phenylglycine-*o*-carboxylic acid in 15% w./v. hydrochloric acid gave the low yield recorded of 4,5,6-trichlorophenylglycine-*o*-carboxylic acid, no other isomers were isolated [d] B.A.S.F., German Patent 231962. [e] B.A.S.F., German Patent 148615. [f] G. Heller and L. Hessel, *J. prakt. Chem.*, **120**, 73 (1929). [g] V. Villiger, *Ber.*, **42**, 3541 (1909). [h] B.A.S.F., German Patent 148615. [i] W. R. Orndorff and E. H. Nichols, *Amer. Chem. J.*, **48**, 483 (1912).

TABLE X

CHLOROINDIGOS

Substituents	Solvent	Color and λ_1 (mμ) in tetrachlorethane		Lit. λ_1 (mμ) or color
4,4'-Dichloro	Chlorobenzene	Blue	610	602.5 tetralin[a,b]
5,5'-Dichloro	Nitrobenzene	Green blue	620	606.5 tetralin[a,c]
6,6'-Dichloro	Nitrobenzene	Mauve	590	561 tetralin[a] red, nitrobenzene[d]
7,7'-Dichloro	Chloroform	Blue violet	600	*j*
4,4',5,5'-Tetrachloro	Nitrobenzene	Green blue	622.5	*e*
4,4',6,6'-Tetrachloro	Tetrachlorethane	Blue mauve	595	*k*
4,4',7,7'-Tetrachloro	Nitrobenzene	Royal blue	610	*f*
5,5',6,6'-Tetrachloro	Nitrobenzene	Blue	605	Blue violet[g]
5,5',7,7'-Tetrachloro	Chlorobenzene	Green blue	617.5	613.5 Carbon tetrachloride[a,h]
6,6',7,7'-Tetrachloro	Tetrachloroethane	Mauve	590	*l*
4,4',5,5',6,6'-Hexachloro	Tetrachloroethane	Royal blue	610	*m*
4,4',5,5',7,7'-Hexachloro	Tetrachlorethane	Blue	615	615.5 tetralin[a]
5,5',6,6',7,7'-Hexachloro	Tetrachloroethane	Blue violet	600	*i*
4,4',6,6',7,7'-Hexachloro	Tetrachloroethane	Blue mauve	595	*n*
Octachloro	Tetrachloroethane	Royal blue	610	Blue violet[i]

[a] J. Formanek, *Angew. Chem.*, **41**, 1133 (1928). [b] L. Gindraux, *Helv. Chim. Acta*, **12**, 921 (1929). [c] C. Mettler, *Ber.*, **38**, 2809 (1905). [d] F. Sachs and E. Sicket, *Ber.*, **37**, 1861 (1904). [e] B.A.S.F. German Patents 234961, 409618.[f] E. Grandmougin and P. Seyder, *Ber.*, **47**, 2365 (1914). [g] German Patent 254467. [h] L. Kalb, *Ber.*, **42**, 3653 (1909). [i] C. Van De Bunt, *Rec. trav. chim.*, **48**, 121 (1929). [j] *Anal.* Calcd. for $C_{16}H_8O_2N_2Cl_2$: C, 58.0; H, 3.0; N, 8.5. Found: C, 58.2; H, 3.0; N, 8.7. [k] *Anal.* Calcd. for $C_{16}H_6O_2N_2Cl_4$: C, 48.0; H, 1.5; N, 7.0. Found: C, 48.0; H, 1.5; N, 6.7. [l] *Anal.* Calcd. for $C_{16}H_6O_2N_2Cl_4$: C, 48.0; H, 1.5; N, 7.0. Found: C, 48.2; H, 1.3; N, 7.0. [m] *Anal.* Calcd. for $C_{16}H_4O_2N_2Cl_6$: C, 40.9; H, 0.9; N, 6.0. Found: C, 41.0; H, 1.0; N, 6.2. [n] *Anal.* Calcd. for $C_{16}H_4O_2N_2Cl_6$: C, 40.9; H, 0.9; N, 6.0. Found: C, 40.8; H, 0.8; N, 6.0.

Acknowledgment.—The authors are indebted to Dr. S. J. Holt for several stimulating discussions and suggestions concerning this problem.

LONDON, W. 1, ENGLAND

[CONTRIBUTION FROM THE NATIONAL RESEARCH COUNCIL OF CANADA, PRAIRIE REGIONAL LABORATORY]

The 3-*o*-Nitrophenyl- and 3-(Phenyl-*p*-azo-phenyl)-2-thiohydantoins of Amino Acids[1]

BY L. K. RAMACHANDRAN[2] AND W. B. MCCONNELL

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The synthesis of the 5-alkyl-3-(*o*-nitrophenyl)-2-thiohydantoins and the 5-alkyl-3-(phenyl-*p*-azo-phenyl)-2-thiohydantoins derived from several of the naturally occurring amino acids is reported. The ultraviolet spectra of the 3-(phenyl-*p*-azo-phenyl)-thiohydantoins have been studied over the region 225–380 mμ and the molecular extinction coefficients for the peaks of absorption recorded.

2,4-Dinitro-1-fluorobenzene and phenyl isothiocyanate are widely used[3] as reagents for studies on the sequence of amino acids in peptides and proteins. The partial destruction of dinitrophenyl amino acids during the hydrolysis of dinitrophenylated proteins is a serious disadvantage in the use of the former reagent. Phenyl isothiocyanate reacts with amino acids to give N-phenylthiocarbamyl derivatives which can undergo ring closure to yield 5-substituted 3-phenyl-2-thiohydantoins.[4] With a peptide, the phenylthiocarbamyl peptide formed undergoes rearrangement to yield the phenylthiohydantoin of the amino acid occupying the N-terminal position and exposing the amino group of the adjacent residue, thus offering a means for the stepwise degradation starting from the N-terminal amino acid. The phenylthiohydantoins are ordinarily identified by paper chromatography[5,6] or by infrared spectrophotometry,[7] and quantitative determinations are made by ultraviolet spectrophotometry.[3]

It appeared that chromatography of the amino acid thiohydantoins could be facilitated if an isothiocyanate containing a chromophore were used in place of phenyl isothiocyanate. Further, the colored derivatives would be expected to have improved absorption properties. Determination of these thiohydantoins by measurement of the absorption in the visible region of the spectrum might also become possible. The feasibility of this ap-

(1) Issued as paper No. 216 in the Uses of Plant Products series and as N.R.C. No. 3896.

(2) National Research Council of Canada Postdoctorate Fellow, 1953–1955.

(3) H. Fraenkel-Conrat, J. I. Harris and A. L. Levey, in "Methods of Biochemical Analysis," Vol. II, Edited by D. Glick, Interscience Publishers, N. Y., 1955, p. 389.

(4) P. Edman, *Acta Chem. Scand.*, **4**, 277 (1950).

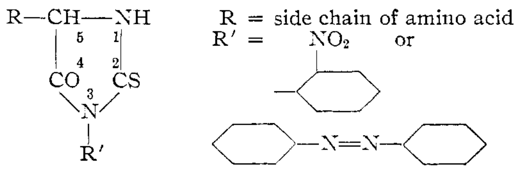
(5) J. Sjoquist, *ibid.*, **7**, 447 (1953).

(6) W. A. Landmann, M. P. Drake and J. Dillaha, THIS JOURNAL, **75**, 3638 (1953).

(7) L. K. Ramachandran, A. Epp and W. B. McConnell, *Anal. Chem.*, **27**, 1734 (1955).

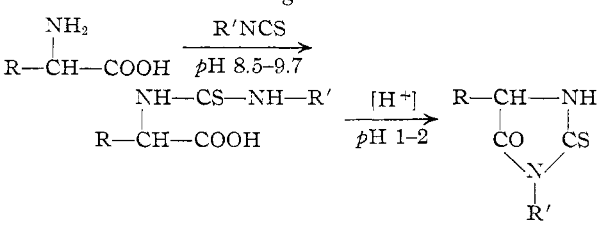
proach has been indicated recently[8,9] by the use of 5-dimethylamino-3,5-dinitrophenyl isocyanate to prepare colored amino acid derivatives. Six hydantoins were described and the usefulness of the reagent demonstrated by application to a di- and tripeptide.

The present communication records thiohydantoin derivatives of several amino acids obtained through the use of *o*-nitrophenyl isothiocyanate and *p*-isothiocyanoazobenzene. The thiohydantoins obtained by the use of the above reagents are represented by I

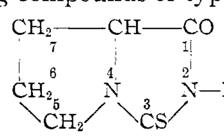


I

and are formed according to the scheme



Proline and hydroxyproline would be expected to yield fused ring compounds of type II



II

o-Nitrophenyl isothiocyanate and *p*-isothiocyanoazobenzene on reaction with amino acids yield crystalline thiohydantoins. The *o*-nitrophenylthiohydantoins are not highly colored and *o*-nitrophenyl isothiocyanate is not therefore considered satisfactory from the points of view mentioned. However, it is worth mentioning that this reagent is far more reactive than phenyl isothiocyanate or *p*-isothiocyanoazobenzene. *p*-Isothiocyanoazobenzene yields thiohydantoins which have an intense color and possess excellent absorption characteristics. The suitability of such a reagent in the Edman degradation of peptides[10] appears worthy of further investigation. Modification of this latter reagent by introduction of hydrophilic substituents could be expected to yield a reagent with better solubility properties more useful in the structural investigation of peptides.

Experimental Methods and Results

Materials.—Commercially available *l*- or *dl*-amino acids were used. *o*-Nitrophenyl isothiocyanate was prepared by the method of Erlenmeyer and Ueberwasser[11] and *p*-isothiocyanoazobenzene according to Bolser and Hartshorn.[12]

(8) G. G. Evans and W. S. Reith, *Biochem. J.*, **56**, 111 (1954).

(9) W. S. Reith and N. M. Waldron, *ibid.*, **56**, 116 (1954).

(10) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(11) H. Erlenmeyer and H. Ueberwasser, *Helv. Chim. Acta*, **23**, 328 (1940).

(12) C. E. Bolser and E. B. Hartshorn, This Journal, **45**, 2349 (1932).

Preparation of the Thiohydantoins.—The reaction with *o*-nitrophenyl isothiocyanate was carried out at room temperature by dissolving one mml. of the amino acid in 5–10 ml. 50% pyridine and adding 0.197 g. of the reagent (10% excess); 0.5 *N* sodium hydroxide was added as required to maintain a *p*H of 8.5. When the consumption of alkali had ceased (94–100%, complete in 10–40 minutes) the reaction mixture was continuously extracted with ether to remove excess reagent and pyridine. The aqueous phase contained the thiocarbamyl derivative in solution. Ring closure was effected by addition of concentrated hydrochloric acid to *p*H 1 and standing at room temperature for 2 days. The precipitated thiohydantoin was then collected by filtration and recrystallized from alcohol–water mixtures; over-all yield 70–80%. All derivatives obtained with this reagent were pale yellow in color.

The conditions described above for ring closure of the thiocarbamyl derivative to the thiohydantoin were satisfactory for most amino acids. Since the thiocarbamyl derivative of glycine is more stable than that from other amino acids, in the case of glycine the aqueous phase was made 2 *N* with respect to HCl and the mixture refluxed for 2 hours, evaporated to small volume and cooled. The thiohydantoin, which crystallized on standing, was recrystallized from aqueous alcohol. No attempt was made to isolate the intermediate thiocarbamyl derivatives.

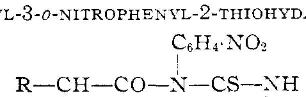
The reaction of amino acids with *p*-isothiocyanoazobenzene was conducted as described above for *o*-nitrophenyl isothiocyanate, except that 85% pyridine was used as solvent and a *p*H of 9.7 was used instead of 8.5. The change in solvent concentration was necessary because of the limited solubility of the reagent in 50% pyridine. Under these conditions asparagine and glutamine reacted slowly but the reaction proceeded faster after adding a little water to the mixture and warming it to 40–45°. Reaction with cystine even under these conditions was negligible. With glycine, the ring closure of the thiocarbamyl derivative to the thiohydantoin could not be effected by refluxing in 2 *N* or 5.7 *N* HCl for 2 hours. Derivatives arising from the use of this reagent are all colored bright orange red.

Measurement of Absorption in the Ultraviolet.—All measurements of absorption were made on solutions in absolute alcohol, at 5 mμ intervals through the region 225–380 mμ. Near the absorption maxima optical densities were recorded at 1 mμ intervals. The spectrophotometer used was a Beckman, Model DU.

5-Alkyl-3-*o*-nitrophenyl-2-thiohydantoins.—The physical constants and analyses for twelve of the thiohydantoin derivatives of amino acids are recorded in Table I. The molecular extinction for the compounds in the region 258–264 mμ is about 10,000, considerably lower than that observed for the 3-phenyl-2-thiohydantoins which is always higher than 14,000.[1] Among the thiohydantoin derivatives obtained, that of glycine alone gave a purple color with NH_3

TABLE I

5-ALKYL-3-*o*-NITROPHENYL-2-THIOHYDANTOINS



Amino acid from which R- derived	M.p., °C.	Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found
dl-Glycine	163–164	45.56	45.72	2.97	2.99
dl-Valine	159–162	51.60	51.73	4.69	4.73
dl-Aspartic acid	210	44.75	44.69	3.07	3.10
l-Asparagine	236	44.90	44.60	3.43	3.37
l-Glutamine	166–168	46.73	46.73	3.92	3.86
l-Cystine	112–113	42.53	42.66	2.86	2.85
dl-Phenylalanine	140	58.71	58.75	4.00	4.03
l-Tyrosine	191	55.98	55.89	3.82	3.88
l-Proline[a]	168–169	51.97	51.71	4.00	4.00
l-Histidine	235	49.20	49.71	3.41	3.61
l-Lysine[b]	89	49.18	49.25	4.13	4.18
dl-Tryptophan	218	59.21	59.49	4.05	4.22

Analyses, %

[a] Fused ring system. [b] The lysine derivative has a O_2N-C_6H_4-NH-CS-NH- grouping on the terminal carbon of the side chain.

TABLE II

5-ALKYL-3-(PHENYL-*p*-AZOPHENYL)-2-THIOHYDANTOINS, $R—CH—CO—N(C_6H_4·N_2·C_6H_5)—CS—NH$ (ring: CH to NH)

Amino acid from which R- derived	Mol. formula	M.p., °C.[a]	Carbon Found	Carbon Calcd.	Hydrogen Found	Hydrogen Calcd.	Sulfur Found	Sulfur Calcd.
			Analyses,[b] %					
dl-Glycine[c]	$C_{15}H_{14}O_2N_4S$	179–180	57.32	57.32	4.85	4.50	10.30	10.20
dl-Alanine	$C_{16}H_{14}ON_4S$	184–186	62.07	61.93	4.65	4.55	10.48	10.33
dl-Valine	$C_{18}H_{18}ON_4S$	258–259	64.24	63.89	4.37	4.36	9.56	9.48
dl-Leucine	$C_{19}H_{20}ON_4S$	204–205	64.72	64.76	5.72	5.72	8.98	9.10
dl-Isoleucine	$C_{19}H_{20}ON_4S$	220–221	65.21	64.76	5.74	5.72	9.10	9.09
dl-Serine[d]	$C_{16}H_{14}O_2N_4S$	151–152	58.00	58.89	4.43	4.32	9.70	9.82
dl-Threonine	$C_{17}H_{16}O_2N_4S$	233–234	60.38	59.99	4.71	4.74	9.42	9.58
dl-Aspartic acid	$C_{17}H_{14}O_3N_4S$	223–224	57.67	57.63	4.03	3.98	9.08	9.05
l-Asparagine	$C_{17}H_{15}O_2N_5S$	Dec. wide range	58.25	57.78	4.25	4.28	9.06	9.20
l-Glutamic acid	$C_{18}H_{16}O_3N_4S$	196–197	58.64	58.69	4.39	4.38	8.60	8.69
l-Glutamine	$C_{18}H_{17}O_3N_5S$	210–211	59.19	58.85	4.57	4.66	8.62	8.71
l-Proline[c,e]	$C_{18}H_{16}ON_4S$	131–132	58.48	64.29	5.28	4.80	9.62	9.56
l-Hydroxyproline	$C_{18}H_{16}O_2N_4S$	140–141	60.71	61.31	4.63	4.58	9.00	9.10
dl-Phenylalanine	$C_{22}H_{18}ON_4S$	254–255	68.92	68.38	4.80	4.70	7.98	8.03
l-Tyrosine	$C_{22}H_{18}O_2N_4S$	224–225	65.38	65.66	4.54	4.51	7.92	7.97
dl-Tryptophan	$C_{24}H_{19}ON_5S$	194–195	67.75	68.00	4.65	4.50	7.65	7.53
dl-Methionine	$C_{18}H_{18}ON_4S_2$	210–211	58.56	58.37	4.95	4.90	17.20	17.30
l-Lysine[c,f]	$C_{32}H_{32}O_2N_8S_2$	164–165	61.44	61.53	5.29	5.16	10.40	10.25
l-Arginine[g]	$C_{18}H_{19}ON_7S$	181–182	56.52	56.66	5.02	5.02	8.37	8.40
l-Histidine[g]	$C_{19}H_{16}ON_6S$	235–236	60.30	60.63	4.31	4.29	8.50	8.52

[a] Decomposition. [b] Nitrogen analyses were attempted by the modified Friederick method (G. E. Secor, M. C. Long, M. D. Kilpatrick and L. M. White, *J. Assoc. Offic. Agric. Chemists*, **33**, 872 (1950)), but results were unsatisfactory for these azo compounds. Sulfur was determined by a modified Gröte method (R. N. Walter, *Anal. Chem.*, **22**, 1332 (1950)). [c] The infrared spectra of the derivatives of glycine, proline and lysine do not show the characteristic absorption bands for the ring C=O group and it would appear that in these the molecular formula $C_{15}H_{14}O_2N_4S$ is for the un-ring closed thiocarbamyl derivative. [d] Purified by chromatography on an ether Supercel column. [e] The analyses recorded, except for sulfur, would correspond to the thiocarbamyl derivative, $C_{18}H_{18}O_2N_4S$, with an additional molecule of water also being present. [f] Both amino groups in lysine have $C_6H_5·N{=}NC_6H_4—NH—CS—$ groups. [g] Recrystallized from water.

TABLE III

ABSORPTION CHARACTERISTICS OF 5-ALKYL-3-(PHENYL-*p*-AZOPHENYL)-2-THIOHYDANTOINS[a]

Amino acid from which thiohydantoin derived	Wave length absorption max., mμ	Molar extinction coefficient	Amino acid from which thiohydantoin derived	Wave length absorption max., mμ	Molar extinction coefficient
Glycine[b]	235	19,300	Glutamine	359	23,900
	361	26,600	Proline[b]	239	20,100
Alanine	265–268	22,200		359	21,800
	322	22,200	Hydroxyproline	239	13,200
Valine	270	23,300		272	11,000
	322.5	19,900		339	13,800
Leucine	269	21,100	Phenylalanine	236	17,700
	324	19,900		359	21,700
Isoleucine	270	21,400	Tyrosine	272.5	22,900
	325	20,500		322.5	19,000
Serine	238	19,900	Tryptophan	269	23,500
	326	25,700		324	20,000
Threonine	270	20,200	Methionine	270	22,600
	324	22,800		324	20,700
Aspartic acid	268	17,700	Lysine[b]	239	36,700
	325	18,900		362.5	48,200
Asparagine	269	21,200	Arginine	269	18,300
	324	19,600		324	17,800
Glutamic acid	269	20,800	Histidine	268	20,700
	324	19,600		322.5	18,500

[a] Compounds used in the absorption measurements are the same as recorded in Table II. [b] As indicated in Table II the thiohydantoin ring system is probably absent in the derivatives of glycine, proline and lysine.

vapor which changed back to pale yellow on exposure to acid fumes.

5-Alkyl-3-(phenyl-*p*-azo-phenyl)-2-thiohydantoins.—The physical constants and analyses for these derivatives are recorded in Table II and molecular extinction coefficients at the peak of absorption in Table III. All derivatives obtained with this reagent were found to be superior to the 3-*o*-nitrophenyl derivatives in both color and intensity of absorption.

In amounts of 5–10 μg. the compounds yield yellow spots easily seen on paper which can be further intensified in color by exposure to acid fumes when the color changes to deep red; the color change was reversible, changing back to yellow on exposure to ammonia vapor.

On the basis of the absorption data the thiohydantoins arising from the use of *p*-isothiocyanoazobenzene may be classified at least into two groups, one major group comprising those of alanine, valine, leucine, isoleucine, aspartic acid, glutamic acid, asparagine, tyrosine, tryptophan, methionine, arginine and histidine, which have absorption maxima at 268–273 and 320–325 mμ, and another comprising those of glycine, proline, lysine, serine and phenylalanine with absorption maxima at 235–240 and 359–362.5 mμ. Analytical evidence and the infrared spectra indicate that the first three in the latter group are the thiocarbamyl derivatives and not the thiohydantoins. It is not understood why the ultraviolet spectra of the derivatives of serine and phenylalanine differ from those in the major group. Glutamine has only one major absorption maximum at 359 mμ. The non-reactivity of cystine toward the reagent and the unusual stability of the thiocarbamyl derivatives of glycine and, to a smaller extent, of those of proline and lysine, restrict the usefulness of the reagent in the study of peptide structure.

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SASKATOON, SASKATCHEWAN