1154. N-2,4-*Dinitro*-6-*sulphophenylamino-acids: Acid Hydrolysis*

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N-2,4-Dinitro-6-sulphophenyl-α-amino-acids undergo hydrolysis in acid solution to give 2,4-dinitro-6-sulphophenol and the corresponding amino-acid. The mechanism of the reaction is discussed.

The reaction of 2,4-dinitro-6-sulphophenyl chloride (DNSP-Cl) (2-chloro-3,5-dinitrophenyl sulphonic acid) with an amino-acid in the presence of base gives the *N*-2,4-dinitro-6-sulphophenylamino-acid (DNSP-amino-acid). A number of these have been prepared and isolated as their bis-*S*-benzylthiouronium salts. The decomposition in acid solution of these DNSP-amino-acids and of some 2,4-dinitro-6-sulphoanilines is examined in this Paper.

Experimental

Melting points were taken on a Kofler hot-stage microsope. Micro-analyses were by Weiler and Strauss, Oxford. Ultraviolet absorption spectra were taken by hand on a Unicam S.P. 500 spectrophotometer using solutions of the bis-*S*-benzylthiouronium salts in 50% ethanol. For all work on these compounds it is advisable to avoid exposure to strong light as photo-decomposition is sometimes rapid.

Preparation of Sodium 2-Chloro-3,5-dinitrophenyl Sulphonate.[1]—Sodium 2-chloro-5-nitrobenzenesulphonate (20 g.) was heated gently under reflux for 3 hr. with a mixture of concentrated sulphuric acid (80 ml.) and nitric acid (S.G. 1·5; 40 ml.). The mixture was cooled and to it was added, cautiously, 10% aqueous sodium hydroxide (120 ml.). A copious pale yellow precipitate of the sodium salt formed, which was recrystallised from water (yield 72%). *S-Benzylthiouronium salt*, m. p. 87°, 156—157, 168·5—169° (polymorphic) (Found: C, 37·6; H, 3·15; N, 12·75. $C_{14}H_{13}ClN_4O_7S_2$ requires C, 37·5; H, 2·9; N, 12·5%).

Preparation of DNSP-Derivatives.[1]—The DNSP-derivatives were prepared by warming sodium 2-chloro-3,5-dinitrobenzenesulphonate with the appropriate compound in alkaline solution. For the preparation of the DNSP-amino-acids, triethylamine was used as base, and in the case of DNSP-sarcosine the reaction was carried out in the cold for 4 days. Removal of excess of base and treatment with *S*-benzylthiouronium chloride gave the *S*-benzylthiouronium salts which were recrystallised from aqueous ethanol. *Bis*-S-*benzylthiouronium salt* of 2,4-dinitro-6-sulphophenol, m. p. 191—192°, $\lambda_{max.}$ 364, 400sh mμ (Found: C, 44·2; H, 3·9; N, 14·2. $C_{22}H_{24}N_6O_8S_3$ requires C, 44·3; H, 4·05; N, 14·1%). 2,4-*Dinitro*-6-*sulphoaniline* S-*benzylthiouronium salt*, m. p. 160°, $\lambda_{max.}$ 335, 380sh mμ (Found: C, 39·2; H, 3·6; N, 16·5. $C_{14}H_{15}N_5O_7S_2$ requires C, 39·15; H, 3·5; N, 16·3%). N-*Methyl*-2,4-*dinitro*-6-*sulphoaniline* S-*benzylthiouronium salt*, m. p. 138—139·5°, $\lambda_{max.}$ 362 mμ (Found: C, 40·8; H, 3·7; N, 15·9. $C_{15}H_{17}N_5O_7S_2$ requires C, 40·6; H, 3·9; N, 15·8%). N,N-*Dimethyl*-2,4-*dinitro*-6-*sulphoaniline* S-*benzylthiouronium salt*, m. p. 134·5—136°, $\lambda_{max.}$ 386 mμ (Found: C, 42·0; H, 4·45; N, 15·0. $C_{16}H_{19}N_5O_7S_2$ requires C, 42·0; H, 4·2; N, 15·3%). N-2,4-*Dinitro*-6-*sulphophenylglycine bis*-S-*benzylthiouronium salt*, m. p. 172—172·5°, $\lambda_{max.}$ 361 mμ (Found: C, 44·2; H, 4·4; N, 15·2. $C_{24}H_{27}N_7O_9S_3$ requires C, 44·1; H, 4·2; N, 15·0%). N-2,4-*Dinitro*-6-*sulphophenylsarcosine bis*-S-*benzylthiouronium salt*, m. p. 163—165°, $\lambda_{max.}$ 384 mμ (Found: C, 45·2; H, 4·4; N, 14·7. $C_{25}H_{29}N_7O_9S_3$ requires C, 45·0; H, 4·4; N, 14·7%). N-2,4-*Dinitro*-6-*sulphophenyl*-DL-α-*alanine bis*-S-*benzylthiouronium salt*, m. p. 162—163°, $\lambda_{max.}$ 364 mμ (Found: C, 45·0; H, 4·0; N, 14·35%). N-2,4-*Dinitro*-6-*sulphophenyl*-β-*alanine bis*-S-*benzylthiouronium salt*, m. p. 162·5°, $\lambda_{max.}$ 364 mμ (Found: C, 45·0; H, 4·6; N, 14·7%). The *N*-2,4-dinitro-6-sulphophenyl-α-aminoisobutyric acid bis-*S*-benzylthiouronium salt, $\lambda_{max.}$ 372 mμ, whilst electrophoretically homogenous, had a rather indefinite m. p. and did not give satisfactory analytical figures. It is probable that the solid was contaminated with some of the mono-*S*-benzylthiouronium salt as the carboxyl group is tertiary in this case.

Electrophoresis.—High-voltage electrophoresis at pH 4·4 (pyridine–acetic acid) was used routinely to follow the preparation, purification, and decomposition of the DNSP-compounds. Electrophoresis at pH 1·9 (formic acid–acetic acid) and pH 6·0 (phosphate) gave further confirmation where needed. The mobilities of the compounds used in this study relative to that of DNSP-OH at the same pH are shown in the Table.

[1] F. Ullmann and E. Herre, *Annalen*, 1909, **366**, 111.

Electrophoretic mobilities of DNSP-compounds (DNSP-OH = 1·0)

	pH 1·9	pH 4·4	pH 6·0		pH 1·9	pH 4·4	pH 6·0
DNSP-Cl	0·90	0·60	0·61	DNSP-β-Alanine	0·67	0·65	0·91
DNSP-NH_2	0·80	0·54	0·51	DNSP-α-Aminoiso-			
DNSP-NHMe	0·84	0·54	0·52	butyric acid	0·68	0·81	0·93
DNSP-NMe_2	0·83	0·54	0·53	DNSP-Sarcosine	—*	0·61*	0·92
DNSP-Glycine	0·71	0·90	0·93	Salicylic acid	0·075	0·67	0·72
DNSP-α-Alanine	0·71	0·91	0·96	2,4-Dinitrophenol	0·004	0·35	0·48

* DNSP-Sarcosine partly hydrolyses during electrophoresis at pH 4·4 and is completely hydrolysed at pH 1·9. Mobility at pH 5·3 is 0·78.

All the DNSP-derivatives except DNSP-Cl are yellow in visible light. On paper, under ultraviolet light (366 mμ), most of the DNSP-derivatives show up black, but DNSP-NH_2 shows a yellow fluorescence. DNSP-Cl is more readily detected if it is converted into DNSP-NH_2 by hanging the completed electropherogram in ammonia vapour. The DNSP-OH spot is bleached by formic acid vapour.

Reaction of 2,4-*Dinitro*-6-*sulphoaniline and its* N-*Methyl and* NN-*Dimethyl Derivatives with Hydrochloric Acid.*—The *S*-benzylthiouronium salt of the 2,4-dinitro-6-sulphoaniline (20—40 mg.) was converted to the sodium salt by treatment with Dowex-50 resin in the Na^+ form. The sodium salt was taken up in 5·7N-hydrochloric acid (20 ml.) and heated at 100° under nitrogen in the dark for 24 hr. The mixture was cooled and diluted with water (80 ml.). The diluted solution was extracted with ethyl acetate (3 × 20 ml.) and the ethyl acetate extract washed once with 10% sodium carbonate solution (10 ml.). The volumes and the optical densities at 370 mμ of the ethyl acetate extract and of the aqueous residue were determined and used to calculate the amount of desulphonation which had occurred.

A portion of the aqueous residue was examined by electrophoresis. A portion of the ethyl acetate extract was examined by thin-layer chromatography on Silica Gel G (Merck) in the systems ethyl acetate–toluene (1 : 1) on activated plates, and methanol–chloroform–water–medicinal paraffin (5 : 5 : 3 : 2).[2] In the latter case the samples were applied to the activated plate which was then equilibrated for 1 hr. in a tank saturated with the vapour of the top phase of this sytem and the chromatogram was then run using the bottom phase.

Hydrolysis of 2,4-*Dinitro*-6-*sulphophenylamino-acids.*—*Qualitative examination.* The DNSP-amino-acid bis-*S*-benzylthiouronium salt in 5·7N-hydrochloric acid (1 mg. per ml.) was heated at 100° in the dark until the solution had become virtually colourless. The solution was taken to dryness and the residue examined by high-voltage electrophoresis (nitro-compound) and by paper chromatography in the systems n-butanol–acetic acid–water (4 : 1 : 5), propan-2-ol–ammonia–water (20 : 1 : 2), and methyl ethyl ketone–t-butyl alcohol–water–diethylamine (5 : 10 : 10 : 1). The chromatograms were sprayed with ninhydrin to reveal amino-acids.

Rate determinations. For the determinations at 50° a thermostat-controlled cell housing fitted to the Unicam S.P. 500 spectrophotometer was used. For the determinations at 100° the solutions were heated in stoppered tubes in a water-bath, samples being withdrawn at intervals. The DNSP-amino-acid bis-*S*-benzylthiouronium salt was dissolved in 5·7N-hydrochloric acid at the appropriate temperature and the decrease in optical density (near the maximum for the particular compound used) with time followed. DNSP-OH has very little absorption in the 350—400-mμ region in 5·7N-hydrochloric acid. Plots of log O.D. (corrected for absorption at $t = \infty$) versus time gave straight lines, which were used to calculate $t_{\frac{1}{2}}$. The rate of decomposition of a DNSP-glycine triethylammonium salt was also determined, and gave similar results to those obtained with the bis-*S*-benzylthiouronium salt.

Reaction of N-2,4-*Dinitro*-6-*sulphophenylsarcosine with Dimethylamine Hydrochloride in Dimethylformamide.*—DNSP-Sarcosine bis-*S*-benzylthiouronium salt (14 mg.) and dimethylamine hydrochloride (25 mg.) in dimethylformamide (1 ml.) were heated at 100° for 1 hr. The dimethylformamide was removed under reduced pressure and the residue examined by electrophoresis at pH 4·4 and by chromatography in the propan-2-ol–ammonia–water system, developing with ninhydrin. An authentic sample of sarcosine dimethylamide prepared from chloroacetyl chloride by reaction first with dimethylamine and then with methylamine [3] was run as a standard. The identity of the DNSP-derivatives was confirmed by electrophoresis at pH 5·3.

[2] A. M. Asatoor, *J. Chromatog.*, 1960, **4**, 144.
[3] S. G. Waley and J. Watson, *Proc. Roy. Soc.*, 1949, *A*, **119**, 499.

Results and Discussion

Unlike the corresponding *N*-2,4-dinitrophenylamino-acids [4] (DNP-amino-acids), the DNSP-amino-acids are readily hydrolysed in acid solution. Thus, DNSP-glycine, on being heated for 4 hr. with 5·7N-hydrochloric acid at 100°, is almost completely converted into 2,4-dinitro-6-sulphophenol (DNSP-OH) and glycine. The other DNSP-α-amino-acids examined (DNSP-sarcosine, DNSP-α-alanine, and DNSP-α-aminoisobutyric acid) behave similarly, giving DNSP-OH as the only coloured product, and the parent amino-acid as the only ninhydrin-positive product.

DNSP-β-Alanine is scarcely attacked by acid under the conditions used for the DNSP-α-amino-acids. On prolonged heating with acid, however, it is converted largely into DNP-β-alanine, but with traces of 2,4-dinitro-6-sulphoaniline and other material present. The action of acid on 2,4-dinitro-6-sulphoaniline and its *N*-methylated derivatives is also slow:

Reactions of 2,4-dinitro-6-sulphoanilines in 5·7N-hydrochloric acid at 100° for 24 hr.

	DNSP-NH_2	DNSP-NHMe	DNSP-NMe_2
Desulphonation (%)	30	70	70
Desulphonated products...	DNP-NH_2	DNP-NMHe *	DNP-NHMe, DNP-NMe_2 (1 : 2)
DNSP-OH	—	Trace	Trace
DNSP-Cl	—	—	Trace

* Precipitates from the reaction mixture. M. p. and infrared spectrum identical with those of an authentic sample.

The DNSP-NHMe product also contained traces of DNSP-NH_2, recognisable by its yellow fluorescence, indicating that demethylation largely precedes desulphonation. (It is not possible to distinguish between DNSP-NHMe and DNSP-NMe_2 by electrophoresis.) DNP-NMe_2 undergoes only about 5% demethylation under comparable conditions. The desulphonation reaction is analogous to that undergone by 2,6-dinitro-4-sulphoaniline in 50% sulphuric acid.[5]

The difference in the rates of reaction of the DNSP-α-amino-acids and the DNP-α-amino-acids is undoubtedly due to the sulphonic acid residue of the DNSP-compounds assisting protonation of the amino-group. This also accounts for the more ready demethylation in the aniline derivatives studied. Further, while the DNSP-α-amino-acids are all hydrolysed comparatively rapidly there are also surprising differences in the rate of hydrolysis between the individual DNSP-amino-acids studied.

Half-lives of DNSP-amino-acids in 5·7N-hydrochloric acid (min.)

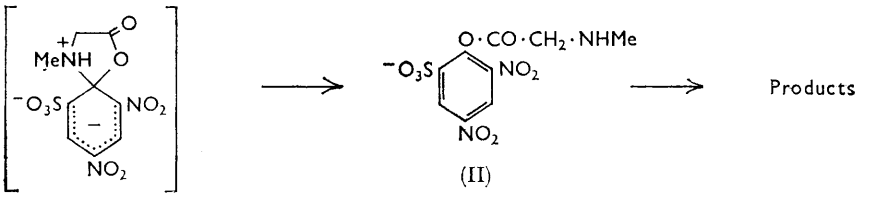
	DNSP-gly	DNSP-α-al	DNSP-α-AIBA	DNSP-sar
At 50°	—	500	10	1
At 100°	45	8	—	—

These rates reflect the relative base-strengths of the amino-groups in these compounds, and this, together with the effect of the neighbouring sulphonic acid group, indicates that the protonation of the amino-group is a rate-determining step in the reaction.

The ready hydrolysis reactions of the DNSP-α-amino-acids compared with the 2,4-dinitro-6-sulphoanilines suggests that the amino-acid carboxyl group participates in the nucleophilic displacement of the protonated amino-group by forming a cyclic intermediate (I)—illustrated for DNSP-sarcosine. The intermediate is analogous to the cyclic Meisenheimer complex formed so readily from 2′-hydroxyethyl 2,4-dinitrophenyl ether and alkali.[6] The cyclic intermediate (I) will immediately rearrange to the ester (II) which would rapidly hydrolyse to give the amino-acid and DNSP-OH.

[4] R. R. Porter, *Methods in Medical Research*, 1951, **3**, 256.
[5] L. H. Welsh, *J. Amer. Chem. Soc.*, 1941, **63**, 3276.
[6] R. J. Pollitt and B. C. Saunders, *J.*, 1964, 1132.

Support for the participation of the ester intermediate (II) is obtained from the reaction of DNSP-sarcosine with dimethylamine hydrochloride in dimethylformamide. Here, DNSP-OH is produced as usual, but in addition to traces of sarcosine, another ninhydrin-positive product with the same chromatographic properties as authentic sarcosine dimethylamide is produced. This could have arisen from the attack of dimethylamine (or dimethylformamide) on the ester (II). Direct nucleophilic displacement on the benzene ring would have given sarcosine and DNSP-NMe_2.



(I) (II)

The acid decomposition of DNP-amino-acids has been studied by workers who were interested in quantitative aspects of the use of dinitrophenylation for the determination *N*-terminal groups of proteins. Of the DNP-amino-acids examined only DNP-glycine, DNP-sarcosine, bis-DNP-cystine, and the DNP-prolines were markedly labile. DNP-Proline and DNP-sarcosine are hydrolysed readily by acid. The latter gives 2,4-dinitrophenol and sarcosine almost quantitatively,[7] but the reactions of DNP-proline are complicated by ring-opening to give α-chloro-δ-DNP-aminovaleric acid and δ-chloro-α-DNP-aminovaleric acid.[8] However, Scanes and Tozer[8] conclude that the actual hydrolysis reaction to give proline and 2,4-dinitrophenol involves DNP-proline itself. Thus, both these imino-acids are hydrolysed readily, probably in an analogous manner to the DNSP-derivatives. DNP-Glycine and bis-DNP-cystine are decomposed more slowly, 40% and 25% being recovered after 8 and 12 hr., respectively, in boiling 5·7N-hydrochloric acid.[4] Acher and Laurilla[9] find that both these compounds give the free amino-acid on decomposition, but Ruske[7] states that glycine gives glycolic acid and no ninhydrin-positive substance. There is a need for reinvestigation of this reaction, but the relative stability of DNP-α-alanine, for example, (80% recovered after 12 hr.[4]) indicates that the decomposition of DNP-glycine is probably not analogous to the hydrolysis of DNSP-derivatives.

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[7] W. Ruske, *Annalen*, 1957, **610**, 156.
[8] F. S. Scanes and B. T. Tozer, *Biochem. J.*, 1956, **63**, 282.
[9] R. Acher and U. R. Laurilla, *Bull. Soc. Chim. biol.*, 1953, **35**, 413.