Post-Aromatic Deoxygenation in Polyketide Biosynthesis: Reduction of Aromatic Rings in the Biosyntheses of Fungal Melanin and Anthraquinone

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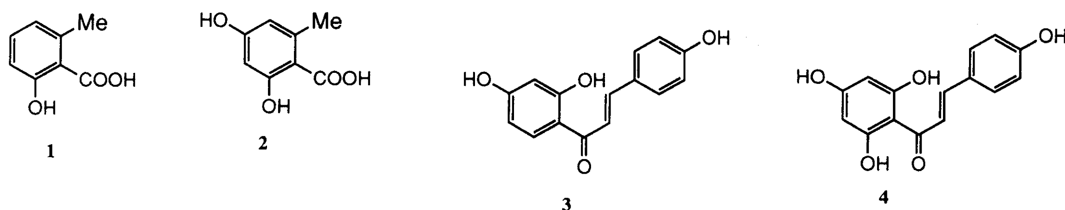
Two enzyme reactions involved in the post-aromatic deoxygenation process of fungal melanin and pigments were studied from the viewpoint of enzymic reduction of aromatic rings. Hydroxynaphthalene reductase that catalyzes reduction of the aromatic rings of 1,3,6,8-tetrahydroxynaphthalene and 1,3,8-trihydroxynapthalene was partially purified from *Phialophora lagerbergii* and characterized. Emodin deoxygenase of *Pyrenochaeta terrestris* that catalyzes deoxygenation of emodin to afford chrysophanol was found to be resolved into two protein fractions with DEAE-cellulose column. The two protein fractions acted synergistically in regard to emodin deoxygenase activity.

Keywords biosynthesis; anthraquinone; fungal melanin; *Phialophora lagerbergii*; *Pyrenochaeta terrestris*

Fatty acids and polyketides are biosynthesized in living organisms from common building units such as acetyl and malonyl CoAs. The oxygen atoms of these precursors are lost in fatty acids during their biosynthesis except for the terminal carboxyl group, while some oxygen atoms are retained in polyketides, in which the assembly pattern of building units is clearly recognized by the presence of oxygen functions at alternate carbons. 6-Methylsalicylic acid (6-MS, **1**) is one of the simplest examples of aromatic polyketides whose skeleton is built from one acetyl and three malonyl CoAs. 6-MS is a deoxygenated analogue of orsellinic acid (**2**) and one of the potential oxygen functions is lost during biosynthesis. 6-MS synthase, an enzyme catalyzing the synthesis of 6-MS (**1**) from acetyl and malonyl CoAs, was characterized to be a single protein[1] and its genomic DNA has been cloned and sequenced.[2] NADPH is required to form 6-MS (**1**) and when NADPH was omitted from the incubation mixture the reaction stopped at the stage of triacetic acid (C_6). This indicates that reduction of a carbonyl group occurs at the C_6 stage of ketoacyl synthase reaction, *i.e.* condensation reaction. The reduced C_6 intermediate bound to the enzyme is then dehydrated to yield an enone intermediate, which further undergoes condensation with a malonyl group followed by cyclization reaction to afford 6-MS (**1**). A similar example of oxygen loss is seen in the biosynthesis of deoxy-type chalcone (**3**) where *p*-coumaroyl and malonyl CoAs are the substrates of the reaction.[3] In contrast to 6-MS synthase the reaction of deoxy-chalcone biosynthesis requires NADPH and two enzymes, chalcone synthase (CHS) and a reductase (chalcone synthetic reductase, CHR).[3,4] In the absence of NADPH the combined enzyme system consisting of CHS and CHR afforded hydroxy-type chalchone (**4**) instead of deoxy-type chalcone (**3**).[4a,b] The hydroxy-type chalcone (**4**) was also formed by the reaction of CHS alone. This indicates that reduction of a carbonyl group takes place at a stage immediately before cyclization and one oxygen atom is lost by dehydration simultaneously with ring cyclization. This is in contrast to 6-MS synthase, and the deoxychalcone synthase system is similar to Type II fatty acid synthase in bacteria and plants, where the enzymes responsible for each reaction step are separable proteins and form a multi-enzyme complex in catalyzing fatty acid biosynthesis.[5]

Contrary to the loss of oxygen atom during the formation of aromatic rings, pre-aromatic deoxygenation, the loss of phenolic hydroxyl group also occurs in the polyketide biosynthesis after the formation of aromatic ring, post-aromatic deoxygenation. Examples of latter are seen in the biosyntheses of fungal melanin and pigments. Fungal melanin is an important compound in infection of phytopathogenic fungi such as *Verticillium dahliae*,[6] *Pyricularia orizae*,[7] *Thielaviopsis basicala*[8] and *Colletotrichum lagenarum*,[9] particularly for their hyphal invasion into plant tissue.[10] Since fungicides such as tricyclazole and pyroquilon were found to exhibit their action by the inhibition of melanin biosynthesis, the pathway of fungal melanin biosynthesis was extensively studied at various levels (Chart 1).[6-9] Incorporation experiments with $(1,2\text{-}^{13}C_2)$ acetate have clearly demonstrated that scytalone (**6**) is formed by the reduction of 1,3,6,8-tetrahydroxynaphthalene (TetHN, **5**) in *Phialophora lagerbergii*,[11] and the biomimetic reduction of TetHN (**5**) with sodium borohydride also supports this biochemical aromatic reduction.[12] Enzymatic reduction of TetHN (**5**) and 1,3,8-trihydroxynaphthalene (TriHN, **7**) was demonstrated recently with a cell free homogenate from *Verticillium dahliae* to give scytalone (**6**) and vemelone (**8**), respectively.[13]



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Another example of aromatic reduction is deoxygenation of an anthraquinone emodin (**10**), in which a phenolic hydroxyl group was lost by aromatic reduction followed by dehydration to give chrysophanol (**11**). An enzyme activity converting emodin (**10**) into chrysophanol (**11**) was rigorously established from *Pyrenochaeta terrestris*.[14] It is taken for granted that chemical reduction of aromatic rings requires a rather strong reaction condition, however, enzymic aromatic reduction proceeds under a fairly mild condition, *i.e.* at room temperature and neutral pH. This led us to study the enzymic reduction of aromatic rings. This paper deals with the partial characterization of enzymes involved in reduction of TetHN (**5**) and TriHN (**7**) and deoxygenation of an anthraquinone emodin (**10**) to afford chrysophanol (**11**).

Results and Discussions

Reduction of Aromatic Ring in Fungal Melanin Biosynthesis: Hydroxynaphthalene Reductase in *Phialophora lagerbergii* In our previous papers on the biosynthesis of scytalone (**6**), a derivative of tetrahydroxynaphthalene, TetHN (**5**) was postulated as an intermediate in the biosynthesis of fungal melanin as well as the immediate precursor of scytalone (**6**).[11] In continuing of our studies on the biosynthesis of scytalone (**6**),[15] we attempted partial purification and characterization of hydroxynaphthalene reductase, because the cell free extract of *Verticillium dahliae*, a phytopathogenic fungus producing melanin, has been shown to catalyze reduction of TetHN (**5**) and TriHN (**7**) into scytalone (**6**) and vermelone (**8**), respectively.[13] A reaction mixture containing an enzyme preparation from *Phialophora lagerbergii*, a substrate, either TetHN (**5**) or TriHN (**7**), and NADPH was incubated under N_2 atmosphere and the enzyme activity was determined by reverse phase HPLC analysis of the reaction products. The activity of hydroxynaphthalene reductase was detected in a crude cell free preparation. The reaction under N_2 atmosphere was essential, because TetHN (**5**) was easily oxidized by atmospheric oxygen to form a naphthoquinone flaviolin and a complex mixture of oxidized products. An active enzyme protein was precipitated with ammonium sulfate at 30—55% saturation and the precipitates were further fractionated successively with DEAE-cellulose, Phenyl Sepharose and HPLC gel filtration columns. The native molecular weight of the enzyme was estimated to be *ca.* 140 kDa from its retention time in the HPLC gel filtration column. pI value was estimated to be 5.9 by an isoelectric focusing experiment. The purified enzyme was not homogeneous and showed two major bands near 31 kDa in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (data not shown). Table I summarizes the results of four-step purification of hydroxynaphthalene reductase. The activity of hydroxynaphthalene reductase was measured at each purification step for the two substrates, TetHN (**5**) and TriHN (**7**). The two activities, reduction of TetHN (**5**) and TriHN (**7**), behaved almost in parallel throughout the four purification steps, indicating that the same protein possesses the activities to reduce both substrates. In our previous studies on the biomimetic reduction of TetHN (**5**) it was firmly established that TetHN (**5**) is solely present as its keto-tautomers (**5a, b**) in a strongly alkaline solution, sodium methoxide in methanol.[15] Contrary to TetHN (**5**), no NMR signals corresponding to a keto-form of TriHN (**7**) were observed in strong alkaline solution, indicating that the chemical properties of TetHN (**5**) and TriHN (**7**) are quite different. The same enzyme, however, catalyzes reduction of both substrates under a neutral condition. The transient formation of a keto-tautomer and its reduction has been suggested in deoxygenation of an anthraquinone emodin (**10**) based on the exchange of proton adjacent to the deoxygenated phenolic hydroxy group.[14b] The enzymic reduction of a carbonyl group of a keto-tautomer (**5c**) is the most plausible mechanism of aromatic ring reduction

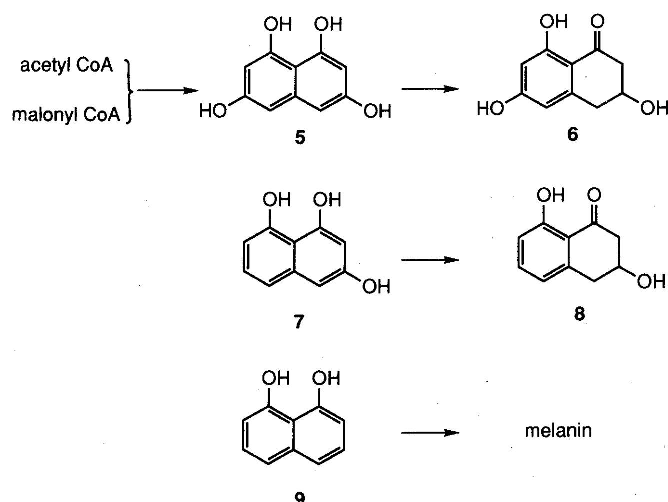

Chart 1. Biosynthetic Scheme of Fungal Melanin

Table I. Partial Purification of Hydroxynaphthalene Reductase

Purification step	Total volume (ml)	Total protein (mg)	1,3,6-TriHN to vermelone: Total activity (mU[a])	Specific activity (mU/mg)	Purification (-fold)	Yield (%)	1,3,6,8-TetHN to scytalone: Total activity (mU[a])	Specific activity (mU/mg)	Purification (-fold)	Yield (%)
Crude homogenate	58	29.3	1570	53.5	1	100	4387	149	1	100
$(NH_4)_2SO_4$ precipitation	3.9	5.84	701	120	2.24	45	668	115	0.77	15
DEAE–cellulose	5.0	1.16	297	257	4.80	19	873	755	5.05	20
Phenyl sepharose	4.4	0.36	78.3	216	4.04	5.0	136	375	2.51	3.1
$G3000SW_{XL}$	0.6	0.045	30.4	676	12.6	1.9	46.8	1040	6.96	1.1

a) 1U: μmol. Products formed/30 min.

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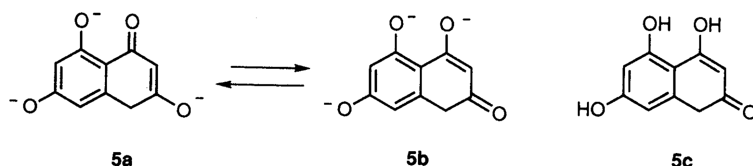
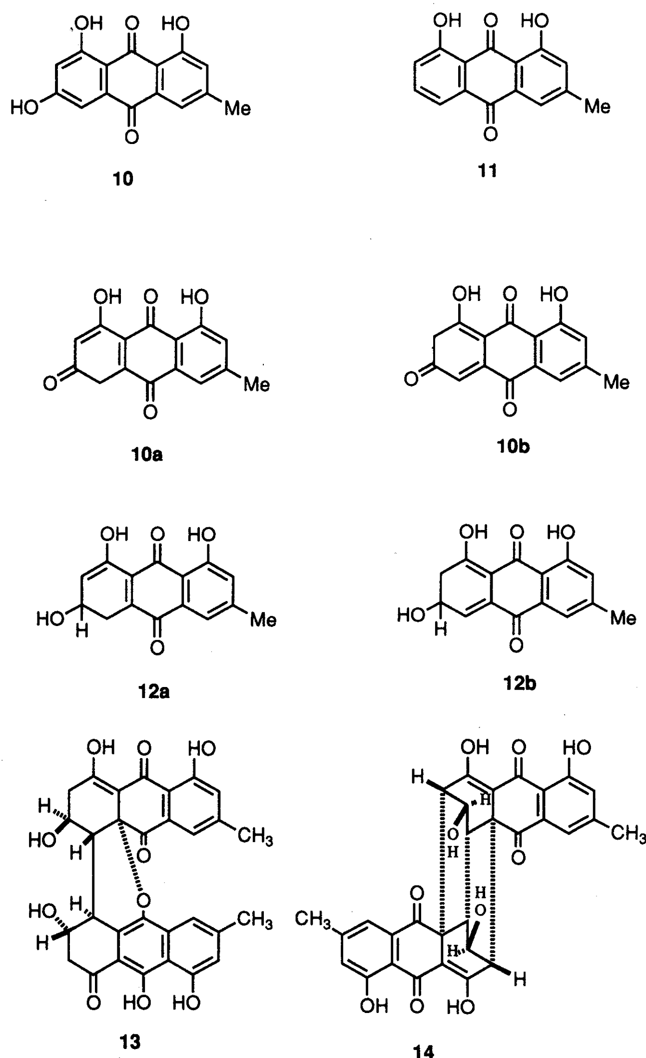


of hydroxynaphthalene. This may indicate that hydroxynaphthalenes are transiently present as their 3-keto-tautomers when they bind to the enzyme and that the hydride from NADPH directly attacks C-3 carbonyl group.

Deoxygenation in Anthraquinone Biosynthesis: Emodin Deoxygenase in *Pyrenochaeta terrestris* In the biosynthesis of ergochromes, fungal pigments produced by *Claviceps purpurea*, anthraquinones emodin (**10**) and chrysophanol (**11**) were shown to be their efficient precursors by feeding experiments with radio-labelled compounds.[16)] The structure of ergochromes indicated that emodin (**10**) was incorporated *via* chrysophanol (**11**), an anthraquinone with one less hydroxy groups than emodin (**10**).[16b,c)] Anderson *et al.* first detected an enzyme activity converting emodin (**10**) into chrysophanol (**11**).[14a)] This conversion is believed to consist of two steps, reduction of a keto-tautomer of emodin (**10a** or **10b**) to afford dihydroemodin (**12a** or **12b**) and subsequent dehydration. The transient formation of the corresponding keto-tautomer of emodin (**10a** or **10b**) in enzymic reaction was supported by the incorporation of deuterium from deuterium oxide in an incubated solution into chrysophanol carbons adjacent to the deoxygenated phenolic hydroxy group.[14b)] The exchange of proton with deuterium was also observed in recovered emodin (**10**) when it was incubated with the enzyme but without NADPH.[14c)] The overall reaction is regarded as the deoxygenation of emodin (**10**) and the enzyme was thus designated emodin deoxygenase.[14a,c)] Since one of the possible tautomers of dihydroemodin (**12b**) is an important hypothetical intermediate in the biosynthesis of anthraquinonoids such as flavoskyrin (**13**) and rugulosin (**14**),[17)] the enzyme capable of catalyzing reduction of the aromatic ring of emodin (**10**) should have a critical role in the biosynthesis of anthraquinonoids. This drew our attention and led us to study the enzyme further in detail. Recently Anderson *et al.* reported the partial purification and characterization of emodin deoxygenase and particularly emphasized the activation of the enzyme reaction with a low molecular weight cofactor.[14c)]



A crude enzyme was prepared according to the procedure reported by Anderson *et al.* from the mycelium of *Pyrenochaeta terrestris*[14a)] and the enzyme activity converting emodin (**10**) into chrysophanol (**11**) was assayed using ^{3}H-labelled emodin (**10**) as a substrate and measuring the amount of chrysophanol (**11**) formed. In a preliminary trial for the fractionation of the enzyme with various types of columns, DEAE-cellulose, HPLC DEAE-5PW and HPLC TSK-gel columns were found to be practically applicable methods of enzyme purification. Desalted ammonium sulfate precipitate (35—65% saturation) was first fractionated with DEAE-5PW and then twice with TSK gel, G3000SW$_{XL}$ linearly connected with G2000SW$_{XL}$ to give a single peak with enzyme activity which corresponds to a molecular weight of 90 kDa. However, SDS–PAGE revealed that the active fraction contained several proteins of different molecular weights. To clarify the participation of a low molecular weight activating factor as suggested by Anderson *et al.*,[14a,c)] the enhancing activities of the eluted fractions from the DEAE-cellulose column were tested against the active fractions of emodin deoxygenase obtained from HPLC. Since some of DEAE column fractions enhanced the activity of HPLC enzyme fraction, the fractions of DEAE-cellulose column chromatography were investigated in detail. The eluents of DEAE cellulose column were collected in faster eluted ED I (fr. 35—51) and slower eluted ED II (fr. 81—98) fractions which showed only low deoxygenation activity (Fig. 1 and Table II). The fractions of active enzyme corresponding to emodin deoxygenase were eluted between ED I and ED II. The activities of ED I and ED II themselves were very low, however, their combined incubation showed a remarkably high activity compared to the control experiments. When ED I and ED II were heated at 95 °C, they lost their synergistic effect

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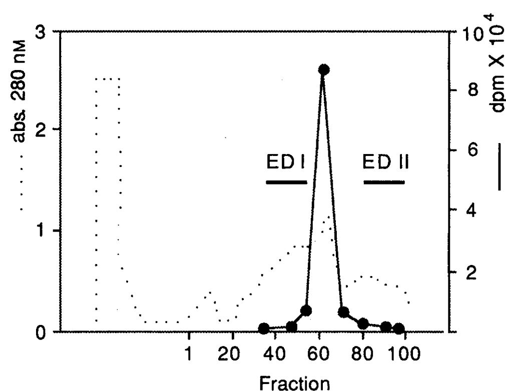

Fig. 1. DEAE-Cellulose Elution Profile and Fractionation of ED I and ED II

Table II. Activity of Emodin Deoxygenase with ED I and ED II

Enzyme solution	Activity (dpm)
ED I	288
ED II	156
ED I+ED II	3542
ED I+boiled ED II	205
Boiled ED I+ED II	117

(Table II). The results of heat treated experiments demonstrated that the enhancing factors contained in ED I and ED II were not low molecular weight compounds but proteins.

At this stage the two fractions (ED I and ED II) were regarded to be a reductase and a dehydratase as in fungal melanin biosynthesis where the two enzymes were obtained separately. Since ED I and ED II were not clearly separated enzymes and the apparent activity was thought to result from the overlap of the two proteins, the following experiments were conducted to test this possibility. Enzyme reactions with ED I and ED II were terminated with either 5 N KOH or 2 N HCl separately and the quantities of chrysophanol (**11**) formed were determined in each reaction. From the known chemical property of germichrysone (**15**), a hydroanthracene, dihydroemodin (**12**) was excepted to be more stable under an acidic condition. If dihydroemodin (**12**) was formed and present in the reaction mixtures it should be quantitatively converted into chrysophanol (**11**) by the treatment with alkali. However the amounts of chrysophanol (**11**) formed were the same in acid and alkali treatments and no positive information was obtained by the experiments to prove the presence of dihydroemodin (**12**) in the reaction mixture with ED I and ED II (Table III). The molecular weights of ED I and ED II were estimated to be *ca.* 90 kDa by HPLC gel filtration which is more or less the same as that reported by Anderson *et al.*[14c] The exact roles of these two proteins are still unknown, however, the participation of low molecular weight factor was not observed in our experiments.

Effects of cofactors, inhibitors and metal ions were tested with the partially purified enzyme (*i.e.* fr. 63 of the DEAE cellulose column) and the results are summarized in Tables IV and V. The critical role of sulfhydryl group is suggested from the inhibitory effects of *p*-mercuriphenylsulfonic acid (PCMPS) and *N*-ethylmaleimide. Strong inhibitory effects of α,α′-dipyridyl and *o*-phenanthroline may indicate that metal ion such as ferrous ion is essential in the reaction, however, externally added ferrous ion was rather inhibitory at a high concentration as high as 1.0 mM. NADPH could not be replaced by NADH and ATP was rather inhibitory to the reaction as reported by Anderson *et al.*[14c] The substrate specificity of emodin deoxygenase was investigated on anthraquinone analogues with a crude enzyme preparation, ammonium sulfate precipitate. Incubations with boiled enzyme as well as incubation without NADPH were carried out as control. Of the substrates tested (see Experimental) germichrysone (**15**), 1,3-dihydroxyanthraquinone (**16**) and emodin anthrone (**17**) were found to be as good substrates as emotin (**10**), while ω-hydroxyemodin (**18**) was less effective in this role. Since emodin anthrone oxygenase that catalyzes oxygenation of emodin anthrone (**17**) into emodin (**10**) was found widely distributed among fungi,[18] emodin anthrone (**17**) was utilized as a substrate

Table III. Incubation Experiments of ED I and ED II with Acid and Alkali Treatments

Enzyme solution	HCl treated (dpm)	KOH treated (dpm)
ED I	1947	1776
ED II	4338	3466
ED I+ED II	27514	25082

Table IV. Effects of Inhibitors and Metal Ions on Emodin Deoxygenase Activity

Inhibitors and metal ions	Concentration (mM)	Relative activity (%)	Inhibition (%)
None	—	100	0
Iodoacetamide	1.0	142	−42
N-Ethylmaleimide	1.0	15	85
	0.5	25	75
PCMPS	1.0	0	100
	0.5	1.2	98.8
α,α′-Dipyridyl	1.0	20	80
	0.5	31	69
o-Phenanthroline	1.0	2.3	97.7
	0.5	5.3	94.7
Aminopterine	1.0	76	24.3
$CoCl_2$	1.0	214	−114
	0.5	231	−131
$CuSO_4$	1.0	0.1	99.9
	0.5	1.0	99.0
$FeSO_4$	1.0	67.3	32.7
$ZnSO_4$	1.0	65.3	34.7

Table V. Effects of Cofactors on Emodin Deoxygenase Activity

Cofactor	Concentration (mM)	Relative activity (%)
None	—	0
NADPH	1.0	100
	5.0	135
NADH	1.0	0
	5.0	0
Ascorbic acid	1.0	0
	5.0	0
NADPH (1 mM)+ATP	1.0	72
	5.0	42

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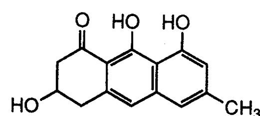

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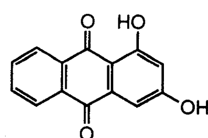

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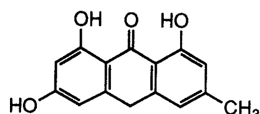

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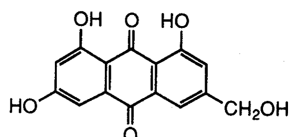

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after it was oxidized into emodin (**10**). The consumption of germichrysone (**13**) by a crude enzyme preparation may reflect the dehydratase activity of emodin deoxygenase to give chrysophanol anthrone, which was further converted into chrysophanol (**11**) by oxidation with emodin anthrone oxygenase.[18] Chrysophanol anthrone was a more efficient substrate for emodin anthrone oxygenase.[18] The enzyme activity of emodin deoxygenase was detected in several *Penicillia* which produce anthraquinones and related metabolites. Two strains of *Penicillium oxalicum* and *Penicillium islandicum* showed the significant activities of emodin deoxygenase, which seems to be commonly found among fungi producing anthraquinones.

Conclusion

The two enzymes so far investigated relate to the deoxygenation of phenolic aromatic polyketides and act in the post-aromatic deoxygenation process. In the enzymic deoxygenation of hydroxynaphthalenes (**5**, **7**) and emodin (**10**) the reduction of aromatic ring with NADPH seems to occur in their keto-tautomers which are transiently formed by the action of enzyme as suggested by Scott and his colleagues for the deoxygenation of emodin (**10**).[14b)] It is just a coincidence that TetHN (**5**) has a unique chemical property showing the presence of keto-tautomers in NMR spectra under a strong alkaline condition and that it is easily reduced by sodium borohydride, since TriHN (**7**) without such property is also reduced by the hydroxynaphthalene reductase. Deoxygenation of emodin (**10**) involves the step of aromatic reduction which is also expected to occur in a keto-tautomer (**10a** or **10b**), though emodin shows no property of giving NMR signals of keto-tautomers. One of the proteins, ED I or ED II, may act to generate the keto-tautomer of emodin (**10a** or **10b**) in facilitating its reduction into dihydroemodin (**12**). The results so far obtained concerning their two proteins are not conclusive and further studies are necessary to clarify the exact reaction mechanisms.

Experimental

General Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. NMR and MS were taken on a JEOL GSX-400 or GSX-500 and JEOL JMS DX-300. SDS–PAGE was carried out with Mini Protean II with 10.5% gel. Isoelectric focussing was carried out with Bi-Lyte (pH range 3—10) and a Rotofor Cell (Bio-Rad) according to the instruction manual of the manufacturer. *Verticillium dahliae* was the kind gift of Dr. I. Yamaguchi of RIKEN and *Phialophora lagerbergii* was obtained from Commonwealth Micological Institute. *Pyrenochaeta terrestris* was the kind gift to Dr. J. A. Anderson. *Penicillium brunneum*, *Penicillium islandicum*, *Penicillium oxalicum* 73-GO-3 and 73-GO-10 were obtained from Dr. S. Udagawa of National Institute of Hygienic Science. Anthraquinone related compounds used for substrate specificity experiments were from our collection.

TetHN (5) TetHN (**5**) was synthesized by the alkaline fusion of chromotropic acid according to the literature.[19] Scytalone (**6**) was obtained from the cultures of *Phialophora lagerbergii* as described.[11]

TriHN (7)[6a)] TriHN (**7**) was synthesized from scytalone (**6**): scytalone (**6**, 3.65 g) was dissolved in 1 N NaOH (50 ml) and the solution was heated at 45 °C under N_2 stream. After the reaction mixture was cooled to room temperature, it was poured into ice water and made acidic with 0.5 M H_3PO_4. The acidic solution was extracted with EtOAc, and the EtOAc solution was washed with H_2O and dried over Na_2SO_4. The reaction product was purified with column chromatography on silica gel to give greenish yellow TriNH (**7**) in a yield of 76%. High resolution MS Calcd for $C_{10}H_8O_3$: 176.0474. Found: 176.0476.

Vermelone (8)[6d)] Vermelone (**8**) was prepared by biological reduction of TriHN (**7**) with the cultures of *Verticillium dhaliae*.[6a,c)] *Verticillium dahliae* grown on potato–carrot–dextrose-agar slant was inoculated to liquid SNC125 medium and cultured stationary at 25—26 °C for 3 weeks.[6a,c)] TriHN (**7**) in ethanol (25 mg/ml) was added to seven culture flasks and incubated for a further 6 d. Mycelia were removed by filtration and the culture medium was made acidic by the addition of 1 N HCl. The acidified medium was extracted with EtOAc and the EtOAc solution was dried over Na_2SO_4. Vermelone (**8**, 4.5 mg) was obtained by silica gel column chromatography with chloroform–acetone (9 : 1) as a solvent. MS *m/z* (rel. int. %): 178 (100, M^+), 160 (78, $M-H_2O$). ^{1}H-NMR ($CDCl_3$) δ: 2.79 (1H, ddd, $J=17.1$, 8.0, 1.0 Hz, C-2ax), 2.98 (1H, ddd, $J=17$, 3.9, 1.0 Hz, C-2eq), 3.02 (1H, br dd, $J=16.0$, 8.0 Hz, C-4ax), 3.23 (1H, br dd, $J=16$, 3.9 Hz), 4.45 (1H, septet like, $J=3.9$, 3.9, 8.0, 8.0 Hz, C-3), 6.75 (1H, dd, $J=7.3$, 1.0 Hz, C-5), 6.84 (1H, br d, $J=7.3$ Hz, C-7), 12.27 (1H, s, –OH). ^{13}C-NMR ($CDCl_3$) δ: 38.34 (C-4), 47.18, (C-2), 66.32 (C-3), 116.13 (C-7), 116.76 (C-8a), 119.73 (C-5), 136.83 (C-6), 141.16 (C-4a), 162.67 (C-8), 202.78 (C-1).

Culture of *Phialophora lagerbergii*[11] *Phialophora lagerbergii* IMI 96735 grown on potato–dextrose-agar (PDA) was inoculated in culture medium containing sucrose (50.0 g), glucose (30.0 g), yeast ext. (1.0 g), $NaNO_3$ (2.0 g), K_2HPO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g) in 1 l H_2O. Preliminary shake culture was carried out on a rotary shaker (200 rpm) at 27 °C for 7 d. Mycelial suspensions of 5 ml was then inoculated into production culture flasks containing the same medium. The cultures were grown on a rotary shaker (200 rpm) for another 7 d and harvested mycella were kept at −80 °C until use.

Assay of Hydroxynaphthalene Reductase An assay mixture in a total volume of 1 ml contained 100 mM potassium phosphate buffer (KPB) (pH 6.8), 1 mM substrate, 0.5 mM NADPH, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA and an appropriate amount of enzyme. An incubation mixture without substrate was preincubated for 15 min under N_2 stream at 27 °C and ethanol solution of the substrate was then added to the incubation mixture, which was further incubated under the same conditions for 30 min. The reaction was terminated by the addition of 0.5 M H_3PO_4. The reaction products were then extracted with EtOAc (2.0 ml) by vortexing. The organic layer (1.4 ml) was washed with H_2O (0.2 ml) by vortexing and 1.2 ml of EtOAc solution was subjected for quantification on HPLC. The solvent was removed and dried in a desiccator. The reaction products were dissolved in MeOH and quantified with reverse phase HPLC (TSKgel ODS-120T, Tosoh) using solvent systems of H_2O–CH_3CN (12 : 88) for scytalone (**6**) and H_2O–CH_3CN (22 : 78) for vermelone (**8**). The two solvent systems contained 2% AcOH to give sharp peaks.

Partial Purification of Hydroxynaphthalene Reductase Frozen mycelium (30 g) in liquid N_2 was mixed with Polyclar AT (50 w/w) in a motor, crushed with a pestle and pulverized. The mycelium was then extracted with 100 mM KPB (pH 6.8, 5 ml/g mycelium) containing 1 mM EDTA and DTT. KPB buffer was previously degassed under reduced pressure in an ultrasonic bath. The extract was filtered through 4 layer of cotton gauze and the filtrate was centrifuged at 10000 × ***g*** for 30 min to give a crude extract (118 ml). Crude extract (115 ml) was subjected to ammonium sulfate precipitation (30—50%) and the pI value of precipitated crude enzyme was measured by isoelectric focussing with a Rotofor (BIO-RAD) according to the manual using BIO-LYTE 3/10. The precipitates in 10 mM KPB were desalted with PD-10 (Pharmacia). The buffer was then loaded on a DEAE-cellulose (DE 52 Whatman) column and active fractions (7.2 ml) were eluted with 10 mM KPB containing 1 mM DDT, 1 mM EDTA and 0.1 M KCl. The pooled active fractions were concentrated to 1.5 ml

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with Centricon 30 (Amicon) and applied to a Phenyl Sepharose (CL-4B Pharmacia) column which had been equilibrated with 100 mM KPB buffer containing 1 mM DTT and EDTA, and 0.4 M ammonium sulfate. Active fractions were eluted with the buffer without ammonium sulfate, pooled, concentrated up to 170 μl with Centricon 30 and loaded on a HPLC gel filtration column (G3000SW$_{XL}$ Tosoh). Active fraction was eluted as a single fraction with a retention time of 20 min which corresponds to 60 kDa.

Cultures of *Pyrenochaeta terrestris* The stock cultures of *Pyrenochaeta terrestris* were maintained on PDA slant. The fungus was precultured in a 100 ml perculture medium in a 500 ml Erlenmeyer flask at 200 rpm on a shaker for 5 d at 30 °C. The preculture medium contained glucose (10 g), neopeptone (5 g) and corn steep liquor (5 g) in 1 l water. The precultured fungus was homogenized with a Waring blender and inoculated into 500 ml Erlenmyer flasks containing 150 ml production culture medium. The production culture medium consisted of soluble starch (5 g) and neopeptone (1 g) in 1 l water. The production cultures were carried out for 4 or 5 d and cultured mycelia were collected by filtration, washed twice with H_2O and then with buffer A, which was composed of 20% (v/v) glycerol, 0.1 M KPB buffer pH 7.4 and 1 mM EDTA. The mycelia were frozen with liquid nitrogen and kept at −20 °C until use.

Assay Method of Emodin Deoxygenase[14)] Emodin deoxygenase was assayed as described by Anderson *et al.* with some modifications.[14a)] The substrate of the reaction was ^3H labelled emodin which had been prepared by the Wilzbach method (carried out by Amersham Co.). Radioactive emodin was purified with an oxalic acid treated silica gel column and reverse phase HPLC (ODS-120T). The specific activity of radioactive emodin was 15 MBq/μmol (55.5 MBq/mg). The assay mixture (total volume 1 ml) consisted of enzyme solution (less than 200 μl), [^3H]emodin (1.65 nmol, 25 kBq in 25 μl ethylene glycol monomethyl ether), 2.5 mM NADPH and buffer C (more than 725 μl to make a final volume of 1 ml). Buffer C contained 0.1 M citrate buffer pH 5.5, 20% glycerol (v/v) and 0.5 M sucrose. The assay mixture was incubated for 45 min at 27.5 °C and 2 N HCl (200 μl) added to terminate the reaction. At the same time non-labelled chrysophanol was added as a carrier. The reaction mixture was extracted with EtOAc (1 ml) by vortexing. EtOAc layer (0.75 ml) was loaded on a silica gel TLC plate and developed with a solvent system of petrol ether : EtOAc : AcOH = 175 : 25 : 2. A zone corresponding to chrysophanol was scraped off from the plate and submitted for radioactivity counting. Enzymic formation of chrysophanol was confirmed by TLC and HPLC as well as by dilution analysis. Cold chrysophanol (21.1 mg) was added to a solution of ^3H labelled chrysophanol (3.5×10^5 dpm) and recrystallized from EtOH to give 11.1 mg chrysophanol with 7.15 dpm/nmol. The second recrystallization gave 2.45 mg chrysophanol with 4.12 dpm/nmol and the third gave 1.27 mg chrysophanol with 4.23 dpm/nmol.

Enzyme Preparation of Emodin Deoxygenase and Its Fraction with HPLC Frozen mycelia were thawed and homogenized three times for 30 s each in a Waring blender at maximum speed with 2—3 times volume of buffer A in the presence of Polyclar AT (50% w/w). The homogenate was filtered through 4 layer of cotton gauze and treated with Dowex I × 2 which had been equilibrated with potassium phosphate buffer (KPB) pH 7.5. The mixture was stirred for 20 min, centrifuged at 3000 rpm and then at 10000 × ***g***. The supernatant was filtered through a cotton plug to give a crude enzyme solution. Active protein was precipitated with 35—65% ammonium sulfate saturation. The precipitate was dissolved in 5 mM KPB pH 7.8 containing 10% glycerol (v/v) and 1 mM DTT and desalted twice with PD-10 (Pharmacia). The solution (2.5 ml) was then loaded on a HPLC DEAE-5PW (Tosoh) column and eluted with the same buffer with a linear gradient of KCl (0—0.5 M). Active fractions (frs. 15—23, 10 ml) eluted were concentrated to 1 ml with Centriprep-30 (Amicon). The enzyme solution was fractionated with TSK gel G3000SW$_{XL}$ + G2000SW$_{XL}$ (0.5 ml/min) and each 0.5 ml was collected as a fraction. Active fractions were eluted with a retention time of 34 min corresponding to a molecular weight of 90 kDa. The most active fraction (fr. 34) was again applied to the same TSK gel column and the active fractions were eluted as a single peak with a retention time of 34 min. The most active fraction of the second TSK gel column was used as an enzyme pool to detect enhancing factors in the fractions of a DEAE-cellulose column.

Fractionation of Emodin Deoxygenase with DEAE-Cellulose Column Desalted ammonium sulfate precipitates (13 ml) were loaded on a DEAE-cellulose (DE 52 Whatman) column and eluted with the same buffer under a linear gradient of KCl (0—0.3 M). Fractions 31—51, 52—80 and 81—98 were pooled as ED-I, active fractions and ED-II, respectively. The enzyme activities of the active fractions eluted as a clear peak at a KCl concentration of 0.1—0.2 M were varied by the runs and the activities were in a range of 5000—40000 dpm in final counting. The solutions of ED I and ED II were applied on TSKgel G3000SW$_{XL}$ + G2000SE$_{XL}$ column, eluted at a rate of 0.5 ml/min and each 0.5 ml was collected as a fraction. Activities of ED I and ED II were measured by a combined incubation assay, and both gave the peak of activity at a retention time of 34 min which corresponds to a molecular weight of *ca.* 90 kDa. Molecular weight was measured from a calibration plot of standard proteins: cytochrome c (12.4 kDa), adenylate kinase (32 kDa), enolase (67 kDa), lactate dehydrogenase (142 kDa) and glutamate dehydrogenase (290 kDa).

Synergistic Effect of ED I and ED II Assay mixture contained 20 μl each of ED I and ED II, [^3H]emodin (1.65 nM), NADPH (2.5 mM) in buffer C (total 1 ml). In heat treated experiments ED I or ED II was heated at 95 °C for 5 min.

Effects of Cofactors, Inhibitors and Metal Ions Incubation experiments were carried out as in the standard assay procedure with tested samples at the concentrations given in Tables IV and V.

Substrate Specificity of Emodin Deoxygenase Substrate specificity experiments were carried out using desalted ammonium sulfate precipitate (35—65% saturation) with a substrate concentration of 2.75 mM in the presence of NADPH (1.5 mM). 1,3-Hydroxyanthraquinone, emodin anthrone, germichrysone, 2-hydroxyanthraquinone, emodic acid, 2-chloroemodin, questin, parietin, penicilliopsin, skyrin, norsolorinic acid, averufin and versicolorin C were tested for substrates of emodin deoxygenase. The reaction was measured by the consumption of the substrates and analyzed by reverse phase HPLC (ODS-120T, Tosoh). Incubation experiments with boiled enzyme and without NADPH were carried out as controls. The formation of 1-hydroxyanthraquinone from 1,3-dihydroxyanthraquinone was confirmed by the HPLC analysis of the product in an incubation experiment on a ten fold scale of normal assay conditions.

Cultures of *Penicillia* and Their Emodin Deoxygenase Activities Tested *Penicillia* were cultured on respective culture media. *Penicillium bruneum*; glucose 50 g, $NaNO_3$ 2 g, K_2PO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 10 mg, $ZnSO_4 \cdot 7H_2O$ 10 mg and $CuSO_4 \cdot 7H_2O$ 5 mg in 1 l H_2O, adjusted to pH 6.8 with dil. HCl before autoclaving. *Penicillium oxalicum*; glucose 30 g, yeast extract 5 g, $NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$, KCl 0.5 g and $FeSO_4 \cdot 7H_2O$ 10 mg in 1 l H_2O, adjusted to pH 6.8 with dil. HCl. *Penicillium islandicum*; glucose 40 g, $NaNO_3$ 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, NaCl 0.5 g and $FeSO_4 \cdot 7H_2O$ 20 mg in 1 l H_2O, pH 5.5 adjusted dil. HCl. Stock cultures were maintained on PDA. Fungi were cultured stationary and harvested when pigmentation started. Mycelia were collected by filtration, washed twice with H_2O and then with buffer A. The mycelia were frozen with liq. N_2 and kept at −20 °C until use. The mycelia were pulverized in liq. N_2 in a mortar under the presence of plyclar AT (50% weight). Buffer A (2 times volume) was added to powdered mycelium and kept at 4 °C for 30 min. The mixture was filtered through 4 layer of cotton gauze and the filtrate was centrifuged at 10000 × ***g***. The activity of each filtrate was measured as in *Pyrenochaeta terrestris.* The activities of the two strains of *Penicillium oxalicum* were more or less the same level as *Pyrenochaeta terrestris*. The activity of *Penicillium islandicum* were significant but at a level 1/10 that of *Pencillium oxalicum* and *Pyrenochaeta terrestris*.

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