PERKIN 1

# NMR studies of tautomerism in the fungal melanin biosynthesis intermediate 1,3,8-trihydroxynaphthalene

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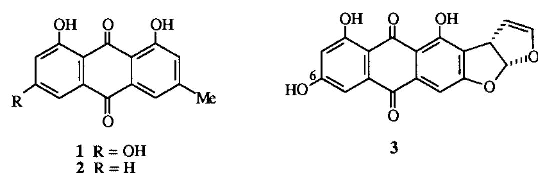
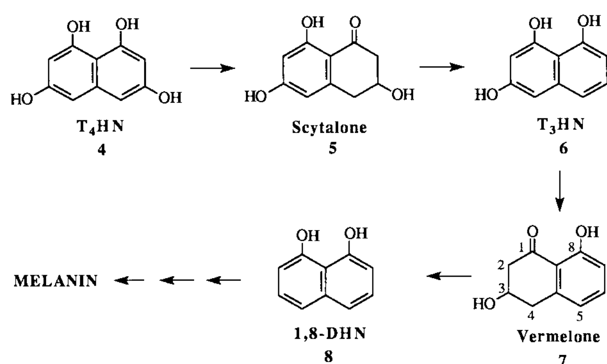
The naphthol reductase catalysed conversion of 1,3,8-trihydroxynaphthalene ($T_3HN$) to vermelone has been studied using a partially purified cell-free enzyme preparation from *Verticillium dahliae*. NMR studies show that in aqueous buffer $T_3HN$ exists as an equilibrium mixture of the parent phenol and a keto-tautomer. 1,3,6,8-Tetrahydroxynaphthalene ($T_4HN$) is a more efficient substrate than $T_3HN$ for the naphthol reductase. 1,3-Dihydroxynaphthalene also acts as a substrate and is converted to 8-deoxyvermelone.

A feature of the biosynthesis of many polyketide-derived metabolites is the loss of oxygen functionality from positions where acetate-derived oxygen would be predicted to be present.[1] Most often this loss of oxygen occurs by reductive modifications analogous to those occurring during fatty acid biosynthesis in the assembly phase of polyketide biosynthesis.[2] However, it is evident that in a number of important metabolites, phenolic oxygen is lost after assembly, cyclisation and aromatisation of the polyketide intermediate. Examples of this include the conversion of emodin **1** to chrysophanol **2**,[3] and the

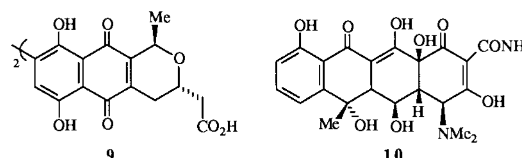



deoxygenation at C-6 of versicolorin A **3**, a key step in aflatoxin $B_1$ biosynthesis[4] and during the biosynthesis of melanin in a number of fungal pathogens.[5] Rice blast fungus, *Pyricularia oryzae*, and a number of other plant pathogens, *e.g. Verticillium dahliae*, and *Colletotrichum lagenarium*, invade their hosts by leaf penetration, a process which requires the production of melanin in their appressoria. Production of fungal melanin occurs by oxidative polymerisation of 1,8-dihydroxynaphthalene **8**. This has been shown (Scheme 1) to be formed in two successive reduction–dehydration steps from the pentaketide-derived 1,3,6,8-tetrahydroxynaphthalene ($T_4HN$ **4**). $T_4HN$ is reduced by a reductase to scytalone **5**† which undergoes enzyme-mediated dehydration to 1,3,8-trihydroxynaphthalene ($T_3HN$ **6**). Repeating the same process leads to 1,8-dihydroxynaphthalene **8** *via* vermelone **7**.‡ Known anti-blast compounds such as tricyclazole act by inhibiting trihydroxynaphthalene reductase,[6] while carpropanide inhibits scytalone dehydratase.[7]

The biosynthesis of polycyclic aromatic antibiotics such as actinorhodin **9** and oxytetracycline **10** also requires loss of oxygen and, while this is commonly regarded as occurring by normal ketoreductase–dehydratase-mediated reductions during assembly of the polyketide precursor,[8] it is noteworthy that




**Scheme 1** Intermediates on the pathway of fungal melanin biosynthesis.




from sequence alignment comparisons the actinorhodin "KR" correlates more closely to the fungal phenolic reductases rather than to bacterial or fungal ketoreductases.[9] This suggests that loss of oxygen in actinorhodin and other antibiotic biosynthetic pathways may also be post-aromatisation processes.

The mechanism of such post-aromatic deoxygenations has been rationalised in part by the existence of the keto-tautomeric form of the *meta*-dihydroxy-substituted aromatic rings in equilibrium with the expected phenolic or fully aromatic form. Indeed, the NMR spectrum of $T_4HN$ in acetone has been reported[10] to contain signals corresponding to *ca.* 30% of the keto-tautomers, *e.g.* **11**, in equilibrium with the phenolic form (Scheme 2). No such signals were observed in the $^1H$ NMR spectrum of $T_3HN$, suggesting that the equilibrium was less favourable towards the keto-forms than with $T_4HN$.[10] Chemical verification of this observation comes from the facile borohydride-mediated reduction of $T_4HN$ to scytalone.[11] Similar results have been reported by Sankawa and co-workers who established that $T_4HN$ exists completely in the form of keto-tautomers in a strongly alkaline solution, sodium methoxide in methanol.[12] Again, no NMR signals corresponding to a keto-form (*e.g.* **12**) of $T_3HN$ were observed under the same

† The IUPAC name for scytalone is 3,6,8-trihydroxy-3,4-dihydronaphthalen-1(2*H*)-one.
‡ The IUPAC name for vermelone is 3,8-dihydroxy-3,4-dihydronaphthalen-1(2*H*)-one.

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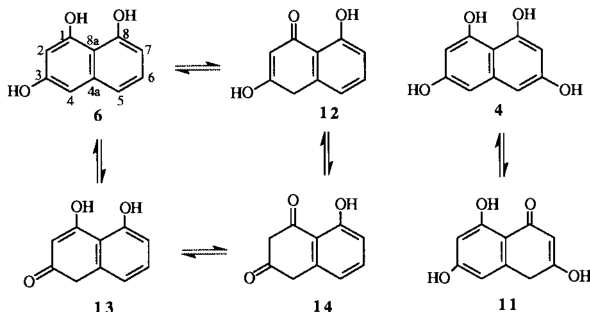
**Table 1** $^{1}H$ NMR spectrum of $T_3HN$ and the variation with pH of $^{1}H$ chemical shifts (keto form in brackets)

| Position | $\delta^a$ (m, $J^b$) | pH 6$^c$ | pH 7$^c$ | pH 8$^c$ |
|---|---|---|---|---|
| 2 (2′) | 6.47 (d, 2.3) | 6.28 (5.31) | 6.20 (5.26) | 6.13 (5.22) |
| 4 (4′) | 6.68 (d, 2.3) | 6.53 (3.55) | 6.51 (3.56) | 6.40 (3.50) |
| 5 (5′) | 7.08 (dd, 8.4, 1.2) | 6.98 (6.68) | 6.98 (6.65) | 6.82 (6.64) |
| 6 (6′) | 7.14 (dd, 7.2, 8.4) | 7.02 (7.16) | 7.12 (7.14) | 6.98 (7.16) |
| 7 (7′) | 6.57 (dd, 7.2, 1.2) | 6.44 (6.55) | 6.40 (6.54) | 6.28 (3.50) |

$^a$ ppm in $d_6$-acetone. $^b$ Hz. $^c$ ppm in deuterated phosphate buffer and $d_6$-acetone.




**Scheme 2** Possible tautomeric forms of $T_3HN$ **6** and $T_4HN$ **4**.

conditions. In contrast to these published observations, we now report that, in buffered aqueous solutions, $T_3HN$ does exist as an equilibrium mixture of tautomeric forms.

## Results and discussion

Naphthol reductase was partially purified from the *brm-1* mutant (which is deficient in dehydratase activity) of *V. dahliae* by assaying the conversion of $T_4HN$ to scytalone by HPLC. In contrast to $T_4HN$, it had been reported that the hydrogens of $T_3HN$ did not undergo rapid exchange and so we hoped to use this enzyme preparation to establish the stereochemistry of reduction of the 1,3-dihydroxy ring of $T_3HN$ by carrying out the enzyme-mediated reduction in deuterated buffer. The axial and equatorial hydrogens at C-2 and C-4 of vermelone could be assigned by detailed analysis of their coupling constants and by comparison with those of scytalone **5**.[13] $^{2}H$ NMR analysis of the vermelone produced, however, indicated that almost complete exchange of all four of the 2- and 4-methylene hydrogens and partial exchange at H-7 had occurred during incubation. This suggested that the H-2 and H-4 hydrogens of $T_3HN$ had exchanged with deuterium in the medium prior to conversion. Incubation of $T_3HN$ with boiled enzyme in pH 7 potassium phosphate buffer in $D_2O$ provided confirmation of this. The resultant $T_3HN$ was re-isolated and purified and its $^{1}H$ and $^{2}H$ NMR spectra were determined. To our initial surprise, these showed extensive incorporation of $^{2}H$ into H-7 and, to a lesser extent, into H-5, but not into H-2 or H-4, indicating that facile reverse exchange of label had occurred from these latter positions during work-up.

In light of this result, the $^{1}H$ NMR spectrum of $T_3HN$ was obtained by dissolving $T_3HN$ in potassium phosphate buffer at pH 7 with the addition of an equal volume of acetone to aid dissolution of the $T_3HN$. The $^{1}H$ NMR spectrum (Fig. 1) showed a total of ten signals (Table 1) which, on the basis of their relative integrations, fell into two sets of five in a ratio of 2 : 1. In addition, twenty individual carbon signals were observed in the $^{13}C$ NMR spectrum (Table 2) and, again, these can be divided into two sets according to their intensities. The major species present could easily be ascribed to the expected phenolic form **6** of $T_3HN$ by comparison of its signals with those observed for $T_3HN$ in deuterio-acetone. At pH 7, broadening of the signals resulted in loss of the meta couplings

**Table 2** $^{13}C$ NMR spectrum of $T_3HN$ at pH 7 in potassium phosphate buffer and $d_6$-acetone (1 : 1)

| Position$^a$ | $\delta^a$ (ppm) | Position$^b$ | $\delta^b$ (ppm) |
|---|---|---|---|
| 1 | 160.4 | 1′ | 183.2 |
| 2 | 101.2 | 2′ | 101.0 |
| 3 | 155.3 | 3′ | 189.0 |
| 4 | 102.4 | 4′ | 39.4 |
| 4a | 138.3 | 4a′ | 104.2 |
| 5 | 116.6 | 5′ | 117.9 |
| 6 | 127.8 | 6′ | 130.5 |
| 7 | 104.7 | 7′ | 106.0 |
| 8 | 155.9 | 8′ | 157.0 |
| 8a | 110.7 | 8a′ | 113.0 |

$^a$ Phenolic form. $^b$ Keto form.

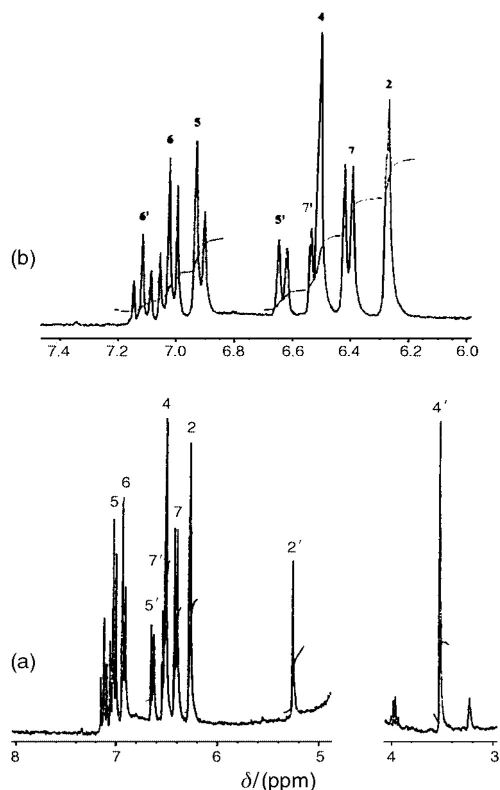



**Fig. 1** (a) 270 MHz $^{1}H$ NMR spectrum of $T_3HN$ in a mixture of pH 7 aqueous potassium phosphate buffer and $d_6$-acetone (1 : 1 v/v), and (b) expansion of the aromatic region.

previously observed for H-2 and H-4, and H-5 and H-7. In addition, all of the signals show a slight downfield shift (0.11–0.27 ppm). The second set contained only three signals assignable to aromatic hydrogens in addition to an olefinic (enolic) singlet (5.26 ppm) and an aliphatic methylene singlet (3.56 ppm). These signals provide clear evidence for the presence of a major keto-tautomer (Scheme 2). Of the two possible tautomers **12** and **13**, the former is favoured due to the possibility of hydrogen bonding to the 8-hydroxy group. The diketo-tautomer **14** can be ruled out. The $^{13}C$ NMR spectra are consistent with the presence of the keto-tautomer, the enolic and ketonic carbons appearing at 101.0, 189.0 and 193.2 ppm respectively, and the methylene carbon at 39.4 ppm. The assignment of the protonated carbons in both tautomers was confirmed by a $^{1}H$–$^{13}C$ COSY experiment.

The relative ratio (2 : 1) implies that the phenolic form is only marginally favoured at neutral pH. The pH profile for the naphthol reductase is shown in Fig. 2. The enzyme is active between pH 5.5 and 8.5 with maximum activity at pH 7. To investigate whether the enzyme activity is related to the proportion of keto-tautomer present, the $^{1}H$ NMR spectra were repeated at

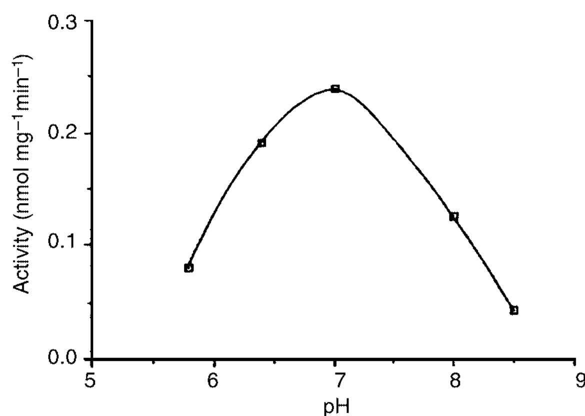


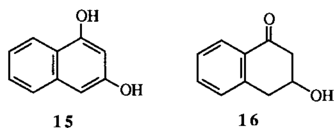

**Fig. 2** Variation of *V. dahliae* phenol reductase activity with pH.

pH 6 and pH 8. At lower pH, the ratio changed to favour the phenolic tautomer to the extent of 10:1. However, at pH 8 the keto-tautomer was favoured in a 2:1 ratio over the phenolic form. Thus it appears that the pH dependence of the enzyme is not related to the extent of tautomerism.

The rates of exchange of the ring hydrogens were examined by carrying out time course experiments in deuterated buffer. At all 3 pH values tested, the 2- and 4-hydrogens exchanged rapidly. The 6-hydrogens, as expected, were resistant to exchange. The half-life for exchange of the 5-hydrogens was *ca.* 48 h at all 3 pH values, and the only significant variation was seen for the 7-hydrogens for which the half-life increased from 3.5 h at pH 6 to 8 hours at pH 8 in accord with expected reactivity of hydrogens *ortho* to a phenolic hydroxy.

It is interesting to speculate whether the reductase acts on tautomer **12** as its preferred substrate, adding hydride at C-3 by a conjugate addition mechanism, or whether it requires the 3-keto-tautomer **13** as its substrate. Although **13** is not present in detectable concentrations, it is possible that its formation is promoted by the presence of the enzyme.

The substrate specificity of the reductase preparation was tested using a range of possible substrates. No reaction was observed with resorcinol, 1,3-dihydroxy-6,8-dimethoxynaphthalene or emodin. However, when 1,3-dihydroxynaphthalene **15** was incubated with the purified enzyme preparation, a new




product was observed by thin-layer chromatography. To characterise this product, the incubation was carried out on a larger scale and the product was isolated. The signals in the $\delta$ 2.5–3.5 region of the $^1$H NMR spectrum of the product were essentially identical to those in the corresponding spectra of scytalone and vermelone, consistent with the product being 8-deoxyvermelone **16** – as was the mass spectrum, which gave a molecular ion at *m*/*z* 162.

The conversion of **15** after 14 hours incubation at 30 °C was *ca.* 6.6%. This is lower than the conversion of $T_4$HN and $T_3$HN under similar conditions, which led to conversions of 50 and 10% respectively. Nonetheless, it is significant that the synthetically useful conversions of this unnatural substrate could be obtained.

The substrate specificity of the reductase merits further investigation. There is evidence from the occurrence of minor metabolites in some fermentations[5] that metabolites with 1,4-dihydroxylation are also present and that they too are reduced in a similar fashion, possibly *via* the corresponding quinones. It should also be noted that the purity of the reductase preparation does not preclude the presence of more than one reductase, perhaps with different affinities for $T_4$HN and $T_3$HN.

## Experimental

$^1$H and $^{13}$C NMR spectra were recorded on a JEOL GX 270 spectrometer. In the case of $^2$H NMR, spectra were recorded on a JEOL GX 400 instrument. Samples which contained water or deuterium oxide were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) whereas all the others were referenced to tetramethylsilane. High performance liquid chromatography was performed on a Gilson 303 system. The HPLC column (25 × 0.46 cm) contained reversed-phase Anachem spherisorb $C_{18}$ S5ODS2 packing material. Preparative thin-layer chromatography was carried out on 20 × 20 cm glass plates coated with silica gel (0.5 or 0.75 cm thickness, Merck, Art. 7747, Kieselgel $PF_{254}$). Bands were visualised by the use of ultra-violet light (254 nm). Flash column chromatography was performed using silica gel (Fluka, 60738 Kieselgel 60 220–440 mesh). Microbiological work was carried out under sterile conditions, in a Biomat Class II microbiologist cabinet. Sterilisation of all the materials prior and subsequent to use was carried out in an autoclave at 15 psi for 15 minutes.

Centrifugations were performed on a Sorvall RC 5C high-speed centrifuge. Shaken cultures were grown on an Innova orbital shaker, manufactured by New Brunswick Scientific Co., Inc., USA. All the UV-visible measurements were carried out on a Pharmacia LKB Ultrospec II UV-visible spectrophotometer. Bradford's protein assay method was used for all the protein assays.[14]

All the solvents were dried and distilled prior to use according to the standard procedures. Nitrogen was dried by passage through a silica-gel–calcium-chloride column.

### Synthesis of 1,3,8-trihydroxynaphthalene

Scytalone (500 mg) which was isolated from *Phialaphora lagerbergii* as previously described[12] was dissolved in trifluoroacetic acid (20 ml) and refluxed for two hours under nitrogen. The solvent was evaporated and the residue was extracted with ethyl acetate. The organic layer was washed with water, brine and dried over $MgSO_4$. Evaporation of the solvent yielded a pale green–brown solid (348 mg, 71%) which was purified twice by preparative TLC eluting with chloroform–acetone–formic acid (89:10:1). Pure 1,3,8-trihydroxynaphthalene (325 mg, 65%, $R_f$ 0.45) was obtained as a pale green–brown solid. $\delta_H$ in $(CD_3)_2CO$, 6.44 (1H, d, *J* 2.4, *H*-2), 6.55 (1H, dd, *J* 7.2, 1.3, *H*-7), 6.65 (1H, d, *J* 2.4, *H*-4), 7.05 (1H, dd, *J* 8.4, 1.3, *H*-5), 7.12 (1H, dd, *J* 8.4, 7.2, *H*-6); $\delta_C$ in $(CD_3)_2CO$, 101.7 (*C*-2), 102.30 (*C*-7), 106.67 (*C*-4), 110.73 (*C*-8a), 118.80 (*C*-5), 127.85 (*C*-6), 139.12 (*C*-4a), 154.94 (*C*-82), 156.04 (*C*-3), 157.06 (*C*-1); *m*/*z* 176 (99.1%, $M^+$), 134 (100%, $M^+ - CH_2{=}C{=}O$), 106 (20.6%), 77 (16.9%).

### Proton exchange of 1,3,8-trihydroxynaphthalene at pH 7, pH 6 and pH 8

Trihydroxynaphthalene (9.0 mg) in $^2H_6$-acetone (0.2 ml) and potassium phosphate buffer (100 mM, pH 7, 0.2 ml) was placed in a NMR tube under nitrogen and the sample was analysed by NMR spectroscopy. Another sample was prepared similarly with potassium phosphate (100 mM, pH 7) buffer in deuterium oxide and monitored by NMR at specific time intervals, *i.e.* at time zero, every 30 minutes up to 5 hours, and then at 8, 12, 24 and 48 hours.

The above experiment was repeated at pH 6 and pH 8 in the same way. Potassium phosphate was used to prepare the buffers.

### Preparation of cell-free extracts

*V. dahliae brm-1* mutant (ATCC 44571) was grown in 500 ml flasks, each containing 160 ml of modified Brandt's sucrose

nitrate medium. Mycelia (280 g), grown for 6–7 days in a total volume of 20 litres culture medium were harvested. Mycelial aliquots (10 g) were suspended in potassium phosphate buffer (100 mM, pH 6.8, 20 ml), containing EDTA (1 mM) and dithiothreitol (1 mM) and mixed with glass beads (30 g). The cells were lysed using a carbon dioxide cooled ball-mill (Braun cell homogeniser, Germany) for 4 minutes. The crude extract was then centrifuged at 38 000 g for 20 min at 4 °C to remove particulate materials and the glass beads. The supernatants (620 ml) were collected. Streptomycin sulfate (1% w/v) was added to the crude extract and stirred on ice for 30 minutes. The solution was centrifuged at 38 000 *g* for 20 min at 44 °C. 35–90% pellets were obtained by fractionating the supernatant liquor with ammonium sulfate and centrifuging at 40 000 *g* for 20 min at 4 °C. The protein pellets were then dissolved in a minimum volume of potassium phosphate buffer (100 mM, pH 6.9), containing EDTA (1 mM), dithiothreitol (1 mM) and glycerol (20%). The enzyme preparations were then stored at −80 °C, until required for purification and other analysis.

### Standard enzyme assay procedure

Before assaying for reductase activity, the enzyme preparations were dialysed for four hours against potassium phosphate buffer (100 mM, pH 6.9), containing EDTA (1 mM) and dithiothreitol (1 mM) at 4 °C.

The protein solution (100 µl, 4.8 mg $ml^{-1}$) was diluted with potassium hydrogen phosphate (100 mM, pH 6.9, 300 µl), containing EDTA (1 mM) and dithiothreitol (1 mM) and incubated with reduced nicotinamide adenine dinucleotide phosphate (NADPH) (17 µg) under nitrogen for 15 min. Tetrahydroxynaphthalene (4 µg) in ethanol (2 µl) was then added to the reaction mixture and the incubation was continued under nitrogen for a further 45 min at 30 °C. Similarly, a control experiment was performed by incubating the substrate in the absence of enzyme. The assay mixture was then acidified to pH 5 using phosphoric acid (2 M), saturated with brine and extracted into ethyl acetate (2 × 500 µl). The ethyl acetate layer was concentrated and analysed by HPLC.

Samples (25 µl) were applied to a $C_{18}$ reversed-phase column (Anachem spherisorb, 25 × 0.46 cm), equilibrated and run isocratically in acetonitrile–water–acetic acid (20 : 78 : 2 v/v) at a flow rate of 1 ml $min^{-1}$. The metabolites were detected at $\lambda$ 254 nm with the sensitivity set to 0.02 AUF. An authentic sample of scytalone, isolated from cultures of *P. lagerbergii* was used as the reference.

### Product analysis

The above dialysed protein (10 ml, 1.25 mg $ml^{-1}$) in potassium phosphate buffer (100 mM, pH 6.9) containing EDTA (1 mM) and dithiothreitol (1 mM) was incubated with NADPH (39 mg) under nitrogen for 15 min. 1,3,6,8-Tetrahydroxynaphthalene (9 mg), dissolved in ethanol (0.4 ml) was added to the incubation mixture and the incubation was continued under nitrogen for 14 hours at 30 °C. The metabolites were then extracted into ethyl acetate as in the above assay. The concentrated ethyl acetate layer was analysed by TLC (acetone–hexane 6 : 4). The product was seen at $R_f$ 0.55 and isolated by preparative TLC using the same solvent system. The pure scytalone (3.2 mg, 36%) was analysed by $^1$H NMR and mass spectrometry. $\delta_H$ 2.62 (1H, ddd, *J* 17.1, 7.8, 1.1, $H$-$2_{ax}$), 2.84 (1H, ddd, *J* 17.1, 3.9, 1.0, $H$-$2_{eq}$), 2.86 (1H, dddd, *J* 16.1, 7.8, 1.1, 1.0, $H$-$4_{ax}$), 3.20 (1H, dddd, *J* 16.1, 7.8, 1.1, 1.1, $H$-$4_{eq}$), 4.31 (1H, septet, *J* 3.9, *H*-3), 6.15 (1H, dd, *J* 2.2, 0.6, *H*-7), 6.28 (1H, dt, *J* 2.2, 1.1, *H*-5); *m*/*z* 194 (100%, $M^+$), 176 (79.3%, $M^+ - H_2O$), 150 (90.3%), 45 (82.4%).

### Purification of NADPH dependent naphthol reductase

Whatmann DE-52 pre-swollen micro-granular anion exchanger (60 g) was equilibrated in potassium phosphate buffer (50 mM, pH 7.5) containing EDTA (1 mM), dithiothreitol (1 mM), glycerol (10% v/v) and sodium chloride (0.2 M) after removal of the fine particles. All the operations were carried out at 4 °C. The protein solution (1690 mg in 257 ml), which was dialysed against the above buffer for four hours at 4 °C, was added to the ion exchanger and stirred slowly for 15 min using a glass rod. The slurry was allowed to settle down for approximately 30 minutes and the supernatant (A) decanted. Further 60 ml of buffer were added to the ion exchanger and again stirred for 15 min. Once the slurry had settled, the supernatant was decanted and combined with the supernatant (A).

Secondly, ion exchanger was stirred with above buffer (80 ml) in 0.8 M sodium chloride as in the previous procedure. Once the slurry had settled, the supernatant (B) was decanted. The procedure was repeated with another volume (60 ml) of above buffer in 0.8 M sodium chloride. The supernatant was decanted and added to the supernatant (B).

Thirdly, the same procedure was followed with the above buffer in 1 M sodium chloride (2 × 80 ml) and the supernatants were collected (supernatant C).

All three supernatants were assayed for protein content and for the enzyme activity, under the standard assay conditions.

### Purification by FPLC

The protein fraction (0.2–0.8 M batch) eluted from DE-52 was dialysed against the Bis-Tris propane (50 mM, pH 6.9) containing EDTA (1 mM) and dithiothreitol (l mM) for four hours at 4 °C and fractionated by Mono Q anion-exchange column chromatography. The protein solution (100 µl) and Bis-Tris propane (50 mM, pH 6.9, 300 µl) buffer, containing EDTA (1 mM) and dithiothreitol (1 mM) were used for each assay. The fractions which contained the enzyme activity were analysed by SDS-PAGE gel electrophoresis. The active enzyme fractions obtained from the Mono Q anion exchange column were pooled and concentrated to 5 ml using an Amicon ultrafiltration kit. Further concentration was achieved by dialysing against a solution of poly(ethylene glycol) (70% w/v). The concentrated protein solution (4.4 mg in 1.0 ml) was then applied in 200 µl aliquots to a Superose-12 (Pharmacia) gel filtration column, equilibrated and eluted with Tris-HCl (50 mM, pH 6.9), containing EDTA (1 mM), dithiothreitol (1 mM) and sodium chloride (150 mM) at a flow rate of 0.25 ml $min^{-1}$. Fractions (1 ml) were collected and assayed for enzyme activity under the standard assay conditions. The protein solution (100 µl) and above buffer (300 µl) were used for each assay.

### Effect of pH on the reductase activity

The enzyme fraction was dialysed against potassium phosphate buffer (10 mM, pH 7), EDTA (1 mM) and dithiothreitol (1 mM) for four hours at 4 °C. The pH 5.8, 6.4, 7, 8, 8.5 and 9 buffers were prepared from potassium phosphate (100 mM), containing EDTA (1 mM) and dithiothreitol (1 mM). The dialysed enzyme (100 µl) was added to each buffer (400 µl) to prepare the pH series. Thus prepared enzyme solutions were assayed under the standard conditions. The products were then analysed by HPLC and the amount of scytalone produced was quantified. The reductase activity was plotted against the pH.

### Conversion of 1,3,8-trihydroxynaphthalene into vermelone using cell-free enzyme preparations

*V. dahliae brm-1* was grown in modified Brandt's sucrose nitrate medium and the ammonium sulfate (35–90%) pellets were prepared as described above. The pellets were dialysed against potassium phosphate buffer (100 mM, pH 7) containing EDTA (1 mM) and dithiothreitol (1 mM). The enzyme preparation (1 ml, 1.2 mg $ml^{-1}$) in the above buffer was pre-incubated with NADPH (4.7 mg) under nitrogen for 15 min. Afterwards, 1,3,8-trihydroxynaphthalene (1 mg) in acetone (20 µl) was added to

the mixture and the incubation was continued overnight under nitrogen at 30 °C. Following the incubation period, the assay mixture was acidified to pH 5 using 2 M phosphoric acid saturated with brine, and the metabolites were extracted into ethyl acetate (2 × 1 ml). The ethyl acetate layer was concentrated and analysed by TLC and HPLC. Vermelone and 1,3,8-trihydroxynaphthalene were visualised at $R_f$ 0.34 and $R_f$ 0.45 respectively in TLC eluted with $CHCl_3$–acetone (9:1 v/v). For HPLC analysis the sample volume (50 µl) was applied to a $C_{18}$ reverse-phase HPLC column (Anachem - spherisorb S5-ODS2, 25 × 0.46 cm) equilibrated and run isocratically in methanol–water (42:58 v/v) at a flow rate of 1 ml $min^{-1}$. The metabolites were detected at 254 nm with sensitivity set to 0.02 AUF.

The enzyme preparation (1.2 mg $ml^{-1}$, 5 ml) in potassium hydrogen phosphate buffer (pH 6.8, 100 mM) containing EDTA (1 mM) and dithiothreitol (1 mM) was incubated with NADPH (35 mg) under nitrogen for 15 min. 1,3,8-Trihydroxynaphthalene (20 mg) was added to the solution and the incubation continued overnight. Following the incubation period, the assay mixture was acidified to pH 5 using 2 M phosphoric acid, saturated with brine and the metabolites were extracted into ethyl acetate (2 × 6 ml). The product was isolated and purified by preparative TLC eluting with chloroform–acetone (9:1 v/v, $R_f$ 0.34). The pure product (3.2 mg, 16%) was analysed by $^1$H NMR spectroscopy and mass spectrometry. $\delta_H$ 2.80 (1H, ddd, *J* 16.9, 7.6, 1.2, *H*-2$_{ax}$), 2.95–3.06 (2H, m, *H*-4$_{ax}$ and *H*-2$_{eq}$), 3.21 (1H, ddd, *J* 16.3, 3. 8, 1.1, *H*-4$_{eq}$), 4.20 (1H, septet, *J* 3.9, *H*-3), 6.74 (1H, dd, *J* 7.4, 1.0, *H*-7), 6.82 (1H, d, *J* 8.3, *H*-5), 7.40 (1H, dd, *J* 7.4, 8.3, *H*-6); *m/z* 198 (100%, $M^+$), 1 (48.7%, $M^+ - CH_3C{=}O$).

#### Synthesis of deuterium-labelled 1,3,8-trihydroxynaphthalene

Trihydroxynaphthalene (150 mg) was dissolved in deuterated trifluoroacetic acid (5 ml) and deuterium oxide (2 ml) under nitrogen and stirred for 2 hours. All the acids were evaporated under high vacuum. The residue was dissolved in ethyl acetate and analysed by TLC. Chloroform–acetone (9:1 v/v, $R_f$ 0.45) was used as the solvent system. The product was purified by preparative TLC using the same solvent system. The pure product (120 mg, 80%) was analysed by $^1$H NMR, $^2$H NMR spectroscopy and mass spectrometry.

#### Monitoring the deuterium exchange of 1,3,8-trihydroxynaphthalene with deuterated trifluoroacetic acid–deuterium oxide by NMR

Trihydroxynaphthalene (15 mg) was dissolved in deuterated trifluoroacetic acid (0.5 ml) and deuterium oxide (0.2 ml) under nitrogen. The mixture was kept in a NMR tube under nitrogen and analysed by NMR spectroscopy at time zero and at every 30 minutes up to 2 hours.

#### Conversion of 1,3,8-trihydroxynaphthalene into vermelone in deuterated buffer

Potassium phosphate buffer (100 mM, pH 7) containing EDTA (1 mM) and dithiothreitol (1 mM) was prepared in deuterium oxide. The pH of the buffer was adjusted using potassium deuteroxide (potassium hydroxide pellets dissolved in deuterium oxide). The enzyme preparation (4.7 mg $ml^{-1}$, 1 ml) was dissolved in the above buffer (5 ml) and incubated with NADPH (35 mg) under nitrogen for 15 min. Trihydroxynaphthalene (20 mg) in acetone (0.4 ml) was added to the mixture and the incubation was continued overnight under nitrogen. Following the incubation period, the assay mixture was acidified to pH 5 using 2 M phosphoric acid saturated with brine, and the metabolites were extracted into ethyl acetate (2 × 6 ml). The isolation and the purification of the product were performed by preparative TLC eluting with chloroform–acetone (9:1, v/v). The pure product 2.3 mg (11.5%) was analysed by $^1$H NMR, $^2$H NMR spectroscopy and mass spectrometry.

#### Incubation of 1,3-dihydroxynaphthalene 14 with the reductase

Reductase enzyme preparation (5 ml, 1.28 mg $ml^{-1}$) in potassium phosphate (100 mM, pH 6.9), containing EDTA (1 mM) and dithiothreitol (1 mM) was incubated with NADPH (35 mg) for 15 min under nitrogen. 1,3-Dihydroxynaphthalene (20 mg) in acetone (500 µl) was added to the mixture and the incubation was continued overnight under nitrogen in the dark. The assay mixture was acidified with 2 M phosphoric acid, saturated with brine and extracted into ethyl acetate (2 × 5 ml). The ethyl acetate layer was concentrated and analysed by TLC (ethyl acetate–petroleum ether 6:4). The product was seen at $R_f$ 0.35. Isolation and purification of the product were accomplished by preparative TLC using the same solvent system to give the pure product *3,4-dihydro-3-hydroxynaphthalen-1(2H)-one* **16** (1.2 mg yield, 6.6%). $\delta_H$ in $CD_2Cl_2$, 2.68 (1H, ddd, *J* 16.7, 7.3, 1.1, *H*-2ax), 2.95 (2H, m, *H*-2eq and *H*-4ax), 3.25 (1H, ddd, *J* 16.3, 4.0, 1.5, *H*-4eq), 4.46 (1H, septet, *J* 4.0, *H*-3), 7.30 (2H, t, *J* 8.5, Ar-H), 7.54 (1H, t, *J* 8.5, Ar-H), 7.98 (1H, d, *J* 8.0, Ar-H); *m/z* 162 (20%, $M^+$), 147 (17.4%, $M^+ - CH_3$), 118 (19.9%), 91 (34.4%), 86 (25%), 57 (32.3%), 43 (100%) ($C_{10}H_{10}O_2$ requires *M*, 162).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