66. Studies Related to Biological Detoxification of Kraft Pulp Mill Effluent. III. The Biodegradation of Abietic Acid with *Mortierella isabellina*[1])

by **James P. Kutney**[2]), **Eugene Dimitriadis, Gary M. Hewitt, Mahatam Singh** and **Brian R. Worth**

Department of Chemistry, University of British Columbia, 2036 Main Mall, University Campus, Vancouver, B.C. V6T 1Y6, Canada

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Summary

A study relating to the biological detoxification of resin acids is described. Abietic acid was transformed into a number of non-toxic hydroxylated derivatives by *Mortierella isabellina*. The biotransformation products have been identified and shown to be less toxic than abietic acid.

Introduction. – Diterpene resin acids, arising from softwoods used in the pulping industry, have been shown to be the major toxicants in kraft pulp mill effluent [1] [2]. Among the toxic resin acids identified are dehydroabietic acid (**1**), isopimaric acid (**2**), abietic acid (**3**) and the chlorodehydroabietic acids.

Abietic acid is a minor raw kraft mill effluent constituent which has a significant toxicity towards rainbow trout (96 h, $LC_{50} = 0.7$ mg/l) [3]. Its degradation by natural mixed microflora proceeds at an intermediate rate between those of acid **1**, which was most easily degraded, and **2** [4]. An *Alcaligenes* species can utilize **3** as its sole C-source and in doing so produces minute yields of 5α-hydroxyabietic acid and other products [5]. *Flavobacterium resinovorum* and *Pseudomonas resinovorans* can also utilize **3** for growth [6].

In earlier work [6] [7] the fungus *Mortierella isabellina* was shown to hydroxylate dehydroabietic and isopimaric acids in a site-specific manner. The hydroxylated derivatives were not toxic to juvenile *Daphnia pulex* or to salmon fry. These results have encouraged us towards further research into the biotransformation of other resin acids. Demonstration that resin acid derivatives formed by *M. isabellina* are non-toxic may eventually lead to supplementation of biobasins with this fungus to promote detoxification.

In the present report, the biotransformation of **3** into a number of non-toxic, hydroxylated products by *M. isabellina* is described.

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[1]) *Key words:* Kraft pulp mill effluent, detoxification, abietic acid, biodegradation, *Mortierella isabellina*.

[2]) Author to whom correspondence should be addressed.

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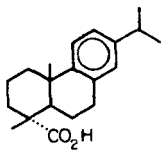
Methods for the quantitative analysis of metabolites. – The conjugated diene in abietic acid (**3**) provides a suitable chromophore for its quantitation by HPLC. and UV. Fortunately, the diene chromophore is not lost or modified by biotransformation with *M. isabellina*. Detection and quantitation of metabolites (except for **7**) was therefore possible using UV. detection at 254 nm.

Samples taken from the fermentor at regular intervals were frozen until ready for extraction. Collected samples were allowed to thaw, treated with ammonium sulfate, and extracted with ethyl acetate. Portions of the extract were analyzed by HPLC. using a reverse phase (C18) column, a methanol/water (0.1% acetic acid) gradient, and the UV. detector operating at both 254 and 280 nm. It was useful to operate at both wavelengths as the diene chromophore of **3** and its hydroxylated derivatives showed λ_{max} near 232, 246 and 248 nm while the triene **7**, also formed, with a λ_{max} at 262, 272, 283, absorbed only weakly at 254 but strongly at 280 nm.



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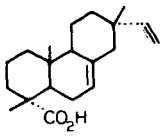

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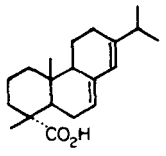

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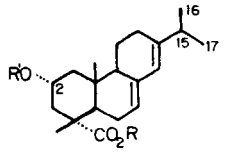
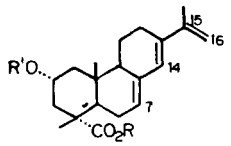
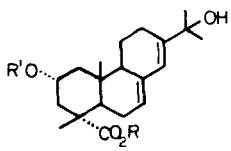


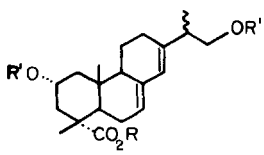
R = H R' = H **4**
R = CH_3 R' = H **5**
R = CH_3 R' = Ac **6**



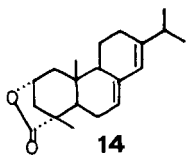
R = H R' = H **7**
R = CH_3 R' = H **8**
R = CH_3 R' = Ac **9**



R = H R' = H **10**
R = CH_3 R' = Ac **11**



R = H R' = H **12**
R = CH_3 R' = Ac **13**



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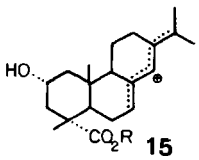

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R = H or CH_3

Although separation of hydroxylated metabolites (see *Fig. 5*) was possible using a solvent gradient *(Table 1, Exper. Part)* the resolution of diols **10** and **12** (see *Fig. 6*) was only possible after reinjection of samples under different conditions *(Table 2, Exper. Part)*.

Samples required analysis within 48 h after extraction as detectable decomposition occurred, even when sealed at 0°, after 2–3 days.

Standard curves for abietic acid (**3**) (*Fig. 1* and *2*) show a linear response between peak area (calculated electronically) and amount injected over the range 5–30 µg. The detection limit estimated from the lower range curve *(Fig. 2)* is approximately 30 ng at 254 nm indicating a high sensitivity for this diene chromophore. The standard curves could be used for the quantitation of **3** in both culture and kraft mill effluent extracts.

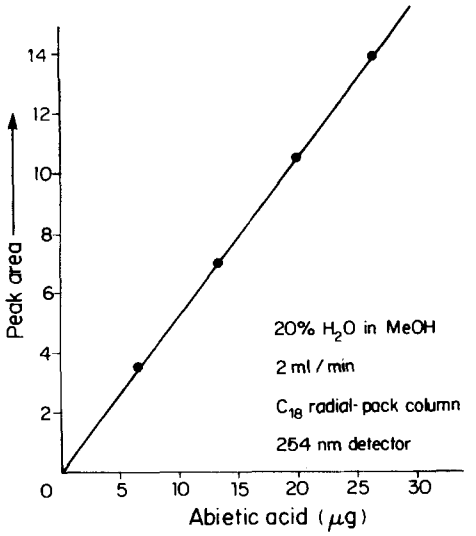

Fig. 1. *HPLC. of abietic acid* (5–30 µg range), *standard curve*

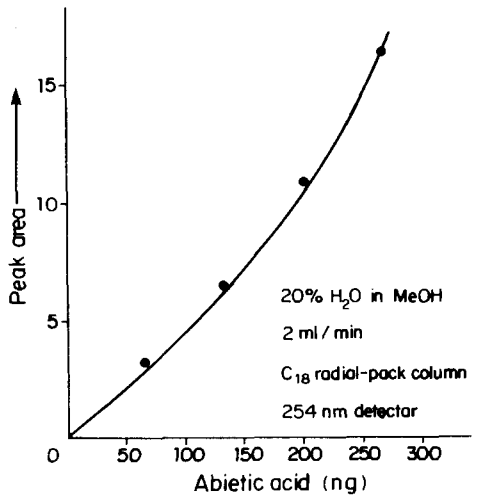

Fig. 2. *HPLC. of abietic acid* (60–250 ng range), *standard curve*

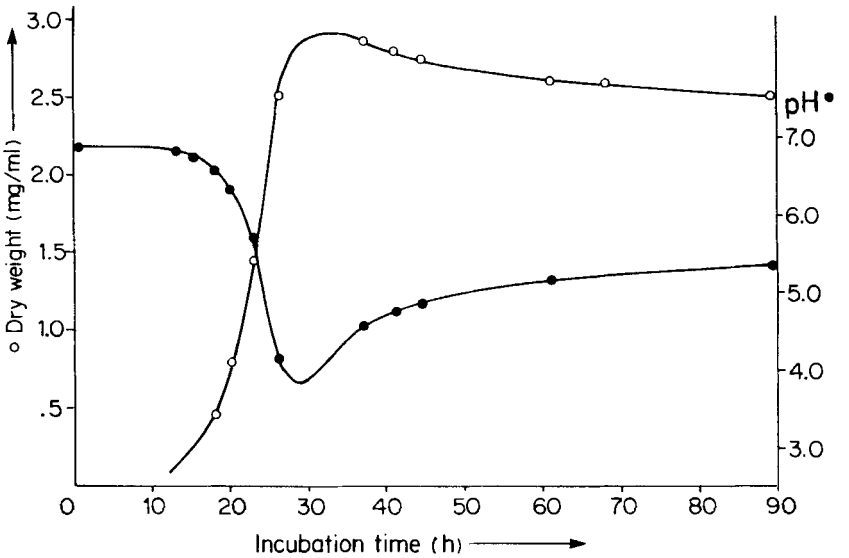

Fig. 3. *Growth and pH curves for a 10 l, abietic acid biotransformation* (550 mg) *by* M. isabellina *at 35°*

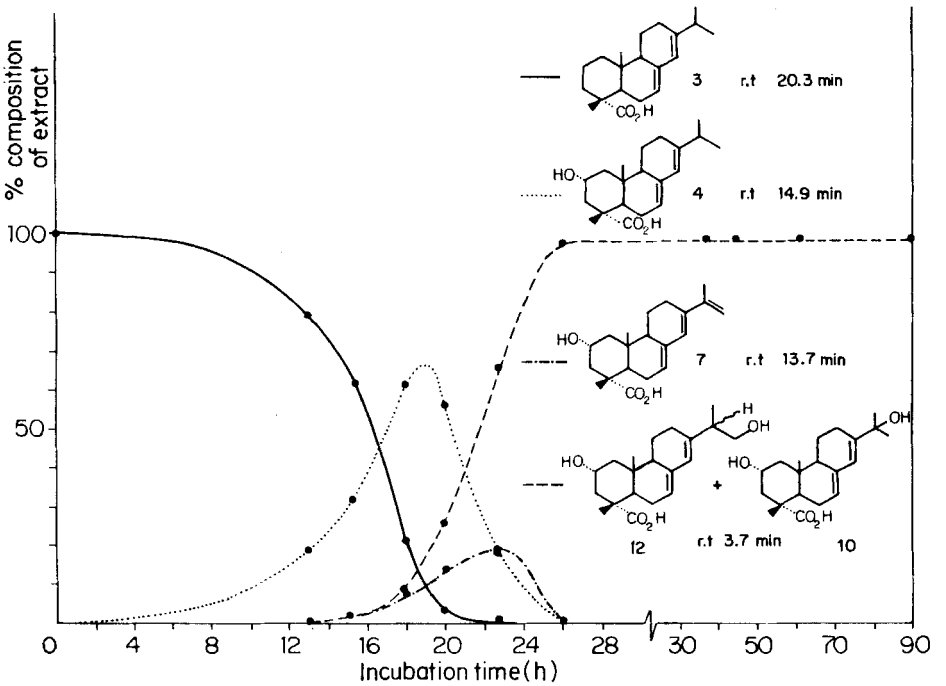

Fig. 4. *Abietic acid – biotransformation with* Mortierella isabellina (50 mg/l abietic acid)

Biotransformation of abietic acid (*3*) by *M. isabellina*. – A solution of **3** (as the sodium salt) was added to a freshly inoculated *M. isabellina* culture. Growth was allowed to proceed for up to 90 h. Samples of *ca.* 100 ml were taken at convenient time intervals and frozen. As with the previous cultures, a rapid growth of the organism occurred after 18–22 h associated with a drop in pH *(Fig. 3)*.

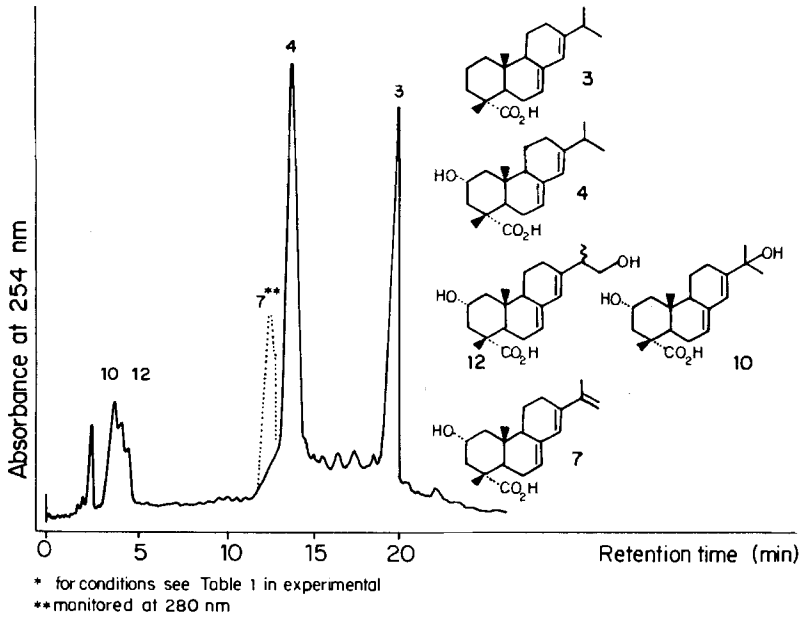

Fig. 5. *HPLC. trace from abietic acid culture at 18 h* (for conditions, see *Table 1* in *Exper. Part*)

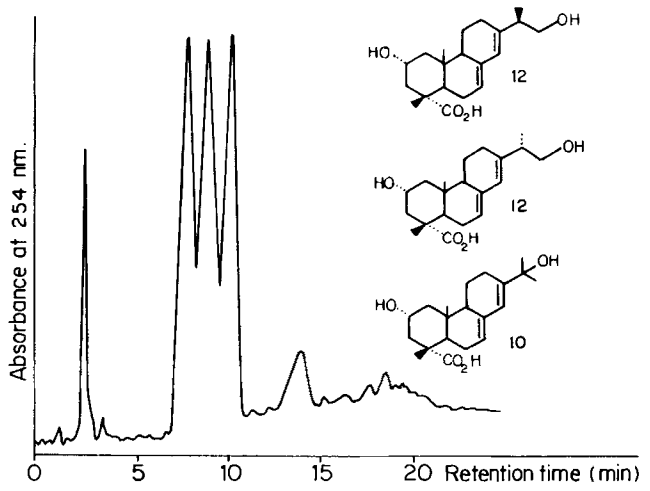

Fig. 6. *HPLC. trace of abietic acid diols* **10** *and* **12** *at 37 h* (for conditions, see *Table 1* in *Exper. Part*)

Analysis of the extracted samples *(Fig. 4)* by HPLC. showed a rapid conversion of **3** to metabolites within 26 h followed by appearance and disappearance of intermediate metabolites.

After 14 h, the major product formed was 2α-hydroxyabietic acid (**4**) which reached a peak concentration in approximately 19 h. Decrease in the concentration of **4** (after *ca.* 4 h lag) was associated with an increase in concentration of diols **10** and **12** and triene **7**. The triene **7**, after reaching a maximal concentration at 23 h was not detected after 27 h. Thereafter only the diol mixture was present and did not appear to be further hydroxylated up to 90 h.

Isolation and identification of metabolites. – Two large scale fermentations (10+12 l, containing 55–60 mg **3**/l) provided sufficient amounts of metabolites for structural determination and toxicity testing.

It was necessary to remove the fungal mycelia by filtration as the cells otherwise interfere in the solvent extraction of the broth. The cell mats were washed with ethyl acetate, and the same solvent was used to extract the broth after it was saturated with sodium chloride. Removal of the solvent was done at $< 40°$ to minimize decomposition of the products. The residue was chromatographed on silica gel to separate the crude metabolites **4** and **7** from **10** and **12** and from starting material **3**. The extract also contained an oily triglyceride which was removed during chromatography. Further purification was achieved by rechromatography on silica gel to provide samples sufficiently pure for toxicity tests. In order to fully characterize the different metabolites, it was necessary to convert the carboxylic acid function to the methyl ester and any primary and or secondary hydroxyl groups to the corresponding acetyl derivatives.

The initial biotransformation product **4**, was treated with diazomethane to give ester **5** which had the molecular formula $C_{21}H_{32}O_3$ confirming the introduction of one O-atom to the abietic acid skeleton. Hydroxyl stretching at $\tilde{\nu}$ 3400 cm^{-1} was

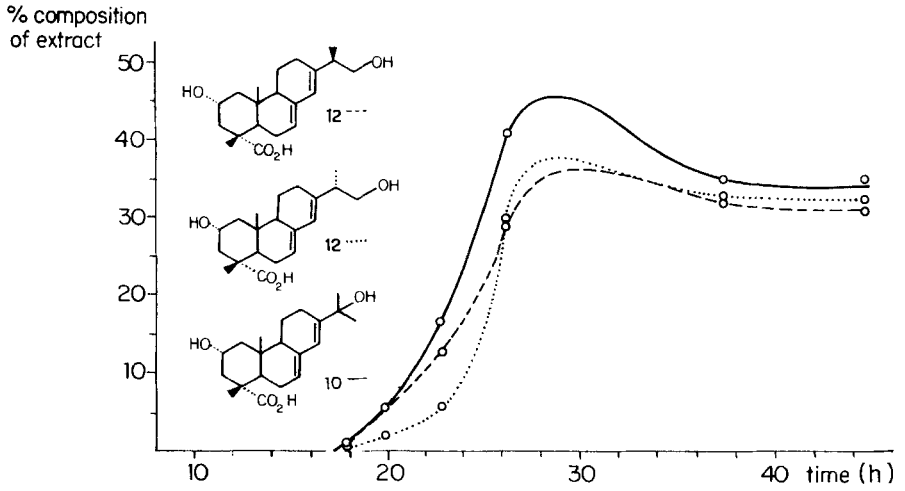

Fig. 7. *Formation of diols from abietic acid*

evident in the IR. spectrum. As with dehydroabietic acid (**1**) [1] and isopimaric acid (**2**) [2] the ^{1}H-NMR. spectrum revealed a nine line ($t \times t$) single proton resonance at δ 3.93. The coupling constants of 4.0 and 11.7 Hz were clearly consistent with the assignment of H–C(2) in the β(axial)-configuration. Thus the hydroxyl group was introduced at C(2α), substantiated by a downfield shift of 1.17 ppm for H–C(2β) in the corresponding acetyl derivative **6**.

In the hope of further confirming 2α-hydroxylation, lactonisation of **4** was attempted by heating with *p*-toluenesulfonic acid in benzene. Isolation of the major product and examination of its ^{1}H-NMR. spectrum showed it to be a mixture of olefinic isomers, presumably due to acid-catalyzed isomerization. However, the otherwise homogeneous mixture (by TLC.) showed the characteristic lactone absorption in the IR. spectrum at 1780 cm^{-1} common to the components of the mixture. The mass spectrum of the isomeric mixture showed the required molecular ion at *m/z* 300.

Attempted oxidation of **4** with pyridinium chlorochromate, used earlier for a dehydroabietic acid metabolite characterization [1], gave extensive decomposition. Although attempts to prepare a lactone and ketone from the first formed metabolite were unsuccessful, its spectral data are in complete agreement with the assigned structure **4**.

The next products of biotransformation, **10** and **12**, were isolated as an apparently homogeneous band by preparative layer chromatography. Diazomethane treatment gave the esters which, also, were not separated by TLC. The mixture of **10** and **12** was just resolvable using reverse phase liquid chromatography (see *Fig. 6*). Separation of **10** and **12** was achieved after acetylation of the mixed esters to the monoacetate **11** and diacetate epimers **13**.

Acetate **11** was isolated as an unstable gum after chromatography on silica. Mass spectral data showed a weak molecular ion for **11** (*m/z* 390) with ions resulting from characteristic losses of water and acetic acid. The ^{1}H-NMR. spectrum showed H–C(2β) deshielded by the acetate at 5.1 ppm ($t \times t$, J=4 and 12 Hz) with 2 H_3C–C(15) and H_3C–C(4) all resonating at 1.33 ppm as a broadened singlet. The downfield shift of 2 H_3C–C(15) is consistent with hydroxylation at C(15). Examination of the IR. spectrum of **11** confirmed the presence of the tertiary hydroxyl with an absorption at 3500 cm^{-1}. Evidently the tertiary hydroxyl of **10** and **11** is very labile, undergoing facile elimination to a stabilized allylic cation like **15** which on loss of a proton can explain the formation and contamination of many of the compounds by triene **7**.

Although the transient species, triene **7**, could not be isolated in high purity, it showed olefinic proton resonances in the ^{1}H-NMR. spectrum at 5.6 (H–C(7)) and 6.2 (H–C(14)) ppm and 4.95 and 5.1 ppm for 2 H–C(16). A broadened singlet at 1.9 ppm was ascribed to H_3C–C(15). A small pure sample isolated by analytical HPLC. showed λ_{max} (MeOH) at 283, 272, 262 nm similar to the diene pattern but shifted to a lower energy absorption by 30 nm. This is consistent with the introduction of a new conjugated double bond. These data support the triene **7** as the unstable metabolite which may be an artifact. This artifact could arise during cultivation of *M. isabellina* where a marked drop in pH from *ca.* 7.0 to as low as 3.7 was observed. This acidity resulted in conditions in which the diol **10**

is unstable *(Fig. 3)*. However, after 30 h the triene was not detected by HPLC., although diol **10** was present. This result can be ascribed to the increase in pH (to *ca.* 4.5) after 24 h, enough to reduce the dehydration process. Alternatively, the disappearance of the triene may be the result of an increase in the concentration or activity of the enzyme responsible for the hydroxylation or hydration at C(15) or C(16). At low fungal concentrations, as would exist in biobasins, this drop in pH would be minimized and therefore the formation of triene should not occur. Formation of diols **12** may arise from remote hydroxylation of a non activated site (C(16) of **4**) or allylic hydroxylation *via* **15**. The formation of diastereoisomers **12** occurs with little stereoselectivity. The three diol peaks, evident in the HPLC. trace *(Fig. 6)*, remain unassigned. Changes occur in the ratio of the three diol peaks during incubation *(Fig. 7)*. As one of the peaks decreases (presumably the tertiary alcohol **10** owing to dehydration), after 30 h at a pH of *ca.* 3.8 the other two diols (diastereoisomers **12**) increase, possibly by hydration of the Δ^{15}-double bond (see *Fig. 4*). The triene **7** is not detected after 27 h when the pH is beginning to increase and dehydration of **10** is possibly decreasing.

The diastereoisomeric methyl esters of **12** were isolated as the diacetyl derivatives **13** which could not be separated by TLC. The ^{1}H-NMR. spectrum revealed features common to both isomers. The olefinic protons resonated as singlets at 5.82 and 5.41 ppm, the H_β–C(2) was deshielded from 3.9 in the alcohols to 5.04 ppm ($t \times t$, $J = 4$ and 12 Hz) in the acetyl derivative **13**. Similarly the methylidene protons on C(16) were deshielded on acetylation from 3.4–3.5 to 3.9–4.1 ppm and, furthermore, were not equivalent in each isomer because of the newly formed chiral centre at C(15). One H–C(16) of each epimer resonated at 4.07 ppm ($d \times d$, $J = 6.7$ and 9.4 Hz). The other H–C(16), appeared at 3.93 ppm as two apparent triplets ($J = 6.7$ Hz) from overlapping doublets of each isomer. A barely resolved pair of doublets at 1.08 ppm ($J = 7$ Hz) was assigned to the methyl groups on C(15) of epimers **13** while H–C(15) resonated at 2.45 ppm as a multiplet. The acetoxy resonances of both isomers were barely separated singlets (2.03 ppm). Other resonances (*e.g.* H_3C–C(10) and $COOCH_3$) were superimposed in both isomers.

The results presented above clearly indicate that **3** is rapidly, efficiently and systematically converted to a number of hydroxylated derivatives by *M. isabellina*. These results further demonstrate the potential of *M. isabellina* in the microbial biodetoxification of resin acids. Preliminary toxicity testing of metabolites **4**, **10**, and **12** indicates that they are non toxic at levels in excess of 30 mg/l to both juvenile *Daphnia pulex* and salmon fry. These results will be published in a separate account. Further work is continuing into the biotransformation of the chlorodehydroabietic acids.

Experimental Part

Abietic acid (3) biotransformation by *M. isabellina*. – *M. isabellina* stock culture was maintained on potato dextrose agar *(Difco)*. Cultures to be used as inocula in biotransformation studies were grown on 100 ml of potato dextrose agar supplemented with 10 mg 3/l in *Roux* bottles at 25°. Inocula were prepared by washing the surface of confluent mature cultures with 50 ml of sterile Aerosol OT solution (*Fisher Scientific*, 450 µl per l distilled water). Resulting spore suspensions were

used to inoculate 10–12 l of dextrose yeast extract broth (5.0 g D-glucose, 0.5 g yeast extract *(Difco)*, 1.0 g KH_2PO_4, 1.0 g $(NH_4)_2H\,PO_4$, 1.0 g NaCl, and 0.5 g $MgSO_4 \cdot 7\,H_2O$ per l tap water) contained in a 15 l fermentor jar *(Labroferm*^{R}, *New Brunswick Scientific)*. Initial spore concentrations were 4.0 to 5.5×10^5 spores per ml as determined by a *Howard* mold counting chamber. A filter-sterilized **3**-solution was added at the time of inoculation for an initial **3** concentration of 55 or 100 mg/l. This solution was prepared by dissolving abietic acid **3** (> 92% purity) in ethanol (1 ml per 100 mg **3**), converting the acid to its sodium salt by addition of 1.05 mol-equiv. of 0.1 M NaOH, and diluting with distilled water.

The fermentor was operated at 35°, 300 rpm, and 200 ml air/l/min.

Culture dry weights were measured using membrane filtered (0.45 μm cellulose acetate filters, *Millipore Corp.*), freeze-dried cells.

UV. spectra [λ_{max} (ε)] were recorded on a *Cary* 15 spectrometer in methanol as solvent. IR. spectra (cm^{-1}) were recorded using *Perkin Elmer* 457 or 710 spectrometers. ^{1}H-NMR. spectra were recorded on a *Varian* XL-100, *Nicolet Oxford* H-270 or *Brüker* WH-400 spectrometers. All ^{1}H-NMR. spectra were done in $CDCl_3$, using tetramethylsilane (TMS) as internal standard and are reported in ppm from TMS. Low resolution MS. (*m/z*) were recorded on either an *Atlas* CH-4B or *AEI-MS* 902 spectrometers and high resolution mass measurements on an *AEI-MS* 50 spectrometer. Microanalyses were done by Mr. *P. Borda*, Microanalytical Laboratory, University of British Columbia.

High performance liquid chromatography (HPLC.) was done using *Waters* instrumentation and included a System Controller, Data Module, *Wisp 710 B*, Model 440 UV. detector and Radial Compression Module containing a reverse phase (C18) analytical column. Solvent systems for monitoring the formation of metabolites (methanol/water, both containing 0.1% acetic acid) are shown in gradient *Table 1*.

Table 1. *HPLC. separation of metabolites*

Time (min)	Flow (ml/min)	% Water	% Methanol	Curve
Initial	2.0	30	70	–
15	2.0	5	95	08
24	2.0	5	95	06
27.0	5.0	30	70	01
27.1	2.0	30	70	01

Table 2. *Solvent gradient conditions for analysis of diols* **10** *and* **12**

Time (min)	Flow (ml/min)	% Water	% Methanol	Curve
Initial	2.0	40	60	–
15.0	2.0	5	95	08
24.0	2.0	5	95	06
27.0	5.0	40	60	01
27.1	2.0	40	60	01

Acetylations were done using an excess of acetic anhydride in pyridine, the excess reagents being removed under vacuum.

Methyl 2α-hydroxyabietate (**5**) was obtained as a resinous solid foam which resisted attempts to crystallise. – UV. (MeOH): 232 (4.32), 240 (4.36), 248 (4.18). – IR. (film): 3450 (br.), 1725, 1250, 1130, 1060, 910. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 0.87 (*s*, 3 H, H_3C–C(10)); 1.01, 1.00 (2 *d*, J = 6 Hz, 6 H, 2 H_3C–C(15)); 1.28 (*s*, 3 H, H_3C–C(4)); 3.65 (*s*, 3 H, CO_2CH_3); 3.93 (*t* × *t*, J = 4 and 11.5 Hz, 1 H, H–C(2)); 5.38 (apparent *d*, J = 4.5 Hz, 1 H, H–C(7)); 5.78 (*s*, 1 H, H–C(14)). – MS.: 332 (M^+), 314, 299, 273, 271, 255 (100), 239, 211, 187, 185, 145, 143, 131, 128, 105, 91. High resolution molecular weight determination: $C_{21}H_{32}O_3$ Calc. 332.2351, Found 332.2348.

$C_{21}H_{32}O_3 \cdot \frac{1}{2}\,H_2O$ Calc. C 73.90 H 9.68% Found C 73.08 H 9.24%

This compound was further characterized as the acetate **6**.

$C_{23}H_{34}O_4$ Calc. C 73.97 H 9.00% Found C 73.76 H 9.15%

Lactonisation of **4**. A solution in benzene (20 ml) of hydroxy acid **4** (15 mg) and *p*-toluenesulfonic acid (5 mg) was heated under reflux until the acid **4** was no longer detected by TLC. (20 min). TLC. showed a mixture of products. The cooled solution was washed with water, dried ($MgSO_4$) and concentrated *in vacuo*. Chromatography on silica allowed the isolation of the major product (8 mg) which was a mixture (^{1}H-NMR.; complex olefinic resonances). – IR. (film): 1780, 1195, 1095, 740. – MS.: 300 (M^+), 298, 254, 239, 211, 197, 136, 135, 121, 119, 105, 91.

Attempted oxidation of **4** *and* **5**. A solution of **4** (15 mg) in dry CH_2Cl_2 (2–3 ml) was added at once to a suspension of pyridinium chlorochromate (25 mg) in CH_2Cl_2 (5 ml) at RT. and the mixture stirred for 2 h when TLC. showed only two products. Standard workup and chromatography on silica gave complex mixtures (^{1}H-NMR.).

A similar oxidation of the methyl ester **5** (10 mg) gave a similar complex mixture which was not investigated further.

Methyl 2α-acetoxy-15-hydroxyabietate (**11**). Metabolite **12** could only be separated from the isomeric diol **10** after methylation and acetylation. The hydroxy acetate **11** was isolated after repeated chromatography on silica as an unstable resinous foam. – IR. (film): 3500 (br.), 1730, 1240, 1135, 1030, 960, 905, 735. – ^{1}H-NMR. ($CDCl_3$): 0.90 (*s*, 3 H, H_3C–C(10)); 1.33 (*s*, 9 H, H_3C–C(4) and 2 H_3C–C(15)); 2.05 (*s*, 3 H, $OCOCH_3$); 3.65 (*s*, 3 H, CO_2CH_3); 5.10 (*t*×*t*, *J*=4 and 12 Hz, 1 H, H_β–C(2)); 5.50 (br. *s*, 1 H, H–C(7)); 6.12 (*s*, 1 H, H–C(14)). – MS.: 390 (M^+), 372, 330, 312, 271, 255, 253, 237, 197, 185, 183, 145, 121, 119, 86, 84. – High resolution molecular weight determination: Calc. 390.2401, Found 390.2407.

$C_{23}H_{34}O_5$ Calc. C 70.74 H 8.78% Found C 70.51 H 8.93%

Methyl 2α,16-diacetoxyabietate (**13**). This metabolite derivative was obtained during the isolation of **11** after chromatography as a viscous oil, epimeric at C(15). – IR. (film): 1735, 1240, 1135, 1030, 735. – ^{1}H-NMR. ($CDCl_3$, 270 MHz): 0.92 (*s*, 3 H, H_3C–C(10)); 1.08 and 1.07 (2 *d*, *J*=6 Hz, 3 H, H_3C–C(15)); 1.33 (*s*, 3 H, H_3C–C(4)); 2.03 and 2.02 (2 *s*, 6 H, 2 $OCOCH_3$); 2.45 (*m*, 1 H, H–C(15)); 3.66 (*s*, 3 H, CO_2CH_3); 3.93 (2 *t*, *J*=6.7 Hz, 1 H, H–C(16)); 4.07 (2 *d*×*d*, *J*=6.7 and 9.4 Hz, 1 H, H–C(16)); 5.01 (*t*×*t*, *J*=4 and 12 Hz, H_β–C(2)); 5.41 (br. *s*, 1 H, H–C(7)); 5.82 (*s*, 1 H, H–C(14)). – MS.: 432 (M^+), 372, 312, 253, 185, 183. – High resolution molecular weight determination: Calc. 432.2512, Found 432.2512.

A small sample hydrolyzed by aqueous methanolic KOH-solution (5 h) gave the diol ester **12**.

$C_{21}H_{32}O_4 \cdot \frac{1}{2} H_2O$ Calc. C 70.60 H 9.31% Found C 69.91 H 9.26%

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