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CONVERSION OF EMODIN TO CHRYSOPHANOL IN A CELL-FREE SYSTEM FROM *PYRENOCHAETA TERRESTRIS*

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Key Word Index—*Pyrenochaeta terrestris*; Sphaeropsidaceae; cell-free system; biosynthesis; emodin; chrysophanol.

Abstract—Incubation of a cell-free extract from *Pyrenochaeta terrestris* with [^{3}H]emodin gave a single major product which was purified and identified as chrysophanol by mass spectrometry. Maximum conversion of [^{3}H]emodin to [^{3}H]chrysophanol was obtained with anaerobic conditions and NADPH, ATP, mercaptoethanol, iron(II) and glycerol. The apparent K_m for emodin in the crude extract was 1.0 ± 0.2 μM. At 4.2 μM, the conversion of emodin was 17 times the conversion of emodinanthrone to chrysophanol plus chrysophanolanthrone. It is proposed that chrysophanol is synthesized in plant and fungal species primarily by dehydroxylation of emodin.

INTRODUCTION

The polyhydroxyanthraquinones emodin and chrysophanol occur in several plant species [1] and have been isolated from fungi [2, 3]. These anthraquinones are usually isolated together with the anthrones emodinanthrone and chrysophanolanthrone [4]. Emodin is derived from acetate [5] via an assumed C_{16} polyketide intermediate.

The secalonic acids, mycotoxins produced by *Penicillium* sp. and by *P. terrestris*, are also derived from a C_{16} polyketide precursor [6]. The structure of the secalonic acids suggests that chrysophanol is an intermediate in their biosynthesis and this role of chrysophanol is supported by the observation that [^{3}H]chrysophanol is efficiently incorporated into secalonic acid D in *P. oxalicum* [7]. Radioactively labeled emodin and emodinanthrone are also incorporated into secalonic acid D, but with lower efficiency than chrysophanol [7, 8].

Three possible pathways for the biosynthesis of chrysophanol are (Fig. 1): (1) direct dehydroxylation of emodin; (2) dehydroxylation of emodinanthrone to chrysophanolanthrone and oxidation of chrysophanolathrone to chrysophanol; and (3) reduction of an intermediate in

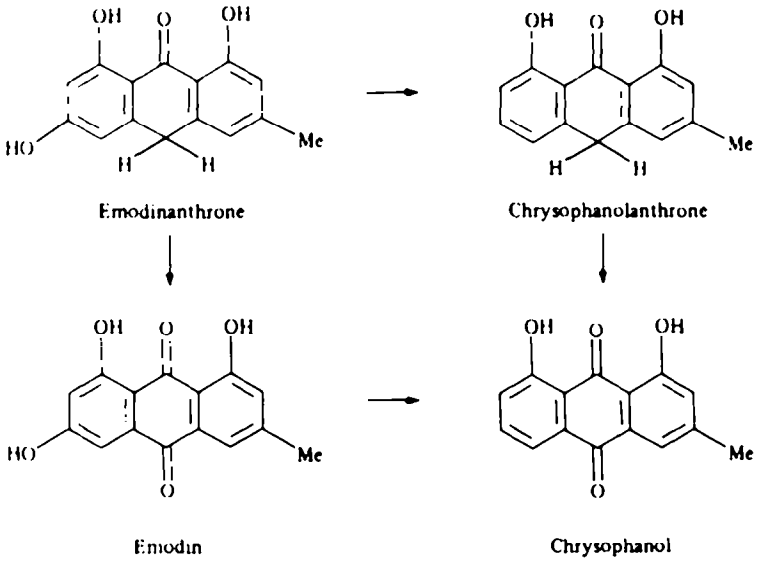

Fig. 1. Possible conversions of emodinanthrone and emodin to chrysophanolanthrone and chrysophanol.

polyketide biosynthesis, release of chrysophanolanthrone, and oxidation of chrysophanolanthrone to chrysophanol. In support of the latter pathway, reduction during addition of C_2 units to the growing polyketide chain results in the absence of a hydroxyl group in the structure of 6-methysalicyclic acid [9, 10].

[^{3}H]Emodin and [^{3}H]emodinanthrone were incubated with a cell-free system from *P. terrestris* and the conversion of the substrates to chrysophanol and chrysophanolanthrone was determined.

RESULTS

Identification of product

A single radioactive product was observed in the radioactivity scan of extracts after incubation of [^{3}H]emodin with the cell-free system from *P. terrestris*. The product cochromatographed with chrysophanol in three TLC systems. After reduction and formation of the adduct with *p*-nitrosodiphenylaniline or after acetylation, the radioactive derivative cochromatographed with the corresponding derivative of chrysophanol.

The crystallized product from an incubation with unlabeled emodin was analysed by electron impact mass spectrometry (EIMS). The spectrum was identical to that of reference chrysophanol: EIMS (direct insertion probe) 70 eV, *m/z* (rel. int.): 255 $[M+1]^+$ (12), 254 $[M]^+$ (100), 237 $[M-OH]^+$ (28), 226 $[M-CO]^+$ (16), 225 $[M-CO-H]^+$ (12), 198 $[M-2CO]^+$ (8), 197 $[M-2CO-H]^+$ (12), 152 $[M-102]^+$ (4), 151 $[M-103]^+$ (60).

Conditions for enzyme activity

Activity in the frozen mycelia and crude extracts was stable for over 4 months at −20°. When mercaptoethanol was omitted from the homogenization buffer, addition of mercaptoethanol to the incubation mixture increased activity 3.5 fold. Addition of 20% v/v glycerol gave 80% recovery of activity after 4 hr dialysis against Buffer A compared to 65% recovery without glycerol.

The conversion after three hours of incubation was 1.2 nmol [^{3}H]chrysophanol (81% conversion) with 1.16 mg/ml protein compared to 0.006 nmol [^{3}H]chrysophanol with boiled control. Addition of NADPH, iron(II), and ATP to the incubation mixture increased the conversion of [^{3}H]emodin to [^{3}H]chrysophanol (Table 1). The three fold increase in conversion with addition of NADPH indicates that NADPH is the cosubstrate for the reductive dehydroxylation. Iron(II) was a metal ion activator and increased activity three fold. The function of ATP, which increased the conversion 40%, is not known. Twice the conversion was obtained when the tube was flushed with N_2 before incubation. A component is therefore sensitive to air.

Effect of concentration of [^{3}H]*emodin on activity*

The rate increased rapidly with increase in concentration of emodin (0.64 mg protein, 0.38–3.05 nmol [^{3}H]emodin, 15 min). The data gave a linear plot of the integrated Michaelis–Menten equation [11]. The coefficient of correlation was 0.91. The K_m and V_{max} were 1.0 ± 0.2 μM and 1.26 nmol/15 min (2.2 pkat/mg), respectively.

Comparison of activity with [3H]*emodin and* [^{3}H]*emodinanthrone*

The conversion of [^{3}H]emodin was 17 times the conversion of [^{3}H]emodinanthrone to chrysophanol plus chrysophanolanthrone at 4.2 μM concentration (Table 2, average of 15 min and 30 min incubations).

DISCUSSION

The low K_m and reasonably good activity with emodin support emodin as the natural substrate for the dehydroxylase in *P. terrestris*. A similar enzyme may catalyze the conversion of emodin to chrysophanol in other fungi and in plants.

Incorporation of [^{3}H]emodin was much greater than the incorporation of [^{3}H]emodinanthrone into chrysophanol plus chrysophanolanthrone *in vitro*. However, in *in vivo* radioactive incorporation experiments with *P. oxalicum* more [^{3}H]emodinanthrone was incorporated into secalonic acid D than [^{3}H]emodin [8]. If chrysophanol is the precursor of the secalonic acids, as indicated by the efficient incorporation of labelled chrysophanol into the secalonic acids [7], the much greater conversion of [^{3}H]emodin than [^{3}H]emodinanthrone observed in the cell-free system should result in a greater amount of labelled secalonic acids from [^{3}H]emodin than from [^{3}H]emodinanthrone. Further *in vivo* and *in vitro* studies

Table 1. Effect of additions on conversion of [^{3}H]emodin to [^{3}H]chrysophanol.

Component added	[^{3}H]Chrysophanol (nmol)
NADPH, Fe(II), ATP	1.00
NADPH, Fe(II), ATP (in air)	0.49
NADPH, Fe(II)	0.60
NADPH, ATP	0.31
Fe(II), ATP	0.28

See Experimental section for assay conditions. The crude extract was dialysed for 8 hr against Buffer A. The mixture contained 0.44 mg protein and 1.52 nmol emodin. The incubation time was 35 min.

Table 2. Conversion of [^{3}H]emodin and [^{3}H]emodinanthrone to chrysophanol plus chrysophanolanthrone

^{3}H-labelled substrate	Incubation time (min)	Chrysophanol plus chrysophanolanthrone (nmol)
Emodin	15	0.55
Emodinanthrone	15	0.038
Emodin	30	1.27
Emodinanthrone	30	0.065

See Experimental section for assay conditions. The incubation mixture contained 0.64 mg protein and 2.5 nmol [^{3}H]emodin or [^{3}H]emodinanthrone.

should be carried out concerning this difference in relative efficiency of conversion of emodin and emodinanthrone into chrysophanol in the cell-free system from *P. terrestris* and into secalonic acid D in cultures of *P. oxalicum*.

The only previous cell-free system which catalysed the biosynthesis of a natural product with a missing aromatic hydroxyl was 6-methylsalicyclic acid synthetase in which the reduction resulting in the absence of a hydroxyl took place during synthesis of the polyketide [9, 10]. The results of this investigation indicate that in *P. terrestris* the missing hydroxyl in chrysophanol is removed after formation of the anthraquinone emodin. It is likely that aromatic dehydroxylations occur in the biosynthetic pathways of other natural products.

EXPERIMENTAL

Preparation of materials. Emodin was purified by crystallization from EtOH. Chrysophanol (Pfaltz and Bauer) was purified by prep. TLC and crystallized from EtOH. [^{3}H]Emodin was prepared by the Wilzbach procedure [12] and was crystallized twice from EtOH. The sp. radio activity was 72 Ci/mol. A mixture of 10 mg of [^{3}H]emodin, 10 mg unlabelled emodin, 1.89 g HOAc, and 0.80 ml 55% aq. HI was refluxed for 3 hr [Frank, B., private communication]. The precipitated [^{3}H]emodinanthrone was filtered and dried under vacuum. The yield was 13 mg and the sp. radio activity was 30 Ci/mol.

Culture methods. P. terrestris strain T-66 was obtained from the American Type Culture Collection. The growth of slants, precultures, and production cultures was carried out as described in ref. [13].

Preparation of enzyme extracts. The cultures grown in production medium were harvested after 3 days. The cells were washed five times with H_2O and once with 0.5 M sucrose, 0.1 M K-Pi buffer, pH 7.4, and 10^{-3} M EDTA. The cells were stored at $-20°$. The frozen cells were mixed with 20% (v/v) glycerol, 0.1 M K-Pi buffer, pH 7.4, 10^{-3} M EDTA, 0.020 M EtSH (Buffer A). The mixture was homogenized for 2 one-min periods with a Virtiz homogenizer at maximum speed. N_2 was bubbled through the mixture during homogenization. The mixture was centrifuged at 25000 g for 30 min. The supernatant was collected and kept frozen at $-20°$.

Protein was determined by the dye-binding method [14] with bovine serum albumin as the standard.

Assay procedure. The assay system was composed of enzyme, [^{3}H]emodin (72 Ci/mol) or [^{3}H]emodinanthrone (30 Ci/mol), 2.5 μmol ATP, 0.45 μmol NADPH, 7.1 μmol EtSH and 3.0 μmol $FeCl_2$. The vol. was brought to 0.6 ml with Buffer A.

Inoculating needles were inserted into a rubber stopper in a 16 × 100 mm test tube that contained the incubation mixture and N_2 was passed through the tube for 90 sec with shaking in an ice bath. The needles were removed and the tubes were shaken on a water bath at 25°.

The reaction was stopped by the addition of 0.2 ml of 3.0 M HCl. The soln was extracted with Et_2O and the Et_2O layer was evaporated to dryness with a stream of N_2. The residue was dissolved in MeOH and the soln was applied to a silica gel G TLC plate. Reference emodin and chrysophanol were applied on top of the sample. The plate was developed in petrol (30–60°)–HCOOEt–88% aq. HCO_2H (90:10:1; System I).

The regions of emodin and chrysophanol were scraped directly into a liquid scintillation vial, Monofluor (National Diagnostics) was added, and the cpm were measured with a Beckman L200 liquid scintillation counter. The efficiency of counting was 35%. The same amount of [^{3}H]emodin used in the incubations was added to an incubation mixture after addition of 0.2 ml 3.0 M HCl. Extraction, separation on TLC and counting was carried out as described for the incubated samples. The nmol chrysophanol was calculated from the equation:

$$\text{nmol chrysophanol} = \frac{\text{cpm chrysophanol}}{\text{cpm emodin (no incubation)}} - \times \text{nmol added emodin}$$

Chrysophanolanthrone, if present, is included in the value for the nmol chrysophanol since these two compounds did not separate in TLC solvent System I.

TLC systems. The migration of the radioactive product and reference chrysophanol in three TLC systems was measured. The adsorbent, solvent system, and R_f values of emodin and chrysophanol were: (1) silica gel G, System I, 0.16, 0.54; (2) octadecylsilane bonded to silica, Me_2CO–H_2O (3:2), 0.43, 0.29; (3) silica gel G impregnated with tartaric acid, petrol (30–60°)–$CHCl_3$ (1:1), 0.06, 0.73. The anthrone did not separate from the anthraquinone in these TLC systems.

Derivatization of 3H-labelled product. The amount of incubation mixture was increased to 31.5 ml containing 34 mg protein, 0.52 μmol [^{3}H]emodin, 5.0 ml Mixture A, and 0.15 mmol $FeCl_2$ in 2.5 ml H_2O. The mixture was incubated for 3 hr, acidified and extracted with Et_2O. The extract was concd to dryness and the residue was dissolved in 5.0 ml MeOH. An aliquot was separated by TLC. The % conversion of [^{3}H]emodin to [^{3}H]chrysophanol was 98% based on the radioactivities of the emodin and chrysophanol bands recovered from the TLC plate.

An aliquot of the radioactive product was mixed with 2 mg unlabelled chrysophanol and reduction to the anthrone was carried out [Frank, B., private communication]. The adduct of the anthrone with *p*-nitrosodiphenylaniline was prepared [15]. The *p*-nitrosodiphenylaniline derivative of chrysophanolanthrone had an R_f value of 0.77 on silica gel G with diisopropylether [14]. Another aliquot with 1.5 mg unlabelled chrysophanol was acetylated with Ac_2O [16]. The acetylated product from chrysophanol had an R_f value of 0.74 on silica gel G with cyclohexane–EtOAc (1:1). The plates were scanned for radioactivity and the locations of the radioactive peaks and derivatives of chrysophanol were compared.

Purification and mass spectrographic analysis of product. An incubation mixture containing 43 mg enzyme, 7.4 μmol emodin, 34 μmol NADPH, 0.14 mmol ATP, 0.36 mmol EtSH, and 0.15 mmol $FeCl_2$ in a final volume of 31.5 ml was incubated for 5 hr at 25° under N_2. The mixture was extracted as described above, the extract was applied to a TLC plate, and the plate was developed with Solvent I. The conversion of emodin to chrysophanol estimated from the intensities of the bands of chrysophanol and emodin was 67%. The chrysophanol was extracted from the silica gel with MeOH. The MeOH extract was evaporated to dryness and the residue was dissolved in EtOH. The product was crystallized from EtOH. The crystalline product was analysed by EIMS.

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