The β-Phenacyl Ester as a Temporary Protecting Group to Minimize Cyclic Imide Formation during Subsequent Treatment of Aspartyl Peptides with HF[1,2]

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The rearrangement of a series of aspartic acid containing peptides to the corresponding cyclic aspartoyl imides by HF was studied. Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Resin (I) produced 99% of the aspartoyl tetrapeptide after 1 h at 0 °C in HF–10% anisole, while Boc-Glu(OBzl)-Asp(OBzl)-Ser(Bzl)-Leu-Resin and Boc-Glu(OBzl)-Asp(OBzl)-Ala-Leu-Resin each gave 25%. Since it was known that aspartyl peptides containing a free β-carboxyl group do not readily undergo the rearrangement, a synthesis was designed in which a temporary β-protecting group was selectively removed after the synthesis of the peptide chain but before the HF cleavage. For that purpose β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate was synthesized and used for the solid-phase synthesis of the tetrapeptides. Treatment of the protected peptide resins with 1 N sodium thiophenoxide in DMF for 8 h at 25 °C quantitatively removed the phenacyl ester. Subsequent cleavage of the peptide resins in HF–anisole and analysis of the resulting peptides by ion-exchange chromatography showed that the level of cyclic imide by-products was reduced as much as 40-fold. Thus, even in the extreme case, the phenacyl ester derivative of I gave only 2.4% of the by-product. The aspartoyl derivatives of the tetrapeptides were isolated and characterized by ir spectroscopy, amino acid analysis, and electrophoretic mobility. The mixtures resulting from mild alkaline hydrolysis of the cyclic imides were separated by ion-exchange chromatography into the α- and β-aspartyl tetrapeptides, which were identified by comparison with synthetic standards of the two isomers.

In the course of a solid-phase synthesis[3] of an active bovine growth hormone (125–133) fragment, a side reaction associated with the -Asp-Gly- sequence was encountered. This reaction was shown to be the formation of a cyclic aspartoyl imide derivative during the HF cleavage step. The sensitivity of aspartic acid amides and esters to imide formation under basic or acidic conditions or at elevated temperature is well known,[4–16] and the rearrangement has even been observed during recrystallization[14] and catalytic hydrogenolysis[17] of peptides containing aspartyl residues. The imide is subject to nucleophilic attack at either carbonyl, with ready formation of a mixture of α- and β-aspartyl peptides. The phenomenon has been observed in classical solution syntheses and in solid-phase syntheses but it has been of particular concern in the latter case because of the marked susceptibility to this rearrangement of -Asp(OBzl)-Ser(Bzl)-[13] and -Asp(OBzl)-Gly-[14] sequences and to a lesser extent of other β-benzyl-aspartyl sequences[15,16] when subjected to HBr–TFA or HF. It was known,[13,14] however, that -Asp-Ser(Bzl)- or -Asp-Gly- sequences containing a free β-carboxylic acid showed little tendency to undergo cyclization under these conditions. Consequently, synthetic strategies to circumvent this rearrangement during solid-phase synthesis were designed in which the protecting group on the β-carboxyl of aspartic acid was removed prior to the exposure to strong anhydrous acid.

Our first application of this principle involved the use of a *tert*-butyl ester for the β-carboxyl protection in combination with the Bpoc group for N^{α} protection and a relatively acid-labile *p*-alkoxybenzyl ester anchoring bond to the solid support.[3] An alternative form of this strategy for the solid-phase synthesis of aspartic acid containing peptides has now been developed in which the aspartyl residue is incorporated into the peptide chain as its β-phenacyl ester and is deprotected by sodium thiophenoxide before cleavage from the resin support by HF (Figure 1).

Phenacyl esters have been used for many years as derivatives of carboxylic acids for identification purposes and more recently as temporary protecting groups for organic synthesis.[18] They were introduced into peptide synthesis by the Zervas laboratory[19,20] and by Ledger and Stewart[21] as potentially useful derivatives for both α-carboxyl and side-chain carboxyl protection. A phenacyl ester linkage has also been adapted to solid-phase synthesis for anchoring the peptide chain to the resin support.[22–24]

Results

The Rearrangement to Cyclic Aspartoyl Peptides and Its Dependence on Sequence. The nonapeptide, H-Arg-Glu-Leu-Glu-Asp-Gly-Thr-Pro-Arg-OH, corresponding to residues 125–133 of bovine pituitary growth hormone, was synthesized as described previously[3] by a standard solid-phase method[25–27] in which aspartic acid was protected as a β-benzyl ester. The peptide was cleaved from the resin with anhydrous HF in the presence of 10% anisole at 0 °C for 1 h. Paper electrophoresis at pH 6.5 (pyridine–0.1 M acetic acid) showed one major neutral component plus two minor components, one negatively and one positively charged. From the structure of the peptide a net negative charge was expected at this pH. When the peptide was treated with mild aqueous base it showed the expected mobility (R_{Asp} 0.27). This agreed with the mobility of the natural peptide and correlated well with empirical charge–mobility relationship suggested by Offord.[28] Amino acid analyses of an acid hydrolysate gave correct ratios, but an enzymatic hydrolysate by leucine aminopeptidase was low in Asp, Thr, Gly, Pro, and Arg. It was concluded that the cleavage reaction in HF had given rise to the cyclic aspartoyl peptide which then opened in base to give a mixture of the α- and β-aspartyl nonapeptides.

In order to identify the residues responsible for the observed loss of negative charge in the nonapeptide it was resynthesized and small portions were removed after each coupling. The peptides were cleaved in HF and the net charge at pH 6.5 of the major component was determined by paper electrophoresis. The data in Table I indicate that the expected net charge was found for all peptides in the series until the first glutamic acid residue was added. This hexapeptide, and all subsequent peptides, contained one negative charge less than expected, suggesting a direct role for glutamic acid. Several synthetic model peptides (Table II) showed that neither H-Glu(OBzl)-Asp(OBzl)-R nor H-Glu(OBzl)-Asp(OBzl)-Gly-R produced appreciable amounts of the rearranged product, but that H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-R did give rise to a major product

Table I. Observed and Expected Net Electrostatic Charge of Peptides at pH 6.5[a]

Peptide-resin precursor[b]	Net electrostatic charge[c] Observed	Expected
H-Pro-Arg(Tos)-R	+1	+1
H-Thr-Pro-Arg(Tos)-R	+1	+1
H-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	+1	+1
H-Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	0	0
H-Glu(OBzl)—Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	0	−1
H-Leu-Glu(OBzl)—Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	0	−1
H-Glu(OBzl)-Leu-Glu(OBzl)—Asp-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	−1	−2
H-Arg(Tos)-Glu(OBzl)-Leu-Glu(OBzl)—Asp-Gly-Thr(Bzl)-Pro-Arg(Tos)-R	0	−1

[a] Paper electrophoresis in pyridine–0.1 M acetic acid buffer, pH 6.5, 1500 V, 1 h. [b] All peptide resins had good amino acid analyses. The peptide resins were in the form of TFA salts. They were cleaved and deprotected in HF–10% anisole, 1 hr, 0 °C, before electrophoresis. The same results were obtained when the peptide resins were protected with N^{α}-Boc. [c] The net electrostatic charge E of the major ninhydrin-positive spot was determined from the observed mobility, m, the molecular weight M of the free peptide, and the charge–mobility relationship of Offord.[28]

Table II. Observed and Expected Net Electrostatic Charge of Peptides at pH 6.5[a]

Peptide-resin precursor	Net electrostatic charge Observed	Expected
H-Glu(OBzl)—Asp(OBzl)-R	−2	−2
H-Glu(OBzl)—Asp(OBzl)-Gly-R	−2	−2
H-Glu(OBzl)—Asp(OBzl)-Gly-Thr(Bzl)-R	−1	−2

[a] See Table I.

Table III. Observed and Expected Net Electrostatic Charge of Peptides at pH 6.5[a]

Peptide-resin precursor	Net electrostatic charge Observed	Expected
H-Gln-Asp(OBzl)-Gly-Thr(Bzl)-R	0	−1
H-Ala-Asp(OBzl)-Gly-Thr(Bzl)-R	0	−1
H-Ala-Asp(OBzl)-Gly-Ala-R	0	−1
H-Asp(OBzl)-Gly-R	−1	−1
H-Asp(OBzl)-Gly-Thr(Bzl)-R	−1	−1

[a] See Table I.

lacking one negative charge, suggesting that a lactone may have formed between the γ-carboxyl of glutamic acid and the hydroxyl of threonine during the HF treatment. However, there was no evidence of lactone formation by infrared spectroscopy or by chemical methods.[11] In addition, paper electrophoresis did not reveal detectable amounts of an N → O shift product. Further model peptides (Table III) then showed that neither the glutamyl nor threonyl residue was essential for the loss of negative charge, since the rearrangement still occurred when both residues were replaced by alanine as in H-Ala-Asp(OBzl)-Gly-Ala-R.

The data from the model peptides all supported the original expectation that the rearrangement involved imide formation at the Asp-Gly bond, with the restriction that both the amino and carboxyl termini of the dipeptide sequence must be in amide linkage for appreciable reaction to occur.

All of the model di- and tripeptides examined after HF cleavage and deprotection actually showed, in addition to the major component, small amounts of ninhydrin-positive material with the electrophoretic mobility expected for the

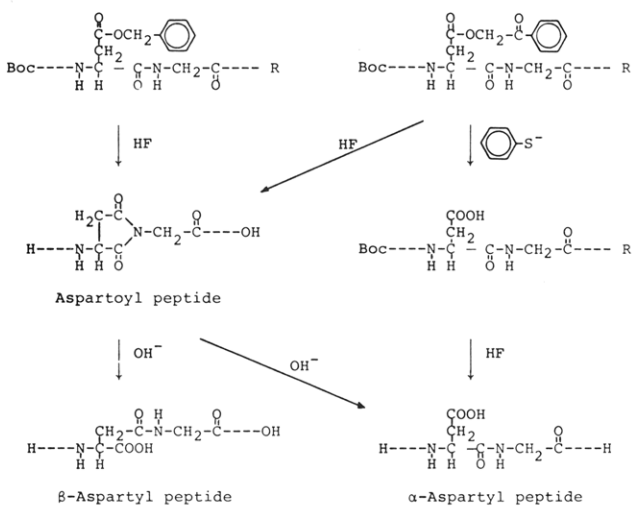

Figure 1. A strategy to minimize imide formation during solid-phase synthesis of aspartic acid containing peptides.

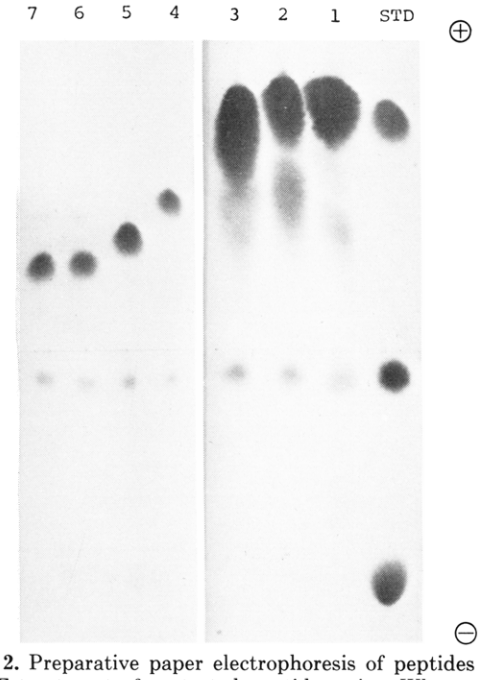

Figure 2. Preparative paper electrophoresis of peptides derived from HF treatment of protected peptide resins. Whatman 3MM paper, 1500 V, 90 min, in pyridine–0.2 M acetic acid, pH 6.5. Sample 1, H-Glu(OBzl)-Asp(OBzl-Gly-R; 2, H-Glu(OBzl)-Asp(OBzl)-Ser(Bzl)-R; 3, H-Glu(OBzl)-Asp(OBzl)-Ala-R; 4, H-Asp(OBzl)-Gly-R; 5, H-Asp(OBzl)-Gly-Thr(Bzl)-R; 6, H-Asp(OBzl)-la-Leu-R; 7, H-Asp(OBzl)-Ser(Bzl)-Leu-R; standard, Asp, Ala, Arg.

cyclic aspartoyl derivative (Figure 2). Preparative electrophoresis was used to quantitate the proportions of product. The peptides were eluted, hydrolyzed, and analyzed for amino acid content. The data of Table IV show again that the bulk of the product migrated in the position expected for the peptide containing a free aspartic acid carboxyl group (either α or β) but, depending on the size and composition of the peptide, 1–12% of a component was found in each sample that migrated in the position of the corresponding peptide with one less negative charge. Analysis showed the presence of each of the amino acids of the parent peptide. This is consistent with a cyclic aspartoyl derivative, but the quantitative number is a maximum value for imide since products derived from other side reactions or incomplete deprotection would not have been distinguished by this simple technique.

Evidence for the Cyclic Imide Structure. When the peptide resin H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-R was cleaved in HF (0 °C, 1 h), the crude peptide showed the loss of one net negative charge (see Table II). The peptide was divided into two equal parts, one of which was treated for 24 h with 1% triethylamine in water and then lyophilized. KBr pellets were prepared and an infrared difference spectrum was measured, using the base-treated peptide as the reference and the untreated peptide as the sample (Figure 3). The sample showed absorption maxima at 1780 and 1710 cm^{-1} which are characteristic of succinimide derivatives.[14,29,30] The infrared spectrum of base-treated, open-chain peptide did not have peaks in this region; instead it absorbed at 1650 cm^{-1}. Both solutions of the peptide were put on an Aminex 50W-X4 cation exchange column and eluted with pyridine acetate buffer (0.1 M, pH 3.20). The base-treated peptide showed two peaks whereas the untreated peptide had only one major peak, which eluted much later from the cation exchange column. The three components were isolated and hydrolyzed and each was shown to contain equimolar amounts of all four amino acids. The above experiments support the conclusion that the side reaction of H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-R in HF was the nucleophilic displacement of the β-benzyl ester of the aspartyl residue by the amide nitrogen of the Asp-Gly peptide bond to form an aspartimide ring. When the cyclized peptide was treated with aqueous base the imide ring was broken, the result being a mixture of α-aspartyl and β-aspartyl tetrapeptides, which could be resolved on the cation exchange column. The peptide that was not treated with base was still in the cyclic imide form and had one less negative charge and was eluted later. Two other tetrapeptides, Glu-Asp-Ala-Leu and Glu-Asp-Ser-Leu, behaved in a similar way.

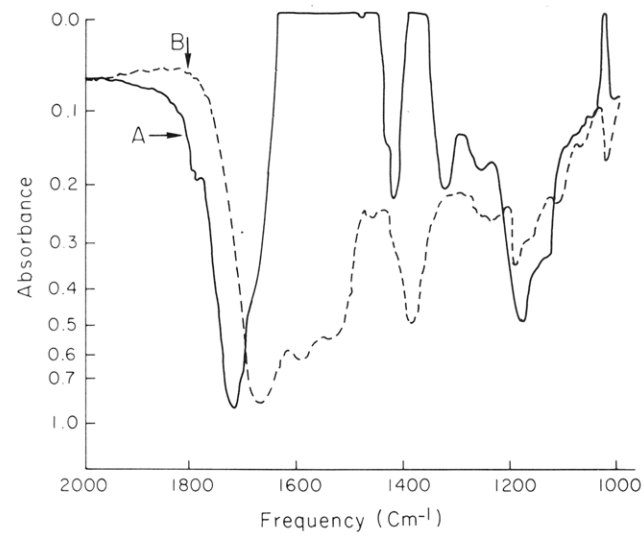

Figure 3. Infrared spectra of aspartyl and aspartoyl tetrapeptides. Curve A (solid line): the difference spectrum with the aspartoyl derivative, H-Glu-Asp-Gly-Thr-OH, in the sample cell and a mixture of α- and β-aspartyl peptides, H-Glu-Asp-Gly-Thr-OH, in the reference cell. Curve B (dashed line): the spectrum of a mixture of α- and β-aspartyl peptides, H-Glu-Asp-Gly-Thr-OH.

The identification of the α-aspartyl and β-aspartyl tetrapeptides was accomplished by enzyme digestion and by comparison with the synthetic α- and β-peptides. Since the β-peptide bond is known to resist leucine aminopeptidase and aminopeptidase M digestions[31,11] any residue C-terminal to the β-peptide bond should be released in much lower yield. The α-aspartyl peptide should be hydrolyzed by both enzymes without difficulty. The results presented in Table V indicate that, for each tetrapeptide, the second peak to be eluted from the ion exchange column by pH 3.20 pyri-

Table IV. Relative Amounts of Aspartyl and Aspartoyl Peptides Following Treatment of Peptide Resins with HF[a]

Peptide resin	Aspartyl peptide, %	Aspartoyl peptide, %
OBzl H-Asp—Gly-R	98.6	1.4
OBzl OBzl H-Glu—Asp—Gly-R	96.6	3.4
OBzl Bzl H-Asp—Gly—Thr-R	98.4	1.6
OBzl OBzl H-Glu—Asp—Ala-R	92.2	7.8
OBzl OBzl Bzl H-Glu—Asp—Ser-R	88.1	11.9
OBzl H-Asp—Ala—Leu-R	95.9	4.1
OBzl Bzl H-Asp—Ser—Leu-R	96.7	3.3

[a] Estimated by amino acid analysis of peptides separated by paper electrophoresis. See Experimental Section.

A standard tetrapeptide, containing only an α-aspartyl bond, was obtained by carrying out the reactions described in Figure 4. The corresponding standard tetrapeptide con-

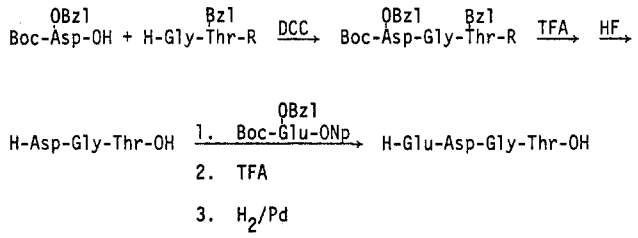

Figure 4. Synthesis of standard H-Glu-Asp-Gly-Thr-OH.

taining only a β-aspartyl bond was prepared from Boc-Asp-OBzl in exactly the same way. Only trace amounts of aspartyl peptide formation (see Table IV) were observed in the synthesis of the tripeptide intermediates. They were removed by preparative paper electrophoresis at pH 6.5. Finally, the glutamyl residue was coupled to the two tripeptides by active ester reaction[32] and the protecting groups were removed with trifluoroacetic acid and catalytic hydrogenolysis.

The two tetrapeptides isolated from the alkaline treatment of the cyclic, aspartoyl tetrapeptide were then identified by comparing their elution times on the long column of the amino acid analyzer (Table VI) with those of the standard α- and β-peptides. This identification agreed with that from the enzymatic digestions.

Table V. Amino Acid Analyses of Enzymatic Hydrolysates of Chromatographically Separated α- and β-Aspartyl Tetrapeptides

	Asp link[a]	H-Glu-Asp-Gly-Thr-OH				H-Glu-Asp-Ala-Leu-OH				H-Glu-Asp-Ser-Leu-OH			
Leucine-aminopeptidase[b]	α	1.0	0.84	0.88	0.97	1.0	1.10	0.95	1.04	1.0	1.06	0.87	1.06
	β	1.0	0.30	0.30	0.31	1.0	0.33	0.36	0.46	1.0	0.35	0.31	0.39
Aminopeptidase M[b]	α	1.0	0.95	1.04	0.90	1.0	0.96	0.99	1.07	1.0	0.89	0.76	1.01
	β	1.0	0.13	0.10	0.22	1.0	0.20	0.14	0.36	1.0	0.19	0.15	0.32

[a] The carboxyl group of aspartic acid that was in peptide linkage was determined by comparing the elution time from the chromatographic column with that of standard tetrapeptides of known structure. [b] The conditions are given in the Experimental Section. These amino acid analyses are for the 24-h digestion time.

dine acetate was completely digested in 24 h to essentially equimolar amounts of each amino acid, whereas the first peak was only 10–30% digested at the aspartyl–X bond. It was concluded that the first peak contained the β-aspartyl peptide. For this analysis 24-h digestion was near optimal, since after 12-h digestion the α-aspartyl peptides were incompletely hydrolyzed, while at 36 h the β-aspartyl bonds were extensively hydrolyzed.

The ion exchange column of the amino acid analyzer using sodium citrate buffers gave a sharp separation of the α, β, and imide forms of these tetrapeptides (Table VI) and allowed an accurate, quantitative determination of the distribution of peptides resulting from the HF cleavage reaction on the three model tetrapeptides (Table VII). Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Resin produced 99% of the aspartoyl tetrapeptide (imide) after 1 h at 0 °C in HF–10% anisole, while Boc-Glu(OBzl)-Asp(OBzl)-Ser-(Bzl)-Leu-Resin and Boc-Glu(OBzl)-Asp(OBzl)-Ala-Leu-Resin each gave 25% imide. The remainder was the α-aspartyl peptide, and no β-aspartyl peptide was detectable. The proportions of the isomers produced by opening the ring of the aspartoyl tetrapeptides at alkaline pH (aqueous triethylamine) was also quantitated (Table VII). Under these conditions, the β-aspartyl isomer predominated and ranged from 71% to 80% of the total.

Table VI. Chromatographic Separation of the Model Tetrapeptides

Peptide	Elution time, min[a]		
	β-Peptide	α-Peptide	Imide
Glu-Asp-Gly-Thr	54	70	116
Glu-Asp-Ala-Leu	102	130	139
Glu-Asp-Ser-Leu	97	131	157

[a] Separations were made on a Beckman Automatic Amino Acid Analyzer (Model 121, long column, 55 cm). Starting buffer was Durrum Pico A for 100 min (pH 3.25, 0.2 N Na^+) followed by Beckman buffer (pH 4.25, 0.2 N sodium citrate). Flow rate for both buffers was 66 ml/h. Column was maintained at 55 °C.

The Use of β-Phenacyl Aspartate to Avoid the Rearrangement. In order to test the proposal that temporary protection of the aspartyl residue by a β-phenacyl ester, followed by deprotection with sodium thiophenoxide before cleavage with HF, would avoid the formation of cyclic imide by-products, it was necessary to synthesize β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate, Boc-Asp(OPac)-OH. The compound was first prepared by a multistep series of reactions starting with the well-characterized β-ben-

Table VII. Distribution of Tetrapeptides[a]

Peptide	Peptides after HF cleavage, %						Peptides in Et_3N hydrolysate of imide, %		
	β-Benzyl-Asp peptide			β-Phenacyl-Asp peptide[b]					
	α	β	Imide	α	β	Imide	α	β	Imide
Glu-Asp-Gly-Thr	1	0	99	97.6	0	2.4	29	71	0
Glu-Asp-Ala-Leu	75	0	25	95.8	0	4.2	26	74	0
Glu-Asp-Ser-Leu	75	0	25	90.5	0	9.5	20	80	0

[a] Using the ion exchange column described in Table VI. [b] The phenacyl ester group was removed in sodium thiophenoxide prior to HF cleavage.

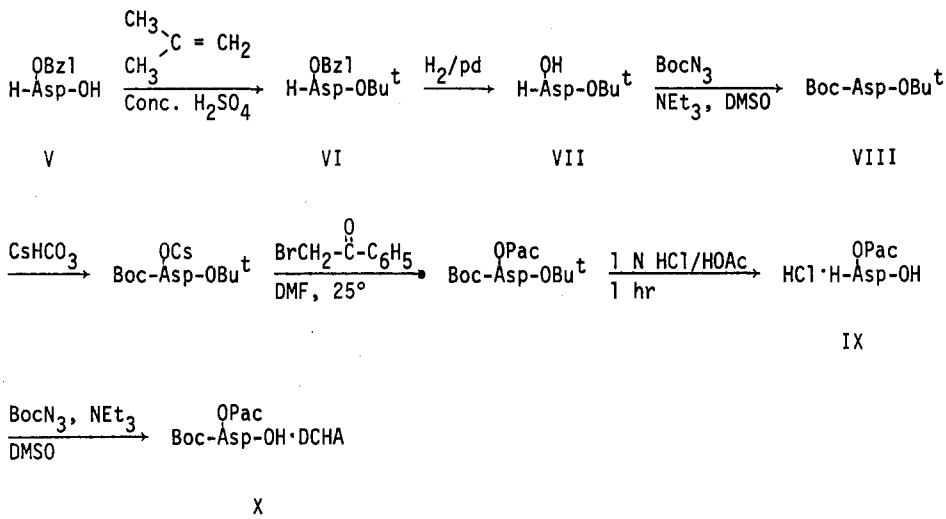

Figure 5. Synthesis of β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate.

zyl L-aspartate[33,34] as shown in Figure 5. The Boc-Asp(OPac)-OH intermediate could also be prepared by a shorter route via lithium copper L-aspartate using the general method of Ledger and Stewart.[21] The phenacyl group was stable to 50% trifluoroacetic acid in methylene chloride and to HF (0 °C, 1 h), but was readily cleaved by nucleophiles such as 1 M sodium thiophenoxide in DMF, with virtually complete retention of the optical purity of the aspartic acid. The rate of removal of the phenacyl ester from Boc-Asp(OPac)-OH by 30 equiv of 1 M sodium thiophenoxide in DMF at 25 °C was followed by withdrawing aliquots at various times, removing the Boc group with TFA, and measuring quantitatively on the amino acid analyzer both the aspartic acid formed and the Asp(OPac) remaining. The latter appeared as a somewhat broadened peak at 259 min in a standard analytical run in which the leucine peak was at 202 min. The operational color factor was 0.35 relative to aspartic acid. The first-order rate constant was found to be 1.0×10^{-3} s^{-1}, corresponding to a half-time of approximately 11 min (Figure 6).

The tetrapeptide resin, H-Glu(OBzl)-Asp(OPac)-Gly-Thr(Bzl)-R, was synthesized by standard solid-phase methods as before, but with the use of the new β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate. The phenacyl group was removed with sodium thiophenoxide (1 M in DMF, 25 °C, 8 h) and the resulting peptide resin, containing a free β-carboxyl group, was then cleaved and fully deprotected by treatment with HF–10% anisole (0 °C, 0.5 h). The crude tetrapeptide prepared in this way contained only 2.4% of the cyclized peptide, no β-peptide, and 97.6% of the α-peptide (Table VII). This result confirmed the validity of this experimental strategy and showed that the phenacyl ester is a suitable temporary protecting group for the β-carboxyl of aspartic acid to minimize the undesired cyclic imide formation.

This procedure also reduced the acid-catalyzed formation of imide in tetrapeptides containing -Asp-Ser- and -Asp-Ala- sequences, but the side reaction was not suppressed to the same extent as in the -Asp-Gly- peptide (Table VII). A more detailed study of the reaction conditions will be necessary before the limits of imide formation can be defined.

Discussion

The results of this study support and extend the information on the now well-established acid-catalyzed side reaction of peptides containing aspartic acid esters that leads to cyclic imides. The rate of the reaction depends on several factors. It proceeds rapidly in the cold in strong anhydrous acids such as HBr in trifluoroacetic acid[13,14] or HF[10] but slowly in trifluoroacetic acid or acetic acid. However, at elevated temperatures it has been observed in acetic acid and even in ethanol[14] or water.[8,9] Under the usual solid-phase synthetic conditions imide formation is a major side reaction only when the β-carboxyl of the aspartyl residue is present as an ester; the free carboxylic acid is much less reactive.[13,14,35] Even when the β-carboxyl is esterified the cyclization to imide does not always occur.

There is clearly a dependence of aspartimide formation on sequence, although this factor has not been extensively examined in any single study. There are certain generalities for which there is agreement, but there are specific examples in which differing conclusions have been drawn by various laboratories. The differences probably can be attributed to variations in time, temperature, reagents, methods of detection, and particularly in the structure surrounding the -Asp-X- sequence. The first study on the possibility of imide formation during solid-phase synthesis[11] showed that Boc-Leu-Asp(OBzl)-Ala-Val-Resin did not lead to detectable levels of imide and that none was produced from

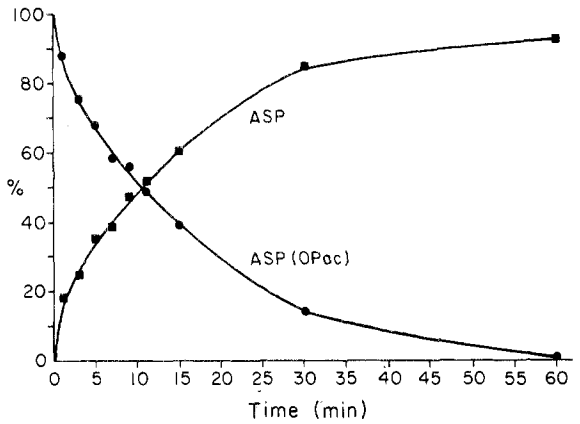

Figure 6. The rate of removal of the β-phenacyl ester from Boc-Asp(OPac)-OH by 1 M sodium thiophenoxide. Sodium thiophenoxide (264 mg, 2.00 mmol) was dissolved in 1.50 ml of dry DMF, added to 21.7 mg (0.062 mmol) of Boc-Asp(OPac)-OH in 0.50 ml of DMF, and stirred at 25 °C. At intervals, 50-μl aliquots were mixed with 150 μl of TFA and after 30 min were diluted with 1.50 ml of pH 2.2 citrate buffer. The concentrations of Asp and Asp(OPac) were determined quantitatively on the amino acid analyzer.

H-Asp(OBzl)-Arg(NO_2)-Val-Tyr(Bzl)-Val-His(Bzl)-Pro-Phe-Resin during the synthesis of angiotensin II. It was concluded from these two examples that this did not appear to be a major side reaction in solid-phase synthesis. However, other peptides were soon found in which the reaction did occur. The -Asp-Ser- sequence of Boc-Gly-Asp(OBzl)-Ser(Bzl)-Gly-Resin was found to be partially cyclized in 1 h at 25 °C by HBr-TFA.[13] The resulting aspartoyl peptide was then converted by alkali to a mixture of the α- and β-aspartyl tetrapeptides. The Ser(Bzl)-containing tetrapeptide, H-Glu(OBzl)-Asp(OBzl)-Ser(Bzl)-Leu-Resin, studied here was found to give 25% of imide after 1 h at 0 °C in HF and Baba et al.[16] obtained a similar result with Z-Asp(OBzl)-Ser(Bzl)-OCH_3 in HF, but in other peptides the -Asp(OBzl)-Ser(Bzl)- sequence was reported to be stable to cyclization.[14] The -Asp-Gly- sequence in Boc-His(Bzl)-Ser(Bzl)-Asp(OBzl)-Gly-Thr-(Bzl)-Phe-Resin was very susceptible to aspartimide formation during treatment with HBr–TFA,[14] and in the present work 99% of the product isolated after HF treatment of H-Glu(OBzl)Asp(OBzl)-Gly-Thr(Bzl)-Resin was the imide. Z-Asp(OBzl)-Gly-OBzl also cyclized in HF,[16] but Kenner showed that Asp(OBu^t)-Gly did not cyclize in TFA.[36]

In general, the resistance to cyclization is correlated with the size of the residue following aspartic acid. Whereas glycine peptides were sensitive, alanine peptides were resistant in HBr[11] but cyclized in HF,[16,37,38] while valine peptides were quite stable in HF.[39] Histidine represents one notable exception. Z-Asp(OBzl)-His-OCH_3[16] was extensively cyclized during HF treatment even at −25 °C in spite of its bulky side chain, suggesting that electronic effects also play a role in this rearrangement.

The present work has brought to light another parameter which had not been recognized before. For an appreciable aspartimide formation to occur both the amino group and the terminal carboxyl group of the -Asp-X-sequence must be blocked. Thus, none of the following peptides were significantly cyclized in 1 h at 0 °C by HF: Glu-Asp, Glu-Asp-Gly, Asp-Gly, Asp-Gly-Thr, Asp-Gly-Thr-Pro-Arg, Asp-Ala-Leu, Asp-Ser-Leu. However, the addition of a single residue at each exposed end of the -Asp-X- sequence led to a susceptible peptide, as in Glu-Asp-Gly-Thr, Ala-Asp-Gly-Thr, Ala-Asp-Gly-Ala, Glu-Asp-Ala-Leu, or Glu-Asp-Ser-Leu. The previously observed[11] stability of Asp-Ala-Val and angiotensin II may, therefore, have been a consequence of the N-terminal position of the Asp(OBzl) residue rather than of a general resistance of the -Asp(OBzl)-Ala- or -Asp-(OBzl)-Arg(NO_2)- sequences. The data of Baba et al.[16] are interesting in this regard because they show rearrangement in the series of dipeptides: Z-Asp(OBzl)-Gly-OBzl, Z-Asp-(OBzl)-Ser(Bzl)-OCH_3, Z-Asp(OBzl)-His-OCH_3, and Z-Asp(OBzl)-Ala-OBzl. These results and ours are compatible if we make two reasonable assumptions: first, that the intramolecular imide formation was rapid compared with acidolytic cleavage of the benzyloxycarbonyl group, in which case their Z group would have prevented protonation of the α-amino group and could have performed the same function as our Glu or Ala residues; and second, that their methyl and benzyl esters were more stable to HF than our oxymethylpolystyrene esters and the rate of imide formation falls between the rate of acidolysis of unsubstituted and substituted benzyl esters. Since the resin ester is an alkyl-substituted benzyl ester it is expected to be more labile to acid. If the resulting C-terminal carboxyl becomes protonated, the nitrogen of the aspartyl peptide bond would become less nucleophilic, and the rate of imide formation would be diminished. The values recorded in Table IV for the extent of imide formation in the di- and tripeptides must be considered as upper limits because in this simple electrophoresis experiment the structures of the products were not actually determined. Other factors could have caused loss of a negative charge or generation of an additional positive charge (e.g., incomplete deprotection or N → O shifts) and have led to an overestimation of imide. In contrast, the quantitative measure of the aspartoyl tetrapeptides by column chromatography is quite reliable.

The reaction mechanism of cyclic imide formation suggested by Ondetti et al.[14] is probably the correct one. It involves protonation of the β-carbonyl oxygen and elimination of ^-OR or ^-OH by nucleophilic attack of the aspartyl amide nitrogen. The overall rate will depend on both steric and electronic effects which in turn depend on the nature of the side chain of the adjacent residue and on the presence or absence of charges at either end of the -Asp-X- sequence. Acylation of anisole via the acylium ion, which had been observed for glutamyl peptides,[38] was not observed for aspartyl peptides in either the previous or present study.

The new approach which we have taken to avoid aspartimide formation depends on the observation[13,14,35] that the acid-catalyzed cyclization reaction was greatly retarded when the β-carboxyl group of the aspartyl residue was free. The incorporation of this idea into a suitable synthetic scheme requires the use of a temporary protecting group for aspartic acid that can be removed selectively before exposure to strong acid. The β-phenacyl ester is suitable for that purpose. It can be used in combination with N^{α}-Boc protection and is quite stable to the conditions of the synthesis. Since phenacyl esters are labile to strong nucleophiles[18] it might be expected that aminolysis by the amino group of a nearby peptide chain could occur at the neutralization step of the synthesis. If this intrachain aminolysis were to occur with alkyl oxygen cleavage, as it does with the strongly nucleophilic sodium thiophenoxide, the products would be the free β-carboxylate and the phenacyl amine; if acyl oxygen cleavage were to occur a crosslinked β-amide would be formed. In either case the reaction would result in a chain termination. The relatively limited number of experiments which have been examined have shown no indication that either of these cleavages has occurred. Stalakatos et al.[19] showed phenacyl esters to be stable to 3.5 N HBr in HOAc and to refluxing TFA, and we also find that β-phenacyl L-aspartate is stable to cleavage by TFA or HF

(0 °C, at least 1 h). However, β-phenacyl aspartyl peptides are readily cyclized to aspartimides in strong anhydrous acids and the phenacyl group must be removed before the HF cleavage step. The deprotection of the phenacyl ester at the end of the synthesis proceeded smoothly and quantitatively ($T_{1/2}$ = 11 min) under the conditions described by Sheehan and Daves.[18] Stalakatos et al.[19] obtained an 80% yield of Z-Gly from Z-Gly-OPac in 30 min at 25°. Thiophenoxide is a strong nucleophile although it is only a weak base, and in these very mild conditions both the benzyl ester and β-lactam of benzyl benzylpencillinate were essentially stable at 5 °C for 18 h.[18] Only 5.6% of the peptide was removed from Boc-Glu(OBzl)-Asp(OPac)-Ser(Bzl)-Val-resin in 24 h at 25 °C by 1 M sodium thiophenoxide in DMF. Shigezane and Mizoguchi[24] synthesized Boc-Leu-Leu-Val-Tyr(Bzl)-resin on an oxyacetyl-resin and cleaved the anchoring phenacyl ester bond with 1 M sodium thiophenoxide in DMF for 24 h at room temperature without noticeable side reaction; the Leu-Leu-Val-Tyr-OEt·HCl which was derived from the product was shown to have the same specific rotation and other properties as a sample synthesized by conventional methods. Nevertheless, it was important to consider the possibility that small amounts of racemization might occur under these conditions. Boc-L-Asp(OPac)-OH was, therefore, deprotected first with TFA and then with 1 M sodium thiophenoxide and the optical purity of the resulting aspartic acid was estimated by the Manning–Moore method.[40] The Leu-Asp diastereomers were present in a ratio of 0.994 LL and 0.006 LD, indicating virtually complete retention of optical purity throughout the eight steps of synthesis and two steps of deprotection.

The usefulness of the β-phenacyl ester method to minimize aspartimide formation was tested by the synthesis of three tetrapeptides. In the case of Boc-Glu(OBzl)-Asp(OPac)-Gly-Thr(Bzl)-Resin the tetrapeptide recovered after deprotection of the phenacyl ester by thiophenoxide and cleavage with HF–10% anisole contained 97.6% of the α-aspartyl peptide, no β-aspartyl peptide, and only 2.4% of the cyclic aspartoyl peptide. This represented a 40-fold decrease in the by-product compared with that obtained when the aspartyl residue was protected with a β-benzyl ester and cleaved directly with HF–anisole. Although the side reaction was not reduced to zero, we consider this to be a very useful result. With the corresponding tetrapeptides containing Ala and Ser(Bzl) in place of Gly, 4.2 and 9.5%, respectively, of aspartoyl peptides were found. It is possible that some imide formation had already occurred before the HF reaction and that these values did not arise from treatment of the peptide containing a free β-carboxyl, although we assume that they did because the isolated H-Glu-Asp-Ala-Leu-OH tetrapeptide (containing a free β-carboxyl and no imide) also yielded 4% of the aspartoyl peptide upon treatment with HF–anisole. In view of the results of Baba et al.[16] it seems probable that a more detailed study of the HF reaction conditions, especially the temperature, may lead to a further reduction of this side reaction. Based on the results reported here we have adopted this phenacyl ester approach for the solid-phase synthesis of peptides that are susceptible to aspartimide formation.

Experimental Section

Sodium thiophenoxide was prepared according to Sheehan and Daves[18] and was recrystallized from DMF by the addition of ether. Methylene chloride was distilled over sodium carbonate; pyridine and triethylamine were distilled over ninhydrin. All other reagents were obtained commercially and used without further purification. Leucine aminopeptidase was purchased from Worthington Biochemical Corp., Freehold, N.J., α-*p*-nitrophenyl γ-benzyl N^{α}-*tert*-butyloxycarbonyl-L-glutamate from Cyclo Chemical Corp., and α-benzyl L-aspartate from Fox Chemical Co., Los Angeles, Calif. All melting points are uncorrected. Amino acid analyses[41] were performed on the Beckman Automatic Amino Acid Analyzer, Model 121. Resin hydrolyzates were carried out in evacuated sealed tubes on 10–20-mg samples of peptide resins with concentrated HCl, glacial acetic acid, and phenol (88%) (2:1:1) at 110 °C for 22–24 h. Peptide hydrolyzates were in 6 N HCl containing a few drops of phenol under similar conditions. Thin layer chromatography was carried out on silica gel G with the solvent system I (CMA), chloroform–methanol–acetic acid (85:10:5), and solvent system II (BAW), 1-butanol–acetic acid–water (4:1:1). Electrophoretic mobilities are expressed as fractions, R_{Asp} or R_{Arg}, of the distances traveled by aspartic acid or arginine in the same electropherograms, with the center of a neutral amino acid spot taken as the point of zero mobility.

Preparative Paper Electrophoresis. Electrophoresis was carried out in a cold room on Whatman 3MM chromatography paper in pyridine acetate buffer, pH 6.5 (0.1 M in acetic acid), at 1500 V for 90 min. Peptides (1–5 mg) were applied on a line at the center of paper. Following electrophoretic separation, these peptides were located by cutting small strips from both edges of the paper and developed with ninhydrin spray. Corresponding portions of the moist undeveloped paper were cut and eluted with 50 ml of the same buffer. The eluates were lyophilized and the residues were carefully extracted three times (1 ml each) with 6 N HCl containing one drop of phenol. The extracts were hydrolyzed and analyzed for amino acid content.

Synthesis of Model Peptides. Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Resin (I). All of the model peptides were synthesized by a standard solid-phase procedure similar to those described earlier.[11,25-27] N^{α}-*tert*-Butyloxycarbonyl-*O*-benzyl-L-threonine-copoly(styrene–1%-divinylbenzene)resin (0.20 mmol Thr/g) was deprotected with 20% TFA in CH_2Cl_2 for 20 min at 25 °C. The resin was filtered, washed four times with CH_2Cl_2, neutralized with 10% Et_3N in CH_2Cl_2 for 10 min, and washed four times with CH_2Cl_2. A threefold excess of Boc-Gly in CH_2Cl_2 was added, followed by a threefold excess of DCC in just enough CH_2Cl_2 to cover the resin. The reaction vessel was shaken for 90 min. The resin was filtered and washed three times each with CH_2Cl_2, DMF, and CH_2Cl_2, once with 10% Et_3N, and three times with CH_2Cl_2. A second coupling with 1 equiv of Boc-Gly and DCC was carried out as before. β-Benzyl N^{α}-Boc-L-aspartate and γ-benzyl N^{α}-Boc-L-glutamate were added by repetitions of the deprotection, neutralization, and double coupling cycle.

Isolation of L-Glutamyl-L-aspartoylglycyl-L-threonine (II). Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Resin (200 mg) was stirred for 1 hr at 0 °C in 10 ml of HF containing 1 ml of anisole. The HF and most of the anisole were removed by vacuum and the residual anisole and derivatives were extracted with ether. The peptide was extracted with 10% aqueous acetic acid and lyophilized. The amino acid analysis of a hydrolysate of the crude peptide gave the following molar ratios: Asp (1.00), Thr (1.05), Glu (1.01), Gly (1.06). Paper electrophoresis at pH 6.5 (pyridine–0.1 M acetic acid) showed a major spot at R_{Asp} 0.49. The crude peptide (10 mg) was put on a preparative cation exchange column (Aminex 50W-X4, 40 × 2.5 cm), and was eluted at 60 ml/h with pyridine acetate buffer (pH 3.20, 0.1 M in acetic acid). The column was maintained at 40 °C by water jacket. The eluate from the column was divided into two streams, with a ratio of 90:10%. The 90% stream was directed to a fraction collector (3 min per fraction) and the other stream went into the coil of the amino acid analyzer to react with ninhydrin. Ninhydrin-positive tubes 114–125 were pooled, concentrated in vacuo, and lyophilized to give 7.5 mg of the aspartoyl derivative of the tetrapeptide, H-Glu-Asp-Gly-Thr-OH. The amino acid analysis of an acid hydrolysate of this cyclized peptide gave the following molar ratios: Asp (1.00), Thr (0.95), Glu (1.02), Gly (1.04).

Alkaline Hydrolysis of the Imide-Containing Tetrapeptide II and Isolation of H-Glu-α-Asp-Gly-Thr-OH (III) and H-Glu-β-Asp-Gly-Thr-OH (IV). Peptide II (4 mg) was dissolved in water (1 ml), and the solution was made alkaline (pH 8–9) by dropwise addition of triethylamine and stirred at room temperature overnight. Water (3 ml) was added to the reaction mixture and the soluon was frozen and lyophilized. The resulting material was put on a preparative cation exchange column to separate the α- and β-peptides. The experimental conditions used in α- and β-peptide separations were essentially the same as those used for the isolation of the aspartoyl derivative (II), as described in the previous paragraph. There were two well-separated ninhydrin-positive peaks which appeared on the recorder at 138 and 198 min. The test

tubes in the fraction collector corresponding to these two peaks were lyophilized. The first peak eluted was shown to be H-Glu-β-Asp-Gly-Thr-OH (IV) and the second was shown to be H-Glu-α-Asp-Gly-Thr-OH (III). The quantities of the β- and α-peptides were 2.8 (71%) and 1.2 mg (29%), respectively, as determined both by integration of the peaks and by the amino acid analyses of the lyophilized peptides. Both peptides III and IV gave essentially identical amino acid analyses and moved together (R_{Asp} 0.19) on paper electrophoresis at pH 6.5 (pyridine–0.1 M acetic acid), but on the long column of amino acid analyzer they were separated into two peaks 16 min apart (see Table VI).

Enzymatic Digestions. The leucine aminopeptidase digestion was carried out as described by Hofmann et al.[42] with small modifications. The peptide (1 mg) was dissolved in 0.025 M, pH 8.5 Tris buffer (0.5 ml), 0.005 M with respect to magnesium chloride, and the pH was readjusted to 8.5 with solid Tris. Leucine aminopeptidase (0.1 mg) in 0.5 ml of 0.1 M Tris buffer, pH 8.5, 0.005 M with respect to magnesium chloride, was preincubated for 3 h at 40 °C, then added to the peptide and the digest was incubated at 40 °C. Aliquots of the digest were withdrawn after 6, 12, 24, and 36 h; digestion was stopped by dilution with 1 N acetic acid (3 ml) and then the samples were lyophilized and analyzed for amino acids. Aminopeptidase M digestion was performed by mixing 0.1 ml of enzyme solution (4 mg in 1 ml of 0.5% NH_4HCO_3) and 0.1 ml of peptide solution (1 mg in 1 ml of 0.5% NH_4HCO_3) and incubating the mixture at 37 °C. Aliquots were withdrawn after 6, 12, 24, and 36 h, lyophilized, and analyzed for the amino acids. Some of the data are given in Table V.

Synthesis of Standard Tetrapeptides. H-Glu-β-Asp-Gly-Thr-OH (IV). The synthesis of the peptide was accomplished by the combination of solid-phase and solution reactions shown in Figure 4. The tripeptide resin, H-β-Asp-Gly-Thr-R (100 mg, 0.25 mmol peptide/g resin) was synthesized by the same procedure as for peptide I but with the use of α-benzyl N^{α}-Boc-L-aspartate for the aspartyl residue. The peptide resin was treated with HF (2 ml) at 0 °C for 1 h with anisole (0.2 ml) as scavenger. After the HF and anisole were removed, the resulting tripeptide was put on paper electrophoresis at pH 6.5 (pyridine–0.1 M acetic acid). The electropherogram showed a major spot with R_{Asp} 0.6 and a neutral spot. The crude peptide (7 mg) was purified by preparative electrophoreses at pH 6.5 using pyridine–0.1 *M* acetic acid as buffer (see preparative electrophoresis in this section). The purified tripeptide was isolated (5 mg). This peptide gave a good amino acid analysis, and was homogeneous on paper electrophoresis at pH 6.5 (R_{Asp} 0.61). This mobility value agreed with the prediction for the tripeptide carrying a negative charge. The crude tripeptide (4 mg, 13 μmol) was dissolved in DMF (4 ml) mixed with α-*p*-nitrophenyl γ-benzyl N^{α}-*tert*-butyloxycarbonyl-L-glutamate (23.9 mg, 52 μmol) and triethylamine (13 μmol in 1 ml of DMF). The ensuing yellowish solution was left at room temperature for 24 h. The solvent was removed under high vacuum and the resulting oily material was treated with TFA (3 ml) for 30 min. The TFA was removed by a water aspirator. The residue was then dissolved in 2 ml of a mixed solvent (water–methanol–acetic acid, 4:2:1) and 5 mg of 5% palladium on barium sulfate was added. The mixture was shaken under hydrogen (40 psi) for 12 h. After hydrogenolysis was completed and the catalyst was filtered off, the solution was diluted with water and was lyophilized. The product was purified on the preparative cation exchange column as described in the isolation of the aspartoyl derivative, II. The desired β-aspartyl tetrapeptide IV (3.3 mg, 58%) eluted at 139 min. An acid hydrolyzate gave good amino acid ratios.

The corresponding α-aspartyl standard, H-Glu-α-Asp-Gly-Thr-OH (III), was synthesized in the same way except that the aspartic acid residue was introduced as β-benzyl N^{α}-Boc-L-aspartate. This tetrapeptide eluted at 197 min on the ion exchange column. The other two pairs of standards, H-Glu-β-Asp-Ala-Leu-OH, H-Glu-α-Asp-Ala-Leu-OH, H-Glu-β-Asp-Ser-Leu-OH, and H-Glu-α-Asp-Ser-Leu-OH, were prepared by these same general procedures. Their elution times are shown in Table VI.

β-Benzyl L-Aspartate (V). The synthesis of compound V was carried out as described by Benoiton:[33] yield 22 g (51%); mp 218–220 °C; $[\alpha]^{25}D$ +28.4° (*c* 1, 1 N HCl) [lit.[33] mp 218–220 °C; $[\alpha]^{20}D$ +28.1° (*c* 1, 1 N HCl); lit.[34] mp 212 °C; $[\alpha]^{20}D$ +28.6° (*c* 1, 1 N HCl)]. The product showed a single spot on TLC in solvent mixture I (CMA), R_f 0.12; on paper electrophoresis at pH 2.3 (1 N acetic acid, 75 min, 1000 V) it had the same mobility, R_{Arg} 0.30, as the authentic β-benzyl L-aspartate (obtained from Fox Chemical Co.).

Anal. Calcd for $C_{11}H_{13}O_4N$: C, 59.18; H, 5.58; N, 6.28. Found: C, 59.44; H, 5.88; N. 6.28.

α-*tert*-Butyl-β-benzyl L-Aspartate Hydrochloride (VI). Isobutene (110 ml) was added to a solution of 12 g (0.052 mol of β-benzyl L-aspartate V in a mixture of 100 ml of dioxane and 10 ml of concentrated sulfuric acid in a 500-ml pressure bottle. The mixture was mechanically shaken at room temperature for 4 h. The solution was poured immediately into a cold mixture of 400 ml of 1 N sodium hydroxide, and the aqueous phase was extracted three times with ether. The combined organic phase was dried over sodium sulfate and evaporated under vacuum to about 5 ml. After the addition of 50 ml of ether, dry hydrogen chloride was bubbled through the solution and crystalline compound VI started to precipitate. Recrystallization from ethyl alcohol gave 11 g (67%): mp 115–117 °C (lit.[43] 115–117 °C); $[\alpha]^{25}D$ +23.1° (*c* 1, EtOH). The product was homogeneous on TLC in solvent mixture I (CMA) (R_f 0.6).

Anal. Calcd for $C_{15}H_{22}NO_4Cl$: C, 57.05; H, 7.02; N, 4.44; Cl, 11.23. Found: C, 57.09; H, 6.88; N, 4.34; Cl, 11.02.

α-*tert*-Butyl L-Aspartate VII. A suspension of α-*tert*-butyl-β-benzyl L-aspartate hydrochloride (VI, 10 g) in 200 ml of ether was treated with 40 ml of 25% potassium carbonate solution, the liberated ester was immediately extracted into ether, and the aqueous solution was washed again with 50 ml of ether. The ether was dried over sodium sulfate and evaporated in vacuo. The oily residue was dissolved in 95% ethanol (125 ml), 1 g of 5% palladium on barium sulfate was added, and the bottle was shaken under hydrogen (50 psi) overnight. After hydrogenolysis was completed, the catalyst was filtered off and the solution was concentrated to 50 ml. When 400 ml of acetone was added, a gel formed which changed to a crystalline precipitate on stirring. The yield was 4.5 g (75%); mp 178–179 °C dec; $[\alpha]^{25}D$ +24.9° (*c* 1, CH_3COOH).

Anal. Calcd for $C_8H_{15}NO_4$: C, 50.78; H, 7.99; N, 7.40. Found: C, 50.43, H, 7.81; N, 7.33.

α-*tert*-Butyl N^{α}-*tert*-Butyloxycarbonyl-L-aspartate (VIII). α-*tert*-Butyl L-aspartate (VII, 4.5 g) was dissolved in 30 ml of dimethyl sulfoxide (redistilled over NaOH pellets), followed by *tert*-butyl azidoformate (4 ml) and triethylamine (6.5 ml). The mixture was stirred for 20 h at room temperature. The solution was diluted with three volumes of water and extracted three times with ether to remove any unreacted *tert*-butyl azidoformate. The aqueous phase was chilled in an ice bath, acidified with citric acid to pH 3.5, and extracted three times with ethyl acetate. The ethyl acetate was extracted three times with small portions of a saturated sodium chloride solution, dried over magnesium sulfate, and evaporated under reduced pressure. The residual oily material was recrystallized from ethyl acetate–hexane: yield 6.5 g (94%); mp 97–98 °C; $[\alpha]^{25}D$ −14° (*c* 1, C_2H_5OH). The resulting material was homogeneous on TLC in solvent system I (CMA), R_f 0.83, and system II (BAW), R_f 0.78.

Anal. Calcd for $C_{13}H_{23}NO_6$: C, 53.96; H, 8.01; N, 4.84. Found: C, 53.69; H, 8.02; N, 4.74.

β-Phenacyl L-Aspartate Hydrochloride (IX). A. Compound VIII (6.4 g, 22.1 mmol) was stirred at 25 °C with α-bromoacetophenone (6.4 g, 32 mmol) in DMF (60 ml), followed by triethylamine (2.9 ml).[18] The solution was kept at room temperature for 2 h and at 55 °C for 6 h. The reaction was monitored by TLC. Slow decomposition of the *tert*-butyl ester bond was detected during the heating period. When the reaction was completed DMF and triethylamine were removed under high vacuum at 35–40°. The residual oily material was treated with anhydrous 1 N HCl in glacial acetic acid for 1 h. The ensuing solution was then concentrated in vacuo, redissolved in 50 ml of ice water, and extracted three times with ether and once with benzene to remove the unreacted α-bromoacetophenone. The aqueous phase was concentrated to about 15 ml and acetone was added to cause the precipitation of β-phenacyl L-aspartate HCl salt (IX): yield 4 g (61%); mp 144–145 °C; $[\alpha]^{25}D$ +15.6° (*c* 1, 1 N HCl); TLC in solvent system I (CMA), R_f 0.05, system II (BAW) R_f 0.39.

Anal. Calcd for $C_{12}H_{14}NO_5Cl$: C, 50.09; H, 4.91; N, 4.87; Cl, 12.32. Found: C, 50.13; H, 4.05; N, 4.90; Cl, 11.96.

B. A second esterification procedure, with much milder reaction conditions, was also used to prepare compound IX. Compound VIII (4.6 g, 15.9 mmol) was dissolved in 95% ethanol (50 ml) and the solution was adjusted to neutrality by slow addition of cesium bicarbonate[44] as measured with a pH meter. During the neutralization some precipitate formed, which was redissolved by addition of 20 ml of water. The final pH of the solution was about 7. The resulting clear solution was then flash evaporated, and after repeated evaporation to dryness with benzene, the cesium salt of compound VIII was obtained as a white solid. This was then dried over P_2O_5 in vacuo overnight. The resulting cesium salt was dissolved in

DMF (50 ml) at 25° and α-bromoacetophenone (3.6 g, 18 mmol) was added. A white precipitate formed within a few minutes and the suspension was stirred overnight at room temperature. The reaction mixture was filtered and the filtrate was concentrated to an oil which was dissolved in 10 ml of ethanol. This crude sample was placed on a Dowex 1 × 2 column (2.7 × 15 cm), OH^- form, which had been equilibrated with ethanol and then was eluted with ethanol. Fractions containing α-*tert*-butyl β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate were located by TLC and evaporated to dryness. The resulting material was treated with anhydrous 1 N HCl in acetic acid at 25 °C for 1 h. Acetic acid was evaporated off to yield a solid material, which was then suspended in 50 ml of H_2O and extracted three times with ether and once with benzene. The aqueous phase was concentrated to a volume of 10–15 ml and acetone was added to cause precipitation of β-phenacyl L-aspartate hydrochloride (IX): yield 3.7 g (80%); mp 144–145 °C; mobilities on TLC were identical with those of the previous synthesis.

C. β-Phenacyl L-aspartate was conveniently prepared as the zwitterion[45] via the lithium–copper salt by the general method A of Ledger and Stewart.[21] The yield after recrystallization from water was 15%. The elemental analysis was correct; the mobilities on TLC and corrected specific rotation were identical with those of preparations A and B; mp 177–179 °C.

β-Phenacyl N^{α}-Boc-L-aspartate DCHA Salt (X). Compound IX (1 g, 5.3 mmol) was dissolved in dimethyl sulfoxide (10 ml) followed by the addition of *tert*-butyl azidoformate (1.5 ml) and triethylamine (1.5 ml). Within a few minutes, a white precipitate formed, which was filtered off. To the remaining filtrate was added 100 mg of anhydrous $MgSO_4$. The filtrate was stirred for 10–12 h (TLC indicated that the reaction was near completion). The reaction mixture was poured into chilled (0 °C) citric acid solution and adjusted to pH 3 with citric acid. The solution was extracted three times with ethyl acetate, and the combined organic phases were washed three times with small portions of a saturated NaCl solution, dried over $MgSO_4$, and evaporated. The resulting oil was dissolved in 5 ml of ethyl acetate and cooled in a dry ice bath. Dicyclohexylamine was added dropwise until the solution became slightly basic to litmus paper. A precipitate formed which was triturated with cold (0 °C) ether and collected by filtration: yield 1.4 g (75%); mp 139–140°; $[\alpha]^{25}D$ +8.0° (*c* 1, ethanol); TLC (CMA), R_f 0.67, (BAW) R_f 0.9.

Anal. Calcd for $C_{29}H_{44}N_2O_7$: C, 65.39; H, 8.33; N, 5.26. Found: C, 65.37; H, 8.08; N, 5.51.

The Boc group was cleaved from X by TFA and the phenacyl group was removed by sodium thiophenoxide treatment (1 M in DMF, 25 °C, 8 h). The resulting aspartic acid was treated with L-leucine *N*-carboxyanhydride and the dipeptide diastereomers were separated chromatographically by the method of Manning and Moore.[40] The mixture contained 99.4% L-Leu-L-Asp and only 0.6% L-Leu-D-Asp.

β-Phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate (50 mg, obtained from compound X by removing DCHA with citric acid) was treated with HF–10% anisole for 1 h at 0 °C. Following removal of HF and anisole, the residue was dissolved in acetic acid (2 ml) and spotted on a TLC plate with β-phenacyl L-aspartate HCl salt (IX) and L-aspartic acid as standards. TLC showed that HF treatment of β-phenacyl N^{α}-*tert*-butyloxycarbonyl-L-aspartate gave a spot identical in R_f value with the β-phenacyl L-aspartate HCl salt. No detectable amount of aspartic acid was observed.

Synthesis of Tetrapeptides via the β-Phenacyl Aspartic Acid Ester. H-Glu-α-Asp-Gly-Thr-OH (II). The same solid-phase synthesis procedure described for peptide I was used for the synthesis of peptide II except that β-phenacyl Boc-aspartate DCHA (X) was used instead of β-benzyl Boc-L-aspartate. The phenacyl group was removed from 50 mg of the fully protected peptide resin by stirring at 25 °C for 8 h with 1 M sodium thiophenoxide (132 mg in 1 ml of DMF). The partially protected peptide resin was filtered, washed well with DMF and CH_2Cl_2, and dried. The peptide was then cleaved and deprotected with HF–10% anisole at 0 °C for 0.5 h. The crude product was separated and quantitated on the amino acid analyzer as described in Table VI. A large peak (97.6% of total) of H-Glu-α-Asp-Gly-Thr-OH eluted at 70 min. This was identical with the standard α-aspartyl peptide that had been prepared for reference. A small peak (2.4%) of cyclized H-Glu-Asp-Gly-Thr-OH eluted at 116 min.

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Registry No.—II, 57718-76-0; III, 57680-10-1; IV, 57680-11-2; V, 2177-63-1; VI, 52615-97-1; VII, 57680-12-3; VIII, 34582-32-6; IX, 57680-13-4; X, 57680-15-6; H-Pro-Arg(Tos)-OH, 57680-16-7; H-Thr(Bzl)-Pro-Arg(Tos)-OH, 57680-17-8; H-Gly-Thr(Bzl)-Pro-Arg(Tos)-OH, 57680-18-9; H-Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-OH, 57680-19-0; H-Glu(OBzl)-Asp(OBzl)-Gly-Thr-(Bzl)-Pro-Arg(Tos)-OH, 57680-20-3; H-Leu-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-OH, 57680-21-4; H-Glu(OBzl)-Leu-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-Pro-Arg(Tos)-OH, 57680-22-5; H-Arg(Tos)-Glu(OBzl)-Leu-Glu(OBzl)-Asp(OBzl)-Gly-Thr-(Bzl)-Pro-Arg(Tos)-OH, 57680-23-6; H-Glu(OBzl)-Asp(OBzl)-OH, 57680-27-4; H-Glu(OBzl)-Asp(OBzl)-Gly-OH, 57680-25-8; H-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-OH, 57680-26-9; H-Gln-Asp(OBzl)-Gly-Thr(Bzl)-OH, 57680-27-0; H-Ala-Asp(OBzl)-Gly-Thr(Bzl)-OH, 57680-28-1; H-Ala-Asp(OBzl)-Gly-Ala-OH 57680-29-2; H-Asp(OBzl)-Gly-OH, 47094-17-7; H-Asp(OBzl)-Gly-Thr(Bzl)-OH, 57680-30-5; H-Glu(OBzl)-Asp(OBzl)-Ala-OH, 57680-31-6; H-Glu(OBzl)-Asp(OBzl)-Ser(Bzl)-OH, 57680-32-7; H-Asp(OBzl)-Ala-Leu-OH, 57694-98-1; H-Asp(OBzl)-Ser(Bzl)-Leu-OH, 57680-33-8; Boc-Glu(OBzl)-Asp(OBzl)-Gly-Thr(Bzl)-OH, 57680-34-9; HF, 7664-39-3; isobutene, 115-11-7; *tert*-butyl azidoformate, 1070-19-5; α-bromoacetophenone, 70-11-1; dicyclohexylamine, 101-83-7.

References and Notes

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(2) Abbreviations used: Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(4-biphenylyl)-2-propyloxycarbonyl; Bzl, benzyl; Bu^t, *tert*-butyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMF, *N,N*-dimethylformamide; Et_3N, triethylamine; Pac, phenacyl; R, resin; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl. Aspartoyl indicates the bivalent amino acid radical derived from aspartic acid, Organic Nomenclature, IUPAC rule 58.3, "Handbook for Chemical Society Authors", *Chem. Soc., Spec. Publ.*, **No. 14** (1961). Aspartoylglycine is the cyclic imide formed by diacylation of the amino group of glycine by the two carboxyl groups of aspartic acid. Other nomenclature and symbols follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241,** 2491 (1966); **242,** 555 (1967); **247,** 977 (1972).
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Ring Contractions of 5-Diazouracils. I. Conversions of 5-Diazouracils into 1,2,3-Triazoles by Hydrolysis and Methanolysis

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The hydrolysis of $O^{5'}$-6(*S*)-cyclo-5-diazouridine (**1**) to 1-(β-D-ribofuranosyl)-1,2,3-triazole-4-carboxamide (**2**) and carbon dioxide was shown to proceed via initial attack at C-2 by using oxygen-18 label in the C-4 position. Similar reactions of $O^{5'}$-6(*S*)-cyclo-5-diazo-2′-deoxyuridine (**5**), 5-diazouracil-6-methanolate (**7**), and 5-diazo-1-methyluracil-6-methanolate (**11**) gave the expected triazole derivatives. The unsuccessful hydrolysis of $O^{5'}$-6(*S*)-cyclo-5-diazo-3-methyluridine (**13**) was shown to be due to the absence of an initial attack by water. Methanolysis of **1** gave **2**, methyl 1-(β-D-ribofuranosyl)-1,2,3-triazole-4-carboxylate (**16**), and methyl carbamate (**17**). Methanolysis of **11** gave 1-methyl-1,2,3-triazole-4-carboxamide (**12**), **17**, and methyl 1-methyl-1,2,3-triazole-4-carboxylate (**18**). Methanolysis of **7** gave methyl *N*-(1,2,3-triazol-4-ylcarbonyl)carbamate (**20**) which established that these ring contractions proceeded via a N-1–C-2 bond cleavage. Diazotization of O^{2}-2′-cyclo-5-amino-5′-deoxyuridine (**28**) gave a product which suggested that these ring contractions require the formation of a tautomeric carbinolamidine prior to nucleophilic attack. Methanolysis of 5-(3,3-dimethyl-1-triazeno)uridine (**36**) gave **2** and **16**. This reaction was probably the result of direct nucleophilic attack on **36** rather than a prior decomposition of the triazeno group to a diazo group since 5-(3,3-dimethyl-1-triazeno)-1,3-dimethyluracil (**38**) was recovered quantitatively under similar reaction conditions. A partial hydrolysis of **11** labeled with oxygen-18 at C-2 showed a retention of isotopic label and suggested that the transition state for ring opening involved a partial C–N bond cleavage rather than the formation of a tetrahedral intermediate. The results are discussed in terms of a mechanism in which a proton at N-3 of the uracil ring must tautomerize to the O-2 position and the diazo ether derivative of this tautomer must be formed prior to ring opening.

Our initial interest in this area involved a structural reinvestigation[1] of 5-diazouracils and their possible synthetic utility for the preparation of 5-substituted uracil derivatives. This had been suggested[2] in the literature and a few reports of nucleophilic displacement reactions had been published.[3–5] Our preliminary studies, to determine the susceptibility of the diazo group of $O^{5'}$-6(*S*)-cyclo-5-diazouridine[1] (**1**) toward nucleophilic displacement, revealed that displacement reactions did not occur at low temperatures. In an effort to find a suitable solvent for conducting displacement reactions at elevated temperatures, we investigated the stability of **1** in acetonitrile at 100°. We observed a ring contraction of **1** to afford 1-(β-D-ribofuranosyl)-1,2,3-triazole-4-carboxamide[6] (**2**). We could find no precedent for this unusual reaction in the literature, which prompted us to initiate a study on the scope and mechanism of this reaction.

Results and Discussion

A solution of **1** in acetonitrile was heated in a stainless steel reaction vessel at 100° and the solution was then allowed to stand at ambient temperature to afford a white solid (**2**). Initial data indicated that **1** had been converted to uridine via a simple nitrogen elimination, since there was observed an absence of absorption bands in the 4.65-μ region of the ir spectrum and specific peaks [B + H (112), B + 2H (113), S (133), M − 30 (214)] in the low-resolution mass spectrum were essentially identical with those reported for uridine.[7] However, the uv spectrum of **2** revealed the absence of any absorption maximum in the 230–346-nm region. Elemental analyses (C, H, N) for **2** were found to be consistent with the empirical formula $C_8H_{12}N_4O_5$ and established that a ring carbonyl group had been lost instead of diatomic nitrogen. The structure of **2** was established on the basis of the following data.

The ^{1}H NMR spectrum (Figure 1) of **2** revealed a pattern of peaks in the δ 3.5–6.5 region which were indicative[8] of a ribofuranosyl moiety. The presence of D-ribose was confirmed by the treatment of **2** with dilute acid, followed by a direct paper chromatographic comparison of the hydrolysate with similarly treated samples of D-ribose, D-arabinose, and D-xylose (Table I). The facile hydrolysis of **2** suggested a *N*-glycosyl bond. The ^{1}H NMR spectrum (Figure 1) of **2** revealed the presence of two broad singlets (δ 7.75 and 7.50) which were exchanged on the addition of deuterium oxide to the ^{1}H NMR sample. This was suggestive of a carboxamide group and additional evidence for the presence of an amide group was obtained by a positive hydroxylamine–ferric chloride test.[9] Only one unassigned absorption peak remained in the ^{1}H NMR spectrum and it was assumed to be an aromatic proton on the basis of its chemical shift (δ 8.80). These data were all consistent with a disubstituted, five-membered heterocycle with three ring nitrogens (triazole). The formation of a triazole could occur by loss of the carbonyl group in the C-2 position of **1** followed by annulation between N-1 and the diazo group. If ring opening and rearrangement had occurred in the proposed manner, then the structure must be 1-(β-D-ribofuranosyl)-1,2,3-triazole-4-carboxamide[10–12] (**2**). A rigorous comparison of this nucleoside with an authentic sample prepared