Melanin Biosynthesis: A Study of Polyphenol Deoxygenation

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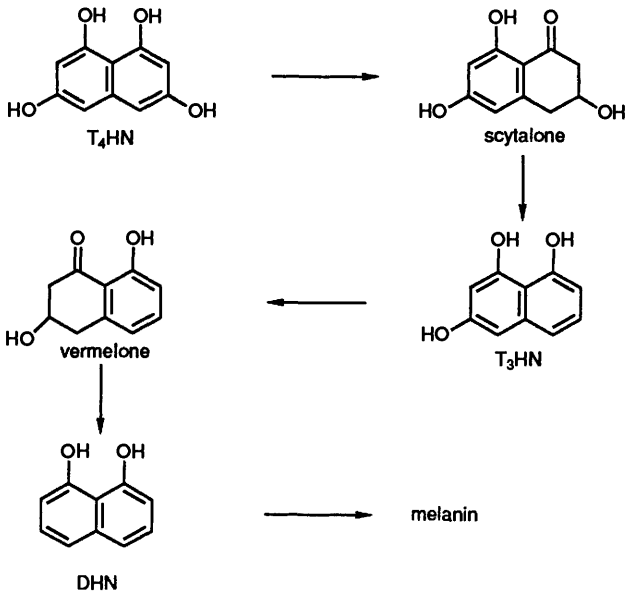
The 1,3,6,8-tetrahydroxynaphthalene (T_4HN) reductase of *Verticillium dahliae* has been studied in a cell-free system. The use of specifically labelled 4(*R*)- and [4(*S*)-^{2}H] NADPH in the reduction of T_4HN to scytalone reveals that the label is specifically transferred in the case of [4(*S*)-^{2}H] NADPH whereas no deuterium transfer occurs with the 4*R*-isomer. This establishes that the T_4HN reductase of *V. dahliae* is a NADPH-dependent dehydrogenase and that it belongs to class B.

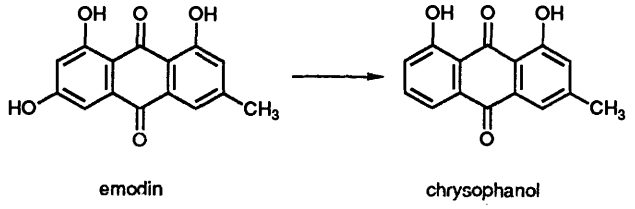
The deoxygenation of phenols is an important step in the biosynthesis of polyketide derived products.[1] During the biosynthesis of melanin, a dark pigment produced by ascomycetes *Pyricularia oryzae* or *Verticillium dahliae*, two successive deoxygenations occur, leading from 1,3,6,8-tetrahydroxynaphthalene (T_4HN)† to 1,8-dihydroxynaphthalene (DHN),[2] the last identified precursor of melanin (Scheme 1). Biosynthetic studies with mutants have established that the deoxygenation is achieved in two steps: a reduction stage in which T_4HN is reduced to scytalone, followed by a dehydration stage leading to T_3HN (the same process leads from T_3HN to DHN *via* vermelone). To date, little is known concerning the reduction step: the enzymes have not been purified or characterized but Wheeler,[3] working on crude cell-free extracts from *V. dahliae*, mentioned that NADPH was necessary. Since diphenols can exist as a mixture of phenolic and ketonic forms,[4] two mechanisms could be considered for the reduction: (i) a hydride transfer on the ketonic forms, catalysed by a $NADP^+$-dependent dehydrogenase; or (ii) a reduction catalysed by a flavin-containing enzyme coupled with a NADPH-regenerating system. Reduction of ketonic groups according to hypothesis (ii) is unprecedented but owing to the unusual structure of the substrate this possibility could not be excluded *a priori*.



Scheme 1. Biosynthesis of melanin.

We thus decided to study the fate of the label in the stereospecific 4(*R*)- and [4(*S*)-^{2}H]NADPH during the reduction by the reductase from *V. dahliae*. A transfer to the reduction products scytalone or vermelone would be a strong argument in favour of hydride transfer catalysed by a $NADP^+$-dependent dehydrogenase whereas washing of the label to the solvent would favour the involvement of a flavin.

While this work was in progress, Anderson *et al.*[5] reported that they observed a transfer of deuterium from labelled NADPH to product while studying a very closely related reaction, the transformation of emodin into chrysophanol by cell-free extract from *Pyrenochaeta terrestris* (Scheme 2).



Scheme 2. Biosynthesis of chrysophanol.

We report here on the study of the reductase from *V. dahliae* with 4(*R*)- and [4(*S*)-^{2}H] NADPH.

Experimental

General Procedure.—^{1}H NMR spectra were recorded on a Bruker AC200 spectrometer and chemical shifts are expressed in ppm relative to TMS. Mass spectra were run on a Nermag-Sidar V_3 spectrometer and UV spectra on a Beckman 34 spectrophotometer. M.p.s were measured on a Kofler hot stage apparatus and are uncorrected. TLC was carried out on silica gel precoated plates from Merck. Protein concentrations were determined with the Bio-Rad protein assay kit[6] with bovine serum albumin as standard.

Materials.—Brm-1 mutant from *V. dahliae* Kleb. strain T_9 was obtained from the American Type Culture Collection (ATCC 44571). A liquid sucrose–nitrate medium[7] was inoculated (10^6 conidies per 500 ml) and shaken at 24 °C for 6 days in the dark. Alcohol dehydrogenase from *Thermoanaerobium brockii* (EC 1.1.1.2) was obtained from the Sigma Chemical Co. Glutathione reductase from Yeast (EC 1.6.4.2) was obtained

† Abbreviations used throughout this paper: T_4HN, 1,3,6,8-tetrahydroxynaphthalene; T_3HN, 1,3,8-trihydroxynaphthalene; DHN, 1,8-dihydroxynaphthalene; and DTT, dithiothreitol (*threo*-1,4-dimercaptobutane-2,3-diol).

from Boehringer. Mycelia from *V. dahliae* (6 day old cultures in liquid medium) were harvested (5 000 g, 15 min), re-suspended in distilled water, lyophilized and stored at −25 °C. Lyophilized cells (20 g per 5 l liquid medium), were suspended in buffer 1 (potassium phosphate buffer 0.1M; 200 ml, pH 6.8; EDTA 1mM; DTT 1mM), frozen in liquid nitrogen and ground manually in a mortar. After being centrifuged (25 000 g, 20 min, 4 °C) the supernatant liquor was fractionated with ammonium sulphate (25–50%). The pellet (30 000 g, 20 min, 4 °C), dissolved in buffer 1, was thoroughly dialysed against buffer 1 and kept frozen at −25 °C before use.

Diethyl 1,3-acetonedicarboxylate was purchased from Janssen. Boron tribromide was obtained from Aldrich; [$^2H_8$]-propan-2-ol (99%) and deuterium oxide (99%) were from CEA (Saclay); and $NADP^+$, NADPH, dithiothreitol, EDTA, and glutathione (reduced form) were obtained from the Sigma Chemical Co.

Ethyl 2,4-*di*(*ethoxycarbonyl*)-3,5-*dihydroxyphenylacetate* (**1**). This was prepared according to the method of Theilacker;[8] m.p. 97–98 °C (lit.,[8] 98 °C); $\delta_H(CDCl_3)$ 1.24 (t, 3 H, Me), 1.37 (t, 3 H, Me), 1.45 (t, 3 H, Me), 3.83 (s, 2 H, CH_2CO), 4.14 (q, 2 H, CH_2), 4.36 (q, 2 H, CH_2), 4.49 (q, 2 H, CH_2), 6.36 (s, 1 H, arom.), 12.0 (s, 1 H, OH), and 12.9 (s, 1 H, OH); $\nu_{max}(CHCl_3)$ 3 420 (OH), 1 735 (CO_2Et), 1 660 ($ArCO_2Et$), and 1 600 cm^{-1} (Ar).

3,5-Dihydroxyphenylacetic acid (**2**). This was prepared according to the method of Theilacker;[8] m.p. 128–130 °C; $\delta_H[(CD_3)_2CO]$ 3.44 (s, 2 H, CH_2), 6.25 (d, *J* 2.06 Hz, 1 H, arom.), 6.32 (d, *J* 2.06 Hz, 2 H, arom.), and 8.17 (s, 1 H, CO_2H) (Found: C, 56.5; H, 4.77. Calc. for $C_8H_8O_4$: C, 57.14; H, 4.76%); ν_{max}(Nujol) 3 550 (OH), 3 300 (CO_2H), 1 700 (CO_2H), and 1 610 cm^{-1} (Ar).

Methyl 3,5-*dimethoxyphenylacetate* (**3**). This was prepared by treatment of compound (**2**) with dimethyl sulphate, b.p. 175–177 °C/20 mmHg (lit.,[9] 155–160 °C/15 mmHg); $\delta_H(CDCl_3)$ 3.57 (s, 3 H, Me), 3.69 (s, 2 H, CH_2), 3.81 (s, 6 H, Me), 6.37 (d, *J* 2.12 Hz, 1 H, arom.), and 6.43 (d, *J* 2.18 Hz, 2 H, arom.) (Found: C, 62.3; H, 6.85. Calc. for $C_{11}H_{14}O_4$: C, 62.86; H, 6.66%); $\nu_{max}(CHCl_3)$ 1 735 ($COOCH_3$) and 1 600 cm^{-1} (Ar).

Methyl 2-*acetyl*-3,5-*dimethoxyphenylacetate* (**4**). This was prepared according to the method of Bycroft;[9] m.p. 63–64 °C (lit.,[9] 64 °C); $\delta_H(CDCl_3)$ 2.51 (s, 3 H, COMe), 3.68 (s, 3 H, CO_2Me), 3.70 (s, 2 H, CH_2), 3.81 (s, 3 H, OCH_3), 3.82 (s, 2 H, OCH_3), 6.35 (d, *J* 1.92 Hz, 1 H, arom.), and 6.41 (d, *J* 1.94 Hz, 1 H, arom.) (Found: C, 62.0; H, 6.35. Calc. for $C_{13}H_{16}O_5$: C, 61.9; H, 6.35%) $\nu_{max}(CHCl_3)$ 1 735 (CO_2Me), 1 680 (COMe), and 1 605 cm^{-1} (Ar).

6,8-*Dihydroxy*-1,3-*dimethoxynaphthalene* (**5**). This was prepared according to the method of Bycroft;[10] m.p. 128–129 °C (lit.,[10] 126–127 °C); $\delta_H(CDCl_3)$ 3.86 (s, 3 H, Me), 3.99 (s, 3 H, Me), 5.07 (br s, 1 H, OH), 6.29 (d, *J* 2.14 Hz, 1 H, 2-H), 6.34 (d, *J* 2.36 Hz, 1 H, 7-H), 6.54 (m, 2 H, 4-H and 5-H), and 9.22 (s, 1 H, OH) (Found: C, 65.4; H, 5.45. Calc. for $C_{12}H_{12}O_4$: C, 65.45; H, 5.45%); $\nu_{max}(CHCl_3)$ 3 600 (OH), 3 400 (OH), and 1 620 cm^{-1} (Ar); λ_{max}(EtOH) 327 (ε 2 860 dm^3 mol^{-1} cm^{-1}), 312 (3 750), 300 (4 400), and 242 (62 400); *m/z* (EI, 70 eV) 220 (M^+, 100%), and 177 ($M - CH_3$, $M - CO$, 46).

1,3,6,8-*Tetrahydroxynaphthalene* (**6**). Boron tribromide (7.8 ml, 82 mmol) was added under argon to a solution of compound (**5**) (3.63 g, 16.5 mmol) in methylene dichloride (80 ml), cooled to −80 °C. The temperature was allowed to rise to room temperature and stirring in the dark was continued for 6 h. Hydrolysis with a saturated solution of potassium and sodium tartrate (100 ml, stirring for 20 min), followed by extraction with ethyl acetate and purification on a silica gel column (chloroform–methanol, 9:1) yielded compound (**6**) (2.44 g, 77%) as yellow crystals, m.p. >250 °C); $\delta_H[(CD_3)_2CO]$ 6.24 (d, *J* 2.1 Hz, 2 H, 2-H and 7-H) and 6.46 (d, *J* 2.1 Hz, 2 H, 4-H and 5-H); δ_H(tautomeric forms) 6.37 (m), 6.21 (d, *J* 2.18 Hz), 5.62 (s), 3.81 (s), and 3.32 (s) (Found: C, 61.9; H, 4.1. Calc. for $C_{10}H_8O_4$: C, 62.5; H, 4.16%). In spite of very careful handling and storage under argon, no improved microanalysis could be obtained for this highly sensitive compound); ν_{max}(Nujol) 3 400 (OH) and 1 590 cm^{-1} (Ar); λ_{max}(EtOH) 337 (ε 9 200 dm^3 mol^{-1} cm^{-1}), and 242 (31 000) (lit.,[11] λ_{max} 241, 287, 297, 310, and 325); *m/z* (EI, 25 eV) 192 (M^+, 100%) and 150 ($M^+ - CH_2{=}C{=}O$, 45).

3,4-*Dihydro*-3,6,8-*trihydroxynaphthalen*-1(2H)-*one* (*Scytalone*) (**7**). Argon was carefully bubbled through the solvents prior to use. Compound (**6**) (200 mg, 1.04 mmol) was dissolved under argon in methanol (10 ml) containing sodium (95.8 mg, 4.17 mmol). Sodium borohydride (78.8 mg, 2.08 mmol) in methanol (5 ml) was then added and the mixture was kept at room temperature overnight. Dropwise addition of a saturated aq. solution of sodium and potassium tartrate (30 ml) and adjustment to pH 7 (2M hydrochloric acid), followed by extraction with ethyl acetate yielded a mixture of compounds (**6**) and (**7**). Purification on a silica gel column (chloroform–methanol, 9:1) yielded compound (**7**) (70.7 mg, 35%) m.p. 185–190 °C (lit.,* 185–190 °C). $\delta_H[(CD_3)_2CO]$ 2.57–3.15 (m, 4 H, 2-CH_2 and 4-CH_2), 4.29–4.37 (m, 1 H, 3-H), 6.16 (d, *J* 2.25 Hz, 1 H, 7-H), 6.29 (m, 1 H, 5-H), and 12.80 (s, 1 H, OH) (Found: C, 61.85; H, 5.25. Calc. for $C_{10}H_{10}O_4$: C, 61.85; H, 5.15%) ν_{max}(Nujol) 3 300 (OH), 1 640 (CO), and 1 595 cm^{-1} (Ar) [lit.,* ν_{max}(KBr) 3 500, 1 640, and 1 590 cm^{-1}; λ_{max}(EtOH) 322 (ε 6 800 dm^3 mol^{-1} cm^{-1}), 283 (13 300), 232 (9 400), and 221 (13 700) [lit.,* 322 (6 600), 285 (12 600), 232 (7 100), and 223 (15 800)]; *m/z* (EI, 70 eV) 194 (M^+, 77%), 176 ($M^+ - H_2O$, 61), and 150 ($M^+ - CH_2{=}CHOH$, 100).

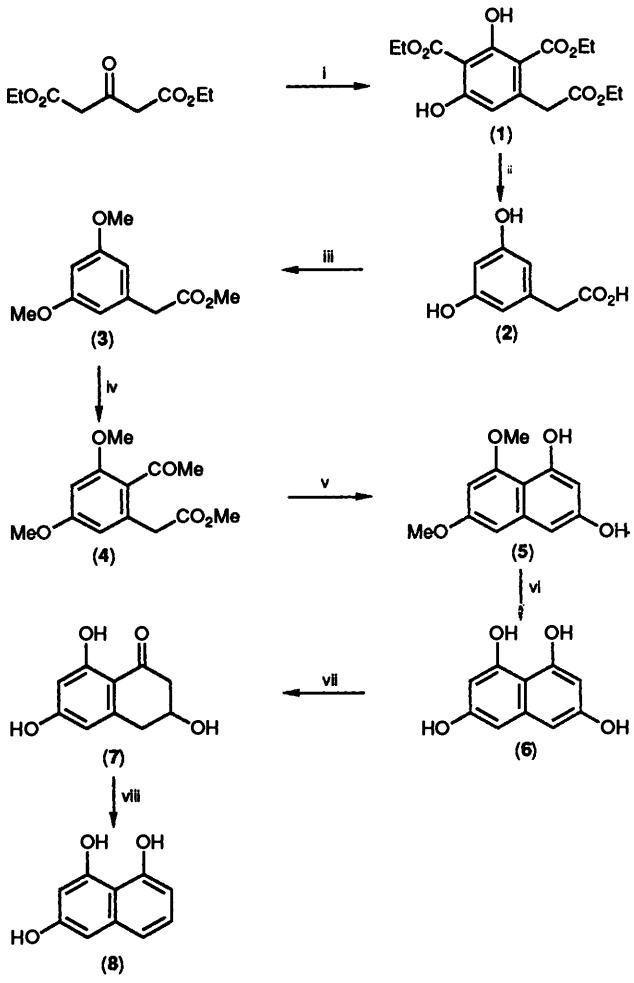
1,3,8-*Trihydroxynaphthalene* (**8**). This was prepared according to the method of Bell *et al.*,[2] m.p. 195–198 °C (lit.,[2] 193–203 °C) $\delta_H[(CD_3)_2CO]$ 6.45 (d, *J* 2.26 Hz, 1 H, 2-H), 6.55 (dd, J_2 6.86, J_3 1.74 Hz, 1 H, 7-H), 6.67 (d, *J* 2.26 Hz, 1 H, 4-H), and 7.06–7.13 (2 dd, 2 H, 5-H and 6-H) (Found: C, 67.85; H, 4.4. Calc. for $C_{10}H_8O_3$: C, 68.18; H, 4.54). In spite of very careful handling and storage under argon, no improved microanalysis could be obtained for this highly sensitive compound); ν_{max}(Nujol) 3 350 (OH) and 1 600 cm^{-1} (Ar); λ_{max}(EtOH) 340 (ε 4 500 dm^3 mol^{-1} cm^{-1}), 329 (4 300), 305 (5 700), 292 (5 300), and 232 (37 900) [lit.,[2] 340 (3 900), 329 (3 700), 304 (5 300), 292 (4 900), and 230 (43 700)]; *m/z* (EI, 70 eV) 176 (M^+, 100%) and 134 ($M^+ - CH_2{=}C{=}O$, 78).

[4(R)-^{2}H]*NADPH*. $NADP^+$ (40 μmol) and [$^2H_8$]propan-2-ol (2 mmol) were incubated in 0.1M tris-HCl buffer (pH 7.8, 5 ml) at 40 °C in the presence of alcohol dehydrogenase from *T. brockii*[12] (10 units). The reaction was monitored at 340 nm and stopped when A_{260}/A_{340} reached 3. Purification on a DE52 column (1.5 × 6 cm) was achieved with an ammonium carbonate linear gradient (0–0.5M). Fractions containing [4-^{2}H]NADPH were analysed by ^{1}H NMR spectroscopy,[13] lyophilized and stored at −20 °C before being used (yield 73%).

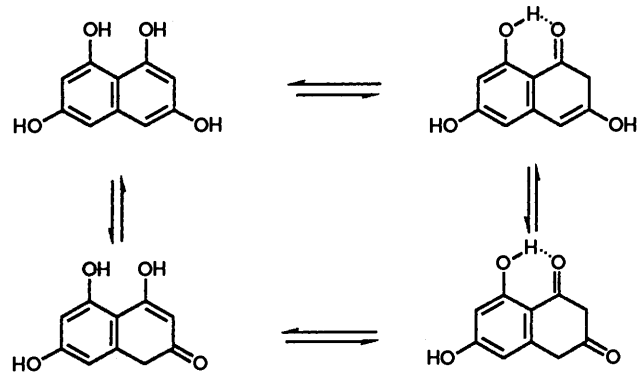
[4(S)-^{2}H]*NADPH*.[14] $NADP^+$ (50 μmol), glutathione (25 μmol, reduced form), DTT (2 mmol) and ammonium carbonate (250 μmol) were exchanged twice in D_2O. Incubation in D_2O [5 ml, pH adjusted to 8.5 (NaOD), 25 °C] with glutathione reductase (30 units) was monitored as previously (yield 75%).

Enzymatic Reduction of Compound (**6**) *with Labelled NADPH without Regenerating System*.—Labelled NADPH (15.6 μmol) and an enzyme preparation from *V. dahliae* (62 mg protein, 3 ml) were pre-incubated at 25 °C for 15 min in buffer (potassium phosphate 0.1M; 11.7 ml, pH 6.8; EDTA 1 mM; DTT 1 mM). Argon was bubbled through the solution over that period. Compound (**6**) (15.6 μmol) in ethanol (300 μl) was added and incubation was continued under argon for a further 5 h. The pH

* B. W. Bycroft, unpublished results.



Scheme 3. Synthesis of T_4HN, scytalone and T_3HN. *Reagents and conditions:* i, Na, 140 °C; ii, 3.5M NaOH, reflux then 3.5M H_2SO_4, reflux; iii, Me_2SO_4, K_2CO_3/acetone, reflux; iv, Ac_2O, $HClO_4$ 60% AcOH; v EtO^-Na^+/EtOH, reflux; vi, BBr_3, CH_2Cl_2 – 80 °C→20 °C; vii, $NaBH_4$, Na/MeOH; viii, 50% KOH, 95 °C.



Scheme 4. Tautomeric forms of T_4HN.

was then adjusted to 5 (H_3PO_4, 1M) and solid NaCl was added until saturation was reached. The mixture was extracted with ethyl acetate and analysed by TLC (chloroform–methanol, 9:1). After elution, compound (7) was recovered and analysed by ^1H NMR spectroscopy and mass spectrometry.

Enzymatic Reduction of Compound (6) with [4-^2H]*NADPH Regenerating System.*—$NADP^+$ (20 μmol) and [$^2H_8$]propan-2-ol (2 mmol) were pre-incubated at 40 °C for 10 min in a potassium phosphate buffer (0.1M, pH 7.4; EDTA 1 mM; DTT 1 mM). Following addition of alcohol dehydrogenase from *T. brockii* (12 units) and incubation (40 °C, 20 min), an enzyme preparation from *V. dahliae* (3 ml, 62 mg protein) and phosphate buffer (11.7 ml) were added and incubated at 25 °C for 10 min under argon. Compound (6) [15.6 μmol dissolved in ethanol (300 μl)] was added and incubation continued for 5 or 22 h. The extraction, purification, and analysis of metabolites was achieved as above.

Hydrogen–Deuterium Exchange of Compound (6).—Compound (6) (2.6 μmol) was dissolved in [$^2H_6$]acetone (0.5 ml) containing t-butyl alcohol (0.4%) as internal reference. Deuterium oxide (99.8%; 20 μl) was added and the exchange of the aromatic protons was monitored by ^1H NMR spectroscopy.

Results and Discussion

Scytalone (7) was synthesized according to the general strategy of Bycroft[15] (Scheme 3). Acylation of 3,5-dihydroxyphenylacetic acid (2) presented more difficulty than expected. Using the conditions previously described[15] (acetic anhydride, 1.3 mol equiv.; BF_3·Et_2O, 8 mol equiv. 60 °C, 2 h) we obtained exclusively the *O*-acylation product. Other conditions (Ac_2O–BF_3·Et_2O in benzene, 50–90 °C, 1–15 h; AcCl–$AlCl_3$ in benzene or nitrobenzene, 80–90 °C, 1–3 h; AcOH–$ZnCl_2$, 200 °C, 30 min; CH_3CN–$ZnCl_2$ in Et_2O, 25 °C, 3 h to 3 days) led to mixtures of starting material and mono- and di-acetylated esters. Obviously the rearrangement of the *O*-acylated product to the *C*-acylated product did not occur during the acylation of the acid (2). When the hydroxy groups were methylated, the acylation of the ester (3) proceeded smoothly leading to ester (4) which readily cyclized upon treatment with sodium ethoxide to yield compound (5). This was then demethylated with boron tribromide in methylene dichloride. T_4HN (6) was highly sensitive to oxidation and had to be handled and stored under argon. Reduction of T_4HN (6)—as its sodium salt in methanol—with sodium borohydride yielded scytalone (7) in moderate yield (*ca.* 35%) along with unchanged starting material. Treatment of scytalone (7) with 50% aq. potassium hydroxide yielded 1,3,8-trihydroxynaphthalene (8) that proved also to be very sensitive to oxidation. However, in contrast with compound (6), the triol (8) could not be reduced to vermelone using sodium borohydride under the same conditions.

The NMR spectrum of T_4HN (6) in acetone contained signals corresponding to tautomeric forms in equilibrium with the phenolic form (Scheme 4) and which represented *ca.* 31% of the total quantity of T_4HN (6): in fact, both aromatic protons exchanged with solvent rapidly at very similar rates in D_2O/$(CD_3)_2CO$ mixtures [$t_{\frac{1}{2}}$ = 7 min (H_2, H_7); 10 min (H_4, H_5)]. Such exchange has also been observed during the study of the emodin→chrysophanol transformation.[5]

No signals indicative of the presence of tautomeric forms could be detected in the ^1H NMR spectrum of T_3HN, suggesting that the equilibrium was less favourable towards the keto form than with T_4HN. Consistently, the exchange of the corresponding proton with solvent was slow.

T_4HN, which is present to a large extent in the keto form, could be reduced with sodium borohydride whereas T_3HN, for which no keto forms can be detected, could not be reduced. This suggests strongly that the readiness of reduction of *meta*-diphenols is linked to the extent of the keto form in equilibrium with the phenolic form. It will be interesting to see if such a correlation exists for the enzymatic reduction of T_4HN and T_3HN with the dehydrogenase from *V. dahliae*.

The dehydrogenase was extracted from a melanin-deficient strain of *V. dahliae*, Brm-1 (ATCC 44571). This strain was

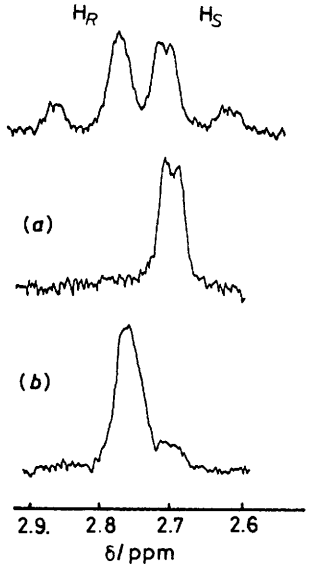


Figure 1. Isotopic purity of 4(*R*)- and [4(*S*)-^{2}H]NADPH.

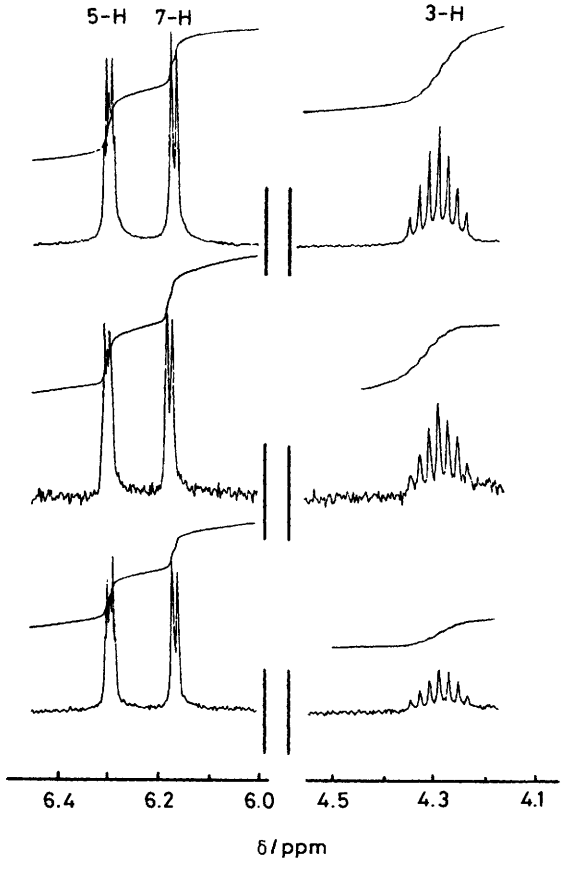


Figure 2. Determination of the extent of deuterium transfer from [4-^{2}H]NADPH during reduction by the reductase from *V. dahliae*.

selected because it is unable to dehydrate scytalone or vermelone, respectively, to T_3HN and DHN and allowed us to follow fractionation simply by testing the transformation of T_4HN (**6**) into scytalone (**7**) or T_3HN (**8**) into vermelone without formation of other metabolites such as DHN or even melanin that could have interfered. Following the incubation, the medium was extracted with ethyl acetate after adjustment of the pH and the extracts were analysed by TLC using synthetic scytalone or vermelone as references.

[4(R)-^{2}H]NADPH was synthesized in good yield using the dehydrogenase from *T. brockii* and [$^2H_8$]propan-2-ol as deuterium source. The deuterium content (>95%) was determined by ^{1}H NMR spectroscopy [Figure 1(*a*)]. [4(*S*)-^{2}H]NADPH was synthesized using glutathione reductase in deuterium oxide.[14] However, in spite of a thorough exchange of the system in deuterium oxide prior to reaction, the deuterium content of [4(*S*)-^{2}H]NADPH was only 85–90% [Figure 1(*b*)].

The incubations of T_4HN (**6**) with labelled NADPH were carried out under argon in the presence of 1 mol equiv. of NADPH and were stopped after 25–30% transformation. The remaining T_4HN (**6**) and the scytalone (**7**) were extracted and purified by column chromatography. The deuterium content of scytalone was estimated by means of its ^{1}H NMR spectra using the aromatic hydrogens as reference. In both cases this determination was confirmed by MS analysis (EI) by monitoring the peaks at *m/z* 194 (M^+), 176 ($M - H_2O$) and 150 ($M - CH_2$=CHOH).

Figure 2(*a*) shows that no incorporation of deuterium occurred during the reduction with [4(*R*)-^{2}H]NADPH whereas incorporation (50%) was observed with [4(*S*)-^{2}H]NADPH [Figure 2(*b*)].

This experiment thus clearly reveals that the transfer of deuterium from [4-^{2}H]NADPH is stereospecific and that the NADPH-dependent tetrahydroxynaphthalene reductase from *V. dahliae* belongs to class B [*i.e.*, transfers specifically the 4(*S*) hydrogen].

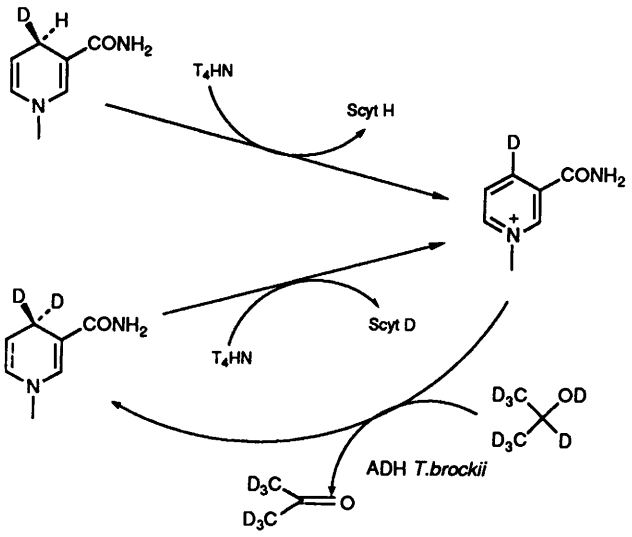
The incomplete incorporation for a 25–30% conversion (50% deuterium in scytalone) is likely due to the 10–15% hydrogen present at the 4(*S*) position of the NADPH produced by glutathione reductase in deuterium oxide, and to an isotope effect.*

Anderson *et al.*, while studying the reduction of emodin to chrysophanol by the cell-free system from *P. terrestris* observed the incorporation of deuterium from [4-^{2}H]NADPH.[5] Labelled NADPH was generated from $NADP^+$ and deuteriated propan-2-ol using the dehydrogenase from *T. brockii*. The incorporation of deuterium amounted to 40% and the authors concluded that NADPH served as the source of the hydride but they did not comment on the stereospecificity of the reaction.

Since the dehydrogenase from *T. brockii* is very specific in generating [4(*R*)-^{2}H]NADPH, this could have suggested that the dehydrogenase from *P. terrestris* belonged to class A. The apparently low incorporation of deuterium into chrysophanol (only 40%), even though the dehydrogenase from *T. brockii* is very stereospecific, could be explained on the basis of the experimental conditions: a large excess of NADPH compared to emodin, a large excess of regenerating system (dehydrogenase and deuteriated propan-2-ol) compared with $NADP^+$, and a low level of transformation of emodin. In addition to this, the long reaction time (30 h) favours non-specific label scrambling through a NADPH/$NADP^+$ exchange. This should have amplified the incorporation of hydrogen present in deuteriated propan-2-ol, leading to a higher incorporation of hydrogen than that which operates in the presence of only 1 equiv. of NADPH without continuous regeneration by the dehydrogenase.

We decided to investigate again the reduction of T_4HN with [4-^{2}H]NADPH generated by *T. brockii* dehydrogenase and deuteriated propan-2-ol under NADPH-regenerating conditions. After incubation in the presence of an almost stoicheiometric amount of NADPH (1.28 mol equiv. T_4HN) for 5 and 22 h, corresponding to 40 and 80% T_4HN reduction, the deuterium content of the scytalone extracted was 10 and 30%,

* Isotope effects of 3–5 are commonly encountered for NADH dehydrogenases.[16]



Scheme 5. Reduction of T_4HN in the presence of a regenerating system.

respectively. This result seems to be in conflict with the lack of incorporation of deuterium into scytalone that we observed with [4(*R*)-^{2}H]NADPH (generated by *T. brockii* reductase) without regenerating system (*vide supra*). However, this apparent discrepancy can be accounted for as follows: as the reaction proceeds, [4-^{2}H]NADPH is released and is recycled immediately into [4,4-^{2}H]NADPH by the *T. brockii* dehydrogenase, leading thus to deuterium incorporation by the T_4HN reductase (Scheme 5). (It is probable that the label scrambling due to non enzymatic NADPH/$NADP^+$ exchange is negligible in our case because of the high transformation levels observed).

Thus, operating under regenerating conditions could have led, without a careful examination of the experimental conditions, to erroneous conclusions concerning the stereospecificity of the reduction.

One can wonder if Anderson's experiment[5] run under regenerating conditions can be interpreted from a stereochemical point of view. It will thus be interesting to study 4(*R*)- and [4(*S*)^{2}H]NADPH stoicheiometric reduction of emodin in order to compare it with the *V. dahliae* reductase stereoselectivity.

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