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STUDIES IN PEROXIDASE ACTION—XXIV[a]

THE PREPARATION AND PEROXIDASE OXIDATION OF 4-PHENYL-2,6-DIMETHYL-ANILINE

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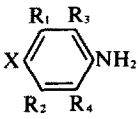
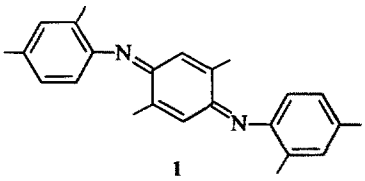
Abstract—4-Phenyl-2,6-dimethylaniline has been prepared in a variety of ways. Oxidation by the peroxidase system gives 4,4'-*diphenyl*-2,6,2',6'-*tetramethylazobenzene* and p-*benzoquinone*-4-(4'-*phenyl*-2',6'-*dimethyl*)*anil*. A mechanism is proposed for this unusual reaction.

The peroxidase oxidation of amines of the type



can proceed very rapidly giving compounds of higher molecular weight.[1] In order that this shall happen with facility the group X is often involved. Among typical examples the following may be cited. (i) If $R_1 = R_2 = R_3 = R_4 = H$ and X = Cl or Br, then Cl^- and Br^- are eliminated. If $R_1 = R_2 = R_3 = R_4 = H$ and X = I, then X is eliminated as I_2 which can be involved in subsequent transiodination reactions. (ii) If $R_1 = R_2 = H$ and $R_3 = R_4 = CH_3$, and $X = CH_3$, the methyl group is eliminated as formaldehyde. If X = cyclohexyl, cyclohexanone is produced.

During the peroxidation of certain aromatic amines, alkyl group migrations have been observed. For example, Holland *et al*[2] have described the oxidation of 2,4-dimethylaniline to give 2,5-dimethyl-*p*-benzoquinonebis-(2',4'-dimethyl) anil. (1).



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The oxidations of 2,3,4-trimethylaniline, 4-t-butyl-2,6-dimethylaniline and 4-cyclohexyl-2,6-dimethylaniline have already been studied and shown to give products containing "migrated" alkyl groups.[3]

Both this mechanism and that proposed for the production of the di-anil (1) involve the migration of the alkyl group as a carbonium ion. The reaction takes place in aqueous buffer solution at room temperature, not ideal conditions for a carbonium ion migration. Furthermore, it is to be expected that a tertiary carbonium ion would be more stable than a secondary carbonium ion and would therefore give a greater yield of the rearranged product. However, only 6% of a "migrated" product is obtained from the oxidation product of 4-t-butyl-2,6-dimethylaniline whereas a 64% yield of a "migrated" product is obtained from the oxidation of 4-cyclohexyl-2,6-dimethylaniline. Furthermore confusion is introduced by the absence of any migration in the oxidation of 4-isopropyl-2,6-dimethylaniline.

The oxidation of 4-phenyl-2,6-dimethylaniline by peroxidase has now therefore been studied in an attempt to elucidate further the mechanism of peroxidase action and the mechanism of migrations in particular. A phenyl group should be able to support more easily than an alkyl group a positive charge and therefore, if the mechanism proposed is correct, should give a greater proportion of "migrated" product.

Initial attempts to synthesise 4-phenyl-2,6-dimethylaniline were not successful. Two references were found reporting the synthesis of this compound. In 1933, Hey and Jackson nitrated 3,5-dimethylbiphenyl with concentrated nitric acid and acetic acid and obtained a yellow oil.[4] The crude oil was reduced and acetylated to give N-acetyl-4-phenyl-2,6-dimethylaniline. Nitration of 3,5-dimethylbiphenyl under these conditions gives a complex mixture of mono- and di-nitrated products and some starting material remains.

A report that 4-phenyl-2,6-dimethylaniline could be obtained by the saponification of the N-acetylaniline (prepared as described below) was also noted.[4] Acidic hydrolysis gave no trace of the amine. Attempted hydrolysis of the compound with dilute, concentrated and ethanolic solutions of sodium and potassium hydroxides all failed. A small quantity of the free amine was obtained by fusion of the N-acetyl derivative with solid caustic alkali, but extensive degradation took place to yield a very impure product. No reference giving the

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conditions for the hydrolysis of this or related compounds could be found in the literature.

The synthesis of useful quantities of 4-phenyl-2,6-dimethylaniline was finally achieved as follows. 5-Cyclohexyl-1,3-dimethylbenzene was synthesised by a Friedel-Crafts' reaction between cyclohexene and *m*-xylene.[3] 3,5-Dimethylbiphenyl was prepared by heating 5-cyclohexyl-1,3-dimethylbenzene with sulphur in quinoline. Nitration of this compound yielded a mixture of isomeric nitrocompounds. Fractional crystallisation gave pure 4-phenyl-2,6-dimethylnitrobenzene and reduction of this compound with stannous chloride according to the method of Hey and Jackson[4] gave pure 4-phenyl-2,6-dimethylaniline. Acetylation gave N-acetyl-4-phenyl-2,6-dimethylaniline.

The structure of the amine was confirmed by synthesis of N-acetyl-4-phenyl-2,6-dimethylaniline from N-chloroacetyl-2,6-dimethylaniline and benzene in the presence of aluminium chloride.[5] 4-Phenyl-2,6-dimethylnitrobenzene was also prepared unambiguously by a Gombert reaction between benzene and 4-nitro-3,5-dimethybenzene diazonium chloride.[6] These reactions are summarised in Fig 1.

The addition of the amine in glacial acetic acid to buffer solution containing peroxidase and hydrogen peroxide produced a green colour, quickly changing to brown and finally depositing a brown solid which was filtered from a pale yellow solution. Analysis of the product by TLC showed the presence of many dark-coloured components, similar to those obtained by the oxidation of 4-aminobiphenyl. Column chromatography on silica and elution with dichloromethane gave three distinct components in low yields. The three components were further purified by preparative thin-layer chromatography. The first two have been identified as follows:

(a) 4,4' - *Diphenyl* - 2,6,2',6' - *tetramethylazobenzene* (**2**) as orange crystals, constituting 6% of the total oxidation product. The compound was identical with that obtained in high yield from the oxidation of the parent amine by lead dioxide. The structure was further confirmed by microanalytical and spectral data.

(b) *Benzoquinone* - 4 - (4' - *phenyl* - 2',6' - *dimethyl*)*anil* (**3**) as a red oil constituting 1·5% of the products. The structure of the compound was elucidated from spectral data and was confirmed by acidic hydrolysis to benzoquinone and 4-phenyl-2,6-dimethylaniline and also synthesis from benzoquinone and the parent amine according to the method of Roberts and Saunders.[7] The compound derived from the peroxidase oxidation and that prepared by the synthetic method had identical spectra and identical behaviour on thin-layer plates in all solvents. The compound could not be prepared in a crystalline state.

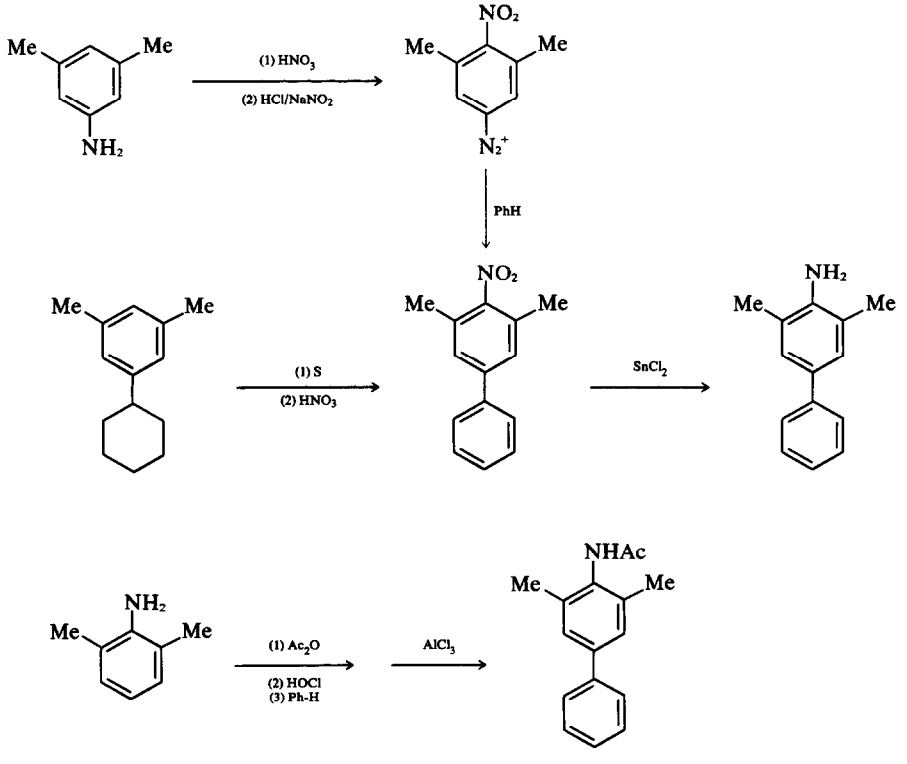
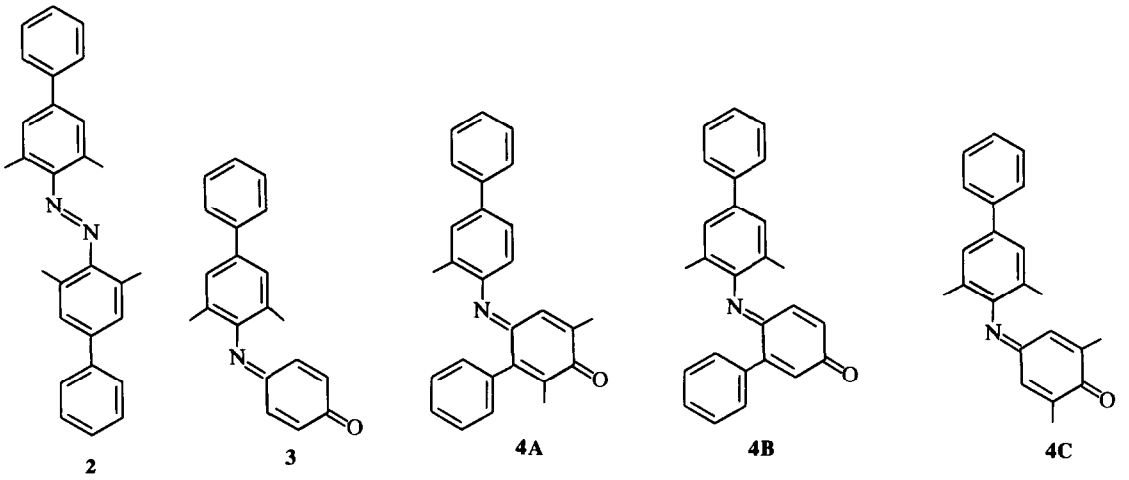


Fig 1. The preparation of 4-phenyl-2,6-dimethylaniline.



2 3 4A 4B 4C

(c) A compound which was obtained in trace amounts as deep purple crystals from ethanol (95%). The extremely small quantity of this compound rendered identification difficult. However, the compound does not undergo acidic hydrolysis under the usual conditions for the hydrolysis of quinone anils and so the structures 4A and 4B (the "migrated" products) and 4C (the 2,6-dimethylbenzoquinone derivative corresponding to 3) can be discounted with certainty.

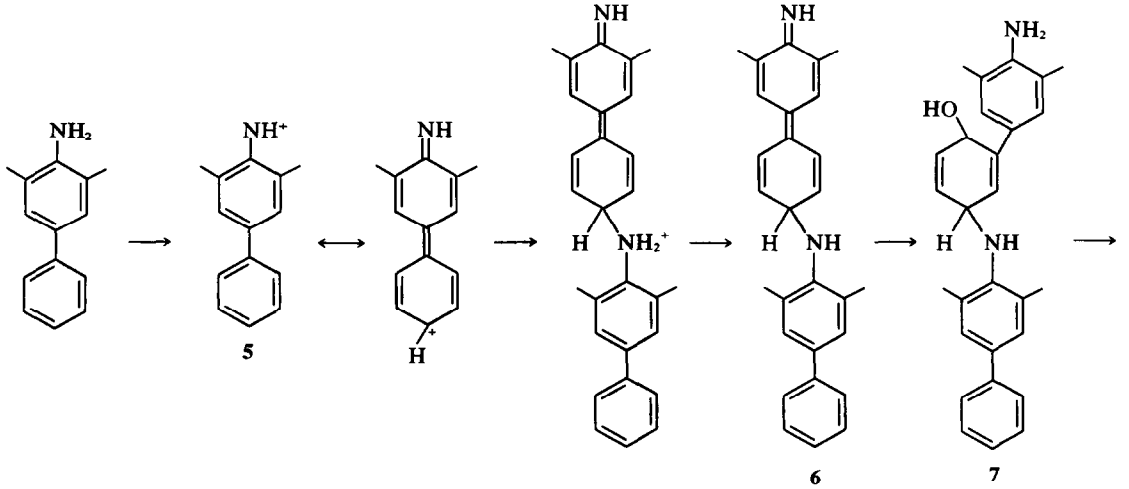
Steam distillation of the pale yellow solution obtained after removal of the solid oxidation products gave a pale yellow distillate which was extracted with ether. TLC revealed the presence of a single compound identical with 2,6-dimethylbenzoquinone.

4-Hydroxy-2,6-dimethylaniline has also been prepared.[8] On oxidation by peroxidase, a single compound, identical with 2,6-dimethylbenzoquinone, was isolated.

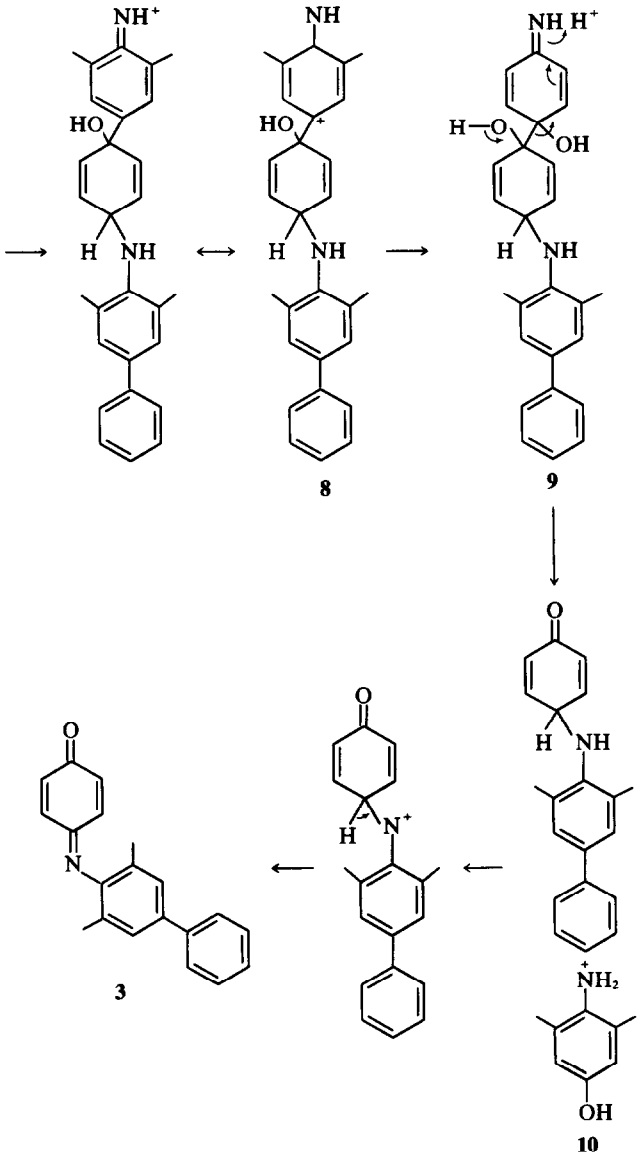
The identification of this residue has made it possible to write a mechanism for the formation of the benzoquinone derivative (3). The amine is oxidised enzymically to the cation 5 which mesomerises and is nucleophilically attacked by a second molecule of amine. Loss of a proton yields the intermediate 6, a quinone imine methide. Addition of water in a similar manner to that proposed in the mechanism of the oxidation of mesidine[9] will give the primary amine 7. Further enzymic oxidation and mesomerism gives the cation 8. Nucleophilic attack by a molecule of water followed by loss of a proton yields the dihydroxy compound (9). Further oxidation and loss of 4-hydroxy-2,6-dimethylaniline followed by oxidation of the secondary amine (10) will give benzoquinone-4-(4′-phenyl-2′,6′dimethyl)anil (3).

It has been shown, as described above, that 4-hydroxy-2,6-dimethylaniline is rapidly oxidised by the peroxidase system to 2,6-dimethylbenzoquinone, the residue found in solution after the peroxidase oxidation of 4-phenyl-2,6-dimethylaniline.

The migration of a phenyl group during the



5 6 7



oxidation of 4-phenyl-2,6-dimethylaniline has not been observed.

The work of Brill and Weinryb[10] on the inhibition of peroxidase by azide ion has shown the presence of a methionine residue at the active centre of the enzyme. Since peroxidase oxidations take place through a ternary complex of enzyme, donor and hydrogen peroxide, the migration most probably occurs while the donor is held within the complex. It is proposed that the alkyl group migrates from the donor to a site within the enzyme, perhaps the methionine group, and then realkylates the now oxidised donor in another position. The mechanism may closely resemble that which has been proposed, while avoiding the concept of a carbonium ion free in aqueous solution.

The migration might then be controlled by steric rather than electronic factors. The results of experiments carried out so far indicate that more bulky groups migrate with greater ease than smaller group (% migration cyclohexyl group > t-butyl > methyl).

Thus, it is concluded that a precise mechanism of alkyl group migration during the peroxidase oxidation of aromatic amimes cannot be written at the present state of our knowledge. The indications are that this is a more common phenomenon than at first was thought, and may be of importance in the biological function of peroxidase.

EXPERIMENTAL

Materials and equipment. All oxidations were carried out using a purified horseradish peroxidase preparation (R = 0·3) supplied by Seravac Laboratories Ltd., and 20 volume H_2O_2.

Preparation of 4-phenyl-2,6-dimethylaniline. 5-cyclohexyl-1,3-dimethylbenzene was prepared as previously described.[2] 5-Cyclohexyl-1,3-dimethylbenzene (20 g) was added to a mixture of sulphur (10 g) and quinoline (20 g) and heated under reflux for 4 h. The product, a dark brown oil, was distilled to yield three fractions, which were identified as indicated.

(a) Quinoline, b.p. 108–112°/16 mm.

(b) A mixture of 5-cyclohexyl-1,3-dimethylbenzene and 3,5-dimethylbiphenyl, b.p. 140–160°/16 mm (15 g).

(c) A dark coloured oil which was not distilled.

The second fraction was chromatographed on silicic acid (Mallinkrodt), using light petroleum (b.p. 40–60°) as eluent. Two bands were eluted as follows:

(a) fast running band consisting of 5-cyclohexyl-1,3-dimethylbenzene (4 g).

(b) slower running band of 3,5-dimethylbiphenyl (9 g). Traces of quinoline were left at the top of the column.

The second band was evaporated to a colourless liquid and distilled, b.p. 155°/16 mm, yield, 8·5 g (53%). NMR spectrum (CCl_4) showed a singlet (6 Me protons) at 7·76τ; singlet (1 aromatic proton) at 3·20τ; singlet (2 aromatic protons) at 2·96τ and multiple peaks (5 aromatic protons) at 2·50–2·90τ.

Fuming HNO_3 (7 ml) was added slowly to a well-stirred mixture of 3,5-dimethylbiphenyl (4 g) in glacial AcOH (10 ml) at room temp. After addition was complete, the mixture was stirred for ½ h. The product was poured onto ice (50 g) and neutralised with Na_2CO_3 aq. The organic components were extracted with ether. The ether was dried (Na_2SO_4) and evaporated to give a yellow oil (4·5 g). The oil was fractionally crystallised and recrystallised (EtOH) to give white crystals of *4-phenyl-2,6-dimethylnitrobenzene* (1·9 g, 34%) m.p. 88°. (Found: C, 73·8; H, 5·8; N, 6·1. $C_{14}H_{13}NO_2$ requires: C, 74·0; H, 5·7; N, 6·2%). NMR spectrum ($CDCl_3$) showed singlet (6 Me protons) at 7·70τ; singlet (2 aromatic protons) at 2·74τ and multiple peaks (5 aromatic protons) at 2·40–2·70τ. IR spectrum (KBr disc) showed prominent bands at 2900, 1585, 1505, 1455, 1360, 1340, 870, 830, 820, 770, 745, 695 cm^{-1}. Mass spectrum: *m/e* 229, (% of base peak 3); 228, (16); 227, (100); 211, (7); 210, (46); 199, (6); 198, (7); 197, (22); 182, (58); 181, (13); 180, (10); 179, (7); 178, (10); 167, (20); 166, (20); 165, (43). M.W. = 227. UV spectrum (95% ethanol): λ_{max} 249 mμ, ($\log_{10}\epsilon$ 4·134); λ_{min} 228 mμ, (3·242).

The compound was identical in every respect with 4-phenyl-2,6-dimethylnitrobenzene prepared by a Gomberg reaction between benzene and 4-nitro-3,5-dimethyl benzene diazonium chloride.

4-Phenyl-2,6-dimethylnitrobenzene (1 g) was dissolve in EtOH (5 ml) and added to a boiling soln of $SnCl_2$ (5 g) i conc HCl (12 ml). The mixture was heated under reflux fo 2 h. After cooling, excess of 10% NaOH aq was added an the mixture extracted with ether. The extracts were drie (Na_2SO_4) and examined by TLC. A single compound wa shown to be present on all supports in all solvents. Th solvent was evaporated to yield 4-phenyl-2,6 dimethylaniline as a pale yellow oil (0·8 g, 92%). NMI spectrum (CCl_4) showed singlet (6 Me protons) at 7·92τ singlet (broad, 2N–H protons) at 6·70τ; singlet (2 aromati protons) at 2·98τ and multiple peaks (5 aromatic protons at 2·50–2·90τ.

Acetylation of 4-phenyl-2,6-dimethylaniline. 4-Phenyl 2,6-dimethylaniline (500 mg) was dissolved in anhy pyridine (5 ml). Ac_2O (2 ml) was added and the mixtur was allowed to stand for ½ h. Water (20 ml) was adde slowly and the white solid filtered off, dried and recrystal lised (EtOH). The yield of N-acetyl-4-phenyl-2,6 dimethylaniline was 520 mg (86%). M.p. and mixed m.p with a sample prepared by the Friedel-Crafts' reactio between N-chloroacetyl-2,6-dimethylaniline and benzene 205°.

Oxidation of 4-phenyl-2,6-dimethylaniline. 4-Phenyl 2,6-dimethylaniline (800 mg) in glacial AcOH (2 ml) wa added to pH 4·8 acetate buffer (2 L), containing peroxid ase (2 mg) and H_2O_2 (2 ml). A brown colour was generate and a brown solid was slowly produced. Peroxidas (1 mg) and H_2O_2 (1 ml) were added every 2 h for 12 h. Th mixture was subsequently stirred for 36 h. The brow solid was filtered from the soln and dried. Further addi tions of peroxidase and H_2O_2 produced no further colou ration in the very pale yellow filtrate. The filtrate wa steam-distilled (200 ml) and the distillate was extracte with ether (2 × 20 ml). The extracts were dried (Na_2SO_4 and evaporated to 5 ml. Examination of the extracts b TLC on silica, using light petroleum (b.p. 40–60°) an Me_2CO (9:1) as eluent showed the presence of a singl compound R_f 0·75, identical with 2,6-dimethyl *p*-benzoquinone.

The residual soln after steam-distilation was mad alkaline with NaOH aq. (10%) and extracted with ethe The extracts were dried (Na_2SO_4), partially evaporate and examined by TLC using the same support and solven system as above. The plate showed a single spot, R_f 0·24 identical with 4-phenyl-2,6-dimethylaniline.

The solid (770 mg) was chromatographed on silica ge using dichloromethane as eluent and the following band were eluted.

(a) A fast-running yellow band; evaporated to a yello solid (49 mg, 6% of the oxidation product). This wa recrystallised (light petroleum, b.p. 40–60°) to giv yellow-orange needles of *4,4'-diphenyl-2,6,2',6' tetramethylazobenzene* m.p. 183°. (Found C, 86·3; H, 6·9 N, 7·1. $C_{28}H_{26}N_2$ requires: C, 86·2; H, 6·7; N, 7·2%). NMI spectrum ($CDCl_3$) showed singlet (12 Me protons) at 7·64 and multiple peaks (14 aromatic protons) at 2,2–2·8τ. IR spectrum (KBr disc) showed prominent bands at 3010 2980, 2960, 1600, 1505, 1465, 1450, 1420, 1320, 1230, 1080 1030, 875, 770, 705 cm^{-1}. Mass spectrum: *m/e* 392, (% o base peak 4); 391, (25); 390, (82); 389, (11); 388, (5), 376 (5), (15); 347, (16); 209, (5); 197, (5); 195, (11); 182, (33) 181, (100); 180, (10); 179, (9); 175, (5); 167, (8); 166, (31) 165, (38); 164, (5); 152, (5), 141, (5); 115, (5). M.W. = 390 UV spectrum (95% EtOH): λ_{max} 248 mμ, ($\log_{10}\epsilon$ 4.148)

344 mμ, (4·172); 469 mμ, (3·169); λ_{min} 234 mμ, (4·143); 285 mμ, (4·135); 422 mμ, (3·130).

The compound was identical in every respect with that obtained from the lead dioxide oxidation of 4-phenyl-2,6-dimethylamiline.

(b) A slow-running red band contaminated with a small quantity of a slower-running purple band; on evaporation and purification by preparative TLC on silica, this gave a red oil which could not be crystallised. The oil was identified by spectral, synthetic and degradative data as *p-benzoquinone-4-(4'-phenyl-2',6'-dimethyl)anil* (12 mg, 1·6% of the oxidation product by weight).

NMR spectrum ($CDCl_3$) showed a singlet (6 Me protons) at 8·00τ; doublet (perturbed, 2 aromatic protons) at 3·53τ (J = 10 c/s); doublet (perturbed, 2 aromatic protons) at 3·27τ (J = 10 c/s) and multiple peaks (7 aromatic protons) at 2·30–2·80τ. IR spectrum (CCl_4 soln) showed prominent bands at 3040, 2960, 1655, 1470, 1315, 1265, 1220, 1185, 1150, 1090, 1010, 875, 700 cm^{-1}. Mass spectrum: *m/e* 289, (% of base peak 8); 288, (22); 287, (100); 286, (11); 259, (14); 258, (64); 245, (17); 244, (8); 178, (17); 163, (28); 152, (17); 128, (14); 115, (17) 102, (8); 91, (8). M.W. = 287. UV spectrum (95% ethanol): λ_{max} 258 mμ; 390 mμ; λ_{min}210 mμ; 320 mμ.

The compound was identical in all respects with that prepared by the condensation of *p*-benzoquinone with 4-phenyl-2,6-dimethylaniline.

(c) A slower-running purple band. The compound was recrystallised (95% EtOH) to give purple plates. The compound has so far not been identified. It was not hydrolysed under the usual conditions for the hydrolysis of quinone anils.

Many further dark-coloured polymeric compounds could be removed from the column in trace amounts by the use of highly polar solvents, such as methanol, but these were not further examined.

Hydrolysis of benzoquinone-4-(4'-phenyl-2',6'-dimethyl)anil (3). Compound 3 (25 mg), derived from the peroxidase oxidation of 4-phenyl-2,6-dimethylaniline, was heated under reflux with dil H_2SO_4 (1·5 M, 5 ml) for 1 h.

The mixture was extracted with ether and the extracts dried (Na_2SO_4). Examination of the ethereal soln by TLC on silica, using light petroleum (b.p. 40–60°) and Me_2CO (9:1) as eluent, showed a single spot, R_f = 0·6, identical with authentic benzoquinone.

After separation of the ethereal soln of benzoquinone, the aqueous soln was made alkaline with Na_2CO_3 aq (0·5M, 20 ml) and extracted once again into ether. Examination of the extract by TLC as for benzoquinone above, showed a single spot, R_f 0·24, identical with 4-phenyl-2,6-dimethylaniline.

Preparation of 4,4'-diphenyl-2,6,2',6'-tetramethylazobenzene. 4-Phenyl-2,6-dimethylaniline (100 mg) was added to a soln of glacial AcOH (1 ml) in ether (20 ml) containing dried lead dioxide (5 g). The mixture was shaken for 5 h, dried and evaporated to yield an orange solid. Recrystallisation (light petroleum b.p. 40–60°) gave orange needles of 4,4'-diphenyl-2,6,2',6'-tetramethylazobenzene (74 mg, 74%), m.p. and mixed m.p. with the orange compound derived from the oxidation of 4-phenyl-2,6-dimethylaniline 165°. Spectral and chromatographic data were identical with those of the orange compound.

Preparation of benzoquinone-4-(4'-phenyl-2',6'-dimethyl)anil. 4-Phenyl-2,6-dimethylaniline (200 mg) in glacial AcOH (0·4 ml) was added to benzoquinone (100 mg) in 25% aqueous Me_2CO (7 ml). A deep red colour developed rapidly and the mixture was heated under reflux for 1 h. The solvents were removed by evaporation, leaving a dark red gum which was purified by preparative TLC on silica, using light petroleum (b.p. 40–60°) and Me_2CO (9:1) as eluent. The major component, a deep red band, R_f 0·6, was removed from the plate, extracted into dichloromethane, filtered and evaporated. The residue, a red oil, could not be crystallised, but was shown by comparison of spectral and chromatographic data to be identical with *p*-benzoquinone-4(4'phenyl-2',6'-dimethyl)anil obtained from the peroxidase oxidation of 4-phenyl-2,6-dimethylaniline.

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