental

The N-carboxyamino acid anhydrides (NCA) were synthesized by the phosgenation procedure. Methods for those of D- and DL-phenylglycine, 1-aminocyclopentanecarboxylic acid, and 1-aminocyclobutanecarboxylic acid[7] have not been previously reported. Published methods for those of glycine, D-tryptophan, and L-alanine were followed. The products were recrystallized from ethyl acetate.

The purity and stability of the anhydrides were determined by titration with standardized alcoholic sodium methoxide. A second method, depending upon reaction with hydroxylamine, was developed and standardized against the titration method. The procedure is as follows: the anhydride sample is dissolved to a concentration of 0.2 to 1.0 mg./ml. in the "working hydroxylamine reagent" of Niedermayer, *et al.*,[8] and stirred by a magnetic stirrer for 6 min. The solution or suspension is filtered, and 2.0 ml. of filtrate is mixed with 1.0 ml. of water and 2.0 ml. of a solution consisting of $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 70 ml. of 1 *N* H_2SO_4. The orange-brown color is read immediately on a colorimeter, such as the Klett, with a 540 mμ filter. The absorbance is linear with concentration. For N-carboxy-D-phenylglycine anhydride, melting at 123 to 125° and giving the theoretical equivalent weight on titration, the absorbance for an initial concentration of 1 mg./ml., read from the fitted line, is 0.900.

D-Phenylglycine was purchased from the L. Perrigo Co., Allegan, Mich. 1-Aminocyclopentanecarboxylic acid was synthesized by the Bucherer–Berg hydantoin method, and 1-aminocyclobutanecarboxylic acid was synthesized by the method of Ingold, *et al.*[9] 6 APA was prepared enzymatically,[10] and it assayed 98% pure by iodometric and hydroxylamine assays.

The concentration of a newly synthesized aminopenicillin was best determined by antimicrobial assays. These were carried out by the cylinder plate method against a Gram-positive organism, *Straphyloccocus aureus* 209P, and against a Gram-negative or-

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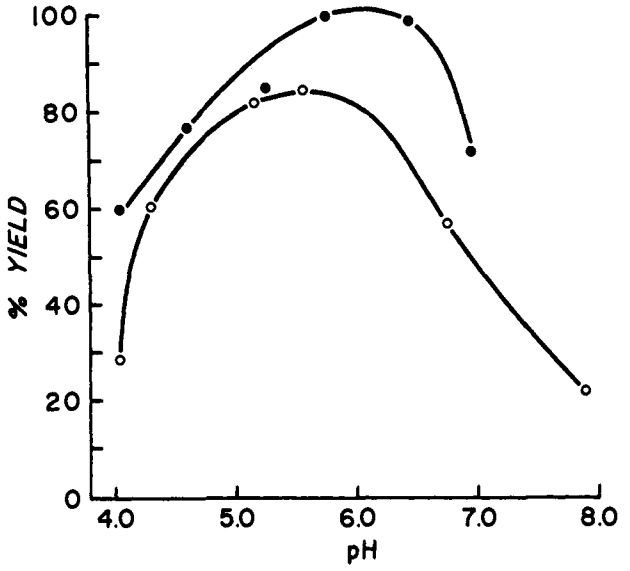

Fig 1.—Dipeptide formation from N-carboxy-D-phenylglycine anhydride as a function of pH: upper curve, 0.04 *M* 6-APA, 0.004 *M* NCA; lower curve, 0.04 *M* 6-APA, 0.02 *M* NCA. Other conditions: 1°, 1 hr., continuous pH adjustment (no buffer).

ganism, *Escherichia coli* 11370. Reaction mixture filtrates were diluted in pH 6.0 phosphate buffer (61.2 m*M* KH_2PO_4 and 9.6 m*M* K_2HPO_4), and in every case a standard curve with α-aminobenzylpenicillin was run simultaneously. Standards were purified as described below. In kinetic determinations, aliquots of reaction mixtures were pipetted directly into the "working hydroxylamine reagent" for β-lactam assay[8] or into one volume of 2 *M* acetic acid before neutralizing and diluting for antimicrobial assay. Both procedures terminated dipeptide formation.

The ranges of penicillin concentration measured in these assays were 0.5–3.0 μg./ml. with *S. aureus* and 3–32 μg./ml. with *E. coli*. 6-APA has 2.5% of the activity of the standard against *E. coli* and 1.3% against *S. aureus*. D-Phenylglycine and its NCA and polymers, as well as the penicilloates and polymers of 6-APA and α-aminobenzylpenicillin, are inactive. Ureido derivatives, which would be extractable into organic solvents from acidified solutions, were not formed. A tripeptide, D-phenylglycylphenylglycylpenicillanic acid, could be distinguished from α-aminobenzylpenicillin by its antimicrobial spectrum and by its higher R_f in chromatographic systems, and it could be easily separated by its much lower water solubility. Thin layer chromatography failed to show additional components in reaction mixtures.

Thin layer chromatography of reaction mixtures, partially purified fractions, and final products was carried out principally with two solvent systems: methanol–pyridine (1:1) and 1-butanol–2-butanol–acetone–0.1 *M* pH 6.0 phosphate buffer–glacial acetic acid (24:12:10:14:3). Ninhydrin was the principal detecting reagent, but in several analyses azide–iodine, toluidine–potassium iodide, and bioautography were used. Paper chromatography was performed with the solvent system 1-butanol–2-butanol–acetone–water (12:12:10:9) on S and S paper impregnated with pH 6 phosphate buffer.

Ionization data were obtained by titrations in water using a Beckman expanded scale pH meter. The pK_a's were determined from the inflection points, and the isoelectric points (P_I) were computed as the average of pK_1 and pK_2. The values of pK_1, pK_2, and P_I for 6-APA were found to be 2.70, 4.92, and 3.81. The corresponding values for α aminobenzylpenicillin were 2.50, 7.20, and 4.85. The values for D-phenylglycine were 2.70, 9.03, and 5.86.

6-[D(−)-α-Amino-α-phenylacetamido]penicillanic Acid (α-Aminobenzylpenicillin).—A mixture of 19.6 g. (0.09 mole) of 6-APA and 24.5 g. (0.14 mole) of N-carboxy-D-phenylglycine anhydride in 2.45 l. of water was adjusted to pH 5.0 by the addition of 10 *N* NaOH. The system was stirred at 1° for 3 hr. and filtered. The filtrate was concentrated 4-fold at 20–24°, giving a precipitate which, on drying, weighed 3.1 g. The filtrate was further concentrated, giving a flocculation of crystalline product which was washed with a small amount of acetone and dried under vacuum at room temperature, yielding 11.7 g.

Anal. Calcd. for $C_{16}H_{19}N_3O_4S \cdot H_2O$: C, 52.3; H, 5.8; N, 11.4; S, 8.7. Found: C, 52.6; H, 5.8; N, 11.4; S, 8.6.

The material separating out on 4-fold concentration was 12% as active against *E. coli* as α-aminobenzylpenicillin, and subsequent studies showed it to be nearly pure phenylglycylphenylglycylpenicillanic acid. Its maximum yield was obtained when the anhydride was used in 50% excess and fell as the ratio of 6-APA to anhydride rose. Formation of the tripeptide directly from the dipeptide could be demonstrated by paper chromatography following a 2-hr. reaction in water at 1° between 0.7 mmole of D-α-aminobenzylpenicillin and 0.6 mmole of D-phenylglycine NCA. Bioautography (*S. aureus*) revealed only two spots, one corresponding to the dipeptide control and the other, with a 40% greater R_f, corresponding to the tripeptide control.

6-(1-Aminocyclopentanecarboxamido)penicillanic Acid.—A mixture consisting of 32 g. (0.15 mole) of 6-APA and 11.5 g. (0.075 mole) of N-carboxy-1-aminocyclopentanecarboxylic acid anhydride in 4 l. of cold water was adjusted to pH 5.2 by the addition of 12.7 g. of solid barium hydroxide and was stirred at 5°. After 2 hr., 5.1 g. of oxalic acid dihydrate was added to the clear solution bringing the pH to 3.8, and the system was filtered. The filtrate was concentrated to 150 ml. and the insoluble fraction was discarded. Half of the filtrate, 66 ml., was lyophilized, giving 9.2 g. of product.

Anal. Calcd. for $C_{14}H_{21}N_3O_4S \cdot H_2O$: C, 48.7; H, 6.7; N, 12.2; S, 9.3. Found: C, 47.9; H, 6.6; N, 12.5; S, 9.2.

The remaining 66 ml. was adjusted to pH 7.5 with 10 *N* KOH and passed through a column of Dowex 1-X10 (bicarbonate), 34 cm. × 1.9 cm. After being washed with 200 ml. of water, the column was eluted with CO_2-saturated water, giving a single peak within 900 ml. of eluate. The peak (252 ml.) was pooled and lyophilized, giving 1.44 g. of product.

Anal. Found: C, 49.0; H, 6.8; N, 12.0; S, 9.2.

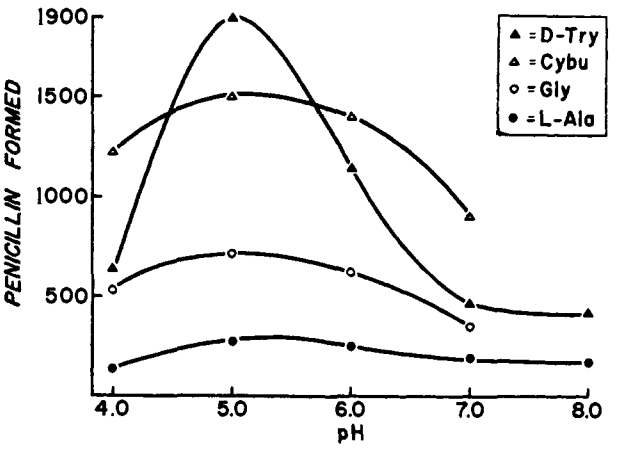

Fig. 2.—Dipeptide formation from various N-carboxyamino acid anhydrides as a function of pH: 0.04 *M* 6-APA, 0.04 *M* NCA, other conditions as in Fig. 1. The ordinate is expressed as μg. of α-aminobenzylpencillin standard per ml.

Results

pH Dependence.—The formation of a dipeptide in water from 6-APA and N-carboxyamino acid anhydrides shows a very clear pH dependence. This relationship is illustrated in Fig. 1. α-Amino benzylpenicillin synthesis (Fig. 1) proceeds optimally at about pH 5.4 when 2 equiv. of the amino acid reacts with 1 equiv. of D-phenylglycine NCA. With a 10-fold excess of the amino acid, the pH optimum shifts upward to about 6.0. With a 50% excess of anhydride (not shown in the figure), the nearly bell-shaped relationship is repeated and the optimal pH is 5.1.

The significant influence of pH on the condensation was found with every N-carboxyamino acid anhydride studied (Fig. 2). When those of glycine (Gly), L-alanine (L-Ala), D-tryptophan (D-Try), and 1-amino-

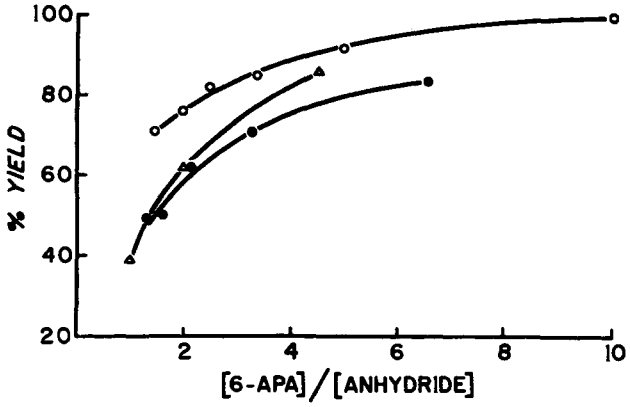

Fig. 3.—α-Aminobenzylpenicillin formation as a function of reactant ratio: O–O, 0.04 *M* 6-APA, 1 hr.; Δ–Δ, 0.04 *M* NCA, 1 hr.; ●–●, 0.074 *M* 6-APA, NCA added stepwise every 30 min.

cyclobutanecarboxylic acid (Cybu) were treated with equivalent amounts of 6-APA, all the reactions showed an optimal pH near 5.0.

Stoichiometry and Kinetics.—Figure 1 shows, in addition to the pH shift, that the higher ratio of amino acid to anhydride results in significantly higher dipeptide yields. Experiments in which either the anhydride or amino acid concentrations were varied initially or in which the anhydride was added stepwise all showed the same trend toward higher yields with larger excesses of 6-APA (Fig. 3).

This relationship also held for the reactions of the other N-carboxyanhydrides. With 6-APA to anhydride ratios of 1, 2, 4, and 10, the conversions from N-carboxy-L-alanine anhydride were in the proportion 1.0, 1.4, 1.9, and 3.2. With 6-APA to anhydride ratios of 1, 2, and 4, the conversions from the anhydride of 1-aminocyclopentanecarboxylic acid were in the proportion 1.0, 1.6, and 2.2.

The replacement, at a fixed pH, of part or all of excess 6-APA by either highly basic or weakly basic amines was studied. In a pH 5.0 system with 18 m*M* 6-APA (pK_2 = 4.92) and 18 m*M* N-carboxy-D-α-phenylglycine anhydride, 18 m*M* aniline (pK_a = 4.85) lowered the dipeptide yield from 42% to 35%, and 18 m*M* isopropylamine (pK_a = 10.72) had no influence. With the same anhydride concentration and pH and the 6-APA concentration raised to 28 m*M*, aniline and methylamine (pK_a = 10.64) lowered the conversion from 64 to 46 and 38%, respectively, while isopropylamine, *t*-butylamine (pK_a = 10.45), and triethylamine (pK_a = 10.75) had no influence. It is apparent that both aniline, mostly un-ionized and with a p*K* close to that of the amino group in 6-APA, and methylamine, largely cationic at pH 5.0, catalyzed reactions competing with α-aminobenzylpenicillin formation.

The synthesis of α-aminobenzylpenicillin is heterogeneous over much of its course in the various aqueous systems described here. The solubility of an NCA in a nucleophilic solvent such as water cannot be determined. The NCA of D-phenylglycine gives every appearance of reacting in a suspended form. The water solubility of 6-APA, which has a very small temperature coefficient between 0 and 25°, ranges between 0.2% (9 m*M*) at its isoelectric point, pH 3.7, to at least 5 molal at pH 7 to 8. At pH 4.8–5.8, within the optimal range for its coupling to anhydride, its solubility is 0.3% (14 m*M*). As the reaction proceeds, forming the substantially more soluble α-aminobenzylpenicillin (2.5% at pH 4.8, its isoelectric point, and 3.5% at pH 5.8), the suspension tends to clear. Hydroxamate analyses after 60 min. account for 90 to 100% of the initial β-lactam as soluble in water when the 6-APA is initially added at 0.8% and the 6-APA to anhydride ratio is 1 or 2. When the ratio is higher, the reaction nevertheless proceeds toward completion and the unused insoluble reactant is recovered unchanged. It appears from Fig. 3 that the excess amino acid participates in the reaction in both soluble and dispersed forms.

Raising the concentration of either reactant above 1% brings a leveling off in the extent of conversion. The addition of various amounts of nonionic, anionic, and cationic detergents had no influence on the reaction in systems with 1.3–2.6% (0.072–1.44 *M*) NCA and 1.6–3.2% (0.114–0.228 *M*) 6-APA.

The decomposition of N-carboxy-D-phenylglycine anhydride was found to be rapid in both the presence and absence of 6-APA. In systems maintained at pH 5.0 by continuous adjustment, the breakdown of NCA followed first-order kinetics in the absence of added amino acid. In the presence of 2 equiv. of 6-APA, the synthesis of dipeptide antibiotic was a second-order reaction, and rate constants could be calculated without correcting for the unimolecular reaction rate. The second-order constants were calculated for both the total amino acid, giving k_{AA}, and for that fraction of the total amino acid present in the free base (anion) form, giving k_{AA^-}. These data are summarized in Table I.

TABLE I

REACTION RATES FOR N-CARBOXY-D-PHENYLGLYCINE ANHYDRIDE

Amino acid	Temperature, °C.	k_1, min.$^{-1}$	k_{AA}, l. mole^{-1} min.$^{-1}$	k_{AA^-}, l. mole^{-1} min.$^{-1}$
None	1	0.16		
None	23	0.24		
6-APA	1		3640	7,110
6-APA	23		6950	13,880

Synthetic reactions carried out at 1–4° generally reached equilibrium within 40 min. At identical pH's, the rates in water, in acetate buffer (ionic strength 0.1) prepared in water, and in acetate buffer prepared in D_2O were very similar, but the rate in phosphate buffer was slightly lower.

Solvent–Water Mixtures.—Irrespective of the relative concentrations of NCA and 6-APA, the presence of large amounts of water-miscible organic solvents depressed the formation of dipeptide. The experiments, summarized in Table II, were carried out on mixtures in which the aqueous component was buffered at pH 5.5 with the intention of fixing the ionization of the attacking amino group within the limits imposed by varying dielectric constant. The solvents fall into two classes, which are independent of dielectric constant. Dioxane (ϵ = 2) and N,N-dimethylacetamide (DMAC) (ϵ = 38) show decreasing yields with increasing solvent concentration. Acetone (ϵ = 21), on the other hand, enhances the conversion at the lower solvent concentrations and depresses it at higher solvent concentrations. N,N-Dimethylformamide (ϵ = 37), which was studied only with equivalent amounts of NCA and 6-APA, behaved similarly to dioxane and

TABLE II

THE EFFECT OF ORGANIC SOLVENTS ON THE SYNTHESIS OF α-AMINOBENZYLPENICILLIN IN AQUEOUS SOLUTION[a]

% solvent, v./v.	% yield from D-phenylglycine NCA		
	Dioxane	DMAC	Acetone
0	75	84	79
20	60	62	86
40	58	50	100
60	55	33	69
80	51	25	65
90	35	28	50

[a] Each system contained 0.04 *M* 6-APA, 0.004 *M* NCA, and 0.01 *M* acetate buffer, pH 5.5. The reactions were run for 30 min. at 1°.

DMAC. Paper chromatography showed the presence of six different products from N-carboxy-D-phenylglycine anhydride and 6-APA in dioxane–water reaction mixtures.

Discussion

These findings show that N-carboxyamino acid anhydrides condense in aqueous systems with 6-aminopenicillanic acid to give dipeptide penicillins. Two critical factors, pH and amino acid concentration, control the extent to which dipeptide synthesis occurs.

Becker and Stahmann[11] found that in the absence of added free amino acid the *polymerization* of N-carboxyglycine anhydride in buffered aqueous sytems occurred with a pH optimum at 6.9. About 90% of the anhydrides of glycine and several other amino acids were polymerized at pH 7.4 and 37°. Above pH 8.9, hydrolysis predominated. From a study of the kinetics of glycylglycine formation by reaction of N-carboxyglycine anhydride with glycine in buffered systems, Bartlett and Jones[4] concluded that the conditions for selective synthesis are very critical and that highest yields of this dipeptide are obtained at pH 10.

The major product of the reaction between an N-carboxyamino acid anhydride and an amino acid depends upon which of several amine-containing compounds is the principal attacking agent: the parent amino acid, liberated from the anhydride by hydrolysis and elimination of carbon dioxide; the dipeptide, formed by condensation with the added amino acid; or the added amino acid itself. The activity of a nucleophile attacking at a carbonyl carbon depends almost entirely on its basicity.[12] With high anhydride to amine ratios, a weakly basic amine and an anhydride releasing a strongly basic amine give high polymers. For the dipeptide to predominate, it is important that the base strength of the attacking amino acid exceeds the basicities of the monomer and the dipeptide and that a high anhydride to amino ratio be avoided, since this would favor a high yield of monomer.

Well-defined pH optima, shown by curves in Fig. 1 and 2, indicate a selective reactivity among several charged species. 6-APA and the various NCA parent amino acids and dipeptides in this study all exist predominantly in cationic, anionic, and dipolar forms, as indicated by infrared spectra of their crystals. The amino group of 6-APA (pK_2 = 4.92) is more weakly basic than that of D-phenylglycine (pK_2 = 9.03) by a factor of about 10,000. However, at a pH near 4.9, half of the total 6-APA is in the free base (anion) form, while only one-ten-thousandth of the hydrolyzed phenylglycine is in its anionic form. The concentration of reactive 6-APA is therefore higher by a factor of at least 5000. Similarly, although the amino group of 6-APA is only about one-two-hundredth as basic as that of the dipeptide (α-aminobenzylpenicillin, pK_2 = 7.20), the concentration of the former is 100 times as high when the total concentrations of the two compounds are equal. By using excess amino acid, a favorable ratio of reactive species is preserved even though the concentration of dipeptide steadily increases. If the pH were raised without changing the relative concentrations of anhydride and amino acid, the more basic dipeptide would become the major attacking agent, and several products, most notably higher peptides, would form. By lowering the concentration of anhydride (Fig. 3), the concentration of dipeptide amine does not increase significantly relative to that of 6-APA at pH 6, and side reactions are practically eliminated.

Our results are the obverse of those of Bartlett and Jones[4] and, accordingly, support their interpretation. They found the dipeptide to be the preferred product at pH 9–10 and tri- and higher peptides the more likely at pH 4.75. This was correlated with the lower basicity of glycylglycine, relative to glycine, and the correspondingly higher concentration of its free base (anion) species at low pH.

It appears that large differences between the second dissociation constants of the desired peptide and the attacking amino acid can be exploited to give controlled peptide synthesis in water. Although the method is not proposed as a general approach to successive condensations, it may prove useful at difficult points within other schemes and also for new varieties of aminoacylation.

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