216. Studies Related to Biological Detoxification of Kraft Pulp Mill Effluent. VII[1])

The Biotransformation of 12-Chlorodehydroabietic Acid with *Mortierella isabellina*

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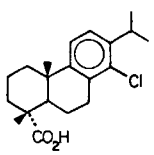
Summary

A detailed study of the biodegradation of one of the fish-toxic chlorinated resin acids, 12-chlorodehydroabietic acid (**3**), is discussed. When **3** is exposed to the fungus *Mortierella isabellina*, it is converted into the monohydroxylated metabolites 2α-hydroxy-12-chlorodehydroabietic acid (**5**) and 16-hydroxy-12-chlorodehydroabietic acid (**8**) after short-term (26 h) incubation, and into the 2α,16-dihydroxy derivatives **11** and **14** after 96 h incubation. These metabolites show low levels of toxicity to fish.

Previously [1] [2], we described the biotransformation of two chlorinated toxicants present in kraft pulp mill effluents, 14-chlorodehydroabietic acid (**1**) and 12,14-dichlorodehydroabietic acid (**2**), to less toxic metabolites using the fungus *Mortierella isabellina*. Herein are details of the biotransformation of 12-chlorodehydroabietic acid (**3**), the third member of the chlorinated dehydroabietic acid series.

Methods for the Quantitative Analysis of Metabolites. – Samples procured after different periods of incubation in fermentors were acidified (pH 2) and extracted twice with AcOEt. After concentrating the organic extracts and drying *in vacuo*, the residue



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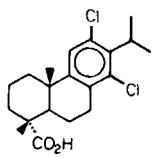

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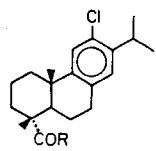

3 R = OH

4 R = 

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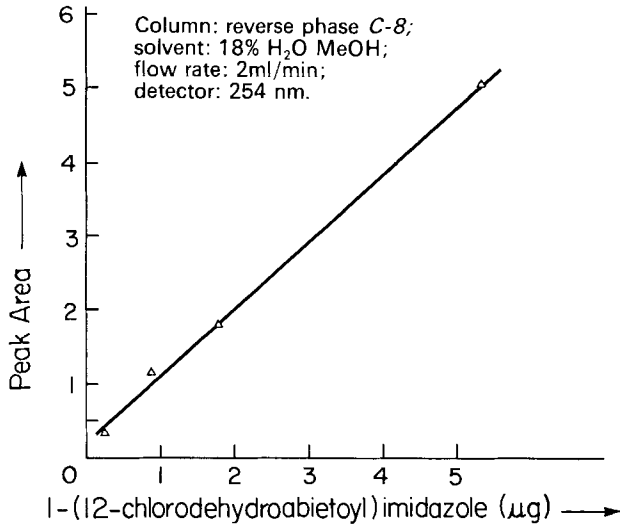


Fig. 1. *Standard Curve in HPLC for 1-(12-Chlorodehydroabietoyl)imidazole* (**4**). Column: reverse phase *C-8;* solvent: 18% H_2O/MeOH; flow rate: 2 ml/min; detector: 254 nm.

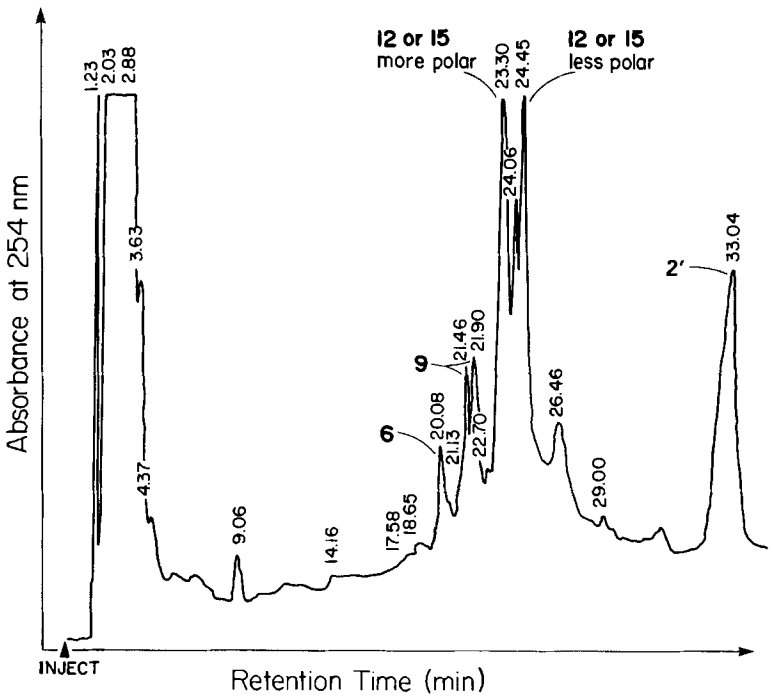
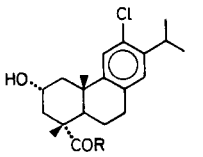


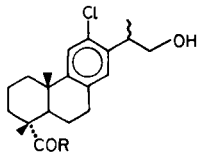
Fig. 2. *Typical HPLC Trace of the Biotransformation Mixture (as Imidazole Derivatives) of* **3**. Column: reverse phase *C-8;* solvent: initial H_2O/MeOH 4:6, at 20 min H_2O/MeOH 1:9, at 30 min H_2O/MeOH 1:9, at 40 min MeOH; flow rate: 2 ml/min; detector: 254 nm (**2′** = Imidazole derivative of **2**).

was treated with 1,1'-carbonyldiimidazole in anhydrous MeCN/THF to form imidazole derivatives of the resin acids [3]. Derivative concentrations were measured by HPLC employing a radial compression reverse phase *C-8* column, a MeOH/H_2O solvent system, and a UV (254 nm) detector. The standard curve for 1-(12-chlorodehydroabietoyl)imidazole (**4**) and a typical HPLC trace of the biotransformation mixture of **3** are shown in *Fig. 1* and *2*, respectively.

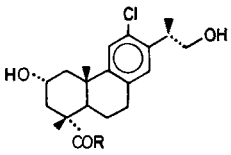
Biotransformation of 3 by *M. isabellina*. – Growth of *M. isabellina* in 12-l-fermentor cultures proceeded as described previously [1] [2]. The time course of the disappearance of **3** and the appearance of the four major metabolites **5**, **8**, **11**, and **14** detected as their imidazole derivatives **6**, **9**, **12**, and **15**, respectively, is depicted in *Fig. 3*. Nearly complete transformation of **3** was achieved within 45 hours (s. **4** in *Fig. 3*). The metabolites **5** and **8** (s. **6** and **9**, resp.) made transitory appearances before being further hydroxylated to diastereoisomers **11** and **14** (s. **12** and **15**, resp.). The dihydroxy compounds persisted at constant levels from 55 hours until the end of incubation at 90 hours. Precipitation of **3** upon addition to acidic cultures and its subsequent adherence to fungal cells may account for the recovery of starting material, up to 20 mg per 12 l of culture, from cells harvested after 90 h of incubation.



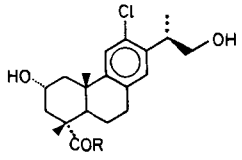
5 R = OH
6 R = 
7 R = OCH_3



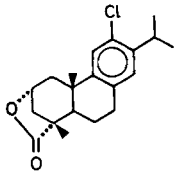
8 R = OH
9 R = 
10 R = OCH_3



11 R = OH
12 R = 
13 R = OCH_3



14 R = OH
15 R = 
16 R = OCH_3



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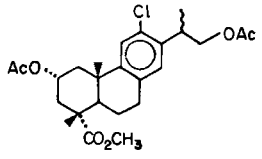

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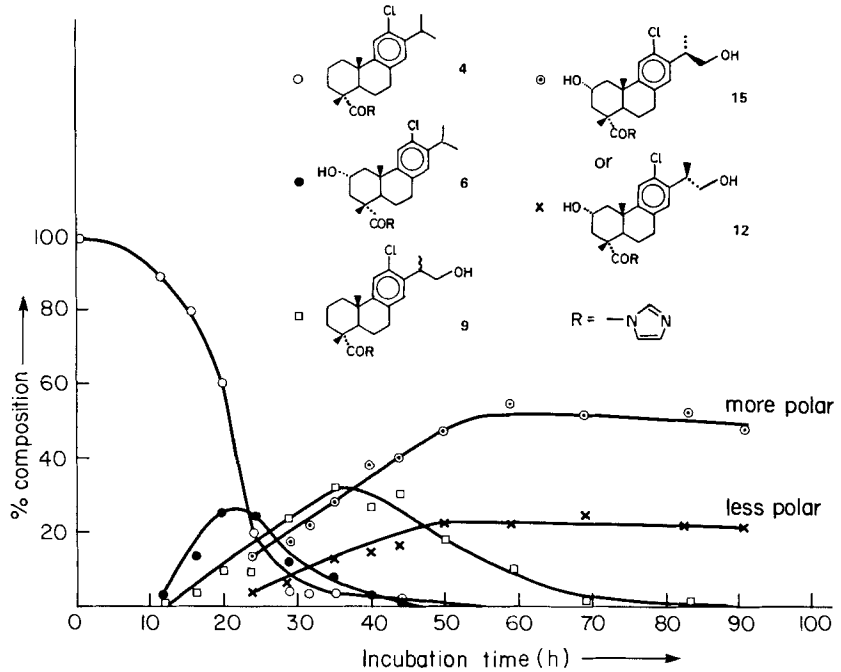

Fig. 3. *Composition of the Biodegradation Mixture (as Imidazole Derivatives) from* **3** versus *Incubation Time*

The site and degree (mono- or dichlorinated) of chlorination in the aromatic ring affect the rate and mode of biotransformation of these substituted dehydroabietic acid derivatives by *M. isabellina*. The 12-chloro derivative **3** is slightly more resistant to bioconversion than the corresponding 14-chloro isomer **1** [2] but much less resistant than **3** the 12,14-dichlorodehydroabietic acid (**2**) [1]. The pattern of metabolite production from **3** is not like that obtained for other natural resin acids or their chlorinated derivatives. Common to all the biotransformations (except for that of **2** where a C(2) keto function occurs) is the transitory appearance of a 2α-monohydroxylated intermediate, for example **5** (s. **6** in *Fig. 3*), which is invariably subsequently hydroxylated in the isopropyl side chain. Metabolism of **3** is unique in that a 16-monohydroxylated metabolite (**8**) is also produced simultaneously in significant concentrations prior to its hydroxylation at C(2) to afford a mixture of **11** and **14**.

Isolation and Identification of Metabolites. – A number of large-scale fermentations (12 l) containing 20 mg/l of **3** were used to obtain sufficient amounts of metabolites for structure elucidation and toxicity evaluation. Short-term fermentations (26 h) were required to produce metabolites **5** and **8**, while longer incubations (90 h) yielded **11** and **14**. For isolation and purification purposes the above metabolites **5**, **8**, **11**, and **14** were converted to their corresponding methyl esters **7**, **10**, **13**, and **16**, respectively, by treatment with diazomethane. Thus, in a typical short-term experiment, incubation of **3** (200 mg) with *M. isabellina* for 26 h afforded **7** (44 mg), **10** (40 mg), **13** and **16** (40 mg),

and recovered substrate **3** (42 mg). The same quantity of **3** (200 mg) after 90 h incubation resulted in **13** and **16** (130 mg, 8:5) and recovered **3** (20 mg).

The methyl ester **7** obtained from metabolite **5** had a molecular formula $C_{21}H_{29}ClO_3$ (high resolution MS), suggesting introduction of one O atom into the 12-chlorodehydroabietic molecule. The presence of a broad band centered at 3500 cm^{-1} in the IR spectrum confirmed a OH function in the molecule. A nine-line resonance (*tt*, $J_{aa} = 12$, $J_{ae} = 4$ Hz) centered at 4.07 ppm in the ^{1}H-NMR spectrum of **7** was consistent with an axial proton, geminal to a OH group and coupled with two adjacent CH_2 groups. Thus, the additional O atom in the form of a OH group is in α position at C(2). This was further confirmed when **5** was refluxed in acetone in the presence of *p*-toluenesulfonic acid and $CaCl_2$ to give the lactone **17** exhibiting the typical strong IR absorption band at 1770 cm^{-1}.

The methyl ester **10** of the second metabolite **8** from the short-fermentation run also had a molecular formula $C_{21}H_{29}ClO_3$. A broad IR absorption band between 3700–3400 cm^{-1} suggested the presence of a OH function.

The ^{1}H-NMR spectrum which showed 2 *d* at 1.24 and 1.26 ppm (3 H), in addition to 2 other CH_3 signals, suggested the absence of one of the CH_3 groups at C(15). Also, the appearance of a 2-H *m* at 3.82–3.66 ppm could be attributed to a CH_2 group derived from the substitution of one of the C(16) H atoms by a OH group. The presence of 2 sets of *d* for the remaining H_3C (17) and two *s* at 1.20 and 1.19 ppm for the $CH_3C(10)$ indicated that the metabolite was a mixture of C(15) diastereomers.

From the longer-period incubation experiments, only two products **13** and **16** were isolated after diazomethane methylation of the crude extract. High resolution MS and microanalyses indicated they were isomeric with a molecular formula $C_{21}H_{29}ClO_4$. Their other spectral properties were very similar (see *Exper. Part*) and consistent with structures **13** and **16**, formulated as diastereomers at C(15). This was further supported by acetylation of a mixture **13**/**16** with Ac_2O in pyridine to afford the diacetate **18** in quantitative yield. Again, some of the ^{1}H-NMR resonances consisted of two sets of signals, presumably due to the slight differences which exist between the two diastereomers. However, with the available data it was not possible to assign conclusively the absolute configuration at C(15) for the two dihydroxy metabolites.

Thus, enzymatic hydroxylation of 12-chlorodehydroabietic acid with *M. isabellina* initially occurs at C(2α) and C(16) to give the metabolites **5** and **8** which then undergo concomitant hydroxylation to the same dihydroxy compounds **11** and **14**. These two dihydroxy compounds resist further transformation over an extended length of time.

Experimental Part

General Remarks. S. [2]. Differing from that: Dried sample extracts were transformed to imidazole derivatives by dissolving them in anh. THF (1.0 ml), adding a solution of 1,1'-carbonyldiimidazole (1.0 ml) from a stock solution (*Aldrich*, 20 mg/ml MeCN) and allowing the reaction to proceed at r.t. for 1 h. The mixture of the resultant imidazole derivatives was analyzed by HPLC (reverse phase *(C-8)* analytical column).

Biotransformation of 12-Chlorodehydroabietic Acid by M. isabellina. The experimental procedure concerning maintenance of culture, time and temperature of the experiment etc. was identical with that previously reported for the 14-chlorodehydroabietic acid case [2].

Isolation and Characterization of Metabolites. In a typical large-scale experiment, a mixture of sterilized dextrose yeast extract broth (9.4 l) and glucose solution (400 ml) placed in a 14-l-fermentor jar was inoculated with spores of *M. isabellina*. This was followed by the addition of the solution of the sodium salt of **3** (200 ml) obtained from 200 mg of **3** and the fermentor was allowed to run for 26 h. The culture was harvested, filtered through *Celite* and the filtrate acidified (pH *ca.* 2) with conc. HCl, saturated with rock salt, and extracted twice with AcOEt. The combined extract was washed with H_2O to neutrality, dried ($MgSO_4$), and concentrated to give the crude extract (305 mg). This crude extract, after treatment with CH_2N_2/Et_2O, was separated by flash column chromatography (*Merck* silica gel *GF 254*, 25 g, 15–40% Et_2O/petroleum ether) to give **7** (44 mg), **10** (40 mg), **13** and **16** (40 mg), and starting substrate ester (42 mg).

Exactly the same experiment as described above was repeated, but for 90 h. Similar workup and purification procedures gave **13** and **16** (130 mg 8:5) and starting material isolated as the methyl ester (20 mg).

Methyl 12-Chloro-2α-hydroxydehydroabietate (**7**) was obtained as colourless crystals, m.p. 76–78° (Et_2O/petroleum ether), after flash chromatography with Et_2O/petroleum ether 1:4. IR (Nujol): 3700–3400, 1720, 840. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.18 (*s*, 1 H, H–C(14)); 6.93 (*s*, 1 H, H–C(11)); 4.07 (*tt*, *J* = 4.0, 12.0, 1 H, H_β–C(2)); 3.67 (*s*, 3 H, CO_2CH_3); 3.3 (*sept.*, *J* = 6, 1 H, H–C(15)); 1.3 (*s*, 3 H, H_3C–C(4)); 1.24 (*s*, 3 H, H_3C–C(10)); 1.22 (*d*, *J* = 6, 3 H, H_3C–C(15)); 1.21 (*d*, *J* = 6, 3 H, H_3C–C(15)). MS: 364 (M^+), 349 ($M^+ - CH_3$), 346 ($M^+ - H_2O$), 271 (100). MS (HR): 364.1799 (calc. for $C_{21}H_{29}{}^{35}ClO_3$ 364.1797).

$C_{21}H_{29}ClO_3$ Calc. C 69.10 H 8.01% Found C 69.25 H 8.14%

Methyl 12-Chloro-16-hydroxydehydroabietate (**10**), a colourless crystalline solid, m.p. 105–109° (Et_2O/petroleum ether), was isolated after flash chromatography with Et_2O/petroleum ether 1:4. IR (Nujol): 3700–3400, 1720, 840. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.22 (*s*, 1 H, H–C(14)); 6.91 (*s*, 1 H, H–C(11)); 3.82–3.66 (*m*, 2 H, H_2C–C(16)); 3.67 (*s*, 3 H, CO_2CH_3); 3.44 (*sext.*, *J* = 6, 1 H, H–C(15)); 1.28 (*s*, 3 H, H_3C–C(4)); 1.26, 1.24 (2 *d*, *J* = 6, 3 H, H_3C–C(15)); 1.20, 1.19 (2 *s*, 3 H, H_3C–C(10)). MS: 364 (M^+), 349 ($M^+ - CH_3$), 333 ($M^+ - OCH_3$, 100). MS (HR): 364.1801 (calc. for $C_{21}H_{29}{}^{35}ClO_3$ 364.1797).

$C_{21}H_{29}ClO_3$ Calc. C 69.10 H 8.01% Found C 68.92 H 8.10%

Methyl 12-Chloro-2α,16-dihydroxydehydroabietate (**13** *and* **16**) were isolated after flash chromatography with 20–40% Et_2O/petroleum ether (gradient).

Less Polar Diastereomer: colourless crystalline solid, m.p. 101–103° (Et_2O/petroleum ether), *ca.* ⅓ of the more polar epimer. IR (Nujol): 3700–3400, 1725, 840. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.23 (*s*, 1 H, H–C(14)); 6.94 (*s*, 1 H, H–C(11)); 4.09 (br. *t*, *J* = 12, 1 H, H_β–C(2)); 3.82–3.64 (*m*, 2 H, H_2C–C(16)); 3.69 (*s*, 3 H, CO_2CH_3); 3.44 (*sext.*, *J* = 6, 1 H, H–C(15)); 1.31 (*s*, 3 H, H_3C–C(4)); 1.25 (*d*, *J* = 6, 3 H, H_3C–C(15)); 1.24 (*s*, 3 H, H_3C–C(10)). MS: 380 (M^+), 349 ($M^+ - OCH_3$, 100). MS (HR): 380.1747 (calc. for $C_{21}H_{29}{}^{35}ClO_4$ 380.1746).

$C_{21}H_{29}ClO_4$ Calc. C 66.20 H 7.67% Found C 66.32 H 7.66%

More Polar Diastereomer: colourless crystalline solid, m.p. 77–79° (Et_2O/petroleum ether). IR (Nujol): 3700–3400, 1725, 840. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.24 (*s*, 1 H, H–C(14)); 6.94 (*s*, 1 H, H–C(11)); 4.08 (*tt*, *J* = 4.0, 12.0, 1 H, H_β–C(2)); 3.8–3.63 (*m*, 2 H, H_2C–C(16)); 3.7 (*s*, 3 H, CO_2CH_3); 3.44 (*sext.*, *J* = 6, 1 H, H–C(15)); 1.3 (*s*, 3 H, H_3C–C(4)); 1.26 (*d*, *J* = 6, 3 H, H_3C–C(15)); 1.23 (*s*, 3 H, H_3C–C(10)). MS: 380 (M^+). MS (HR): 380.1766 (calc. for $C_{21}H_{29}{}^{35}ClO_4$ 380.1746).

$C_{21}H_{29}ClO_4$ Calc. C 66.20 H 7.67% Found C 65.85 H 7.65%

Methyl 2α,16-Diacetoxy-12-chlorodehydroabietate (**18**). A mixture of **13**/**16** (25 mg), pyridine (1.5 ml) and Ac_2O (1.0 ml) was stirred for 16 h at r.t., diluted with MeOH (5 ml) and concentrated under vacuum. The residue was dissolved in toluene and evaporated to dryness (thrice) to get rid of pyridine. The thick oily mass thus obtained was passed through a short silica gel column and crystallized from Et_2O/petroleum ether (m.p. 41–45°) to give **18** in quant. yield. IR (Nujol): 1730, 860, 740. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.2 (*s*, 1 H, H–C(14)); 6.92 (*s*, 1 H, H–C(11)); 5.2 (*tt*, *J* = 4.0, 12.0, 1 H, H_β–C(2)); 4.2–4.09 (*m*, 2 H, H_2C–C(16)); 3.7, 3.69 (2 *s*, 3 H, CO_2CH_3); 3.63–3.58 (*m*, 1 H, H–C(15)); 2.08 (*s*, 3 H, AcO–C(2α)); 2.05 (*s*, 3 H, AcO–C(16)); 1.36 (*s*, 3 H, H_3C–C(4)); 1.3, 1.29 (2 *s*, 3 H, H_3C–C(10)); 1.27, 1.25 (2 *d*, *J* = 6, 3 H, H_3C–C(15)). MS: 464 (M^+), 405 ($M^+ - CO_2CH_3$), 404 ($M^+ - CH_3CO_2H$, 100). MS (HR): 464.1964 (calc. for $C_{25}H_{33}{}^{35}ClO_6$ 464.1965).

$C_{25}H_{33}ClO_6$ Calc. C 64.55 H 7.15% Found C 64.34 H 7.07%

12-Chlorodehydroabieto-18,2α-lactone (**17**). The mixture of hydroxy acid **5** (30.4 mg), *p*-toluene sulfonic acid (15 mg), and $CaCl_2$ (15 mg) in anh. acetone (25 ml) was refluxed for 30 min, then cooled, filtered, and concentrated under vacuum. The anal. sample was prepared by prep. TLC on silica gel: **17** as a thick oil. IR (Nujol): 1770, 840, 740. ^{1}H-NMR ($CDCl_3$, 400 MHz): 7.18 (*s*, 1 H, H–C(14)); 6.93 (*s*, 1 H, H–C(11)); 4.8–5.0 (*m*, $W_{½}$ = 12, 1 H, $H_β$–C(2)); 3.3 (*sept.*, *J* = 6, 1 H, H–C(15)); 1.32 (*s*, 3 H, H_3C–C(4)); 1.25 (*s*, 3 H, H_3C–C(10)); 1.22 (*d*, *J* = 6, 3 H, H_3C–C(15)); 1.21 (*d*, *J* = 6, 3 H, H_3C–C(15)). MS: 332 (M^+). MS (HR): 332.1525 (calc. for $C_{20}H_{25}{}^{35}ClO_2$ 332.1536).

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