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Preparation of Tetrahydroagathic Acid: A Serum Metabolite of Isocupressic Acid, a Cattle Abortifacient in Ponderosa Pine

MASSOUD GARROSSIAN, DALE R. GARDNER,* KIP E. PANTER, AND LYNN F. JAMES

Poisonous Plant Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, 1150 East 1400 North, Logan, Utah 84341

Isocupressic acid (**1**) was used to synthetically prepare a mixture of (8*S*,13*R,S*)-labda-15,19-dioic acid (tetrahydroagathic acid) (**5**) via a two-step oxidation procedure followed by hydrogenation of the double bonds at C13 and C8. Reduction of the C8,17 double bond was stereospecific producing only the 8*S* isomer and confirmed by the nOe interaction between the resulting C17 and C20 methyl groups. The 13*R* and 13*S* isomers of **5** were separated and analyzed by HPLC/MS, and (13*S*)-tetrahydroagathic acid was isolated and identified by comparison to a standard prepared by hydrogenation of naturally occurring (13*S*)-dihydroagathic acid (**4**). (13*R,S*)-dihydroagathic acid was prepared by selective sodium metal-catalyzed hydrogenation of the C13,14 allylic double bond of agathic acid (**3**). The prepared compounds were then used as standards to confirm the presence of **4** and **5** and their respective 13*R* and 13*S* isomers in bovine serum samples. Tetrahydroagathic acid was shown to be the only metabolite detected in serum samples taken from a suspected cattle abortion case submitted for diagnosis; and, thus, **5** could be a valuable diagnostic marker for pine needle-induced abortions.

KEYWORDS: Isocupressic acid; *Pinus ponderosa*; cattle abortion; diterpene acids; tetrahydroagathic acid

INTRODUCTION

Consumption of ponderosa pine (*Pinus ponderosa* Laws) needles causes late-term abortions in cattle and is a serious poisonous plant problem in foothill and mountain rangelands of the western U.S. (*1*−*4*). Pine needle abortion often occurs during the late winter or early spring when cattle are in their third trimester of pregnancy. Late winter storms often force animals into pine tree stands for shelter, increasing their consumption of pine needles and exacerbating the abortion problem (*3*−*5*). Cows may abort as soon as 24 h after consumption of the pine needles, but typically abortions occur in 3−4 days, and up to two weeks after exposure to the pine needles. The abortion often results in the birth of small weak calves and retained placentas in cows that have aborted. Stillborn calves may occur if pine needles are ingested during the second trimester or early third trimester. The incidence of pine needle-induced abortion increases the closer cows are to their expected date of parturition. Cow deaths are not uncommon, often resulting from endometritis, septicemia, or other complications of premature parturition and retained placenta. The direct economic losses from pine needle abortions have been estimated to be as high as $20 million per year (*6, 7*).

Isocupressic acid (**1**) was recently identified as the abortifacient agent in ponderosa pine needles (*8*). However, analysis of rumen and serum samples from both in vitro and in vivo studies documented the rapid metabolism of isocupressic acid to at least four compounds via a series of reduction and oxidation steps (*9, 10*). The identified metabolites (**Figure 1**) include imbricatoloic acid (**2**), agathic acid (**3**), dihydroagathic acid (**4**), and a fourth compound tentatively identified as tetrahydroagathic acid (**5**). The pine needle toxin is thought to increase uterine vascular tone resulting in decreased placental perfusion and reduced blood flow to the fetus, subsequently initiating the parturition process (*11*−*14*). Apparently isocupressic acid does not appear to act directly at the site of the uterine artery muscle (*15*), and the specific abortion mechanism caused by isocupressic acid or its metabolites remains unknown at this time.

The detected metabolites are present in the serum at parts per million levels, and thus the proposed compound (**5**) has not been isolated in sufficient quantities for complete structural characterization. Based on the further need for sufficient quantities of isocupressic acid metabolites for future experiments we sought to develop methods for their preparation from isocupressic acid, which can be isolated in large quantities from ponderosa pine needles or bark. We present here the characterization of (8*S*,13*S*)-tetrahydroagathic acid (**5**) prepared from naturally occurring isocupressic acid and its confirmation as a metabolite, along with its corresponding 13*R* isomer, in serum of cattle after consumption of plant material containing isocupressic acid.

* To whom correspondence should be addressed [telephone (435) 752-2941; fax (435) 753-5681; e-mail gardnerd@cc.usu.edu].

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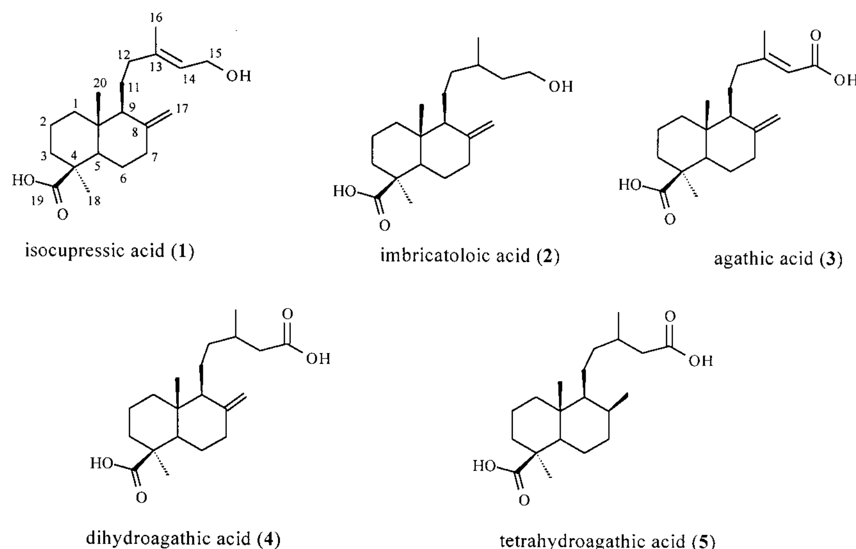


Figure 1. Structure of isocupressic acid and identified and proposed serum metabolites.

MATERIALS AND METHODS

General Methods. All reaction solvents were dried and distilled by standard methods prior to use. Reaction materials were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were generally used without further purification. Triphenylphosphine was recrystallized before use by dissolving 20 g in boiling benzene (50 mL). Absolute ethanol was added slowly until the solution turned slightly cloudy. After the solution cooled, the triphenylphospine was filtered and dried under vacuum at 76 °C. Isocupressic acid (**1**) was isolated from the bark of *Pinus ponderosa* using previously described methods (*8*).

Gas chromatography/mass spectrometry analyses of the isolated or prepared diterpene acids were conducted using a Finnigan MAT GCQ (San Jose, CA) system consisting of a 30 m × 0.25 mm i.d. DB-5ms capillary column (J&W Scientific, Folsom, CA). Samples (50−100 μg) were derivatized to their corresponding TMS esters after they were dissolved in pyridine (200 μL), adding *N*,*O*-bis(trimethylsilyl)trifluoroactamide (50 μL) (BSTFA, Pierce Chemical, Rockford, IL) and heating at 70 °C for 30 min. Samples (1−2 μL) were injected splitless with an injector temperature of 225 °C. The initial column temperature was 100 °C for 1 min, then raised from 100 to 200 °C at 40 °C/min and from 200 to 300 °C at 5 °C/min, and then held at 300 °C for 1.5 min.

Reversed-phase liquid chromatography/mass spectrometry (HPLC/MS) was preformed using a system consisting of an HP 1100 (Agilent Technologies, Wilmington, DE), binary solvent pump and autosampler, a 100 × 2 mm i.d. Betasil C18 reversed-phase HPLC column (Keystone Scientific, Bellefonte, PA), and a Finnigan LCQ mass spectrometer. The mobile phase was a mixture of methanol (A) and 20 mM ammonium acetate (B) at a flow rate of 0.5 mL/min starting with 50% A for 1 min, followed by a linear gradient increasing to 100% A in 20 min. Ionization was achieved using an atmospheric pressure chemical ionization (APCI) source with a vaporizer temperature of 450 °C and corona discharge current of 5 μA. The capillary inlet temperature and voltage were 150 °C and −30V, respectively. Operational parameters of the mass spectrometer were optimized using the standard "autotune" procedure to optimize the signal [*m*/*z* 319, $(M - H)^-$] for a standard solution of isocupressic acid infused into a flow (0.5 mL/min) of 60:40 methanol/20 mM ammonium acetate. The mass spectrometer was set to scan negative ions in a mass range of 150−1000 amu. Maximum ion trap inject time was 200 ms and two micro scans were averaged for each data point. Under these conditions the two most prominent negative ions observed for isolated or prepared diterpene acids were $(M - H)^-$ and $(M + OAc)^-$.

Synthesis of Agathic Acid (3). A solution of isocupressic acid (1.00 g, 3.1 mmol) in methylene chloride (45 mL) was placed into a 100-mL round-bottomed flask containing oven-dried 4 Å molecular sieves (2.0 g) and equipped with a rubber septum and a magnetic stir bar. While the flask contents were stirring, 4-methylmorpholine *N*-oxide (549.7 mg, 4.7 mmol), and tetrapropylammonium perruthenate (55 mg, 0.16 mmol) were added, and after 1 h the solution was filtered through a plug of silica gel (slurry packed in EtOAc) and concentrated under reduced pressure to give 0.88 g (89% yield) of the corresponding aldehyde (**1a**, **Figure 2** - scheme 1) (*16*).

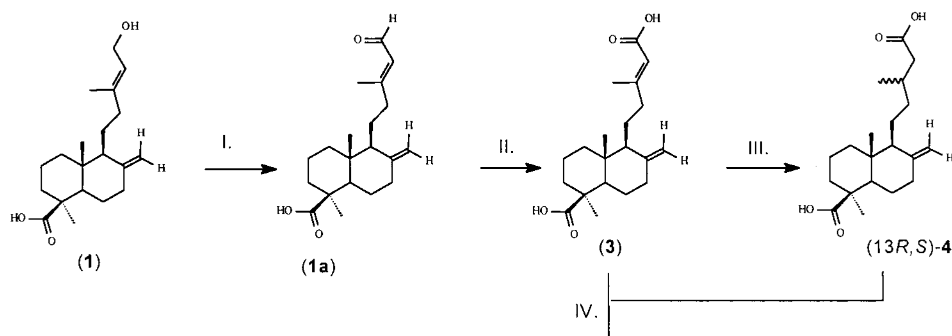
The crude aldehyde (**1a**) (650 mg, 2.0 mmol) was added to a mixture of *tert*-butyl alcohol (25.0 mL) and 2-methyl-2-butene in a 100-mL round-bottomed flask equipped with a condenser and magnetic stirrer, and it was cooled to −5 °C (*17*). Within a 15-min time period, a 1.25 M aqueous solution of KH_2PO_4 (15 mL, 13.8 mmol) was added. Then 10 mL of sodium chlorite solution (1.65 g, 13.8 mM) was added within 5 min. The reaction mixture was stirred for 30 min at −5 °C, and then for 15 h at room temperature. The reaction was quenched by slowly adding dilute HCl (0.7 M) until pH 3 was attained. The solution was extracted with methylene chloride (3 × 25 mL), and the organic layers were combined and dried over $MgSO_4$. Removal of the solvent by rotary evaporation gave 0.68 g (100% yield) agathic acid (**3**), which was identical with authentic compound by GC/MS and ^1H NMR.

Preparation of Tris (Triphenylphosphine) Chlororhodium Catalyst. To a solution of freshly recrystallized triphenylphosphine (6.0 g, 0.6 M excess) in hot absolute ethanol (175 mL) was added a solution of rhodium trichloride trihydrate (1.0 g) in hot absolute ethanol (35 mL), and the mixture was refluxed for 45 min. The hot solution was filtered, and the burgundy-red crystals were washed with degassed diethyl ether (50 mL) and dried under vacuum at room temperature to yield 3.1 g (84% yield); mp 156−158 °C (lit. mp 157−158 °C) (*18*).

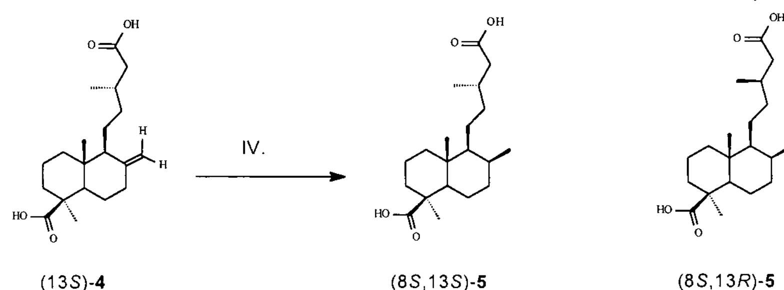
Conversion of Agathic Acid to (13*R*,*S*)-Dihydroagathic Acid (4). Agathic acid (**3**) (140 mg) was added to a 50-mL round-bottomed flask (equipped with a condenser and magnetic stirrer) containing absolute ethanol (14 mL) and sodium metal (800 mg). The reaction mixture was refluxed for 3 h. After the mixture cooled, deionized water (25 mL) was added, and the reaction mixture was extracted twice with methylene chloride (40 mL). The organic portions were combined and dried over Na_2SO_4. The solvent was removed under reduced pressure to give 129 mg (92% yield) of (13*R*,*S*)-dihydroagathic acid. The 13*R*,*S* isomers coeluted on GC/MS analysis with retention time and mass spectrum identical to that of the naturally occurring (13*S*)-dihydroagathic acid (**4**) isolated from *P. ponderosa* needles. Analysis by LC/MS resolved the two compounds with (13*R*)-dihydroagathic acid (Rt 4.19 min) and (13*S*)-dihydroagathic acid (Rt 5.26 min) being identified by comparison of retention time with that of naturally occurring (13*S*)-dihydroagathic acid.

Preparation of (8*S*,13*R*,*S*)-Tetrahydroagathic Acid. To a high-pressure hydrogenation reactor, agathic acid (**3**) (211 mg, 0.65 mmol) and dry benzene (30 mL) were added, and the solution was degassed for 10 min using dry, ultrapure argon. While under argon the tris(triphenylphosphine) chlororhodium catalyst (10 mg; 1.08×10^{-2} mmol) was added (*19*), and dry hydrogen gas was introduced for 3

Scheme 1. Conversion of isocupressic acid (**1**) to (13*R*,*S*)-tetrahydroagathic acid (**5**).



Scheme 2. Conversion of (13*S*)-dihydroagathic acid (**4**) to (13*S*)-tetrahydroagathic acid (**5**).



I. $Pr_4N^+RuO_4^-$ catalytic oxidation (*6*).

II. Sodium chlorite oxidation (*7*).

III. Sodium metal reduction (*4*).

IV. Tris(triphenylphosphine)chlororodium homogeneous hydrogenation (*9*).

Figure 2. Reaction schemes for production of tetrahydroagathic acid (**5**).

min to displace all argon. The pressure of the reaction vessel was increased to 60 psi hydrogen, and then it was placed into an oil bath and the temperature was maintained at 50−55 °C. After 14 h GC/MS analysis (TMS derivative) indicated complete conversion of **3** to **5**. To purify the product from the rhodium catalyst, the solvent was removed at reduced pressure; the residue was dissolved in chloroform (40 mL) and extracted with 0.75 N NaOH (2 × 30 mL). The aqueous layers were combined and adjusted to pH 2 by dropwise addition of 1 N HCl. The aqueous acid solution was then extracted with chloroform (2 × 50 mL). The organic layers were combined, dried with anhydrous Na_2SO_4, and filtered, and the solvent was removed under reduced pressure by rotary evaporation to give 135 mg of (8*S*,13*R*,*S*)-tetrahydroagathic acid (**5**).

The individual 13*R* and 13*S* isomers of **5** were detected by HPLC and were separated by semipreparatory HPLC. Aliquots (10 mg/mL) were injected onto a 250 × 10 mm i.d. C18 reversed-phase Beckman Ultrasphere column and eluted with a mixture of 20 mM ammonium acetate and methanol starting with 50% methanol for 2 min followed by a linear gradient to 100% methanol after 30 min. Fractions (4 mL) were collected throughout the run and then analyzed by APCI negative ion MS. Repeated runs were combined to give 10 mg of a 9:1 enriched 13*S*/13*R* isomer which was identical to that prepared by hydrogenation of naturally occurring 13*S*-dihydroagathic acid, (**Figure 2** - scheme 2) isolated from *Pinus ponderosa* needles by LC/MS analysis.

(8*S*,13*S*)-Labda-15,19-dioic acid (tetrahydroagathic acid) (5). Colorless resin. IR (drifts, KBr) 3400−3000 broad, 2960, 2842, 2677, 1697, 1471, 1411, 1388, 1268, 1182, 946 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 0.72 (3H, s, H-20), 0.85 (1H, m, H-1), 0.89 (3H, d, $J = 7.4$ Hz, H-17), 0.98 (3H, d, $J = 6.7$ Hz, H-16), 1.0 (2H, m, H-3,9), 1.08 (1H, m, H-5), 1.09 (1H, m, H-12), 1.22 (1H, m, H-11), 1.22 (3H, s, H-18), 1.32 (1H, m, H-12), 1.4 (2H, m, H-2), 1.42 (1H, m, H-11), 1.43 (1H, m, H-7), 1.68 (1H, m, H-6), 1.70 (1H, m, H-7), 1.78 (1H, m, H-1), 1.80 (1H, m, H-2), 1.88 (1H, m, H-6), 1.90 (1H, m, H-8), 1.98 (1H, m, H-13), 2.12 (1H, m, H-3), 2.25 (2H, m, H-14). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.7 (CH_3, C-20), 14.8 (CH_3, C-17), 18.8 (CH_2, C-6), 19.0 (CH_2, C-2), 21.5 (CH_3, C-16), 23.7 (CH_2, C-11), 29.0 (CH_3, C18), 29.2 (CH, C-8), 31.1 (CH, C-13), 35.0 (CH_2, C-7), 36.3 (CH_2, C-12), 37.9 (CH_2, C-3), 38.9 (C-10), 39.8 (CH_2, C-1), 41.8 (CH_2, C-14), 43.9 (C-4), 53.3 (CH, C-9), 57.5 (CH, C-5), 180.0 (C=O, C-15), 184.6 (C=O, C-19). Methylabda-15,19-dionate (tetrahydroagathic acid dimethyl ester) was prepared by reaction of (**5**) with idomethane, K_2CO_3, and dimethylformamide, room temperature (1 h), followed by extraction with water and ethyl acetate. The ethyl acetate fraction was dried with Na_2SO_4, and the solvent was removed by evaporation under N_2 flow at 70 °C. HRCIMS (DIP, isobutane), obsd *m*/*z* 367.28567 (MH^+), $C_{22}H_{39}O_4$ requires 366.28483.

RESULTS AND DISCUSSION

Preparation of (13*R*,*S*)-Tetrahydroagathic Acid (5). Isocupressic acid (**1**) was the first choice as the starting material for the preparation of tetrahydroagathic acid (**5**), as it was readily available from previous preparatory extractions of ponderosa pine needles and bark (*8*). The conversion of **1** to **5** (**Figure 2** - scheme 1) proceeded via a two-step oxidation, followed by reduction of both double bonds to yield (13*R*,*S*)-**5**. Lin et al. (*9*) previously used a similar procedure in the preparation of **3** and **4**; however, their use of pyridinium chlorochromate in the initial oxidation of **1** led to an equal mixture of the *E* and *Z* isomers of the corresponding aldehyde (**1a**). The use of the tetrapropylammonium perruthenate oxidation (*16*) followed by the sodium chlorite oxidation method (*17*) maintained the C13, 14 cis configuration in the conversion of **1** to **3**. From **3** it was then possible to selectively reduce the C13,14 allylic double bond to first provide (13*R*,*S*)-dihydroagathic acid (**4**) (step 2a, scheme 1) which then could be further reduced to **5;** or both

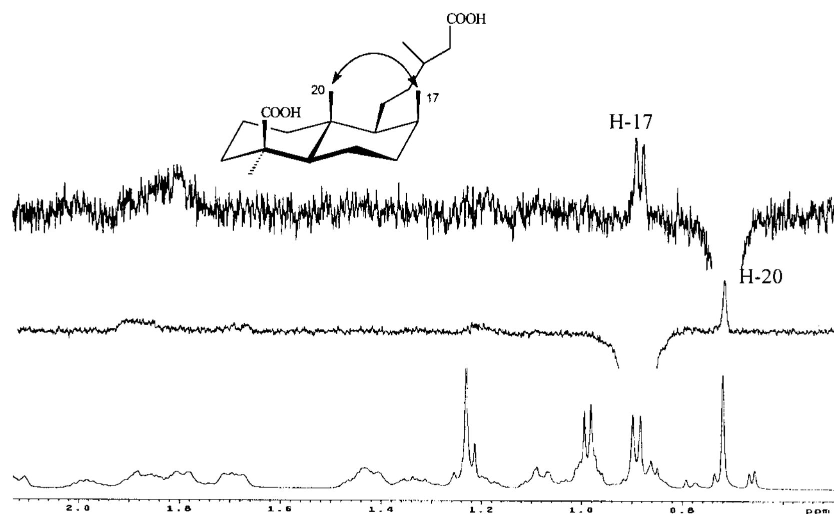


Figure 3. Observed nOe difference spectra and dipolar interaction between C17 and C20 confirming the assignment as the 8*S* isomer.

double bonds of **3** could be reduced in a single step using the homogeneous catalytic hydrogenation procedure (*18, 19*) to produce (13*R,S*)-**5**.

Use of the large tris(triphenylphosphine)chlororhodium catalyst for general hydrogenation was nonspecific for the C13,14 double bond but was stereospecific with respect to the reduction of the C8,17 double bond, producing only the 8*S* isomer and a final mixture of (8*S*,13*R,S*)-tetrahydroagathic acid. The proposed 8*S* configuration was confirmed from the nOe interaction between C-20 and C-17 (**Figure 3**).

A simpler procedure was to start with naturally occurring (13*S*)-dihydroagathic acid, a minor diterpene acid in ponderosa pine needles, and convert it directly to (8*S*,13*S*)-tetrahydroagathic acid by reduction of the C8,17 exomethylene group (scheme 2). Because of a limited supply of (13*S*)-**4** this procedure was only used to provide a few mg of analytical standard material that was useful for later identification of the 13*R* and 13*S* isomers by HPLC/MS.

GC/MS analysis of the TMS derivatives was useful in following each reaction step, but it was not suitable for separating the 13*R,S* isomers. HPLC/MS analysis of products from schemes 1 and 2 produced the expected products as shown in **Figure 4**, and enabled the assignment of the later eluting compound as (13*S*)-**5** by comparison with the single product from scheme 2 and the (13*R,S*) mixture produced by scheme 1.

Identification of (13*R,S*)-Tetrahydroagathic Acid in Bovine Serum. Previous isolation and analysis of bovine serum from animals after consumption of *P. ponderosa*, *Juniperus communis*, or isocupressic acid, identified **3** and **4** as metabolites by direct comparison of GC/MS data (retention time, mass spectra, and coelution) with the natural occurring compounds (*9, 10*). A third metabolite, tetrahydroagathic acid (**5**), was proposed, based on mass spectral data alone, but could not be confirmed at the time because no naturally occurring compounds were available (*10*). After preparation of standards of known configuration we reexamined the serum metabolites to confirm their identity.

A pooled serum sample taken 52−60 h after a cow was dosed with **1** was extracted using the previous described procedures (*10*) and then analyzed by HPLC−MS (**Figure 5**). Analysis of selected ion chromatograms for **4** (*m/z* 394, M + 59) and **5** (*m/z* 396, M + 59), and comparison to the standard compounds confirmed their presence in the serum. Both the 13*R* and the 13*S* isomers were observed for compounds **4** and **5**. Lin et al. (*9*) had previously reported the presence of (13*R,S*)-**4** as a serum

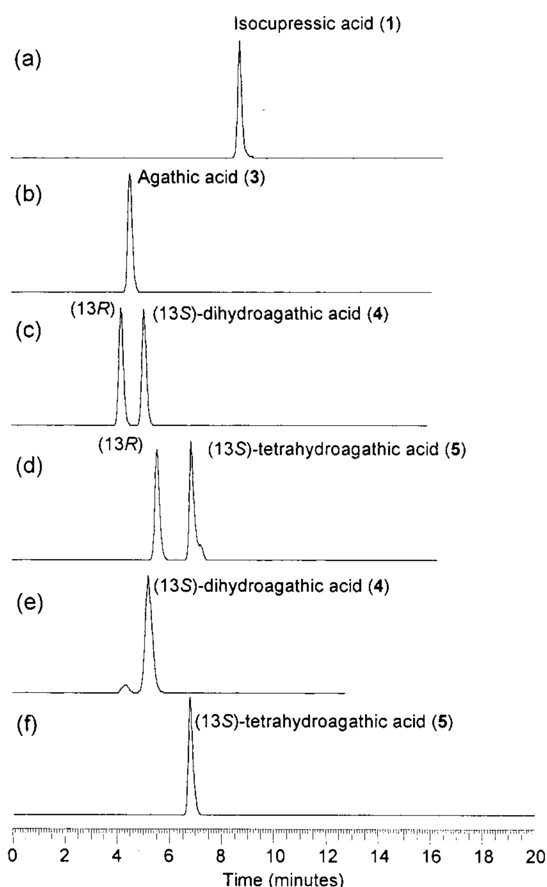


Figure 4. HPLC/MS ion chromatograms for reaction products: (a) starting material; (b) agathic acid (**3**) product prepared from (**1**); (c) dihydroagathic acid product prepared from (**3**); (d) the final product, tetrahydroagathic acid, prepared from (**4**); (e) naturally occurring (13*S*)-dihydroagathic acid isolated from ponderosa pine needles; and (f) (13*S*)-**5** isomer prepared from naturally occurring (13*S*)-**4**.

metabolite of isocupressic acid in vitro and reported the *S/R* ratio was about 2:1. We could directly observe the individual *R* and *S* isomers by HPLC and found *S/R* to be about 3:1, demonstrating that the reductive enzyme is stereoselective but not stereospecific. However, in vivo reduction of C8,17 is stereospecific as only the (8*S*)-**5** isomer was observed.

The physiological role of the serum metabolites of isocupressic acid is not known at this time. The observed

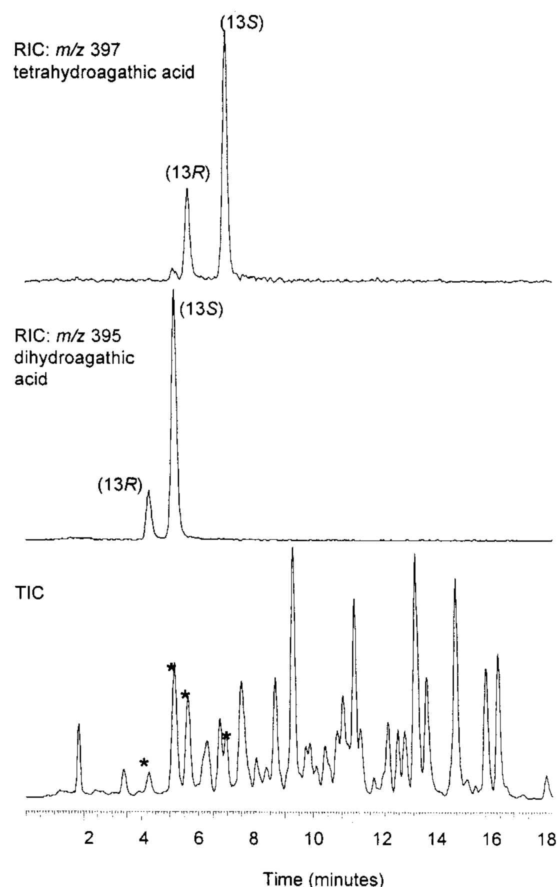


Figure 5. HPLC/MS analysis and detection of (13*R,S*)-dihydroagathic acid (**4**) and (13*R,S*)-tetrahydroagathic acid (**5**) in bovine serum. Figures (a) and (b) are the reconstructed ion chromatograms selected to isolate the $(M - H)^-$ ions for tetrahydoagathic acid (*m*/*z* 397) and dihydroagathic acid (*m*/*z* 395), respectively, and are compared to the total ion chromatogram (c) from which these ions were extracted. (*) Peaks in the total ion chromatogram corresponding to dihydro- and tetrahydroagathic acid.

metabolites might result from a detoxification process, or one or more metabolites could be key compounds in inducing abortions after consumption of ponderosa pine needles by late-term pregnant cattle. Further research is needed to establish the exact role of each of the metabolites. The developed synthetic procedures might be useful in providing sufficient materials for continued animal studies which must be done in cattle because isocupressic acid appears to be not abortifacient in other animal species.

Serum metabolites of isocupressic acid may have diagnostic value as well, and because tetrahydroagathic acid is observed for longer periods of time in the serum than the other metabolites are (*10*), it may be the most important compound to use as a diagnostic marker for pine needle-induced abortion. A recent diagnostic case provides an example. The animals in question had been exposed to pine trees for 2−3 days prior to abortions, and serum samples were taken 1−2 days postabortion (personal communication, Dr. Merl Raisbeck, University of Wyoming, Wyoming State Veterinary Diagnostic Laboratory). Samples were submitted, extracted, and analyzed by GC/MS using methods previously described (*10*). Of the four isocupressic acid metabolites previously identified in experiments with cows fed a controlled diet, only compound **5** was observed in these diagnostic samples taken from actual cattle abortions in the field. Four of the five samples contained higher concentrations of **5** when compared to that of a control sample taken from a test animal at 48-h postdosage (dosed 2.2 kg of ponderosa pine needles) (**Table 1**). The detection of tetrahydroagathic acid in the serum of these five animals confirmed the animals' consumption of isocupressic acid-containing plant material, and the relative concentrations demonstrate a likelihood that the animals consumed quantities sufficient to induced the abortions.

Table 1. Detection of Tetrahydroagathic Acid in Serum from Cattle Reported to Abort after Consumption of Ponderosa Pine Needles

serum sample	THAA (units/g)[a]	DHAA (units/g)[a]
4691-00-6G	10.4	
4691-00-8	35.3	
4691-00-9	60.4	
4691-00-11	5.5	
4691-00-26	32.1	
control[b]	8.3	17.0

[a] THAA = tetrahydroagathic acid (**5**); DHAA = dihydroagathic acid (**4**); units/g = (peak area counts/sample weight) × 10^{-3}. [b] Control serum was taken 48 h post-dosage from test animal treated with 2.2 kg of ground ponderosa pine needles, a dose sufficient to induce abortion in cattle (*5*).

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