In Vitro Biotransformations of Isocupressic Acid by Cow Rumen Preparations: Formation of Agathic and Dihydroagathic Acids

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Received August 6, 1997[⊗]

Isocupressic acid [15-hydroxylabda-8(17),13*E*-dien-19-oic acid] (**1**) was incubated under anaerobic conditions for 48 h in an in vitro ruminal fluid mixture and was transformed into two metabolites. The two metabolites were identified by GC/MS as agathic acid [labda-8(17),13(*E*)-diene-15,19-dioic acid] (**4***E*) and dihydroagathic acid [labda-8(17)-ene-15,19-dioic acid] (**6**). Metabolite identities were confirmed by chemical conversions of isocupressic acid (**1**) and imbricataloic acid (**5**) into **4***E* and **6**, respectively. Structures of synthetic metabolites were confirmed by ^{1}H and ^{13}C NMR, specific rotation, GC/MS, and high-resolution mass spectrometry. Plasma obtained from cows that were fed Ponderosa pine needles contained (13*R*,*S*)-dihydroagathic acid (**6**) but not isocupressic acid (**1**) or **4***E*. The results suggest that isocupressic acid (**1**) is metabolically oxidized to agathic acid (**4***E*), subsequently reduced to (13*R*,*S*)-dihydroagathic acid (**6**) in the rumen, and then absorbed into the bloodstream of cattle.

Premature parturition in cattle due to the ingestion of Ponderosa pine [*Pinus ponderosa* Dougl. ex Laws (Pinaceae)] needles is a major economic problem in the Western United States and Canada.[1] Previous investigations have attempted to isolate and characterize the compounds in *P. ponderosa* responsible for pine needle abortion. A new class of vasoactive lipids[2] and isocupressic acid[3] [15-hydroxylabda-8(17),13(*E*)-dien-19-oic acid] (**1**) were identified as possible causative agents in pine needle abortion in pregnant cows. However, the effects of pine needle ingestion on domestic ruminants such as cattle, Bison, sheep, and goats are variable with regard to the parturition response and/or the presence of vasoconstrictive principles present in plasma samples.[4−6] The species differences observed in the pine needle abortion phenomenon may result from variability in the differences in digestive systems among species, from differences of possible metabolic transformations of ingested precursor abortifacient compounds, or from a combination of these factors.

Ruminants, including cattle, possess a four-compartment stomach. The largest compartment, the rumen, contains a mixed and variable population of symbiotic microorganisms effective in fermenting fibrous feedstuffs into nutrients usable by the host animal. The environment in the rumen is generally anaerobic, neutral to slightly acidic (between pH 5.5−7.0), and at a temperature between 38 and 41 °C. During digestion, the rumen microflora cooperatively catalyze enzymatic transformations of nutrients, fiber, and other ingested substances. Digested materials ultimately pass into the bloodstream of the ruminant animal where they are broadly distributed, bound, further metabolized, and ultimately excreted.

The mechanism whereby pine-needle ingestion induces abortion in cattle has been shown to involve a reduction in blood flow to the gravid uterus, resulting in a marked and progressive decrease in nutrients and oxygen available to the fetal calf.[4,7] This is consistent with the observation that mechanical compression of the uterine artery supplying blood to the gravid horn during late gestation induces premature parturition in the ewe.[8] Further, Ford et al. demonstrated that plasma obtained from pregnant cows that had ingested pine needles contained a component that produced a dose-dependent and sustained contraction of an intact arterial bed (placentome) of the isolated perfused bovine.[9,10] Because this vasoconstrictive activity was absent in the plasma of pregnant cows that had not ingested pine needles, abortifacient principles apparently pass through the rumen digestive system and are absorbed into the bloodstream where they effect a decrease in uterine blood flow. Furthermore, while diesters of α,ω-alkanediols[2] affected the contractility and tone of the placentome, isocupressic acid (**1**) did not.[11]

This paper describes the results of an in vitro rumen metabolism study in which isocupressic acid (**1**) was converted into two metabolites, agathic acid [labda-8(17),13(*E*)-diene-15,19-dioic acid] (**4***E*) and dihydroagathic acid [labd-8(17)-ene-15,19-dioic acid] (**6**). We further report the presence of one of the rumen incubation metabolites, dihydroagathic acid (**6**), as the sole diterpene product detected in plasma from pine-needle-fed cows.

Results and Discussion

In vitro incubations of ruminal fluid were conducted with mixtures containing isocupressic acid (**1**) in con-

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.

S0163-3864(97)00372-8 CCC: $15.00 © 1998 American Chemical Society and American Society of Pharmacognosy
Published on Web 01/23/1998

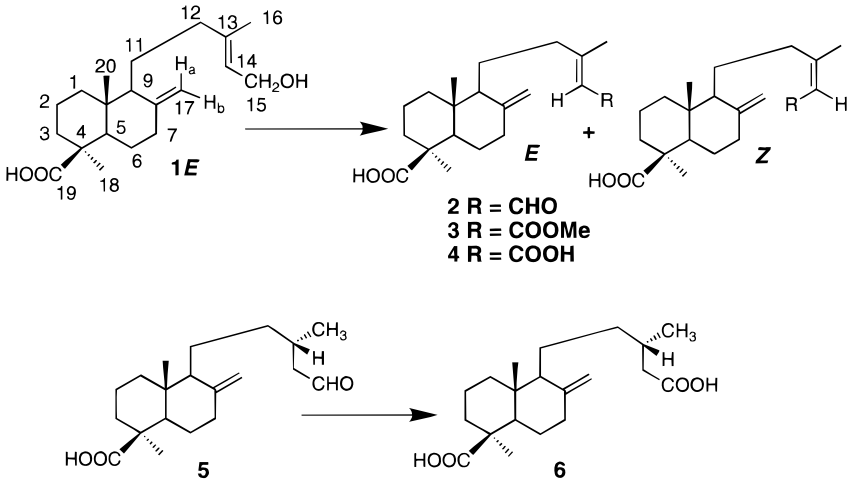

Figure 1. Structures of isocupressic acid (**1**) and its derivatives and metabolites (**4***E* and **6**).

centrations of 3.3, 16.5, and 33 μg/mL. Incubations were continued for 48 h, after which time they were extracted with CH_2Cl_2, derivatized with diazomethane, and examined by both gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) for the presence of isocupressic acid and/or metabolites. Control incubations contained no interfering substances or metabolites. Isocupressic acid (**1**) was completely converted into other products in all incubations and at all concentrations used. A single new metabolite peak with t_R 8.87 min was obtained in incubations containing 3.3 μg/mL of **1**, while two new metabolite peaks at t_R 8.87 and 9.17 min were found in extracts of incubations containing higher concentrations of **1**. The peak at t_R 8.87 min gave an apparent molecular ion at *m/z* 364 and major fragment ions at *m/z* 304, 305, and 121. In contrast, the peak eluting at t_R 9.17 min gave an apparent molecular ion at *m/z* 362 with additional prominent ions at *m/z* 189, 302, 303, and 121. These molecular weights are consistent with metabolites of **1** (molecular ion of the methyl ester, *m/z* 334) in which the alcohol at the 15-position was oxidized to a carboxylic acid (**4**) (*m/z* 362 for dimethyl ester) and where the double bond at C-13, C-14 has been subsequently reduced (**6**) (*m/z* 364 for dimethyl ester). Mass spectral fragments showed identical base peaks at *m/z* 121 for the A rings of the diterpene metabolites and for the starting material, isocupressic acid (**1**). The prominent *m/z* 189 peak occurs in the spectra of isocupressic acid (**1**) and **4**, but not in the spectrum of **6**. This suggests that a 13,14-double bond occurs in the structure of **4** but not in the structure of **6**.

With higher concentrations of **1**, both metabolites were observed, suggesting that they were formed as a consequence of stepwise conversion of **1** to **4** and **6**. The very small amounts of metabolites obtainable by ruminal fluid incubations precluded the isolation of sufficient amounts for proper spectral analysis and structure elucidation. Therefore, we sought to prepare putative rumen metabolites **4** and **6** by chemical means to confirm their identities.

Isocupressic acid (**1**) was isolated from *P. ponderosa* needles and then completely characterized by mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy and was used to prepare **4**. Oxidation of **1** with pyridinium chlorochromate (PCC) in CH_2Cl_2 gave the corresponding diterpene aldehyde, **2***E* (Figure 1), which was spectrally identical with previously reported **2***E* isolated from *Cryptomeria japonica*.[26] When the PCC oxidation was repeated, an equal mixture of **2***E* and **2***Z* was obtained. After separation by Si gel column chromatography, the isomers were compared spectrally to reveal the same properties for **2***E* as before.[26] All diterpene skeletal proton and carbon shifts for the *Z* isomer were the same as for the *E* isomer except for signals for the side chain. The ^{13}C NMR spectrum of a mixture of the **2***E* and **2***Z* isomers displayed new peaks at δ 31.17 and 24.82 for C-12 and Me-16, respectively, for the *Z* isomer. A new three-proton peak at δ 1.97 was observed for Me-16 of the **2***Z* isomer. A full differentiation of the *E* and *Z* isomer profiles is given for the subsequent products, **4***E* and **4***Z*.

The direct oxidations of conjugated aldehydes to their corresponding carboxylic acids are difficult reactions to achieve.[12] Therefore, the mixture of **2***E* and **2***Z* isomers was first converted to the corresponding *E,Z*-cyanohydrin mixture and then oxidized to the corresponding acyl cyanides with MnO_2. Methanolysis gave a mixture of the **3***E* and **3***Z* monomethyl esters. After methylation with CH_2N_2, peaks at t_R 8.90 min and t_R 9.17 min, were shown by GC/MS, each exhibiting a molecular ion at *m/z* 362. The peak eluting at t_R 9.17 min was identical both in retention time and in mass spectral fragmentation to that obtained in rumen metabolism experiments. Compound **3***E* is the C-15 methyl ester of the natural product, agathic acid (**4***E*).[13,14]

After saponification of the **3***E* and **3***Z* mixture with NaOH, the products **4***E* and **4***Z* were separated by Si gel column chromatography. HRFABMS (dimethyl ester) of **4** gave $[M + Na]^+$ *m/z* 385.2350 for $C_{22}H_{34}O_4$-Na (calcd 385.2354). As with all compounds, ^{1}H and ^{13}C NMR spectral assignments of the *E* and *Z* isomers were confirmed by HMBC and HMQC correlations and by comparison with well-defined spectral properties of isocupressic acid.[3,25] These isomers showed essentially identical NMR spectral properties for ring skeletal proton and carbon signals and only differed in signals attributable to the side chain. Comparison with NMR spectral properties of other *E* and *Z* isomers of labdane diterpenes[15,16] and from the literature[13,14,27] confirmed the structure of **4***E*. Isomer **4***Z* was unknown. The NMR spectrum of **4***Z* was nearly identical with that for

4*E* except for signals for the C-16 methyl group at δ 1.88 (^{1}H NMR) and δ 25.6 (^{13}C NMR) and C-12 at δ 33.7 (^{13}C NMR). The GC/MS properties of **4***E* dimethyl ester were identical in all respects to those for the t_R 9.17 min metabolite obtained from rumen incubations, which confirmed the identity of the rumen metabolite as **4***E*.

Zinkel and Magee previously reported[17] that *P. ponderosa* needles contain labdane diterpenes including imbricataloic acid (**5**), which contains an aldehyde moiety at C-15, but no 13,14-double bond. Imbricataloic acid (**5**) was isolated from *P. ponderosa* needle extracts as a colorless oil. HRFABMS of **5** gave *m/z* $[M + Na]^+$ 343.2255 for $C_{20}H_{32}O_3Na$ (calcd 343.2249). The ^{1}H NMR spectrum showed a broad 1H-singlet at δ 11.73 for the carboxylic acid at C-19 and a 1H-triplet aldehyde proton signal at δ 9.75, which confirmed the aldehyde functional group at C-15. A prominent three-proton doublet signal centered at δ 0.97 was attributed to the C-16 methyl group, whereas two 1H signals at δ 4.83 and 4.47 were assigned to the C-17 hydrogens of **5**. HMBC and HMQC spectral correlations and comparison of the spectral data obtained in the present investigation with literature values confirmed the structure of the isolated compound as imbricataloic acid (**5**).[18,19]

The oxidation of **5** to the second putative rumen metabolite, dihydroagathic acid (**6**), was carried out with $KMnO_4$ in acetone. Following chromatographic purification, **6** gave HRFABMS *m/z* $[M + Na]^+$ 359.2205 for $C_{20}H_{32}O_4Na$. The specific rotation $[[\alpha]^{29}_D$ +34°] was almost identical with that of dihydroagathic acid (**6**), as reported in the literature (+33.8°).[20] The aldehyde peak at δ 9.75 in the ^{1}H NMR spectrum of **5** was absent and replaced by new signals at δ 11.90 (^{1}H NMR, 2H, s) and δ 180.1 (^{13}C NMR, for a new carboxylic acid moiety at C-15).

The GC/MS spectrum of the dimethyl ester of **6** gave t_R 8.87 min and an MS fragmentation identical with the major metabolite obtained from rumen digestion. The MS of **6** dimethyl ester exhibited prominent peaks at *m/z* 364, 304, 305, and 121 and a very small ion at *m/z* 189. By comparison with synthetic compounds, the metabolites produced during rumen incubations with isocupressic acid were identified as agathic acid (**4***E*) and dihydroagathic acid (**6**).

With the knowledge that in in vitro rumen incubations isocupressic acid (**1**) was completely converted into **4***E* and **6**, we attempted to identify labdane diterpene compounds occurring in plasma obtained from cows that were fed *P. ponderosa* needles. Plasma samples obtained from pregnant cows fed pine needles were extracted with CH_2Cl_2. Controls consisted of plasma from cows receiving normal diets. Methylene chloride extracts were concentrated and worked up directly or subjected to alkaline saponification and subsequent CH_2Cl_2 extraction following acidification. Methylated concentrated extracts prepared with diazomethane or BSTFA [bis(trimethylsilyl)trifluoroacetamide] upon GC and GC/MS analysis revealed the presence of a single peak at t_R 8.87 min with a fragmentation pattern displaying prominent ions at *m/z* 364, 305, 304, and 121, identical with those for **6**. No peaks at t_R 9.17 or t_R 8.90 for **4***E* or isocupressic acid, respectively, were observed in extracts. Quantitative GC analysis of plasma extracts showed that the concentrations of **6** were 11.36 $\pm$ 0.09 mg/L for the simple CH_2Cl_2 extract and 10.94 $\pm$ 0.07 mg/L for extracts that were saponified. It was impossible to determine the amounts of **6** or other ingested terpenes that were deposited in or bound to tissues after ingestion.

By GC, the metabolite in plasma extracts possessed a retention time identical to both (13*R,S*)-**6** and (13*S*)-**6** prepared synthetically. Therefore, to establish the stereochemical purity of **6** in plasma of pregnant cows fed Ponderosa pine needles, the metabolite was isolated and completely characterized. Both HRFABMS and the ^{1}H NMR spectrum were essentially identical with those for (13*S*)-dihydroagathic acid obtained by oxidation of imbricataloic acid or (13*R,S*)-**6** obtained by sodium reduction of an *E,Z*-mixture of agathic acid (**4**).[21] Clear differences in the isolated metabolite and (13*S*)-dihydroagathic acid were evident in the specific rotation $[[\alpha]^{30}_D$ + 39.1° (*c* 0.8, EtOH)] and in the ^{13}C NMR spectrum, in which nearly all signals were displayed as twin peaks. On the basis of the comparison with (13*S*)-dihydroagathic acid (**6**) and synthetic (13*R,S*)-**6**, the ratio of **6** *S/R* in the metabolite isolated from plasma was about 2:1.

The work reported here shows that isocupressic acid (**1**) from *P. ponderosa* is subject to metabolic alteration by the microflora of cow rumen. Gardner et al.[22] used much shorter, 8 h rumen incubations to show that 15-*O*-acetyl-(**1**) and 15-*O*-succinyl (**1**) acids are completely hydrolyzed to isocupressic acid (**1**). No other metabolites of isocupressic acid were apparently observed. Following the rumen incubations in this study, it was observed that the C-15 alcohol functional group and the 13,14-double bond of isocupressic acid (**1**) were metabolically altered.

The isolation of (13*R,S*)-**6** from plasma of pregnant cows fed Ponderosa pine needles and the detailed chemical manipulations reported here enable the following conclusions. Under the conditions used in our work, the C-15 alcohol functional group is first oxidized to an aldehyde (**2**) by an alcohol dehydrogenase and then to agathic acid (**4***E*) by the action of an aldehyde dehydrogenase. All of the proposed enzymes can function under the anaerobic environment used in our experiments and are present in the rumen. Double bond reduction is likely accomplished by hydride 1,4-addition to either the α,β-unsaturated aldehyde or an acid. The reductase catalyzing the reduction of the C-13,C-14-double bond is apparently nonspecific. Furthermore, it is likely that the plasma metabolite **6** arose from metabolic transformations of isocupressic acid (**1**) and not imbricataloic acid (**5**) present in pine needles. Imbricataloic acid (**5**) isolated from *P. ponderosa* needles was isomerically pure by analytical comparison with previously isolated **5** and by chemical conversion to (13*S*)-dihydroagathic acid (**6**). The identification of dihydroagathic acid but not isocupressic acid in the plasma of cows that had eaten *P. ponderosa* pine needles suggests that dihydroagathic acid might be responsible for the observed vascular effects attributed to the ingestion of pine needles by cattle.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker WM-360

MHz instrument. HMQC and HMBC experiments[23,24] were conducted using a Bruker AMX-600 spectrometer. NMR spectra were recorded in $CDCl_3$ or CD_3OD, and chemical shifts are given in δ value downfield from TMS. FABMS were measured with a VG ZAB-HF mass spectrometer. Gas chromatography/mass spectrometry (GC/MS) was conducted with a Trio-1 mass spectrometer operating at 50 eV, linked with a Hewlett-Packard 5890 A gas chromatograph using a DB-1 methylsilicone column (15 m × 0.32 mm i.d.) (J&W Scientific, Folsom, CA). For analysis, the starting temperature was 50 °C, which was increased at a rate of 20 °C/min to 250 °C and held at the final temperature of 250 °C for 20 min. Gas chromatography (GC) was performed with a Shimadzu GC-14A instrument equipped with FID using a 30 m × 0.53 mm column (1.5 μm film thickness) with a methylsilicone phase (AT-1, Alltech Associates, Inc., Deerfield, IL), linked to a Shimadzu CR 501 recorder. The column was programmed at 150 °C for 1 min and at 150−250 °C at 5 °C/min, with a final temperature of 250 °C for 15 min. The injector and detector temperatures were 250 and 325 °C, respectively. Optical rotations were measured with a JASCO DIP-140 digitial polarimeter and a JASCO DIP-1000 digitial polarimeter. Samples of CH_2Cl_2 extracts (from plasma and ruminal fluid incubations) were dissolved in 100 μL of CH_3OH, and 100 μL of freshly prepared diazomethane were added. After methylation, samples were analyzed by GC and GC/MS.

Plant Material. *P. ponderosa* bark (790.4 g) (June 1994) and needles (winter 1989) were collected in Custer County, MT. Voucher specimens of the plant material are on deposit at the Botany department of Montana State University, Bozeman.

Plasma Sampling. Plasma was taken from pregnant cows in October 1991, after they were fed Ponderosa pine needles. Blood samples were collected into heparinized tubes daily from each cow beginning on day 257 of gestation and continuing through the day of parturition. After collection, blood samples were centrifugated immediately, and the collected plasma was stored at −20 °C until being extracted. For analysis, samples were acidified to pH 2 with 6 N HCl and extracted with CH_2Cl_2. The combined CH_2Cl_2 layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under N_2. These were derivatized with diazomethane prior to GC or GC/MS analysis.

Isolation of Dihydroagathic Acid from the Plasma of Pine-Needle-Fed Cows. A sample of 2.8 L of plasma from pregnant cows after pine needle ingestion (2.5 kg/day for 3 days) was acidified to pH 2 and exhaustively extracted with 1.4 L of CH_2Cl_2. The combined CH_2Cl_2 extract was partitioned with 270 mL of 5% $NaHCO_3$. After acidification to pH 2 with 1 N HCl, the $NaHCO_3$ layer was extracted with 270 mL of diethyl ether. The ether extract was dried over anhydrous Na_2SO_4, filtered, and concentrated to give an oil (58 mg). Repeated Si gel column chromatography on elution with petroleum ether−EtOAc (6:1) or CH_2Cl_2−CH_3OH (60:1) gave 2 mg of (13*R*,*S*)-dihydroagathic acid (**6**) as a colorless oil: $[\alpha]^{30}_D$ +39.1° (*c* 0.8, EtOH); ^{1}H NMR ($CDCl_3$, 360 MHz) was essentially identical with that for (13*S*)-**6**; ^{13}C NMR ($CDCl_3$, 90 MHz) was identical with that for (13*S*)-**6** except that all signals were twinned; GC t_R 17.5 min; HRFABMS *m/z* [M − H + 2 Na]$^+$ 381.2033 (calcd for $C_{20}H_{31}O_4Na_2$, 381.2017).

Isolation of Isocupressic Acid (1). *P. ponderosa* bark (790.4 g) was milled to 2 mm in a blender and exhaustively extracted with CH_2Cl_2 in a glass Soxhlet extraction apparatus to give 54.8 g of a dark brown viscous oil after concentration of the extract. A 35.4 g sample of the crude extract was dissolved in CH_2Cl_2 (300 mL) and stirred with 0.75 M NaOH (320 mL) for 24 h at room temperature. The CH_2Cl_2 phase was removed and concentrated to 9.9 g of yellow oil. The aqueous alkali fraction was acidified to pH 2 with 1 N HCl and extracted five times, each with 300 mL of CH_2Cl_2. The combined extracts were concentrated to 21.6 g of a dark brown oil that was fractionated by flash column chromatography over 40 mm Si gel (Baker) eluted under 7 psi N_2 pressure with hexanes−EtOAc (4:1) with 10 mL fractions being collected. Fractions containing isocupressic acid (**1**) were monitored by TLC (hexanes−EtOAc, 3:2) and combined. Repeated flash column chromatography as before afforded 2.88 g of isocupressic acid (**1**) as a white solid: mp 109−110 °C (lit.[25] mp 117−119 °C); $[\alpha]^{25}_D$ +53.4° (*c* 1.0, $CHCl_3$) [$[\alpha]^{25}_D$ +52.9° (*c* 0.94, $CHCl_3$)]; ^{1}H NMR ($CDCl_3$, 360 MHz) and ^{13}C NMR ($CDCl_3$, 90 MHz) spectra were as previously reported;[25] EIMS (70 eV) [M + TMSi + methyl ester]$^+$ *m/z* 406 (1), 391 (4), 316 (15), 301 (19), 241 (35), 189 (23), 121 (100); CIMS (CH_4) [M + H + TMSi + methyl ester]$^+$ *m/z* 407 (2), 391 (10), 345 (10), 317 (100), 257 (62); HRFABMS *m/z* [M + Na]$^+$ 343.2240 (calcd for $C_{20}H_{32}O_3Na$, 343.2249).

In Vitro Metabolism of Isocupressic Acid (1) by Ruminal Fluid. Ruminal fluid was collected in July 1995 from cows at the Fort Keogh Livestock and Range Research Laboratory, Miles City, MT. The anaerobic incubation medium contained the following components: grass hay, 500 mg; methanol, 800 μL; McDougall's buffer, 15 mL; phosphate buffer, 7.5 mL; and ruminal fluid, 7.5 mL. Three different concentrations of isocupressic acid (**1**) were added to incubation mixtures: 100 μg (3.3 μg/mL), 500 μg (16.5 μg/mL), and 1000 μg (33 μg/mL). Controls were composed of incubation mixtures without grass hay and/or methanol. All media were prepared and inoculated under an atmosphere of CO_2. Cultures were incubated in duplicate, anaerobically at 39 °C. After 48 h of incubation, mixtures were centrifuged at 10000*g* for 15 min, and supernatants were taken from culture broths for detection of metabolites.

Extraction of in Vitro Ruminal Digestion of Isocupressic Acid (1). Samples were acidified with 6 N HCl to pH 2 and extracted with CH_2Cl_2. The combined CH_2Cl_2 layers were dried over anhydrous Na_2SO_4, filtered, and concentrated under N_2.

Oxidation of Isocupressic Acid (1) to 2. A solution of 60 mg of isocupressic acid in 1 mL of CH_2Cl_2 was added dropwise to 245 mg of pyridinium chlorochromate (PCC) in 1 mL of CH_2Cl_2 and stirred at room temperature for 2 h. The reaction mixture was diluted with ether (10 mL) and filtered. The black solid was washed twice with ether. The combined filtrate was dried over anhydrous Na_2SO_4, filtered, and evaporated to give an oil (31.2 mg). Purification by Si gel column chromatography gave 3.4 mg of **2***E* as a colorless oil: $[\alpha]^{27}_D$ +43.9°

(*c* 1.1, CH_3OH) [lit.[26] $[\alpha]_D$ +47.5° (*c* 0.8, CH_3OH)]; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 90 MHz) were as previously reported;[26] HRFABMS *m/z* [M + Na]$^+$ 341.2092 (calcd for $C_{20}H_{30}O_3Na$, 341.2092).

Conversion of 2 to Labda-8(17),13-diene-15,19-dioic Acid (4). A 50 mL, two-necked round-bottomed flask containing 32 mg of **2**, 27 mg of NaCN, 2 mL of CH_3OH, two drops of acetic acid, and 183 mg of MnO_2 was stirred under N_2 at room temperature overnight. After filtration through Celite, the filtrate was concentrated and diluted with water. The mixture was extracted with diethyl ether, and the combined ether extracts were dried and concentrated. The crude product (24.4 mg) was purified by Si gel column chromatography to give 14.5 mg of a mixture of **3***E* and **3***Z*: HRFABMS (dimethyl ester) gave *m/z* [M + Na]$^+$ 385.2350 (calcd for $C_{22}H_{34}O_4Na^+$ 385.2354). GC/MS of the combined methyl esters gave peaks at t_R 8.90 (*m/z* 362) and t_R 9.17 (*m/z* 362). A 9 mg mixture of 13,14-(*E,Z*)-**3** in 0.2 mL of CH_3OH was mixed with 2 mL of 2% NaOH and stirred at room temperature. The mixture was acidified with 1 N HCl and extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4 to give an oil that was purified over Si gel column chromatography (7 × 0.5 cm CH_2Cl_2−CH_3OH, 25:0.5) to give 2 mg of ***4E*** and 1 mg of ***4Z***.

Labda-8(17),13(*E*)-diene-15,19-dioic acid (***4E***) was obtained as a colorless oil: $[\alpha]^{29}_D$ +55.5° (*c* 2.0, EtOH) [lit.[20] $[\alpha]_D$ +56.1° (EtOH)]; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR ($CDCl_3$, 90 MHz) were as reported;[13,14,27] EIMS (50 eV) [M + dimethyl ester]$^+$ *m/z* 362 (1), 347 (5), 303 (7), 302 (10), 288 (6), 235 (2), 221 (4), 189 (31), 173 (6), 161 (14), 149 (6), 122 (14), 121 (100); HRFABMS *m/z* [M + Na]$^+$ 357.2041 (calcd for $C_{20}H_{30}O_4Na$, 357.2041).

Labda-8(17),13(*Z*)-diene-15,19-dioic acid (***4Z***) was obtained as a colorless oil: $[\alpha]^{28}_D$ +14.8° (*c* 1.8, EtOH); ^1H NMR (CD_3OD, 600 MHz) δ 5.63 (1H, br s, H-14), 4.91 (1H, s, H-17b), 4.69 (1H, s, H-17a), 2.50 (2H, m, H-12), 2.38 (1H, m, H-7), 2.10 (1H, m, H-3), 1.83−2.01 (5H, m, H-1, H-2, H-6, H-7), 1.88 (3H, d, J = 1.2 Hz, Me-16), 1.65−1.71 (2H, m, H-9, H-11), 1.46−1.56 (2H, m, H-2, H-11), 1.33 (1H, dd, J = 3, 12 Hz, H-5), 1.19 (3H, s, Me-18), 1.12 (1H, m, H-1), 1.02 (1H, dt, J = 4.2, 13.2 Hz, H-3), 0.59 (3H, s, Me-20); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 181.3 (C-19), 169.9 (C-15), 162.1 (C-13), 149.5 (C-8), 117.4 (C-14), 107.1 (C-17), 57.8 (C-5), 57.6 (C-9), 45.2 (C-4), 41.7 (C-10), 40.5 (C-1), 40 (C-7), 39.4 (C-3), 33.7 (C-12), 29.6 (C-18), 27.6 (C-6), 25.6 (C-16), 23.8 (C-11), 21.2 (C-2), 13.4 (C-20); EIMS (50 eV) [M + dimethyl ester]$^+$ *m/z* 362 (2), 347 (6), 303 (11), 302 (14), 288 (6), 235 (8), 221 (5), 189 (37), 173 (7), 161 (18), 149 (9), 122 (13), 121 (100); HRFABMS *m/z* [M + Na]$^+$ 357.2036 (calcd for $C_{20}H_{30}O_4Na$, 357.2041).

Reduction of (*E,Z*)-Agathic Acid (4) to (13*R,S*)-Dihydroagathic Acid (6). To a refluxing and stirred solution of (*E,Z*)-agathic acid (70 mg) in 8 mL of butanol was added 380 mg of sodium metal. After 30 min, the solution was cooled, diluted with water (10 mL), and concentrated in vacuo to remove butanol. Acidification, extraction with diethyl ether, drying, and evaporation afforded 120 mg of a viscous residue. Purification by silica gel column chromatography (petroleum ether−EtOAc 6:1 or CH_2Cl_2−CH_3OH 25:1) gave 40 mg of (13*R,S*)-dihydroagathic acid (**6**) as a colorless oil: $[\alpha]^{26}_D$ +44.5° (*c* 1.1, EtOH); HRFABMS *m/z* [M − H + 2 Na]$^+$ 381.2017 (calcd for $C_{20}H_{31}O_4Na_2$, 381.2017); ^1H NMR was closely comparable to that for (13*S*)-**6**; ^{13}C NMR identical with that for (13*S*)-**6** except that all signals were twinned.

Isolation of Imbricataloic Acid (5). *P. ponderosa* needles were extracted as described for the bark. After repeated chromatograpy over silica gel, 21 mg of imbricataloic acid (**5**) was obtainted as a colorless oil: $[\alpha]^{27}_D$ +43.3° (*c* 1.7, $CHCl_3$); ^1H NMR ($CDCl_3$, 600 MHz) was as previously reported;[18,19] ^{13}C NMR ($CDCl_3$, 90 MHz) δ 203.1 (C-15), 183.2 (C-19), 148.1 (C-8), 106.4 (C-17), 56.5 (C-9), 56.3 (C-5), 50.8 (C-14), 44.2 (C-4), 40.6 (C-10), 39.2 (C-1), 38.7 (C-7), 38 (C-3), 36.1 (C-12), 29.0 (C-18), 28.9 (C-13), 26.0 (C-6), 21.2 (C-11), 20.2 (C-16), 19.9 (C-2), 12.8 (C-20); HRFABMS *m/z* [M + Na]$^+$ 343.2255 (calcd for $C_{20}H_{32}O_3Na$, 343.2249).

Oxidation of Imbricataloic Acid (5) to Dihydroagathic Acid (6). $KMnO_4$ (23 mg, 0.125 mmol), dissolved in a mixture of 0.5 mL of H_2O and 0.5 mL of acetone, was added dropwise to 40 mg (0.125 mmol) of imbricataloic acid (**5**) in 2 mL of acetone. The reaction mixture was stirred at room temperature for 2 h, diluted with 3.5 mL of H_2O, and extracted with diethyl ether. The ether layer was then extracted with 5% $NaHCO_3$. The $NaHCO_3$ extract was washed with ether, and the $NaHCO_3$ aqueous layer was acidified with 0.1 N HCl and reextracted with ether. The ether extract was dried over $MgSO_4$ and concentrated to an oil (12 mg). The crude product was purified by silica gel column chromatography (7 × 0.5 cm, petroleum ether−EtOAc, 10:1) to give 4 mg of labd-8(17)-ene-15,19-dioic acid (dihydroagathic acid) (**6**) as a colorless oil: $[\alpha]^{29}_D$ +34° (*c* 1.7, EtOH) [lit.[20] $[\alpha]_D$ +33.8° (*c* 1, EtOH)]; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 90 MHz) were closely comparable to the reported spectral data;[26,28] EIMS (50 eV) [M + dimethyl ester]$^+$ *m/z* 364 (1), 305 (7), 304 (14), 289 (2), 235 (4), 221 (8), 189 (3), 181 (7), 161 (13), 149 (5), 122 (20), 121 (100); HRFABMS *m/z* [M + Na]$^+$ 359.2205 (calcd for $C_{20}H_{32}O_4Na$, 359.2198).

Acknowledgment. We acknowledge financial support through a USDA grant, and financial support through a fellowship afforded by the National Science Council, Taipei, Taiwan. U.S. Department of Agriculture, Agricultural Research Service, Northern Plains Area, is an Equal Opportunity/Affirmative Action employer and all Agency services are available without discrimination.

References and Notes

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NP970372U