93. Studies Related to Biological Detoxification of Kraft Pulp Mill Effluent. VI[1][2]. The Biodegradation of 12,14-Dichlorodehydroabietic Acid[3] with *Mortierella isabellina*

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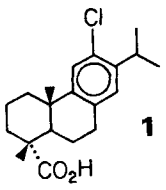
Summary

A detailed study of the biodegradation of one of the fish toxic chlorinated resin acids, 12,14-dichlorodehydroabietic acid[3] (**3**) is presented. When exposed to the fungus *Mortierella isabellina*, **3** is converted into a number of hydroxylated and keto derivatives which show low levels of toxicity to fish. These metabolites were isolated and characterized.

We have previously [1–2] commented on the fish toxic nature of the chlorinated derivatives of dehydroabietic acid **1**, **2**, **3**, and synthesized these compounds unambiguously. We have demonstrated [2] that the fungus *Mortierella isabellina* converts 14-chlorodehydroabietic acid (**2**) into a series of hydroxylated derivatives with low levels of toxicity to fish. This publication describes our studies with this organism and 12,14-dichlorodehydroabietic acid (**3**).



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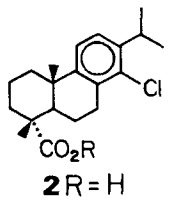

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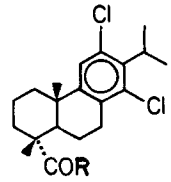

3 R = OH

4 R = 

[1]) Part V: [1].

[2]) Key Words: Diterpenes, dehydroabietic acid, chlorinated derivatives, 12,14-dichlorodehydroabietic acid, fish toxic diterpenes.

[3]) The correct IUPAC-name is 12,14-dichloro-8,9,11,12-tetradehydro-7,8-dihydroabietic acid.

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Methods for the quantitative analysis of metabolites. – A number of samples of different incubation times were taken from the fermentor, acidified (pH 2) and each extracted with ethyl acetate. After concentrating the organic extracts and drying *in vacuo*, the residue was treated with 1,1'-carbonyldiimidazole in acetonitrile tetrahydrofuran to form imidazole derivatives of the acids (see **4**). These latter compounds were analyzed by HPLC. and a UV. (254 nm) detector. A typical HPLC. trace of the biodegradation mixture and the standard curve for 1-(12,14-dichlorodehydroabietoyl)imidazole (**4**) are shown in *Figures 1* and *2* respectively.

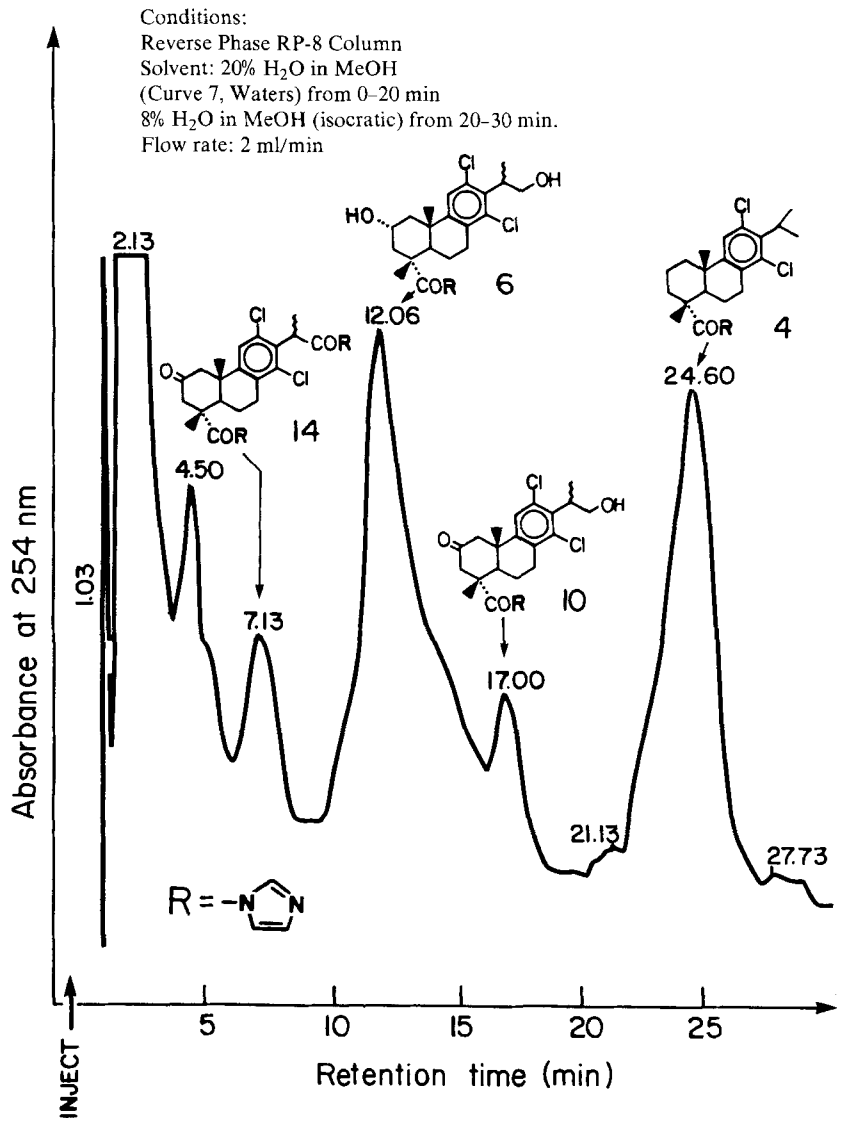


Fig. 1. *HPLC. trace of the biodegradation mixture of 12,14-dichloro-dehydroabietic acid by* Mortierella isabellina

Biodegradation of *3* by *M. isabellina*. – Growth of *M. isabellina* in fermentor cultures proceeded as described previously [2]. Culture growth to maximal dry biomass levels of 2.8 mg/l within 25 h caused a decrease in pH from 6.9 to as low as 2.8. Extended incubation beyond the growth phase gave decreased biomass yields and increased pH. Ten separate fermentors were monitored. The rate and the time of onset of the growth phase varied with the initial spore concentration, higher levels giving earlier and more rapid growth. The nature of the metabolites was not altered by this variance.

The time course of the disappearance of **3** and the appearance of the major metabolites detected as their imidazolides **6**, **10**, and **14** is shown in *Figure 3*. Persistence of **3** up to 62 h of incubation indicates that this is the most recalcitrant of the five compounds within the resin acid family which have been fed to *M. isabellina*. Nearly total removal of unchlorinated resin acids was achieved by 24 h [3] [4], while **2** was no longer detectable by 37 h [2]. It therefore seems that chlorination not only increases toxicity of resin acids, but may also increase their resistance to biodegradation.

Chlorination also affects the nature of the metabolites formed by *M. isabellina*. Previously, a mono-hydroxylated metabolite substituted at C(2) was a major metabolite formed during the growth phase of cultures fed with the fish toxic diterpenes [2–5]. Its appearance was transitory as it was further hydroxylated upon extended incubation. Such an intermediate may have occurred during biodegradation of **3** since a small transitory peak at 16 to 20 h of incubation was detected by HPLC. analysis of culture samples. However, this minor metabolite proved difficult to isolate for full characterization. Further departures from previously observed routes of biodegradation are exemplified by the production of the ketonic metabolites which were isolated as the methyl esters **11** and **15**. Oxidation beyond the alcohol level had not been previously observed for resin acid biodegradation by *M. isa-*

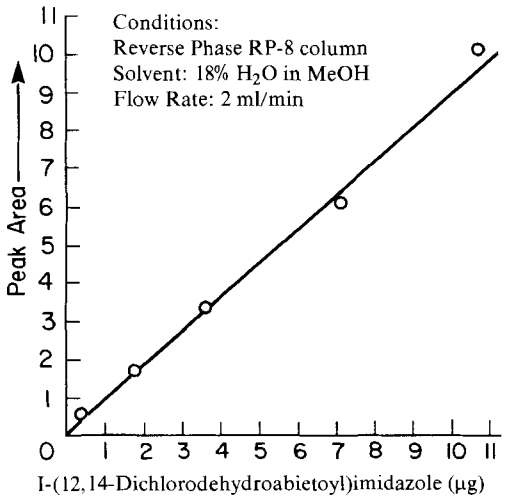


Fig. 2. *HPLC. peak areas of 1-(12,14-dichlorodehydroabietoyl)imidazole as measure of its quality*

bellina. Whether these compounds arise directly from **3** or arise from the preformed dihydroxy acid is not known.

Isolation and characterisation of metabolites. – For the isolation, see *Exper. Part.*

The methyl ester **7** of the major metabolite **5** had a molecular formula $C_{21}H_{28}Cl_2O_4$ indicating the introduction of two additional O-atoms into **3**. The IR. spectrum indicated the presence of hydroxyl groups (absorption band 3400–3700 cm^{-1}) while the ^{1}H-NMR. spectrum revealed a nine line resonance at 4.1 ppm for a single proton geminal to a hydroxyl group. The splitting pattern ($t \times t$, J(aa) = 12, J(ae) = 4 Hz) was consistent with an axial proton coupled with two adjacent methylene groups. Thus one of the O-atoms was present at C(2α) in the form of hydroxyl, confirmed by a downfield shift of 1.15 ppm for H_β–C(2) in the corresponding acetate **8**. This assignment derived further support from the fact that when **5** was refluxed with a catalytic amount of p-toluenesulfonic acid in acetone; the lactone **17** with the expected spectral properties was obtained.

The ^{1}H-NMR. spectrum of **7** further revealed the absence of one of the methyl signals of the isopropyl group. This was suggestive of the position of the second hydroxylation and was supported by the appearance of a two-proton double doublet (J = 7 Hz) centered at 3.9 ppm. The presence of the hydroxyl group at C(16) was confirmed by the downfield shift (0.6 ppm) of the C(16) methylene-protons signal upon acetylation of **7** to **8**.

Another minor metabolic product showed the molecular formula $C_{21}H_{26}Cl_2O_4$ (MS.). This implied the introduction of two O-atoms into the starting substrate with loss of two H-atoms. The ^{1}H-NMR. spectrum revealed the loss of one methyl signal of the isopropyl unit and exhibited a new two-proton double doublet (J = 7 Hz)

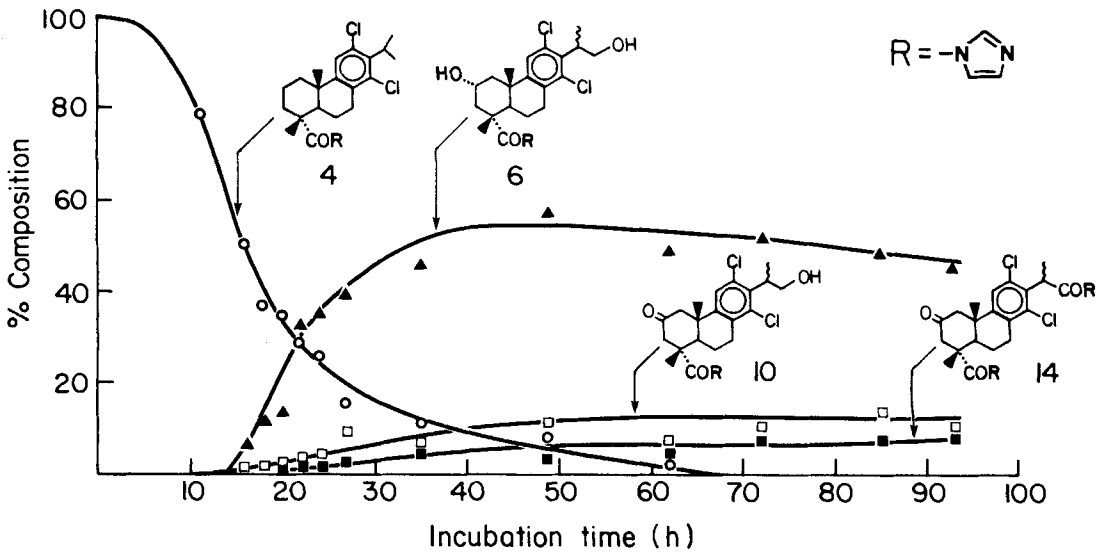
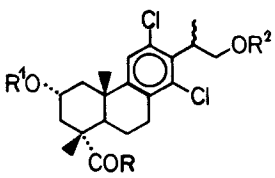


Fig. 3. *Time course of disappearance of 12,14-dichlorodehydroabietic acid and of appearance of the major metabolites*

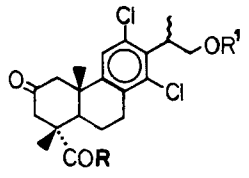
centered at 3.9 ppm, indicative of hydroxylation at C(16). This suggestion was confirmed on acetylation of this metabolite with the corresponding downfield shift of the C(16) methylene resonance observed in the ^{1}H-NMR. spectrum. The second additional O-atom could arise either from the hydroxylation of a primary or secondary C-atom and subsequent oxidation leading to an aldehyde or a ketone. The absence of an aldehydic proton signal in the ^{1}H-NMR. however, excluded the possibility of a primary C-atom oxidation. This was confirmed by the IR. spectrum which showed a strong saturated ketone absorption band (1708 cm^{-1}) in addition to hydroxyl (3400–3600) and saturated ester (1720) absorptions.

To ascertain the position of the ketone function, the dihydroxy ester **7** was oxidized with $NaBrO_3$ in the presence of cerium ammonium nitrate according to the procedure of *Oshima et al.* [6]. This gave a product in which the secondary C(2α)-hydroxyl group was oxidized in preference to the C(16) primary hydroxyl group and which proved to be identical to the metabolic product under discussion. The structure **9** for this compound was now established.

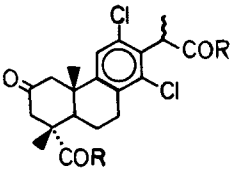
The third metabolic product, also isolated as a minor component after diazomethane treatment of the original mixture and chromatographic separation, possessed the molecular formula $C_{22}H_{26}Cl_2O_5$ (MS.). The absence of hydroxyl group absorption and the presence of strong bands at 1708 and 1720 cm^{-1} in the IR. spectrum suggested the presence of saturated ketone and ester groups. The ^{1}H-NMR. spectrum allowed the tentative assignment of structure **15** to this product. Thus the absence of one of the methyl groups of the normal isopropyl side-chain coupled with two three-proton singlets at 3.67 (C(4) ester methyl) and 3.72 ppm (new ester methyl) suggested that oxidation to the carboxyl level of the missing methyl group had occurred in the fermentation process.



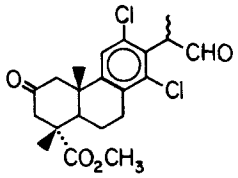
5 R=OH, R^1=R^2=H
6 R=  , R^1=R^2=H
7 R=OCH_3, R^1=R^2=H
8 R=OCH_3, R^1=R^2=Ac



9 R=OH, R^1=H
10 R=, R^1=H
11 R=OCH_3, R^1=H
12 R=OCH_3, R^1=Ac



13 R=OH
14 R=
15 R=OCH_3



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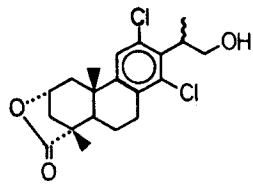

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Confirmatory evidence for **15** was provided by comparison of spectral data with that of a synthetic sample obtained by a two-step oxidation process of the dihydroxyester **7**. Firstly, pyridinium chlorochromate oxidation of **7** afforded the aldehyde **16** which was then treated with alkaline silver oxide followed by methylation with diazomethane. The resultant keto diester **15** was identical to the product obtained from biotransformation, thereby establishing the structure of the original metabolic product as **13**.

Preliminary studies with *Daphnia pulex* indicate that the above-mentioned metabolites show considerably reduced toxicity. More detailed investigations, together with toxicities to fish fry, will be published in a separate account.

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Experimental Part

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

General remarks. Dried sample extracts were transformed to imidazolide derivatives by dissolving them in anhydrous THF (1.0 ml), adding a solution of 1,1'-carbonyldiimidazole (1.0 ml) from a stock solution (*Aldrich*, 20 mg/ml CH_3CN) and allowing the reaction to proceed at r.t. for 1 h. The mixture of the resultant imidazolides was analyzed by HPLC. 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 *RP 8* 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 *Varian XL-100* and a *Bruker WP-80, 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.

Isolation and characterisation of metabolites. A number of large scale fermentations containing 20 mg/l of 12,14-dichlorodehydroabietic acid (**3**) were required to get sufficient amounts of metabolites for structure elucidation and toxicity measurements.

In a typical large scale experiment, a mixture of sterilized dextrose yeast extract broth (11 l) and glucose solution (500 ml) placed in a 14 l fermentor jar was inoculated with the spores of *M. isabellina* followed by the addition of the solution of the sodium salt of **3** (400 ml) prepared from 235 mg of acid. The fermentor was allowed to run for a period of 93 h. The culture was harvested, and filtered through *Celite*. The resulting filtrate was acidified (pH ≈ 2) with conc. HCl-solution, saturated with NaCl and extracted with AcOEt (2 × 1 l). The combined extract was washed with water to neutrality, dried ($MgSO_4$) and concentrated to give the crude extract (520 mg). A rapid short path column chromatography (silica gel, MeOH/$CHCl_3$ 5:95) gave a mixture of metabolites (200 mg) and unreacted **3** (20 mg). The metabolite mixture was then treated with diazomethane and chromatographed again on silica gel preparative plates using ether/petroleum ether 1:1 to give several products as noted below.

Methyl 12,14-dichloro-2α,16-dihydroxyabieta-8,11,13-trien-18-oate (**7**). This compound representing a mixture of diastereoisomers was obtained as a white solid by crystallization (ether/petroleum ether, 140 mg, 56%), m.p. 72–78°. – IR. ($CHCl_3$): 3700–3400, 1720, 840. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.18 (*s*, 1 H, arom.); 4.18 (*m*, 1 H, H–C(15)); 4.1 (*t* × *t*, J = 4 and 12, 1 H, H_β–C(2)); 3.98 and 3.9 (2*d*, J = 7, 2 H, H_2C–C(16)); 3.7 (*s*, 3 H, CO_2CH_3); 1.4 (br. *d*, J = 7, 3 H, H_3C–C(15)); 1.3 (*s*, 3 H, H_3C–C(4)); 1.25 (*s*, 3 H, H_3C–C(10)). – MS.: 414 (M^+), 383, 366, 339, 321, 305, 269, 257, 239. – High resolution molecular weight determination: Calc. (^{35}Cl) 414.1364 Found 414.1364.

$C_{21}H_{28}Cl_2O_4$ Calc. C 60.84 H 6.81% Found C 60.38 H 6.62%

Preparation of methyl 2α,16-diacetoxy-12,14-dichloroabieta-8,11,13-trien-18-oate (**8**). A mixture of the dihydroxyesters **7** (35 mg), pyridine (2 ml) and acetic anhydride (1.0 ml) was stirred over night at r.t. The reaction mixture was diluted with methanol (10 ml) and concentrated under reduced pressure. The residue was diluted with toluene and concentrated again. The process was repeated several times to remove pyridine and the thick oil thus obtained after preparative chromatography on silica gel gave **8** (31 mg, 73.6%) as a white solid, m.p. 42–46° (ether/petroleum ether). – IR. ($CHCl_3$): 1725, 840, 770. – ^{1}H-NMR. ($CDCl_3$, 100 MHz): 7.22 (br. *s*, 1 H, arom.); 5.25 (*t*×*t*, *J* = 12 and 4, 1 H, H_β–C(2)); 4.58, 4.54 (2*d*, *J* = 7, 2 H, H_2C–C(16)); 4.14 (*m*, 1 H, H–C(15)); 3.72 (*s*, 3 H, CO_2CH_3); 2.06 (*s*, 3 H, AcO–C(2α)); 2.02 (*s*, 3 H, AcO–C(16)); 1.42 (br. *d*, *J* = 7, 3 H, H_3C–C(15)); 1.38 (*s*, 3 H, H_3C–C(4)); 1.28 (*s*, 3 H, H_3C–C(10)). – MS.: 498 (M^+), 483, 438, 425, 362, 239. – High resolution molecular weight determination: Calc. (^{35}Cl) 498.1575 Found 498.1590.

$C_{25}H_{32}Cl_2O_6$ Calc. C 60.22 H 6.45% Found C 60.45 H 6.50%

Methyl 12,14-dichloro,16-hydroxy,2-oxoabieta-8,11,13-trien-18-oate (**11**). This substance representing a mixture of C(15) epimers was isolated in approximately 5% yield as a viscous oil. – IR. (film): 3700–3400, 1720, 1708, 850. – ^{1}H-NMR. ($CDCl_3$, 100 MHz): 7.1 (*s*, 1 H, arom.); 4.1 (*m*, 3 H, H–C(15) and H_2C–C(16)); 3.76 (*s*, 3 H, CO_2CH_3); 1.35 (br. *d*, *J* = 7, 3 H, H_3C–C(15)); 1.28 (*s*, 3 H, H_3C–C(4)); 1.24 (*s*, 3 H, H_3C–C(10)). – MS.: 412 (M^+). – High resolution molecular weight determination: Calc. (^{35}Cl) 412.1207 Found 412.1210

$C_{21}H_{26}Cl_2O_4$ Calc. C 61.14 H 6.34% Found C 60.54 H 6.21%

Preparation of methyl 16-acetoxy,12,14-dichloro,2-oxoabieta-8,11,13-trien-18-oate (**12**). The title compound (6.2 mg, 78%) was obtained as a viscous liquid by the acetylation of **11** following the procedure described for **7**. – IR. (film): 1725, 1708, 850. – ^{1}H-NMR. ($CDCl_3$, 100 MHz): 7.12 (br. *s*, 1 H, arom.); 4.56, 4.52 (2*d*, *J* = 7, 2 H, H_2C–C(16)); 4.1 (*m*, 1 H, H–C(15)); 3.76 (*s*, 3 H, H_3COOC–C(4)); 2.02 (*s*, 3 H, AcO–C(16)); 1.4 (br. *d*, *J* = 7, 3 H, H_3C–C(15)); 1.3 (*s*, 3 H, H_3C–C(4)); 1.26 (*s*, 3 H, H_3C–C(10)). – MS.: 454 (M^+), 439 ($[M-CH_3]^+$), 394, 381. – High resolution molecular weight determination: Calc. (^{35}Cl) 454.1313 Found 454.1315.

Methyl 12,14-dichloro-2-oxoabieta-8,11,13-trien-16,18-dioate (**15**). This compound was isolated in approximately 4% yield as a white solid, m.p. 134–139° (ether/petroleum ether). – IR. ($CHCl_3$): 1720, 1708, 840. – ^{1}H-NMR. ($CDCl_3$, 400 MHz): 7.05 (*s*, 1 H, H–C(11)); 4.45 (*qa*, *J* = 7, 1 H, H–C(15)); 3.72 (*s*, 3 H, H_3COOC–C(15)); 3.67 (*s*, 3 H, H_3COOC–C(4)); 1.47 (br. *d*, *J* = 7, 3 H, H_3C–C(15)); 1.28 (*s*, 3 H, H_3C–C(4)); 1.25 (*s*, 3 H, H_3C–C(10)). – MS.: 440 (M^+), 425 ($[M-CH_3]^+$), 405 (basis peak), 394, 381, 365. – High resolution molecular weight determination: Calc. (^{35}Cl) 440.1156 Found 440.1153.

$C_{22}H_{26}Cl_2O_5$ Calc. C 59.98 H 5.94% Found C 59.48 H 6.03%

Preparation of 12,14-dichloro-2α,16-dihydroxyabieta-8,11,13-trien-18-oic acid 2,4-lactone (**17**). To the hydroxy acid **5** (43.2 mg) dissolved in anhydrous acetone (25 ml) was added 20 mg of *p*-toluenesulfonic acid and the reaction mixture was refluxed for 30 min. The mixture was concentrated under vacuum, the residue was taken up in CH_2Cl_2 and the extract washed with water and dried ($MgSO_4$). Removal of solvent and chromatography on silica gel (ether/petroleum ether 1:1) furnished the white crystalline **17**, m.p. 72–76° (ether/petroleum ether) in 73% yield. – IR. ($CHCl_3$): 3600–3400, 1770, 840. – ^{1}H-NMR. ($CDCl_3$, 100 MHz): 7.06 (*s*, 1 H, H–C(11)); 4.9 (*m*, W/2 = 12, 1 H, H_β–C(2)); 4.05 (*m*, 3 H, H–C(15) and H_2C–C(16)); 1.36 (br. *d*, *J* = 7, 3 H, H_3C–C(15)); 1.32 (*s*, 3 H, H_3C–C(4)); 1.28 (*s*, 3 H, H_3C–C(10)). – MS.: 382 (M^+), 351, 323, 317, 307, 239. – High resolution molecular weight determination: Calc. (^{35}Cl) 382.1102 Found 382.1102.

$C_{20}H_{24}Cl_2O_3$ Calc. C 62.80 H 6.31% Found C 62.71 H 6.50%

Oxidation of dihydroxy ester **7**. *Formation of 16-formyl-12,14-dichloro-3-oxoabieta-8,11,13-trien-18-oate* (**16**) *and of dicarboxylate* **15**. A solution of **7** (50.32 mg, 0.12 mmol) in CH_2Cl_2 (5 ml) was added to a suspension of pyridinium chlorochromate (75 mg, 0.3 mmol) in CH_2Cl_2 (5 ml) at r.t. and the mixture was stirred for 1 h. Dilution with anhydrous ether (25 ml), filtration and concentration of the filtrate *in vacuo* afforded the crude reaction product. Chromatography on silica gel (ether/petroleum ether 30–60°) gave the keto-aldehyde **16** as a white solid (41.24 mg, 83%) m.p. 107–112° (ether/petroleum

ether 30–60°). – IR. ($CHCl_3$): 1720, 1705, 840. – ^{1}H-NMR. ($CDCl_3$, 80 MHz): 9.8 (*s*, 1 H, CHO); 7.2 (*s*, 1 H, H–C(11)); 4.19 (*qa*, $J=7$, 1 H, H–C(15)); 3.76 (*s*, 3 H, H_3COOC–C(4)); 1.5 (*d*, $J=7$, 3 H, H_3C–C(15)); 1.3 (*s*, 3 H, H_3C–C(4)); 1.26 (*s*, 3 H, H_3C–C(10)). – MS.: 410 (M^+), 396, 381, 347, 321, 166. – High resolution molecular weight determination: Calc. (^{35}Cl) 410.1051 Found 410.1049.

$C_{21}H_{24}Cl_2O_4$ Calc. C 61.44 H 5.87% Found C 61.28 H 5.43%

The aldehyde **16** (25.63 mg) was oxidized with alkaline silver oxide (prepared by the addition of a solution of 30 mg of silver nitrate in 10 ml water to a solution of 15 mg NaOH in 10 ml water) following the procedure of *Campaigne et al.* [7]. The reaction mixture was acidified with HCl (6N) extracted with ether and the organic extract treated with diazomethane. Excess diazomethane was removed by addition of a few drops of acetic acid. After drying ($MgSO_4$), ether was removed to give the diester **15** (83%). This synthetic product was identical with the metabolite isolated and described above.

Oxidation of the dihydroxy ester **7** *and formation of the keto ester* **11**. A mixture of $NaBrO_3$ (7.5 mg, 0.05 mmol), diol **7** (20.12 mg, 0.05 mmol), and $(NH_4)_2Ce(NO_3)_6$ (3.82 mg, 0.005 mmol) was refluxed in CH_3CN/H_2O 7:3 (10 ml) for 1 h. The reaction mixture was cooled, diluted with ether (50 ml) and washed with sat. $NaHCO_3$- and sat. NaCl-solution. After drying of the ethereal extract ($MgSO_4$), and concentration *in vacuo* the resultant residue was purified by prep. TLC. to afford the hydroxy keton **11** (40%).

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