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CONVERSION OF EMODIN TO PHYSCION BY A CELL-FREE PREPARATION OF *ASPERGILLUS PARASITICUS*

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Key Word Index—*Aspergillus parasiticus*; hyphomycetes; phialosporae; biosynthesis; cell-free system; methylation; emodin; physcion.

Abstract—Cultures of *Aspergillus parasiticus* contained 1.2 mg/litre of physcion. A cell-free extract of a 3-day-old culture of *A. parasiticus* catalysed the conversion of [^{3}H]emodin and *S*-adenosyl-L-[Me-^{14}C]methionine to ^{3}H- and ^{14}C-labelled physcion. Methylation of emodinanthrone was 9% of the methylation of emodin. Methylation of norsolorinic acid or versicolorin A, compounds associated with aflatoxin biosynthesis, was not detected.

INTRODUCTION

Physcion (6-methylemodin) and physcionanthrone are found in higher plants including *Rhamnus*, *Polygonum*, *Cassia* and *Rumex* [1] and have been identified in cultures of *Aspergillus* sp. [2, 3]. The likely precursors of physcion and physcionanthrone are emodin and emodinanthrone. However, the *in vitro* 6-methylation of these compounds has not been reported.

Several compounds isolated from *Aspergillus* species that are, or are related to, intermediates in the biosynthesis of the aflatoxins contain hydroxyl groups at positions 1, 6 and 8, i.e. the positions of the hydroxyls in emodin, but differ from the structure of emodin at positions 2 and 3 [4]. Solorinic acid, the 6-methylated derivative of one of these compounds, norsolorinic acid, has been identified in cultures of a mutant of *A. parasiticus* (NOR) which accumulates norsolorinic acid [5]. 6,8-Dimethyl derivatives of nidurufin [6] and versicolorins A and B [7] have been isolated from *Aspergillus* sp.

Since both types of polyhydroxyanthraquinones occur in *Aspergillus* sp., the same transmethylase may methylate the 6-hydroxyl of emodin and the polyhydroxyanthraquinones related to aflatoxin biosynthesis. This work reports the presence of physcion in cultures of an aflatoxin-producing strain of *A. parasiticus* and investigates the transmethylase activity of a cell-free system from the organism toward emodin and two polyhydroxyanthraquinones proposed to be intermediates in aflatoxin biosynthesis.

RESULTS AND DISCUSSION

The major pigment in cultures of *A. parasiticus* NRRL 5862 (SU-1) was identified as physcion (EIMS). Based on the absorbance of the purified physcion at 436 nm, the cultures contained 4 nmol physcion/litre. A trace of chrysophanol (0.05 nmol/litre) was identified in the same fractions as physcion by co-migration with reference chrysophanol in three TLC solvent systems.

[^{3}H]Emodin and S-adenosyl-[Me-^{14}C]methionine ([^{14}C]SAM) were converted to physcion in a cell-free extract from *A. parasiticus* NRRL 5862. The radioactive product comigrated with physcion through three successive TLC separations. The molar ratios of [^{3}H]emodin: [Me-^{14}C] of [Me-^{14}C]SAM in the product were: solvent A, 0.81; *iso*-propanol, 0.76; RP-TLC (octyldecylsilane bonded to silica, acetone–water, 3:1), 0.85.

The product from a second incubation co-crystallized with physcion. The mCi/mol ^{14}C and the molar ratio of [^{3}H]emodin: [Me-^{14}C] of [Me-^{14}C]SAM through preparative TLC with solvent A and three crystallizations were: TLC: 0.17, 0.66; 1st cryst.: 0.15, 0.64; 2nd cryst.: 0.10, 0.52; 3rd cryst.: 0.11, 0.48. The ^{3}H/^{14}C ratio was lower in this incubation than the above incubation. The ratio moles anthraquinone: moles methyl group of 0.5–0.8 supports the monomethylation of emodin by SAM.

The activity of the transmethylase for polyhydroxyanthraquinones involved in the biosynthesis of aflatoxin was investigated (Table 1). When emodin was the substrate, a single higher R_f peak at the position of added physcion was observed in the radioactivity scan of the extract of the incubation mixture. No radioactive peaks in the region of methylated products (above R_f 0.25) were observed with norsolorinic acid or versicolorin A as substrates.

The cpm of the region above R_f 0.25 (Table 1) shows the formation of physcion from emodin. Emodinanthrone was methylated in the cell free system. After subtraction of radioactivity in the sample without added substrate, the ratio of cpm with emodinanthrone as substrate to cpm with emodin was 0.09 (Table 1). This value for relative 6-*O*-methyltransferase activity is similar to the ratio of 8-*O*-methyltransferase activity with emodinanthrone and emodin as substrates in *A. terreus* of 0.05 [8].

The observation that emodinanthrone was methylated in the cell free system supports emodinanthrone as the precursor of physcionanthrone in *Aspergillus* sp. [3]. Transmethylation of emodinanthrone is probably also the source of physcionanthrone in higher plants.

The formation of methylated product was the same with versicolorin A or norsolorinic acid as without added substrate (Table 1). Norsolorinic acid and versicolorin A are therefore not substrates or are poor substrates for the

Table 1. Methylation in a cell-free system from *A. parasiticus* NRRL 5862 by [Me-^{14}C]SAM

Additions	Product(s) (cpm)
None	204
Emodin	2527
Emodin (boiled extract)	30
Emodinanthrone	408
Norsolorinic acid	186
Versicolorin A	212

The assay mixture contained 0.36 mg protein, 7.14 nmol [Me-^{14}C]SAM (56 Ci/mol), and 25 nmol substrate in 0.6 ml. Incubation was at 30° for 6 hr. See Experimental section for other conditions.

enzyme that catalyses the methylation of emodin.

The structures of emodin, norsolorinic acid and versicolorin A are the same except at positions 2 and 3 [4]. The bulky groups on norsolorinic acid and versicolorin A apparently interfere with the binding of these compounds to the emodin SAM methyltransferase. The failure to detect transmethylase activity for norsolorinic acid or versicolorin A suggests that the enzyme is specific for emodin and is not involved in methylation of C_{20}-derived metabolites.

A. parasiticus NRRL 5862 produces all four major aflatoxins [J. W. Bennett, private communication] and therefore contains C_{20} polyketide synthetase activity and associated activities. The identification of physcion demonstrates the presence of C_{16} polyketide synthetase activity. The emodin SAM transmethylase activity in the cell free extract accounts for the presence of physcion as the major C_{16} polyketide-derived product in cultures of *A. parasiticus* and also accounts for the failure to detect emodin, which was converted to physcion by the action of the enzyme.

EXPERIMENTAL

Materials. [^{3}H]Emodin (135 Ci/mol) was prepared by the Wilzbach procedure [9]. [Me-^{14}C]SAM (56 Ci/mol) was from ICN. Chrysophanol and physcion were separated from commercial grade chrysophanol (Pfaltz and Bauer) by prep. TLC on silica gel G (all TLC was carried out on glass plates precoated with silica gel G unless indicated otherwise) with petrol (30–60°)–ethyl formate–88% aq. HCO_2H (90:10:1) (solvent A), and crystallized from 96% (w/w) EtOH. The purified compounds were identified by MS. Emodin was obtained from Aldrich Chemical Company and was recrystallized from 96% (w/w) EtOH. Emodinanthrone was prepared as described previously [10]. Versicolorin A and norsolorinic acid were provided by J. W. Bennett, Tulane University.

Culture methods. A. parasiticus NRRL 5862 was provided by J. W. Bennett, Tulane University. Flasks containing 100 ml of culture medium [11] were inoculated with spores from 12-day potato-dextrose agar slants. Cultures were shaken at 220 rpm at 25°.

Derivatives of emodin in cultures of A. parasiticus. The cultures of *A. parasiticus* NRRL 5862 were incubated for 9 days. The cultures were combined (1.5 l.) and homogenized in a Waring blender and the homogenate extracted with Et_2O. The Et_2O extract was dried (Na_2SO_4) and the Et_2O removed in a rotary evaporator.

The sample was applied to a 2.8 × 20 cm column of silica gel (60–200 mesh, J. T. Baker). Wet C_6H_6 (200 ml) and then 200 ml C_6H_6–EtOAc (19:1) were run through the column. The eluates were collected and the solvent was removed in a rotary evaporator. The pigments were separated by prep. TLC with solvent A. The absorbance spectrum of each pigment was measured in MeOH.

The MeOH soln of the major pigment was evaporated to dryness under a stream of N_2 and the compound crystallized from 96% (w/w) EtOH. EIMS (direct probe) 70 eV, *m/z* (rel. int.): 285 $[M+1]^+$ (18), 284 $[M]^+$ (100), 255 $[M-CO-H]^+$, 254 $[M-CO-2H]^+$, 241 $[M-CO-Me]^+$ (7); identical to that of authentic physcion.

The R_f of a second pigment present in trace amount was compared with reference chrysophanol on adsorptive TLC (*iso*-PrOH and solvent A) and reverse phase TLC (octyldecylsilane bonded to silica, Me_2CO–H_2O, 4:1).

Methylation of emodin, norsolorinic acid and versicolorin A by SAM. Cultures grown for 72 hr were filtered through cheesecloth. The mycelia were washed × 4 with H_2O and twice with 0.5 M sucrose, 0.10 M KPi buffer, pH 7.4, 10^{-3} M EDTA. The mycelia were stored at −20°. The mycelia were suspended in 0.10 M KPi buffer, pH 7.4, 0.02 M EtSH, 20% glycerol, and the mixture was homogenized at maximum speed for 5 × 1 min with a Virtiz homogenizer. The mixture was centrifuged at 25 000 *g* for 30 min and the supernatant was collected and stored at −20°.

A mixture of supernatant, substrate, and [Me-^{14}C]SAM was incubated for 6 hr (see Table 1 for details), acidified with 0.2 ml 3.0 M HCl, and then extracted with Et_2O. The Et_2O extract was concd to dryness under N_2. The residue was dissolved in MeOH and applied to a silica gel G TLC plate. The plate was developed with petrol (30–60°)–ethyl formate–88% aq. HCO_2H (80:20:1).

The plates were scanned with a Packard radioactivity scanner and the region of the plate above R_f 0.25 was scraped from the plate and transferred to a liquid scintillation vial. Monofluor (National Diagnostics) scintillation cocktail was added and the radioactivity was measured (counting efficiency 95%) with a Beckman 7000 liquid scintillation counter.

Incubation with [^{3}H]*emodin and* [Me-^{14}C]*SAM.* ^{3}H- and ^{14}C-labelled product was obtained by incubating 36 nmol [Me-^{14}C]SAM, 53 nmol [^{3}H]emodin and 0.18 mg protein in 3.0 ml total vol. for 5 hr at 30°. The mixture was acidified with 0.6 ml 3.0 M HCl and extracted with Et_2O. The Et_2O extract was evaporated to dryness in a rotary evaporator. The residue was dissolved in MeOH. The soln and 5 μmol unlabelled physcion were co-spotted on a TLC plate and the plate was developed with solvent A. The physcion band was scraped off and physcion was eluted from the gel with MeOH. The soln was concd to dryness under a stream of N_2 and the residue was dissolved in MeOH.

The counts in the ^{3}H and ^{14}C channels of an aliquot of the MeOH soln were determined. In order to determine the cpm from ^{14}C in the ^{3}H channel and the efficiency, [^{14}C]toluene and [^{3}H]toluene of known sp. radioactivity were added in succession, and the sample was counted after each addition.

The remainder of the sample was separated successively on adsorptive TLC (*iso*-PrOH) and RP-TLC (octyldecylsilane bonded to silica, Me_2CO–H_2O, 3:1), and the radioactivity due to ^{3}H and ^{14}C of an aliquot was measured after each TLC separation. The counting efficiency was determined after each TLC separation: ^{3}H 25–30%, ^{14}C 65–73%.

Recrystallization to constant sp. radioactivity. Another incubation of the enzyme with [^{3}H]emodin and [Me-^{14}C]SAM was carried out and the product was extracted and purified

by prep. TLC with solvent A as described above. The dried sample was dissolved in 96% (w/w) EtOH and an aliquot was removed and counted as described above. Unlabelled physcion (35 μmol) was dissolved in the remainder of the soln. Physcion was crystallized (×3) from 96% (w/w) EtOH. After each crystallization, the crystals were weighed and dissolved in EtOH. An aliquot was removed for measurement of ^{3}H and ^{14}C. The counting efficiencies, which were low because of the amount of physcion added, were 13–14% for ^{3}H and 37–39% for ^{14}C.

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