547. *A Coloured Maleimide and its Interaction with Cysteine.*

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The preparation of *N*-2,4-dinitroanilinomaleimide is described. Cyclization of the cysteine addition compound of this maleimide, involving the amino-group of cysteine and resulting in scission of the succinimide ring, has been investigated. Evidence is presented that other maleimides react in a like manner.

N-Ethylmaleimide reacts rapidly with thiols forming a stable thio-ether link, and has had frequent application as a reagent for thiol groups of proteins.[1] However, as a marker of such groups it is of little value as it forms colourless derivatives. We report the preparation of a coloured maleimide, *N*-2,4-dinitroanilinomaleimide, which we have used to label protein thiols,[2] and we also describe a novel reaction with cysteine which appears to be general also for other maleimides.

Compound	Solvent	Absorption bands in the region 1550—1800 cm.$^{-1}$
N-Ethylmaleimide [a]	$CHCl_3$	1712, 1594
N-(1-Naphthyl maleimide) [a]	$CHCl_3$	1745sh, 1724, 1602
N-2,4-Dinitroanilinomaleamic acid (I)	Nujol	1695, 1615sh, 1580
N-2,4-Dinitroanilinomaleimide (II)	CH_2Cl_2	1750, 1620, 1605sh
	Nujol	1730, 1620, 1595sh
p-Phenylazomaleanil	CH_2Cl_2	1715, 1595
1,6-Dihydro-3-hydroxy-6-oxo-1-phenylpyridazine (IV)	Nujol	1650, 1590, 1560
[IX; R = H, R′ = 2,4-$(NO_2)_2C_6H_3$·NH]	Nujol	1665, 1640sh, 1610, 1580
(IX; R = CO_2Me; R′ = *p*-Ph·N_2·C_6H_4·NH)	Nujol	1745, 1650, 1590
	CH_2Cl_2	1740, 1680, 1590

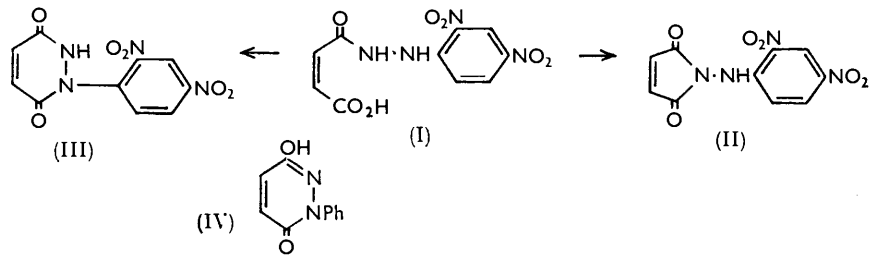
[a] Tsou, Barnett, and Seligman, *J. Amer. Chem. Soc.*, 1955, **77**, 4613.

Reaction of 2,4-dinitrophenylhydrazine with maleic anhydride gave the hydrazide (I) which was dehydrated in hot acetic acid. The latter step could result in the formation of either the maleimide (II) or the pyridazine derivative (III).

[1] Boyer, in " The Enzymes," (eds. Boyer, Lardy, and Myrback, Academic Press, New York, 1959), p. 511.

[2] Clark-Walker, unpublished work.

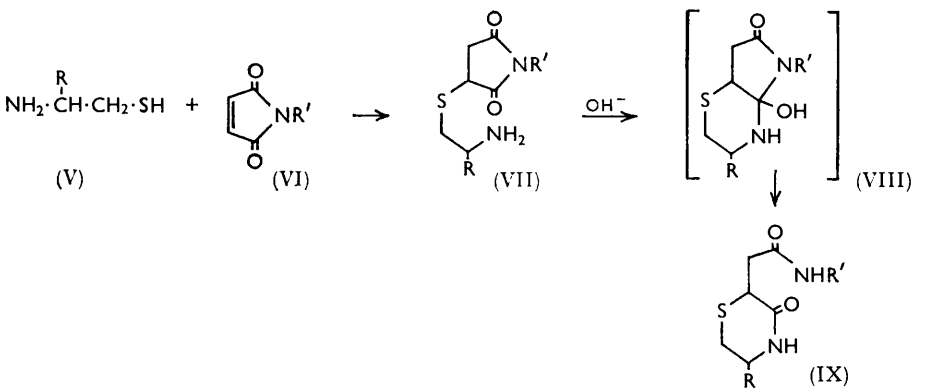
A decision in favour of the former was made on the basis of the infrared spectrum (see Table). The band at 1750 cm.$^{-1}$ in dichloromethane lies in the region associated with known maleimide-carbonyl frequencies. In contrast, the pyridazine derivative (IV)



which is obtained [3,4] by condensation of maleic anhydride and phenylhydrazine does not absorb above 1700 cm.$^{-1}$. We find that compound (IV), unlike maleimides and our product (II), gives a positive test with ferric chloride and is unreactive towards cysteine. It appears from these results that the effect of the nitro-groups on the benzene ring decreases the nucleophilic power of the α-nitrogen atom, causing condensation to occur at the β-nitrogen, whereas, in their absence the reaction occurs at the α-nitrogen.

Compound (II) was then tested for its reactivity towards cysteine and glutathione. These thiols added readily at room temperature and over the tested pH range of 3—8. However, chromatography with butanol–acetic acid–water indicated that at alkaline pH the cysteine addition compound (VII) [R = CO_2H, R′ = 2,4-$(NO_2)_2C_6H_3$·NH] rapidly gave a new substance with a greater R_F value and unreactive to ninhydrin. The glutathione addition compound showed no such change.

Attempts to isolate the resultant ninhydrin-negative compound proved unsuccessful as the product was always contaminated with the maleamic acid resulting from the hydrolysis of unchanged imide (II). By replacing cysteine with 2-mercaptoethylamine a similar masked amino-compound could be separated from all contaminants by ether-extraction under acid and alkaline conditions. A similar compound was prepared from



p-phenylazomaleanil [5] and methyl-L-cysteine. The infrared spectra of the compounds are given in the Table. The carbonyl absorptions (1665, 1650, and 1680 cm.$^{-1}$) are substantially lower than those of the corresponding maleimides. The absorptions fall in the region associated with amide-carbonyl and indicate that the five-membered succinimide ring no longer exists. This evidence together with the affinity for non-polar solvents and

[3] Biquard and Grammaticakis, *Bull. Soc. chim. France*, 1942, **9**, 675.
[4] Druey, Huni, Meier, Ringier, and Staehelin, *Helv. Chim. Acta*, 1954, **37**, 510.
[5] Nayler and Whiting, *J.*, 1955, 297.

ninhydrin-negativity indicates that the amino-group of the mercaptoethylamine, methylcysteine, and (by analogy) cysteine addition compounds has become involved in cyclization with the adjacent carbonyl group of the succinimide ring, leading to the formation of a substituted 1,4-thiazine (IX).

We have also observed the cyclization to occur with cysteine addition compounds of *N*-ethyl-, *N*-phenyl-, *N*-*p*-methoxyphenyl-,[6] and *N*-*p*-nitrophenyl-maleimide.[6]

Experimental

Chromatography.—Ascending paper chromatography was used with Whatman No. 1 paper with butanol–ethanol–ammonia (*d* 0·88)–water (200 : 47 : 3 : 88) and butanol–acetic acid–water (120 : 30 : 50). The solvents were renewed after 7 days.

Infrared Analyses.—Infrared spectra were obtained on a Perkin–Elmer recording spectrophotometer model 137 for Nujol mulls or 0·01M-solutions in dichloromethane.

Cyclization of Cysteine Product.—A solution of the maleimide (0·01 molar) in acetone was treated with an equal quantity of cysteine (0·01 molar) at room temperature for 10 min., then chromatographed in both the acid and the alkaline solvent for 5 hr. Cyclization was associated with disappearance of a free amino-group and was tested for by the use of a ninhydrin dipping reagent (0·3% in chloroform).

Preparations.—N-2,4-*Dinitroanilinomaleamic acid* (I). Maleic anhydride (16 g.) and 2,4-dinitrophenylhydrazine (30 g.) were refluxed in chloroform (350 ml.) with occasional shaking for ½ hr. After cooling, the orange solid was collected. Four crystallizations from acetone–benzene gave the *maleamic acid* as yellow needles, m. p. 194° (decomp.) (Found: C, 41·0; H, 3·1; N, 18·7. $C_{10}H_8N_4O_7$ requires C, 40·5; H, 2·7; N, 18·9%).

N-2,4-*Dinitroanilinomaleimide* (II). Compound (I) (5 g.) was refluxed in " AnalaR " acetic acid (50 ml.) for 10 min. The yellow *imide* crystallized (4 g.). Five crystallizations from acetone–benzene yielded pale yellow needles, m. p. 237° (decomp.) (Found: C, 43·3; H, 2·5; O, 34·1. $C_{10}H_6N_4O_6$ requires C, 43·2; H, 2·25; O, 34·5%), soluble in acetone but insoluble in benzene and water. In dilute alkali it gave a dark brown solution from which the amic acid could be recovered.

N^2-(2,4-*Dinitrophenyl*)-α-(*tetrahydro*-3-*oxo*-1,4-*thiazin*-2-*yl*)*acethydrazide* [IX; R = H, R′ = 2,4-$(NO_2)_2C_6H_3$·NH]. Compound (II) (1·5 g.) in acetone (20 ml.) was treated with an excess of 2-mercaptoethylamine hydrochloride (0·65 g.) in water (5 ml.). After 15 min. 0·1M-ammonium carbonate buffer (pH 9; 50 ml.) was added and the solution set aside. After 8 hr. ether was added; a precipitate was formed and was collected. Crystallization from acetone–water gave the *product* as yellow rods (0·8 g.) (Found: C, 41·0; H, 4·0; O, 27·4; S, 8·5. $C_{12}H_{13}N_5O_6S$ requires C, 40·6; H, 3·7; O, 27·0; S, 9·0%). The compound was unchanged when heated in toluene at 100° for 7 hr.

Methyl tetrahydro-5-*oxo*-6-(N^2-*phenylazophenylhydrazinocarbonylmethyl*)-1,4-*thiazine*-3-*carboxylate* (IX; R = CO_2Me, R′ = *p*-Ph·N_2·C_6H_4·NH). *p*-Phenylazomaleanil (0·2 g.) in acetone (10 ml.) was treated with an excess of methyl-L-cysteine (0·1 g.) in water (1 ml.). After 8 hr. at room temperature 5% aqueous ammonia and toluene were added until two phases formed. The toluene extract was washed with dilute mineral acid. Evaporation and crystallization of the residue from aqueous alcohol gave the *thiazine* as orange needles, m. p. 159—160°, $[\alpha]_D^{20}$ +16·8° (1% in $CHCl_3$) (Found: C, 58·3; H, 4·9; N, 13·3; S, 7·6. $C_{20}H_{20}N_4O_4S$ requires for C, 58·2; H, 4·9; N, 13·6; S, 7·8%).

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Nedlands, Western Australia. [*Received, November 30th*, 1960.]

[6] Roderick, *J. Amer. Chem. Soc.*, 1957, **79**, 1710.