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STUDIES IN PEROXIDASE ACTION—XVI*

A CHEMICAL SYNTHESIS OF THE MESIDINE PEROXIDATION PRODUCT

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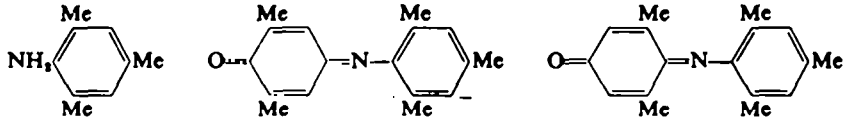
Abstract—Mesidine, a standard used in connection with peroxidase oxidations, has been converted by an unambiguous chemical synthesis into the product identical with that obtained by the direct enzymic oxidation.

A STANDARD substrate of importance in the investigations of peroxidase reactions is mesidine (I) which is oxidized by the enzyme[1] cleanly and almost quantitatively to the dark purple crystalline 2,6-dimethyl-*p*-benzoquinone-4-(2′,4′,6′-trimethyl) anil (II).

Mesidine has therefore been suggested from time to time in connection with possible methods for investigating peroxidase activity.[2-6] Chapman and Saunders based their evidence for the structure II on the following observations:



I II III

(1) Hydrolysis to mesidine (I) and 2,6-dimethyl-*p*-benzoquinone.

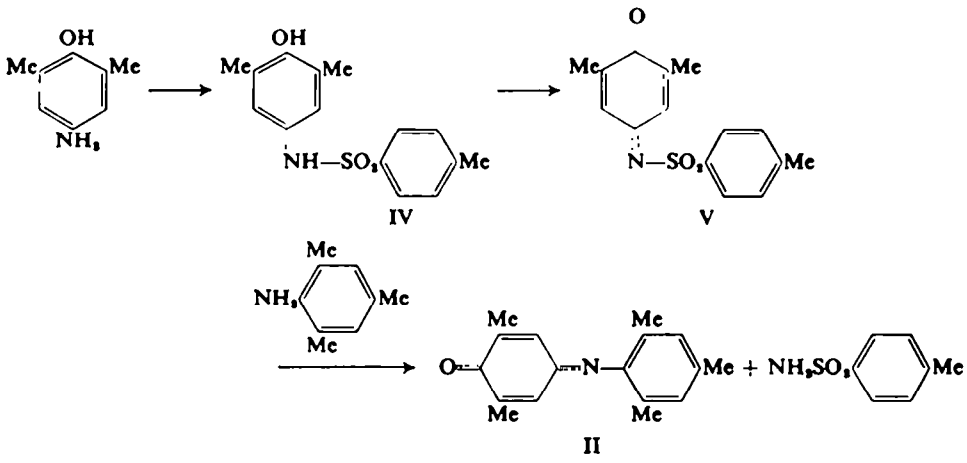
(2) Chemical synthesis by condensing I with 2,6-dimethyl-*p*-benzoquinone. Here it was assumed that the steric effects of the two ortho-methyl groups must necessarily lead to structure II.

(3) Item (2) does not clearly distinguish between structures II and III. However, Chapman and Saunders noted that 2,3,6-trimethyl-*p*-benzoquinone reacted with only one molecule of mesidine presumably at C_4, strongly suggesting that 2,6-dimethyl-*p*-benzoquinone condenses at position 4. Furthermore mesidine fails to condense with duroquinone, presumably because of the steric effects of the four *o*-methyl-groups. The main structural features of II are confirmed by the IR spectrum. The absence of bands in the 3500–3200 cm^{-1} region showed that there were no NH or OH groups. A strong band at 1645 cm^{-1} confirms the quinonoid C=O and a band at 1612 cm^{-1} may be due to C=N. The band at 858 cm^{-1} is due to the 1,2,3,5-substituted benzene ring.

* Part XV *Tetrahedron* **23**, 473 (1967).

[1] N. B. Chapman and B. C. Saunders, *J. Chem. Soc.* 496 (1941).
[2] B. C. Saunders, Second International Congress of Biochemistry, Paris (1951).
[3] Y. Avi-Dor and K. G. Paul, *Acta Chem. Scand.* **7**, 444, (1953).
[4] G. M. K. Hughes, B. M. Roberts and B. C. Saunders *Chem. & Ind.* 471 (1954).
[5] K. G. Paul and Y. Avi-Dor, *Acta Chem. Scand.* **8**, 649 (1954).

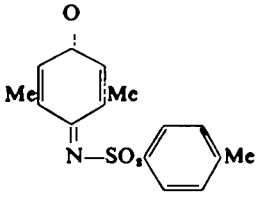
Nevertheless further evidence in favour of structure II is desirable. Adams *et al.* have investigated the reactions of *p*-benzoquinone dibenzene sulphonimide and amines[6] and from their proposed mechanism for the reaction, it seemed to us possible to synthesize II by a method which would further substantiate its structure. This we have achieved by the following set of reactions:



2,6-Dimethyl-4-nitrophenol was catalytically reduced to the amino-compound which gave 2,6-dimethyl-4-toluene-*p*-sulphonamidophenol (IV) in high yield. Compound IV was oxidized by lead tetracetate in glacial acetic acid to 2,6-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (V). The IR spectrum of V showed complete absence of OH and NH groups, whereas bands corresponding to C=N, C=O and SO_2 were prominent.

Condensation of V with mesidine in dioxan containing 20% acetic acid was effected in 3 minutes at 100°. Toluene-*p*-sulphonamide separated and chromatography of the dark purple mother liquor gave dark purple needles, $C_{17}H_{19}NO$, m.p. 92–94°, having UV and IR spectra, identical with those of the enzymic oxidation product.

By a similar sequence of reactions, 3,5-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (VI) was prepared from 3,5-dimethylphenol. IR spectrum again showed absence of OH and NH groups. Reaction of VI under a variety of conditions



yielded only intractable tars from which no crystalline material could be obtained,

[6] R. Adams and K. A. Schowalter, *J. Amer. Chem. Soc.* **74**, 2597 (1952).

thus providing some further evidence against III as a possible formula for the enzymic product.

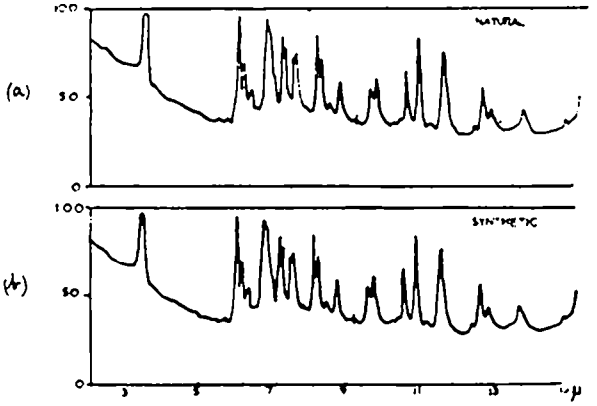


FIG. 1. IR spectrum (Nujol) of the oxidation product of mesidine.
(a) Enzymic
(b) Synthetic.

EXPERIMENTAL

Peroxidase oxidation of mesidine. Mesidine (5 g) dissolved in distilled water (1 l.) containing sufficient glacial AcOH to give a soln of pH 4·5 was oxidized.[1] The product was filtered off without suction, to avoid loss of volatile formaldehyde, yield 4·2 g, m.p. 84·88° which was raised to 92–93° by recrystallization from aqueous EtOH. (Found: C, 80·9; H, 7·72; N, 5·59. Calc. for $C_{17}H_{19}NO$: C, 80·7; H, 7·51; N, 5·54%.) UV (95% EtOH) λ_{max} 484–91 275 mμ, (log ε 3·04, 4·443); λ_{min} 376, 239 mμ, (log ε 2·556, 3·519). IR spectrum (Nujol) showed no prominent bands in the 3400–3000 cm^{-1} region, but possessed prominent bands at 1645, 1612, 1545, 1365, 1325, 1315, 1218, 1209, 1137, 1042, 1022, 912, 858, 787, 775 and 727 cm^{-1}.

Examination of the filtrate. To the filtrate (10 ml) was added an aqueous soln of 5,5-dimethylcyclohexane-1,3-dione (dimedone; 10 ml, 10%) and the mixture stoppered and left for 2 hr at room temp. A white crystalline ppt was filtered off, m.p. 184–186°. Recrystallization from EtOH raised m.p. to 189°, which was not depressed by admixture with authentic sample of the dimedone derivative of formaldehyde, m.p. 189°.

2,6-*Dimethyl-4-nitrophenol* was prepared from 2,6-dimethylphenol,[7,8] m.p. 171–172°. (Lit. m.p. 169°, 171°.) *O-Benzoyl derivative*, m.p. 121°. (Found: C, 66·15; H, 5·12; N, 5·4. $C_{16}H_{13}NO_4$ requires: C, 66·4; H, 4·85; N, 5·2%.)

2,6-*Dimethyl-4-aminophenol hydrochloride.* 2,6-Dimethyl-4-nitrophenol (5·1 g) dissolved in EtOH (25 ml) was catalytically reduced with H in the presence of Raney Ni (500 mg). When uptake ceased, the catalyst was filtered off and ether (500 ml) saturated with dry HCl was added slowly to the filtrate. The precipitated amine hydrochloride was recrystallized from a mixture of dry ether and EtOH, yield 4·21 g (80%). The 2,6-*dimethyl-4-aminophenol hydrochloride* sublimes above 220° without melting. (Found: C, 55·8; H, 7·01; N, 8·0. $C_8H_{12}ClNO$ requires: C, 55·5; H, 6·93; N, 8·1%.)

2,6-*Dimethyl-4-toluene*-p-*sulphonamidophenol.*[9] Toluene-*p*-sulphonyl chloride (6·4 g) dissolved in dry pyridine (10 ml) was added slowly to 2,6-dimethyl-4-aminophenol hydrochloride (5·75 g) dissolved in dry pyridine (25 ml). The mixture, after standing at 15° for 24 hr, was poured onto a mixture of ice (200 g) and conc HCl (100 ml). After 12 hr, the product was filtered off, dissolved in 10% NaOHaq (200 ml), filtered, and decolourized with charcoal, again filtered and the filtrate acidified as before. The crude product, m.p. 126°, was filtered, dried and recrystallized from AcOEt and 80–100°

[9] Cf Ref. 9.

[1] K. Auwers and T. Markovitz, *Ber. Dtsch. Chem. Ges.* **41**, 2332 (1908).

[7] E. C. S. Jones and J. Kenner, *J. Chem. Soc.* 1842 (1931).

[8] R. Adams and J. H. Looker, *J. Amer. Chem. Soc.* **73**, 1145 (1951).

light petroleum (equal volumes). Yield of 2,6-*dimethyl-4-toluene*-p-*sulphonamidophenol* 7·1 g (72%), m.p. 129°. (Found: C, 61·5; H, 6·24; N, 4·8. $C_{14}H_{17}NO_3S$ requires: C, 61·8; H, 5·85; N, 4·81%.) IR spectrum (Nujol) showed prominent bands at 3240, 1488, 1388, 1325, 1315, 1238, 1160, 1150, 1092 1030, 975, 905 and 735 cm^{-1}.

2,6-*Dimethyl*-p-*benzoquinone-4-toluene*-p-*sulphonimide*. 2,6-Dimethyl-4-toluene-*p*-sulphonamidophenol (2·12 g) dissolved in glacial AcOH (15 ml) was added slowly to $Pb(OAc)_4$ (3·14 g) wet with glacial AcOH (5 ml). After stirring for 90 min at 15°, ethylene glycol (0·2 ml) was added and stirring continued for 20 min. No product crystallized out.

The solvent was removed under reduced press below 50° and the pale yellow product repeatedly washed till free from traces of Pb salts, yield of crude product 1·94 g, m.p. 88–94°. The dried crude product was dissolved in dry AcOEt (5 ml), filtered, and 60–80° light petroleum (14 ml) was added; 1·32 g of 2,6-*dimethyl*-p-*benzoquinone-4-toluene*-p-*sulphonimide* as pale yellow plates m.p. 99·5–101° were precipitated. Addition of further light petroleum (20 ml) gave 200 mg yellow plates, m.p. 97–98°. Repeated recrystallization of the first fraction did not raise the m.p. (Found: C, 62·6; H, 5·59; N, 4·79. $C_{14}H_{15}NO_3S$ requires; C, 62·4; H, 5·59; N, 4·79. $C_{14}H_{15}NO_3S$ requires: C, 62·3; H, 5·2; N, 4·84%.) IR spectrum (Nujol) showed no bands in the 3300 cm^{-1} region but prominent bands at 1655, 1645, 1610, 1535, 1315, 1307, 1187, 1170, 1090, 1020, 940, 913, 827, 813, 786, 721 and 690 cm^{-1}.

Reduction of 2,6-*dimethyl*-p-*benzoquinone-4-toluene*-p-*sulphonimide*. To 2,6-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (110 mg) dissolved in 40% aqueous EtOH (10 ml) was added an $NaHSO_3$aq (5 ml). The pale yellow soln became colourless immediately. The EtOH was removed under reduced press; a white crystalline ppt appeared, which was filtered off, washed and dried, yield 87 mg; m.p. 127–128°, not depressed by mixture with an authentic sample of 2,6-dimethyl-4-toluene-*p*-sulphonamidophenol.

Reaction of mesidine with 2,6-*dimethyl*-p-*benzoquinone-4-toluene*-p-*sulphonimide*. To 2,6-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (240 mg) dissolved in dioxan (4 ml) was added mesidine (112 mg) dissolved in glacial AcOH (0·5 ml). A dark red soln was formed immediately, and the reaction mixture was warmed for 3 min at 100°. The solvent was removed under reduced press, and the dark red residue treated with benzene (15 ml) giving a dark red soln and a ppt which was filtered and characterized as toluene-*p*-sulphonamide, m.p. 137°. The combined filtrates were evaporated and the dark red glass dissolved in 40–60° light petroleum (5 ml) and chromatographed on an alumina column developed with the same solvent. The eluate from the single dark red band gave dark red needles (176 mg), m.p. 88–90°, raised by recrystallization to 92–94° not depressed by mixture with the dark red enzymic oxidation product. (Found: N, 5·24. for $C_{17}H_{19}NO$: N, 5·54%.) IR spectrum (Nujol) showed no bands in the 3300 cm^{-1} region but prominent bands at 1645, 1612, 1545, 1365, 1325, 1315, 1218, 1209, 1137, 1042, 1022, 912, 858, 787, 775 and 727 cm^{-1}.

3,5-*Dimethyl-4-aminophenolhydrochloride*. 3,5-Dimethyl-4-nitrophenol (4·1 g) dissolved in EtOH (40 ml) was catalytically reduced by H in the presence of Adams catalyst (200 mg). When uptake ceased, the catalyst was filtered off and to the filtrate was added ether (400 ml) saturated with dry HCl. A white crystalline ppt of 3,5-*dimethyl-4-aminophenol hydrochloride* was recrystallized from a mixture of dry ether and EtOH yielding white needles (2·82 g) which sublimed above 180° without melting. (Found: N, 8·15. $C_8H_{12}ClNO$ requires: N, 8·1%.)

3,5-*Dimethyl-4-toluene*-p-*sulphonamidophenol*. To 3,5-dimethyl-4-aminophenol hydrochloride (1·52 g) dissolved in pyridine (15 ml) was added toluene-*p*-sulphonyl chloride (1·56 g) dissolved in pyridine (10 ml). After 24 hr, the pyridine was removed under reduced press, the residue was dissolved in EtOH (40 ml) and poured onto a mixture of ice (200 g) and conc HCl (15 ml) and set aside for 12 hr. The product was filtered off washed, and dissolved in 10% NaOHaq (125 ml), decolourized with charcoal, filtered and the filtrate poured onto ice-acid mixture as before. The product was filtered off dried, and recrystallized from an AcOEt–40–60° light petroleum mixture, when it yielded colourless needles of 3,5-*dimethyl-4-toluene*-p-*sulphonamidophenol* (2·4 g) m.p. 190°. (Found: C, 62·0; H, 5·8; N, 5·3. $C_{14}H_{17}NO_3S$ requires: C, 61·8; H, 5·85; N, 4·81%.) IR spectrum (Nujol) showed prominent bands at 3405, 3285, 1601, 1315, 1300, 1252, 1179, 1155, 1080, 1032, 896, 848, 815, 715 and 705 cm^{-1}.

Oxidation of 3,5-*dimethyl-4-toluene*-p-*sulphonamidophenol*. 3,5-Dimethyl-4-toluene-*p*-sulphonamidophenol (1·4 g) dissolved in glacial AcOH (25 ml) was added to $Pb(OAc)_4$ (2·01 g) wet with glacial AcOH. The mixture was stirred at 15° for 90 min, then ethylene glycol (0·2 ml) was added and stirring was continued for a further 20 min. The solvent was removed under reduced press (below 50°) and the residue heated with water, filtered and washed free from Pb salts. The pale yellow product

was dried, and recrystallized from a mixture of AcOEt–40–60° light petroleum yielding pale yellow plates of 3,5-*dimethyl*-p-*benzoquinone*-4-*toluene*-p-*sulphonimide* (1·2 g), m.p. 110°. (Found: C, 62·3; H, 5·4; N, 4·84. $C_{15}H_{15}NO_2S$ requires: C, 62·3; H, 5·2; N, 4·84%.) IR spectrum (Nujol) showed no bands in the 3400–3000 cm^{-1} region, but prominent bands at 1601, 1562, 1328, 1155, 1080, 1055, 940, 912, 822, 815, 773 and 712 cm^{-1}.

Reduction of 3,5-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (100 mg) dissolved in aqueous EtOH by sodium hyposulphite yielded colourless needles (60 mg) m.p. 189–190° not depressed by mixture with an authentic sample of 3,5-dimethyl-4-toluene-*p*-sulphonamidophenol.

Condensation of mesidine and 3,5-*dimethyl*-p-*benzoquinone*-4-*toluene*-p-*sulphonimide*. To 3,5-dimethyl-*p*-benzoquinone-4-toluene-*p*-sulphonimide (200 mg) dissolved in glacial AcOH (5 ml) was added mesidine (100 mg) dissolved in glacial AcOH (1 ml). The mixture, maintained at 15°, became dark brown and on standing for 2 hr became black. The solvent was removed under reduced press and the residue dissolved in 40–60° light petroleum (25 ml). No ppt appeared, and the soln was chromatographed on an alumina column. No distinct bands developed and the dark brown eluate yielded a tar from which no crystalline material could be obtained.

The reaction was repeated, using similar quantities of reactants in chloroform, benzene and dioxan as solvent, but intractable tars were always obtained.

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