# Formation and Degradation of Urea Derivatives in the Azide Method of Peptide Synthesis. Part 2.[1] Acidolytic Degradation of Urea Derivatives †

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Treatment of Boc-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)·NH·CO-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-Lys-(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-$NH_2$ with trifluoroacetic acid for deprotection yielded not the desired octadecapeptide-related urea but an $N^{\alpha}$-blocked tetradecapeptide. This unexpected degradation has been studied with Z-Gly-NH·CH($CH_2$Ph)·NH·CO-Gly-OBu$^t$ as a model compound to demonstrate that the urea derivative of general formula $R^1CO{\cdot}NH{\cdot}CHR^2{\cdot}NH{\cdot}CO{\cdot}NHR^3$ decomposes to give $R^1CO{\cdot}NH_2$ and $NH_2{\cdot}CO{\cdot}NHR^3$ in the absence of scavenger and to give $R^1CO{\cdot}NH{\cdot}CHR^2R^4$ and $NH_2{\cdot}CO{\cdot}NHR^3$ in the presence of scavenger $R^4H$ (anisole or 2-mercaptoethanol) when treated with an acid reagent, such as formic acid, trifluoroacetic acid, hydrogen fluoride, or hydrogen bromide in acetic acid. The urea derivative, which is a side product in the azide method of peptide synthesis, is thus degraded to smaller fragments which usually differ in size and nature from the desired peptide, so that the danger of contamination of peptide with urea may be eliminated if the coupling product is treated with acid before purification.

In the azide method of peptide synthesis ($R^1CON_3 + R^2NH_2 \longrightarrow R^1CO{\cdot}NHR^2$) the formation of a urea ($R^1NH{\cdot}CO{\cdot}NHR^2$) is an inevitable side reaction and, as suggested in our preceding paper,[1] it may be extremely difficult to separate the urea from the desired peptide, especially when $R^1$ and $R^2$ are fairly large and complex. This could be a serious problem not only in the synthesis but also in the biological characterisation and clinical use of physiologically active peptides, since the urea could well have undesirable effects. In view of this, we decided to synthesise the urea derivative H-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)·NH·CO-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-$NH_2$ {[$Gly_1$]-ACTH(1—18)-$NH_2$-4,5-urea} (1), one of the ureas which could be produced as side products in the synthesis of [Gly$^1$]-ACTH(1—18)-$NH_2$.[2] When the synthesis was attempted in the manner described below, however, the product was not the desired octadecapeptide-related urea (1) but an $N^{\alpha}$-blocked tetradecapeptide. This indicated that a bond next to the urea linkage had been cleaved during the treatment with acid for deprotection at the final step. The present paper describes our studies on this acidolytic degradation of urea derivatives.

*Attempted Synthesis of* [Gly$^1$]-ACTH(1—18)-$NH_2$-4,5-urea (1).—The synthesis was performed essentially as described for [Gly$^1$]-ACTH(1—18)-$NH_2$.[2] The *N*-hydroxysuccinimide ester of Boc-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)·NH·CO-Glu(OBu$^t$)-His-Phe-Arg-Trp-Gly-OH (2)[1] was coupled with an octapeptide, H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-$NH_2$ acetate

† The results described in this paper were presented at the 11th Symposium on Peptide Chemistry, Kanazawa, Japan, October 1973 (Proceedings of the 11th Symposium on Peptide Chemistry, ed. H. Kotake, Protein Research Foundation, Osaka, Japan, 1974, p. 35).

All the amino-acid residues have the L-configuration. Abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochem. J.*, 1967, **102**, 23; 1967, **104**, 17; 1972, **126**, 773); Z = benzyloxycarbonyl, Boc = t-butoxycarbonyl, DCC = dicyclohexylcarbodi-imide, HOSu = *N*-hydroxysuccinimide, TFA = trifluoroacetic acid, DMF = *NN*-dimethylformamide, ACTH = adrenocorticotrophic hormone (corticotrophin).

[1] Part 1, K. Inouye, K. Watanabe, and M. Shin, preceding paper.

[2] H. Otsuka, M. Shin, Y. Kinomura, and K. Inouye, *Bull. Chem. Soc. Japan*, 1970, **43**, 196.

(3),[3] and the product was then deprotected with TFA (25 °C; 60 min) in the presence of anisole and 2-mercaptoethanol as scavengers. The resulting material was purified on a carboxymethylcellulose column (Figure 1) and by partition chromatography on a Sephadex G-25 column with a butan-1-ol–acetic acid–pyridine–water system as solvent. The final product [compound (1a)] was homogeneous to ninhydrin and the Ehrlich reagent on t.l.c., and clearly distinguished from [Gly[1]]-ACTH(1—18)-$NH_2$. Amino-acid analysis not only revealed the absence of methionine, as expected, but also showed that one residue each of Ser, Gly, and Tyr was missing. The absence of tyrosine was also suggested by the fact that the u.v. spectrum of (1a) in alkali was identical with that in acid within the precision of measurement. These results indicated that at least three amino-acid residues had been split off from the *N*-terminal portion of the desired compound (1). In addition, compound (1a) was completely lacking in *in vivo* steroidogenic activity.* According to available knowledge of ACTH peptides,[4] this means that the amino-end of (1a) may be blocked in some way.

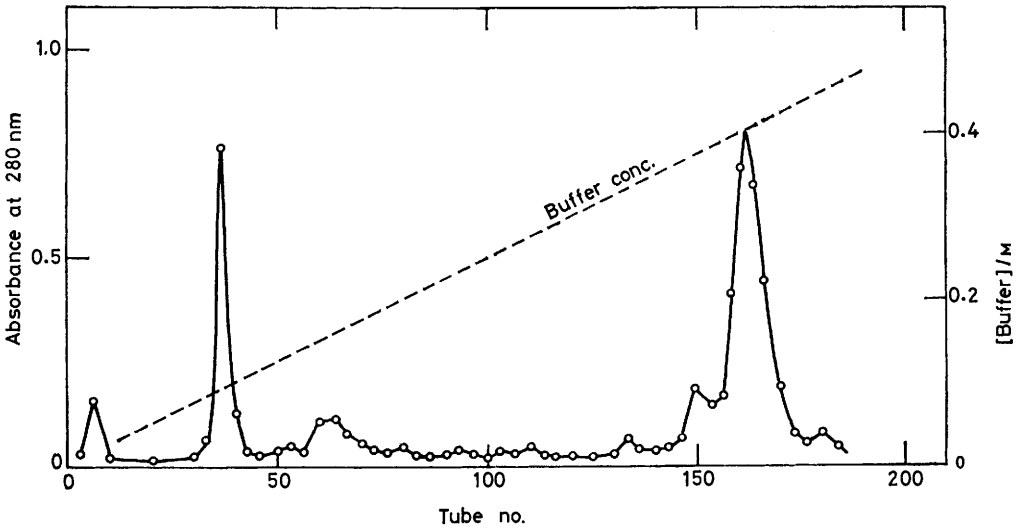



FIGURE 1 Carboxymethyl cellulose-column chromatography of product derived from protected [Gly[1]]-ACTH(1—18)-$NH_2$-4,5-urea by treatment with TFA: material, 100 mg; column, CM-52 (Whatman), 2 × 20 cm; buffer, 0—0.5M-ammonium acetate (pH 6.5), 1 800 ml; 9 ml fractions

*Acidolytic Breakdown of Urea Derivatives.*—The above unexpected degradation seemed to have occurred during the TFA treatment for removal of the protecting groups. To confirm this, the decapeptide-related urea (2) and an octapeptide-related urea H-Lys(Boc)-Pro-Val-Gly-NH·CH([$CH_2$]$_4$·NH-Boc)NH·CO-Lys(Boc)-Arg-Arg-$NH_2$ (4), derived from the corresponding $N^{\alpha}$-Z-derivative [1] by catalytic hydrogenolysis, were treated with TFA under the same conditions. The crude product from (2) was chromatographed on a silica gel column with an ethyl acetate–acetic acid–water system as solvent to give two major products. One was reactive to ninhydrin and amino-acid analysis revealed the presence of Ser, Gly, and Tyr in equimolar ratios. The other was reactive to the Ehrlich reagent, but not to ninhydrin. This was found to contain His, Arg, Glu, Gly, Phe, and Trp as expected. The product from (4) also contained two major components (t.l.c.), which could be separated by partition chromatography on a Sephadex G-25 column with a butan-1-ol–acetic acid–pyridine–water system as solvent. One was reactive to ninhydrin and composed of Lys, Pro, Gly, and Val in equimolar ratios. The other was reactive to both ninhydrin and the Sakaguchi reagent, and contained Lys and Arg in the molar ratio 0.6 : 2.

The above results showed that the urea derivative is highly susceptible to the action of TFA; the molecule is split at a bond or bonds next to the urea linkage to afford two major fragments. In view of the fact that the fragment derived from the carboxy-side (*C*-fragment) of compound (2) was not reactive to ninhydrin, the amino-end of this fragment must be blocked in some way. It is noteworthy that in the case of the *C*-fragments of both (2) and (4) the recovery of their *N*-terminal residues in amino-acid analysis was remarkably low.

*Isolation and Identification of Degradation Products.*—For further information on the acidolytic breakdown of urea derivatives, we carried out a detailed investigation of Z-Gly-NH·CH($CH_2$Ph)·NH·CO-Gly-OBu$^t$ (5) [1] and related compounds. These model compounds were treated with TFA in the presence or absence of a scavenger, such as anisole or 2-mercaptoethanol, and the products were examined by t.l.c. in chloroform-methanol–acetic acid (90 : 10 : 3). In the absence of scavenger, compound (5), Z-Gly-NH·CH($CH_2$·$C_6H_4$·OH)·NH·CO-Gly-OBu$^t$ (6),[1] and a symmetrical urea [Z-Gly-NH·CH($CH_2$Ph)·NH]$_2$CO (7),[1] all seemed to give the same

* The *in vivo* steroidogenesis assay was performed by Dr. A. Tanaka and K. Odaguchi of this laboratory.

[3] H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Japan*, 1966, **39**, 882.

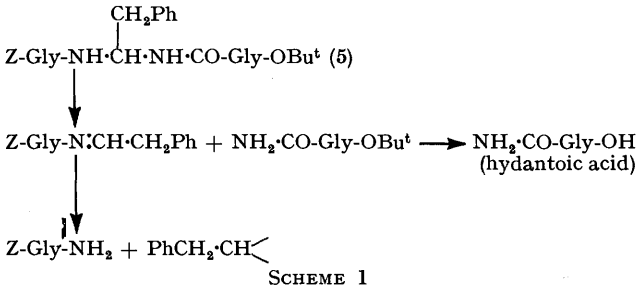
[4] H. Otsuka and K. Inouye, *Pharmac. Therap.* (*B*), 1975, **1**, 501, and references cited therein.

major product, which behaved like Z-Gly-$NH_2$, and a number of minor products. In the presence of scavenger, however, the nature of the main product seemed to depend on the scavenger used. These and other products were isolated and identified as follows.

Compound (5) was treated with TFA (25 °C; 30 min) in the absence of scavenger and the crude product was extracted with water in the presence of ethyl acetate. The aqueous extract yielded a crystalline material ($C_3H_6N_2O_3$) with m.p. 173—175° (decomp.). This was identified as hydantoic acid ($NH_2$·CO·NH·$CH_2$·$CO_2H$) by comparison of its i.r. spectrum with that of an authentic sample. The yield was 59% after recrystallisation. The ethyl acetate solution contained a main component which reacted positively to the HBr–ninhydrin test (see Experimental section) and was isolated crystalline after purification on a silica gel column. N.m.r. and elemental analysis data indicated that this product might be Z-Gly-$NH_2$, and this was confirmed by comparison (m.p., chromatographic behaviour, and i.r. spectrum) with authentic material. The yield was 59%. The acidolysis of the symmetrical urea (7) also produced Z-Gly-$NH_2$, but not hydantoic acid.

In the presence of 2-mercaptoethanol or anisole as a scavenger treatment of compound (5) with TFA yielded two kinds of products; one insoluble in ether and negative to the HBr–ninhydrin test, and the other soluble in ether and positive to the same test. The ether-insoluble product was hydantoic acid, obtained in quantitative yield. The ether-soluble product, which seemed to contain a molecule of the scavenger, was obtained crystalline after chromatography. The product formed in the presence of 2-mercaptoethanol had the formula $C_{20}H_{24}N_2O_4S$. N.m.r. data were consistent with the partial structure Z-Gly-NH·CH($CH_2$·Ph)–. This compound was, therefore, identified as Z-Gly-NH·CH($CH_2$·Ph)·S·$CH_2$·$CH_2$·OH (8). Similarly, the product formed in the presence of anisole was found to be Z-Gly-NH·CH($CH_2$Ph)·$C_6H_4$·OMe (9). The yields of (8) and (9) after chromatographic purification were 64 and 58%, respectively. Neither showed any optical rotation.

The acidolytic degradation of compound (5) occurs not only in TFA but also in some other acid reagents, such as formic acid, hydrogen fluoride, M-hydrogen chloride in ethyl acetate, and 25% hydrogen bromide in acetic acid,* currently used for removal of acid-labile protecting groups in peptide synthesis. Compound (5) seemed stable in acetic acid, but it decomposed slowly in 80% acetic acid. In formic acid compound (5) had decomposed completely within 45 min at room temperature, whereas in 80% formic acid the reaction mixture contained three major components (t.l.c.; HBr–ninhydrin test): starting material (5), Z-Gly-$NH_2$, and an unknown. The unknown component disappeared upon continued treatment with concomitant increase in the amount of Z-Gly-$NH_2$. This finding, as well as those described above, supports the idea that the acidolysis of (5) may proceed stepwise in a manner such as that shown in Scheme 1. In the presence of scavenger RH (2-mercaptoethanol or anisole) the intermediate Z-Gly-N:CH·$CH_2$Ph may be stabilised by the formation of an addition product, Z-Gly-NH·CH($CH_2$·Ph)R.† Scheme 1 is consistent with the fact that both (8) and (9) were optically inactive. In the absence of scavenger, however, the intermediate compound further decomposes to yield Z-Gly-$NH_2$ and $PhCH_2$·CH<, and the latter might be responsible for a number of unidentified minor products. Bergmann and Zervas showed that compounds of general formula $R^1CO$·NH·$CHR^2$·$NH_2$,HCl were hydrolysed in boiling water to give aldehydes $R^2CHO$ and amides $R^1CO$·$NH_2$.[5] However, we could not isolate phenylacetaldehyde in any form (*e.g.* 2,4-dinitrophenylhydrazone, oxime, or dimedone derivative) from the reaction mixture of compound (5) and TFA. It is therefore unlikely that the degradation of (5) in TFA involves some hydrolytic process.




Scheme 1

Since the acidolytic degradation of the compound of general formula $R^1CO$·NH·$CHR^2$·NH·CO·$NHR^3$ is apparently independent of the nature of the groups $R^1$, $R^2$, and $R^3$, as has been demonstrated with urea derivatives including compounds (2) and (4), the main degradation routes may be formulated more generally as shown in Scheme 2.

In the absence of scavenger:

$$R^1CO\cdot NH\cdot CHR^2NH\cdot CO\cdot NHR^3 \longrightarrow R^1CO\cdot NH_2 + >CHR^2 + NH_2\cdot CO\cdot NHR^3$$

In the presence of scavenger $R^4H$:

$$R^1CO\cdot NH\cdot CHR^2\cdot NH\cdot CO\cdot NHR^3 + R^4H \longrightarrow R^1CO\cdot NH\cdot CHR^2R^4 + NH_2\cdot CO\cdot NHR^3$$

Scheme 2

Apparently the total acidic hydrolysis of the urea derivative can also proceed stepwise, with the formation of an amide $R^1CO$·$NH_2$ and a carbamoylamino-acid $NH_2$·CO·$NHR^3$ as intermediates (Scheme 3). The fact

$$R^1CO\cdot NH\cdot CHR^2\cdot NH\cdot CO\cdot NHR^3 \xrightarrow{H_2O} R^1CO\cdot NH_2 + R^2CHO + NH_2\cdot CO\cdot NHR^3$$

$$R^1CO\cdot NH_2 \xrightarrow{H_2O} R^1CO_2H + NH_3$$

$$NH_2\cdot CO\cdot NHR^3 \xrightarrow{H_2O} R^3NH_2 + CO_2 + NH_3$$

Scheme 3

* In the case of HF or HBr–AcOH, H-Gly-$NH_2$ instead of Z-Gly-$NH_2$ was a product from compound (5).

† Very slow release of Z-Gly-$NH_2$ was observed with (8) and (9) when treated with TFA for a long time.

[5] M. Bergmann and L. Zervas, *J. Biol. Chem.*, 1936, **113**, 341.

that the recovery in amino-acid analysis of the amino-acid residue whose α-amino-function had constituted a part of the urea group was significantly low may be ascribed to the stability of the carbamoylamino-acid toward acidic hydrolysis. As an example, the result of the hydrolysis of hydantoic acid ($R^3NH_2$ = glycine in $NH_2$·CO·$NHR^3$) is shown in Figure 2, in which the hydrolysis, determined by the ninhydrin method [6] with an equimolar mixture of glycine and ammonium hydrochloride as reference, was found to be much slower than that expected for normal peptide bonds.

The present results have demonstrated that the urea derivatives formed as side products in the azide method of peptide synthesis are degraded by acid to smaller fragments, which usually differ from the desired peptide in chemical and physicochemical properties. In the preceding paper [1] we suggested that the peptide might be

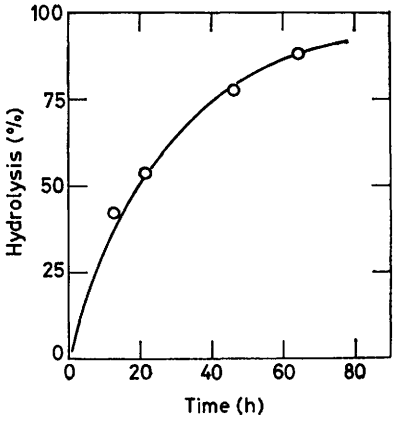



FIGURE 2 Hydrolysis of hydantoic acid with 6M-HCl at 110 °C as determined by ninhydrin method

contaminated with the related urea when their molecules were fairly large and complex. Riniker and Schwyzer [7] had also posed the same problem in their synthetic studies on the urea derivatives of angiotensin II analogues. However, we now conclude that there is little or no danger of such contamination if the coupling product is treated with acid under the deprotection conditions prior to its purification.

## EXPERIMENTAL

T.l.c. was performed on precoated silica-gel plates (Merck Kieselgel $60F_{254}$) or cellulose plates (Merck Cellulose F) with the following solvent systems (ratios by volume): A, chloroform–methanol (9 : 1); B, chloroform–methanol–acetic acid (90 : 10 : 3); C, butan-1-ol–acetic acid–water (4 : 1 : 2); D, butan-1-ol–acetic acid–pyridine–water (15 : 3 : 10 : 12); E, butan-1-ol–acetic acid–pyridine–water (15 : 3 : 10 : 15). In some cases the silica-gel plate was sprayed with hydrobromic acid (sp. gr. 1.48), then heated at 150 °C for a few minutes (to dryness), and finally sprayed with ninhydrin (HBr–ninhydrin test). Kieselgel 60 (Merck) was used for column chromatography throughout. Samples for amino-acid analysis were hydrolysed with constant-boiling hydrochloric acid in evacuated sealed tubes at 110 °C for 24 h.

*Attempted Synthesis of* H-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)·NH·CO-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-$NH_2$ {[$Gly^1$]-ACTH(1—18)-$NH_2$-4,5-urea} (1).—Boc-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)·NH·CO-Glu($OBu^t$)-His-Phe-Arg-Trp-Gly-OH (2) (150 mg, 0.1 mmol) [1] was converted into the *N*-hydroxysuccinimide ester hydrochloride as described [8] for the related decapeptide active ester, and this was added to a solution of H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-$NH_2$ acetate (3) [prepared from the $N^{\alpha}$-Z-derivative (160 mg, 0.1 mmol) by catalytic hydrogenolysis] [3] and triethylamine (0.05 ml) in DMF (3 ml). The mixture was kept at 4 °C for 40 h, then introduced into ethyl acetate–ether (1 : 1; 100 ml) and the precipitate was collected (270 mg). For deprotection a sample (100 mg) was treated with TFA (5 ml) in the presence of anisole (0.1 ml) and 2-mercaptoethanol (0.15 ml) at 25 °C for 60 min. The mixture was evaporated *in vacuo* and the residue was passed through a small column of Amberlite CG-400 ($AcO^-$ form) with portions of water. The aqueous solutions were combined and lyophilised, and the residue (100 mg) was chromatographed on a carboxymethylcellulose column in an ammonium acetate buffer (Figure 1). The fractions corresponding to a major peak (tubes 157—174) were combined and lyophilised, and the residue (56 mg) was subjected to partition chromatography on a column (2.0 × 43 cm) of Sephadex G-25 (medium) with butan-1-ol–acetic acid–pyridine–water (15 : 3 : 10 : 15) as solvent. Fractions (3 ml) were collected and those containing the major product as a single component (tubes 12—40) were combined and evaporated *in vacuo*. The residue was chromatographed again on a carboxymethylcellulose column in the same manner to afford pure material (32 mg); $[\alpha]_D^{24}$ −63.6 ± 2.5° (*c* 0.4 in 0.1M-AcOH); $\lambda_{max.}$ (0.1M-HCl) 280 nm ($E_{1\,cm}^{1\%}$ 23.0), $\lambda_{sh}$ 289 nm (18.8); $\lambda_{max.}$ (0.1M-NaOH) 280 nm ($E_{1\,cm}^{1\%}$ 24.3), $\lambda_{sh}$ 289 nm (20.0); amino-acid analysis [figures in parentheses are theoretical values for compound (1)]: Lys 2.89 (3), His 0.95 (1), Arg 2.90 (3), Ser not detected (1), Glu 0.79 (1), Pro 1.14 (1), Gly 1.88 (3), Val 1.00 (1), Met not detected (0), Tyr not detected (1), Phe 0.98 (1). The product was homogeneous (Ehrlich reagent and ninhydrin) on t.l.c. in system E.

*Acidolytic Breakdown of Urea Derivatives.*—(a) *Treatment of* Boc-Gly-Tyr-Ser-NH·CH($CH_2$·$CH_2$·SMe)NH·CO-Glu-($OBu^t$)-His-Phe-Arg-Trp-Gly-OH (2) *with TFA*. Compound (2) (30 mg) was treated with TFA (1 ml) at 25 °C for 60 min in the presence of anisole (0.02 ml) and 2-mercaptoethanol (0.02 ml). The precipitate formed on addition of ether was treated with Amberlite CG-400 ($AcO^-$) in 60% ethanol for 30 min. The resin was filtered off and the filtrate evaporated *in vacuo* (30 mg). The residue contained two major components (t.l.c. on silica gel, in system C). Separation was attempted by partition chromatography on a column (3.4 × 150 cm) of Sephadex G-25 (medium) with butan-1-ol–acetic acid–water (4 : 1 : 2) as solvent and by chromatography on a carboxymethylcellulose column (2.0 × 15 cm; Whatmans CM-52) using an ammonium acetate buffer (pH 6.5) with a linear concentration gradient (0—0.2M), but was not achieved. The material (18 mg)

[6] S. Moore and W. H. Stein, *J. Biol. Chem.*, 1954, **211**, 907; C. H. Hirs, *Methods. Enzymol.*, 1967, **11**, 325.

[7] B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, 1964, **47**, 2375; B. Riniker, *Metabol. Clin. Exp.*, 1964, **13**, 1247.

[8] K. Inouye, F. Shinozaki, M. Kanayama, and H. Otsuka, *Bull. Chem. Soc. Japan*, 1976, **49**, 3615.

recovered from the CM-52 column was then chromatographed on a silica gel column (5 g) with ethyl acetate–acetic acid–water (3 : 1 : 1) as solvent. Fractions (3 ml) were collected and their absorption at 280 nm was measured. The fractions corresponding to two peaks, tubes 6—11 (I) and 13—25 (II), were combined and evaporated *in vacuo*. Fraction (I) yielded a ninhydrin-positive and Ehrlich-negative material (9 mg) with the following amino-acid ratios: Ser 0.87, Gly 1.00, Tyr 0.85, $NH_3$ 1.43. Fraction (II) yielded a ninhydrin-negative and Ehrlich-positive material (11 mg) whose amino-acid ratios were: His 0.94, Arg, 0.68, Glu 0.84, Gly 1.00, Phe 1.02, $NH_3$ 0.93, Trp (0.42).

(b) *Treatment of* H-Lys(Boc)-Pro-Val-Gly-NH·CH([$CH_2$]$_4$·NH-Boc)·NH·CO-Lys(Boc)-Arg-Arg-$NH_2$ (4) *with TFA*. Z-Lys(Boc)-Pro-Val-Gly-NH·CH([$CH_2$]$_4$·NH-Boc)·NH·CO-Lys(Boc)-Arg-Arg-$NH_2$ (50 mg) [1] was hydrogenolysed over palladium in methanol for 3 h in the presence of a few drops of acetic acid. The resulting $N^{\alpha}$-free compound, which was homogeneous to ninhydrin on t.l.c. (silica gel; system C) was treated with TFA (1 ml) at 25 °C for 60 min in the presence of anisole (0.02 ml) and 2-mercaptoethanol (0.02 ml). The product was precipitated by addition of ether and treated with Amberlite CG-400 ($AcO^-$) in 50% ethanol (53 mg). It was then subjected to partition chromatography on a column (2.0 × 40 cm) of Sephadex G-25 (medium) with butan-1-ol–acetic acid–pyridine–water (15 : 3 : 10 : 15) as solvent. Fractions (3 ml) were collected and their peptide content was estimated by the ninhydrin method.[6] The fractions corresponding to two major peaks, tubes 31—90 and 91—130, were separately combined and evaporated *in vacuo* to give fractions (I) and (II), respectively. For further purification both were rechromatographed as above. Fraction (I) yielded a homogeneous material (17 mg) which was reactive to ninhydrin but not to the Sakaguchi reagent and had amino-acid ratios: Lys 0.93, Pro 0.92, Gly 0.93, Val 1.00, $NH_3$ 0.57. Fraction (II) afforded another pure material (15 mg), which was reactive to both ninhydrin and the Sakaguchi reagent and had the following amino-acid ratios: Lys 0.62, Arg 2.00, $NH_3$ 0.95.

*Isolation and Identification of Degradation Products.*—(a) *Treatment of* Z-Gly-NH·CH($CH_2$Ph)·NH·CO-Gly-OBu$^t$ (5) *with TFA*. (i) *Isolation of hydantoic acid*. Compound (5) (200 mg) was treated with TFA (2 ml) at 25 °C for 30 min, after which the mixture was evaporated *in vacuo*. The residue was dissolved in ethyl acetate and the solution was extracted with water. Evaporation of the extract afforded a crystalline residue, which was recrystallised from ethanol–ether; yield 29 mg (59%); m.p. 173—175° (decomp.) [Found: C, 30.65; H, 5.25; N, 23.65; O, 40.95. Calc. for hydantoic acid ($C_3H_6N_2O_3$): C, 30.5; H, 5.1; N, 23.7; O, 40.65%]. The i.r. spectrum (Nujol) of the product was identical with that of hydantoic acid.

(ii) *Isolation of* Z-Gly-$NH_2$. The ethyl acetate solution obtained in (i) was evaporated *in vacuo* and the residue was chromatographed on a column of silica gel (20 g) with chloroform–methanol (95 : 5) as solvent. Fractions (2.5 ml) were examined by t.l.c. (silica gel in system A) (HBr–ninhydrin) and those containing a main component (tubes 46—63) were combined and evaporated *in vacuo* to give a crystalline residue (51 mg), which was recrystallised from methanol–ether; yield 34 mg (40%); m.p. 137—139° $\tau$ ($CD_3OD$) 2.68 (5 H, s, Ph), 4.89 (2 H, s, PhC$H_2$·O), and 6.21 (2 H, s, NH·C$H_2$·CO) [Found: C, 57.9; H, 5.85; N, 13.15. Calc. for Z-Gly-$NH_2$ ($C_{10}H_{12}N_2O_3$): C, 57.7; H, 5.8; N, 13.45%]. The i.r. spectrum was identical with that of Z-Gly-$NH_2$ and a mixed m.p. with an authentic sample showed no depression.

(b) *Treatment of* [Z-Gly-NH·CH($CH_2$Ph)·NH]$_2$CO (7) *with TFA*. Compound (7) (50 mg) was treated with TFA (1 ml) at 25 °C for 60 min. The TFA was evaporated off *in vacuo* and the residue was chromatographed on a silica gel column (15 g) with chloroform–methanol (95 : 5) as solvent. Fractions (3.5 ml) containing a main component (tubes 13—21) were combined and evaporated *in vacuo*. The residue was crystallised from methanol–ether to give Z-Gly-$NH_2$ (18 mg, 49%), m.p. 133—135°.

(c) *Treatment of* Z-Gly-NH·CH($CH_2$Ph)·NH·CO-Gly-OBu$^t$ (5) *with TFA in the presence of anisole*. Compound (5) (0.3 g) and anisole (0.4 ml) were dissolved in TFA (1 ml) and the mixture was kept at 25 °C for 60 min, then evaporated *in vacuo*. To the residue was added ether (30 ml), and the precipitate was collected, giving hydantoic acid (74 mg, 100%), m.p. 173—175° (decomp.). The filtrate was evaporated and the residue chromatographed on a silica gel column (40 g) with chloroform (200 ml) and chloroform–methanol systems (197 : 3, 200 ml; 97 : 3, 300 ml) as solvent. Fractions (7 ml) were collected and examined by t.l.c. (silica gel in system B) (HBr–ninhydrin); those containing a major component (tubes 35—53) were combined and evaporated *in vacuo*. After rechromatography as above the *product* was crystallised from ether; yield 150 mg (58%); m.p. 143—147°, no rotation; $\tau$ [$(CD_3)_2SO$] 2.68 (5 H, s, *Ph*$CH_2$·O), 2.83 (5 H, s, *Ph*$CH_2$·CH), and 4.98 (2 H, s, PhC$H_2$O) [Found: C, 71.8; H, 6.4; N, 6.65. Z-Gly-NH·CH($CH_2$Ph)·$C_6H_4$·$OCH_3$ ($C_{25}H_{26}N_2O_4$) requires C, 71.75; H, 6.25; N, 6.7%].

(d) *Treatment of* Z-Gly-NH·CH($CH_2$Ph)·NH·CO-Gly-OBu$^t$ (5) *with TFA in the presence of 2-mercaptoethanol*. Compound (5) (0.3 g) and 2-mercaptoethanol (0.4 ml) were dissolved in TFA (1 ml). The mixture was kept at 25 °C for 60 min and then evaporated *in vacuo*. To the residue was added ether (30 ml), and the crystalline precipitate was filtered off [hydantoic acid (71 mg, 97%)]. The filtrate was evaporated and the residue was chromatographed on a silica-gel column as in (c). The fractions containing a major component (tubes 54—85) were combined and evaporated *in vacuo*. The resulting *material* was purified by rechromatography and by crystallisation from ether; yield 155 mg (64%); m.p. 90—91°; no rotation; $\tau$ [$(CD_3)_2$-SO] 2.69 (5 H, s, *Ph*$CH_2$), 2.80 (5 H, s, *Ph*$CH_2$·CH), and 5.11 (2 H, s, PhC$H_2$·O) [Found: C, 62.0; H, 6.3; N, 7.15; S, 8.45. Z-Gly-NH·CH($CH_2$Ph)·S·$CH_2$·$CH_2$·OH ($C_{20}H_{24}N_2O_4S$) requires C, 61.85; H, 6.25; N, 7.2; S, 8.25%].

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