The Synthesis of Peptides in Aqueous Medium. VII. The Preparation and Use of 2,5-Thiazolidinediones in Peptide Synthesis

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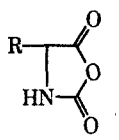
Optically active *N*-thiocarboxyamino acid anhydrides, NTA's (**4**), have been prepared and used for the stepwise synthesis of peptides in aqueous solution. Generally thiocarboxyanhydrides of good optical purity were obtained by the recrystallization of products from the reaction of alkoxythiocarbonyl L-amino acids (**3**) with phosphorus tribromide. Alternative syntheses of these anhydrides were provided by the cyclization of L-amino acid and L-amino thio acid thio carbamates, or by the reaction of an L-amino thio acid, L-thioproline, with phosgene. Salts of amino acid thiocarbamates were stable to electrophoresis at pH 11, whereas the carbamate salts decomposed. Using conditions similar to those reported for *N*-carboxyanhydrides (NCA's), addition of an NTA to an aqueous solution of an amino acid or peptide at pH 9–9.5 at 0–4° led to high yields of the peptide homolog. The increased stability of the thiocarbamates permitted the reaction to be carried out at a lower pH than was the case with the NCA's, generally affording higher yields but still leading to by-products analogous to those observed with the NCA's. In contrast to the NCA's, the NTA's led to 1–20% of epimeric peptide in the product. Quantitation of small amounts of racemate derived from alanine NTA was made by nmr spectral comparison of the low intensity peaks in the alanine *C*-methyl doublet in a diastereomeric by-product with the ^{13}C-satellite peaks of the *C*-methyl doublet of the major product. Racemization occurring during reaction of proline NTA was estimated using a previously reported method in which the incorporation of tritium from labeled water was measured. The NTA's which should prove most useful in peptide synthesis are those of glycine and alanine which gave significantly higher yields of product than the NCA's, and histidine NTA which, in contrast to the NCA, was used successfully for controlled peptide synthesis.

The use of the α-amino acid *N*-carboxyanhydrides (NCA's), **1**, in the synthesis of peptides in aqueous solution is complicated by the fact that below pH 11 the instability of peptide carbamates leads to overreactions *via* decarboxylation, whereas at pH 11 overreaction *via* the NCA anion, formation of hydantoic acids, and hydrolysis become troublesome side reactions.[1] Hydantoic acid formation was a problem even at pH 10.2 with the NCA of glycine and occasionally with that of alanine. Further, histidine NCA rearranged to a fused imidazolone. A more stable carbamate analog would permit peptide condensation to be carried out at lower pH and this, in turn, would suppress those side reactions arising from reactions of the anhydride with base. Moreover, the production of a more stable carbamate ion should suppress the acid-catalyzed formation of overreaction products.

It was thought that analogs of the NCA's in which the ether oxygen is replaced by sulfur might solve some of these problems because the related thiocarbamates could be expected to show a greater stability at a given pH than would the carbamates. A few free dithiocarbamic acids were known,[2] and although the free monothiocarbamic acids had not been reported,[3] we assumed that they would have a stability intermediate between the carbamates and dithiocarbamates. Therefore, it should be possible to carry out peptide syntheses at a lower pH with NTA's than with the NCA's. The use of 2-thiono-5-thiazolidinones, **2**, in peptide synthesis has been reported,[4,5] but a considerable amount of racemization accompanied peptide formation.[5] The present



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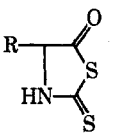

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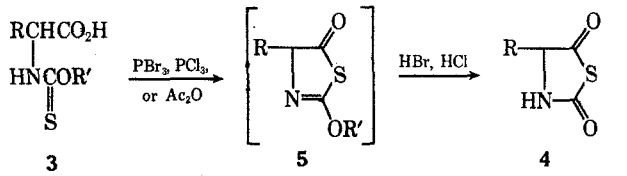
paper describes the synthesis of optically active *N*-thiocarboxyanhydrides (NTA's), *i.e.*, derivatives of 2,5-thiazolidinedione (**4**, R = H), and their use in stepwise peptide synthesis in aqueous solutions.[6]

The *N*-thiocarboxyanhydride of glycine has been prepared by the reaction of the thionourethan, *N*-(ethoxythiocarbonyl)glycine (**3**, R = H; R′ = Et), with phosphorus tribromide or trichloride.[7–9] Recently, the syn-



3 **5** **4**

(1) R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, H. Barkemeyer, D. F. Veber, W. J. Paleveda, Jr., T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.*, **32**, 3415 (1967).

(2) (a) A. Y. Yakubovich and V. A. Klimova, *J. Gen. Chem. USSR*, **9**, 1777 (1939); *Chem. Abstr.*, **34**, 3685 (1940). (b) H. Korner, *Chem. Ber.*, **41**, 1901 (1908).

(3) E. E. Reid, "The Chemistry of Bivalent Sulfur," Vol. IV, Chemical Publishing Co., New York, N. Y., 1962, p 196.

(4) (a) J. D. Billimoria and A. H. Cook, *J. Chem. Soc.*, 2323 (1949); (b) A. C. Davis and A. L. Levy, *ibid.*, 2419 (1951).

(5) A. H. Cook and A. L. Levy, *ibid.*, 651 (1950).

(6) (a) For a preliminary communication, see R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Denkewalter, and R. Hirschmann, *J. Amer. Chem. Soc.*, **90**, 3254 (1968). (b) We are grateful to Dr. Dieter Ziebarth of the Institute für Krebsforschung of the Deutsche Akademie der Wissenschaften zu Berlin for making available a copy of his recent thesis, "Uber 2,5-Dioxothiazolidine. Ein Beitrag zur Peptidsynthese," Humbolt Universität, Berlin, 1968, in which he describes the preparation of some NTA's and the formation of racemic peptides when the NTA's were condensed in basic aqueous solution.

(7) (a) P. Aubert and E. B. Knott, *Nature*, **166**, 1039 (1950); (b) P. Aubert, R. A. Jeffreys, and E. B. Knott, *J. Chem. Soc.*, 2195 (1951).

(8) J. L. Bailey, *ibid.*, 3461 (1950). **4** was formulated as the isomeric 2-thiono-5-oxazolone, i, a structure which was implicated in the formation of polyalanine by the thermal decomposition of lead alanine dithiocarbamate [G. Losse and H. Weddige, *Justus Liebigs Ann. Chem.*, **636**, 144 (1960)].

i

(9) (a) H. G. Khorana, *Chem. Ind. (London)*, 129 (1951); (b) G. W. Kenner and H. G. Khorana, *J. Chem. Soc.*, 2076 (1952). Khorana proposed an NTA as a product in the acid cleavage of a peptide N-terminal alkoxythiourethan.

TABLE I

N-(ALKOXYTHIOCARBONYL) AMINO ACIDS, ROCHNCHRCO$_2$H (S)

Amino acid	L-Ala	D-Allo-isoleu	L-Arg	Gly	L-His	L-Ileu	L-Leu	L-Phe	L-Pro	L-Val
R′	CH_3	CH_3	CH_3	CH_3	C_2H_5	C_2H_5	CH_3	C_2H_5	CH_3	CH_3
Mp, °C	114–115	44–52	212–220 dec	80–82	212 dec	67–69	68–70	85–88	93–94	63–66
$[\alpha]^{25}_{589}$[a]	−19.3	−66			+24	+15.9	−31.3	+80.8	−126[b]	−8.35
Calcd, %										
C	36.81	46.80	38.70	32.20	44.44	49.29	46.80	56.89	44.44	46.80
H	5.56	7.37	6.50	4.73	5.39	7.81	7.37	5.97	5.82	7.36
N	8.58	6.82	22.57	9.38	17.28	6.39	6.82	5.53	7.41	6.82
S	19.64	15.62	12.90		13.19	14.62	15.62	12.66	16.92	15.63
Found, %										
C	37.32	46.96	38.89	32.50	44.56	49.43	46.71	56.85	44.49	47.02
H	5.64	7.45	6.31	4.75	5.47	7.76	7.26	6.14	5.68	7.40
N	8.43	7.05	22.61	9.36	17.58	6.68	5.93	5.81	7.37	6.82
S	20.27	16.03	13.20		13.49	13.76	16.17	12.35	17.03	15.52

[a] *c* 1 (CH_2Cl_2) except as otherwise noted. [b] *c* 1 ($CHCl_3$).

thesis of DL-phenylalanine NTA (**4**, R = $C_6H_5CH_2$) was reported.[10] Glycine or DL-alanine thioanhydride has been used to prepare glycylglycine ethyl ester,[8] DL-alanylglycine,[9a] and a glycine polymer.[11] The thioanhydride has also been postulated as the intermediate in the hydrogen chloride catalyzed cleavage of the N-terminal amino acid of a *N*-(ethoxythiocarbonyl)peptide in analogy with the Edman degradation.[9]

Greater stability of amino acid thiocarbamates compared to carbamates was indeed indicated by electrophoresis. The electrophoretic behavior of glycine carbamate[12] (see below) at pH 11 at room temperature is that of glycine indicating decomposition of the carbamate while glycine thiocarbamate moved with about twice the mobility of glycine indicating the greater stability of the thiocarbamate. Phenylalanine thiocarbamate showed a similar stability at pH 11, but when the electrophoresis was carried out at pH 9 at room temperature streaking was observed, suggesting thiocarbamate decomposition during the electrophoresis at the lower pH.

Preparation of the NTA's.—Because optically active NTA's had not heretofore been prepared, a variety of methods were explored for the synthesis of NTA's of L-amino acids. Of the methods outlined below, cyclization of the thionourethan **3** (method A) was the most convenient,[7] and could in several instances be used to give material of good optical purity. Methods B and C also gave NTA's of good optical purity, but the preparations involved more steps and led to lower yields. The peptides reported in this paper were synthesized with NTA's prepared *via* method A unless otherwise specified.

1. Cyclization of *N*-Alkoxythiocarbonyl Amino Acids. Method A.—A number of optically active *N*-alkoxythiocarbonyl amino acids were prepared by the reaction of xanthate esters and L-amino acids in alcoholic base (Table I). Generally these derivatives could be crystallized except as noted. The optical purity of the *N*-alkoxythiocarbonyl derivatives of the amino acids was investigated in three cases. The preparation of *N*-ethoxythiocarbonylproline was carried out in ethanol-tritiated water. Examination of the recovered crystalline derivative for nonexchangeable tritium showed that less than 0.006% racemization had taken place. When *N*-(ethoxythiocarbonyl)phenylalanine was treated with sodium methoxide in methanol under the conditions of synthesis, the optical rotation of the compound remained unchanged. Finally, repeated recrystallization of *N*-(ethoxythiocarbonyl)-L-leucine as the quinine salt led to no change in rotation of the recovered compound. Therefore, the crystalline alkoxycarbonyl amino acids are thought to be of excellent optical purity.

Aubert reported that alkoxythionocarbonylglycines (**3**, R = H) could be cyclized to the 2-alkoxy-5-thiazolone **5** with acetic anhydride.[13] Application of this reaction to *N*-(methoxythiocarbonyl)-L-leucine led to an oil which differed in its chromatographic behavior from both the thionourethan and the NTA. The infrared spectrum was consistent with the 5-thiazolone structure **5** (R = Me, R′ = *i*-Bu). Exposure of this oil to hydrogen chloride led to the formation of largely racemized leucine NTA. On the other hand, the rotation of phenylalanine NTA was essentially unchanged after treatment in THF with hydrogen chloride or phosphorus trichloride for 1 hr at room temperature. These results suggested that the racemization observed in the above leucine NTA occurred at the intermediate 5-thiazolone stage. Indeed, the thiazolone **5** is analogous to the azlactones, which have been cited as a major pathway for racemization of *N*-acyl amino acid derivatives.[14] Reaction of *N*-(methoxythiocarbonyl)-L-leucine with phosphorus tribromide at −30° led to a mixture from which the related 5-thiazolone and a partially racemized NTA could be isolated by silica gel chromatography.

The *N*-(alkoxythiocarbonyl) amino acids were best cyclized to the NTA's **4** by reaction with phosphorus tribromide for 5–10 min at 0°. In general, these conditions led to crystalline NTA's of relatively high optical purity. Addition of nucleophiles which should acceler-

(10) I. Z. Siemion, D. Konopińska, and A. Dżugaj, *Rocz. Chem.*, **43**, 989 (1969).

(11) J. H. Bradbury and J. D. Leeder, *Text. Res. J.*, **30**, 118 (1960); *Chem. Abstr.*, **54**, 8092*d* (1961).

(12) A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

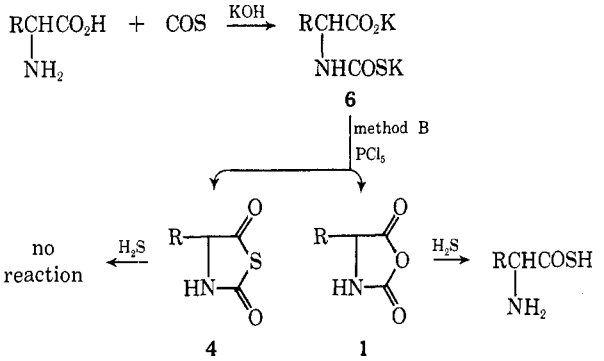
(13) P. Aubert, E. E. Knott, and L. A. Williams, *ibid.*, 2185 (1951).

(14) (a) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, Wiley, New York, N. Y., 1961, pp 832–836, and references therein; (b) I. Antonovics and G. T. Young, *Chem. Commun.*, 398 (1965).

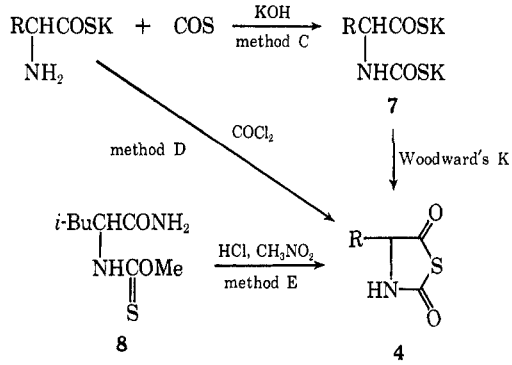
ate the cleavage of the intermediate oxazolone, such as sodium iodide, imidazole, or 1,2,4-triazole,[15] showed slight improvements in the yield and optical rotation of the NTA.

Although hydrogen chloride in the previously cited experiment did not racemize a preformed NTA, treatment of NTA with hydrogen bromide at room temperature led to a drop in optical activity in the product. Nevertheless, the use of PBr_3 proved to be advantageous because the greater reactivity of phosphorus tribromide permitted NTA formation to be carried out for a shorter period of time, thus reducing exposure to acidic conditions. The overall advantage of the use of phosphorus tribromide may be attributed to the greater nucleophilic activity of bromide ion in the cleavage of the thiazolone ether **5**. In an attempt to circumvent some of the above problems in the preparation of the NTA's, other methods of synthesis were examined.

2. From the Amino Acid Thiocarbamate. Method B.—Amino acid carbamates have been converted to NCA's with thionyl chloride.[12] Reaction of phenylalanine with carbonyl sulfide in a basic medium led to the salt of the amino acid thiocarbamate (**6**, R = $C_6H_5CH_2$).[16] Treatment of a suspension of this salt in tetrahydrofuran with phosphorus pentachloride gave a mixture of phenylalanine NCA (**1**, R = $C_6H_5CH_2$) and NTA (**4**, R = $C_6H_5CH_2$). Addition of hydrogen sulfide rapidly cleaved the NCA, thus permitting the isolation of the unchanged NTA by extraction with ethyl acetate. The products had an optical purity comparable to those prepared by route A.



3. Other Routes.—The problem of contamination of the NTA with NCA in method B could be avoided by use of an amino thio acid. Optically active thioleucine was prepared from the NCA with hydrogen sulfide and then converted to the potassium salt of thioleucine thiocarbamate (**7**, R = *i*-Bu) in analogy to method B. The salt was cyclized directly in aqueous solution with Woodward's Reagent K to the NTA (method C). Alternatively, the amino thio acid thiophenylalanine was converted to the NTA of good optical purity with phosgene (method D).



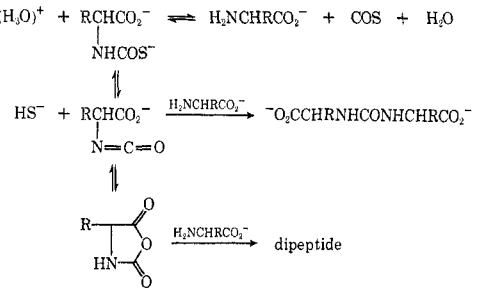
Finally, in analogy with a known scheme[9] for peptide degradation, *N*-methoxythiocarbonylleucine amide (**8**) was cyclized to the NTA with hydrogen chloride (method E). The anhydride was, however, largely racemized. The results of these methods are outlined in Table II.

Optical Purity.—The optical purity of selected NTA's was estimated by hydrolysis to the amino acid and determination of the amount of the D isomer present in the product. In general, the crystallization of the NTA's permitted the isolation of anhydrides with an optical purity ≧98%. This was not true of the NTA of leucine. Rotations of samples of the latter varying from $[\alpha]^{25}_{589}$ −30 to −55° remained essentially unchanged upon crystallization. To determine the optical purity of the NTA of leucine, $[\alpha]_{589}$ −57.4°, a sample was treated with silver nitrate to give silver sulfide and leucine. The crude product was treated with phenylalanine NCA. The resulting dipeptide contained 3% LD isomer by comparison on tlc[17] with dipeptide similarly prepared from DL-leucine and spotted at various concentrations. In the case of the NTA of proline, acid hydrolysis gave a quantitative yield of proline. The crude reaction product was assayed with D-amino acid oxidase and was found to contain about 2% D-proline.

Use of NTA's in Peptide Synthesis.—The reaction of *N*-thiocarboxyanhydrides with amino acids and peptides in aqueous solution was examined to determine yields and extent of racemization in peptide formation. The experimental conditions were similar to those used for the stepwise synthesis of peptides with NCA's,[1] (Scheme I) except that the pH was lower. After

(15) The addition of nucleophiles has catalyzed amino acid active ester condensations in some solvents: H. C. Beyerman and W. M. van den Brink, *Proc. Chem. Soc.*, 266 (1963); T. Wieland and W. Kahle, *Justus Liebigs Ann. Chem.*, **691**, 212 (1966).

(16) An aqueous solution of phenylalanine thiocarbamate gave rise to phenylalanine and a trace of phenylalanylphenylalanine on standing, which was detected by electrophoresis at pH 11. Upon tlc the dipeptide resolved into two spots corresponding to LL- and DL-phenylalanylphenylalanine. Phenylalanine carbamate did not give rise to peptide formation under these conditions, whereas phenylalanine dithiocarbamate, which was prepared from phenylalanine and carbon disulfide, did form the dipeptide. Further, a solution of phenylalanine thiocarbamate and radioactive phenylalanine in addition gave rise to a ninhydrin negative spot on tlc corresponding to hydantoic acid. A possible mechanism for the formation of these products is as follows.



This type of ring formation has been suggested previously by T. Wieland, R. Lambert, and H. U. Land, *ibid.*, **597**, 181 (1956). It may be noted that this route would seem to offer another pathway to peptide formation under prebiotic conditions.

(17) E. Taschner, J. F. Biernat, and T. Sokolowski, *Peptides, Proc. Eur. Symp.*, 5th, 1962 (1963).

TABLE II
AMINO ACID N-THIOCARBOXYANHYDRIDES[a]

Amino acid	Method	Yield, %	Mp, °C	$[\alpha]^{25}_{589}$[b]	Calcd, % C	H	N	S	Found, % C	H	N	S
L-Ala[c]	A	47	91–93	−164	36.62	3.81	10.68		36.50	3.61	10.65	
L-Arg[d]	A	62.5	115–117	−124.5[f]	28.29	4.41	18.85	10.79	28.57	4.40	18.99	10.73
Gly[e]	A	66	108–109		30.77	2.58	11.96	27.38	30.96	2.61	11.99	27.57
L-His[d,e]	A	72.5		−7.0[g]	30.20	2.90	15.10		30.16	3.00	14.76	
L-Leu	A	68	77–78	−57.2	48.53	6.40	8.09	18.51	48.67	6.34	8.02	18.80
	B	13		−56.0								
	C	45	76–77	−56.7								
	E	28		−34.5								
L-Phe[e]	A	47	109–111	−154	57.94	4.37	6.75	15.46	58.10	4.31	6.67	15.70
	B	25	111–112	−153								
	D	29[h]		−155, −154								
L-Pro	A	21	44.5–45	−157[i]	45.80	4.55	8.96	20.40	45.96	4.43	8.90	19.71
L-Val[e]	A	67	80–82	−82	45.26	5.70	8.80	20.14	45.09	5.83	8.93	20.44

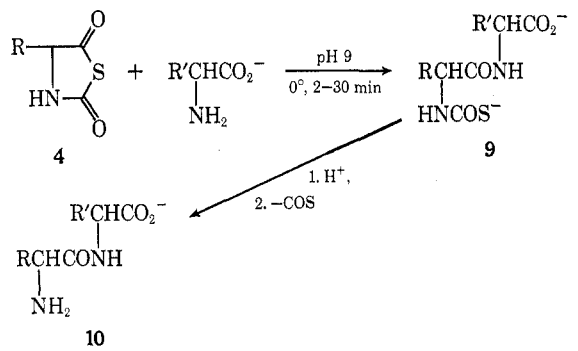
[a] The anhydrides were prepared from the methyl thionourethans unless otherwise indicated. [b] c 1 (CH_2Cl_2) unless otherwise indicated. [c] Prepared in the presence of added imidazole. [d] As the N-thiocarboxyanhydride hydrobromide. [e] Prepared from the ethyl thionourethan. [f] c 2 (DMSO). [g] c 2 (methyl carbitol) at 365 nm. [h] Two crops of 9.2 and 22%. [i] c 1 ($CHCl_3$, EtOH free).

TABLE III
PEPTIDES, PREPARED WITH NTA'S IN COMPARISON WITH OTHER METHODS

Reactants: Carboxy-anhydride	Nucleophile	pH: NTA[a]	pH: NCA[a]	Product	Isolated yield: NTA, %	NCA, %
Gly	Phe	9.5	10.5	Gly-Phe	93	50[b]
Gly	Phe-Leu	9.0	10.2	Gly-Phe-Leu	75[c]	37
Ala	Leu-Phe	9.5	10.2	Ala-Leu-Phe	92	70
Ala	Ser-Val (Bzl)	9.15	10.1	Ala-Ser-Val (Bzl)	68[d]	55[d]
His	Phe-Asp-Ala-Ser-Val (Bzl)	9.0		His-Phe-Asp-Ala-Ser-Val (Bzl)	24[e]	
Boc-His·N_3	Phe-Asp-Ala-Ser-Val[f] (Bzl)			Boc-His-Phe-Asp-Ala-Ser-Val	79[d]	

[a] The NCA or NTA was used in 10% excess unless otherwise specified. [b] Disappearance yield. More than 20% of the hydantoic acid was indicated by tlc. [c] The NTA was used in 20% excess. [d] Small amounts of impurities were indicated. [e] 3.8 equiv of the NTA were used. [f] The reaction was run in DMF–Et_2O.

SCHEME I
STEPWISE SYNTHESIS OF PEPTIDES WITH NTA'S



cessation of a rapid uptake of base (2–30 min), the solution was acidified to cleave the carbonyl sulfide protecting group. The carbonyl sulfide was swept from the reaction mixture with nitrogen. Representative reactions of NTA's and NCA's with amino acid or peptide nucleophiles are compared in Table III. The yields refer to isolated products unless otherwise indicated. Alanine NTA and, especially, glycine NTA gave higher yields of the desired peptides than did the NCA's.

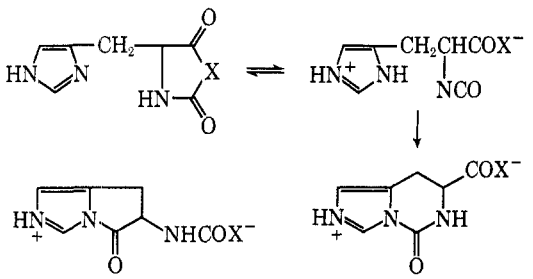
A comparison of the products from the reaction of phenylalanine NCA and of NTA with ^{14}C-arginine is given in Table IV. The reactions were evaluated by paper-strip electrophoresis as previously described.[1] A higher yield of peptide was obtained with the NTA at pH 9.5 than with the NCA at pH 10. Furthermore, the NTA left less unchanged arginine and afforded less of the overreaction product, Phe-Phe-Arg. The amount of hydantoic acid formed was not changed significantly. The yields in Tables III and IV support the expectation that a greater stability of the thiocarbamate should permit efficient peptide condensation to be carried out at a lower pH.

TABLE IV
REACTION PRODUCTS FROM PHE NTA AND PHE NCA WITH LABELED ARGININE

Reactants	pH, NTA	Product	Yield,[a] %
Phe-NCA + ^{14}C-Arg	10.0	Phe-Arg	89.2
		Arg	3.5
		Phe-Phe-Arg	4.0
		Hydantoic acid[b]	2.8
Phe-NTA + ^{14}C-Arg	9.5	Phe-Arg	94.2
		Arg	2.2
		Phe-Phe-Arg	0.3
		Hydantoic acid[b]	2.7

[a] Yields based on radioactivity counts from fractions from paper electrophoresis. [b] $HO_2CCH(CH_2C_6H_5)NHCO\cdot Arg\cdot OH$.

A striking difference was noted between the NCA and the NTA of histidine. The former failed to yield histidyl peptides at pH 10.2, whereas the NTA was used, for example, to prepare the C-terminal hexapeptide of ribonuclease[18] (Table III). Inspection of molecular models suggested that the imidazole nitrogen is in an ideal position to abstract the NH proton from the nitrogen of the anhydride ring. This intramolecular, base-catalyzed ring opening which would lead to an isocyanate parallels the mechanism for isocyanate formation which had been proposed[1] to explain the hydantoic acid by-products in NCA reactions. In the case of the NCA and NTA derived from histidine, the intermediate isocyanate can be expected to undergo further intramolecular reaction to form the imidazopyrimidine **11** (X = O or S, respectively), and indeed the NCA gave a noncrystalline product which was formulated as **11** (X = O) on the basis of its ir and nmr spectra. When the NTA of histidine was treated with aqueous alkali a crystalline product was obtained after acidification which had an elemental analysis and ir spectrum consistent with structure **11** (X = S). An alternate structure, **12**, was discarded on the basis of its infrared spectrum and of its expected ease of decarboxylation. We believe that the NTA, unlike the NCA, of histidine is useful in controlled peptide synthesis because the equilibrium be-



12 **11**

tween anhydride and isocyanate is shifted to the left when X = S. It was also possible to prepare histidyl peptides using compound **11** (X = S). The reaction proceeded slowly at room temperature but **11**, unlike the NTA, failed to give histidyl peptides at an appreciable rate at 0°.

In the reaction of glycine NTA with L-phenylalanyl-L-leucine a 75% yield of the isolated tripeptide (Table III) was obtained whereas the NCA gave about half that amount. In Table V, the distribution of products is shown for the reaction carried out between the ^{14}C-labeled dipeptide used in 5% excess and glycine NTA. Although the yield in NCA reactions decreases sharply when the reaction was carried out at a pH below 10,[1] the NTA reaction is optimal either below the pH range studied or between 8.5 and 9.2 (see Table V). The data are consistent with the results expected for the greater stability of the thiocarbamate. Thus, overreaction is suppressed even at pH 8.5 as judged by the lack of a substantial increase in the amount of unchanged nucleophile (Phe-Leu) indicating that it is not being inactivated by reaction with any carbonyl sulfide derived from the decomposition of the product thiocarbamate. The increase in residual nucleophile at pH 10 can be ascribed to the loss of NTA *via* hydrolysis and polymerization. In the NCA reaction, the yield of hydantoic acid rose with pH.[1] In the pH range examined for the NTA case (Table V), the yield of hydantoic acid remained essentially unchanged. That the NTA does form the anion is suggested by the increase in overreaction products at high pH due to anionic oligomerization of the NTA. However, ring opening may be less favored for the reasons discussed in the case of NTA histidine. If it is assumed that the NTA has about the same solubility at the pH's studied and that the nucleophile competes relatively effectively against hydroxide ion for any isocyanate, a second mechanism for hydantoic acid formation may be required.[19]

In stepwise peptide condensation, the NTA's gave a significant amount of the epimeric product,[20] whereas the NCA's had given optically pure products. Using the NTA's in aqueous solutions, from less than 1 to as high as 20% of the D isomer appeared in the resulting peptide. The reaction of L-histidine NTA hydrobromide with L-alanylglycine led to a mixture which was analyzed directly by nmr. The analysis of D-His-L-Ala-Gly in L-His-L-Ala-Gly could be made by comparison of the separated alanine methyl doublets of the two diastereomeric products using 100-MHz nmr.[21] The product contained 75% of His-Ala-Gly, which consisted of 93% of the LL isomer and 6.7% of the DL isomer based on nmr examination of the freeze-dried crude product. Similarly, reaction of L-histidine NTA with D-alanylglycine gave a 58% yield of tripeptide, 83% of which was the LD isomer and 17% of which was the DD isomer. The

TABLE V
PRODUCTS FROM THE REACTION GLYCINE NTA WITH L-PHENYLALANYL-^{14}C-L-LEUCINE[a]

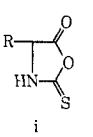
Product	% At PH 8.5	% At pH 9.2	% At pH 10.0
Gly-Phe-Leu	78.9	76.3	50.0
Hydantoic acid[b]	5.95	5.55	5.15
Phe-Leu	12.28	11.64	33.7
Gly-Gly-Phe-Leu	3.03	6.33	8.47
(Gly)$_3$Phe-Leu	0.07	0.34	2.14

[a] The reaction was carried out with a 5% deficiency of Gly NTA. [b] HSOCCH$_2$NHCO-Phe-Leu·OH.

(18) S. R. Jenkins, R. F. Nutt, R. S. Dewey, D. F. Veber, F. W. Holly, W. J. Paleveda, Jr., T. Lanza, Jr., R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, M. J. Dickinson, J. Sondey, R. Hirschmann, and E. Walton, *J. Amer. Chem. Soc.*, **91**, 505 (1969).

(19) Possibly the hydantoic acid is formed by direct attack of the nucleophile on the carbamate carbonyl. Alternatively, the hydantoic acid could be formed *via* the isocyanate if the ring opening were catalyzed by the solvent.

(20) The late Professor Weygand had kindly offered the interesting suggestion that the racemization might be attributed to the presence of a 2-*thiono*-5-oxazolone, i, as an isomeric impurity in the NTA. Although we have no reference sample, two considerations argue against the presence of i in our cyclic anhydrides. It should be detectable by nmr or uv spectroscopy.



i

For example, a marked difference in the anisotropic magnetic field around the C=S bond compared to that around the C=O bond has been reported for thioamides and amides [H. Paulsen and K. Todt, *Angew. Chem. Int. Ed. Engl.*, **5**, 899 (1966)]. Histidine NTA shows only the expected peaks although it gives rise to 10% of the D epimer on reaction. Further, whereas the uv of glycine NTA shows only end absorbtion, that of glycine thionourethan, which would contain the major chromophore of a 2-thiono-5-oxazolone system, shows $\lambda_{max}^{0.01\ N\ HCl}$ 240 mμ (ε 12,000).

(21) B. Halpern, D. E. Nitecki, and B. Weinstein, *Tetrahedron Lett.*, 3075 (1967).

identification of the peaks could be determined by comparison of the positions of the methyl doublets of the diastereomers in the two preparations.

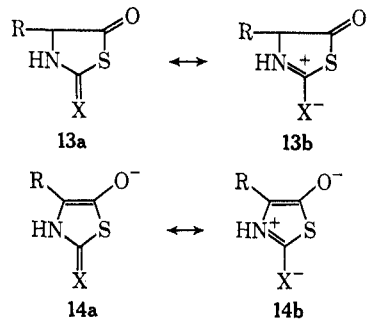
In contrast to the racemization found in the above crude tripeptide, that in the *purified* form of the C-terminal hexapeptide of ribonuclease prepared using histidine NTA was considerably lower. This hexapeptide was also prepared using α-*tert*-butyloxycarbonyl histidine azide. In both cases these intermediates were converted to the N-terminal heptapeptide at which point they were purified by chromatography on silica gel. Enzymatic hydrolysis of the purified heptapeptide with aminopeptidase M showed only about 2% residual peptide and similar amino acid analyses in either case. The fact that the above histidine containing heptapeptide appears to be of good optical purity may be due to purification effected in its isolation or it may indicate that polypeptides react appreciably faster with the L than with a D anhydride. A greater reactivity of L-amino acid NCA's with amino acids or peptides of like configuration has been observed previously.[22,23]

Far less racemization was observed with alanine NTA. Reaction of L-alanine NTA with L-phenylalanine led to a crude dipeptide which by nmr analysis contained 1.4% of the DL isomer. In this case, the peak areas of the alanine methyl doublet of the low intensity epimeric product were compared with the methyl doublets in the ^{13}C satellites of the major product. The satellite peaks provided an internal standard which was directly available for the comparison of the low intensity peaks.[24]

Reaction of L-arginine NTA hydrobromide with L-phenylalanine led to diastereomeric dipeptides which could be separated by chromatography on silica gel. The amounts of the fractionated products were then determined by means of their ultraviolet absorption. The ratio of L-Arg-L-Phe to D-Arg-L-Phe was 95:5. A similar experiment with D-phenylalanine led to a ratio of L-Arg-D-Phe to D-Arg-D-Phe of 84:16. Again these results suggest a preferred reaction between amino acids and NTA's of like configuration.

To estimate the extent of racemization occurring during the peptide forming reaction only, our previously described[25] hydrogen isotope exchange method was employed. The reaction of L-proline NTA with L-phenylalanine was carried out in tritiated water, and a sample of the dipeptide was examined for uptake of "permanently bound" tritium. A reaction carried out at pH 9.35 gave 0.114% of one tritiated hydrogen in the dipeptide and a reaction at pH 10.0 gave 0.129%. A similar reaction carried out in D_2O gave a dipeptide with 0.495% excess of one deuterium in the dipeptide indicating a H/T isotope effect of 4.6. Since it is possible that hydrogen exchange could occur in part with retention, the figures represent the maximum racemization that occurred during the condensation step. Proline NTA appeared to give rise to the lowest level of racemization of the NTA's studied. A sample of L-proline NTA (Table II) which had shown 2.9% of D-proline after acid hydrolysis, therefore, on the basis of the above isotope exchange experiment, might be expected to yield a maximum of 3.2% of a D-prolyl peptide.

Some explanation for the difference between the levels of racemization of the NTA's and NCA's is in order. Tyrosine NCA has been reported to show less than 0.004% racemization when the condensation was carried out in aqueous solution at pH 10.[25] At the other extreme, the tyrosine cyclic anhydride containing two sulfur atoms, the 2-thiono-5-thiazolidine-2,5-dione **2** (R = p-$HOC_6H_4CH_2$), was completely racemized in a reaction with glycine.[4] In this case the high level of racemization could be attributed to an expected greater



double bond character of structure **13b** where X = S than for X = O.[26-28] Systems having such increased urethan C–N double bond character would show a greater tendency to racemize at the 4 position giving rise to the hydroxythiazole anion ring system **14** (X = S). In the NTA's no such C–S double bond can occur. However, two reasons can be offered for the relative ease of enolization in the thiazolidine system of the NTA's *vs.* the oxazolidine system of the NCA's. It has been postulated that the larger delocalization energy of thiophene relative to furan can be attributed to sulfur d-orbital participation and to differences in oxygen and sulfur electronegativities.[26] Furthermore, some decrease in bond angle strain of sp^2 carbon in a five-membered ring could be attained by the change of the heteroatom from oxygen to sulfur.[26] In the present case the thiazole system should similarly be favored over the oxazole analog by sulfur d-orbital participation and by the possibility of formation of a slightly less strained anion. That the observed racemization in the NTA's cannot be attributed to bond angle strain alone is indicated by the fact that N-protected amino acid thio esters exhibit racemization in peptide synthesis[29] and in fact the blocked amino acid esters of thiophenol show higher rates of racemization in the presence of triethylamine than do the corresponding esters of the more acidic p-nitrophenol.[30]

In view of these studies, the usefulness of the NTA's in controlled peptide synthesis is restricted to the NTA of glycine, of alanine, which affords products of good optical purity, and of histidine. In addition, the method

(22) P. D. Bartlett and R. H. Jones, *J. Amer. Chem. Soc.*, **79**, 2153 (1957).

(23) M. Idelson and E. R. Blout, *ibid.*, **80**, 2387 (1958).

(24) The use of the ^{13}C satellites as an internal standard has been employed to relate aromatic hydrocarbons of greatly different concentrations: F. F. Caserio, *Anal. Chem.*, **38**, 1802 (1966).

(25) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschmann, *J. Amer. Chem. Soc.*, **88**, 3163 (1966).

(26) E. Kooyman in "Organosulfur Chemistry," M. J. Janssen, Ed., Interscience, New York, N. Y., 1967, Chapter 1.

(27) This sulfur probably exists in the thiono form: A. R. Katritsky and J. M. Lagowski, *Advan. Heterocycl. Chem.*, **2**, 61 (1964).

(28) Hindered rotation in simple thionocarbamate esters has been observed by R. A. Bauman, *J. Org. Chem.*, **32**, 4129 (1967).

(29) In peptide synthesis varying levels of racemization have been seen with thiol esters: H. Determann and T. Wieland, *Justus Liebigs Ann Chem.*, **670**, 136 (1963); F. Weygand, A. Prox, and W. König, *Chem. Ber.*, **99**, 1451 (1966).

(30) B. Liberek and Z. Grzonka, *Tetrahedron Lett.*, 159 (1964).

should prove useful in situations when the purification of a desired diastereoisomer is readily accomplished or when optical purity is relatively unimportant as in the preparation of a reference compound in connection with sequence studies.[31]

Experimental Section

Methoxythiocarbonyl-L-alanine (Alanine Thionourethan), 3 (R, R′ = Me).—A solution of 71.5 g (0.80 mol) of L-alanine in 69 ml (0.80 mol) of a 45% solution of aqueous potassium hydroxide was stirred under nitrogen at 25° while 97.5 g (0.80 mol) of *O,S*-dimethyl dithiocarbonate (dimethyl xanthate)[32,33] in 90 ml of methanol was added. The mixture was held at 45° while nitrogen was passed through the mixture to remove methyl mercaptan. The exit gas was passed through a scrubber containing potassium hydroxide in aqueous ethanol until after 1 hr the exit gas gave only a weak test for mercaptan (yellow precipitate with aqueous lead acetate). The reaction mixture was concentrated and the syrup was taken up in water and extracted with ether. The aqueous layer was acidified with 6 *N* HCl and extracted with ethyl acetate. The extract was washed with saturated aqueous NaCl, dried with Na_2SO_4, and concentrated to an oil which solidified to give 118 g of the urethan. Recrystallization from ethyl acetate and hexane afforded 74 g (56.7%) of methoxythiocarbonyl-L-alanine: ir (CH_2Cl_2) 3534 (NH), 1724 (-CO_2H), 1528 cm^{-1} (NH); mp 114–115° (Table I).

Alkoxythiocarbonyl derivatives of other amino acids were prepared in a similar manner (Table I). The ethoxythiocarbonyl derivatives were prepared using *O,S*-diethyl dithiocarbonate (Eastman Organic Chemicals).

Optical Purity of Ethoxythiocarbonyl-L-leucine 3 (R = *i*-Bu; R′ = Et).—The salt prepared from 2.19 g (10 mmol) of L-ethoxythiocarbonyl-L-leucine, $[\alpha]^{25}_{589}$ −27.9° (*c* 1, CH_2Cl_2), and 3.24 g (10 mmol) of quinine was fractionally crystallized from benzene–hexane to yield three crops: A, 1.76 g; B, 1.475 g; C, 0.925 g. The three fractions were individually dissolved in ethyl acetate and washed with dilute hydrochloric acid. Concentration of the ethyl acetate extracts led to crystalline residues with the following properties.

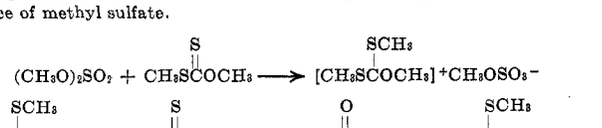
Sample	Mp, °C	$[\alpha]^{25}_{589}$ (*c* 1, CH_2Cl_2)
Starting material	79–81	−27.9 ± 0.4°
A	79–80	−27.5
B	79–80	−28.2
C	79–80	−28.3

Stability of Ethoxythiocarbonyl-L-phenylalanine, 3 (R = $C_6H_5CH_2$; R′ = Et), in Alkali.—A solution of 5.0 g of ethoxythiocarbonyl-L-phenylalanine, mp 85–88°, $[\alpha]^{25}_{589}$ +80.8° (*c* 1, CH_2Cl_2), in 20 ml of ethanol and 10 ml of water was brought to pH 10 with a 50% solution of NaOH and heated under reflux in a nitrogen atmosphere for 18 hr. The solution was concentrated *in vacuo* and diluted with 25 ml of water, acidified, and extracted twice with ethyl acetate. The combined extract was washed with saturated NaCl, dried, and concentrated to an oil which crystallized. Trituration with hexane gave 4.16 g (83%) of ethoxythiocarbonyl-L-phenylalanine, mp 82–85°, $[\alpha]^{25}_{589}$ +77.8° (*c* 1, CH_2Cl_2). Recrystallization from benzene–hexane gave the thionourethan, $[\alpha]^{25}_{589}$ +81.3° (*c* 1, CH_2Cl_2).

(31) T. E. Beesley, R. E. Harman, T. A. Jacob, C. F. Homnick, R. A. Vitali, D. F. Veber, F. J. Wolf, R. Hirschmann, and R. G. Denkewalter, *J. Amer. Chem. Soc.*, **90**, 3255 (1968).

(32) M. Delepine, *Bull. Soc. Chim. Fr.*, **7**[4], 404 (1910).

(33) *O,S*-Dimethyl dithiocarbonate prepared from dimethyl sulfate and potassium *O*-methyl dithiocarbonate occasionally developed a band in its ir spectrum at 5.90 μ. This decomposition could be avoided if the xanthate ester were stirred with a small amount of triethylamine for 1 hr. The decomposition was attributed to the following rearrangement catalyzed by a trace of methyl sulfate.



A similar observation was made for thioglycolic acid by E. Bülmann, *Justus Liebigs Ann. Chem.*, **364**, 314 (1909).

Preparation of Ethoxythiocarbonyl-L-proline in Tritiated Aqueous Ethanol.—A solution of 5.75 g of L-proline was prepared in 5 ml of tritiated water containing 3.3 g of 85% potassium hydroxide. To this was added 7.5 g of *O,S*-diethyl dithiocarbamate along with 5 ml of ethanol. The mixture was stirred for 2 hr at 65–75° and overnight at room temperature. The mixture was concentrated to dryness *in vacuo* and reconcentrated four times with H_2O (in order to exchange labile hydrogen). This residue was taken up in 60 ml of water, extracted three times with ether, acidified with HCl, and extracted three times with ethyl acetate. The ethyl acetate extract was washed three times with saturated aqueous NaCl, dried ($MgSO_4$), and concentrated to an oil, which was triturated to give 7.4 g of solid. This material was recrystallized from ethyl acetate and hexane to give 5.2 g of thionourethan. The specific activity of the tritiated solvent after dilution by exchangeable hydrogen was 7.55×10^6 cpm/mg-atom H. The specific activity of hydrogen in the product corresponded to 2.44×10^2 cpm/mmol of thionourethan.

Preparation of L-Alanine NTA, 4 (R = Me).—A solution of 26.12 g (0.16 mol) of methoxythiocarbonyl-L-alanine and 10.92 g (0.16 mol) of imidazole in 200 ml of THF was stirred under nitrogen. Phosphorus tribromide (18.2 ml, 0.19 mol) from a freshly opened bottle was added over 2–3 min while the reaction temperature was held at 25–35°. Initially, a strong exothermic reaction occurred and a thick precipitate formed which made temperature control difficult. The mixture thinned considerably as the last two thirds of the phosphorus tribromide was added. The reaction mixture was then poured into an ice cold mixture of 800 ml of a saturated solution of $NaHCO_3$ and 800 ml of ethyl acetate. The organic layer was washed successively with 1 *N* hydrochloric acid, 10% $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4, and concentrated to an oil. The oil was crystallized from ethyl acetate hexane to give 9.85 g (47%) of L-alanine NTA: mp 91–93° (Table II); ir (CH_2Cl_2) 3559 (NH), 1758, 1718 cm^{-1}. A number of the other thiocarboxyanhydrides were similarly prepared (Table II).

L-Histidine NTA Hydrobromide, 4 (R = $C_3H_3N_2CH_2 \cdot HBr$).—Ethoxythiocarbonylhistidine was prepared by the procedure used for the alanine derivative. A slurry of 10 g (0.041 mol) of ethoxythiocarbonyl-L-histidine in 250 ml of THF was stirred at room temperature while a freshly prepared ice cold solution of 5 ml (0.052 mol) of phosphorus tribromide in 50 ml of THF was added rapidly. The ethoxythiocarbonylhistidine dissolved and a precipitate separated. After an additional 3 min this was collected and washed with ether in a drybox. Some residual material which remained on the flask walls was stirred with 3 ml of phosphorus tribromide in 300 ml of THF, and the resulting precipitate brought the yield of crude NTA to theory (15 g). The NTA was dissolved in 125 ml of methyl carbitol and 375 ml of ethyl acetate was added to give 10.5 g (72% recovery) of histidine NTA hydrobromide, $[\alpha]^{25}_{589}$ −7.0° (*c* 2, methyl carbitol).

Histidine NTA By-product 11.—The NTA of histidine hydrobromide (2.80 g) was added over 2 min at 0° to 100 ml of a 0.2 *M* solution of potassium borate at pH 10.2 with magnetic stirring. Stirring was continued for 10 min more after the addition was complete and the mixture was brought to pH 4 with sulfuric acid at 0°. The product (1.6 g) was removed by filtration and washed with water. An aliquot was crystallized for analysis by purification *via* the sodium salt, ir (methyl carbitol) 1705 cm^{-1} (NCONH).

Anal. Calcd for $C_7H_7O_2N_3S$: C, 42.64; H, 3.58; N, 21.32; S, 16.26. Found: C, 42.43; H, 3.34; N, 21.46; S, 16.82.

L-Arginine NTA Hydrobromide, 4 [R = $(CH_2)_3NHC(NH_2)_2^+$].—Methoxythiocarbonyl-L-arginine was prepared from the free base by the usual procedure and the crude concentrate from this reaction could be crystallized from water. A suspension of 10 g of methoxythiocarbonyl L-arginine in 240 ml of THF was stirred and a cold solution of 15 ml of phosphorus tribromide in 35 ml of THF was added rapidly. An oil separated which crystallized and was collected after 3 hr. Recrystallization from 2-propanol and ether led to 7.5 g of a hygroscopic white solid, $[\alpha]^{25}_{589}$ −18.7° (*c* 1, CH_2Cl_2), mp 115–117°.

L-Leucine NTA, 4 [R = $(CH_3)_2CHCH_2$], *via* the Methyl Enol Ether, Isobutyl-2-methoxy-5-thiazolone.—A solution of 4.11 g (0.020 mol) of the methoxythiocarbonyl-L-leucine in 15 ml of benzene was stirred with 1.9 ml (0.020 mol) of acetic anhydride for 5 hr at room temperature. The solution was diluted with 20 ml of ethyl acetate and washed with 20 ml of water, twice with 20-ml portions of 5% $NaHCO_3$, and with 10 ml of saturated aqueous NaCl. The organic phase was dried over Na_2SO_4 and con-

centrated to give 0.42 g of an oil, ir (CH_2Cl_2) 1730 (s, C=O), 1631 cm^{-1} (N=C). Tlc revealed one component as detected by iodine vapor with an R_f 0.46 (benzene), whereas leucine NTA showed an R_f of 0.09 and methoxythiocarbonyl-L-leucine remained at the origin.

The enol ether (0.16 g, 0.855 mmol) prepared above was allowed to react with 1.0 ml of 1.7 *N* HCl in THF at room temperature for 20 min. The solution was then diluted with 5 ml of ethyl acetate and washed successively with water, aqueous $NaHCO_3$, and saturated NaCl. From the organic layer was obtained 0.067 g (45% yield) of leucine NTA, mp 79.5–81° (ethyl acetate–hexane), $[\alpha]^{25}_{589}$ −27° (*c* 1, CH_2Cl_2). The ir spectrum of this product was identical with that of a specimen prepared from methoxythiocarbonyl-L-leucine with phosphorus tribromide.

L-Leucine NTA. Direct Preparation from *N*-Methoxythiocarbonyl-L-leucine.—A solution of 8.12 g (39.6 mmol) of *N*-methoxythiocarbonyl-L-leucine and 2.72 g (40 mmol) of imidazole in 32 ml of THF was treated with 4.56 ml (48 mmol) of phosphorus tribromide with ice bath cooling such as to keep the reaction temperature below 40°. A precipitate formed, and after about 10-sec reaction time the product was quenched into an ice-cold stirred mixture of 200 ml of ethyl acetate and 200 ml of 10% aqueous $NaHCO_3$. The organic layer was washed with cold 1 *N* HCl, 5% aqueous $NaHCO_3$, and with saturated aqueous NaCl. The organic layer was dried and concentrated to yield 6.45 g (93.4%) of a colorless oil which rapidly crystallized. This product showed a strong spot for leucine NTA (R_f 0.25) and a smaller spot (R_f 0.80) corresponding to the 2-methoxythiazolone upon tlc on silica gel in chloroform–methanol (9:1). Recrystallization from 7 ml of ethyl acetate and 80 ml of hexane gave 4.67 g (68%) of L-leucine NTA, mp 77–78°, $[\alpha]^{25}_{589}$ −57.2° (*c* 1.035, CH_2Cl_2).

When a similar reaction was carried out at −30° for 0.5 hr, an oil was obtained which showed the thiazolone and the NTA by tlc. The product mixture was chromatographed on silica gel in benzene to give 230 mg of an oil which corresponded in its R_f to that of the thiazolone. A second fraction (160 mg) was obtained which when rechromatographed gave 22 mg of leucine NTA $[\alpha]^{25}_{589}$ −34.5 (*c* 0.345, CH_2Cl_2), mp 73.5–75°. Another reaction was performed at 4–10° for 20 min, and gave the crude NTA in 85% yield, $[\alpha]^{25}_{589}$ −52.4 (*c* 1, CH_2Cl_2). Repeated recrystallizations from ethyl acetate–hexane gave successive rotations of −54.0 and −52.7° (*c* 1, CH_2Cl_2). A similar difficulty in obtaining optically pure NTA by recrystallization was observed in other experiments.

Leucine NTA [1.73 g, 10 mmol, $[\alpha]^{25}_{589}$ −57.4° (*c* 1, CH_2Cl_2)] was added to a solution of silver nitrate (5.1 g, 30 mmol) in 10 ml of water and 10 ml of dioxane. The mixture was stirred overnight at room temperature and filtered to remove the precipitate of silver sulfide. The filtrate was adjusted to pH 6 with triethylamine and concentrated to a semisolid. The residue was triturated with ethanol and the resulting white crystals (0.63 g, 4.8 mmol) were collected after 1.5 hr. The filtrate was made alkaline with NaOH and concentrated to remove triethylamine.

A portion of the above crystalline leucine (131 mg, 1.00 mmol) was treated with 210 mg (1.10 mmol) of L-phenylalanine NCA under the usual conditions for controlled peptide synthesis.[1] The product was examined by tlc on silica gel in butyl alcohol–acetic acid–water (10:1:3) for the presence of L-phenylalanyl-D-leucine in addition to the major product, L-phenylalanyl-L-leucine. No LD-dipeptide (<1%) was detected. Comparison standards were made from the dipeptide obtained from the reaction of L-phenylalanine NCA with DL-leucine to give the LL-dipeptide (R_f 0.62) and the LD-dipeptide (R_f 0.46).

A similar experiment was carried out on the above filtrate from the hydrolysis. One half of the filtrate was made up to 10 ml. Comparison by tlc with standard solutions showed a concentration of leucine of 0.037 ± 0.012 *M*. Reaction with phenylalanine NCA led to a dipeptide mixture showing 15% L-phenylalanyl-D-leucine in the mother liquors. This amount would correspond to 3 ± 1% D-leucine of the leucine in the hydrolysate.

Preparation of Amino Acid Thiocarbamates, Dipotassium Phenylalanine Thiocarbamate (9, R = $C_6H_5CH_2$).—To a suspension of 33 g of L-phenylalanine in 50 ml of methanol was added 50 ml of a 4 *N* solution of methanolic potassium hydroxide. The resulting solution was cooled to 0° and 15 ml of carbonyl sulfide (The Matheson Co., Rutherford, N. J.) which had been condensed at −80°, was distilled into the solution while a second portion (53 ml) of methanolic potassium hydroxide was added. After 1 hr, the solution was concentrated *in vacuo* to give a syrup, which was taken up in anhydrous ethanol and concentrated to dryness *in vacuo*. This operation was repeated to remove traces of water, which inhibited crystallization. The residue was then taken up in 150 ml of methanol and filtered and 200 ml of 2-propanol was added slowly to give 59.4 g (48.6%) of a white granular precipitate. Electrophoresis at pH 11 in 0.1 *N* phosphate buffer on S & S 598 paper at 600 V showed a strong spot (ninhydrin) migrating toward the anode with about twice the mobility of phenylalanine as well as a weak spot corresponding to phenylalanine itself. A sample was recrystallized for analysis from hot ethanol with 2-propanol added.

Anal. Calcd for $C_{10}H_9K_2NO_3S$: C, 39.85; H, 3.01; K, 25.94; N, 4.65; S, 10.60. Found: C, 39.08; H, 3.68; K, 23.46; N, 4.22; S, 9.12.

The electrophoresis of the disodium salt of glycine thiocarbamate which was similarly prepared was carried out as above and showed a single spot by ninhydrin at 17.2 cm while glycine and disodium glycine carbamate showed spots at 6.8 cm.

L-Leucine NTA [4, R = $(CH_3)_2CHCH_2$–] *via* Leucine Thiocarbamate. Method B.—Dipotassium leucine thiocarbamate (5.34 g, prepared in analogy with phenylalanine thiocarbamate), 40 ml of THF, and 4.4 g of phosphorus pentachloride were stirred at 0° under nitrogen to give a translucent gel. After 5 min hydrogen sulfide was bubbled through the mixture while the temperature was raised to 25°. After 1 hr, 25 ml of ethyl acetate and 25 ml of water were added to the opaque mixture. The organic layer was washed with water and sodium bicarbonate, dried over $MgSO_4$, and concentrated to give 2.04 g of a syrup. Silica gel chromatography led to 0.45 g (13%) of crystalline leucine NTA (see Table II).

L-Leucine NTA *via* Thioleucine Thiocarbamate. Method C.—A solution of 20 ml of 2,6-lutidine in 200 ml of THF was saturated with hydrogen sulfide at −10°, and leucine NCA (7.9 g) was added with stirring. A heavy precipitate developed after 3 hr. The solid was collected, washed with ethanol and ether, and then dried *in vacuo* to give 6.63 g (90%) of thioleucine. A sample was recrystallized from water.

Anal. Calcd for $C_6H_{13}NOS$: C, 48.95; H, 8.90; N, 9.52; S, 21.78. Found: C, 49.10; H, 9.15; N, 9.55; S, 22.24.

A solution of 0.43 g (2.9 mmol) of the above thioleucine in 5 ml of water was stirred at 0–5°, and a slight excess of carbonyl sulfide was passed into the solution while 2.5 ml of a 2.5 *N* sodium hydroxide solution was added dropwise so as to maintain the pH at 10. The solution was stirred 2 hr at ambient temperature, and the resulting crude thiocarbamate solution was used directly in the following step.

The solution (pH 8.8) was cooled to 0°, and 5 ml of ethyl acetate was added. Then 0.76 g (3.0 mmol) of *N*-ethyl-5-phenylisoxazolium-3-sulfonate (Woodward's reagent K) was added with rapid stirring. The mixture showed a pH of 8. After 10 min the organic layer was separated, washed with water, and dried to give an oil which crystallized upon the addition of hexane to give 0.23 g (45%) of leucine NTA, $[\alpha]^{25}_{589}$ −56.7° (*c* 0.96, CH_2Cl_2) (Table II).

Dipotassium thioleucine thiocarbamate was also prepared and isolated from alcohol in a manner similar to the preparation of phenylalanine thiocarbamate. Upon electrophoresis at pH 11 the product showed a single fast moving spot relative to thioleucine. However, when the reaction with Woodward's K was carried out upon isolated thioleucine thiocarbamate, only a 29% yield of NTA was obtained, $[\alpha]^{25}_{589}$ −28.9° (*c* 1, CH_2Cl_2).

A reaction of the thiocarbamate with phosphorus pentachloride in THF gave a 23% yield of NTA, $[\alpha]^{25}_{589}$ −1.9° (*c* 1, CH_2Cl_2).

Leucine NTA *via* Leucine Amide. A. From Leucine Amide.—A solution of 2.6 g (20 mmol) of L-leucine amide and 2.17 ml (23 mmol) of *O*,*S*-dimethyl dithiocarbonate in methanol (5 ml) was stirred under nitrogen at room temperature for 3.5 hr and then heated to 50° for 0.5 hr. Evolution of methyl mercaptan as detected by a yellow precipitate with lead acetate had virtually ceased. The reaction mixture was taken up in ethyl acetate (10 ml), washed with 10% NaCl solution, dried over Na_2SO_4, and concentrated to a tacky noncrystalline residue of the thionourethan amide, ir (CH_2Cl_2) 1689 cm^{-1} (amide C=O).

Hydrogen chloride was bubbled through a solution of crude thiourethan derived from leucine amide (0.45 g) in nitromethane (5 ml) for 1 hr at room temperature. After 2 hr the mixture was partitioned between ethyl acetate (5 ml) and water (5 ml). The organic layer was separated, washed with water (5 ml), dried over sodium sulfate, and concentrated. The residue was crystal-

lized from cyclohexane to give 0.17 g of leucine NTA as colorless needles, mp 74–75°, $[\alpha]^{25}_{589}$ −35.4° (*c* 1.06, CH_2Cl_2). Its infrared spectrum was identical with that of leucine NTA prepared *via* the thionourethan.

B. From Methoxythiocarbonylleucine Amide.—In an alternate route, 2.05 g (10 mmol) of L-methoxythiocarbonylleucine was dissolved in ether (15 ml) and ammonia was bubbled through the solution for a few minutes. An oil separated. The mixture was concentrated *in vacuo*. Acetonitrile (10 ml) and 2.06 g (10 mmol) of dicyclohexylcarbodiimide were added, and the mixture was stirred overnight at room temperature. The mixture was concentrated and the residue was extracted with three 10 ml portions of ether. The ether extract was filtered and concentrated to give an oil, 2.08 g.

The above thiourethan (1.0 g) was treated with HCl to give 0.29 g of leucine NTA, mp 81–82°, $[\alpha]^{25}_{589}$ 0.0° (*c* 1.02, CH_2Cl_2). Its infrared spectrum was identical with that of leucine NTA prepared as described above.

Phenylalanine NTA (4, R = $PhCH_2$–) from Thiophenylalanine and Phosgene. Method D.—A suspension of thiophenylalanine (5.44 g, 0.030 mol) in dioxane (125 ml) was stirred at room temperature while phosgene (0.031 mol) was introduced below the surface of the slurry to yield a clear solution after 15 min. After 1 hr the solution was concentrated, and ethyl acetate (75 ml) and hexane (150 ml) were added to give a solution which was decanted from a small amount of oil and concentrated. The solid residue was crystallized from ethyl acetate (17 ml) and hexane (17 ml) to give 0.50 g (81%) of the NTA, $[\alpha]^{25}_{589}$ −155° (*c* 1, CH_2Cl_2). Addition of further hexane (20 ml) gave a further 1.36 g, $[\alpha]^{25}_{589}$ −154° (*c* 1, CH_2Cl_2).

Peptide Syntheses Using NTA's.—In general the reactions were carried out in a Waring Blendor as previously described for the reaction of NCA's,[1] but with the following modifications. The amino acid or peptide was dissolved in 0.45 *M* boric acid, and the solution was adjusted to pH 9.5 in the case of amino acids and to pH 9.0 for peptides. In some of the earlier runs, standard glass equipment was used. The powdered NTA was added over a 15–60-sec period at 0° while concentrated aqueous potassium or sodium hydroxide was added subsurface in order to maintain the initial pH. At the end of the reaction, which was ascertained by the cessation of a significant uptake of base (generally 5–20 min), the pH was lowered to 3–5, and the mixture was swept with nitrogen to remove carbonyl sulfide.

Glycyl-L-phenylalanine.—An aqueous solution of 0.181 g (1.1 mmol) of L-phenylalanine was treated with 0.131 g (1.5% excess) of glycine NTA in a blender while the pH was maintained with saturated barium hydroxide. The solution was neutralized with sulfuric acid, and the precipitate was filtered. An aliquot of the filtrate was placed directly on a Beckman amino acid analyzer and the peaks were compared with those of glycylphenylalanine and of phenylalanine. The intensity of the peaks indicated Gly-Phe, 92.5%, Phe, 2.25%, and a third peak presumed to be Gly-Gly-Phe, about 2%. Residual phenylalanine was thought to have been converted to the hydantoic acid by-product. The solution was concentrated and the residue was crystallized from aqueous ethanol to give 1.49 g (74.5%) of dipeptide.

Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.46; H, 6.35; N, 12.61. Found: C, 59.08; H, 6.60; N, 12.54.

A second crop of 0.23 g (11.5%) was obtained which showed a second but weak spot by tlc corresponding to a trace of Gly-Gly-Phe.

Glycyl-L-phenylalanyl-L-leucine. A. *Via* **Glycine NTA.**—In a three-necked flask equipped with a paddle stirrer, combination pH electrode, and a nitrogen inlet was placed 10 ml of 0.45 *M* boric acid and 0.556 g (1.9 mmol based on 95% peptide content by amino acid analysis) of L-Phe-L-Leu. The solution was cooled to 4° and adjusted to pH 9.5 with 45% potassium hydroxide. The solution was stirred vigorously while 0.246 g (2.1 mmol) of glycine NTA was added. The pH was held at 9.5 with potassium hydroxide. After the reaction was complete (about 5 min), the mixture was brought to room temperature and filtered. The filtrate showed a disappearance yield[1] by tlc on silica gel of 90–95% in butyl alcohol–acetic acid–water (10:1:3). The solution was brought to pH 5.5 with 50% H_2SO_4 and the resulting precipitate was collected to give 0.430 g (64%) of the tripeptide, R_f 0.45 (10:1:3), $[\alpha]^{25}_{589}$ −12.5° (*c* 1.04, HOAc). An amino acid analysis showed a ratio of $Gly_{1.00}Phe_{0.99}Leu_{1.00}$.

Anal. Calcd for $C_{17}H_{25}N_3O_4$: C, 60.88; H, 7.51; N, 12.53; neut equiv, 335. Found: C, 60.95; H, 7.40; N, 12.76; neut equiv, 323.

B. *Via* **Glycine NCA.**—A solution of 1.112 g (4 mmol) of L-phenylalanyl-L-leucine was treated with 0.424 g (4.2 mmol) of glycine NCA in a blender under the usual conditions. The crude product showed a disappearance yield of about 85%. Acidification of the solution led to precipitation of 0.450 g (35.4%) of the tripeptide, which corresponded in R_f to that prepared above.

C. *Via* **Glycine NTA (Variation of By-products with pH).**—A series of reactions were carried out similar to the above but at various pH levels. Visual comparison of the spot intensities of the products by tlc (10:1:3) indicated the following by-products along with glycine and the tripeptide. The sample of the over-reaction product, Gly-Gly-Phe-Leu was prepared by a reaction of glycine NTA with the above tripeptide. A solution of this product was then diluted to the appropriate concentration for comparisons.

pH	Phe-Leu, %	Gly-Gly-Phe-Leu, %
8.5	<2	>10, <20
9.0	2	<10
9.5	>5, <10	>10
10.0	>10, <20	>10, <20

D. Glycyl-L-phenylalanyl-^{14}C-L-leucine.—Glycine NTA (0.95 equiv) was added to a stirred solution of L-phenylalanyl-^{14}C-L-leucine at a concentration of 1 mmol in 5 ml of 0.4 *N* borate buffer at 0–2°. The reaction was carried out at pH 8.5, 9.2, and 10.0. The product was examined by tlc on silica gel in the butyl alcohol–acetic acid–water system (10:1:3). The plates were scanned directly and the spots on the plates were extracted and their radioactivity was redetermined by means of a scintillation counter, by a previously outlined procedure.[1] The assays determined by the use of the scintillation counter are summarized in Table V.

L-Alanyl-L-phenylalanyl-L-leucine. A. *Via* **Alanine NTA.**—In the apparatus described for the preparation of glycylphenylalanylleucine, a mixture of 0.556 g (2.0 mmol) of L-phenylalanyl-L-leucine and 10 ml of 0.45 *M* aqueous boric acid was chilled to 4° and adjusted to pH 9.05 by addition of 0.106 ml of 50% aqueous sodium hydroxide. L-Alanine NTA (0.276 g, 2.1 mmol) was added in one portion with vigorous stirring along with addition of 0.294 ml of 50% aqueous sodium hydroxide as required to maintain the pH in the range 9.05 ± 0.05. Thin layer chromatography of the reaction mixture showed a "disappearance yield"[1] of 98%. Acidification to pH 5.1 with 50% aqueous sulfuric acid yielded a solid product (0.510 g). This was dissolved in 5 ml of water by addition of 2.5 *N* sodium hydroxide and then acidified with acetic acid. The crystalline precipitate was collected, washed with water, and dried to give 0.420 g (63.4%) of L-alanyl-L-phenylalanyl-L-leucine which moved as a single spot component upon tlc (R_f 0.54, 10:1:3), amino acid analysis, $Ala_{0.98}Phe_{0.97}Leu_{1.00}$.

Anal. Calcd for $C_{18}H_{27}N_3O_4$: C, 61.87; H, 7.79; N, 12.03; neut equiv, 349. Found: C, 62.14; H, 7.70; N, 12.29; neut equiv, 347.5, pK_2 = 7.4.

The optical purity of the crude tripeptide was examined in another experiment carried out essentially as described above; tlc analysis again showed a 98% disappearance yield. A sample of the filtrate prior to acidification (0.04 ml, corresponding to 8 μmol) in 0.1 ml of a solution of 1.0 mg of leucine aminopeptidase in 0.5 ml of tris buffer was held at 37° for 18 hr. Comparison with standard solutions showed that 1% of the tripeptide in the sample remained unhydrolyzed. A sample of D-alanyl-L-phenylalanyl-L-leucine (prepared by reaction of D-alanine NCA with the dipeptide) showed little or no hydrolysis with the enzyme under the same conditions.

B. *Via* **Alanine NCA.**—Reaction of a 10% excess of L-alanine NCA with L-phenylalanyl-L-leucine at pH 10.2 under the usual conditions[1] gave 52% of a product which upon reprecipitation as above led to a 36% yield of a single spot tripeptide with an amino acid analysis of $Ala_{1.00}Phe_{1.00}Leu_{0.98}$: equiv wt, found 338; pK_2 = 7.3.

Comparison of L-Alanine NCA and NTA in a Reaction with *O*-Benzyl-L-seryl-L-valine. Formation of L-Alanyl-*O*-benzyl-L-seryl-L-valine.—Reaction of *O*-benzylserine NCA with a 0.2 *M* solution of valine in 0.45 *M* borate buffer led to a solution of crude *O*-benzyl-L-seryl-L-valine which was used directly for the following experiments. In two other runs the disappearance yields of the dipeptide were 88 and 95%.

A. With L-Alanine NCA.—A 20-ml aliquot (3.7 mmol) of the above solution of crude *O*-benzyl-L-seryl-L-valine was treated with 459 mg (3.99 mmol) of alanine NCA at pH 10.1. The resulting solution showed a 90% disappearance yield by tlc (10:1:3). The solution was filtered and acidified to pH 5.9. The precipitate was collected, washed with water, and dried to give 748 mg (55%) of crude tripeptide. Reprecipitation of this product from a sodium hydroxide solution with acetic acid gave 673 mg (49%) of the tripeptide, R_f 0.44 (10:1:3), amino acid analysis, $Ala_{0.99}Ser_{1.00}Val_{1.00}$.

Anal. Calcd for $C_{18}H_{27}N_3O_5$: C, 59.16; H, 7.45; N, 11.50; neut equiv, 365. Found: C, 58.96; H, 7.51; N, 11.39; neut equiv, 347, $pK_2 = 7.6$ (in 50% v/v aqueous methanol).

B. With L-Alanine NTA.—A similar experiment was carried out between another 20-ml aliquot of the dipeptide solution and 512 mg (3.91 mmol) of alanine NTA at pH 9.15 to give a 95% disappearance yield, and precipitation with acetic acid led to 923 mg (67.8%) of the crude tripeptide. Reprecipitation led to 830 mg (61%) of the tripeptide, R_f 0.44 (10:1:3), amino acid analysis, $Ala_{1.02}Ser_{0.99}Val_{1.00}$.

Anal. Found: C, 59.28; H, 7.73; N, 11.44; neut equiv, 354, $pK_2 = 7.6$.

Formation of L-Valyl-L-histidyl-L-phenylalanyl-L-aspartyl-L-alanyl-*O*-benzyl-L-seryl-L-valine. A. *Via* Histidine NTA.—A solution of crude pentapeptide prepared by the reaction of phenylalanine NCA on 10.1 g (20 mmol) of aspartylalanyl-*O*-benzylserylvaline was treated with an excess of histidine NTA hydrobromide (21.45 g, 77.3 mmol) in 100 ml of borate buffer at pH 9. The product showed a disappearance yield of 92% (10:1:3). The mixture was extracted with butyl alcohol at pH 3.5, and the residue (14.15 g) from the organic extract was fractionated on a dry silica gel column in 1-propanol–water (71:29) to give 2.41 g of starting pentapeptide and 3.62 g (23.7%) of the product hexapeptide, R_f 0.24 in ethyl acetate–pyridine–acetic acid–water (10:5:1:3).

The above hexapeptide (3.06 g, 4.0 mmol) was treated with 0.63 g (4.4 mmol) of valine NCA in 20 ml of 0.45 *M* borate buffer in the usual manner. The crude heptapeptide was reprecipitated from a basic solution with acetic acid. Hydrogenation in 75% acetic acid with 10% Pd–C led to the unprotected peptide,[34] amino acid analysis, $Val_{2.02}His_{0.99}Phe_{0.99}Asp_{1.01}Ala_{1.01}Ser_{1.00}$.

Anal. Calcd for $C_{35}H_{51}N_9O_{11}\cdot 4H_2O$: C, 49.70; H, 7.03; N, 14.90; equiv wt 423. Found: C, 49.37; H, 6.41; N, 14.37; equiv wt, 418.

Enzymatic cleavage of the peptide with aminopeptidase M (Rohm and Haas GmbH, Darmstadt) was carried out analogous to a reported procedure.[35] The reaction mixture was compared with standards of diluted peptide solution by tlc in butyl alcohol–acetic acid–water (10:2.5:6) and showed 2% residual peptide, amino acid analysis, $Val_{1.98}His_{0.95}Phe_{1.01}Asp_{1.04}Ala_{1.02}Ser_{1.00}$.

B. *Via tert*-Butoxycarbonyl-L-histidine Azide.—A solution of 54 mg (0.20 mmol) of *tert*-butoxycarbonyl-L-histidine hydrazide[36] in 2 ml of DMF was cooled to −30° and 0.4 ml of 2 *N* hydrogen chloride in THF was added. Isoamyl nitrite (0.026 ml, 0.20 mmol) was added and the solution was stirred at −20° for 30 min. Complete disappearance of the starting hydrazide was evident by tlc in methanol. The solution was cooled to −40° and adjusted to an apparent pH of 8 with triethylamine. A solution of 107 mg (0.20 mmol) of the free seryl pentapeptide, which had been deblocked by the above hydrogenation procedure, was added at −20° in 1.5 ml of DMF. The pH was brought to 8 and the mixture was stored at −10° for 3 days with an occasional readjustment of the pH to 8. The reaction mixture was filtered, the filtrate was concentrated *in vacuo*, and the residue was triturated with ethanol to give 79% of the crude hexapeptide.

The crude *N*-*tert*-butoxycarbonyl hexapeptide (100 mg) was suspended in ethyl acetate in an ice bath and hydrogen chloride was passed through the mixture for 10 min. The mixture was allowed to stand for 30 min and then was swept with nitrogen. The precipitate was collected to give an 86% yield of the deblocked hexapeptide. Peptide (149.2 mg) prepared in this manner was treated with a 50% excess of *tert*-butoxycarbonyl valine *N*-hydroxysuccinimide (93.6 mg) in DMF. The reaction was kept slightly alkaline by small additions of triethylamine for 2 days. The product was isolated by silica gel chromatography in 32% yield. The *tert*-butoxycarbonyl group was removed with HCl in ethyl acetate under conditions similar to the above to give the deblocked heptapeptide, amino acid analysis, $Val_{2.03}His_{0.97}Phe_{1.01}Asp_{1.03}Ala_{1.00}Ser_{0.97}$.

A sample of this heptapeptide was cleaved by aminopeptidase M according to the procedure that was used for the peptide prepared *via* histidine NTA. Residual peptide amounting to about 2% remained after incubation, amino acid analysis, $Val_{2.07}His_{0.94}Phe_{0.97}Asp_{0.98}Ala_{1.02}Ser_{1.02}$.

Histidylalanylglycine. Racemization in the Use of L-Histidine NTA Hydrobromide. A.—Reaction of L-alanyl-glycine with 2 equiv of L-histidine NTA hydrobromide ($[\alpha]^{25}_{589}$ −7.7°) at pH 9.4–9.55 at 4° led to a crude product which was examined directly by 100-MHz nmr. The alanine methyl doublet peaks of L-alanylglycine at τ 8.40 (d, J = 7.3 Hz), of L-histidyl-L-alanylglycine at 8.53 (d, J = 7.1 Hz), and of D-histidyl-L-alanylglycine, at 8.66 (d, J = 7.2 Hz) were found in the ratios of 25:70:5. The ratios of the peak areas were determined from a spectrum of C.A.T. of 27 scans in this region at 100 ml in D_2O. The peaks at τ 8.66 were attributed to the methyl doublet of D-histidyl-L-alanyl-glycine, and this position corresponded to the doublet of L-histidyl-D-alanyl-glycine below.

B. With D-Alanyl-glycine.—A similar reaction was carried out on D-alanylglycine. Here the alanine methyl doublets were in the ratios: D-alanylglycine, 42; D-histidyl-D-alanyl-glycine, 10; L-histidyl-D-alanyl-glycine, 48.

Alanylphenylalanine. Determination of the Racemization with L-Alanine NTA.—A reaction was carried out similar to the above using L-phenylalanine and a 10% excess of L-alanine NTA at pH 9.5. The freeze-dried crude product was examined by nmr in D_2O. The product showed the doublet of the alanine methyl group at τ 8.16 (J_{HH} = 7, $J_{^{13}CH}$ = 132 Hz) attributed to L-alanyl-L-phenylalanine and at τ 8.42 (J_{HH} = 7 Hz) attributed to D-alanyl-L-phenylalanine. The intensities of the alanine methyl doublets for the dipeptide and its epimer were determined from a C.A.T. of 1660 scans of this region in a 100-MHz nmr spectrum. The ratio of the upfield ^{13}CH satellite doublet[24] of the methyl doublet of the LL isomer to the central methyl doublet of the DL-isomer was 1:2.5, which would indicate a ratio of LL to DL peptide of 98.6 to 1.4.

Arginylphenylalanine. Determination of Racemization in the Reaction of L-Arginine NTA Hydrobromide with L- and D-Phenylalanine. A. With L-Phenylalanine.—A reaction of L-phenylananine with a 23% excess of arginine NTA hydrobromide was run at pH 9.5 as above, but in this case the diastereomeric dipeptides were separated by dry column chromatography[1] on silica gel H (E. Merck, Darmstadt) in chloroform–methanol 9:1, and the ultraviolet spectra of aliquots of the total fractions were compared at 258 mμ. The ratio of L-Arg-L-Phe to D-Arg-L-Phe was 95:5. A third fraction corresponded to phenylalanine and represented 2% of the total dipeptide fraction.

B. With D-Phenylalanine.—An identical experiment with D-phenylalanine gave a ratio for L-arginyl-D-phenylalanine to D-arginyl-D-phenylalanine of 84:16. A third fraction, phenylalanine, was obtained, which represented 5% of the dipeptide fractions.

Phenylalanylarginine from Phenylalanine NTA and ^{14}C-Arginine.—A stock solution of ^{14}C-labeled L-arginine was prepared by dissolving 3.484 g of L-arginine (20 mmol, 0.5 mCi) in 80 ml of water, adjusting the pH to 3 with 10 *N* H_2SO_4, and making up the solution to 100 ml with water. A 10-ml aliquot of the arginine stock solution (2 mmol, 50 μCi) was pipetted into a Waring Blendor along with 8 ml of water. The solution was stirred under N_2 at 24.5° and the pH was adjusted to 9.5 with saturated barium hydroxide solution. Phenylalanine NTA (456 mg, 2.2 mmol) was added while the pH was maintained at 9.5. After 20 min the pH was raised to 10.6. The solution was filtered and made up to 50 ml. Electrophoresis at pH 2 separated the peptides and the hydantoic acid derived from phenylalanine NTA and arginine. The paper strip was cut into sections and the radioactivity was determined as per cent of total on the strip. For details see ref 1. A similar reaction was run with phenylalanine NCA at 0–2°, pH 10.0. The results are outlined in Table VI.

L-Proline NTA.—A solution of 94.6 g (0.50 mol) of methoxythiocarbonyl-L-proline, $[\alpha]_{589}$ −126° (*c* 1, $CHCl_3$), which was prepared by the usual procedure, in 350 ml of THF was cooled to −35° and 270.7 g (1.00 mol) of phosphorus tribromide was added. After 4 hr at −35° the reaction was diluted with ethyl acetate and extracted at 0° with 375 ml of ice water. The organic

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TABLE VI
DISTRIBUTION OF PRODUCTS IN THE PHE-ARG REACTION[a]

Product	From Phe-NTA, %	From Phe-NCA, %
Arginine	2.2	3.5
H-Phe-Arg·OH	94.2	89.2
H-Phe-Phe-Arg·OH	0.3	4.0
Hydantoic acid	2.7	2.8

[a] Traces of radioactivity between these spots bring the total to 100%.

layer was washed three times with 5% aqueous $NaHCO_3$, three times with saturated aqueous NaCl, dried over $MgSO_4$, and concentrated to give 33 g (21%) of crude proline NTA. One recrystallization from ether gave material with a rotation of $[\alpha]_{589}$ −155.1° (*c* 1, $CHCl_3$) and three further crystallizations gave proline NTA of constant rotation, $[\alpha]_{589}$ −157 ±0.5° (*c* 1, $CHCl_3$). The final recrystallized proline NTA was used for the following racemization study.

Racemization in the Preparation of Prolylphenylalanine. A. In Tritiated Water.—A solution of 0.826 g (5.0 mmol) of phenylalanine in 50 ml of 0.5 *M* potassium borate in tritiated water was adjusted to pH 9.35 at 0°. Proline NTA (0.807 g, 5.8 mmol) was added while the pH was maintained at 9.35. The peptide was precipitated at pH 4.5 and recrystallized from water to constant activity. This product corresponded by tlc to peptide prepared *via* proline NCA.[1] A similar experiment was carried out at pH 10.0. At pH 9.35, 0.114% of 1 equiv of tritium was incorporated, and at pH 10.0, 0.129%.

B. In D_2O.—A solution of 0.66 g (4.0 mmol) of phenylalanine in 40 ml of 0.5 *M* borate buffer in D_2O which was prepared from boric acid anhydride and sodium deuterioxide was adjusted to a pH of 10.0 using a combination glass–calomel electrode set for a meter reading of 9.6.[37] A sample of the dipeptide was repeatedly recrystallized to free it of labile deuterium. This product was burned, and the water was reduced to hydrogen and then examined by mass spectroscopy.[38] Deuterium appeared at 0.0275% above natural abundance, which would correspond to an excess of 0.495% deuterium for one hydrogen position in the dipeptide.

A sample of L-proline NTA was hydrolyzed in dilute hydrochloric acid to proline, which was identified by tlc. The solution was concentrated and the product was assayed for D-proline by D-amino acid oxidase using a Warburg manometric technique[39] with an increased ratio of enzyme to substrate. Controls containing 0.5, 1.0, and 2.9% D-proline showed 0.34, 0.94, and 2.88% D-proline, whereas the above sample showed 2.08% D-proline (each an average of two runs).

Registry No.—Table I—L-Ala, 19777-64-1; D-alloisoleu, 26686-26-0; L-Arg, 26686-27-1; Gly, 26686-28-2; L-His, 19777-65-2; L-Ileu, 26686-30-6; L-Leu, 26686-31-7; L-Phe, 26686-32-8; L-Pro, 26686-33-9; L-Val, 26686-34-0; Table II—L-Ala, 16964-94-6; L-Arg, 26731-59-9; Gly, 16874-97-8; L-His, 26731-60-2; L-Leu, 26607-56-7; L-Phe, 26686-38-4; L-Pro, 26686-39-5; L-Val, 26731-61-3; **3** (R = *i*-Bu; R′ = Et), 26686-40-8; **9** (R = $C_6H_5CH_2$), 26686-41-9; **11**, 26686-47-5; glycyl-L-phenylalanyl-L-leucine, 15373-56-5; L-alanyl-L-phenylalanyl-L-leucine, 26686-43-1; L-Ala-*O*-benzyl-L-Ser-L-Val, 26731-62-4; L-Val-L-His-L-Phe-L-Asp-L-Ala-*O*-benzyl-L-Ser-L-Val, 6169-58-0; L-histidyl-L-alanylglycine, 26731-63-5; L-alanyl-L-phenylalanine, 3061-90-3; L-Arg-L-Phe, 2047-13-4.

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Steroidal β-Lactams.[1] II. Synthesis of Pregnane and *D*-Homo Compounds

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The conversion of *A*-norprogesterone (**1**) into 3,4-dinor-5-aza-*B*-homopregnane-2,20-dione (**20**) and its *D*-homo isomer, 17α-methyl-3,4-dinor-*B*-homo-*D*-homo-5-azaandrostane-2,17a-dione (**16**) is described.

The synthesis of a new steroidal ring system possessing a fused β-lactam as ring A has been recently described.[1] In that case, the substituent at C_{17} was a hydroxyl group, and we then became interested, from both the chemical and biological points of view, in the synthesis of a steroidal β-lactam bearing a pregnane side chain at C-17.[2] In this paper, we wish to describe the results of our efforts to convert *A*-norprogesterone (**1**)[3] into such a compound.

Our initial step in the synthesis was protection of the C-20 carbonyl of **1** as a hydroxyl function. We expected that treatment of **1** with sodium borohydride would lead to selective reduction at C-20, since α,β-unsaturated ketones reduce more slowly than saturated ketones (unhindered).[4] Indeed, reduction of **1** with sodium borohydride in methanol at 0° gave **2** in 80–90% yield. This compound has been previously prepared during the synthesis of **1**, by the ring A contraction method starting with 20β-hydroxy-4-pregnen-3-one.[3] Treatment of **2** with the permanganate–periodate combination[5] transformed the ring A α,β-unsaturated ketone system into a keto acid that cyclized and was isolated as the lactonol **3**. Room temperature acetylation selectively esterified the 20β-hydroxy group to give **4**. The methyl ester **5**, prepared by treatment of **4** with

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