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Article

Hydroxylation of the Labdane Diterpene Cupressic Acid by *Fusarium graminearum*

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Culturas do fungo *Fusarium graminearum* sob agitação na presença do diterpeno ácido cupréssico [ácido 13-hidróxi-(17),14-labdadien-19-óico], obtido de *Araucarea angustifolia elegans*, produziram quatro derivados diterpênicos hidroxilados os quais foram identificados por métodos químicos e físicos como sendo os ácidos 3β,13-diidróxi-8(17),14-labdadien-19-óico, 7α,13-diidróxi-8(17),14-labdadien-19-óico, 8,13,17-triidróxi-14-labdaden-19-óico e 13,14,17-triidróxi-14-labdaden-19-óico.

Cupressic acid [13-hydroxy-8(17),14-labdadien-19-oic acid], a diterpene obtained from *Araucarea angustifolia elegans* was biotransformed by *Fusarium graminearum* producing four hydroxylated diterpene derivatives, which were identified by chemical and physical methods as 3β,13-dihydroxy-8(17),14-labdadien-19-oic acid, 7α,13-drihydroxy-8(17),14-labdadien-19-oic acid, 8,13,17-trihydroxy-14-labden-19-oic acid and 13,14,15-trihydroxy-8(17)-labden-19-oic acid.

Keywords*: Fusarium*, biotransformation, labdane diterpene, 8(17),14-labdadien-19-oic acid

Introduction

Biotransformation of natural products by fungi is an old tool, now becoming fashionable again to selectively introduce functional groups in different positions of carbon skeletons.[1-3] Diterpenes have received special attention as a substrate[4,5] for biotransformation, since some of these substances are endogenously produced by fungal species; gibberellic acid, for instance, is an endogenous metabolite of the *Fusarium* species in some sexual stage.[6-8] Besides gibberellic acid derivatives, labdane diterpenes have also been used as substrates for biotransformation by *Fusarium*[5,8,9] in an attempt to convert them into analogous forskolins, which are important bioactive compounds.[5,10,11] We recently discovered that the plant *Araucaria angustifolia elegans* produces high yields of cupressic acid and abietane diterpenes.[12] An antifungal bioassay to test these diterpenes against *Leucoagaricus gongylophorus*, a fungus living symbiotically with the leaf-cutting ant *Atta sexdens rubropilosa*, revealed them to be inactive compounds.[12] We therefore decided to study the ability of fungi from Fusaria group to metabolize these diterpene acids. This paper reports the results we obtained from a fermentation experiment using *Fusarium graminearum.*

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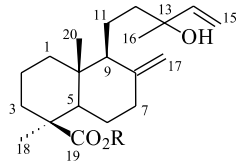
Results and Discussion

Isolation procedures, structural identification and antifungal bioassay of cupressic acid and other diterpenes will be published elsewhere. The biotransformation products **2-6** were esterified with diazomethane prior to isolation and structural identification. The ^{13}C NMR data (Table 1) obtained for the compounds **2a**-**5b**, compared with the extensive NMR study of the cupressic acid methyl ester[12] and other related labdane diterpenes,[13-16] were the most important elucidative elements, and were supported by other 2D spectra used for confirmation. The most important features observed in the NMR data for **1a**, which were used for purposes of comparison for the identification of the hydroxylated bioproducts, were the presence of the two double bonds in C-8(17) (δ 148.1 and 106.4) and C-14 (δ 145.1 and 111.5) and a shielded methylene carbon signal at δ 19.9, which were ascribed to C-2.

A broad band at 3398 cm^{-1}, originated from a hydroxyl group, was detected in the IR absortion spectrum of **2a**. In conjunction with this IR information, the peak at *m/z* 332 ($M^{+\bullet}$ - H_2O) indicated the molecular formula $C_{21}H_{34}O_4$. In



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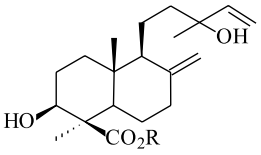
1a: R = CH_3



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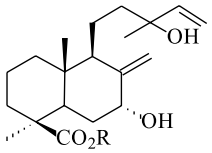
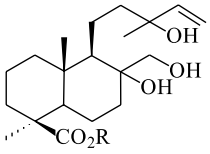
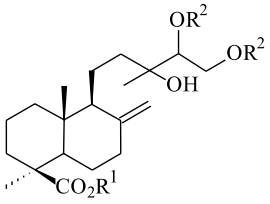


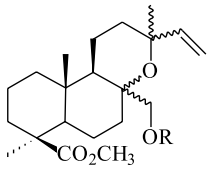
3: R = H
3a: R = CH_3



4: R = H
4a: R = CH_3



5: R^1 = H, R^2 = H
5a: R^1 = CH_3, R^2 = H
5b: R^1 = CH_3, R^2 = Ac



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the ^{1}H NMR spectrum, compound **2a** showed the presence of hydrogen from two double bonds, as in the precursor **1**, and a pair of doublets for a carbynolic hydrogen (δ 3.17). Only six methylene carbons were detected in the ^{13}C NMR spectrum (DEPT135° experiment) of **2a**. In addition, the shielded methylene signal at *c.a.* δ 20 was not present in its ^{13}C NMR spectrum. These NMR data indicate that hydroxylation occurred in ring A. The split pattern shown by the carbynolic hydrogen (dd) may be attributed to a hydrogen in C-1 or C-3. In comparison with the signals for C-4 and C-10 for compound **1a**, C-4 is 5.1 ppm deshielded and C-10 showed the same chemical shift, indicating the positioning of the hydroxyl group at C-3 in **2a**. The β orientation for this hydroxyl was deduced from the split observed for H-3 (dd, *J* 3 and 8 Hz), which was corroborated by TOCSY NMR experiments. Moreover, the expected γ-gauche shielding effect of an α-hydroxyl group at C-3 over C-5 and C-1,[13,14] which was detected almost in the same δ

Table 1. ^{13}C NMR data of compounds **1a** - **5b***

C	1a	2a	3a	4a	5a	5b
1	39.2	37.5	38.8	37.8	39.5	39.2
2	19.9	27.6	20.0	21.3	19.6	20.0
3	38.2	78.4	38.2	37.3	38.2	38.3
4	44.3	49.4	43.9	44.2	44.3	44.4
5	56.4	56.1	48.6	56.3	56.3	56.5
6	26.2	25.9	32.4	18.9	26.3	26.4
7	38.7	38.3	74.0	39.6	39.2	38.8
8	148.1	147.3	149.1	75.8	148.4	148.2
9	56.5	55.5	50.2	60.1	56.9	56.8
10	40.4	40.0	40.5	39.5	40.5	40.6
11	17.9	18.1	17.5	18.4	17.3	17.2
12	41.4	41.3	41.0	43.7	36.9	37.7
13	73.4	73.8	73.5	74.3	74.8	73.6
14	145.1	145.1	145.1	144.7	74.1	76.3
15	111.5	111.5	111.7	111.9	63.3	63.1
16	27.5	28.6	28.6	28.6	22.7	23.4
17	106.4	106.7	109.8	62.6	106.2	106.4
18	27.4	23.5	28.6	29.2	28.8	28.9
19	177.7	nd	177.6	177.5	177.8	177.7
20	12.5	12.3	11.6	13.0	12.6	12.6
OCH_3	51.1	51.1	51.2	51.2	51.2	51.3

(*): $CDCl_3$, 100 MHz. Multiplicities were obtained from DEPT-135. Assignments were done based on HMQC and HMBC NMR experiments

as the one in **1a**, was not observed. Thus, the new diterpene produced by fermentation is 3β-hydroxy-cupressic acid or 3β,13-dihydroxy-8(17),14-labdadien-19-oic acid (**2**), which appears to be a new substance.

Based on the IR (ν 3399 cm^{-1}) and MS (*m/z* 332, $M^{+\cdot}$ - H_2O) data, we concluded that compound **3a** is a diol isomer of **2a**. Furthermore, in comparison with **1a**, the chemical shifts of the carbons involved in ring A and the side chain at C-9 were almost the same. The ^{13}C and ^{1}H NMR spectra also showed that **2a** contained both double bonds present in precursor **1a**. The main differences observed were in the carbons in ring B. The positioning of the hydroxyl group at C-7 was based on the deshielding influence of the hydroxyl on the double bond carbons at C-8(17) ($\Delta\delta$ 1.0 and 3.4, respectively) compared to **1a**, and on the HMBC correlation of H-7 (δ 4.40) and C-17 (δ 109.8). This positioning, as well as the a configuration for the hydroxyl group, were confirmed by the γ-gauche shielding effect of 7.8 and 6.3 ppm over carbons C-5 and C-9, respectively.[14,16] Also, the NMR signal of the H-7 nuclei is a triplet due to an equatorial-equatorial and equatorial-axial coupling with 2H-6, and shows nOe with ^{1}H-17a. These data indicated a structure of 7-hydroxy-cupressic acid or 7α,13-dihydroxy-8(17),14-labdadien-19-oic acid (**3**) for this new diterpene produced in the biotransformation experiment.

The last two hydroxylated products were also a pair of isomers. The IR (ν 3425-3280 cm^{-1}) and ^{13}C NMR [d 75.8 (C), 60.1 (CH_2) and 74.3 (C) for **4a** and 74.8 (C), 74.1 (CH) and 63.3 (CH_2) for **5a**][17] data, together with the absence of signals in the ^{13}C spectra that could be ascribed to the double bonds at C-8(17) for **4a** and C-14 for **5a**, indicated that these two isomers were the triol derivatives of **1a**. The assignments of the ^{13}C chemical shifts shown in Table 1 were obtained from an analysis of HMQC and HMBC experiments of compounds **4a** and **5a** and from the acetylated derivative **5b**. The configurations of the stereogenic carbons in triols **4a** and **5a** are not easily established,[17] but our NMR data indicated that only one major triol isomer was produced in each case.

Triols **4a** and **5a** may have been produced *via* epoxidation of the double bonds, followed by hydrolization of the epoxide. The epoxide would have been a possible intermediate to the partial 8,13-oximonoil labdane structure present in the forskolin derivatives.[10] In our experiment, we obtained a fraction from the culture extract whose NMR and GCMS data indicated a mixture of the many isomers represented by structure **6**. We are now studying the most suitable conditions to optimize the production of these tetrahydropiran ethers.

The carboxyl group at C-19 in precursor **1** apparently was not responsible for the induction of hydroxylation at C-3, since sclareol ([8,13-dihydroxy-14-labdene]), a biosynthetic parent compound of **1**, produced hydroxylated compounds at C-3 under microbial fermentation using other filamentous fungi.[18,19]

Experimental

General experimental procedures. The ^{1}H and ^{13}C NMR experiments were recorded using a VARIAN GEMNI or a BRUKER DRX spectrometer, which were operated, respectively, at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C, using deutero chloroform ($CDCl_3$) as solvent, with TMS as the internal standard. MS data were collected using a Micromass Platform II instrument (Micromass, Wythenshawe, Manchester, UK) equipped with an EI/CI ion source and coupled to a Carlo Erba GC 8000 series. The column used was a DB-1 30 m x 0.5 μm x 0.25 μm from J&W.

Isolation of the starting material **1**. Cupressic acid (**1**) was isolated from the resin exudated from *Araucarea angustifolia*. The crystalline resin was extracted with chloroform and the diterpenes were isolated by extensive silica gel column chromatographic procedures (n-heptane:AcOEt + 0.5 % acetic acid in a gradient elution). Compound **1** was characterized by NMR spectral data.

Microorganism, media and culture conditions. *Fusarium graminearum* was isolated from the soil in St. Paul, Minnesota, USA in February of 1982 and stored in sterilized soil. Samples of this isolate were stored under 4 °C in Dr. Mirocha's collection (Mycotoxin Laboratory, University of Minnesota, Department of Plant Pathology, College of Agriculture, St. Paul Campus) under number 38. The fungus was seeded in a Petri dish containing PDA (potato-dextrose-agar) and allowed to grow for 4 days. Six 5-liter Erlenmeyer flasks, each containing 600 mL of liquid medium (80g glucose, 0.48g NH_4NO_3, 5.0g KH_2PO_4, 1.0g $MgSO_4$, 0.1g $FeSO_4$, 0.015g Cu SO_4, 0.161g $ZnSO_4$, 0.01g $MnSO_4$, and 0.1g $(NH_4)_2MoO_4$ dissolved in 1.5L of distilled water) were inoculated with pieces of the PDA culture containing micelium and were allowed to grow in a shaker (110 rpm) at 25 °C. Substrate **1** (600mg dissolved in acetone, 100mg per flask) was added after 6 days of growth. The growth was interrupted after an additional 14-day period.

Extraction, purification and analysis of microbial transformation products. The fermentation was harvested by filtering the mycelium and the culture filtrate was acidified to pH 3 and extracted with ethyl acetate. The solvent was evaporated under vacuum in a rotary distiller and the residue thus obtained was methylated with diazomethane. The resulting methyl esters were chromatographed on a silica gel column and eluted with gradient mixtures of n-hexane and ethyl acetate. Final purification

of products **2a** and **3a** was achieved on a silica gel column isocratically eluted with petrol ether and ethyl acetate [4:1] with 1% of acetic acid. Triols **4a** and **5a** were eluted from the column with petrol ether and ethyl acetate [2:1] with 1% of acetic acid. The methyl ester **5a** was acetylated with acetic anhydride in pyridine and the acetate derivative purified by silica gel CC. The following physical data were used to identify the hydroxylated diterpenes:

2a:White oil; IR ν/cm^{-1}: 3398, 2944, 2869, 1718, 1643, 1446, 1377, 1228, 1156, 914 and 733 (KBr); EIMS: *m/z*(%) 350($M^{+\bullet}$ not detected, $C_{21}H_{34}O_4$), 332(3, $M^{+\bullet}$- H_2O), 314(4, $M^{+\bullet}$- 2xH_2O), 299(4, $M^{+\bullet}$- 2xH_2O - $^{\bullet}CH_3$), 282(2), 255(8), 239(8), 201(10), 187(12%), 173(13), 159(22), 145(20), 133(32), 121(35), 119(78), 107(76), 105(67), 93(78), 81(70), 79(66), 71(62), 67(53) and 55(100); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 3.17 (dd, *J* 9 and 3 Hz, H-3), 5.85 (dd, *J* 17 and 11 Hz, H-14), 5.13 (dd, *J* 17 and 1 Hz, H-15a), 5.20 (dd, *J* 11 and 1 Hz, H-15b), 1.21 (s, H-16), 4.47 (brs, H-17a), 4.83(brs, H-17b), 1.19(s, H-18), 0.52 (s, H-20), 3.61 (s,OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1.

3a: White oil; IR ν/cm^{-1}: 3399, 2947, 2873, 1720, 1645, 1441, 1379, 1230, 1153, 913 and 739 (KBr); EIMS: *m/z*(%) 350($M^{+\bullet}$ not detected, $C_{21}H_{34}O_4$), 332(2, $M^{+\bullet}$- H_2O), 314(3, $M^{+\bullet}$- 2xH_2O), 299(5, $M^{+\bullet}$- 2xH_2O - $^{\bullet}CH_3$), 282(3), 255(10), 239(6), 145(21), 133(27), 131(32), 123(93), 121(52), 109(68), 107(64), 105(47), 93(68), 91(69), 81(100), 79(88), 71(57) and 67(63); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 4.40(t, *J* 1 Hz, H-7), 5.90(dd, *J* 17 and 11 Hz, H-14), 5.06(dd, *J* 17 and 1 Hz, H-15a), 5.21(dd, *J* 17 and 1 Hz, H-15b), 1.23(s, H-16), 4.67(brs, H-17a), 5.07(brs, H-17b), 1.18(s, H-18), 0.49(s, H-20), 3.62(s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1.

4a: White oil; IR ν/cm^{-1}: 3410, 2941, 2870, 1720, 1622, 1435, 1365, 1200, 1130 and 740 (KBr); EIMS: *m/z*(%) 368($M^{+\bullet}$ not detected, $C_{21}H_{36}O_5$), 350(1, $M^{+\bullet}$- H_2O), 332(2, $M^{+\bullet}$- 2xH_2O) and 299 (4, $M^{+\bullet}$- 2xH_2O - $^{\bullet}CH_3$); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.86(dd, *J* 11 and 17 Hz, H-14), 5.03(dd, J 17 and 1 Hz, H-15a), 5.21(dd, *J* 11 and 1 Hz, H-15b), 1.24(s, H-16), 3.41(d, *J* 10 Hz, H-17a), 3.62(d, *J* 10 Hz, H-17b), 1.18(s, H-18), 0.51(s, H-20), 3.60(s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1.

5b: White oil; IR ν/cm^{-1}: 3371, 2950, 2889, 1740, 1716, 1632, 1417, 1388, 1210, 1125 and 801 (KBr); EIMS: *m/z* (%) 452($C_{25}H_{40}O_7$, $M^{+\bullet}$), 434(1, $M^{+\bullet}$- H_2O), 392(3, $M^{+\bullet}$- AcOH), 374(5, $M^{+\bullet}$- AcOH - H_2O), 314(7, $M^{+\bullet}$- 2xAcOH - H_2O), 289(4), 262(5), 229(9), 188(8), 181(12), 161(11), 147(13), 133(15), 121(100), 107(32), 93(35), 81(42) and 79(23); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.80(dd, *J* 9 and 3 Hz, H-14), 4.48(dd, *J* 13 and 3 Hz, H-15a), 4.11(dd, *J* 13 and 9 Hz, H-15b), 1.12(s, H-16), 4.85(brs, H-17a), 4.50(brs, H-17b), 1.09(s, H-18), 0.52(s, H-20), 3.52(s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz): Table 1.

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