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# BIOSYNTHETIC STUDIES ON CHLORINATED ANTHRAQUINONES IN THE LICHEN *NEPHROMA LAEVIGATUM*

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**Key Word Index**—*Nephroma laevigatum*; Nephromataceae; lichen; tracer studies; chlorinated anthraquinones; polyketide pathway.

**Abstract**—Eight anthraquinones were produced by the lichen *Nephroma laevigatum* Ach. during laboratory incubation. They were characterized by $^{1}$H NMR, $^{13}$C NMR and mass spectrometry as emodin, 7-chloromodin, 7-chloro-1-*O*-methylemodin, 7-chloro-1-*O*-methyl-$\omega$-hydroxyemodin, 5-chloroemodin, 5-chloro-1-*O*-methylemodin, 5-chloro-1-*O*-methyl-$\omega$-hydoxyemodin and 5-chloro-$\omega$-hydroxyemodin. The 5-chloroemodins are new lichen substances, although 5-chloro-1-*O*-methylemodin was isolated previously from a fungus. Radiolabelled 7-chloroemodin derivatives were obtained upon incubation of the lichen with Na [2-$^{14}$C]acetate, demonstrating the utilization of acetate in their formation. 7-Chloro-1-*O*-methylemodin, enriched with $^{13}$C, was isolated following incubation with Na [1-$^{13}$C]acetate. Analysis of its $^{13}$C NMR spectrum showed that the lichen anthraquinones are biosynthesized through the polyketide pathway. Efficient incorporation of label from Na$^{36}$Cl into 7-chloroemodin and 7-chloro-1-*O*-methylemodin showed that the lichen is capable of chlorinating preformed anthraquinones.

## INTRODUCTION

Anthraquinones are an important group of naturally occurring pigments that are widely distributed in nature. They are particularly prominent in fungi [1–3], higher plants [4] and lichens [5–10]. The majority of anthraquinones are derived by way of the polyketide pathway, primarily by use of acetate (acetyl-CoA) as the initial carbon source in their biosynthesis [11, 12]. Emodin (**1**) is known to be derived from acetate in higher plants [13], lower fungi [1, 14] and basidiomycetes [3, 12]. In the basidiomycete genera *Cortinarius* and *Dermocybe*, feeding experiments with $^{13}$C-acetate have established the polyketide origin of **1** and its transformation products [15]. It has been suggested that lichen anthraquinones are derived from acetate, as is the case with fungi [16–20].

We report here the first experimental evidence for the biogenesis of lichen anthraquinones from acetate. We have established, using $^{14}$C- and $^{13}$C-labelled acetate, the derivation of **1**, 7-chloroemodin (**2**) and 7-chloro-1-*O*-methylemodin (**3**) in the lichen *Nephroma laevigatum*. New 5-chloro-substituted anthraquinones were also isolated from the lichen when fed with Na [1-$^{13}$C]acetate; these compounds were not identified in our previous examination of field-collected *N. laevigatum* [21].

In our earlier work on this foliose lichen, we had characterized six pigments (**1–4**) from a sample collected in British Columbia, Canada [21]. *Nephroma laevigatum* may often be found growing in coastal areas of Europe, U.S.A. and Canada [22, 23]. In British Columbia, the lichen is fairly common on shoreline rocks on local islands, but is absent from the continental mainland. The lichen has a characteristic yellow-orange medulla, suggestive of the presence of quinonoid-like pigments. As early as 1898, the constitution of these pigments was considered indicative of hydroxyanthraquinones [24–26].

## RESULTS AND DISCUSSION

Lichen thalli of *N. laevigatum* were collected from shoreline rocks on Gabriola Island, British Columbia, in October, 1994. In three separate experiments, the lichen was incubated with aqueous solutions of Na [2-$^{14}$C]acetate (1.85 MBq), Na [1-$^{13}$C]acetate (0.12 M solution) and Na$^{36}$Cl (0.925 MBq). Isolation and characterization of the labelled anthraquinones follow the methods described in our earlier work [21].

The primary constituent in the mixture of pigments obtained from the Na [2-$^{14}$C]acetate incorporation experiment was compound **2** (TLC; $R_f$ 0.5; chloroform–methanol, 9:1). The TLC pattern revealed the presence of two additional compounds, which were subsequently isolated and characterized as **1** ($R_f$ 0.8) and **3** ($R_f$ 0.7). Five products were identified in the pigment mixture obtained from the Na [1-$^{13}$C]acetate incorporation experiment: Compound **3** ($R_f$ 0.7), 5-

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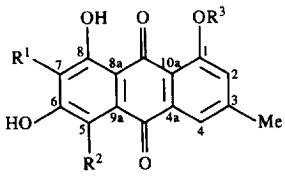
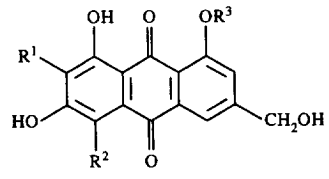


| | $R^1$ | $R^2$ | $R^3$ | |
|---|---|---|---|---|
| **1** | H | H | H | Emodin |
| **2** | Cl | H | H | 7-Chloroemodin |
| **3** | Cl | H | Me | 7-Chloro-1-*O*-methylemodin |
| **5** | H | Cl | Me | 5-Chloro-1-*O*-methylemodin |
| **6** | H | Cl | H | 5-Chloroemodin |



| | $R^1$ | $R^2$ | $R^3$ | |
|---|---|---|---|---|
| **4** | Cl | H | Me | 7-Chloro-1-*O*-methyl-*w*-hydroxyemodin |
| **7** | H | Cl | Me | 5-Chloro-1-*O*-methyl-*w*-hydroxyemodin |
| **8** | H | Cl | H | 5-Chloro-1-*O*-hydroxyemodin |

Fig. 1. Anthraquinones isolated from lichen isotope-labelling studies.

chloro-1-*O*-methylemodin (**5**) ($R_f$ 0.65), 5-chloroemodin (**6**) ($R_f$ 0.55), 5-chloro-1-*O*-methyl-$\omega$-hydroxyemodin (**7**) ($R_f$ 0.3) and 5-chloro-$\omega$-hydroxyemodin (**8**) ($R_f$ 0.25). Finally, the pigment mixture from the $Na^{36}Cl$ incorporation experiment contained **2** ($R_f$ 0.5), **3** ($R_f$ 0.7) and 7-chloro-1-*O*-methyl-$\omega$-hydroxyemodin (**4**) ($R_f$ 0.35). The structures of these compounds are shown in Fig. 1.

The identity of **2** was established by $^1$H NMR and mass spectra. The spectral data are consistent with our previous results [21, 27]. Table 1 provides the specific activities and percentage incorporations for $^{14}$C and $^{36}$Cl-labelled **2**.

Compound **1** was characterized by $^1$H NMR and mass spectra. The spectral data are indistinguishable from those of authentic material [21, 27]. The specific activity and incorporation level for $^{14}$C-labelled **1** are shown in Table 1. The amount of **1** produced in the Na [2-$^{14}$C]acetate incubation experiment was insufficient for a $^{13}$C NMR spectrum to be taken.

Table 2. $^{13}$C NMR data and isotope enrichments for 7-chloro-1-*O*-methylemodin (**3**)*

| C | $\delta$ | Percentage enrichment† |
|---|---|---|
| 1 | 160.0 | 3.1 |
| 2 | 120.5 | 0.7 |
| 3 | 147.8 | 3.7 |
| 4 | 120.0 | 0.7 |
| 4a | 131.8 | 5.2 |
| 5 | 106.4 | 0.6 |
| 6 | 161.0 | 4.9 |
| 7 | 118.0 | 0.0 |
| 8 | 160.2 | 3.1 |
| 8a | 112.4 | 0.0 |
| 9 | 186.2 | 6.0 |
| 9a | 113.5 | 0.0 |
| 10 | 182.0 | 1.9 |
| 10a | 135.6 | 3.8 |
| OMe | 56.6 | 0.0 |
| Me | 21.8 | −0.5 |

*The lichen was fed 0.12 M Na [1-$^{13}$C]acetate. The spectrum was recorded in DMSO-$d_6$ at 125 MHz. Chemical shifts ($\delta$) are reported in ppm from TMS internal standard.

†Percentage $^{13}$C enrichments (>1.1% natural abundance) were calculated from the ratios in peak heights obtained from the $^{13}$C NMR spectra of labelled and unlabelled products, respectively.

Compound **3** was identified by $^1$H NMR, $^{13}$C NMR and mass spectra. The results of an NOE experiment performed on **3** were consistent with the assigned structure. Irradiation of the H-2 proton resulted in an enhancement of the methoxyl protons at C-1. The percentage incorporation of $^{14}$C label into **3** was 20% of the level for **2**, and the specific activity of $^{14}$C-labelled **3** was 25% of the values reported for **2** and **1** (Table 1).

The $^{13}$C NMR spectrum of **3** isolated from the feeding experiment shows specific incorporation at carbon atoms 1, 3, 4a, 6, 8, 9 and 10a (Table 2). This is entirely consistent with its formation from an octaketide precursor, itself derived from acetate. The $^{13}$C isotopic enrichments were measured by comparing the peak intensities in both the natural abundance and enriched spectra after normalization [3, 12, 28]. The enrichment values are consistent with levels obtained from biosynthetic studies of anthraquinones in *Penicillium islandicum* [28] and *Dermocybe* spp. [29–30].

Compound **5** had a mass spectrum similar to that of the 7-chloro-isomer; the $^1$H NMR spectrum was quite

Table 1. Properties of radiolabelled anthraquinones isolated from *N. laevigatum*

| Compound | Isotope | Yield (%)* | Dpm | Dpm mg$^{-1}$ | % Incorp.† |
|---|---|---|---|---|---|
| **1** | $^{14}$C | 2 mg (0.02) | 8188 | 4094 | 0.01 |
| **2** | $^{14}$C | 30 mg (0.23) | 112 716 | 3757 | 0.10 |
| **2** | $^{36}$Cl | 5 mg (0.06) | 18 200 | 3638 | 0.04 |
| **3** | $^{14}$C | 22 mg (0.17) | 23 804 | 1082 | 0.02 |
| **3** | $^{36}$Cl | 5 mg (0.06) | 2010 | 402 | 0.004 |

*Yields are based on lichen dry wt.

†Percentage (absolute) incorporation ([dpm of isolated product] [dpm of isotope fed to lichen]$^{-1}$ × 100).

different, however. The location of the chlorine at C-5 was based on the chemical shift for H-7 (6.78 ppm), which is characteristic for an aromatic proton situated between two aromatic hydroxyl groups. The location of the methoxyl group was determined by an NOE experiment; irradiation of the methoxyl protons at C-1 resulted in an increase in the signal intensity for the H-2 proton.

Compound **5** had previously been isolated from the fungus *Phialophora alba* [31]. Although two other 5-chloro-substituted anthraquinones are known from the fungal genus *Dermocybe* [18], they have not previously been reported from lichens collected in the field.

Compound **4**, a metabolite previously isolated by us [21], was also obtained from our lichen feeding experiments. We could not, however, isolate sufficient quantities for accurate measurements of isotope enrichments. The structure of **4** was proved by $^1$H NMR, NOE experiments and mass spectra. The location of the methoxyl protons at C-1 was confirmed by their irradiation, which produced an increase in the $^1$H NMR signal intensity of the H-2 proton.

Compound **6** was confirmed by $^1$H NMR and mass spectra. The CI mass spectra of **6**, and **6** triacetate, indicated that it was a monochloroemodin. The $^1$H NMR spectrum showed an aromatic signal consistent with an H-7 proton (6.70 ppm).

Compound **7** was confirmed by $^1$H NMR, NOE experiments and mass spectra. The CI mass spectrum was similar to that of **4**; however, the presence of an H-7 proton shift (6.78 ppm) indicated that the chlorine must be at C-5. The position of methoxyl protons was shown by an NOE experiment; irradiation of the methoxyl protons resulted in an increase in the $^1$H NMR signal intensity of H-2.

Compound **8** was confirmed by $^1$H NMR and mass spectra. The EI mass spectrum gave a parent molecular ion corresponding to the molecular mass of **8**. The presence of an H-7 proton shift (6.63 ppm) was readily apparent from the $^1$H NMR spectrum, and indicated that the chlorine must be at C-5.

Until now only a few 5-chloroanthraquinones have been isolated from fungi [18, 31]. Steglich *et al.* proposed a biogenetic scheme in which late chlorination of an anthraquinone in ring position 5 (Fig. 1) might take place in the genus *Dermocybe* [18]. However, no isotope incorporation experiments in *Dermocybe* with $^{36}Cl^-$ have been performed, nor has a chloroperoxidase (chlorinating enzyme) been isolated from a basidiomycete (or lichen).

It is thus presently unclear why *N. laevigatum* would produce the 5-chloro-substituted emodins in the Na [1-$^{13}$C]acetate incorporation study, while they were not evident, by TLC, in either the sample selected for tracer studies, the other two incubation studies, or in all the previous work on the wild lichen. The use of 0.12 M Na acetate in the $^{13}$C study may have altered the pH or the ionic strength of the environment sufficiently to perturb the metabolism of the lichen, or change the characteristics of the chlorinating enzyme.

Finally, the 5- and 7-chloro-regioisomer pairs differ in $R_f$ by only 0.10; it is entirely conceivable that the 5-chloro-isomers are present in the wild lichen, but were not previously detected due to their low concentrations and $R_f$ values so close to their 7-chloro counterparts.

## EXPERIMENTAL

*General.* Mps: uncorr.; EIMS; 70 eV; $^1$H NMR: 400 MHz; $^{13}$C NMR: 125 MHz; DMSO-$d_6$, DMF-$d_6$ and $Me_2CO$-$d_6$ were used in the NMR and NOE experiments (TMS as int. standard); CC: BDH flash silica gel 60 (230–400 mesh); prep. 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 $\mu$m, flow rate 1 ml min$^{-1}$); radioactivity measurements: liquid scintillant (EcoLume, ICN); UV spectra: EtOH (UV grade).

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

*Feeding experiments.* The fresh lichen (80 g) was carefully cleaned of moss, soil and other debris and washed × 2 with sterile distilled $H_2O$. For each experiment, lichen (20 g) was placed in a plastic dish (245 × 245 × 20 cm). The isotope (Na [1-$^{13}$C]acetate, 1 g, 99 atom % $^{13}$C; Na [2-$^{14}$C]acetate, 1.85 MBq; Na$^{36}$Cl, 0.925 MBq) was dissolved in a sufficient amount of sterile distilled $H_2O$ to moisten the lichen thoroughly but not submerge it (*ca.* 100 ml). After several min, all the liquid had been absorbed by the lichen. The incubating lichen was maintained in an indoor greenhouse at 27° for 5 days (16 hr light and 8 hr dark cycles), after which it was dried in air and weighed.

*Isolation.* The lichen was extracted successively with 500 ml $Me_2CO$ and 500 ml MeOH. The combined extracts were concd to a brown–red solid. The solid was purified by CC on Sephadex LH-20, using a gradient of $CHCl_3$–MeOH (9:1) to MeOH. Frs (20 ml) were collected and analysed by TLC. All compounds were further purified by prep. TLC ($CHCl_3$–MeOH, 4:1), and repeatedly recrystallized from a suitable solvent until purity (>99%) could be established on the basis of RP-HPLC. Radiolabelled compounds were recrystallized to constant sp. activity, and the purity checked by RP-HPLC.

*Identification.* All compounds were characterized by UV, MS and $^1$H and $^{13}$C NMR spectra. Reported yields are based on dry wt of lichen. Autoradiographs of radiolabelled compounds were made by exposing 2D silica gel TLC plates of anthraquinones and isotope to X-ray film, after development in $CHCl_3$–MeOH (4:1) and $Me_2CO$–HOAc (4:1). After 2 months exposure, the X-ray film was developed and radioactive spots, corresponding to $^{14}$C- and $^{36}$Cl-labelled anthraquinones, respectively, were marked. Radioactive 'halos'

representing $Na^{36}Cl$ and Na [2-$^{14}$C]acetate were clearly distinguishable from radiolabelled anthraquinones. Development in either solvent system did not result in migration of the isotopes from their original point of application. It was therefore concluded that the purified radiolabelled anthraquinones were free from any contamination by reagents used in lichen feeding studies.

*Products from Na* [2-$^{14}$C]*acetate incorporation experiment*

*Emodin* (**1**). Compound **1** (2 mg, 0.02% dry wt) was obtained as orange crystals (EtOAc); mp 256–257°. The $^1$H NMR spectrum was in agreement with the lit. [21, 27].

7-*Chloroemodin* (**2**). Compound **2** (30 mg, 0.23% dry wt) was obtained as orange crystals (EtOAc); mp 281–283°. The $^1$H and $^{13}$C NMR spectra were in agreement with the lit. [21, 27].

7-*Chloro*-1-O-*methylemodin* (**3**). Compound **3** (22 mg, 0.17% dry wt) was obtained as orange crystals (EtOAc); mp 289–291°. The $^1$H and $^{13}$C NMR spectra were in agreement with the lit. [21].

*Products from Na* [1-$^{13}$C]*acetate incorporation experiment*

7-*Chloro*-1-O-*methylemodin* (**3**). Compound **3** (3 mg, 0.02% dry wt) was obtained as orange crystals (EtOAc); mp 288–290°. The $^1$H and $^{13}$C NMR (Table 2) spectra were in agreement with the lit. [21].

5-*Chloro*-1-O-*methylemodin* (**5**). Compound **5** (3 mg, 0.02% dry wt) was obtained as orange crystals (EtOAc); mp 252–253°; UV (EtOH) $\lambda_{max}$ nm (log $\varepsilon$): 228 (4.30), 259 (4.33), 314 (4.25), 440 (4.02); CIMS *m/z* (rel. int.): 321 $[MH]^+$ (33), 319 (100); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 2.47 (3H, *s*, Me-3), 3.92 (3H, *s*, OMe-1), 6.78 (1H, *s*, H-7), 7.38 (1H, *s*, H-2), 7.58 (1H, *s*, H-4). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 21.8 (Me-3), 56.5 (OMe-1), 112.6 (C-7), 112.6 (C-8a), 113.4 (C-9a), 118.6 (C-5), 119.8 (C-4), 120.0 (C-2), 130.6 (C-4a), 134.8 (C-10a), 145.8 (C-3), 160.2 (C-1), 160.2 (C-8), 161.0 (C-6), 183.6 (C-10), 191.8 (C-9).

5-*Chloroemodin* (**6**). Compound **6** (2 mg, 0.01% dry wt) was obtained as orange crystals (EtOAc); CIMS *m/z* (rel. int.): 307 $[MH]^+$ (33), 305 (100); EIMS (70 eV) *m/z* (rel. int.): 306 $[M]^+$ (22), 304 (100); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 2.40 (3H, *s*, Me-3), 6.68 (1H, *s*, H-7), 7.08 (1H, *s*, H-2), 7.38 (1H, *s*, H-4).

5-*Chloroemodin triacetate*. EIMS (70 eV) *m/z* (rel. int.): 390 $[M]^+$ (9), 338 (27), 348 (9), 346 (25), 306 (51), 304 (100).

5-*Chloro*-1-O-*methyl*-ω-*hydroxyemodin* (**7**). Compound **7** (2 mg, 0.01% dry wt) was obtained as salmon coloured crystals (MeOH); UV (EtOH) $\lambda_{max}$ nm (log $\varepsilon$): 218 (4.50), 257 (4.22), 322 (4.08), 496 (3.56); CIMS *m/z* (rel. int.): 337 $[MH]^+$ (33), 335 (100); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 3.92 (3H, *s*, OMe-1), 4.63 (2H, *d*, $J$ = 5.0 Hz, C$\underline{H}_2$OH-3), 5.75 (1H, *s*, CH$_2$O$\underline{H}$-3), 6.78 (1H, *s*, H-7), 7.46 (1H, *s*, H-2), 7.73 (1H, *s*, H-4).

5-*Chloro*-1-O-*methyl*-ω-*hydroxyemodin triacetate*. CIMS *m/z* (rel. int.): 463 $[MH]^+$ (33), 461 (100); EIMS (70 eV) *m/z* (rel. int.): 420 $[M]^+$ (20), 418 (46), 378 (18), 376 (55), 336 (2), 334 (7), 318 (42), 316 (100), 306 (20), 304 (51).

5-*Chloro*-ω-*hydroxyemodin* (**8**). Compound **8** (2 mg, 0.01% dry wt) was obtained as orange crystals (MeOH); EIMS (70 eV) *m/z* (rel. int.): 322 $[M]^+$ (33), 320 (100); $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 4.54 (2H, *d*, $J$ = 5.0 Hz, C$\underline{H}_2$OH-3), 5.45 (1H, *s*, CH$_2$O$\underline{H}$-3), 6.63 (1H, *s*, H-7), 7.12 (1H, *s*, H-2), 7.54 (1H, *s*, H-4).

*Products from $Na^{36}Cl$ incorporation experiment*

7-*Chloroemodin* (**2**). Compound **2** (5 mg, 0.06% dry wt) was obtained as orange crystals (EtOAc); mp 281–282°. The $^1$H NMR spectrum was in agreement with the lit. [21, 27].

7-*Chloro*-1-O-*methylemodin* (**3**). Compound **3** (5 mg, 0.06% dry wt) was obtained as orange crystals (EtOAc); mp 289–291°. The $^1$H NMR spectrum was in agreement with the lit. [21].

7-*Chloro*-1-O-*methyl*-ω-*hydroxyemodin* (**4**). Compound **4** (1 mg, 0.01% dry wt) was obtained as pink crystals (EtOH); mp >290°. The $^1$H NMR spectrum was in agreement with the lit. [21].

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