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CHLORINATION OF ANTHRAQUINONES BY LICHEN AND FUNGAL ENZYMES

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Key Word Index—biohalogenation; chlorination; chloroperoxidase; anthraquinones; lichen; fungus; *Nephroma laevigatum*; Nephromataceae; *Caldariomyces fumago*.

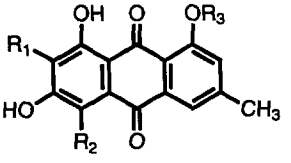
Abstract—Chlorination of the anthraquinones, emodin and 7-chloroemodin, was studied with an enzyme preparation from the lichen *Nephroma laevigatum*, and a commerically available fungal chloroperoxidase (EC 1.11.1.10). The fungal enzyme converted emodin to a mixture of 5-chloro, 7-chloro and 5,7-dichloroemodin, while converting the 7-chloro isomer to 5,7-dichloroemodin in nearly quantitative yield. The lichen enzyme, however, chlorinated emodin only at C-7 and failed to chlorinate 7-chloroemodin further. In non-enzymic controls, emodin gave the same set of products as the fungal enzyme but 7-chloroemodin did not undergo further chlorination. The chemical structures of the products were confirmed from UV, EI-mass and ^{1}H NMR spectral data. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chlorinated natural products are widely distributed and are particularly common in lichens, fungi, marine algae and invertebrates [1–4]. *In vivo* chlorination of secondary metabolites is commonly mediated by chloroperoxidases, which are also capable of catalysing the bromination and iodination of various substrates [5, 6]. While several haloperoxidases, including chloroperoxidases, have been obtained from algae [7], bacteria [8] and lower fungi [9], their presence in higher plants and bryophytes has yet to be fully established. Furthermore, the mechanisms for biohalogenation remain poorly understood, and there are few detailed biosynthetic studies of chlorinated compounds [10, 11].

The single report of a lichen haloperoxidase is that of a bromoperoxidase isolated from *Xanthoria parietina* [12]. The investigators found that the enzyme contained vanadium, essential for catalytic activity, and was remarkably thermostable, maintaining full enzymic activity at 50°. In addition, the bromoperoxidase had a high affinity for bromide ion and was only active at low concentrations. The enzyme was also inhibited by chloride and fluoride ions, and exhibited a pH optimum at 5.5.

Our previous investigation of the foliose lichen *Nephroma laevigatum* established the *in vivo* incorporation of exogenous $Na^{36}Cl$ into chlorinated derivatives of emodin (**1**): 7-chloroemodin (**2**) and 7-chloro-1-*O*-methyl-emodin (**3**) [13]. This paper deals with the *in vitro* chlorination of emodin (**1**) and 7-chloroemodin (**2**) with an active fraction isolated from *N. laevigatum*, and with a commercially available fungal chloroperoxidase.



	R_1	R_2	R_3
1	H	H	H
2	Cl	H	H
3	Cl	H	CH_3
4	H	Cl	H
5	Cl	Cl	H

RESULTS AND DISCUSSION

A semi-purified enzyme preparation was obtained from *N. laevigatum* as described in the Experimental. In order to determine whether the active fraction was capable of chlorinating anthraquinones *in vitro*, emodin (**1**) and 7-chloroemodin (**2**) were incubated with the enzyme preparation from the lichen. For comparison, a

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commercial fungal chloroperoxidase (from *Caldariomyces fumago*) was also tested for its capacity to catalyse the chlorination of **1** and **2**.

The results of the incubation experiments are given in Table 1. Incubation of emodin (**1**) with the lichen enzyme preparation at pH 3.4 resulted in the formation of 7-chloroemodin (**2**), but not 5,7-dichloroemodin (**5**); similarly, incubation of 7-chloroemodin (**2**) with the lichen enzyme failed to give any **5**. In contrast, the commercial fungal chloroperoxidase catalysed the chlorination (at pH 3.4) of emodin (**1**) to 7-chloroemodin (**2**), 5-chloroemodin (**4**) and 5,7-dichloroemodin (**5**). In addition, 7-chloroemodin (**2**) was chlorinated to 5,7-dichloroemodin (**5**) by the fungal enzyme. The latter reaction occurred with extraordinary efficiency: after 40 hr at ambient temperature, the substrate (**2**) had been completely converted to the product (**5**). The non-enzymic control reaction also converted **1** to **2** and **4**, as well as producing a small amount of **5**. Surprisingly, 7-chloroemodin (**2**) was not chlorinated in the control reaction (Table 1).

The time course of the incubations suggests that chlorination in both the lichen and fungal enzyme reactions are, indeed, catalysed by the chlorinating enzymes. The products, therefore, do not appear to be synthesized by electrophilic chlorine generated solely from hydrogen peroxide and chloride ion in the surrounding incubation medium. 7-Chloroemodin (**2**) and 5-chloroemodin (**4**) were detected, by TLC, at least 20 hr earlier in the enzyme reactions than in the control reaction (Table 1). This observation indicates that both enzymes increased the reaction rates for chlorination of **1**. Finally, 5,7-dichloroemodin (**5**) was produced from **1** at the same rate for both the fungal enzyme and control reactions, but was made from **2** only in the fungal chloroperoxidase incubation. This result suggests that the fungal enzyme may utilize different mechanisms for the chlorination of emodins, depending on the chemical structure of the initial substrate and the reaction conditions.

The results of the biohalogenation experiments are consistent with the known chemistry of *N. laevigatum*. Production of 5-chloroemodin (**4**) and 5,7-dichloroemodin (**5**) in the lichen enzyme incubation study would be unexpected, since neither **4** nor **5** has been found in natural lichen populations of *N. laevigatum* [14]. It is thus presently unclear why this lichen would produce 5-chloro-substituted emodins (e.g. **4**) when fed sodium [1 − ^{13}C]acetate, as was observed in our previous investigations on the *in vivo* biosynthesis of chlorinated anthraquinones in *N. laevigatum* [13]. It is further unclear why the chlorinating enzyme, once isolated from the lichen, appears incapable of effecting chlorination of emodin (**1**) at ring position C-5. A typical electrophilic chlorination reaction should provide both 7- and 5-chloro products (e.g. **2** and **4**). This expectation was observed in the fungal chloroperoxidase and non-enzymic reactions. The sodium [1 − ^{13}C]-acetate incubation experiment conducted previously was not supplemented with exogenous chloride ion [13], but the lichen enzyme incubation medium utilized in the present study contained 40 mM potassium chloride. It is conceivable that excess chloride ion inhibits the ability of this lichen enzyme to effect chlorination of **1** at C-5. It is also possible that chlorination of **1** at C-5 can be catalysed by an enzyme normally present in the lichen, but that particular enzyme may not have been removed from the lichen tissue during extraction, or was denatured during purification or during the incubation studies.

In light of these speculations, it is interesting to note that in their study of the bromoperoxidase from *X. parietina*, Plat *et al.* failed to mention the complete absence of brominated compounds in lichens, and did not address the question of why a brominating (and iodinating) enzyme should be present in the lichen [12]. Furthermore, halogenated secondary metabolites have yet to be identified in *X. parietina* or other *Xanthoria* spp. [15]. Interestingly, a bromoperoxidase isolated from the green alga *Penicillus capitatus* was later shown to become a chloroperoxidase at a lower pH [16, 17]. Had the vanadium-containing bromoperoxidase from *X. parietina* been studied at a lower pH, it might also have shown chloroperoxidase activity [18].

EXPERIMENTAL

General. Mps: uncorr.; ^{1}H NMR: 400 MHz. DMSO-d_6 was used in the NMR experiments (TMS as int. standard); EI-MS: 70 eV (probe); CC: flash silica gel 60 (230–400 mesh, BDH); prep. TLC or TLC: Merck Kieselgel 60 GF_{254} layers (0.1 × 20 × 20 cm) on glass plates; HPLC: Waters reversed-phase C_{18} column (3.9 × 30 cm, 10 μm, flow rate 1 ml min^{-1}); UV spectra: EtOH (UV grade); DEAE–Sephadex A 50

Table 1. Reaction products from incubation experiments with emodin (**1**) and 7-chloroemodin (**2**)

Substrates (mg)	Incubation time (hr)	Product (mg, % yield, time of first detection in hr)		
		Lichen enzyme reaction	Fungal enzyme reaction	Non-enzymic reaction
1 (3)	68	**2** (1, 30, 20)	**2** (1, 30, 20)	**2** (1, 30, 40)
			4 (1, 30, 20)	**4** (1, 30, 40)
			5 (1, 27, 68)	**5** (0.5, 14, 68)
2 (3)	68		**5** (3, 90, 40)	

(Sigma); chloroperoxidase (partially purified lyophilized powder from *C. fumago*, Sigma).

Plant material. Nephroma laevigatum Ach. was collected on Gabriola Island, British Columbia (Canada) in October 1994. Two voucher specimens are deposited in the Botany Department Herbarium at the University of British Columbia.

Isolation of lichen chlorinating enzyme. The fresh lichen (400 g) was carefully cleaned of moss, soil and other debris and washed several times with deionized water. The lichen was homogenized in a blender with 2 l 0.1 M KH_2PO_4 buffer (pH 5.8). The homogenate was filtered through cheese cloth and centrifuged. The extraction process was repeated $\times 3$, and the filtered extracts centrifuged. The supernatants were combined, adjusted to 40% saturation with $(NH_4)_2SO_4$, and left at 0° for 24 hr. After centrifugation, the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$, and left at 0° for 24 hr. The pellet was suspended in 0.1 M KH_2PO_4 buffer (pH 5.8), and then dialysed overnight (2 changes) against 10 mM KH_2PO_4 (pH 5.8) (fr. 1). The 60% $(NH_4)_2SO_4$ soln was centrifuged, the pellet suspended in 0.1 M KH_2PO_4 buffer (pH 5.8), and dialysed overnight (2 changes) against 10 mM KH_2PO_4 buffer (pH 5.8) (fr. 2). Frs 1 and 2 were assayed by the dimedon method for haloperoxidases [5]. Fr. 1 displayed little activity. Fr. 2 was purified on a DEAE–Sephadex A 50 column by gradient elution with 0.1–0.2 M KH_2PO_4 buffer (pH 6.0). The column frs (5 ml) were assayed, the active portions combined, and dialysed overnight (2 changes) against 10 mM KH_2PO_4 buffer (pH 5.0). The partially purified enzyme preparation was lyophilized, and the crude enzyme (in 25 ml of 0.1 M KH_2PO_4 buffer, pH 5.0) assayed once again. Two isolations and semi-purifications of lichen enzyme were undertaken. The yield of crude lichen enzyme was 6 mg for both purifications (a total of 12 mg) with a spec. activity of 30 nkat mg^{-1} for each purification. The protein content and spec. activity were calculated according to the Bradford dye-binding method [19] and dimedon assay [5], respectively (one kat of activity is defined as 1 mol of monochlorodimedon chlorinated per second [5]). The crude enzyme preparation was used in the chlorination experiments without further purification.

Biohalogenation experiments. Substrates **1** (3 mg, 11 μmol) and **2** (3 mg, 10 μmol) were dissolved separately in 20 ml of DMF. The solns were buffered with 14 ml of 0.2 M KH_2PO_4 (resulting in an incubation medium composed of 34 ml of 6:4 0.2 M KH_2PO_4–DMF, pH 3.4). Each soln was prepared in triplicate. To each stirred soln was added 100 mg of KCl (1.4 mmol). To each of a pair of solns containing **1** or **2** was added 100 nkat of *C. fumago* chloroperoxidase; 100 nkat of crude *N. laevigatum* enzyme was added to a second pair. To initiate the chlorination process, 17 μl (280 μmol) of 50% aq. H_2O_2 was added to each of the four enzyme reactions, as well as to the two non-enzymic controls. The six mixtures were maintained at ambient temp. (25°), examined periodically by TLC ($CHCl_3$–MeOH 4:1), and the reactions were stopped after 68 hr of stirring by removal of solvent under vacuum.

Isolation and characterization. The reaction mixts were concd *in vacuo* to give yellow–orange solids. The solids were triturated $\times 3$ with 100 ml portions of EtOAc to separate products from inorganic material and denatured enzyme. The EtOAc extracts were concd *in vacuo* to give mixts of chlorinated products. Purification was effected by silica gel CC (gradient of 9:1 $CHCl_3$–MeOH to MeOH), and finally prep. TLC ($CHCl_3$–MeOH 4:1). All final products were recrystallized, and their purities verified by RP-HPLC. Chemical structures were characterized by UV, EI-MS and ^1H NMR. The spectral data of the isolated products (**2**, **4**, **5**) were identical with those reported previously [13, 14, 20].

Products from lichen enzyme reaction

7-*Chloroemodin* (**2**). Compound **2** (1 mg, 30% yield), orange crystals (EtOAc), mp 281–282°; TLC: R_f 0.50 ($CHCl_3$–MeOH 4:1).

Products from fungal enzyme reaction

7-*Chloroemodin* (**2**). Compound **2** (1 mg, 30% yield), orange crystals (EtOAc), mp 281–282°; TLC: R_f 0.50.

5-*Chloroemodin* (**4**). Compound **4** (1 mg, 30% yield), orange crystals (EtOAc), TLC: R_f 0.60.

5,7-*Dichloroemodin* (**5**). Compound **5** produced from **1** (1 mg, 27% yield) and from **2** (3 mg, 90% yield) was obtained as red crystals (MeOH); mp 268–270°; TLC: R_f 0.20.

Products from non-enzymic (control) reaction

7-*Chloroemodin* (**2**). Compound **2** (1 mg, 30% yield), orange crystals (EtOAc), mp 281–282°; TLC: R_f 0.50.

5-*Chloroemodin* (**4**). Compound **4** (0.5 mg, 30% yield), orange crystals (EtOAc), TLC: R_f 0.60.

5,7-*Dichloroemodin* (**5**). Compound **5** (0.5 mg, 14% yield), red crystals (MeOH), mp 268–270°; TLC: R_f 0.20.

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