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Biotransformation of furanocoumarins by *Cunninghamella elegans*

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Abstract Biotransformation of Furanocoumarins; psoralen (**1**), bergapten (**2**), xanthotoxin (**3**) and imperatorin (**4**) was explored by *Cunninghamella elegans* NRRL 1392, revealing the metabolism of psoralen (**1**) and bergapten (**2**) into bergaptol (**5**), while xanthotoxin (**3**) and imperatorin (**4**) were converted into xanthotoxol (**6**). On the other hand unexpected conversion of xanthotoxin (**3**) into 3,4 dihydroxanthotoxin (**7**) occurred. The structure of the isolated pure metabolites was established using physical and spectroscopic techniques including, melting points, IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

1. Introduction

Furanocoumarins represent a large class of compounds which are active against herbivores and with wide spread occurrence in *Apiacae* and *Rutaceae* and at least other 13 plant families: *Amaranthaceae, Compositae, Cyperaceae, Dipsacaceae, Fabaceae, Goodeniaceae, Guttiferae, Moraceae, Pittosporaceae, Rosaceae, Samydaceae* and *Solanaceae*.[1] Furanocoumarins were reported to be used in the treatment of skin disorders such as psoriasis and skin depigmentation.[2]

Microbial transformation studies of Furanocoumarins, which provide the mammalian metabolic pathway, are scarce.[3,4] Conducted biotransformation studies on the biotransformation of Furanocoumarins isoimperatorin and imperatorin by the use of *Glomerella cingulata* give the corresponding reduced acid 6, 7-furano-5-prenyloxy-hydrocoumaric acid and, xanthotoxol in high yields.[3] Meanwhile, imperatorin treated with *Aspergillus flavus* in growth media produced metabolites which were elucidated as xanthotoxol, *E*-trichoclin, *Z*-trichoclin, *E*-imperatorin acid, and *Z*-imperatorin acid.[4] These findings have renewed the interest in studying mammalian metabolism of Furanocoumarins, using *C. elegans* NRRL 1392; ANOVA microbial models of mammalian metabolism for several xenobiotics.[5,6] The major metabolites were isolated by different chromatographic techniques and structurally elucidated by IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

2. Experimental

2.1. General

General psoralen was obtained from Fluka Chemical Company Inc., Milwankee, Wi, while imperatorin, xanthotoxin and bergapten from Cairo Company for pharmaceutical industry, Egypt. IR spectra were recorded on FTIR – 800 Ipc series, Shimadzu 24 Corporation. ^{1}H and ^{13}C NMR

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spectra were obtained on JEOL JNM-ECA 500 and 125 MHz, respectively. The chemical shift values were reported as ppm using tetramethyl-silane (TMS) as an internal standard and coupling constants are expressed in Hz. Negative mode spray ionization (ESIMS) mass spectra were obtained on Thermo-Finnigan mass spectrometer (See Fig. 1).

TLC was performed using silica gel plates F-254 for TLC (5 × 10 mm), (Sigma Chemical Co.,) and precoated silica gel plastic sheets, (20 × 20 mm) with fluorescent indicator (E. Merck). Column chromatography was carried out using flash silica gel for column (Merck) and Sephadex LH-20 (Pharmacia, Sweden).

2.2. Fungal biotransformation protocol

2.2.1. Microorganisms[7] and culture conditions and components[8]

C. elegans was obtained from Northern Regional Research Laboratories (NRRL, Peoria, Illinois).[7] The fermentation experiments[8] were performed using a medium consisting of 10 g dextrose, 10 ml glycerol, 5 g yeast NaCl and 1000 ml distilled water. The PH was adjusted to 6.0 before sterilization at 121 °C for 15 min.

2.2.2. Cultivation of microorganism[9]

The cultures were initiated to grow from lyophilized state by adding 1 ml of sterile water to microorganism vials. A few drops of suspended cells were streaked on Sabouraud-dextrose plates to check the purity. Pure cultures were maintained on slants of either potato-dextrose agar or Sabouraud-dextrose agar. The fresh slants were incubated for few days at room temperature before storage in a refrigerator at (4 °C). Fermentation liquid cultures were initiated by transferring a few drops of suspended cells or a loopful into 25 ml sterile liquid medium present in a 125 ml Erlenmeyer flask then placing it on a gyratory shaker operating at 250 rpm and at 27 °C. The temperature was maintained at 27 °C.

2.2.3. Transformation[7]

Stage I was initiated from two-week old slants (under aseptic conditions) by transferring microbial cells into a 250 ml Erlenmeyer flask, containing 50 ml of sterile liquid medium and allowed to grow for 24–72 h at 27 °C on a gyratory shaker. Stage II cultures were obtained by transferring 5 ml of stage culture to each of 250 ml flasks containing 50 ml of fresh liquid medium. Both substrate and organism controls were treated in the same way. Cultures were allowed to grow for 24 h, before the addition of the acetonic substrate solution (5 mg/0.25 ml per flask). Samples (5 ml) of the liquid culture were periodically (1, 3, 5, 7, 10 and 15 days) withdrawn from each culture and extracted successively with chloroform or ethyl acetate then each extract was separately evaporated to dryness under reduced pressure and kept for further analysis. The obtained residues were dissolved in few drops of a suitable organic solvent and Thin layer chromatographer on silica gel G plates (E. Merck, F 254) using solvent systems I: Chloroform: Methanol (9.5: 0.5, v/v), solvent systems II: Chloroform: Methanol (9: 1, v/v), solvent systems III: Hexane: ethyl acetate (2: 1, v/v). The plates were dried and visualized under UV-light before and after spraying with 10% sulfuric acid in methanol or alcoholic KOH.[10]

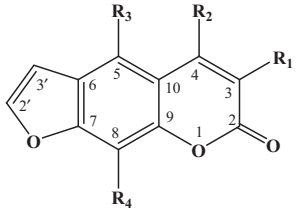

Figure 1 Structures of investigated furanocoumarins and the resulted metabolites.

2.2.4. Fermentation of psoralen

Fermentation of psoralen **(1)** with *C. elegans*. Psoralen (**1**, 40 mg, 5 mg/0.25 ml acetone per eight 250 ml flask containing 50 ml ferment liquid media) was inoculated into stage II cultures of *C. elegans* as mentioned previously. After 10 d, the fermentation was stopped, cells were removed by filtration and the broth was extracted twice with an equal volume of ethyl acetate. The EtOAc residue (27 mg) was chromatographed on preparative precoated silica *G* plastic sheets (E. Merck, F 254), alongside a reference spot of psoralen **(1)** and bergaptol **(5)**. Zone No. 4 which corresponded was scraped off the plates, extracted with ethyl acetate and evaporated. The residue was recrystallized from ethyl acetate and weighed to give the pure metabolite **(5)** as green powder (2 mg, in 5% yield). It showed a faint green florescence under UV, black color when sprayed with 10% sulfuric acid reagent and heated for 10 min to 110 °C with an R_f = 0.73 in solvent system V; m.p. = 286–289 °C, soluble in ethyl acetate and methanol.

2.2.5. Fermentation of bergapten

Fermentation of bergapten **(2)** by *C. elegans*. **Bergapten (2**, 1 g) dissolved in acetone (25 ml), was equally distributed amongst 50, 500ml flasks containing stage II cultures of *C. elegans* as mentioned previously. After 7 days, the fermentation was stopped and cells were removed by filtration and the broth was extracted twice with an equal volume of ethyl acetate. The EtOAc residue (300 mg) was chromatographed on the top of a silica gel (18.9 g, 25 cm × 1 cm) column. Gradient elution method was adopted, using hexane, then hexane: ethyl acetate with increasing amount of ethyl acetate till 75%. About 60 fractions (5 ml each) were collected and monitored on precoated silica gel G TLC plates, using solvent system V. Fractions eluted with hexane: EtOAc (2: 1, v/v) showed one major spot. Those fractions were pooled and evaporated under vacuum to give a residue of 82 mg. The residue was dissolved in 3 ml ethyl acetate and applied on preparative silica gel G plates (E. Merck, F 254) alongside with a reference spots of bergapten **(2)** using solvent system V. The zone location was defined after the exposure to UV light and spraying the reference spots with 10% sulfuric acid in methanol. Zone No. 1 was scraped off the plates, extracted with ethyl acetate and solvent was evaporated. The residue was crystallized from ethyl acetate several times and weighed to give an olive green powder of metabolite **(5)** 11 mg in a yield of 13.4%.

2.2.6. Fermentation of xanthotoxin

Fermentation of xanthotoxin **(3)** by *C. elegans*. **(3**, 1 g) Xanthotoxin dissolved in acetone (25 ml), was equally distributed amongst 50, 500 ml flasks containing stage II cultures of *C. elegans* as mentioned previously. After 7 days, the fermentation was stopped and the cells were removed by filtration. The fermentation broths were extracted twice with an equal volume of chloroform and ethyl acetate successively and each solvent was distilled off under reduced pressure to about 600 mg and 210 mg residue respectively. The chloroformic residue was chromatographed on silica gel (24 g, 25 cm L × 1 cm D) column, using the gradient elution method starting with chloroform then chloroform: methanol with increasing amount of methanol till 10% methanol. Thirty fractions (10 ml each) were collected and chromatographed on precoated silica gel G plates using solvent system No. I and II. Similar fractions were combined, fractions eluted with chloroform: methanol (9.7: 0.3 v/v) showed the pure metabolite (**7**); 3,4-dihydro-xanthotoxin, 60 mg in a yield of 0.6% as golden yellow feathery crystals in chloroform. It showed a green florescence under UV, brown color when sprayed with KOH and bluish black color when sprayed with anisaldehyde/sulfuric acid reagent with an R_f value = 0.32 using solvent system I; soluble in chloroform and methanol.

The ethyl acetate residue was chromatographed on silica gel (sigma) column. Gradient elution method was adopted using hexane then hexane: ethyl acetate as eluent, which yielded 60 fractions (5 ml each) for bergapten column. Fraction eluted with hexane: EtOAc (2:1) gave 42 mg of xanthotoxol (**6**). The residue was chromatographed on silica gel (24 g, 25 cm L × 1 cm D) column, using gradient elution method starting with chloroform then chloroform: methanol with increasing amount of methanol till 10% methanol. Thirty fractions (10 ml each) were collected and chromatographed on precoated silica gel G plates using solvent system Nos. I and II. Similar fractions were combined, fractions eluted with chloroform: methanol (9.7: 0.3 v/v) showed the metabolite (**6**), 20 mg in a yield of 0.2%. It showed a faint green florescence under UV, black color when sprayed with 10% sulfuric acid reagent and heated for 10 min to 110 °C with an R_f = 0.75 in solvent system V and soluble in ethyl acetate and methanol.

2.2.7. Fermentation of imperatorin

Fermentation of imperatorin (4) by *C. elegans*. **imperatorin (4**, 1 g) dissolved in acetone (25 ml), was equally distributed amongst 50, 500ml flasks containing stage II cultures of *C. elegans* as mentioned previously. After 7 days, the fermentation was stopped and cells were removed by filtration and the broth was extracted twice with an equal volume of ethyl acetate. The EtOAc residue (200 mg) was dissolved in 5 ml ethyl acetate and applied on preparative silica gel G plates (E. Merck, F 254) with a reference spot of the extract and the metabolite (**6**), the plates were developed in solvent system (V) and zone location was visualized under UV light and spraying the reference spots with 10% sulfuric acid in methanol. Zone No. 3 was scraped off the plates, extracted with ethyl acetate, and evaporated to dryness under vacuum, crystallized from ethyl acetate several times and weighed to give metabolite (**6**) (5 mg, in a yield of 0.04%. It showed a faint green florescence under UV, black color when sprayed with 10% sulfuric acid reagent and heated for 10 min to 110 °C with an R_f = 0.75 in solvent system V and soluble in ethyl acetate and methanol.

Psoralen (**1**) CIMS(m/z): 186 $[M^+]$– m.p. 165–166 °C $[M]^+$ m/z 186.0300 – ^{1}H NMR ($CDCl_3$): δ 6.38 (1H, *d*, *J* = 9.8, Hz, H-3), 7.46 (1H, brs, H-8), 7.68 (1H, s, H-5), 7.70 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.80 (1H, *d*, *J* = 9.8, Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 99.8 (C-8)(*d*), 106.4 (C-3′) (*d*), 114.6 (C-3) (*d*), 115.4 (C-10)(s), 119.9 (C-5) (d), 124.9 (C-6)(s), 144.1 (C-4′) (d), 146.9 (C-2′) (d), 152.0 (C-9) (s), 156.4 (C-7) (s), 161.0 (C-2) (s). UV λ_{max}^{MeOH} nm (ε): 246(22600), 291 (10400), 327 (6400)-IR (KBr) cm^{-1}: 1625, 1635, 1577, 1136, 824.

Bergapten (**2**) CIMS(NH_4) (m/z): 234 $[M + NH_4]^+$ – UV λ_{max}^{nm} 223, 243, 249, 259, 268, 311 – ^{1}H NMR ($CDCl_3$) δ: 6.26 (1H, H-3, *d*, *J* = 9.8), 8.16 (1H, H-4, *d*, *J* = 9.8), 4.26 (3H, hydrogens of methoxy group attached to C-5, s), 7.16 (1H, H-8, s), 7.60 (1H, H-2′, *d*, *J* = 2.5), 7.01 (1H, H-3′, *d*, *J* = 2.5) – ^{13}C NMR ($CDCl_3$) δ: 160.3 (C-2), 112.6 (C-3), 139.6 (C-4), 149.7 (C-5), 60.4 (C-5 attached methoxy group), 114.0 (C-6), 158.3 (C-7), 94.0 (C-8), 152.7 (C-9), 106.4 (C-10), 144.9 (C-2′), 105.2 (C-3′).

Bergaptol (5) ESIMS (m/z): 201$[M-1]^+$, m.p. 286.5–289.5 °C – Proton NMR spectral data (Acetone-d_6 + DMSo-d_6) displayed resonance at δ 6.17 (1H, H-3, *d*, *J* = 9.9), 8.24 (1H, H-4, *d*, *J* = 9.9), 11.09 (H-5, hydroxy proton), 6.98 (1H, H-8, s), 7.75 (1H, H-2′, *d*, *J* = 2.4) 7.47 (1H, H-3′, *d*, *J* = 2.4). ^{13}C NMR spectral data (Acetone-*d*6 + DMSo-*d*6) displayed resonances at δ 160.5 (C-2), 111.0 (C-3), 139.6 (C-4), 148.5 (C-5), 112.8 (C-6), 157.7 (C-7), 90.8 (C-8), 153.4 (C-9), 104.05 (C-10), 144.6 (C-2′), 104.9 (C-3′). IR (KBr) cm^{-1}: 3300, 1689, 1583, 1251, 825.

Xanthotoxin (**3**) CIMS (NH_4) (m/z): 234 $[M + NH_4]^+$ – UV λ_{max}^{nm} 223, 242, 248, 269, 273, 313 – IR υ_{max} cm^{-1} 3100. 3070, 3050, 1726, 1620, 1602, 1575, 1540, 885 – ^{1}H NMR ($CDCl_3$): δ 6.35 (1H, H-3, *d*, *J* = 9.5), 7.75 (1H, H-4, *d*, *J* = 9.5), 7.34 (1H, H-5, s), 4.30 (3H of methoxy group attached to C-8), 7.67 (1H, H-2′, *d*, *J* = 2.4), 6.8 (1H, H-3′, *d*, *J* = 2.4) – ^{13}H NMR ($CDCl_3$): δ: 160.7 (C-2), 114.8 (C-3), 144.4 (C-4) – 112.9 (C-5), 126.3 (C-6), 147.7 (C-7), 132.6 (C-8), 61.2 (C-8) (attached methoxy group), 143.9 (C-9), 116.5 (C-10), 146.7 (C-2′), 105.2 (C-3′).

Xanthotoxol (6) ESIMS (m/z): 201$[M-1]^+$, m.p.243–244 °C – IR υ_{max} cm^{-1} 3070, 3050, 1726, 1620, 1602, 1575, 1540, 885 – ^{1}H NMR spectral data (Acetone-d_6) displayed resonance at δ 6.3 (1H, H-3, *d*, *J* = 9.4), 8.0 (1H, H-4, *d*, *J* = 9.4), 7.4 (1H, H-2′, *d*, *J* = 2.3), 6.95 (1H, H-3′, *d*, *J* = 2.3). ^{13}C NMR spectral data (Acetone-*d*6) displayed resonance at δ 159.6 (C-2), 114.7 (C-3), 145.41 (C-4), 110.3 (C-5), 125.7 (C-6), 147.21 (C-7), 130.2 (C-8), 139.81 (C-9), 116.4 (C-10,145.03 (C-2′), 106.87 (C-3′).

3,4-Dihydroxanthotoxin (7) ESIMS (m/z): 217 $[M-1]^+$ – m.p. 109–110 °C – IR υ_{max} cm^{-1} 3100, 3070, 3050, 1726, 1620, 1602, 1540, 885 – ^{1}H NMR ($CDCl_3$): δ 2.74 (1H, H-3, *t*), 3–0367 (1H, H, 4, *t*), 7.0176 (1H, H-5, S), 4.2 (3H, methoxy group attached to C-8), 7.48 (1H, H-2′, *d*, *J* = 1.55), 6.6 (1H, H-3′, *d*, *J* = 1.5) – ^{13}C NMR ($CDCl_3$): δ 179.7 (C-2), 25.9 (C-3), 34.2 (C-4), 114.7 (C-5), 123.1 (C-6), 144.4 (C-7), 131.6 (C-8), 60.7 (C-8 methoxy group), 142.8 (C-9), 121.7 (C-10), 143.9 (C-2′), 106.7 (C-3′).

Imperatorin (**4**) CIMS (m/z): 269.08 $[M-1]^+$, m.p. 97.5–99 °C. ^{1}H NMR ($CDCl_3$): δ 1.72 (3H, s), 1.74 (3H, s), 5.01 (2H, *d*, *J* = 7.3 Hz), 5.61 (1H, *t*, like, *J* = 7.3 Hz), 6.37 (1H, *d*, *J* = 9.3 Hz, H-3), 6.81 (1H, *d*, *J* = 2.4 Hz, H-3′), 7.36 (1H, s, H-5), 7.69 (1H, *d*, *J* = 2.4 Hz, H-2′), 7.76 (1H, *d*, *J* = 9.3 Hz, H-4). ^{13}C NMR ($CDCl_3$): δ 18.1 (q), 25.8(q),

70.2(t), 106.7(d), 113.1(d), 114.7(d), 116.1(s), 119.8(d), 125.9(s), 131.7(s), 139.8(s), 143.9(s), 146.6(d), 148.6(s), 160.6(s), 144.3(d).

3. Results and discussion

In the recent study *C. elegans* converted psoralen (**1**) into the metabolite (**5**). Results of IR studies were in coincidence with those of standard psoralen[11] (see experimental), it displayed a molecular ion peak at m/z 201 in negative ESI mass spectral $[M-1]^+$, and showed melting point 286.5–289.5 °C.[12] These findings suggested the hydroxylation of psoralen (**1**) to obtain bergaptol (**5**).

The metabolism of bergapten (**2**)[13] and xanthotoxin (**3**)[13] revealed two metabolites (**5&6**). Studying the ^{1}H NMR spectra (see experimental) of these metabolites revealed the disappearance of the methyl group signal at C-8 or C-5 of xanthotoxin (**3**)[13] and Bergapten (**2**),[13] resonating at δH 4.30–4.26 ppm and the presence of another singlet signal of C-OH at δH 9.47 ppm and δH 11.09 ppm, respectively (D_2O exchange experiment) .The signals of carbon atoms of the metabolites (**6**) and (**5**) in the ^{13}C NMR spectra (see experimental) confirmed the demethylation of these metabolites by the disappearance of the methyl group signal of C-8 or C-5 resonating at 61.2 and 60.4 ppm for xanthotoxin (**3**)[13] and bergapten (**2**)[13] respectively and appearance of new high downfield signals at δ 130.2 ppm and δ 148.5 ppm for metabolites (**6**) and (**5**), respectively, which represented a hydroxylated carbon. The nature of those metabolites was further confirmed with previously reported data.[9] They displayed a molecular ion peak at m/z 201 in negative ESI mass spectral $[M-1]^+$, and melting point values at 243–244 °C and 202 °C for metabolites (**6**) and (**5**), respectively. Thus, these two metabolites were identified as xanthotoxol (**6**) and bergaptol (**5**), respectively.

The metabolite isolated from imperatorin (**4**)[13] was identical to xanthotoxol (**6**), when examined by TLC method on silica gel G plates, using solvent system V and spraying with 10% sulfuric acid followed by heating at 110 °C for 10 min.[13] The PESI of the metabolites isolated from xanthotoxin (**3**)[13] and imperatorin (**4**)[13] showed the same pseudo molecular ion at m/z 201 which is consistent with data reported for xanthotoxol.[9]

In addition to the production of phenolic derivatives metabolite, *C. elegans* was able to ferment xanthotoxin (**3**)[13] into another metabolite (**7**). Examination of ^{1}H NMR and ^{13}C NMR signals of this metabolite (see experimental) revealed that it is distinguishable from those of reference sample of xanthotoxin[13] by the disappearance of olefinic proton doublet signals resonating at δH 6.35, 7.75 ppm (J = 9.5) of H-3 & H-4. Two shielded triplet proton signals resonating at δH 2.75 and 3.04 (J = 8.4), respectively can be observed in the metabolite (**7**) spectrum. Each of these signals is integrated for two protons. Furthermore, the olefinic carbon signals at δC 114.8, 144.4 of C-3 and C-4 of xanthotoxin (**3**)[13] are replaced by two saturated carbon signals at δC 25.9, 34.2, respectively, supporting the reduction reaction and the downfield shift of C-2 carbonyl from δC 160.7 to 179.7 indicated the reduction (hydrogenation) of C3–C4 double bond.

Upon comparison of IR spectrum of this metabolite with that ofxanthotoxin,[14] it showed no bands below 3000 cm^{-1} for vinyl C–H stretching vibration and an aliphatic CH stretching vibration at $>$3000 cm^{-1}. On the other hand, the band at 1575 cm^{-1} corresponding to conjugated enone is no longer observed. These findings support the reduction of C3–C4 bond (see experimental). Negative mode ESIMS showed a pseudo molecular ion at m/z 217 and melting point values at 109–110 °C provide conclusive evidences of this reduction reaction. This metabolite was identified as 3,4-dihydroxanthotoxin.

In the present study, *C. elegans* was able to metabolize the investigated Furanocoumarins as xauthotoxin,[13] bergapten[13] and imperatorine[13] into corresponding phenolic derivatives via, dealkylation reaction, which is a very common reaction in *C. elegans* xenobiotic metabolism.[15] It is specific for these types of coumarins yet it has a broad substrate specification. Reduction reaction takes place for xanthotoxin resulting in the formation of dihydroderivatives, while bergapten and imperatorin need further investigation. However, a hydroxylation reaction takes place for psoralen[11] resulting in the formation of bergaptol.

4. Conflict of interest

We have no conflict of interest.

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