36. *Degradative Studies on Peptides and Proteins. Part III.* Synthesis of Some 2-Thiohydantoins as Reference Compounds.*

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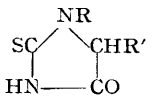
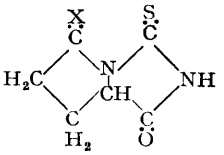
A number of 2-thiohydantoins have been synthesised as reference compounds for the recent method [1] of stepwise degradation of peptides from the end bearing a free amino-group.

The stepwise degradation of polypeptides from the end bearing a free amino-group by means of *N*-acyldithiocarbamates,[1] as well as the Schlack and Kumpf procedure [2] and its modifications [3, 4] from the end bearing a free carboxyl group, requires as reference compounds 2-thiohydantoins corresponding to all the amino-acids likely to be present in the polypeptide. Many of these have long been known; [5] those corresponding to histidine, arginine, ornithine, lysine, proline, methionine sulphone, cysteic acid, and *S*-methylcysteine have not been synthesised and characterised previously, although a few have been obtained as amorphous products which have not been analysed.[6] Various routes to these compounds were investigated and no one proved universally satisfactory. Improved methods for the synthesis of 2-thiohydantoins corresponding to glutamine and sarcosine have also been sought.



(I)



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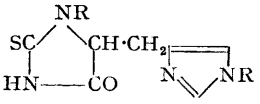

(III)

5-2′-Carbamoylethyl-2-thiohydantoin has recently been prepared in 45% yield by Swan [7] by the careful hydrolysis of the 1-acetyl derivative. We first examined the action of aqueous and ethanolic ammonia on 1-acetyl-5-2′-methoxycarbonylethyl-2-thiohydantoin (I; R = Ac, R′ = $MeO_2C{\cdot}[CH_2]_2$), which was in turn prepared in good yield from L-glutamic acid γ-methyl ester by the general method of Johnson and Nicolet.[8] Paper chromatography indicated a mixture of products. An alternative approach was suggested by the independent demonstrations by Rudinger [9] and Swan and du Vigneaud [10] that aqueous ammonia converted 5-oxo-1-toluene-*p*-sulphonylpyrrolidine-2-carboxylic acid into *N*-toluene-*p*-sulphonylglutamine. Similar treatment of 5′-oxopyrrolidino(1′ : 2′-1 : 5)-2-thiohydantoin (II; X = O) [11] gave a mixture. Replacement of aqueous by ethanolic ammonia, however, converted the compound (II; X = O) into the amide (I; R = H, R′ = $NH_2{\cdot}CO{\cdot}[CH_2]_2$) in excellent yield.

5-4′-Glyoxalinylmethyl-2-thiohydantoin (III; R = H) was readily obtained by acid hydrolysis in aqueous ethanol of 1-acetyl-5-(1-acetyl-4-glyoxalinylmethyl)-2-thiohydantoin (III; R = Ac).[7]

Crystalline pyrrolidino(1′ : 2′-1 : 5)-2-thiohydantoin (II; X = H_2) was forthcoming

* Part II, preceding paper.

[1] Elmore and Toseland, *J.*, 1954, 4533.
[2] Schlack and Kumpf, *Z. physiol. Chem.*, 1926, **154**, 125.
[3] Waley and Watson, *J.*, 1951, 2394; Tibbs, *Nature*, 1951, **168**, 910; Kjaer and Eriksen, *Acta Chem. Scand.*, 1952, **6**, 448; Kenner, Khorana, and Stedman, *J.*, 1953, 673; Baptist and Bull, *J. Amer. Chem. Soc.*, 1953, **75**, 1727.
[4] Dautrevaux and Biserte, *Compt. rend.*, 1955, **240**, 1153.
[5] Ware, *Chem. Rev.*, 1950, **46**, 403.
[6] Edward and Nielsen, *Chem. and Ind.*, 1953, 197.
[7] Swan, *Australian J. Sci. Res.*, 1952, **5**, *A*, 711.
[8] Johnson and Nicolet, *Amer. Chem. J.*, 1913, **49**, 197.
[9] Rudinger, *Chem. Listy*, 1954, **48**, 235, 244; *Coll. Czech. Chem. Comm.*, 1954, **19**, 365, 375.
[10] Swan and du Vigneaud, *J. Amer. Chem. Soc.*, 1954, **76**, 3110.
[11] Johnson and Guest, *Amer. Chem. J.*, 1912, **47**, 242.

from the acid-catalysed ring-closure of the non-crystalline *N*-benzoylthiocarbamoyl-L-proline ethyl ester, which was obtained by the interaction of benzoyl *iso*thiocyanate and L-proline ethyl ester. This thiohydantoin was previously obtained in an amorphous state (no analysis given) by Edward and Nielsen [6] using Johnson and Nicolet's method.[8]

5-3′-Guanidinopropyl-2-thiohydantoin {I; R = H, R′ = NH_2·C(:NH)·NH·$[CH_2]_3$}, previously obtained in an amorphous state (no analysis given) by Edward and Nielsen,[6] was afforded crystalline by the acid-catalysed ring-closure of α-*N*-acetylthiocarbamoyl-L-arginine. The hydrochloride of 5-3′-aminopropyl-2-thiohydantoin (I; R = H, R′ = NH_2·$[CH_2]_3$) was synthesised from DL-ornithine in a similar manner, although the intermediate α-*N*-acetylthiocarbamoyl-DL-ornithine could not be crystallised. This method, when applied to the synthesis of 5-4′-aminobutyl-2-thiohydantoin (I; R = H, R′ = NH_2·$[CH_2]_4$) from L-lysine, gave a complex mixture from which the desired compound was separated, albeit in minute yield, by chromatography on powdered cellulose. Application of Johnson and Nicolet's [8] method to ε-benzyloxycarbonyl-L-lysine proved more satisfactory. Short hydrolysis of the oily intermediate 1-acetyl-5-4′-benzyloxycarbonylaminobutyl-2-thiohydantoin (I; R = Ac, R′ = Ph·CH_2·O·CO·NH·$[CH_2]_4$) with dilute hydrochloric acid afforded the hydrochloride of the desired 5-4′-aminobutyl-2-thiohydantoin (I; R = H, R′ = NH_2·$[CH_2]_4$) directly in modest yield. The previously reported "lysine 2-thiohydantoin," [4, 6] from its mode of preparation and its chromatographic behaviour, is probably 5-4′-acetamidobutyl-2-thiohydantoin (I; R = H, R′ = Ac·NH·$[CH_2]_4$).

1-Methyl-2-thiohydantoin (I; R = Me, R′ = H) was described by Komatsu [12] as a yellow crystalline solid, m. p. 214—215°, produced by heating a mixture of potassium thiocyanate and sarcosine ethyl ester hydrochloride in ethanol. This method, in our hands, gave a minute yield and we attribute this to the sluggish isomerisation of sarcosine ethyl ester thiocyanate to *N*-thiocarbamoylsarcosine ethyl ester.[13] In view of the recent discovery of sarcosine in ground-nut protein,[14] several other routes were examined. Although methyl *N*-acetyldithiocarbamate appeared to react with sarcosine in aqueous ethanol at pH 10, acid treatment of the gummy product failed to give identifiable material. *N*-Acetylthiocarbamoylsarcosine ethyl ester, obtained as a gum from the reaction between methyl *N*-acetyldithiocarbamate and sarcosine ethyl ester, and *N*-benzoylthiocarbamoylsarcosine ethyl ester, obtained as a crystalline solid from the reaction between benzoyl *iso*thiocyanate and sarcosine ethyl ester, both afforded 1-methyl-2-thiohydantoin, albeit in poor yield, when heated with dilute hydrochloric acid.

Stepwise degradation of polypeptides containing cystine residues is complicated by the presence of the disulphide bridge since, unless both amino-groups of cystine are free, no thiohydantoin is liberated. Thus Rovery, Fabre, and Desnuelle [15] found no *N*-terminal residue in chymotrypsinogen by the Edman procedure. Rupture of the disulphide bridge by oxidation with performic acid [16] is commonly employed and Bettelheim [17] found that oxidised chymotrypsinogen possessed one *N*-terminal cysteic acid residue, *i.e.*, one amino-group of cystine is free in chymotrypsinogen. Thus we have found it necessary to synthesise the 2-thiohydantoins corresponding to cysteic acid and methionine sulphone (since methionine is also oxidised by performic acid). The synthesis of 5-sulphomethyl-2-thiohydantoin (I; R = H, R′ = HO_3S·CH_2) was accomplished through *N*-acetylthiocarbamoylcysteic acid. The thiohydantoin was found to be unstable in hot concentrated aqueous solution; cysteic acid was detected among the degradation products. For this reason cyclisation of the intermediate *N*-acetylthiocarbamoylcysteic acid was carried out in dilute solution and the time of reaction affording optimum yield was determined spectrophotometrically. 5-2′-Methylsulphonylethyl-2-thiohydantoin (I; R = H, R′ = $MeSO_2$·$[CH_2]_2$) was easily made by Johnson and Nicolet's method.[8] An alternative method of cleaving cystine disulphide bridges involves reduction with sodium in liquid

[12] Komatsu, *Mem. Coll. Sci. Kyoto Imp. Univ.*, 1914, **1**, 69.
[13] Elmore, Toseland, and Tyrrell, *J.*, 1955, 4388.
[14] Haworth, MacGillivray, and Peacock, *Nature*, 1951, **167**, 1068.
[15] Rovery, Fabre, and Desnuelle, *Biochim. Biophys. Acta*, 1953, **12**, 547.
[16] Sanger, *Biochem. J.*, 1949, **44**, 126.
[17] Bettelheim, *J. Biol. Chem.*, 1955, **212**, 235.

ammonia followed by alkylation.[18] We have accordingly synthesised 5-methylthiomethyl-2-thiohydantoin (I; R = H, R′ = MeS·CH_2) from *S*-methylcysteine by Johnson and Nicolet's method.[8]

Experimental

1-*Acetyl*-5-2′-*methoxycarbonylethyl*-2-*thiohydantoin*.—A solution of γ-methyl hydrogen L-glutamate (2·4 g.) and ammonium thiocyanate (1·5 g.) in acetic anhydride (45 c.c.) and acetic acid (5 c.c.) was heated at 100° for 10 min., then poured into ice-water (250 c.c.). A yellow oil separated and was extracted into chloroform (40 c.c.). The extract was washed with 10% aqueous sodium hydrogen carbonate and water, dried ($CaSO_4$), and evaporated under reduced pressure to half its volume. Addition of light petroleum (b. p. 40—60°) caused the *thiohydantoin* (2·2 g.) to crystallise. Twice recrystallised from chloroform–light petroleum (b. p. 40—60°), it had m. p. 102° (Found: C, 44·1; H, 5·2; N, 10·7. $C_9H_{12}O_4N_2S$ requires C, 44·2; H, 5·0; N, 11·5%).

5-2′-*Carbamoylethyl*-2-*thiohydantoin*.—5′-Oxopyrrolidino(1′ : 2′-1 : 5)-2-thiohydantoin [11] (3 g.) was dissolved in saturated ethanolic ammonia (60 c.c.). After 5 min., the product (2·8 g.) separated, having m. p. 189°. Recrystallised from ethanol–light petroleum (b. p. 40—60°), it had m. p. 193° (Found: C, 38·8; H, 4·9; N, 22·0; S, 17·1. Calc. for $C_6H_9O_2N_3S$: C, 38·5; H, 4·9; N, 22·5; S, 17·1%). When aqueous ammonia was used, several products were shown to be present by paper chromatography.

5-4′-*Glyoxalinylmethyl*-2-*thiohydantoin Hydrochloride*.—1-Acetyl-5-(1-acetyl-4-glyoxalinylmethyl)-2-thiohydantoin (0·5 g.) was heated in ethanol–10N-hydrochloric acid (40 c.c.; 1 : 1) under reflux for 2 hr. On cooling, the *product* (0·26 g.) separated as pale yellow needles. Recrystallised from aqueous ethanol, it had m. p. 285—288° (decomp.) (Found: C, 36·1; H, 3·7; N, 24·3; S, 13·8. $C_7H_9ON_4SCl$ requires C, 36·1; H, 3·9; N, 24·1; S, 13·8%).

Pyrrolidino(1′ : 2′-1 : 5)-2-*thiohydantoin*.—L-Proline ethyl ester hydrochloride from L-proline (1 g.) was dissolved in dry acetone (15 c.c.) and treated with triethylamine (1·2 c.c.). After 15 minutes' shaking, the suspension was added to a solution of benzoyl *iso*thiocyanate [prepared by boiling a solution of ammonium thiocyanate (0·7 g.) and benzoyl chloride (0·7 c.c.) in acetone (15 c.c.) for 5 min.], and the mixture was boiled for a further 15 min., then poured into water. The oil which separated was extracted into ethyl acetate, and solvent was removed. The residual oil was heated in ethanol (30 c.c.) and 3N-hydrochloric acid (30 c.c.) for 3 hr. The white solid, which was deposited when the solution was concentrated under reduced pressure, was dissolved in ether, and the solution was washed with saturated aqueous sodium hydrogen carbonate, then water, and dried (Na_2SO_4). Removal of ether afforded the crystalline *thiohydantoin* (380 mg.), which, after recrystallisation from ethanol–light petroleum (b. p. 40—60°), had m. p. 161—163° (Found: C, 46·2; H, 5·3; N, 18·2. $C_6H_8ON_2S$ requires C, 46·1; H, 5·2; N, 17·9%).

α-N-*Acetylthiocarbamoyl*-L-*arginine*.—L-Arginine monohydrochloride (1·05 g.) was brought into reaction with methyl *N*-acetyldithiocarbamate (1·49 g.) in aqueous ethanol at pH 8·8. After removal of ethanol and extraction of excess of methyl *N*-acetyldithiocarbamate, the solution was evaporated to 10 c.c. and set aside at 0°. α-N-*Acetylthiocarbamoyl*-L-*arginine* (511 mg.) separated as its monohydrate and was recrystallised from water; it then darkened above 230° and finally melted at 252° with effervescence (Found: C, 36·5; H, 6·4; S, 10·9. $C_9H_{17}O_3N_5S,H_2O$ requires C, 36·8; H, 6·6; S, 11·0%).

5-3′-*Guanidinopropyl*-2-*thiohydantoin Hydrochloride*.—A solution of α-N-acetylthiocarbamoyl-L-arginine (200 mg.) in 2N-hydrochloric acid (2 c.c.) was heated under reflux for 1·5 hr. The solution was evaporated under reduced pressure to a syrup and then evaporated several times with ethanol. Final dissolution in ethanol and addition of ether afforded the crystalline *product* (150 mg.), m. p. 208—209° (effervescence) (Found: C, 33·4; H, 5·2; N, 27·4. $C_7H_{14}ON_5SCl$ requires C, 33·4; H, 5·6; N, 27·8%).

5-3′-*Aminopropyl*-2-*thiohydantoin Hydrochloride*.—A solution of DL-ornithine monohydrobromide (4 g.) in 50% aqueous pyridine (40 c.c.) was adjusted to pH 9 with triethylamine. After addition of methyl *N*-acetyldithiocarbamate (9·2 g.), the solution was heated under reflux for 3 hr., during which triethylamine (2 c.c.) was added in 6 portions. After 2 days at room temperature, the solution was filtered and concentrated under reduced pressure to an oil which was shaken with water and then extracted with benzene. The aqueous layer was acidified, extracted with three portions of ethyl acetate, then concentrated to a gum, which partially

[18] Gordon and du Vigneaud, *Proc. Soc. Exp. Biol. Med.*, 1953, **84**, 723.

solidified. The latter was dissolved in ethanol, and ether was added carefully to precipitate triethylamine hydrochloride (2 g.); further addition of ether precipitated a gum which was separated and heated in 5N-hydrochloric acid (25 c.c.) for 3 hr. Solvent was removed under reduced pressure; the residual gum was extracted with ethanol, and a small amount of insoluble material was removed. Addition of ether to the filtrate caused the *thiohydantoin hydrochloride* (860 mg.) to crystallize. It softened at 180° and was completely molten at 210° (Found : C, 32·7; H, 6·2; N, 19·5. $C_6H_{12}ON_3SCl,\frac{1}{2}H_2O$ requires C, 32·9; H, 6·0; N, 19·2%). Paper chromatography in three solvent systems and paper electrophoresis at pH 5·9 provided adequate evidence of its homogeneity.

5-4′-*Aminobutyl*-2-*thiohydantoin Hydrochloride*.—ε-*N*-Benzyloxycarbonyl-L-lysine (3·5 g.) and dry ammonium thiocyanate (2·1 g.) were heated under reflux for 20 min. in acetic anhydride (22·5 c.c.) and acetic acid (2·5 c.c.). The cooled mixture was poured into cold water (250 c.c.), and the resultant oil was extracted into chloroform. Solvent was removed under reduced pressure and the residue was heated in 3N-hydrochloric acid (50 c.c.) for 40 min. The solution was cooled and extracted with chloroform. The residual oil, after removal of chloroform, was further hydrolysed under similar conditions. The combined hydrolysates were evaporated to an oil under reduced pressure. The latter was extracted with ethanol, and the insoluble residue of the *thiohydantoin hydrochloride* (320 mg.) recrystallised from aqueous acetone; it then had m. p. 235—237° (Found : C, 37·4; H, 6·6; N, 19·0. $C_7H_{14}ON_3SCl$ requires C, 37·6; H, 6·3; N, 18·8%). Paper chromatography of the ethanol-soluble fraction indicated the presence of at least two substances, one of which was indistinguishable from the above thiohydantoin. The other was probably 5-4′-benzyloxycarbonylaminobutyl-2-thiohydantoin since, after treatment with 50% (w/v) hydrobromic acid in acetic acid at room temperature for 30 min. followed by precipitation with dry ether, paper chromatography in two solvents revealed the presence of a single substance having an R_F identical with that of 5-4′-aminobutyl-2-thiohydantoin hydrochloride. This was also obtained similarly to 5-3′-aminopropyl-2-thiohydantoin hydrochloride except that tri-*n*-butylamine replaced triethylamine. The resulting gum from 3·4 g. of lysine monohydrochloride was chromatographed on powdered cellulose (32 × 6·5 cm.) irrigated with *sec.*-butanol saturated with water, and a product (13 mg.) having m. p. 217—219° was separated. Although rather crude, this was indistinguishable by paper chromatography from the authentic thiohydantoin, gave a positive reaction with phosphotungstic acid, and had the expected light absorption.

N-*Benzoylthiocarbamoylsarcosine Ethyl Ester*.—The synthesis of this *compound* proceeded in 47% yield from sarcosine ethyl ester hydrochloride and benzoyl *iso*thiocyanate as described for the preparation of pyrrolidino(1′ : 2′-1 : 5)-2-thiohydantoin. Recrystallised from ethanol, it had m. p. 132° (Found : C, 55·9; H, 5·9; N, 10·3; S, 11·6. $C_{13}H_{16}O_3N_2S$ requires C, 55·7; H, 5·8; N, 10·0; S, 11·4%).

1-*Methyl*-2-*thiohydantoin*.—(*a*) *N*-Benzoylthiocarbamoylsarcosine ethyl ester (300 mg.) was heated in ethanol–10N-hydrochloric acid (1 : 1) for 2 hr. Solvents were removed under reduced pressure and the residue was shaken for 30 min. with sodium hydrogen carbonate solution. The insoluble residue, recrystallised from aqueous ethanol, had m. p. 224—226° (42 mg.) (Found : C, 36·5; H, 4·3. Calc. for $C_4H_6ON_2S$: C, 36·9; H, 4·7%).

(*b*) A solution of sarcosine ethyl ester (from 1 g. of hydrochloride) and methyl *N*-acetyldithiocarbamate (1 g.) in ethanol–ether (25 c.c.; 1 : 1) was kept at 37° for 7 days. Concentration of the solution precipitated unchanged methyl *N*-acetyldithiocarbamate which was removed. The filtrate was further evaporated to a gum, which was heated for 2 hr. with 2N-hydrochloric acid and left overnight at room temperature. The solution was extracted with ether, and the extract was dried and evaporated under reduced pressure. Fractional crystallisation of the residue from aqueous ethanol yielded 1-methyl-2-thiohydantoin (15 mg.), m. p. 217—219°, undepressed on admixture with the product from (*a*) and chromatographically indistinguishable from it.

(*c*) Sarcosine ethyl ester hydrochloride (1·5 g.) and potassium thiocyanate (1·5 g.) were heated in ethanol under reflux for 6 hr. Potassium chloride was removed and the filtrate was evaporated to a yellow oil, which was treated with 3N-hydrochloric acid for 2 hr. under reflux. Continuous ether-extraction of the solution afforded the crystalline thiohydantoin (17 mg.), m. p. 214—216°, chromatographically identical with the products from (*a*) and (*b*).

5-*Sulphomethyl*-2-*thiohydantoin*—A mixture of cysteic acid (3·4 g.) and methyl *N*-acetyldithiocarbamate (4·5 g.) in water (20 c.c.) and ethanol (20 c.c.) was kept at 40° and pH 8·5 for 24 hr., sodium hydroxide solution being added as necessary. The solution was evaporated under reduced pressure and extracted thoroughly with benzene. The aqueous layer was evaporated under reduced pressure to dryness, and the residual gum was dissolved in water (150 c.c.) and

passed through a column (30 × 2 cm.) of Dowex 50 resin (50—100 mesh) in the H^+-cycle. The column was washed with water 150 c.c.), and the combined percolate and washings were evaporated under reduced pressure to a gum. The latter in 2N-hydrochloric acid (150 c.c.) was heated for 1 hr. and then evaporated under reduced pressure to a white, deliquescent, crystalline *acid* (3 g.) (Found: C, 20·6; H, 3·6; N, 12·4. $C_4H_6O_4N_2S_2,H_2O$ requires C, 21·0; H, 3·5; N, 12·3%).

1-*Acetyl*-5-2′-*methylsulphonylethyl*-2-*thiohydantoin*.—DL-Methionine sulphone (4 g.) and ammonium thiocyanate (3·4 g.) were heated under reflux for 30 min. in acetic anhydride–acetic acid (20 c.c.; 9 : 1), then cooled and poured into water (100 c.c.), and the precipitated crystalline *product* (3·77 g.) was filtered off, washed with water, and dried. Recrystallised from dilute aqueous ethanol (charcoal), it had m. p. 195·5°, unchanged on further recrystallisation (Found: C, 36·6; H, 4·6. $C_8H_{12}O_4N_2S_2$ requires C, 36·6; H, 4·6%).

5-2′-*Methylsulphonylethyl*-2-*thiohydantoin*.—1-Acetyl-5-2′-methylsulphonylethyl-2-thiohydantoin (3 g.) was hydrolysed by hot 2N-hydrochloric acid (30 c.c.) for 1·5 hr. On cooling, the *product* (2·45 g.) crystallised and had m. p. 227—228° (decomp. after softening and darkening from about 200°). It recrystallised from water in cream-coloured needles, m. p. 228—229° (decomp. as before) (Found: C, 32·7; H, 4·4; N, 12·3; S, 28·4. $C_6H_{10}O_3N_2S_2$ requires C, 32·4; H, 4·5; N, 12·6; S, 28·8%).

5-*Methylthiomethyl*-2-*thiohydantoin*.—*S*-Methylcysteine (811 mg.) and ammonium thiocyanate (912 mg.) in 9 : 1 acetic anhydride–acetic acid (10 c.c.) were heated under reflux for 25 min. The mixture was cooled and poured into water. After being kept at 0° overnight, the crude product was collected and heated with 2N-hydrochloric acid (10 c.c.) for 1 hr. (hydrogen sulphide was copiously evolved). On cooling, an orange mass was precipitated; twice recrystallised from water (charcoal), it yielded colourless crystals (56 mg.) of *product*, m. p. 169—170° (Found: C, 34·4; H, 4·5; N, 15·3. $C_5H_8ON_2S_2$ requires C, 34·1; H, 4·6; N, 15·9%).

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