Two Antimicrobial Alkaloids from Heartwood of *Liriodendron tulipifera* L.

C. D. HUFFORD ˣ, M. J. FUNDERBURK *, J. M. MORGAN, and L. W. ROBERTSON

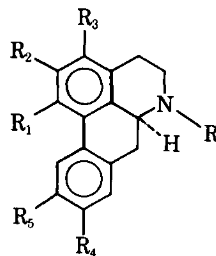
Abstract ☐ Alcoholic extracts of the heartwood of *Liriodendron tulipifera* have demonstrated antimicrobial activity against *Staphylococcus aureus, Mycobacterium smegmatis, Candida albicans,* and *Aspergillus niger.* The antimicrobial activity was associated only with the alkaloidal fraction. Separation of the active alkaloidal fraction by chromatography led to the isolation and identification of dehydroglaucine and liriodenine as the active components. Several other alkaloidal derivatives were prepared and tested. In addition to the active alkaloids, michelalbine was also identified in the tertiary nonphenolic base fraction along with the lignan, lirioresinol-B-dimethyl ether, and two *N*-acetylnoraporphine alkaloids from the nonbasic fraction.

Keyphrases ☐ *Liriodendron tulipifera* L.—isolation and identification of two antimicrobial alkaloids from heartwood ☐ Alkaloids, antimicrobial—isolation and identification of dehydroglaucine and liriodenine from heartwood of *Liriodendron tulipifera* ☐ Antimicrobial activity—dehydroglaucine and liriodenine, isolated and identified from *Liriodendron tulipifera* ☐ Liriodenine—isolated from *Liriodendron tulipifera,* screened for antimicrobial activity ☐ Dehydroglaucine—isolated from *Liriodendron tulipifera,* screened for antimicrobial activity

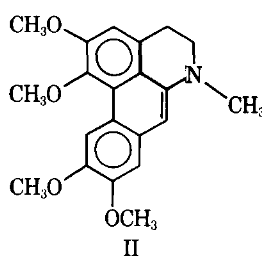
Liriodendron tulipifera (Magnoliaceae), the tulip tree or yellow-poplar tree, is one of two species of the genus *Liriodendron* (1). Chemical constituents isolated from this tree include a lignan diglucoside from the inner bark (2), alkaloids from the heartwood (3–5) and from the leaves (6), cytotoxic sesquiterpene lactones from the root bark (7) and leaves (8), and a cyclitol from the leaves (9).

As part of a program of random screening of higher plants for antimicrobial activity, it was observed that ethanolic extracts of the heartwood of *L. tulipifera* showed consistent and reproducible inhibitory activity against *Staphylococcus aureus, Mycobacterium smegmatis, Candida albicans,* and *Aspergillus niger.*

This paper reports the isolation and identification of the active antimicrobial alkaloids from the heartwood, the testing of several alkaloidal derivatives, and the isolation of two nonbasic aporphine alkaloids and a lignan from the nonbasic fraction of the heartwood.



Ia: R = CH_3, $R_1 = R_2 = R_4 = R_5 = OCH_3$, R_3 = H
Ib: R = $COCH_3$, $R_1 = R_2 = R_3 = OCH_3$, $R_4 + R_5 = OCH_2O$
Ic: R = $COCH_3$, $R_1 = R_2 = OCH_3$, $R_4 + R_5 = OCH_2O$, R_3 = H



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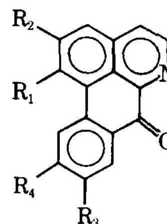
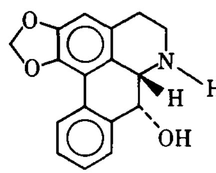


IIIa: $R_1 + R_2 = OCH_2O$, $R_3 = R_4$ = H
IIIb: $R_1 = R_2 = R_3 = R_4 = OCH_3$



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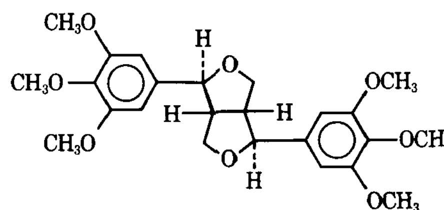

V

EXPERIMENTAL[1]

Plant Material—The yellow heartwood was separated from the sapwood of *L. tulipifera* and air dried before grinding[2]. Other plant parts (leaf, stem bark, root bark, root, fruit, and flower) were collected, but alcoholic extracts showed no antimicrobial activity.

Extraction and Separation of Alkaloids—The air-dried ground heartwood (2.2 kg) was extracted by percolation at room temperature with alcohol USP until a negative alkaloid test of the percolate was observed. Removal of the solvent at reduced pressure and at 40° left 71 g of residue that exhibited antimicrobial activity. A 35-g sample of the alcohol-soluble residue was partitioned between 125 ml each of ether and 2% citric acid. The ether layer was extracted twice more with 125 ml of 2% citric acid, filtered to remove some interfacial solids (5.8 g of alkaloid negative, no antimicrobial activity), dried (sodium sulfate), and evaporated to dryness, giving 8.6 g of ether solubles that had no antimicrobial activity.

[1] Melting points were determined on a Thomas-Hoover Uni-Melt melting-point apparatus and are uncorrected. Elemental analyses were performed by Midwest Microlabs, Ltd., Indianapolis, Ind. IR spectra were run in potassium bromide or chloroform, using a Perkin-Elmer 257 or Beckman IR-33 IR spectrometer. NMR spectra were recorded on a Jeolco C-60-HL spectrometer using deuterated chloroform as the solvent and tetramethylsilane as the internal standard; chemical shifts are reported in δ (parts per million) units. UV spectra were obtained in methanol on a Beckman Acta III spectrophotometer. Optical rotations were determined on a Perkin-Elmer 141 automatic polarimeter. Circular dichroism measurements were performed on JASCO J-40 recording spectrophotometer using methanol as the solvent. Mass spectral data were obtained on a DuPont-CEC 492 spectrometer.

[2] The tree was cut in the summer of 1973 in southern Lafayette County, Miss. The tree was identified by Dr. Maynard W. Quimby and a voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Mississippi.

Table I—Chromatographic Separation of Tertiary Nonphenolic Base Fraction

Fraction Number	Eluent	Weight of Residue, mg	Remarks
1–11	$CHCl_3$	65	Nonalkaloidal, inactive[a]
12–19	$CHCl_3$ 1% CH_3OH in $CHCl_3$	400	Crystalline residue, glaucine, dehydroglaucine, active
20–32	1% CH_3OH in $CHCl_3$	728	Amorphous residue, inactive[a]
33–40	1% CH_3OH in $CHCl_3$	118	Yellow solid, liriodenine, active
41–51	2% CH_3OH in $CHCl_3$	206	Crystalline residue, michelalbine, inactive[a]
52–65	2% CH_3OH in $CHCl_3$	84	Amorphous residue, inactive[a]
66–90	16% CH_3OH in $CHCl_3$	30	Amorphous residue, inactive[a]
Wash	50% CH_3OH in $CHCl_3$	300	Amorphous residue, inactive[a]

[a] No activity was observed against any of the test organisms.

The aqueous citric acid layers were combined, adjusted to pH 9–10 with ammonia, and extracted three times each with 1 liter of chloroform. The aqueous layer was neutralized, and a portion was evaporated to dryness and found to have no antimicrobial activity. The remaining aqueous layer was then acidified with acetic acid; a saturated aqueous solution of ammonium reineckate was added to a portion, but no precipitation occurred. No precipitate was noted with Valser's or Mayer's reagent.

The combined chloroform layers were dried (sodium sulfate) and evaporated to dryness to give 6.2 g of chloroform solubles that showed all of the antimicrobial activity.

The active chloroform-soluble residue (6.2 g) was separated into tertiary phenolic and nonphenolic fractions by dissolving the residue in 250 ml of chloroform and extracting three times each with 250 ml of 5% sodium hydroxide solution. After drying, the chloroform solution was evaporated to leave 4.7 g of tertiary nonphenolic alkaloids that possessed all of the antimicrobial activity.

The combined aqueous solution of the base layers was treated with an excess of ammonium chloride until a cloudy suspension was noted. This suspension was extracted three times with an equal volume of chloroform. The chloroform layer, after washing with water and drying (sodium sulfate), was evaporated to give 1.4 g of tertiary phenolic bases that had no antimicrobial activity.

Isolation of Antimicrobial Alkaloids from Tertiary Nonphenolic Base Fraction—A 2-g portion of the crude nonphenolic base fraction was dissolved in chloroform and chromatographed over 200 g of aluminum oxide (Woelm, neutral, grade III). The solvents used were 300 ml of chloroform, 500 ml of 1% methanol in chloroform, 300 ml of 2% methanol in chloroform, and 400 ml of 16% methanol in chloroform; finally the column was washed with 50% methanol in chloroform. The fractions (20 ml each) were evaporated in tared flasks, combined according to their weights and to their similarity on TLC[3], and then assayed (Table I).

(+)-Glaucine (Ia) and Dehydroglaucine (II) from Fractions 12–19—TLC analyses[3] of these fractions indicated the presence of two alkaloidal constituents. These were obtained pure by chromatography (2.4-g sample compiled from several columns as described previously) over 200 g of silica gel G[4] using ether as the eluent.

The first 125 ml of eluent contained no alkaloids, but the next 150 ml yielded a crude alkaloid (115 mg). This alkaloid was crystallized from alcohol to yield 59 mg of slightly colored plates, mp 113–115°. Subsequent recrystallizations raised the melting point to 121–122° (pale-yellow plates). The mass spectrum exhibited a parent ion at m/e 353. The UV spectrum showed maxima at 260 and 332 nm, while NMR indicated a 1H singlet at δ 9.60 (ArH) and a 3H singlet at δ 3.01 (NCH_3). These data are characteristic of dehydroaporphine alkaloids (10), and the physical data agree with those reported for dehydroglaucine (11). Direct comparison (melting point, TLC[3], UV, and IR) of a sample of dehydroglaucine prepared from glaucine by potassium permanganate oxidation (11) with that obtained from the separation confirmed the identity.

The next 1 liter of eluent yielded no alkaloids; but the following 1 liter yielded the second alkaloid (1.78 g), which was crystallized from alcohol–hexane to yield 0.855 g of needles, mp 119–120°. This was identified as (+)-glaucine by direct comparison (melting point, mixed melting point, TLC[3], IR, and circular dichroism) with an authentic reference sample of (+)-glaucine. (+)-Glaucine was reported previously to be the major alkaloidal constituent of the heartwood (3).

Glaucine methiodide was prepared by stirring a solution of glaucine in acetone with excess methyl iodide. After 30 min the crystalline methiodide was collected, mp 220–221° [lit. (12) mp 219°].

After the glaucine had been eluted, the column was washed with 50% methanol in ether. All fractions were assayed, and only the fraction containing dehydroglaucine was active. Dehydroglaucine was subsequently assayed and was shown to be the antimicrobial agent present in fractions 12–19 (Table II).

Liriodenine (IIIa) from Fractions 33–40—Crystallization of the residue of these fractions (118 mg) from chloroform yielded 85 mg of yellow needles, mp 280–281°. The melting point, IR, and UV data were consistent with that reported for the yellow alkaloid, liriodenine, previously reported from the heartwood (13). Direct comparison (melting point, mixed melting point, IR, and UV) with an authentic sample of liriodenine confirmed the identity. Antimicrobial assay showed liriodenine to be the active component present in these fractions (Table II).

Liriodenine oxime (13) and methiodide (14) were prepared as described in the references and were tested for antimicrobial activity (Table II).

Michelalbine (IV) from Fractions 41–51—Crystallization of the residue in these fractions from chloroform–hexane gave 92 mg of colored needles, mp 191–195°. Recrystallization yielded white needles, mp 199–200°. Chloroform solutions of this alkaloid were observed to undergo air oxidation very easily and become yellow. TLC analyses[3] indicated that liriodenine was formed.

By passing a stream of air through a solution of the alkaloid in *tert*-butanol (15), liriodenine was formed (TLC). Comparison of the physical data of the isolated compound with those of michelalbine (16, 17) indicated them to be the same. Direct comparison (melting point, mixed melting point, TLC[3], UV, IR, and circular dichroism) with an authentic sample of michelalbine confirmed the identity.

Dehydroglaucine (II) and 1,2,9,10-Tetramethoxyoxoaporphine (Oxoglaucine) (IIIb) from Oxidation of Glaucine (Ia)—To a stirred solution of 214 mg of glaucine in 15 ml of acetone was added a solution of 237 mg of potassium permanganate in 25 ml of acetone dropwise over 1 hr. After stirring at room temperature for an additional 4 hr, the suspension was filtered[5] and evaporated to dryness. TLC analysis (silica gel G, ether) showed the presence of mostly dehydroglaucine plus trace amounts of glaucine and oxoglaucine. The dehydroglaucine was obtained pure by chromatography over 10 g of silica gel G[4], using ether as the eluent. Crystallization from alcohol yielded 53 mg of dehydroglaucine, mp 122–123°; m/e 353.162 (calculated m/e for $C_{21}H_{23}NO_4$, 353.163). The physical data agree with that reported previously (11).

In another experiment, a crude fraction (2.4 g, one spot by TLC) of glaucine (in 75 ml of acetone) was oxidized by adding a solution of potassium permanganate in acetone (6 g/450 ml) dropwise over

[3] TLC analyses were carried out on aluminum oxide G-coated plates using 4% methanol in chloroform as the solvent, and the spots were visualized with Dragendorff's reagent.

[4] Silica gel G (for TLC according to Stahl) was slurried with water first, dried at 110° for 12 hr, and sieved through an 80-mesh sieve before use.

[5] Through Celite.

Table II—Antimicrobial Activity of Extracts, Fractions, and Compounds

Sample[a]	Zone Diameter, mm					
	Staph. aureus	*B. subtilis*	*M. smegmatis*	*C. albicans*	*S. cerevisiae*	*A. niger*
Alcohol extract	4	N.T.[b]	10	2	N.T.	5
Tertiary nonphenolic fraction	8	N.T.	12	5	N.T.	9
Fractions 12–19	3	N.T.	2	1	N.T.	—
Fractions 33–40	5	N.T.	10	3	N.T.	10
Liriodenine	5	8	11	3	5	11
Liriodenine methiodide	6	6	14	17	15	4
Dehydroglaucine	4	7	8	6	6	—
Oxoglaucine methiodide	9	9	10	10	11	—

[a] All samples were also tested against *E. coli* and *P. aeruginosa* and were found to be inactive. All other fractions also were assayed, but none showed any activity against any of the microorganisms listed in this table. Glaucine, glaucine methiodide, liriodenine oxime, michelalbine, and oxoglaucine were tested, but none showed any activity against any of the microorganisms. [b] N.T. = not tested.

2 hr, followed by stirring for 6 hr, and then adding another 6 g of potassium permanganate in 450 ml of acetone, followed by stirring for an additional 12 hr. The suspension was filtered[5] and the clear orange solution was evaporated to dryness. Crystallization from a small volume of chloroform yielded 0.578 g of orange needles of oxoglaucine, mp 224–225° dec. Recrystallization yielded orange needles, mp 229–230° dec., of oxoglaucine whose identity was confirmed by a direct comparison (melting point, mixed melting point, TLC[3], and IR) with an authentic sample[6].

Oxoglaucine methiodide was prepared by refluxing oxoglaucine in acetone with methyl iodide as described previously (18).

Chromatography of Nonbasic Fraction—When examined by TLC[7], the ether-soluble fraction indicated the presence of several alkaloidal spots that were different from any of the ones isolated from the basic fraction. The 8.69-g ether-soluble fraction was dissolved in chloroform and chromatographed over 200 g of silicic acid[8], using chloroform with increasing amounts of methanol as eluent. Elution with 2% methanol in chloroform yielded a 2.6-g fraction that was still a mixture. No other column fraction indicated the presence of alkaloids. A portion of this fraction (1.3 g) was further purified by chromatography over 65 g of silica gel G[4], using ether as the eluent. Fractions (8 ml) were collected, analyzed by TLC[7], and then combined.

Fractions 7 and 8 (382 mg) were combined, and crystallization from benzene–hexane gave nearly colorless crystals (200 mg), mp 112–114°. Recrystallization from alcohol two more times gave a pure sample, mp 118–120°. The mass spectrum showed a parent ion peak at *m/e* 446.194 (calculated *m/e* 446.190 for $C_{24}H_{30}O_8$). The formula was also supported by elemental analysis. The UV spectrum showed absorption maxima at λ_{max} 207 (log ϵ 4.79) and 268 (log ϵ 4.16) nm with a shoulder at 230 (log ϵ 4.16) nm. The NMR spectrum was extremely simple for a proton count of 30, suggesting a symmetrical molecule. The physical data were in agreement with the published data for lirioresinol-B-dimethyl ether (V) (2, 19–21), and a direct comparison (melting point, mixed melting point, TLC[7,9], and IR) with an authentic sample confirmed the identity.

Fractions 12–20 (130 mg) were treated with ether, and the crystalline material obtained was recrystallized from benzene–hexane and then alcohol to yield colorless needles (63 mg), mp 216–217°, for which Structure I*b* has been proposed (22).

Fractions 25–35 (217 mg) contained solid material which was crystallized from benzene–hexane and then alcohol to yield 90 mg of white needles, mp 283–284°. This alkaloid has been identified as (+)-*N*-acetylnornantenine (I*c*) (22).

Antimicrobial Assay—Routine qualitative evaluation of antimicrobial activity of extracts, fractions, and pure compounds was accomplished using an agar well diffusion assay. All extracts and fractions were tested for activity against the following microorganisms: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Mycobacterium smegmatis* (ATCC 607), *Pseudomonas aeruginosa* (ATCC 15442), *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763), and *Aspergillus niger* (ATCC 16888). Purified compounds were also tested against *Bacillus subtilis* (ATCC 6633). Bacterial test organisms were cultured in eugonagar[10] and eugonbroth[10], and fungi and yeasts were cultured in mycophil[10] agar and broth.

Plates for assay were prepared by uniformly seeding sterile, partially cooled, molten agar with dilutions of test organisms grown in broth or suspensions of conidia produced on agar slants (*A. niger*). The seeded agar medium was dispensed into 100 × 15-mm sterile petri dishes (15 ml/dish). Cylindrical plugs were removed from the solidified agar plates, using a sterile cork borer, to produce wells having a diameter of approximately 11 mm. Then 100 μl of a solution or suspension of an extract, fraction, or pure compound was added to each well. The extracts and fractions were tested as solutions or suspensions in a concentration of 20 mg/ml; pure compounds were tested at 1 mg/ml. When solvents other than water were required to dissolve extracts or compounds, solvent blanks were run against each test organism.

Plates prepared as described were incubated as follows. Bacteria were grown at 37° for 24 hr before reading, except *M. smegmatis* which grows more slowly and requires incubation at 37° for 48 hr. Fungi or yeasts were incubated at 25° for 24 hr before growth was evident.

Antimicrobial activity was recorded as the width (in millimeters) of the clear zone of inhibition surrounding the agar well. Although no attempt was made to quantitate the diffusion assay rigidly, conditions were held sufficiently constant so that successful fractionation methods were evidenced by an increase in the size of the zones of inhibition as purification was achieved.

Quantitative assay of the pure compounds against selected test organisms involved utilization of a twofold serial dilution in eugonbroth or mycophil broth. The concentration of pure alkaloids in the initial dilution tube was 50 μg/ml. Streptomycin sulfate[11] (50 μg/ml in tube 1) and amphotericin B[12] (50 μg/ml in tube 1) were used as standard antibiotics for comparison with the activities of the alkaloids against bacterial and yeast species, respectively. Cultures used in the serial dilution assay included *Staph. aureus, B. subtilis, M. smegmatis, C. albicans,* and *S. cerevisiae.* The values reported in Table III represent readings taken after incubation times of 24 hr for all organisms except *M. smegmatis,* which was read at 72 hr. The concentration of the tube of highest dilution that was free from growth was recorded as the minimum inhibitory concentration (micrograms per milliliter).

RESULTS AND DISCUSSION

As part of a program of screening higher plants for antimicrobial activity, it was observed that an alcoholic extract of the heartwood of *L. tulipifera* showed inhibitory activity against several microorganisms (Table II). This activity has been traced to the alkaloids, dehydroglaucine (II) and liriodenine (III*a*), by systematic fractionation guided by antimicrobial assay.

Liriodenine previously was reported to be a constituent of the heartwood (12). Liriodenine was found to have a broad antimicro-

[6] Oxoglaucine was reported previously as a naturally occurring alkaloid of the heartwood (3, 13), but none was isolated from this collection. TLC examination showed only traces present.
[7] TLC analyses of this fraction were carried out on silica gel G-coated plates using ether or acetone–methanol–benzene (1:1:8) as the solvent.
[8] Mallinckrodt, 100 mesh.
[9] Lirioresinol-B-dimethyl ether gives a positive test with Dragendorff's reagent.

[10] BBL.
[11] Nutritional Biochemical.
[12] Calbiochem, "A" grade.

Table III—Minimum Inhibitory Concentration (Micrograms per Milliliter) of Active Compounds

Compound	*Staph. aureus*	*B. subtilis*	*M. smegmatis*	*C. albicans*	*S. cerevisiae*
Liriodenine	3.1	0.39	1.56	6.2	6.2
Liriodenine methiodide	6.2	3.1	3.1	0.78	3.1
Oxoglaucine methiodide	25	25	25	1.56	25
Dehydroglaucine	25	25	25	25	50
Streptomycin sulfate	3.1	1.56	0.78	—	—
Amphotericin B	—	—	—	0.78	0.78

bial spectrum, being active against Gram-positive bacteria, an acid-fast bacterium, and yeast-like and filamentous fungi (Table II). Liriodenine has activity comparable to streptomycin sulfate against *Staph. aureus, B. subtilis,* and *M. smegmatis* (Table III) under the same test conditions. Liriodenine methiodide was also prepared and tested. It has a similar spectrum of activity but is more active than liriodenine against *C. albicans* and *S. cerevisiae* and has activity comparable to that of amphotericin B. On the other hand, liriodenine oxime has no activity. It is apparent from the data in Table III that liriodenine and liriodenine methiodide show *in vitro* activity comparable to that of streptomycin sulfate and amphotericin B and thus warrant further investigation of their potential usefulness as antibiotics.

Dehydroglaucine, not previously reported from *L. tulipifera,* showed no activity against *A. niger.* Otherwise, it has a similar spectrum of activity to liriodenine and liriodenine methiodide but is not as potent.

1,2,9,10-Tetramethoxyoxoaporphine (oxoglaucine) (III*b*) has been reported as a constituent of the heartwood (3), but only traces were found[6]. However, oxoglaucine was prepared from glaucine; when tested, it showed no antimicrobial activity. On the other hand, oxoglaucine methiodide is active, particularly against *C. albicans.* Liriodenine methiodide was also more active than liriodenine against *C. albicans.*

Michelalbine (IV) was identified in the tertiary nonphenolic base fraction but showed no antimicrobial activity. Although it is a constituent of other plants of the family Magnoliaceae, this is the first report of it being a constituent of *L. tulipifera.*

The nonbasic fraction of the heartwood was also investigated and a lignan, lirioresinol-B-dimethyl ether (V), was identified along with two new naturally occurring, nonbasic aporphine alkaloids. The structure elucidation of these alkaloids was previously reported (22). One was identified as (+)-*N*-acetylnornantenine (I*b*), and Structure Ic has been proposed for the other.

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