127. Studies Related to Biological Detoxification of Kraft Pulp Mill Effluent. IV[1]). The Biodegradation of 14-Chlorodehydroabietic Acid with *Mortierella isabellina*

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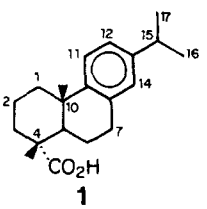
Summary

One of the fish-toxic chlorinated resin acids, 14-chlorodehydroabietic acid (**5**), found in kraft mill effluent is examined. When exposed to the fungus *Mortierella isabellina*, **5** is converted into a number of hydroxylated derivatives which show low levels of toxicity to fish. These biotransformation products were isolated and characterized.

In the following publication of this series [2] and in previous studies [1] [3] [4] we had commented on the fish-toxic nature of several resin acids (**1–3**) and the chlorinated derivatives of dehydroabietic acid (**4–6**). The earlier investigations illustrated



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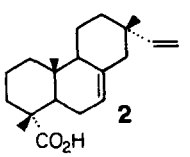

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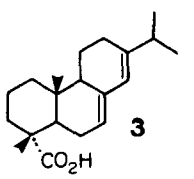

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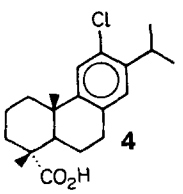

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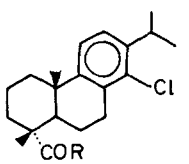
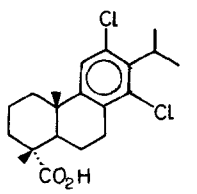


5 R = OH

5a R = —N⟨N



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[1]) For Part III, see [1].

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our approach to microbial detoxification of such compounds, and it was clear that the fungus *Mortierella isabellina* was effective in converting dehydroabietic acid (**1**) [3], isopimaric acid (**2**) [4], and abietic acid (**3**) [1] into metabolites with much lower toxicity to fish. In continuing our studies, we would now like to present results with this organism and 14-chlorodehydroabietic acid (**5**) (*cf.* [1]) described in the following publication [2].

In general, the fermentation methods employed were similar to those described earlier [1] [3] [4], and the method of analysis involved the HPLC. technique with imidazole derivatives developed earlier and described in the following publication [2].

Methods for the quantitative analysis of metabolites. – Samples taken from the fermenter were frozen until ready for extraction. After thawing, aliquots of each sample were acidified (pH 2–3) with phosphoric acid, saturated with sodium chloride and extracted with ethyl acetate. After evaporation, the residue was treated with 1,1'-carbonyldiimidazole in acetonitrile/tetrahydrofuran. This conversion of

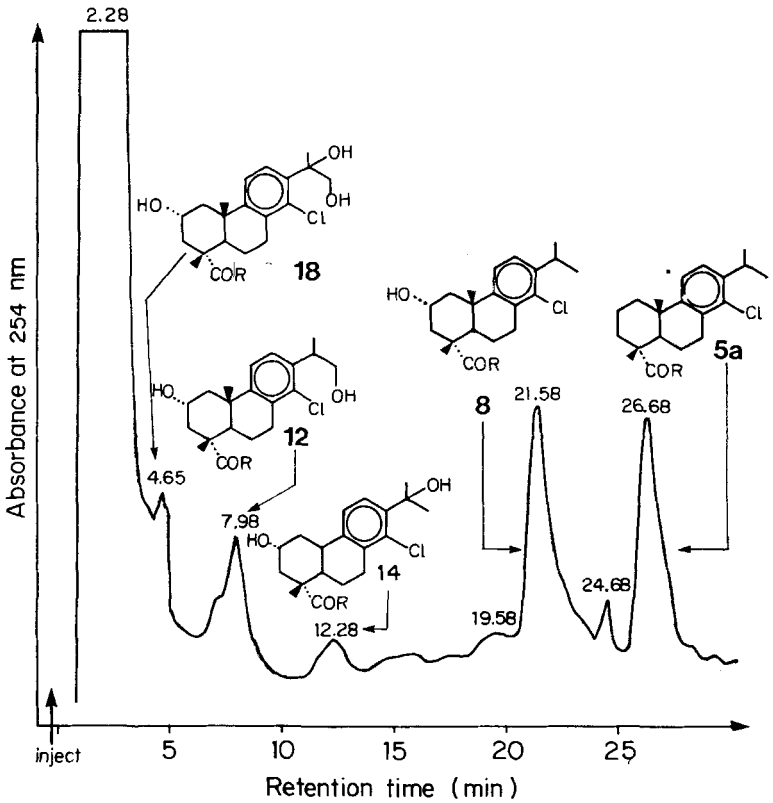


Fig. 1. *HPLC. of the biodegradation mixture (as imidazole derivatives) from* **5**. Conditions: Reverse phase *RP-18* column; solvent: H_2O/MeOH 1:3 (curve 7, *Waters*) from 0–20 min, H_2O/MeOH 1:9 (isocratic) from 20–30 min; flow rate: 2 ml/min

acids to their imidazole derivatives was rapid, quantitative and gave a chromophore which was more responsive to the UV. detector (254 nm) used in the HPLC. analysis than the free acids.

Separation and analysis of the imidazole derivatives of the metabolites was achieved by HPLC. using methanol/water mixtures and a reverse phase column. A typical HPLC. trace of the biodegradation mixture is shown in *Figure 1.*

Standard curves for 1-(14-chlorodehydroabietoyl)imidazole (**5a**) (see *Fig. 2*) shows a linear response between peak area (calculated electronically) and amount injected over the range of interest. The standard curve could be used for the quantitation of **5** in both the culture and kraft pulp mill extracts.

Biodegradation of *5* by *M. isabellina.* – Growth of *M. isabellina* proceeded as described previously for the biodegradation of dehydroabietic acid (**1**) [4], isopimaric acid (**2**) [4] and abietic acid (**3**) [1], except that the initial lag-phase was extended by approximately 5 h. Rapid growth occurred between 25 and 35 h of incubation to achieve maximal dry biomass levels greater than 2.6 mg/l. Culture growth was accompanied by a decrease in pH from 6.9 to 3.9. Extended incubation into the stationary growth phase caused, as with the other acids, decreased biomass yields and increased pH up to approximately 5.3.

Addition of **5** as the sodium salt was made at a lower level than previously (20 mg/l culture medium), due to decreased solubility. During the growth, samples were taken at convenient intervals up to 90 h and frozen.

The major product initially formed was the 2α-hydroxy acid **7** which reached a peak concentration after about 19–20 h. Thereafter, **7** showed a steady drop in concentration (see **8** in *Fig. 3*) accompanied by an increase in the concentration of

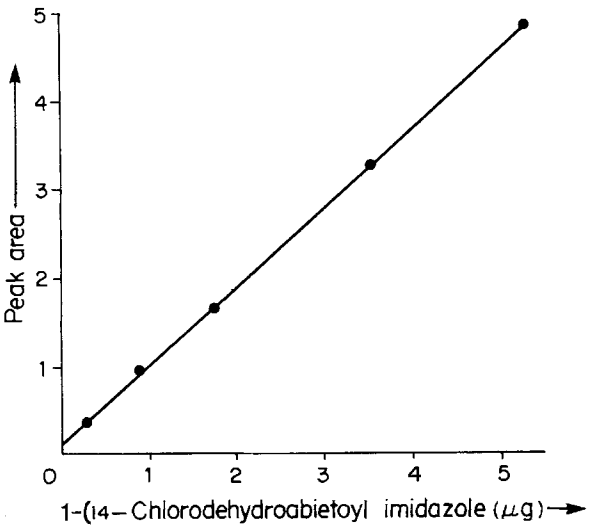


Fig. 2. *HPLC. peak area* versus *amount of injected* **5a**. Conditions: Reverse phase *RP-18* column; solvent: H_2O/MeOH 15:85; flow rate: 2 ml/min.

the 2*a*,15-dihydroxy-, 2*a*,16-dihydroxy- and 2*a*,15,16-trihydroxy acids **13**, **10** and **16**, respectively (see **14**, **12** and **18**, respectively, in *Fig. 3*).

The data, as seen in *Figure 3*, reveal that **10**, **13** and **16** accumulated to a maximum concentration in the time period of 30–40 h and were not further metabolized. Both the acid **5** and metabolite **7** persisted up to 30 h, longer than the other resin acids studied, presumably due to a slower rate of biodegradation or a slower growth of the organism.

Isolation and identification of metabolites. – A number of large-scale fermentations (12–24 l) containig 20 mg/l of **5** were required to provide sufficient metabolites for structural determination and toxicity testing. Short term fermentations, required to isolate metabolite **7**, were sometimes accompanied by deposition of acid on the upper walls of the fermenter, resulting from decreased solubility due to a drop in pH from 6.9 to approximately 4.

Fungal mycelia were readily removed by filtration and washed with solvent to remove any metabolites. The solvent (ethyl acetate) was then used to extract the salt-saturated and acidified filtrate. Chromatography on silica gel gave a separation of **5** and **7** from the diols and triols **10**, **13** and **16**. For further purification, the alcohols were esterified with diazomethane and rechromatography on silica gel gave pure hydroxy ester **9**. The polar di- and trihydroxy esters of **10**, **13** and **16** were best separated by chromatography of their acetyl derivatives **11**, **15** and **17**, respectively.

The first formed metabolite **7**, isolated as methyl ester **9**, had a molecular formula $C_{21}H_{29}ClO_3$ from accurate mass determination, confirming the introduction of one O-atom into the diterpene acid skeleton. An IR. spectrum of **9** confirmed a hydroxyl function with a broad absorption at 3300 cm^{-1}.

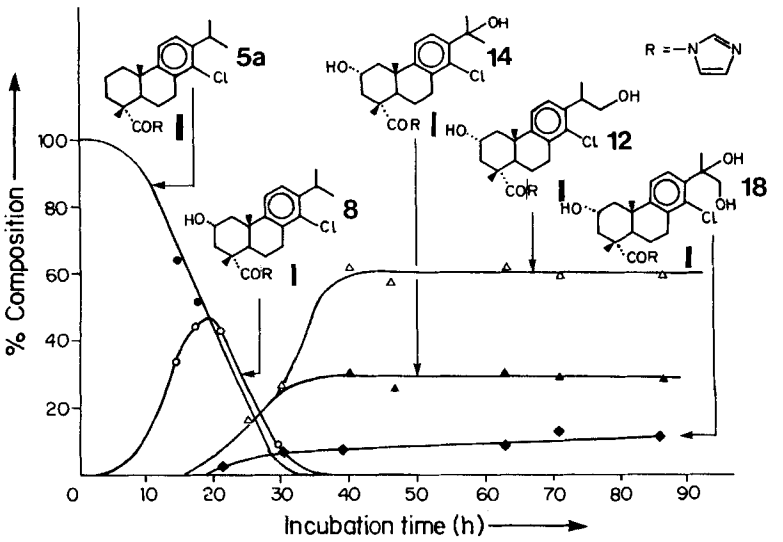
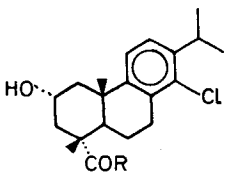
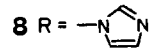


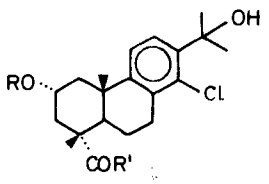
Fig. 3. *Composition of the biodegradation mixture from* **5** versus *incubation time*



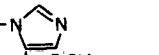
7 R = OH

8 R = 

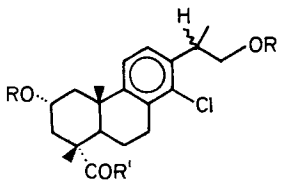
9 R = OCH_3



13 R = H, R' = OH

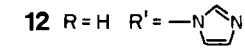
14 R = H R' = 

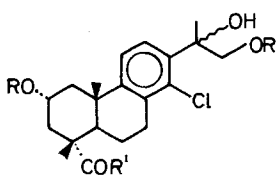
15 R = $COCH_3$, R' = OCH_3



10 R = H, R' = OH

11 R = $COCH_3$ R' = OCH_3

12 R = H R' = 



16 R = R' = OH

17 R = $COCH_3$ R' = OCH_3

18 R = H R' = 

As with previous diterpene acids, a nine line ($t \times t$) resonance was evident at 4.1 ppm in the ^{1}H-NMR. spectrum of **9**. The coupling constants of 4.0 and 11.5 Hz were consistent with axial/equatorial and diaxial coupling of H_β–C(2) with neighbouring protons at C(1) and C(3). The methyl ester resonance appeared at 3.71 ppm, and the H–C(15) septuplet resonated at 3.44 ppm. Other protons in rings A and B remain unassigned.

The second major metabolite **10**, isolated as the diacetyl ester **11**, was subjected to mass spectrometric determination, and the latter revealed that two O-atoms had been introduced into **5**. Separation of the two diastereomers of **11** or **10** was not evident in TLC. or HPLC. examinations. The mixture **11** could not be induced to crystallize, but the ^{1}H-NMR. spectrum showed an isomeric ratio of approximately 2 : 1.

The ^{1}H-NMR. spectrum of **11** revealed that one of the aromatic protons (presumably H–C(12)) was now shielded by 0.08 ppm due to proximity to an acetoxy group at C(16). A resonance at 5.22 ppm ($t \times t$, J = 4.3 and 11.5 Hz) was clearly due to H_β–C(2) deshielded by acetylation at C(2). The complex multiplet at 4.17 ppm was assigned to H_2C(16), with the complexity resulting from the adjacent asymmetric centre at C(15) in both diastereomers. Two ester methyl resonances were evident at 3.70 and 3.71 ppm, with H–C(15) being obscured at 3.68 ppm. Two acetoxy resonances were distinguishable at 2.07 and 2.03 ppm, the former assigned to AcO–C(2) and the latter to AcO–C(16). Irradiation of H–C(15) at 3.68 ppm caused a collapse of two doublets at 1.26 and 1.28 ppm to two singlets which were attributed to H_3C(17) of both isomers. No hydroxy absorptions were evident in the IR. spectrum. The mass spectrum showed loss of acetic acid from the molecular ion giving the base peak at m/z 404 presumably due to a styrene-like system, followed by loss of a methyl radical from C(4).

Chromatography of the mixture of acetyl esters provided the monoacetylhydroxy ester **15**, derived from **13**, as a crystalline product. An accurate mass deter-

mination of **15** and a molecular ion at *m/z* 422 for $C_{23}H_{31}ClO_5$ confirmed the introduction of two O-atoms into **5**.

Furthermore, fragmentation of **15** showed the loss of methyl, water and acetic acid from the molecular ion with the base peak at *m/z* 347 ($M^+ - CH_3 - HOAc$). The ^{1}H-NMR. spectrum revealed a down field shift of 0.3 ppm for one of the aromatic protons, presumably H–C(11), due to the neighbouring OH at C(15). A resonance at 5.22 ppm ($t \times t$, $J = 4.0$ and 11.5 Hz) was assigned to H_β–C(2), while the OH group at C(15) resulted in the disappearance of H–C(15) and in two deshielded singlets at 1.73 and 1.72 ppm arising from 2 H_3C–C(15). The sole acetoxy resonance from AcO_α–C(2) appeared as a sharp singlet at 2.07 ppm was used to assign the acetoxy resonances in **11**.

Finally, the most polar metabolite **16** was isolated in low yield as a mixture of both epimers in the form of its diacetyl-hydroxy ester **17**. The epimers **17** showed no separation by TLC. nor could they be induced to crystallize. An accurate mass determination confirmed that three O-atoms were introduced into **5**.

The weak molecular ion of **17** fragmented with loss of methyl and also a loss of CH_2OAc both from the C(15) benzylic position. The IR. spectrum showed a hydroxy absorption at 3450 cm^{-1} due to the tertiary OH at C(15), as well as ester absorptions at 1730 and 1720 cm^{-1}. The ^{1}H-NMR. spectrum exhibited deshielding of both aromatic protons, one resonating at 7.58 (H–C(11)) and the other at 7.22 ppm due to the proximity of the hydroxy and acetoxy groups at C(15) and C(16), respectively. A resonance at 5.24 ppm ($t \times t$, $J = 4.0$ and 11.5 Hz) was clearly due to H_β–C(2), while the protons at C(16) resonated at 4.73 and 4.53 ppm (2 *d*, $J = 11.5$ Hz). Confirmation of the presence of both epimers was indicated by two methyl ester signals at 3.74 and 3.73 ppm and two AcO–C(16) signals at 2.05 and 2.04 ppm. Again the two epimers showed one acetoxy resonance at 2.09 ppm for AcO_α–C(2), and the two pairs of singlets at 1.73 and 1.72 ppm and 1.31 and 1.30 ppm were ascribed to methyl groups at C(15) and C(10) of both epimers.

Thus, enzymatic hydroxylation of the chlorinated diterpene acid **5** again follows the established course with *M. isabellina*, albeit more slowly. Initial hydroxylation occurs at C(2α) giving metabolite **7** which then undergoes concomitant hydroxylation at C(15) and/or C(16) leading finally to metabolites **10**, **13** and **16**. These secondary metabolites appear to form at approximately similar rates and are not metabolized further.

Preliminary studies with *Daphina pulex* indicates that all the metabolites show greatly reduced toxicity (90 h $LC_{50} > 20$ mg/l). These results, together with toxicities to fish fry, will be published in a separate account. Work is continuing with 12,14-dichlorodehydroabietic acid (**6**) and 12-chlorodehydroabietic acid (**4**) to complete this study.

Experimental Part

Biotransformation of 14-chlorodehydroabietic acid (5) by M. isabellina. The stock culture of *M. isabellina* (University of British Columbia culture collection in Botany # 129) was maintained on potato dextrose agar *(Difco)* slants. Inocula for biotransformation studies were grown on potato dextrose agar (100 ml) supplemented with approximately equal amounts of dehydroabietic acid (**1**), isopimaric acid (**2**), abietic acid (**3**) and 12,14-dichloroabictic acid at a total resin acid level of 20 mg/l. The cultures were grown in *Roux* bottles at room temp. for 7 to 36 days. Inocula were prepared by washing the surface of the mature, confluent cultures with a solution of Aerosol OT (*Fisher Scientific;* 450 µl/l of distilled water). The resulting spore suspensions were used to inoculate 12 l of sterile dextrose yeast extract broth (5.0 g of D-glucose, 0.5 g of yeast extract *(Difco)*, 1.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2HPO_4$, 1.0 g of NaCl and 0.5 g of $MgSO_4 \cdot 7H_2O$ per l of tap water) contained in a 15-l-fermenter jar (*Labroferm®, New Brunswick Scientific*). Initial spore concentrations as determined using a *Howard* mould counting chamber ranged from 7×10^4 to 1×10^6 spores/ml. At the time of inoculation a steam sterilized solution of **5** was added to give a concentration of 20 mg/l. This solution was prepared by dissolving **5** in ethanol

(2 ml/100 mg of **5**) and converting it to the sodium salt by addition of 1.05 mol-equiv. of 0.1M NaOH, and diluting with water to 50 ml. Since some acid came out of solution at the acidic pH's encountered during fungal growth and deposited on the glass-fermenter surface above the culture, periodic shaking of the fermenter was required to resuspend the acid. Siliconizing the glass did not appreciably reduce acid adhesion.

Incubation conditions in the fermenter were $35 \pm 0.5°$, 350 rpm agitation and 250 ml/l/min aeration. Growth was monitored by measuring the dry biomass yield and pH of samples taken at irregular intervals. Dry weights were determined by membrane filtration (0.45 µm cellulose acetate filter, *Millipore Corp.*), freeze drying and weighing of time-course samples.

General Remarks. Dried sample extracts were transformed to imidazole derivatives in vials (4 ml) using CH_3CN/THF (HPLC. grade, dry, 3 ml) and excess 1,1'-carbonyldiimidazole (*Aldrich*, 20 mg per sample). Reaction was allowed to proceed for 1 h before analysis with occasional shaking.

Acetylations were done using an excess of acetic anhydride in pyridine overnight, with addition of methanol followed by removal of reagents under vacuum.

High performance liquid chromatography (HPLC.) was done using *Waters* instruments and included a system controller, data module, *Wisp 710B*, model *440 UV* detector and radial compression module using a reverse phase (C_{18}) analytical column. Solvents used for analysis were methanol/water.

IR. spectra (cm^{-1}) were recorded using *Perkin-Elmer 457* or *710* spectrometers. ^{1}H-NMR. spectra (ppm) were measured on a *Bruker WP-80* and *WH-400* spectrometer with $CDCl_3$ solutions using tetramethylsilane (TMS) as internal standard (= 0 ppm); coupling constants J in Hz. Low resolution MS. (*m/z*) were recorded on either an *Atlas CH-4B* or *AEI-MS 902* spectrometer and high resolution mass measurements on an *AEI-MS 50* spectrometer. Microanalyses were done by Mr. *P. Borda*, Microanalytical Laboratory, University of British Columbia.

Methyl 14-chloro-2α-hydroxydehydroabietate (**9**) was isolated after diazomethane methylation and chromatography on silica gel (ether/petroleum ether 1:9) as colorless needles, m.p. 150.5–151.5° (ether/hexanes). – IR. (nujol): 3300 br., 1715, 1235, 1130, 1040, 840. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.23 (*d*, J = 8.3, 1 H, H–C(12)); 7.14 (*d*, J = 8.3, 1 H, H–C(11)); 4.10 ($t \times t$, J = 4.0 and 11.5, 1 H, H_β–C(2)); 3.71 (*s*, 3 H, CO_2CH_3); 3.44 (*sept.*, J = 7, 1 H, H–C(15)); 3.0 ($d \times d$, J = 6.5 and 18.0, 1 H); 2.81 ($d \times d \times d$, J = 8, 11 and 18, 1 H); 2.66 ($d \times d \times d$, J = 2, 3.5 and 12, 1 H); 2.19 ($d \times d$, J = 2.5 and 12.5, 1 H); 1.32 (*s*, 3 H, H_3C–C(4)); 1.24 (*s*, 3 H, H_3C–C(10)); 1.23 (*d*, J = 7, 3 H, H_3C–C(15)); 1.21 (*d*, J = 7, 3 H, H_3C–C(15)). – MS.: 364 (M^+), 363, 271 (100), 229, 121. High resolution molecular weight determination: Calc. (^{35}Cl) 364.1805, found 364.1792.

$C_{21}H_{29}ClO_3$ Calc. C 69.12 H 8.01 Cl 9.72% Found C 69.34 H 8.13 Cl 9.96%

Methyl 2α,16-diacetoxy-14-chlorodehydroabietate (**11**) was isolated after methylation with diazomethane, acetylation with acetic anhydride/pyridine and chromatography on silica gel (ether/hexanes 1:4) as a viscous oil consisting of the two diastereomers. – IR. (film): 1730, 1230, 1145, 1040, 910, 840, 770. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.16 (*d*, J = 8.3, 1 H, H–C(12)); 7.08 (*d*, J = 8.3, 1 H, H–C(11)); 5.22 ($t \times t$, J = 4.3 and 11.5, 1 H, H_β–C(2)); 4.17 (*m*, 2 H, H_2C(16)); 3.71, 3.70 (3 H, CO_2CH_3); 3.68 (*m*(obscured), 1 H, H–C(15)); 3.02 ($d \times d$, J = 6.5 and 18.3, 1 H); 2.81 ($d \times d \times d$, J = 8.5, 11 and 18.5, 1 H); 2.64 ($d \times d \times d$, J = 1.5, 2.3 and 12, 1 H); 2.20 (*m*, 1 H); 2.07 (*s*, 3 H, AcO_α–C(2α)); 2.03 (*s*, 3 H, AcO–C(16)); 1.35 (*s*, 3 H, H_3C–C(4)); 1.29 (*s*, 3 H, H_3C–C(10)); 1.26, 1.28 (2 *d*, J = 7, 3 H, H_3C–C(15)). – MS.: 464 (M^+), 404 (100), 329, 269. High resolution molecular weight determination: Calc. (^{35}Cl) 464.1966; found 464.1979.

$C_{25}H_{33}ClO_6$ Calc. C 64.58 H 7.15 Cl 7.62% Found C 64.80 H 7.38 Cl 7.44%

Methyl 2α-acetoxy-14-chloro-15-hydroxydehydroabietate (**15**) was isolated, after methylation and acetylation, by chromatography on silica gel (ether/hexanes 1:4). – IR. (nujol): 3500 br., 1720, 1240, 1130, 850, 750. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.46 (*d*, J = 8.5, 1 H, H–C(12)); 7.16 (*d*, J = 8.5, 1 H, H–C(11)); 5.22 ($t \times t$, J = 4.0 and 11.5, 1 H, H_β–C(2)); 3.71 (*s*, 3 H, CO_2CH_3); 3.02 ($d \times d$, J = 6.5 and 18.5, 1 H); 2.81 ($d \times d \times d$, J = 8.5, 11.5 and 18.5, 1 H); 2.64 ($d \times d \times d$, J = 2.0, 4.0 and 12.3, 1 H); 2.18 ($d \times d$, J = 2.5 and 12.5, 1 H); 2.07 (*s*, 3 H, AcO); 2.01 ($d \times d \times d$, J = 2.5, 4.5 and 12.5, 1 H); 1.73, 1.72 (2 *s*, 3 H, 2 H_3C–C(15)); 1.37 (*s*, 3 H, H_3C–C(4)); 1.30 (*s*, 3 H, H_3C–C(10)). – MS.: 422 (M^+), 407, 347 (100), 287. High resolution molecular weight determination: Calc. (^{35}Cl) 422.1859, found 422.1856.

$C_{23}H_{31}ClO_5$ Calc. C 65.32 H 7.39 Cl 8.38% Found C 65.29 H 7.49 Cl 8.21%

Methyl 2a,16-diacetoxy-14-chloro-15-hydroxydehydroabietate (**17**) was isolated as the minor polar derivative after methylation and acetylation followed by chromatography on silica gel (ether/hexanes 3:7): colorless viscous oil of two diastereomers. – IR. (film): 3450 br., 1730, 1720, 1240, 1050, 840, 740. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.58 (*d*, J = 8.8, 1 H, H–C(12)); 7.22 (*d*, J = 8.8, 1 H, H–C(11)); 5.24 ($t \times t$, J = 4.0 and 11.5, 1 H, H_β–C(2)); 4.73 (*d*, J = 11.5, 1 H, H–C(16)); 4.55, 4.53 (2*d*, J = 11.5, 1 H, H–C(16)); 3.74, 3.73 (2*s*, 3 H, CO_2CH_3); 3.03 ($d \times t$, J = 5.3 and 17.8, 1 H); 2.81 (*m*, 1 H); 2.66 ($d \times t$, J = 2.0 and 11.8, 1 H); 2.19 (*m*, 1 H); 2.09 (*s*, 3 H, AcO_α–C(2)); 2.05, 2.04 (2*s*, 3 H, AcO–C(16)); 1.73, 1.72 (2*s*, 3 H, H_3C–C(15)); 1.37 (*s*, 3 H, H_3C–C(4)); 1.31, 1.30 (2*s*, 3 H, H_3C–C(10)). – MS.: 480 (M^+), 465, 407, 347, 287 (100), 207, 162. High resolution molecular weight determination: $C_{25}H_{33}ClO_7$ Calc. (^{35}Cl) 480.1915, found 480.1915; $C_{24}H_{30}ClO_7$ ($M^+ - CH_3$) Calc. 465.1680, found 465.1663; $C_{22}H_{28}ClO_5$ ($M^+ - CH_2OAc$) Calc. 407.1647, found 407.1636.

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