Lignans from *Larrea divaricata*

OLE GISVOLD [x] **and EMIL THAKER**

Abstract □ A phytochemical reinvestigation of the phenolics of *Larrea divaricata* led to the isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin. Partially demethylated dihydroguaiaretic acid was detected *via* the use of partially demethylated dihydroguaiaretic acid synthesized from dihydroguaiaretic acid. Techniques are given for the preparation of norisoguaiacin, dihydroguaiaretic acid, and nordihydroguaiaretic acid. Sodium molybdate complexes were used to effect the isolation and preparation of norisoguaiacin. Some biological properties of the phenolic compounds studied are given.

Keyphrases □ *Larrea divaricata*—isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin, biological screening □ Lignans from *L. divaricata*—isolation and characterization of phenolics, biological screening □ Anticancer agents, potential—isolation and characterization of dihydroguaiaretic acid, norisoguaiacin, and 3′-demethoxyisoguaiacin from *L. divaricata*, screening

Previous phytochemical investigations of *Larrea divaricata* resulted in the isolation of nordihydroguaiaretic acid (1) and two incompletely characterized flavones (2). Subsequent to its isolation and characterization, nordihydroguaiaretic acid was used as an antioxidant. A review of its properties has been reported (3) and its activities at the molecular level are outstanding (4, 5).

Several years ago a reinvestigation of the phenolics of *L. divaricata* was initiated to resolve the complex mixture indicated by preliminary TLC. These studies were stimulated by the report (6) that marked regression of a malignant melanoma occurred in an elderly man who had consumed a tea prepared from *L. divaricata* over several months.

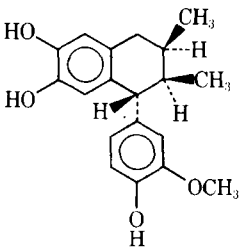
Because nordihydroguaiaretic acid is a lignan, one would suspect that biogenetically related compounds might be present in the phenolics of *L. divaricata*. Four such compounds were isolated: norisoguaiacin (I), a lignan containing one catechol moiety and probably formed from the cyclization of guaiaretic acid followed by partial demethylation or by the cyclization of partially demethylated guaiaretic acid, which is more lipid soluble than nordihydroguaiaretic acid; dihydroguaiaretic acid (II); partially demethylated dihydroguaiaretic acid[1] (III), conceivably a biogenetic intermediate in the biosynthesis of nordihydroguaiaretic acid; and a minor product, 3′-demethoxyisoguaiacin (IV).

EXPERIMENTAL

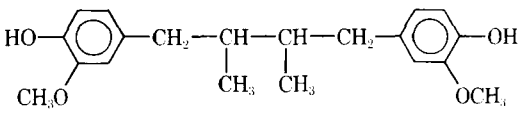
TLC analyses using silica gel were used to follow the isolations and to detect the purity of the isolates. The mobile phase used was benzene–isopropyl alcohol–acetic acid–water (25:5:2:10).

Isolation of Nordihydroguaiaretic Acid—A primary extract of 50 g of the leaves and very small twigs of *L. divaricata*[2] was prepared by shaking overnight with 500 ml of toluene containing 10% ether, 10% isopropyl ether, 10% acetone, 7.5% methyl ethyl ketone, 10% methanol, 10% ethanol, or 7.5% isopropyl alcohol. This primary extract was concentrated under vacuum using a rotovapor pump, a bath temperature of 50°, and a temperature inside the distilling flask of 25°. A final volume of 250 ml was desirable in most cases. During the concentration, considerable amounts of resinous material separated containing most of the degradation products, flavonoid pigments, and some less soluble phenolics. Upon standing or following centrifugation, a clear solution separated and was seeded with nordihydroguaiaretic acid crystallized from toluene. In some cases, nordihydroguaiaretic acid began to crystallize in a few hours; in other instances, this occurred after standing overnight. Even upon prolonged standing, not all of the nordihydroguaiaretic acid crystallized. It may be almost colorless or various shades of yellow, depending on the amount of flavonoids present. The mother liquor also contains the more lipid-soluble phenolics, pigments, waxes, and possibly other lipids.

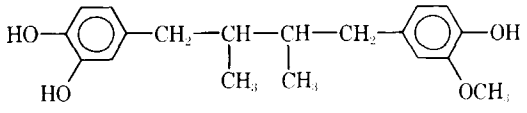
The insoluble substances that separated when the primary extract was concentrated contained considerable amounts of nordihydroguaiaretic acid together with norisoguaiacin, partially demethylated dihydroguaiaretic acid, dihydroguaiaretic acid, yel-



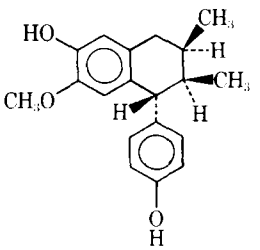
I: norisoguaiacin



II: dihydroguaiaretic acid



III: partially demethylated dihydroguaiaretic acid



IV: 3′-demethoxyisoguaiacin

[1] From a nomenclature standpoint, this new phenolic should be called nordihydroguaiaretic acid and the nordihydroguaiaretic acid of the literature would better be named bisnordihydroguaiaretic acid.

[2] American West Botanical Co., Tucson, Ariz. Voucher specimens have been deposited in the Botany Department, University of Minnesota, Minneapolis, Minn.

low pigments, and, no doubt, minor amounts of other lignans. These substances were dissolved in 30 ml of ether and diluted with 500 ml of toluene. This solution was processed in the same way as was the original primary extract with similar results. This process was repeated as often as necessary to obtain some crystalline nordihydroguaiaretic acid from the toluene concentrate. The amount obtained depended on the source of the original *L. divaricata*.

The NMR spectrum for nordihydroguaiaretic acid tetramethyl ether in deuterated chloroform was as follows: a sharp doublet centering at δ 0.87 (J = 6.5 Hz) and integrating for six protons, a complex multiplet ranging from δ 1.5 to 2.0 and integrating for two methine protons, a multiplet of eight lines ranging from δ 2.04 to 3.04 and integrating for four methylene protons, a sharp singlet at δ 3.85 accounting for 12 methoxy protons, and a multiplet between δ 6.5 and 7.0 and integrating for six aromatic protons. These data support the known structure for nordihydroguaiaretic acid tetramethyl ether.

Preparation of Norisoguaiacin—Toluene solutions of the phenolics of *L. divaricata* from which much of the nordihydroguaiaretic acid had separated were concentrated *in vacuo* to remove all of the solvent. The resinous mass obtained was dissolved in ethylene dichloride and diluted with three parts of ligroin[3] to obtain a saturated solution containing about 1% of phenolics. After standing overnight, 400 ml of this solution, now free of nordihydroguaiaretic acid, was shaken with 2 × 100 ml of 5% sodium molybdate containing 0.4% sodium hydroxide and 3% sodium pyrosulfite[8]. These extractions removed, as red molybdate complexes, all phenolics containing a catechol moiety together with very small amounts of other phenolics that accompanied these aqueous molybdate extractions, probably as a mechanical dispersion. The latter were removed readily in a liquid–liquid extraction apparatus using *n*-hexane[4]. Norisoguaiacin was recovered from its molybdate complex by continuous extraction with ether. This ether extract was decolorized by charcoal; TLC, using silica gel, showed the presence of only one spot. Norisoguaiacin was crystallized directly from this preparation.

Norisoguaiacin is soluble in ether, and the addition of *n*-hexane followed by the slow removal of ether yields a crystalline product, mp 148–149°. It gave a single spot on a silica gel TLC plate and a single peak by GLC, using the trimethylsilyl ethers and a OV-1 column. The IR spectrum showed aromatic bands at 1500 and 1600 cm^{-1} and phenolic hydroxyls at 3500–3600 cm^{-1}. The NMR spectrum in deuterated acetone or deuterated methanol showed a doublet of a CH_3 group centering at δ 0.87 (J = 6.5 Hz), a complex multiplet ranging from δ 1.50 to 3.2, a sharp doublet at δ 3.62 (J = 6.5 Hz), a sharp singlet centering at δ 3.75, a multiplet ranging from δ 6.2 to 6.85, and a sharp singlet at δ 7.18 that can be exchanged with D_2O. The mass spectrum gave a molecular ion peak at 314.

Anal.—Calc. for $C_{19}H_{22}O_4$: C, 72.61; H, 7.00. Found: C, 72.90; H, 6.86. Methoxyl—Calc.: 9.87. Found: 9.87.

Preparation of Norisoguaiacin Trimethyl Ether (Dimethylisoguaiacin)—Norisoguaiacin was methylated in the usual way using potassium carbonate and dimethyl sulfate. The crude methylated product was recrystallized several times from methanol, mp 100–101°, $[\alpha]D^{23}$ −46° (c 5.2% in $CHCl_3$). The NMR spectrum revealed a doublet centering at δ 0.90 (J = 6.5 Hz) and integrating for six protons, a multiplet ranging from δ 1.50 to 3.24 and integrating for four protons, three singlets appearing between δ 3.50 and 4.00 and integrating for 13 protons (*i.e.*, 12 methoxy protons and one dibenzylic proton), and aromatic protons appearing as multiplets between δ 6.24 and 6.95 and integrating for five protons.

Anal.—Calc. for $C_{22}H_{28}O_4$: C, 74.15; H, 7.86. Found: C, 74.25; H, 8.06. Methoxyl—Calc.: 34.83. Found: 34.27.

The physical constants of trimethylnorisoguaiacin agree with those of dimethylisoguaiacin in the literature (9). The NMR data reported are new and support the stereochemistry previously reported for dimethylisoguaiacin.

Norisoguaiacin formed a triphenylurethane derivative when prepared from phenylisocyanate in the usual way, mp 137–139°.

Anal.—Calc. for $C_{40}H_{37}N_3O_7$: C, 71.52; H, 5.55; N, 6.26. Found: C, 71.39; H, 5.78; N, 6.05.

Partial Demethylation of Dihydroguaiaretic Acid—Dihydroguaiaretic acid was partially demethylated with boiling hydrobromic acid (48%). Recovery of the half-demethylated dihydroguaiaretic acid in 40% yield was effected in a manner similar to that used for the preparation of norisoguaiacin, mp 87–88°, $[\alpha]D^{25}$ 0°.

Partially demethylated dihydroguaiaretic acid readily formed a triphenylurethane derivative when prepared in the usual way, mp 198–200°.

Anal.—Calc. for $C_{40}H_{39}N_3O_7$: C, 71.02; H, 5.80. Found: C, 70.62; H, 6.07.

Partially Demethylated Dihydroguaiaretic Acid—The mother liquors obtained from the isolation of crystalline norisoguaiacin yielded some amorphous resinous material. This material, although it gave only one spot on a TLC silica gel plate, refused to crystallize. However, it gave two peaks of about equal intensity by GLC using the trimethylsilyl ethers and an OV-1 column. The retention time of one peak was identical to that obtained for the trimethylsilyl ether of norisoguaiacin and the other was identical to that obtained for the trimethylsilyl ether of the partially demethylated dihydroguaiaretic acid.

Preparation of 3′-Demethoxyisoguaiacin—The ethylene dichloride–ligroin (1:3) solution that had been treated with the aqueous sodium molybdate contained much dihydroguaiaretic acid and what appeared to be a fair amount of a phenolic that was somewhat slower moving on a TLC silica gel plate than was dihydroguaiaretic acid. Preparative thick-layer chromatography, using silica gel and the same mobile phase utilized for the TLC studies, led to the isolation of several hundred milligrams of an ether-soluble phenolic which was crystallized from hot 5% aqueous acetic acid. Fine needles were obtained, mp 96–98°.

Anal.—Calc. for $C_{19}H_{22}O_3$: C, 76.51; H, 7.38. Found: C, 76.40; H, 7.50.

The NMR spectrum for 3′-demethoxyisoguaiacin supported the presence and stereochemistry of a tetrahydronaphthalene moiety that is the same as that found in norisoguaiacin. It also showed the presence of only one methoxy group and two phenolic hydroxy groups.

The mass spectrum showed a molecular ion peak at 298. The fragmentation pattern supported the presence of a methyl group at each of the 2- and 3-positions of the hydroaromatic ring of this lignan because a significant peak was obtained at M−56. This also was the case with norisoguaiacin.

Isolation of Dihydroguaiaretic Acid—The toluene–*n*-hexane (1:2) solutions saturated with the phenolics from which some norisoguaiacin had separated was diluted with *n*-hexane until a ratio of 1 part of toluene to 5 parts of *n*-hexane was obtained. The total volume first used was 1000 ml. It was shaken with 30-ml portions of 5% aqueous sodium carbonate until very small amounts of extractives were obtained. The organic solution then was washed once with 25 ml of 5% potassium biphosphate. Removal of the solvents under vacuum left a resinous mass which, upon standing several days, developed large amounts of crystalline material. This material appeared to be about 95% of one substance by GLC and TLC analyses. This crystalline mass was stirred with a small volume of toluene and *n*-hexane (1:1) and filtered. The crystalline mass was washed on the filter with small amounts of this solvent mixture. This product was recrystallized from ether and *n*-hexane, mp 88° [lit. (10) mp 88°]; the mixed melting point with an authentic specimen of *meso*-dihydroguaiaretic acid showed no depression.

Anal.—Calc. for $C_{20}H_{26}O_4$: C, 72.7; H, 7.9. Found: C, 73.13; H, 8.02.

Dihydroguaiaretic acid formed a diphenylurethane derivative when prepared from phenylisocyanate in the usual way, mp 165°.

Anal.—Calc. for $C_{34}H_{36}N_2O_6$: C, 71.81; H, 6.38; N, 4.93. Found: C, 71.71; H, 6.54; N, 5.14.

Preparation of *meso*-Diethyldihydroguaiaretic Acid—Dihydroguaiaretic acid was ethylated in the usual way using potassium carbonate and diethyl sulfate. Recrystallizations from methanol gave white needles, mp 98–99° [lit. (11) mp 98–99°]. The NMR spectrum revealed a sharp doublet centering at δ 0.87 (J = 6.5 Hz) and integrating for six protons of the two methyl groups in the two ethoxy groups, a complex multiplet ranging from δ 1.5 to 2.0 of the methine protons and overlapped by this triplet, a multiplet of eight lines ranging from δ 2.04 to 3.04 and integrating for four methylene protons, a sharp singlet at δ 3.85 accounting for six

[3] Apcothinner, Apco Oil Corp., Oklahoma City, Okla.
[4] Skellysolve-B.

methoxy protons, a quartet centering at δ 4.08 and partially overlapped by the methoxy resonance at δ 3.85 accounting for four methylene protons of the ethoxy groups, and a multiplet of aromatic protons ranging from δ 6.50 to 7.00 accounting for six protons. These data support the known structure for this compound.

GLC—A gas chromatograph[5] equipped with a hydrogen flame-ionization detector and a circular stainless steel column, 1.8 m (6 ft) long and 0.3 cm (0.12 in.) o.d., was used. The column packing was Chromosorb 40 (60–80 mesh) coated with 3% OV-1; the injection port and flame-ionization detector temperatures were both 310° and the oven temperature was 215°. The carrier gas (nitrogen), hydrogen, and air flow rates were 30, 35, and 500 ml/min, respectively.

The free phenolics of *L. divaricata* could not be used because they decomposed. Their trimethylsilyl ethers were suitable, and silylation of the total phenolics gave at least nine peaks. The peak due to nordihydroguaiaretic acid was the largest, followed by an almost equivalent peak for dihydroguaiaretic acid. The other peaks were progressively much smaller and were due to norisoguaiacin, partially demethylated dihydroguaiaretic acid, probably other biogenetically related lignans, and the flavonoids. Demethylation of the total phenolics followed by silylation and GLC analysis revealed one major peak, nordihydroguaiaretic acid, and a second smaller peak, bisnorisoguaiacin. Similar results were obtained from the completely methylated phenolic resin examined by GLC. These results indicate that, other than the flavonoids, the phenolics of *L. divaricata* have only two carbon skeletons, *i.e.*, the one present in nordihydroguaiaretic acid and the one found in norisoguaiacin. It also indicated that most lignans present contain catechol or partially methylated catechol moieties.

The retention times of the silyl ethers of nordihydroguaiaretic acid, dihydroguaiaretic acid, guaiaretic acid, norisoguaiacin, and partially demethylated dihydroguaiaretic acid are 11.5, 8.6, 9.35, 6.5, and 10.8 min, respectively. Those of tetramethylnordihydroguaiaretic acid and dimethylisoguaiacin are 6.9 and 5.0 min, respectively.

Biological Activity—The minimum inhibitory concentration of norisoguaiacin for *Streptococcus* sp., *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa* was 100 μg/ml in tryptone agar[6]. It is as active against the beef heart mitochondrial NADH oxidase system and succinoxidase system as nordihydroguaiaretic acid (12).

The minimum inhibitory concentration of dihydroguaiaretic acid for *Strep.* sp., *Staph. aureus*, and *B. subtilis* was 10 μg/ml in tryptone soy agar.

Partially demethylated dihydroguaiaretic acid is as active against the beef heart mitochondrial NADH system as is nordihydroguaiaretic acid or norisoguaiacin.

Antioxidant Activity—Using the active oxygen method stability test at 110°, with lard having a stability of 6 hr, 0.01% norisoguaiacin gave a stability of 55.5 hr whereas the stability of nordihydroguaiaretic acid at the same concentration was 73 hr[7].

DISCUSSION

The total phenolics together with small amounts of lipid substances produced by *L. divaricata* were 16% from older plants and 21% from young and vigorously growing plants collected in the vicinity of Tucson, Ariz. This was determined by extracting the dried small twigs and leaves with toluene containing 10% ether.

Norisoguaiacin and partially demethylated dihydroguaiaretic acid are as active as nordihydroguaiaretic acid against the beef heart mitochondrial NADH oxidase system and succinoxidase system as is nordihydroguaiaretic acid. Thus, the biscatechol structure found in nordihydroguaiaretic acid is not an essential requirement for the described activities. Nordihydroguaiaretic acid has been called the "Penicillin of hydroquinones and the most potent cancer antimetabolite in vitro" (4). It also is effective *in vivo* against Ehrlich ascites tumor in mice when combined with ascorbic acid. In view of the reported anticancer activity for nordihydroguaiaretic acid, it is desirable to determine if dihydroguaiaretic acid, norisoguaiacin, 3′-demethoxyisoguaiacin, or partially demethylated dihydroguaiaretic acid possesses similar properties.

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[x] To whom inquiries should be directed.

[5] Perkin-Elmer 900.

[6] S. R. Rolfing, 3M Co., St. Paul, Minn., personal communication.

[7] W. E. Lundberg, Hormel Institute, Austin, Minn., personal communication.