59015-40-6; **13b**, 59015-41-7; **14a**, 59015-42-8; **14b**, 59015-43-9; **15a**, 59015-44-0; **15b**, 59015-45-1; **16a**, 59015-46-2; **16b**, 59015-47-3; **24**, 59015-48-4; **26**, 59015-49-5; *N*-(phenylpropargyl)phthalimide, 4656-94-4; *trans*-cinnamoyl chloride, 17082-09-6; *cis*-cinnamoyl chloride, 59015-50-8; phenylpropiolyl chloride, 7299-58-3; *N*-acetyl-*N*-(*cis*-cinnamyl)acetamide, 59015-51-9; *trans*-cinnamyl chloride, 21087-29-6.

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Use of Substituted Benzyl Esters as Carboxyl-Protecting Groups in Solid-Phase Peptide Synthesis

Ross L. Prestidge, David R. K. Harding, and William S. Hancock*

Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand

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Benzyl esters have been widely used for the protection of side-chain carboxyl groups in peptide synthesis. This paper describes the evaluation of two substituted benzyl esters of glutamic acid in solid-phase peptide synthesis. The γ-*p*-chlorobenzyl ester of glutamic acid was found to be significantly more stable to trifluoroacetic acid cleavage than the benzyl ester, and yet it could be removed without difficulty by liquid HF at 0 °C. Hence it is recommended for side-chain protection of aspartic acid and glutamic acid residues in longer syntheses. Peptides with side-chain carboxyl groups protected by *p*-nitrobenzyl esters were prepared by solid-phase peptide synthesis followed by cleavage from the resin with HBr in acetic acid. Two protected peptides were synthesized by this approach, the tripeptide H-Gly-Glu(γ-OBzl-*p*-NO_2)-Ala-OH, and the amino-terminal hexapeptide from the acyl carrier protein of *E. coli*.

Protection of the side-chain carboxyl groups of aspartic and glutamic acids in peptide synthesis has been most commonly achieved by benzyl esters.[1] This protection is very suitable, in that it is fairly stable to the conditions of peptide synthesis, and it can be removed at the end of the synthesis by strongly acidic or reducing conditions.[2]

There are important reasons, however, for seeking alternative carboxyl-protecting groups, for it has been shown[3–5] that benzyl esters are not completely stable to the conditions commonly used to remove the *t*-Boc[6,7] group during peptide synthesis. This lability gives rise to a cumulative loss of side-chain protection, and increases the possibility of branching of the peptide chain, particularly during a long synthesis.

An important potential use of more stable carboxyl-protecting groups is in the synthesis of protected peptides, which can be used in fragment syntheses and semisynthesis of proteins.[8–12] In an attempt to develop a simple method for the preparation of protected peptides, it was decided to examine the synthesis of a fully protected peptide using standard solid-phase techniques. If the side chains and amino terminus of a peptide were blocked by groups stable to acidolysis, the synthesis could be performed on a chloromethylated resin, with the usual *t*-Boc group for α-amino protection, and using HBr in acetic acid for the cleavage of the peptide from the resin.

This paper describes the evaluation of two acid-stable carboxyl-protecting groups: the *p*-chlorobenzyl ester as a group of moderately increased stability for use in longer syntheses, and the *p*-nitrobenzyl ester as a much more stable group for the synthesis of protected peptides with HBr

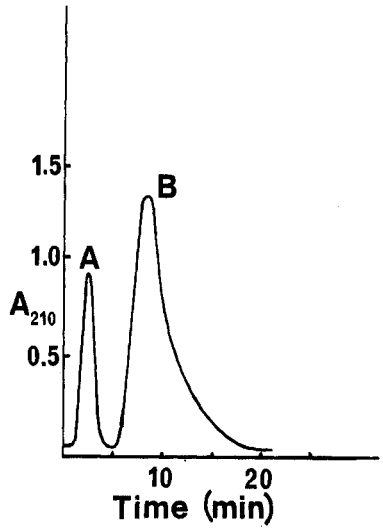

Figure 1. High-pressure liquid chromatography of a sample of Glu(γ-OBzl) partially cleaved by trifluoroacetic acid–dichloromethane (1:1). Peak A represents benzyl alcohol and impurities present in the solvent and was not quantitated. Peak B is Glu(γ-OBzl). The cleavages and analyses were carried out as described in the Experimental Section.

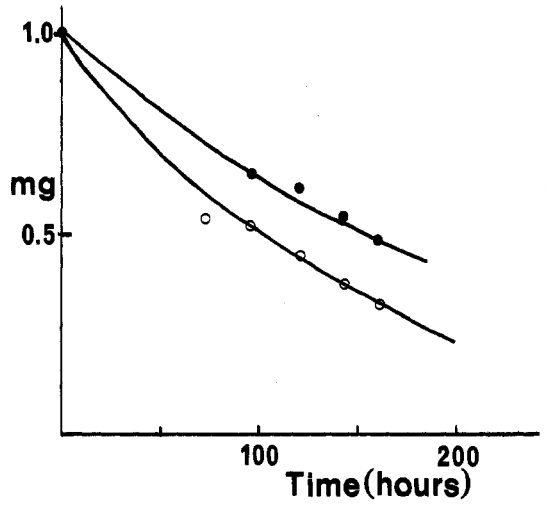

Figure 2. A study on the hydrolysis of glutamic acid monoesters by trifluoroacetic acid–dichloromethane (1:1) at 45 °C. The rate of hydrolysis was quantitated by the amount of monoester remaining using the procedures described in the Experimental Section, Glu(γ-OBzl) (○) and Glu(γ-OBzl-*p*-Cl) (●).

cleavage from the resin. In a recent communication[13] we have described a new facile method for the preparation of the substituted benzyl esters of aspartic and glutamic acids. The ready availability of these amino acid derivatives has allowed us to examine the use of more stable esters of glutamic acid in the solid-phase synthesis of several model peptides. These studies have led to the synthesis of the amino-terminal hexapeptide of the acyl carrier protein of *E. coli* in a fully protected form, suitable for use in semisynthesis.

Results and Discussion

The criteria for a more stable side-chain protecting group are that it should be readily prepared, that it should be significantly more stable to the conditions of peptide synthesis than benzyl ester protection, and that it should be readily removed at the end of the synthesis. The acid stability of the benzyl ester group can be conveniently increased by the introduction of electron-withdrawing substituents into the aromatic ring. For example, Merrifield[14] investigated the use of chlorinated benzyloxycarbonyl (Z) groups for the protection of lysine residues, and found that (4-Cl-Z)Lys was three times more stable than Z-Lys to trifluoroacetic acid in dichloromethane (1:1). Other chlorinated derivatives were found to be even more stable, some to the point of being difficult to remove by HF cleavage. Similarly Li[15] employed the 4-bromobenzyl ester for the protection of Glu residues, and claimed it to be four times as stable as the benzyl ester, but detailed evidence as to the suitability of this protecting group in peptide synthesis was not presented.

Following the above criteria, the *p*-chlorobenzyl ester was selected for investigation. It can be readily prepared by the copper-catalyzed hydrolysis method of Prestidge et al.,[13] and the starting material, *p*-chlorobenzyl alcohol, is commercially available. The stability of this ester to trifluoroacetic acid (TFA) hydrolysis was studied, and compared with the stability of the benzyl ester. In order to provide a measurable extent of hydrolysis in a reasonable time, the studies were performed at 45 °C. The samples from these hydrolyses were analyzed by high-pressure liquid chromatography (HPLC) on a Porasil silica column with potassium phosphate buffer (pH 4.0, 0.02 M) as the eluent. A typical chromatogram is shown in Figure 1. It should be noted that an aqueous buffer system was used in conjunction with a silica column. The aqueous system minimized tailing of the benzyl esters, and should prove to be a useful method for the analysis of very polar compounds on HPLC. The time course of the cleavage of para-substituted benzyl esters of glutamic acid is shown in Figure 2.

The *p*-chlorobenzyl ester was found to be twice as stable as the benzyl ester to hydrolysis by TFA–CH_2Cl_2 (1:1) at 45 °C. From the temperature dependence of Hammett ρ factors[16] it can be predicted that this stability difference will be significantly greater at room temperature. The *p*-chlorobenzyl ester was also found to be completely removed by liquid HF at 0 °C for 30 min and can therefore be recommended for the synthesis of large peptides. Although the data reported by Merrifield[3] imply that benzyl ester protection of Asp and Glu is adequate for moderate sized peptides, it should be emphasized that such protection is inadequate for small acidic proteins such as the acyl carrier protein (ACP) from *E. coli*. This protein contains 14 glutamic and 7 aspartic acid residues out of a total of 77. Using the method of Merrifield[3] it can be calculated that in a synthesis of ACP using benzyl ester protection of Asp and Glu, 5.6% of the chains would be prematurely deprotected at a glutamic residue and 2.75% at an aspartic residue. These side reactions should be reduced to an acceptable level by the use of a more stable protecting group such as *p*-chlorobenzyl.

Although many approaches to the synthesis of protected peptide fragments have been suggested, involving alternative resins and cleavage techniques,[17–31] none has found a wide application.

One disadvantage is that many of the modified resins involve lengthy syntheses.[18,19,23,24,30] More significantly, the effect of these modifications on the properties of the resin has not been evaluated, so that the routine use of such resins must await further evaluation of their behavior, particularly in longer synthesis. It has been observed that for mild, selective cleavage procedures, yields can decrease with larger peptides,[31,32] so that these methods may not be suitable for the synthesis of large protected peptides.

Most of the published approaches to solid-phase fragment synthesis involve changes in the standard procedures of synthesis. These new procedures are not only time consuming but also often contain difficulties which would make them unsuitable for routine use, or limitations which would restrict their applicability to a wide range of syntheses. For example, resins which form a labile bond to the first amino acid require the use of even more labile α-amino protection during the synthesis[23,24] which is inconvenient for routine use. Resins

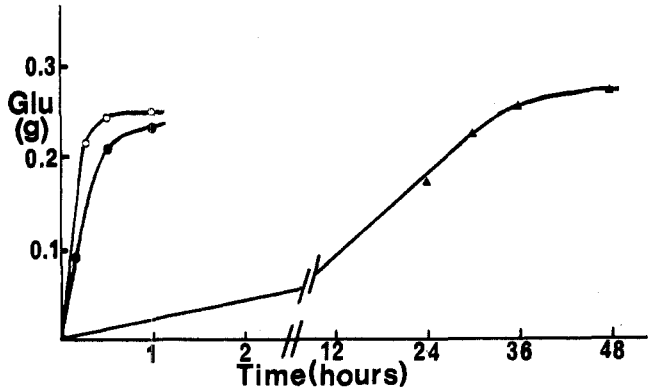

Figure 3. A study on the hydrolysis of glutamic acid monoesters by HBr and acetic acid. The rate of hydrolysis was quantitated by the amount of Glu liberated using the procedures described in the Experimental Section and gave values for Glu from Glu(γ-OBzl) (○), from Glu(γ-OBzl-*p*-Cl) (●), and from Glu(γ-OBzl-*p*-NO_2) (▲).

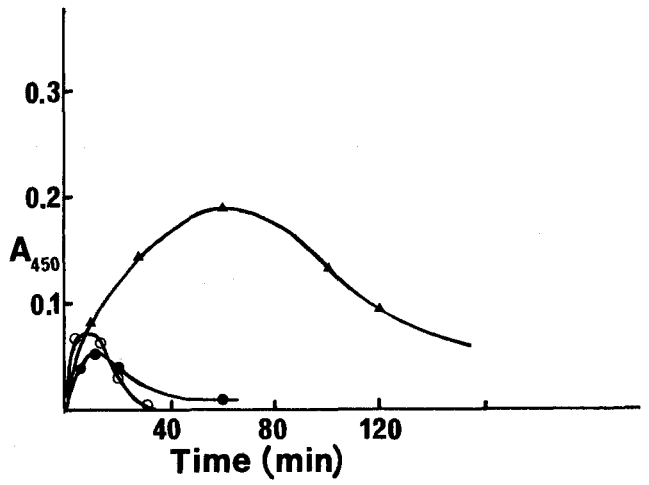

Figure 4. A study on the cleavage of protected tripeptides H-Gly-Glu(OBzl-X)-Ala-OH from the resin with HBr and acetic acid. The amount of peptide cleaved was quantitated by uv measurements, using the procedures described in the Experimental Section and gave values for X = H (○), X = *p*-Cl (●), and X = *p*-NO_2 (▲).

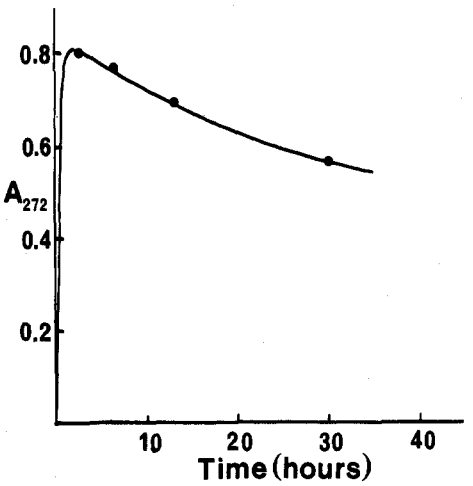

Figure 5. A study on the cleavage of the protected hexapeptide, ACP_{1-6} from the resin with HBr and acetic acid. The amount of peptide cleaved was quantitated by uv measurement, using the procedures described in the Experimental Section.

where the bond to the peptide chain is activated in the penultimate step involve treating the peptide with such reagents as peroxides or methyl iodide.[20-22] This activation step is not compatible with peptides which contain Trp, Cys, or Met, and may also cause racemization.[33]

An attractive approach to the solid-phase synthesis of protected peptides is to use standard methods of peptide synthesis with the exception that the side chains are protected by acid-stable groups. A range of protecting groups are known which are stable to HBr in acetic acid; for example, Lys(TFA), Arg(NO_2), His(DNP) Cys(Acm). If a suitable acid-stable group could be found for carboxyl protection, and the amino terminus was protected by trifluoroacetylation, HBr cleavage would release a peptide with only one free functional group, the carboxyl-terminal COOH, which could be used to form an active ester for fragment condensation.

For this reason the stability of *p*-chlorobenzyl and *p*-nitrobenzyl esters to HBr cleavage was investigated, and the results of these studies are shown in Figure 3. The *p*-chlorobenzyl ester was found to be only twice as stable to HBr as the benzyl ester, and this stability was not sufficient for the isolation of peptides protected with this group. The *p*-nitrobenzyl ester, however, was 50 times as stable as the benzyl ester, and was used for the synthesis of two protected peptides. The stability of *p*-nitrobenzyl esters to HBr cleavage is well known,[34,35] but little use appears to have been made of this property in peptide syntheses.[36,37]

The tripeptide H-Gly-Glu(OBzl-*p*-NO_2)-Ala-OH was prepared by this method, but the rate of loss of the *p*-nitrobenzyl ester was much greater than expected. This was attributed to the fact that the cleaved protected peptide was not stable to HBr/acetic acid. The data are given in Figure 4, and it can be seen by comparison with Figure 3 that benzyl, *p*-chlorobenzyl, and *p*-nitrobenzyl side-chain protection were all cleaved from this peptide at rates six times greater than that of the corresponding amino acid esters. Further analysis of the products by electrophoresis at pH 2.1 and 6.5 showed a variety of cleavage products formed on the disappearance of the protected peptide. It has been suggested[38,39] that glycine, having no side chain, facilitates intramolecular reactions in peptides containing this residue, and it may have accelerated the acidolytic decomposition of this protected tripeptide. A similar rearrangement has been observed for the aspartylglycine sequence under acidic conditions.[38] The lability of this peptide to acidolysis was confirmed by the observation that the purified peptide decomposed to the extent of approximately 50% on standing in 50% acetic acid for 24 h at 20 °C. The protected peptide, however, was isolated and purified in modest overall yield. In view of these results, and other observations,[40] it is recommended that treatment of peptides with HBr/acetic acid should be minimized. For example, two 20-min cleavages have been found to give excellent results for removal of peptides from the resin. The peptide was shown to be homogeneous on high-voltage paper electrophoresis at pH 2.1 and 6.5. The peptide was neutral at pH 6.5, which demonstrated that the *p*-nitrobenzyl ester had been retained in the cleavage reaction. The success obtained with these model peptides encouraged us to proceed to the synthesis of a larger peptide.

The 1-6-hexapeptide from the acyl carrier protein of *E. coli* was synthesized in a fully protected form using *p*-nitrobenzyl protection for the two side-chain carboxyl groups. This peptide is seen as a stringent test of synthetic technique, as it contains three carboxyl groups, two hydroxyl groups, an amino group, and a guanidino function within six residues. Serine and threonine were protected as benzyl ethers, glutamic acid as *p*-nitrobenzyl esters, the amino terminus by acetylation, and the arginine residue as the nitro derivative. The time course for cleavage of the protected peptide from the resin by HBr in acetic acid was studied by uv measurement of aliquots of the reaction mixture and the results are shown in Figure 5. It was found that two 20-min cleavages gave a good yield of peptide with minimal loss of *p*-nitrobenzyl ester protection.

Assuming $\epsilon_{275} = 1.0 \times 10^4$ for both Glu(OBzl-*p*-NO_2) and Arg(NO_2), the rate of deprotection of glutamic acid residues was calculated to be 2.1×10^{-4} min^{-1} per residue. This corresponds to a rate of deprotection of this peptide which is about half that of the free amino acid. The peptide was purified by gel filtration on Sephadex G10 and recrystallization from methanol.

It was observed in this preparation that *p*-nitrobenzyl esters confer the additional advantage of greater crystallinity, as the crude peptide could be purified by recrystallization to give a product homogeneous by electrophoresis at pH 2.1 and 6.5 and TLC in three solvents. Electrophoresis at pH 6.5 (R_f 0.35 relative to glutamic acid) showed that the glutamic, α-amino, and arginine residues were still protected. Amino acid analysis was satisfactory for the protected hexapeptide (Arg 1.0, Ile 1.0, Glu 2.2, Thr 0.8 and Ser 0.8) except for low values for serine and threonine, which can be attributed to destruction during acid hydrolysis. Moritz and Wade[41] have reported that during acid hydrolysis in the presence of Arg(NO_2), Thr and Ser can be destroyed to the extent of 40%.

In summary, we propose the use of *p*-chlorobenzyl esters for side-chain carboxyl protection in the synthesis of moderately sized peptides and proteins, in that this protecting group is easily used in solid-phase peptide synthesis, readily cleaved by HF, and gives a useful increase in stability to trifluoroacetic acid. We also propose the use of acid-stable side-chain protecting groups, including the *p*-nitrobenzyl ester protection for Asp and Glu, for the synthesis of protected peptides using conventional techniques. The *p*-nitrobenzyl ester has both the advantages of facile preparation and removal, in this case by hydrogenolysis.[35] It is hoped that this approach will greatly reduce the effort required for the synthesis of protected peptides by the solid-phase method, thus leading to a wider use of semisynthesis. For example, the protected ACP hexapeptide prepared by this method can now be used in fragment condensation, as all reactive groups are blocked with the exception of the hydroxyl group of Ser and Thr, which should not need to be blocked for a single active ester coupling.

Experimental Section

All melting points were determined on a Reichert hot-stage apparatus and are uncorrected. Amino acid analyses were carried out on a Beckman 120C amino acid analyzer. Thin layer chromatography (TLC) was done on precoated silica gel plates (Eastman Chromagram). The *t*-Boc group was removed with HCl vapor, and TLC plates were visualized with ninhydrin. Peptide syntheses were performed on a Schwarz-Mann automated peptide synthesizer, using the procedure of Hancock et al.[42] High-pressure liquid chromatography (HPLC) was performed on a Waters Model 6000 chromatograph, and monitored by a Cecil CE212 variable wavelength uv monitor. *p*-Chlorobenzyl alcohol and *p*-nitrobenzyl alcohol were supplied by Fluka, and polystyrene–divinylbenzene resins and protected amino acids were supplied by Schwarz-Mann. All solvents were of analytical grade, or purified as in previous studies.[42]

HF cleavages were performed in an all-Daiflon HF reaction apparatus Type II (Protein Research Foundation, Japan). Mass spectrometry was performed on an AEI MS-9 instrument at a potential of 70 eV.

Synthesis of Substituted Benzyl Monoesters of Aspartic and Glutamic Acids. Amino acid diester tosylates were synthesized by the method of MacLaren, Savige, and Swan.[43] These diesters were selectively hydrolyzed by Cu(II) in the presence of base to give the side-chain esters, using the procedure recently published.[13] In a typical synthesis glutamic acid di(*p*-nitrobenzyl) ester *p*-toluenesulfonate (11 g, 20 mmol) was dissolved in ethanol (180 cm^3) and aqueous $CuSO_4 \cdot 5H_2O$ (20 g, 80 mmol in water, 350 cm^3) was added. The pH was raised to 8.0 and maintained at that value for 60 min with 1 M NaOH. The pH was then lowered to 3.0 with 3 M HCl, and the precipitate of the copper complex of Glu(γ-OBzl-*p*-NO_2) was filtered off and washed with water, ethanol, and ether. This complex was then dissolved in boiling aqueous ethylenediaminetetraacetic acid disodium salt (10 g in 100 cm^3 of water) and the product precipitated immediately. The solution was cooled and the product filtered off and washed with water and ethanol. The product (3.1 g, 54%) was obtained as a light brown powder, mp 158–159 °C (lit. 158–159 °C).[13]

Synthesis of *tert*-butyloxycarbonyl amino acid substituted benzyl esters was performed by the dimethyl sulfoxide procedure of Stewart and Young.[44] In a typical synthesis, Glu(γ-OBzl-*p*-NO_2) (9.40 g, 33 mmol) was suspended in dimethyl sulfoxide (200 cm^3) and *tert*-butyloxycarbonyl azide (6 cm^3) and triethylamine (9 cm^3) were added. The mixture was stirred at room temperature for 3 days, by which time it was homogeneous. The product was isolated by the standard procedure as a viscous yellow-brown oil (11.55 g, 94%) which was homogeneous by TLC on silica plates with 1-butanol–pyridine–water (2:2:1, R_f 0.72) and was used without further purification. The *t*-Boc *p*-chlorobenzyl ester of glutamic acid was synthesized by the same procedure, and was isolated as an oil which crystallized on standing overnight to give the product in 63% yield. The product was recrystallized from ether–petroleum ether: mp 80–81 °C; TLC (1-butanol–pyridine–water, 2:2:1) R_f 0.82, (ethanol–aqueous NH_3, 9:1) R_f 0.78. Anal. Calcd for $C_{17}H_{22}ClNO_6$ (371.41): C, 54.98; H, 5.97; N, 3.77; Cl, 9.55. Found: C, 54.91; H, 5.84; N, 4.10; Cl, 9.50.

Stability of Monoesters to Trifluoroacetic Acid Cleavage. Samples of the monoester (1 mg) were dissolved in trifluoroacetic acid–dichloromethane (1:1, 0.1 cm^3) and held at 45 °C in sealed tubes. At predetermined time intervals a tube was dried with a stream of N_2 and the residue dissolved in potassium phosphate buffer, 0.02 M, pH 4.0 (25 cm^3). These solutions were examined by HPLC on a Corasil I pellicular silica column (2 ft × 0.125 in.), eluting with the potassium phosphate buffer at 4 cm^3/min, and 2300 psi. The elution was monitored by uv absorption at 210 nm and calibrated with standard samples. The results are shown in Figure 2, and a typical chromatogram is given in Figure 1.

The rates of deprotection with TFA–CH_2Cl_2 (1:1) at 45 °C were Glu(γ-OBzl) 6.6×10^{-3} min^{-1}, Glu(γ-OBzl-*p*-Cl) 4.1×10^{-3} min^{-1}. Hence the *p*-chlorobenzyl ester is 1.6 times as stable to trifluoroacetic acid under these conditions as is the benzyl ester.

Stability of Monoesters of HBr–Acetic Acid. The monoester (500 mg) was suspended in HBr in acetic acid (33% w/w, 25 cm^3) and allowed to react at room temperature. Aliquots were taken at various times, dried by a stream of N_2, and dissolved in water (3 cm^3). A portion of this solution (10 μl) was spotted onto silica gel plates which were developed with 1-butanol–pyridine–acetic acid–water (15:10:3:12) and visualized with ninhydrin. The spots corresponding to glutamic acid and the monoester were cut out and eluted with boiling ethanol, and the absorbance of the ninhydrin color read at 565 (Glu) or 520 nm (monoester) and compared with standards. The results are given in Figure 3. The heterogeneity of the reaction mixture made rigorous kinetic analysis difficult, and therefore the time at which glutamic acid reached a stable maximum concentration was taken as the completion of the reaction: Glu(γ-OBzl) 1.5 h, Glu(γ-OBzl-*p*-Cl) 3 h, Glu(γ-OBzl-*p*-NO_2) 48 h.

Stability of Monoesters to HF Cleavage. The monoester (100 mg) was suspended in liquid HF (10 cm^3) for 30 min at 0 °C in the HF line. The HF was then evaporated off under reduced pressure, and the residue dissolved in water (100 cm^3). Aliquots of this solution (50 μl) were chromatographed on silica TLC plates with 1-butanol–pyridine–water (2:2:1). Both the benzyl and *p*-chlorobenzyl esters were completely cleaved by HF under these conditions. The *p*-nitrobenzyl ester was stable, and no free glutamic acid was detected by TLC, confirming the observation of Marglin.[39]

Synthesis of Protected Tripeptides. Peptides of the sequence H-GLY-Glu(OBzl-X)-Ala-OH, where X is H, *p*-Cl, or *p*-NO_2, were synthesized by standard solid-phase techniques.[42] *t*-Boc-alanine substituted polystyrene–1% divinylbenzene resin (1 g, 660 μmol/g) was used in each case. The *t*-Boc group was used for α-amino protection, and 50% trifluoroacetic acid in dichloromethane was used for deprotection. The coupling reagent used was dicyclohexylcarbodiimide.

Portions of the peptide resins (500 mg) were cleaved with HBr in acetic acid (30% w/w, 20 cm^3). Samples (1 cm^3) were taken at various time intervals, dried with a stream of N_2, dissolved in water (0.5 cm^3), and analyzed by high-voltage paper electrophoresis at pH 6.5 and 2.1 in pyridine–acetic acid–formic acid buffers. The electrophoretigrams were visualized with ninhydrin and the appropriate spots cut out and eluted with boiling ethanol. The absorbances of the ninhydrin colors were read at 450 nm (for protected peptides) or 400 nm (for deprotected peptides). The results are shown in Figure 4.

The tripeptide H-Gly-Glu(OBzl-*p*-NO_2)-Ala-OH was prepared by HBr cleavage of the appropriate peptide resin (509 mg) with HBr in acetic acid (33% w/w, 7 cm^3) for 1 h at room temperature. The cleavage products were evaporated under reduced pressure and the residue containing the peptide was dissolved in 50% acetic acid (3 cm^3) and

purified by gel filtration on a Sephadex G10 column (26 × 270 mm) in the same solvent. The peptide was then purified by ion exchange on a Sephadex SP-C25-120 column (16 × 95 mm) with an ammonium acetate buffer (pH 4.5, 0.01 M). The peptide was eluted from the ion exchange column as a single symmetrical peak, and gave only one spot on high-voltage paper electrophoresis at pH 6.5. The pooled peak was desalted on a Sephadex G10 column (26 × 270 mm) in 50% acetic acid and lyophilized. The peptide was recrystallized from ethanol–ether to give a white, crystalline powder (12 mg, 13% yield based on picric acid analysis of the resin peptide[45]), mp 190.5–192 °C. Amino acid analysis after acid hydrolysis (HCl–propionic acid, 1:1, for 2 h at 130 °C) gave Gly 1:1, Glu 1.0, Ala 1.1. A satisfactory elemental analysis could not be obtained, possibly because of the extremely hygroscopic nature of the peptide. Consequently the material was acetylated to increase the volatility[46] and submitted for mass spectrometry. Although the sample gave no molecular ion, a fragment was observed at *m/e* 345.0932 consistent with the loss of two water molecules from the desired tripeptide, and all further fragment peaks were consistent with the proposed structure. The peptide was homogeneous on high-voltage paper electrophoresis at pH 2.1 and 6.5 (R_f 0.75 and 0.0, respectively, relative to glutamic acid).

Synthesis of the Protected 1–6-Hexapeptide from the Acyl Carrier Protein of *E. coli*. The peptide H-Ser(Bzl)-Thr(Bzl)-Ile-Glu(OBzl-*p*-NO_2)-Glu(OBzl-*p*-NO_2)-Arg(NO_2)-resin was synthesized by standard solid-phase techniques as above. The peptide was cleaved from a portion of the resin (100 mg) using HBr in acetic acid (33% w/w, 2 cm^3). Samples (5 μl) were taken at various time intervals, diluted to 5 cm^3 with distilled water, and their uv absorption at 275 nm was measured. The results are given in Figure 5.

The hexapeptide Ac-Ser-Thr-Ile-Glu(OBzl-*p*-NO_2)-Glu(OBzl-*p*-NO_2)-Arg(NO_2)-OH was prepared by acetylation of the peptide resin (800 mg) with acetic anhydride–triethylamine (5 g of each, a 50-fold excess over the amount of peptide on the resin) for 30 min at room temperature, followed by two HBr cleavages (HBr, 33% in acetic acid, 12 cm^3) for 20 min at room temperature. The peptide was purified by gel filtration on a Sephadex G10 column (26 × 280 nm) in 50% acetic acid, and recrystallization from methanol. The peptide was obtained as an off-white powder (2.44 mg, 34.8% yield of the amount of peptide resin, mp 158–162 °C). Its purity was examined by the TLC on silica gel followed by visualization with the chlorine–starch–KI peptide spray.[47] The peptide was found to be homogeneous in the following systems: ethanol–aqueous ammonia (9:1 v/v, R_f 0.54), methanol (R_f 0.79), and 1-butanol–pyridine–acetic acid–water (15:10:3:12, R_f 0.78). On high-voltage paper electrophoresis in pyridine–acetic acid–formic acid buffers at pH 2.1 and 6.5 the product behaved as a fully protected peptide, migrating with a net charge of 0 and −1, respectively.

The peptide was hydrolyzed with HCl–propionic acid (1:1 v/v) in the presence of anisole (0.1 cm^3) at 130 °C for 30, 60, and 120 min. Amino acid analysis of the hydrolysate gave Arg 1.0, Ile 1.0 (extrapolated to infinite time), Glu 2.2, Thr 0.8, and Ser 0.8 (extrapolated to zero time).[41]

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Registry No.—*t*-Boc-*p*-chlorobenzyl glutamate, 59092-58-9; H-Gly-Glu(OBzl-*p*-NO_2)-Ala-OH, 59092-59-0; Ac-Ser-Thr-Ile-Glu(OBzl-*p*-NO_2)-Glu(OBzl-*p*-NO_2)-Arg(NO_2)-OH, 59092-60-3.

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