Acid Stability of Several Benzylic Protecting Groups Used in Solid-Phase Peptide Synthesis. Rearrangement of *O*-Benzyltyrosine to 3-Benzyltyrosine[1]

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Received November 28, 1972

Abstract: The acid stabilities of ten amino acid derivatives bearing benzylic side-chain protecting groups were determined quantitatively by ion-exchange chromatography. The apparent first-order rate constants for loss of the benzylic groups were measured in 50:50 (v/v) trifluoroacetic acid–dichloromethane at 20°. The derivatives and their rate constants (k, 10^{-8} sec^{-1}) follow in order of increasing stability: *O*-benzyl-L-tyrosine (636) < N^{ϵ}-benzyloxycarbonyl-L-lysine (396) ≪ *O*-benzyloxycarbonyl-L-tyrosine (89) ~ *S*-(4-methoxybenzyl)-L-cysteine (82) ≪ *O*-benzyl-L-serine (10.7) ~ L-aspartic acid β-benzyl ester (9) ~ L-glutamic acid γ-benzyl ester (7) ~ *O*-benzyl-L-threonine (6.9) ≪ *O*-(2,6-dichlorobenzyl)-L-tyrosine (<0.1) ~ *S*-(4-methylbenzyl)-L-cysteine (<0.03). These data have been used to estimate the extent of side-chain deprotection and possible side-chain branching to be expected during the solid-phase synthesis of large peptides. During treatment with 50% TFA–CH_2Cl_2, *O*-benzyltyrosine partly underwent debenzylation to furnish tyrosine in 56% yield and partly underwent intramolecular rearrangement to provide 3-benzyltyrosine in 44% yield. In 50% HF–anisole at 0°, *O*-benzyltyrosine rearranged to 3-benzyltyrosine in about 15% yield and *O*-(2,6-dichlorobenzyl)tyrosine rearranged to 3-(2,6-dichlorobenzyl)tyrosine in only 5% yield. During deprotection and cleavage of model peptides from the resin with 80% HF–anisole, 13–20% of the *O*-benzyltyrosine residues were converted into 3-benzyltyrosine residues. Although *O*-benzyloxycarbonyltyrosine furnished only tyrosine on treatment with 50% TFA–CH_2Cl_2 or 50% HF–anisole, it is probably unsuitable for peptide synthesis because the protecting group can be removed by nucleophilic amines. Since *S*-(4-methylbenzyl)cysteine was quite stable in 50% TFA–CH_2Cl_2 and was completely deprotected by 50% HF–anisole in 1 hr at 0°, the 4-methylbenzyl group is recommended for S-protection of cysteine residues during solid-phase peptide synthesis.

Solid-phase synthesis of a large peptide requires a careful choice of protecting groups. During the coupling of an activated amino acid to a support-bound peptide, the α-amino group is usually protected by the *tert*-butyloxycarbonyl (Boc) group. After the coupling reaction, this temporary N^{α}-protecting group is removed by acidolysis with a moderately acidic medium such as dilute HCl in acetic acid or trifluoroacetic acid in dichloromethane. During the synthesis, the side-chain functional groups of several amino acids, including lysine, aspartic acid, glutamic acid, serine, threonine, tyrosine, and cysteine, are usually masked by benzylic protecting groups.[2] At the end of the synthesis, these side-chain protecting groups are also removed by acidolysis, but with a strongly acidic medium such as HBr in trifluoroacetic acid or liquid HF. The benzylic groups must not be removed by the moderately acidic medium used for N^{α} deprotection, since a peptide that has lost a side-chain protecting group may subsequently couple with an activated amino acid to form a branched peptide. This paper deals with the problem of side-chain branching and ways to minimize it.

The extent of side-chain branching in reducing the yield of the desired peptide is determined by the amino acid sequence of the peptide, the stability of the side-chain protecting groups, and the stability of the bonds holding the side-chain branches to the main chain. Solid-phase synthesis of a peptide chain usually proceeds stepwise from the C-terminus to the N-terminus, and removal of the N^{α}-Boc group is required at each step except the last. Thus the protected side chain of the residue at position P from the N-terminus of the desired peptide is treated with acid $P - 1$ times during the synthesis. Therefore, the extent of deprotection and branching will be greater at a side chain located near the C-terminus than at an identical side chain near the N-terminus. Side chains near the C-terminus that branch early in the synthesis will bear long branches; all side chains that branch later in the synthesis will bear shorter branches.

At the end of the synthesis, the use of purification techniques based on differences in molecular weight, shape, and charge usually permits separation of the desired peptide from branched peptides containing many side-chain residues in either one long peptide chain or several shorter ones. But present purification techniques are generally not powerful enough to separate a large peptide from branched peptides containing only a few side-chain residues. Thus the use of side-chain protecting groups that are completely stable to the moderately acidic medium used for removal of the N^{α}-Boc group should significantly increase the purity of the desired peptide by preventing side-chain branching.

As a guide to the usefulness of certain benzylic protecting groups in solid-phase synthesis, the stabilities of *S*-(4-methoxybenzyl)cysteine, *S*-(4-methylbenzyl)-cysteine, aspartic acid β-benzyl ester, glutamic acid γ-benzyl ester, *O*-benzylserine, *O*-benzythreonine, *O*-benzyltyrosine, *O*-(2,6-dichlorobenzyl)tyrosine, *O*-benzyloxycarbonyltyrosine, and N^{ϵ}-benzyloxycarbonyl-

(1) (a) Preliminary communication: B. W. Erickson and R. B. Merrifield in "Chemistry and Biology of Peptides" (Proceedings of the 3rd American Peptide Symposium, Boston, Mass., June 1972), J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 191. (b) This work was supported in part by Grant AM 01260 from the U. S. Public Health Service and by funds from the Hoffman-La Roche Foundation.

(2) R. B. Merrifield, *Advan. Enzymol. Relat. Areas Mol. Biol.*, 32, 221 (1969).

lysine were quantitatively measured in 50:50 (v/v) trifluoroacetic acid–dichloromethane (50% TFA), a medium often used for removal of the Boc group.[3] The presence of the free α-amino and α-carboxyl groups permitted separation of the side-chain-protected amino acid and the free amino acid by ion-exchange chromatography and detection by ninhydrin assay.

Two kinetic studies have shown that the reaction rate of a functional group bound to a copoly(styrene–divinylbenzene) bead is about half of the reaction rate of that functional group in solution. The apparent first-order solvolysis rate in benzyl alcohol at 25° with *N*-methylimidazole catalysis was 2.3 times slower for 2,4-dinitrophenyloxycarbonyl–copoly(styrene–2% divinylbenzene) beads than for 2,4-dinitrophenyl 4-isopropylbenzoate in solution.[4] Similarly, the apparent first-order rate for loss of the benzyloxycarbonyl (Z) group in 50% TFA at 20° was two times slower for oligo(N^{ϵ}-Z-lysyl)-valyloxymethyl–copoly(styrene–1% divinylbenzene) beads than for N^{ϵ}-Z-lysine in solution.[5] These studies demonstrate a relatively close agreement between rates measured in solution and in the solid phase. The relative stabilities of side-chain-protected amino acids in solution are expected to accurately predict their relative stabilities under solid-phase conditions.

The acidolysis of a protected amino acid, X, in a great molar excess of 50% TFA follows the rate law

$$kt = \ln (X_0/X_t) \quad (1)$$

where k is the apparent first-order rate constant and X_t is a quantity (concentration, corrected peak area) directly proportional to the moles of X remaining at time t. If the protected amino acid undergoes exclusive cleavage of the protecting group to yield the free amino acid, Y, then the sum of the moles of X and Y is constant with time. Since only the protected amino acid is present initially, it follows that

$$X_0 = X_t + Y_t \quad (2)$$

and thus

$$kt = \ln [1 + (Y_t/X_t)] \quad (3)$$

Determination of the apparent first-order rate constant from eq 1 requires the accurate measurement of the moles of X remaining after several time intervals. Since several of the amino acid derivatives examined in this study suffered less than 2% deprotection in 100 hr, use of eq 1 would require the determination of X_0/X_t, the ratio of two nearly equal but separetely measured quantities. Relatively small errors due to nonquantitative sampling, transfer, or analysis would significantly affect this ratio. In contrast, use of eq 3 is based on Y_t/X_t, the ratio of a small quantity to a large quantity, where both quantities can be measured during a single chromatographic separation under identical sampling conditions. This ratio is inherently more accurate, since it is independent of the volume of the reaction solution sampled, the volume of buffer used for dilution, or the volume of solution taken for chromatographic analysis.

The apparent first-order rate constants for deprotection of ten benzylic amino acid derivatives (Table I) were determined from the least-squares lines of best fit through data points plotted on a grid of ln (X_0/X_t) *vs.* time (Figure 1). Equation 3 was used to calculate the rate constants for all of the derivatives except Tyr(Bzl), which required the use of eq 1. Table I also shows the rate of deprotection of each derivative relative to that of Thr(Bzl), a derivative of satisfactory stability, and the calculated percentage of each benzylic group that would be lost on treatment with 50% TFA for 20 min, which is sufficient time to remove completely the N^{α}-Boc group during solid-phase synthesis.[6]

O-Benzylthreonine is sufficiently stable to survive prolonged exposure to 50% TFA during solid-phase synthesis. Only 1% of this derivative was deprotected after treatment with 50% TFA for 40 hr, which is equivalent to 120 solid-phase cycles based on deprotection for 20 min/cycle. The stabilities of Glu(OBzl), Asp(OBzl), and Ser(Bzl) are essentially the same as that of Thr(Bzl). Cys(4-CH_3OBzl) and Tyr(Z) were more than an order of magnitude less stable, and Lys(Z) and Tyr-

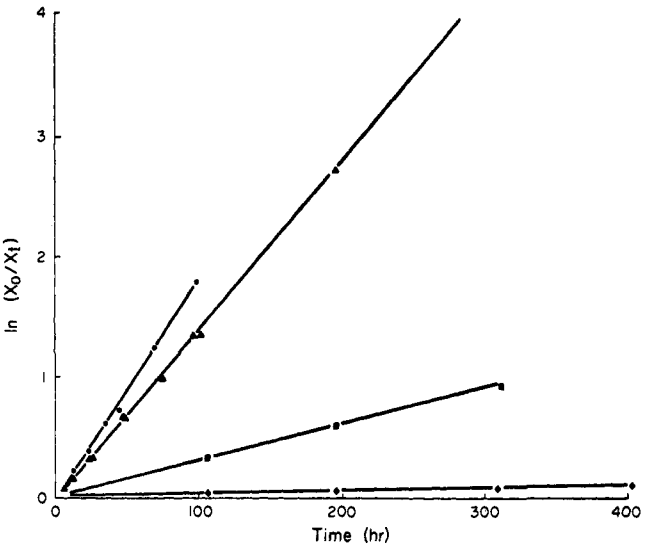

Figure 1. Calculated least-squares lines of best fit for the apparent first-order loss of benzylic groups in 50% TFA at 20°: (●) Tyr(Bzl), (▲) Lys(Z), (■) Cys(4-CH_3OBzl), (◆) Thr(Bzl).

Table I. Deprotection of Benzylic Amino Acid Derivatives in 50% TFA

Benzylic amino acid derivative	k,[a] 10^{-8} sec^{-1}	k_{rel}	% loss per cycle[b]
Tyr(Bzl)	636 ± 14 (5)	94	0.76
Lys(Z)	395.6 ± 4.0 (10)	59	0.47
Tyr(Z)	88.8 ± 7.2 (5)	13	0.11
Cys(4-CH_3OBzl)	81.6 ± 4.2 (2)	12	0.098
Ser(Bzl)	10.7 ± 1.4 (3)	1.6	0.013
Asp(OBzl)	9	1	0.011
Glu(OBzl)	7	1	0.009
Thr(Bzl)	6.7 ± 1.5 (3)	[1.0]	0.0083
Lys(3-ClZ)[c]	0.485 ± 0.023 (3)	0.072	0.00062
Tyr(2,6-Cl_2Bzl)	<0.12	<0.018	<0.00014
Cys(4-CH_3Bzl)	<0.03	<0.005	<0.00004

[a] Apparent first-order rate constant and 90% confidence limits for loss of the benzylic group in 50% (v/v) TFA–CH_2Cl_2 at 20°; degrees of freedom are shown in parentheses. [b] Based on treatment with 50% TFA for 20 min/cycle. [c] Reference 5.

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(6) S. Karlsson, G. Lindeberg, J. Porath, and U. Ragnarsson, *Acta Chem. Scand.*, **24**, 1010 (1970).

(Bzl) were nearly two orders of magnitude less stable than Thr(Bzl).

The usefulness of a side-chain-protecting group during the synthesis of a large peptide can be appraised by comparing its deprotection rate to that of the N^{α}-Boc group or by estimating the fraction of the peptide that would be branched due to loss of that protecting group. A side-chain-protecting group might be considered sufficiently stable for use in solid-phase synthesis if less than 0.05% of that group is lost during removal of more than 99.95% of the N^{α}-protecting group. Thus, when the N^{α}-deprotection reagent is in great excess, the ratio of the apparent first-order rate constant for loss of the N^{α}-protecting group to that for loss of the side-chain group should be at least

$$\frac{k_{\alpha}}{k_{sc}} = \frac{\ln (0.0005)}{\ln (0.9995)} = 1.5 \times 10^{4} \quad (4)$$

Deprotection of N^{α}-Z-glycine benzylamide in 0.1 *M* HBr in acetic acid is known[7] to proceed about 1700 times slower than deprotection of N^{α}-Boc-glycine benzylamide. Assuming that the deprotection of N^{ϵ}-Z-lysine in 50% TFA at 20° is also about 1700 times slower than deprotection of an N^{α}-Boc peptide, then from Table I deprotection of Tyr(Bzl) is about 10^{3} times slower and deprotection of Tyr(Z) and Cys(4-CH_3OBzl) is about 8×10^{3} times slower than loss of N^{α}-Boc. Thus the protection of tyrosine residues as Tyr(Bzl) or Tyr(Z), the masking of lysine residues as Lys(Z), or shielding of cysteine residues as Cys(4-CH_3OBzl) would not prevent significant side-chain branching when Boc is used for N^{α}-protection. In contrast, aspartic acid, glutamic acid, serine, and threonine are adequately protected as the *O*-benzyl derivatives, since loss of the *O*-benzyl group from these residues is estimated to be $6\text{–}10 \times 10^{4}$ times slower than loss of N^{α}-Boc.

An alternate method for appraising the usefulness of a protecting group involves estimating the fraction of peptides that would undergo side-chain branching due to premature loss of that group during synthesis of a model peptide. Three large peptides derived from the human immunoglobulin Eu[8] are useful models in this context. The peptides are the 108-residue variable region of the light chain, the 214-residue light chain, and the 440-residue heavy chain, which is one of the largest peptides sequenced to date. The molar percentages of tyrosine residues in these peptides (4.6, 4.2, and 4.0%, respectively) agree satisfactorily with the average molar percentage of tyrosine residues (3.6%) found in representative sequences of 108 different kinds of peptides and proteins.[9] The agreement is also adequate for the molar percentages of aspartic acid (4.6, 4.7, and 3.5% *vs.* 5.2%), glutamic acid (2.8, 4.7, and 5.3% *vs.* 5.3%), threonine (9.3, 8.4, and 7.4% *vs.* 6.5%), and lysine (6.5, 7.0, and 7.0% *vs.* 7.0%). However, the values for cysteine (1.9, 2.3, and 2.5% *vs.* 3.4%) are relatively low and those for serine (14.8, 15.0, and 11.9% *vs.* 7.8%) are relatively high.

For each type of side-chain-protected residue, the molar percentage of the final peptide mixture that would lose none of the protecting groups and thus would not undergo side-chain branching at these residues was calculated from the deprotection rates in Table I, the number *n* of these residues, and their actual distribution within the model peptide. For example, the variable region of the light chain contains five tyrosine residues, at positions 36, 49, 86, 77, and 91 from the N-terminus. If Tyr^{91} were introduced as a Tyr(Bzl) residue, the benzyl group would be subjected to a 20-min treatment with 50% TFA to remove the N^{α}-Boc group during each of 90 subsequent cycles of the synthesis. The total number of these acidic cycles for all five tyrosine residues is 344. From Table I, 0.76 mol % of Tyr(Bzl) is deprotected during a 20-min treatment with 50% TFA. Thus the fraction of initial chains that would maintain complete tyrosine protection during synthesis of the 108-residue peptide is given by the expression

$$(1 - 0.0076)^{344} = 0.9924^{344} = 0.0719$$

Thus at least 7.2 mol % of the resin-bound chains would still contain five Tyr(Bzl) residues upon complete assembly of the model peptide. The remaining 92.8 mol % of chains would have lost one or more *O*-benzyl groups from the five tyrosine residues and would possibly have undergone branching at a tyrosine hydroxyl group.

This predicted value for the molar percentage of the peptide maintaining complete protection of Tyr(Bzl) residues is only approximate. It would be somewhat larger (a) if the rate constant for deprotection of resin-bound Tyr(Bzl) residues were two to three times smaller than the value measured in solution, (b) if a medium of lower acidity, such as 20–25% TFA,[3,10,11] were used for deprotection of the N^{α}-Boc groups, or (c) if a slightly more acid-labile group, such as 4-methoxybenzyloxycarbonyl or furfuryloxycarbonyl, were used for N^{α}-protection. Yet even this approximate value is useful in predicting the utility of the Tyr(Bzl) group in solid-phase synthesis of a large peptide. The results of many calculations of this type are shown in Table II.

When using Boc for N^{α}-protection and a 20-min treatment with 50% TFA for N^{α}-deprotection, about 90% of the chains formed during synthesis of the 108-residue peptide using Lys(Z) residues would be branched at lysine. This prediction prompted a search for more stable protecting groups for lysine. Five ring-chlorinated derivatives of Lys(Z) were found to be 60–1000 times more stable than Lys(Z) in 50% TFA.[5] N^{ϵ}-(3-Chlorobenzyloxycarbonyl)lysine was the derivative most stable in 50% TFA that was also completely deprotected by treatment with HF at 0° for 1 hr. The results shown in Table II for Lys(3-ClZ) indicate that less than 5% of the chains would be branched at lysine during synthesis of the 440-residue heavy chain.

When using Boc for N^{α}-protection, use of the 4-methoxybenzyl group for S-protection of cysteine residues is predicted to be marginally effective in preventing branching at cysteine during synthesis of the 108-residue peptide and ineffective for the larger model peptides. Thus a more stable group is needed for S-protection of

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Table II. Calculated Yields of Peptides that Maintain Complete Protection of a Residue Type for Solid-Phase Syntheses of Three Peptides from the Human Immunoglobulin Eu

Peptide	Variable region of light chain				Complete light chain				Complete heavy chain			
Molecular weight	11,788 d				23,399 d				48,492 d			
Sequence	1–108				1–214				1–440			
			Yield,[b] mol %				Yield,[b] mol %				Yield,[b] mol %	
Protected residue	n	Cycles[a]	50% TFA	1% TFA	n	Cycles[a]	50% TFA	1% TFA	n	Cycles[a]	50% TFA	1% TFA
Tyr(Bzl)	5	344	7.2	94.9	9	1031	0.0	85.4	18	4,203	0.0	52.7
Lys(Z)	7	475	10.6	95.6	15	1824	0.0	84.1	31	7,595	0.0	48.6
Tyr(Z)	5	344	69.2	99.3	19	1031	33.3	97.8	18	4,203	1.1	91.4
Cys(4-CH_3OBzl)	2	109	90.0	99.8	5	648	53.0	98.7	11	2,500	8.7	95.2
Ser(Bzl)	16	774	90.5	99.8	32	3389	64.7	99.1	53	11,223	23.7	97.2
Asp(OBzl)	5	270	97.0	99.9	10	1060	88.9	99.8	14	3,707	66.1	99.2
Glu(OBzl)	3	227	98.0	99.9	10	1407	88.4	99.8	24	6,007	59.1	99.0
Thr(Bzl)	10	480	96.1	99.9	18	1807	86.1	99.7	33	7,318	54.5	98.8
Lys(3-ClZ)	7	475	99.7	99.9	15	1824	98.9	99.9	31	7,595	95.7	99.9
Tyr(2,6-Cl_2Bzl)[c]	5	344	99.9	99.9	9	1031	99.8	99.9	18	4,203	99.4	99.9
Cys(4-CH_3Bzl)[d]	2	109	99.9	99.9	5	648	99.9	99.9	11	2,500	99.8	99.9

[a] Sum of the acid exposures of the n individual residues, where the acid exposure of the residue at position P is $P - 1$ cycles. [b] Mole per cent of the peptides having all n of the residues still protected upon complete assembly of the resin-bound peptide; based on deprotection by 50% TFA (for rate k, see Table I) or by 1% TFA (assumed rate of $k/50$) for 20 min/cycle. [c] Assuming that $k = 0.12 \times 10^{-8}$ sec^{-1} in 50% TFA. [d] Assuming that $k = 0.03 \times 10^{-8}$ sec^{-1} in 50% TFA.

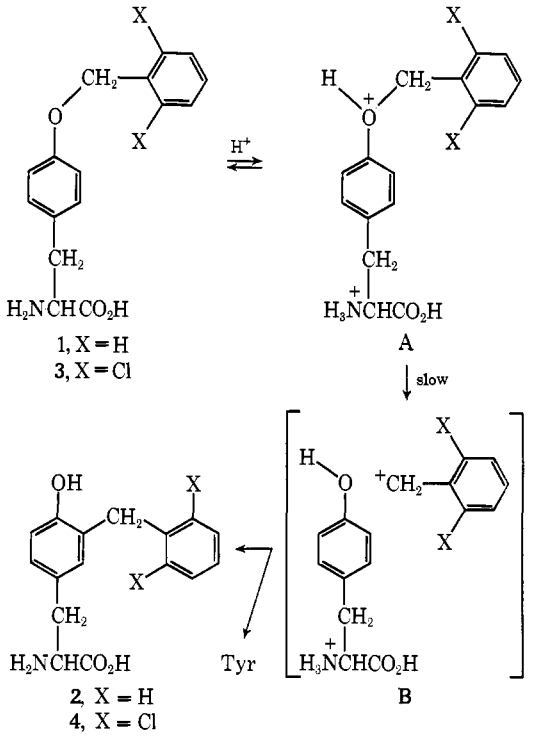
cysteine during the solid-phase synthesis of large peptides.

S-(4-Methylbenzyl)cysteine was found to be at least 2700 times more stable in 50% TFA than Cys(4-CH_3-OBzl). Removal of the 4-methylbenzyl group with 50% HF–anisole at 0° was 98% complete after 30 min and complete after 60 min. Thus the 4-methylbenzyl group is recommended for S-protection of cysteine residues during solid-phase peptide synthesis.

Yamashiro, Noble, and Li[12] have recommended the 3,4-dimethylbenzyl group for S-protection of cysteine. They found that treatment of N^{α}-acetyl-*S*-(3,4-dimethylbenzyl)cysteinamide with 50% TFA for 21 hr removed only about 0.2% of the 3,4-dimethylbenzyl group, but treatment of the *S*-(4-methoxybenzyl) analog under these conditions removed 27% of the 4-methoxybenzyl group. The 3,4-dimethylbenzyl group was completely removed by treatment with HF for 15 min at 0°.

The fraction of chains predicted to lose one or more benzyl groups from the Ser(Bzl) residues is about 10% for the 108-residue peptide, about 35% for the 214-residue peptide, and about 75% for the 440-residue peptide. Since a relatively large percentage of the residues in natural peptides are serine residues, the use of a protecting group for serine more stable than *O*-benzyl would improve the purity of large synthetic peptides when using Boc for N^{α}-protection. The *O*-benzyl group would provide adequate protection for aspartic acid, glutamic acid, and threonine residues during synthesis of the 108-residue peptide. Yet the side-chain protection of these residues would be marginally acceptable for the 214-residue peptide and unacceptable for the 440-residue peptide. Thus the strategy of using Boc for N^{α}-protection and benzyl for side-chain protection of serine, aspartic acid, glutamic acid, and threonine residues is expected to be only marginally useful for the solid-phase synthesis of very large peptides.

The reaction of *O*-benzyltyrosine (**1**) with 50% TFA at 20° furnished not only tyrosine but also a second ninhydrin-positive compound identified as 3-benzyltyrosine (**2**) by chromatographic comparison with an authentic sample. Iselin[13] has previously obtained



3-benzyltyrosine by hydrolysis of *O*-benzyltyrosine with 6 *M* hydrochloric acid at 110° for 18 hr. Further, ring-benzylated derivatives of 2-fluorophenol have been isolated from the solvolysis of benzyl 2-fluorophenyl ether in trifluoroacetic acid at room temperature.[14]

The rate of deprotection of *O*-benzyltyrosine in 50% TFA [$k = (636 \pm 14) \times 10^{-8}$ sec^{-1}] was the fastest rate observed (see Table I). The molar ratio of tyrosine to tyrosine plus 3-benzyltyrosine was constant (56.1% ± 0.5%) for the first 99 hr of reaction, during which 83% of the Tyr(Bzl) reacted (Figure 2). When the acidolysis was conducted in 50:45:5 (v/v/v) trifluoroacetic acid–dichloromethane–anisole so that 100 mol of anisole

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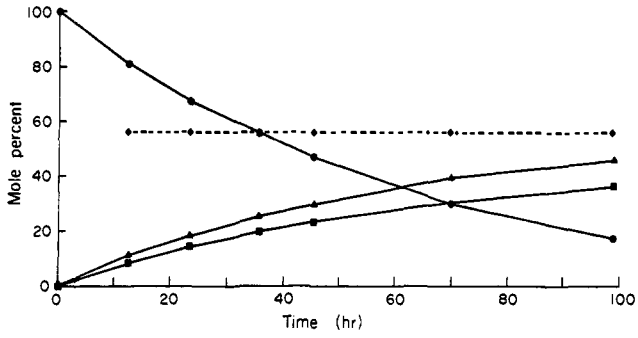

Figure 2. Reaction of *O*-benzyltyrosine with 50:50 (v/v) trifluoroacetic acid–dichloromethane at 20°. Mole per cent of *O*-benzyltyrosine (●), tyrosine (▲), 3-benzyltyrosine (■); molar ratio of tyrosine to tyrosine plus 3-benzyltyrosine (◆).

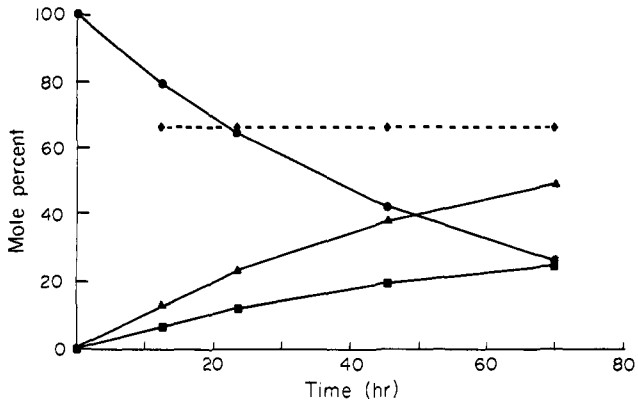

Figure 3. Reaction of *O*-benzyltyrosine with 50:45:5 (v/v/v) trifluoroacetic acid–dichloromethane–anisole at 20°. Mole per cent of *O*-benzyltyrosine (●), tyrosine (▲), 3-benzyltyrosine (■); molar ratio of tyrosine to tyrosine plus 3-benzyltyrosine (◆).

was present initially per mole of Tyr(Bzl) (Figure 3), little change was observed in the rate of deprotection [$k = (688 \pm 9) \times 10^{-8}$ sec^{-1}] or the molar ratio of tyrosine to tyrosine plus 3-benzyltyrosine (66.2% ± 0.2% over 70 hr of reaction). Similar results have been reported for the rearrangement[15] of benzyl phenyl ether to 2-benzylphenol with aluminum bromide in chlorobenzene and the rearrangement[16] of benzyl 4-tolyl ether to 2-benzyl-4-methylphenol in liquid antimony trichloride.

The constancy of the molar ratio of tyrosine to tyrosine plus 3-benzyltyrosine indicates that both tyrosine and 3-benzyltyrosine are produced from a common intermediate that is formed after the rate-determining step. A plausible mechanism is dissociation of the O-protonated derivative A to give the intermediate B, which competitively undergoes either association to 3-benzyltyrosine or further dissociation to tyrosine and benzylic by-products. This mechanism is similar to that proposed by Spanninger and von Rosenberg[17] for the intramolecular rearrangement of *sec*-butyl phenyl ether to 2-*sec*-butylphenol with aluminum bromide in chlorobenzene.

The benzyl group is unsatisfactory for O-protection of tyrosine not only because of its partial rearrangement to give 3-benzyltyrosine but also because of its rapid removal in 50% TFA. Since the N^{ϵ}-2,6-dichlorobenzyloxycarbonyl group of lysine is about 1000 times more stable in 50% TFA than the N^{ϵ}-benzyloxycarbonyl group,[5] the 2,6-dichlorobenzyl group was examined as a potential protecting group for tyrosine. *O*-(2,6-Dichlorobenzyl)tyrosine (**3**) was at least 5000 times more stable than Tyr(Bzl) in 50% TFA. Since **3** was completely stable in 50% TFA for at least 350 hr at 20°, the extent of its intramolecular rearrangement to 3-(2,6-dichlorobenzyl)tyrosine (**4**) could not be assessed under these conditions.

The extent of rearrangement in HF was then examined. Both Tyr(Bzl) and Tyr(2,6-Cl_2Bzl) were completely deprotected by treatment with HF for 10 min at 0°. In both cases the products were about 60 mol % of tyrosine and 40 mol % of the 3-benzylated tyrosine. Most of the latter was formed by intermolecular benzylation of free tyrosine rather than by intramolecular rearrangement of the O-protected tyrosine, because treatment of the protected tyrosine with 50% HF–anisole for 10 min at 0° reduced the formation of 3-benzyltyrosine to 15 mol % and the production of 3-(2,6-dichlorobenzyl)tyrosine to only 5 mol %. Treatment of *O*-benzyltyrosine with 90% TFA–anisole saturated with HBr formed 20 mol % of 3-benzyltyrosine. In addition, the formation of 3-(2,6-dichlorobenzyl)tyrosine was not reduced below 5 mol % by the presence of a potential nucleophile, such as iodide ion or any alkyl sulfide, in the 50% HF–anisole. Thus the 2,6-dichlorobenzyl group is better than the benzyl group for O-protection of tyrosine residues during solid-phase synthesis, but it only partially alleviates the intramolecular benzylation of the phenolic ring.

The acid-catalyzed O-to-C rearrangement of the benzyl group occurs to a significant extent not only for free Tyr(Bzl) but also during acidic deprotection of *peptides* containing Tyr(Bzl) residues. Tyrosine-containing peptides related to angiotensin have been synthesized by the solid-phase method using the benzyl group for O-protection of tyrosine; in each case a by-product was isolated that contained each of the expected amino acids except tyrosine.[18] We found that each of these by-products contained one residue of 3-benzyltyrosine. The peptide hydrolysates were analyzed on a 0.9 × 13 cm column of sulfonated polystyrene using 0.35 *M* citrate buffer at pH 7.0 as eluent and column temperatures of 86–90° to overcome π-bonding of the 3-benzyltyrosine to the polystyrene. The completed peptides had been side-chain deprotected and cleaved from the resin with 4:1 (v/v) HF–anisole for 1 hr at room temperature, but even in the presence of anisole as a trap for electrophiles, 13–20% of the *O*-benzyl groups rearranged to the phenolic ring. The molar ratio of the desired peptide containing tyrosine to the by-product containing 3-benzyltyrosine was 4:1 for Asn-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu ([Val^5]-angiotensin I), 4:1 for Asn-Arg-Val-Tyr-Val-His-Pro-Phe ([Val^5]-angiotensin II), and 7:1 for Val-Tyr-Val-His-Pro-Phe.

The use of the *O*-benzyloxycarbonyl group for tyrosine protection was expected to eliminate this undesired

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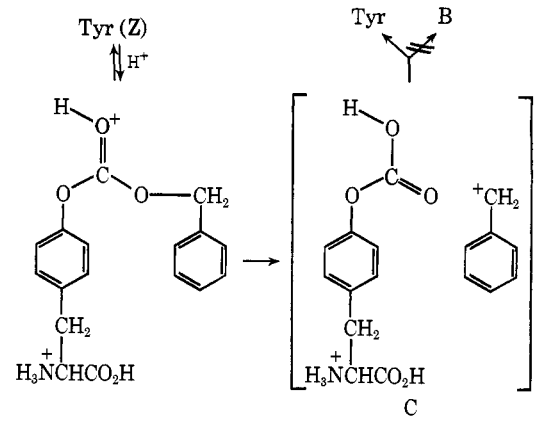
intramolecular benzylation, since reaction species C



was judged more likely to dissociate without benzylation of the electron-deficient ring of the carbonated tyrosine than to lose CO_2 and give B, which would partially yield 3-benzyltyrosine. Boc-Tyr(Z) has been used in the solid-phase synthesis of angiotensin analogs because it is more soluble in dichloromethane than Boc-Tyr(Bzl).[19] As expected, treatment of *O*-benzyloxycarbonyltyrosine[20] with 50% HF–anisole at 0° afforded tyrosine as the sole product. Since the rate of deprotection of Tyr(Z) in 50% TFA is only seven times slower than that of Tyr(Bzl), the Z group also is too acid labile for O-protection of tyrosine when using Boc for N^{α}-protection.

Side-chain protecting groups for solid-phase peptide synthesis must be stable not only to acids but also to amine nucleophiles. Tyr(Z), being a phenyl carbonate, was not expected to be stable to nucleophiles. Indeed, when it was treated with an excess of 1:10 (v/v) ethyldiisopropylamine–dichloromethane, most of the Z group was removed within 2 hr. The nucleophile in this system was not the sterically hindered tertiary amine but the free α-amino group of Tyr(Z), which reacted with the phenyl carbonate linkage of another Tyr(Z) molecule to liberate free tyrosine. Although the premature deprotection of a Tyr(Z) residue within a resin-bound peptide should be slower because the nucleophilic amino groups freed during the neutralization step are also resin-bound, some deprotection can be expected under solid-phase conditions. Tyr(Z) residues could also be deprotected by other nucleophiles, such as the mercaptan often added during the N^{α}-deprotection step to protect tryptophan residues from oxidative destruction. Thus an O-protecting group is still needed that is completely stable to both 50% TFA and amine nucleophiles during the synthesis and cleanly removable by HF at the end of the synthesis.

An alternate strategy for the solid-phase synthesis of large peptides is the use of a highly acid-labile group for N^{α}-protection, such as the 2-nitrophenylsulfenyl group or the 2-(4-biphenylyl)-2-propyloxycarbonyl (Bpoc) group. N^{α}-Bpoc, which is about 3000 times less stable than N^{α}-Boc in 80% aqueous acetic acid,[21] can be removed within 10 min under mildly acidic anhydrous conditions, such as 0.5% TFA–CH_2Cl_2.[22] The calculated yields per residue type shown in Table II are substantially higher using 1% TFA than using 50% TFA for N^{α}-deprotection. Thus each of these peptides is expected to suffer acceptably small percentages of side-chain deprotection (and possible branching) during solid-phase synthesis when using 1% TFA for N^{α}-deprotection and the following protected residues: Ser(Bzl), Thr(Bzl), Asp(OBzl), Glu(OBzl), Lys(3-ClZ), Tyr(2,6-Cl_2Bzl), and Cys(4-CH_3Bzl).

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Experimental Section

Instrumentation. Melting points were determined in glass capillary tubes with a Thomas-Hoover melting point apparatus. Nuclear magnetic resonance spectra were observed in TFA with a Varian A-60 spectrometer; chemical shifts are expressed in parts per million downfield from internal tetramethylsilane. A Beckman Model 120B amino acid analyzer was used for the automated ion-exchange separation and ninhydrin assay of amino acids and protected amino acids. Elemental analyses were performed by Mr. S. T. Bella of the Microanalytical Laboratory, The Rockefeller University.

Materials. All solvents and bulk chemicals were reagent grade. Dichloromethane was distilled from sodium carbonate and stored in amber bottles. Anisole was dried by storage over activated molecular sieves (Linde Type 4A). Ethydiisopropylamine (Aldrich Chemical Co.), bp 126–129°, was distilled from sodium hydride through a 20-cm Widmer column. Trifluoroacetic acid (TFA; Halocarbon Chemical Co.) was distilled and stored in 100-ml amber bottles sealed with polyethylene-lined screw caps. The protected amino acids were obtained commercially or prepared by conventional procedures; all showed a single spot on tlc and a single peak on amino acid analysis (see Table III). The pH 7 citrate buffer (0.35 *M*) was prepared by dissolving sodium citrate dihydrate (34.3 g), polyoxyethylene lauryl alcohol (BRIJ-35, 1.3 g), and phenol (1.0 g) in distilled water (1.00 l.) and adjusting the pH to 7.00 with a few drops of 12 *M* aqueous HCl.

Table III. Conditions for Amino Acid Analysis[a]

Amino acid derivative	Column temp, °C	Retention time, min: Amino acid derivative	Retention time, min: Free amino acid
Thr(Bzl)	55	21	15
Ser(Bzl)	55	26	15
Asp(OBzl)	55	31	15
Glu(OBlz)	55	32	15
Lys(Z)	55	32	55
Cys(4-CH_3OBzl)	55	41	15
Cys(4-CH_3Bzl)	55	46	15
Tyr(Bzl)	86	54	18
Tyr(Z)	86	68	18
3-BzlTyr	86	34	
Tyr(2,6-Cl_2Bzl)	96	112	18
3-(2,6-Cl_2Bzl)Tyr	96	55	

[a] A 0.9 × 13 cm column of sulfonated polystyrene (Beckman PA-35) was eluted with pH 7 citrate buffer at 65 ml/hr.

Kinetic Experiments. The side-chain-protected amino acid (50 μmol) was dissolved in 50:50 (v/v) trifluoroacetic acid–dichloromethane (5.00 ml); 0.5-ml portions of this solution were kept at 20 ± 1° in oven-dried tubes sealed with Teflon-lined screw caps. At time intervals from 5 min to as long as 600 hr after mixing, the solution within a single tube was freed of CH_2Cl_2 and most of the TFA by evaporation under a gentle stream of dry air and the residue was dissolved in pH 7 citrate buffer; this quenching procedure required less than 1 min. An aliquot of this solution was analyzed for both the protected amino acid and the free amino acid; the analytical conditions and retention times are given in Table III. The integrated peak areas were corrected for the relative ninhydrin response of a standard mixture of these compounds. Although the protected amino acids were generally quite stable in the pH 7 citrate buffer for several hundred hours, glutamic acid γ-benzyl ester and aspartic acid β-benzyl ester underwent hydrolysis in this medium at rates faster than their rates of acidolysis in 50% TFA. There-

fore, the rate constants for these esters were determined from samples analyzed immediately after quenching.

Deprotection with 50% HF–Anisole. Hydrogen fluoride (1.5 ml, 75 mmol) was distilled into a fluorocarbon vessel containing a mixture of the protected amino acid (20 μmol) and dry anisole (1.5 ml, 14 mmol) precooled to −78°. The vessel was surrounded with a 0° ice–water bath and the reaction mixture was stirred magnetically for the reaction period. The bath was removed, the HF was evaporated under water aspiration, and the anisole was distilled off under high vacuum. The residue was dissolved in TFA (0.1 ml) and pH 7 citrate buffer (5.0 ml); part of this solution was analyzed as described in Table III.

***S*-(4-Methylbenzyl)-L-cysteine.** Triethylamine (7.75 ml, 55.5 mmol) and 4-methylbenzyl bromide (3.42 g, 18.5 mmol) were added to a solution of L-cysteine hydrochloride monohydrate (3.25 g, 18.5 mmol) in 2:1 (v/v) ethanol–water (30 ml). The mixture was stirred at room temperature for 12 hr and filtered; the filter cake was washed well with water and crystallized from 3:2 (v/v) ethanol–water to provide *S*-(4-methylbenzyl)-L-cysteine as a white solid: mp 209–211° dec; nmr (TFA) 2.37 (s, 3 H, CH_3), 3.1–3.3 (m, 2 H, $C^{\beta}H_2$), 3.84 (s, 2 H, CH_2Ar), 4.1–4.3 (m, 1 H, $C^{\alpha}H$), and 3.84 ppm (s, 4 H, Ar); tlc, see Table IV.

Table IV. Thin Layer Chromatography[a]

Compd	R_f CMA	R_f BMA
Cys	0.04	0.17
Cys(4-CH_3Bzl)	0.19	0.42
Tyr	0.04	0.25
3-BzlTyr	0.10	0.33
3-(2,6-Cl_2Bzl)Tyr	0.12	0.37
Tyr(Bzl)	0.19	0.44
Tyr(Z)	0.21	0.45
Tyr(2,6-Cl_2Bzl)	0.22	0.48

[a] Silica gel plates (0.25 mm) were developed with 17:2:1 (v/v/v) chloroform–methanol–acetic acid (CMA) or 7:2:1 (v/v/v) benzene–methanol–acetic acid (BMA); the plates were dried and redeveloped in the same solvent mixture; spots were visualized by spraying with 0.2% ninhydrin in 1-butanol and heating.

Anal. Calcd for $C_{11}H_{15}NO_2S$: C, 58.64; H, 6.71; N, 6.22. Found: C, 58.88; H, 6.82; N, 6.12.

3-Benzyl-L-tyrosine (2) was prepared by the method of Iselin[18] and isolated as the hydrochloride, mp 242–245° dec (lit.[18] mp 239–243°). The amino acid analysis of 3-BzlTyr was difficult under standard conditions. It was eluted with pH 5.28 citrate buffer at 55° from the 0.9 × 5 cm column of PA-35 sulfonated polystyrene commonly used for separation of basic amino acids as a very broad peak at 34 min (11 min width at half-height). This peak was obscured by peaks due to lysine (26 min), histidine (31 min), and ammonia (39 min). Satisfactory separation was achieved by using a longer column at a higher column temperature with a more basic buffer; the elevated column temperature was necessary to sharpen the 3-BzlTyr peak by overcoming π-bonding of the 3-BzlTyr to the polystyrene resin. Thus, by elution of an 0.9 × 13 cm column of PA-35 at 86° with pH 7 citrate buffer, 3-BzlTyr appeared as a sharp, cleanly resolved peak (34 min) before the peaks for lysine (52 min), ammonia (71 min), and arginine (81 min) and after the peak for histidine plus the acidic and other neutral amino acids (15–20 min).

The by-product formed during acidolysis of Tyr(Bzl) was identical with 3-benzyltyrosine by ion-exchange chromatography (Table III) and thin layer chromatography (Table IV). After treatment of Tyr(Bzl) with 50% HF–anisole for 10 min at 0°, the molar ratio of tyrosine to 3-BzlTyr was 85:15; with HF alone the ratio was 6:4. After treatment of Tyr(Bzl) with an HBr saturated solution of 9:1 (v/v) TFA–anisole for 2 hr at room temperature, the ratio was 8:2. No 3-BzlTyr was formed during hydrogenolysis of Tyr(Bzl) with 5% Pd on barium sulfate as catalyst.

***O*-(2,6-Dichlorobenzyl)-L-tyrosine (3).** Cupric chloride dihydrate (1.70 g, 10.0 mmol) and methanol (20 ml) were added to a solution of L-tyrosine (3.60 g, 20.0 mmol) in 1.0 *M* aqueous sodium hydroxide (40.0 ml, 40.0 mmol). The blue-green solid dissolved on brief stirring. A solution of 2,6-dichlorobenzyl bromide (4.50 g, 18.8 mmol) in methanol (25 ml) was added. The deep-blue mixture was stirred at room temperature for 13 hr and filtered. The filter cake was washed with 1:1 (v/v) methanol–water, methanol, and ether (two 50-ml portions each), and dissolved in a hot mixture of acetic acid (50 ml), 1.0 *M* aqueous HCl (17 ml), and ethylenediaminetetraacetic acid (2.92 g, 10.0 mmol). The hot blue solution was filtered to remove undissolved EDTA and diluted with cold water (400 ml). The gelatinous solid was collected, washed well with water, and dried *in vacuo* to afford *O*-(2,6-dichlorobenzyl)-L-tyrosine in 41% yield as a white solid (2.64 g): mp 211–212° dec; tlc, see Table IV; nmr, see Table V.

Table V. Nmr Data for Tyrosine Derivatives in TFA[a]

Proton assignment	$C^{\alpha}H$	$C^{\beta}H_2$	CH_2Ar	Aryl
Integration, multiplicity	1 H, m	2 H, m	2 H, s	
Tyrosine derivative	Chemical shift[b]			
Tyr(Bzl)	3.6	4.8	5.39	7.1–8.0 (9 H, m)
Tyr(2,6-Cl_2Bzl)	3.5	4.6	5.53	6.8–7.7 (7 H, m)
Tyr(Z)	3.6	4.7	5.40	6.9–7.6 (9 H, m)
3-BzlTyr	3.6	4.8	4.20	6.9–7.8 (8 H, m)
3-(2,6-Cl_2Bzl)Tyr	3.4	4.6	4.48	[c]

[a] The protons for CF_3CO_2H, RNH_3^+, RCO_2H, and $ArOH$ exchange rapidly and appear as a single peak. [b] Parts per million downfield from internal tetramethylsilane. [c] 6.82 (1 H, s, C^2H), 7.25 (2 H, s, C^5H and C^6H), 7.43 (1 H, t, J = 5 Hz, $C^{4'}H$), and 7.57 ppm (2 H, d, J = 5 Hz, $C^{3'}H$ and $C^{5'}H$).

Anal. Calcd for $C_{16}H_{15}Cl_2NO_3$: C, 56.49; H, 4.44; N, 4.12. Found: C, 56.10; H, 4.58; N, 3.92.

3-(2,6-Dichlorobenzyl)-L-tyrosine (4). Treatment of *O*-(2,6-dichlorobenzyl)-L-tyrosine (0.34 g, 1.00 mmol) with HF (3 ml, 0.15 mol) for 10 min at 0° furnished a residue that was slurried with ethyl acetate (10 ml), filtered, and freed of solvent. The residual glass (0.38 g) was chromatographed on a 20 × 20 × 0.2 cm plate of Merck silica gel F-254 using two developments with 7:2:1 (v/v/v) benzene–methanol–acetic acid. The major uv-active band at R_f 0.32–0.42 was extracted with ethyl acetate and freed of solvent; the residue was recrystallized from 25% aqueous methanol to furnish **4**: mp 196–199°; tlc, see Table IV; nmr, see Table V. Although the molecular ion was not seen, the mass spectral fragmentation pattern was consistent with the proposed structure.

After treatment of Tyr(2,6-Cl_2Bzl) (11 mg, 32 μmol) for 5 min at 0° (a) with HF (1.5 ml, 75 mmol) and anisole (1.5 ml, 14 mmol), (b) with HF (1.35 ml, 68 mmol), anisole (1.35 ml, 12.5 mmol), and thiodiglycolic acid (0.30 g, 2.0 mmol), or (c) with HF (1.5 ml, 75 mmol), anisole (0.90 ml, 8.3 mmol), TFA (0.16 ml, 2.0 mmol), and tetra-*n*-butylammonium iodide (0.37 g, 1.0 mmol), the molar ratio of tyrosine to 3-(2,6-Cl_2Bzl)Tyr was 95:5. With HF alone for 10 min at 0°, the ratio was 6:4.

***O*-Benzyloxycarbonyl-L-tyrosine** was obtained by the procedure of Overell and Petrow[20] as white crystals from water: mp 203–205° dec (lit.[20] mp 215° dec); tlc, see Table IV; nmr, see Table V.

Anal. Calcd for $C_{17}H_{17}NO_5$: C, 64.75; H, 5.43; N, 4.44. Found: C, 65.12; H, 5.39; N, 4.45.

Treatment of Tyr(Z) (12.5 mg, 40 μmol) for 30 min at 0° with 50% HF–anisole (3 ml) furnished only tyrosine and no (>0.2%) 3-BzlTyr. Similar treatment of Tyr(Z) with HF (3 ml) afforded tyrosine and 3-BzlTyr in the molar ratio 9:1.

Instability of Tyr(Z) with an Amine Nucleophile. A solution of Tyr(Z) (3.58 mg, 11.4 μmol, 1.0 equiv) in TFA (5.0 μl, 0.25 mmol, 22 equiv) and dichloromethane (4.57 ml) was diluted with ethyldiisopropylamine (0.425 ml, 2.5 mmol, 220 equiv). At 5 min and again at 110 min after dilution, part of the solution was freed of solvent by evaporation under a stream of dry air; the residue was dissolved in pH 7 citrate buffer and immediately analyzed under the conditions given in Table III. The concentration of Tyr(Z) observed at 110 min was only about 25% of that seen at 5 min. At 110 min a substantial quantity of free tyrosine was present. In control experiments Tyr(Z) was shown to be stable to the analysis conditions.