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# ALLELOCHEMICALS IN FOLIAGE OF UNFAVORED TREE HOSTS OF THE GYPSY MOTH, *Lymantria dispar* L.

## 1. Alkaloids and Other Components of *Liriodendron tulipifera* L. (Magnoliaceae), *Acer rubrum* L. (Aceraceae), and *Cornus florida* L. (Cornaceae)

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**Abstract**—Early theories on plant chemical defense against herbivory emphasized that apparent and unapparent plants were primarily defended by different types of compounds. More and more evidence suggests that both quantitative and qualitative defenses are found in apparent plants and that they can play a defensive role against herbivores. A survey of the literature on the gypsy moth suggests not only that there is a large variety of qualitative compounds, as well as the expected quantitative ones, but that unfavored hosts of the gypsy moth are associated with the presence of alkaloids. Foliage of three tree species, *Liriodendron tulipifera* L., *Acer rubrum* L., and *Cornus florida* L., was examined to confirm the presence of alkaloids and other major secondary metabolites. The known sesquiterpene lactone, lipiferolide, and the sugar derivative, liriodendritol, were components of *L. tulipifera* leaves, along with a bisphenylpropanoid previously found only in nutmeg. Alkaloid content [i.e., (−)-*N*-methylcrotsparine content] was low and leaves tested positive for HCN. Leaves of *A. rubrum* L. were examined for the presence of gramine, but none could be detected. No alkaloids were detected in *Cornus florida*.



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**Key Words**—*Lymantria dispar*, Lepidoptera, Lymantriidae, *Liriodendron tulipifera*, *Acer rubrum*, *Cornus florida*, gypsy moth, alkaloids, *N*-methylcrotsparine, 1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane, bisphenylpropanoid.

## INTRODUCTION

The general theory of defensive chemistry of plants developed by Feeny (1976) and Rhoades and Cates (1976) suggests that "apparent plants" rely primarily on "quantitatively acting compounds" for their defense against herbivorous insects, rather than reliance on the "qualitatively acting compounds," which tend to be found in "unapparent plants." The role of alkaloids as defensive compounds is well known (Robinson, 1979; Wink, 1987). In experiments using primarily either unapparent plants or synthetic diets, alkaloids have been shown to be deterrent or toxic to a variety of insect herbivores (Bentley et al., 1984; Miller and Feeny, 1983; Zuniga and Corcuera, 1987; Wink, 1985, 1987). However, there is also evidence now that many apparent plants may gain protection from herbivores by producing qualitative compounds (Miller and Feeny, 1983; Bentley et al., 1984; Lindroth et al., 1986; Scriber et al., 1987; Mattson et al., 1988).

The influence of alkaloid distribution in plants on plant defense and in ecological interactions with other plant species has been discussed for arctic plants (Jung et al., 1979) and for tropical-temperate ecosystem comparisons (Levin, 1976; Levin and York, 1978). However, these reviews provided little or no insight into the relevance of alkaloid distribution to the defense of apparent and unapparent plants. In a survey of the literature, Barbosa and Krischik (1987) showed that many eastern North American tree genera contain many qualitative compounds that typically act as plant defenses. This survey also found that genera that are marginal and unfavored hosts of the gypsy moth (*Lymantria dispar* L.) contain alkaloids more often than favored hosts. If alkaloids are found to be important defenses in apparent plants, this would fail to support the qualitative-quantitative dichotomy suggested by the "plant apparency" theory.

The overall objective of our research is to determine if alkaloids in gypsy moth hosts do provide defense against this herbivore. The initial phase of this research, which is reported here, comprised an attempt to confirm the presence of alkaloids in the leaves of three eastern U.S. tree species that are not favored as hosts by the gypsy moth: tulip tree (*Liriodendron tulipifera* L.), flowering dogwood (*Cornus florida* L.), and red maple (*Acer rubrum* L.). Each of these species has been reported to contain alkaloids (Barbosa and Krischik, 1987). Leaves of the three species were to be used for the isolation, identification, and determination of the concentrations of specific alkaloids. Seasonal changes in

alkaloid content were to be measured so that subsequent experiments using artificial diets to determine the effects of alkaloids on the gypsy moth could be conducted at ecologically relevant concentrations.

Our primary interest was in the tulip tree, from which 40 different secondary metabolites, including many alkaloids, have been reported (Scriber et al., 1987; Ziyaev et al., 1987). One tulip tree alkaloid, glaucine, had been included in a group tested against the gypsy moth, but it was found to be only slightly toxic and did not affect larval consumption rates (Miller and Feeny, 1983). Earlier studies indicated that sesquiterpene lactones from the tulip tree showed antifeedant properties (Doskotch et al., 1981 and references therein). More recently five fractions of varying polarity, from an extraction of tulip tree leaves, were tested for feeding deterrency and their effect on the survival of *Papilio glaucus* larvae (Lindroth et al., 1986). Only one fraction was especially active, and this relatively nonpolar extract was thought to contain the sesquiterpene lactones. The fraction that was alkaloid-containing did not alter performance of the penultimate instar but did cause a moderate decline in neonate survival.

The study reported here extends the research noted above by identifying and quantifying specific alkaloids and other metabolites found in the tulip tree during the period of feeding by the gypsy moth. Since alkaloids have been reported to be present in the unfavored gypsy moth hosts, red maple and flowering dogwood, these species were also investigated for the presence of alkaloids.

## METHODS AND MATERIALS

*Foliage Collections.* Leaves of *Liriodendron tulipifera*, *Acer rubrum*, and *Cornus florida* were collected by hand or using pole pruners, from a woodlot at the Beltsville Agricultural Research Center, USDA. Gypsy moth populations had been low in nearby areas in previous years, and none were observed during sampling for this study. Identified voucher specimens of each species were deposited at the Norton-Brown Herbarium, University of Maryland, College Park.

Two sampling procedures were used. For initial isolation of alkaloids and other compounds, bulk collections were made (e.g., 476 g of air-dried tulip tree leaves in June 1986, a similar bulk sample of dogwood leaves in June 1986, and 2 kg of fresh red maple leaves in September 1987) in which leaves from several trees were combined into single, large samples. To detect chronological trends and tree-to-tree variability in the concentration of these compounds, a more detailed sampling scheme was used. In 1987, trees were sampled weekly, starting on March 29 and extending through the larval development time of the

gypsy moth. Two 15-g foliage samples were taken from each of four trees of each species each week. Leaves of each tree were randomly sampled in such a way that the same area of the tree was never sampled twice. A ring marked with 20 equally spaced compass directions was placed around the base of the trunk. Using a random numbers table, two different compass directions were picked each week. Each 15-g foliage sample was taken from an area of the crown intersecting a plane vertical to one of the chosen compass directions. Each sample was returned to the laboratory in a plastic zip-lock bag and divided into three 5-g subsamples. Each subsample was either air dried for about three days, placed in methanol, or freeze dried.

*Isolation Procedures* (Liriodendron tulipifera). Two separate methods were employed, one for nonalkaloidal material and the other for alkaloids. For nonalkaloidal material, dried and ground leaves were extracted (Soxhlet) with hexane and then chloroform (24 hr each) and the extracts condensed to small, gummy residues in vacuo (in a rotary evaporator). These residues were processed via chromatographic procedures (see Results).

To check for the presence of HCN, we prepared Feigl-Anger test strips (D. Seigler, personal communication; Feigl and Anger, 1966). A 1% (w/v) solution of 4,4-tetramethyldiaminodiphenylmethane in chloroform and a 1% (w/v) solution of copper ethyl acetoacetate in chloroform were prepared. Equal volumes of the two solutions were mixed, and strips of Whatman No. 3MM filter paper were soaked for about 2 min in the mixture and dried. We tested the following materials for HCN: six leaves of tulip tree, two leaves of *Catalpa speciosa* Warder, potassium cyanide, and deionized water. Each leaf was ground individually in a mortar and pestle and placed in a 5.3-ml (3-dram) glass vial with enough deionized water to fill the vial to one third of its volume. Each vial was corked, and a test strip was hung in the vial. After 24 hr, we graded the color of each test strip using the Methuen Handbook of Colour (Kornerup et al., 1984).

For the isolation of alkaloids, sufficient 10% aq. $NaHCO_3$ was added to the plant material to wet it and then a 1:1 1-butanol–toluene solution was added, approximately 10 ml/g of plant. This was stirred well and allowed to stand for 24 hr for large extractions, or for 5 hr with constant stirring for small (5-g) plant samples. The mixture was filtered and the alkaloids extracted into acid. Either 1 M $H_2SO_4$, 1 M HCl, or pH 4 tartaric acid solution was used. The acidic solution was extracted with $CHCl_3$ to remove nonalkaloidal materials and then made basic to pH 9 with $NH_4OH$ or NaOH pellets. This solution was then extracted four times with equal volumes of $CHCl_3$, the $CHCl_3$ solutions were combined, dried over anhydrous $Na_2SO_4$, and the alkaloid residue weighed. Thin-layer chromatography (silica gel, chloroform–methanol 7:3 elution, and iodoplatinate visualization) was used to analyze the mixed alkaloid residue. Although extraction yields of alkaloids were low and highly variable, this var-

iability did not appear to be associated with the method of leaf preservation, i.e., immersion in methanol, freeze drying, or air drying.

*Isolation Studies* (Acer rubrum *and* Cornus florida). The 2-kg sample of *Acer rubrum* (collected in September 1987) was percolated with MeOH–EtOH and the dried extract partitioned repeatedly between 0.5 M HCl and $CHCl_3$. The acid layer was altered to a pH of 9 with 5 M NaOH (in ice) and extracted with $CHCl_3$ (×3). The $CHCl_3$ extract was dried over anhydrous sodium sulfate and evaporated to yield a crude alkaloid fraction (4.4 mg), which showed one low $R_f$ spot by TLC (Silica gel, $CHCl_3$–MeOH 7:3, iodoplatinic acid spray). Analysis of the individual 5-g *Acer rubrum* subsamples, collected from May to June 1987 was undertaken using the same procedures.

A similar large-scale extraction and isolation of an alkaloid residue was attempted on air-dried leaves of *Cornus florida*.

## RESULTS

L. tulipifera *Non-alkaloidal Metabolites*. The hexane extract residue (10 g) from the 476-g bulk sample of dry leaves was chromatographed (flash column, silica gel, hexane followed by increasing amounts of ethyl acetate), and various fractions were examined by TLC and [$^1$H]NMR spectroscopy. Most fractions contained only alkane or lipid-like material, and only the most polar eluting fractions were combined to yield 74 mg of residue. Preparative layer chromatography (silica gel, ethyl ether–chloroform–hexane 3:1:1) was used to isolate a small amount of lipiferolide, **1** (Figure 1), a sesquiterpene lactone (more easily isolated from the chloroform extract; see below) and 40 mg of a yellow oil whose molecular formula was determined to be $C_{23}H_{30}O_6$ by high-resolution mass spectrometry. The [$^1$H]NMR, IR, and UV spectra, were the same as those reported in the literature for the bisphenylpropanoid, **2** (Figure 1), namely, 1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane (Isogai et al., 1973; Forrest et al., 1974). The [$^{13}$C]NMR spectrum, not previously reported, was also consistent with structure **2** (Figure 1): 153.9 and 153.1 ppm (2s; C3, C5, C2′, C6′), 137.3 (d; C$\beta$′), 135.4 and 134.8 (2s; C1, C1′, C4, C4′), 115.7 (t; C$\gamma$′), 107.5 and 106.4 (2s; C2, C3′, C5′, C6), 79.6 (d; C$\beta$), 60.7 (q; C4 OMe), 56.3 and 56.2 (2q; C3, C5, C2′, and C6′ OMe's), 43.7 and 40.4 (2t; $\alpha$ and $\alpha$′), 19.7 (q; C$\gamma$).

When the chloroform extract of the same bulk sample had been evaporated to a small volume, 1.3 g of crystalline material precipitated. This was identified as liriodendritol, i.e., 1,4-di-*O*-methylinositol, **3** (Figure 1), by comparison of mp, [$^{13}$C]NMR and mass spectra with literature values (Angyal and Bender, 1961; Breitmaier and Voelter, 1978). The mother liquor was evaporated to dryness (34.2 g) and the residue partitioned between aqueous methanol and hexane.

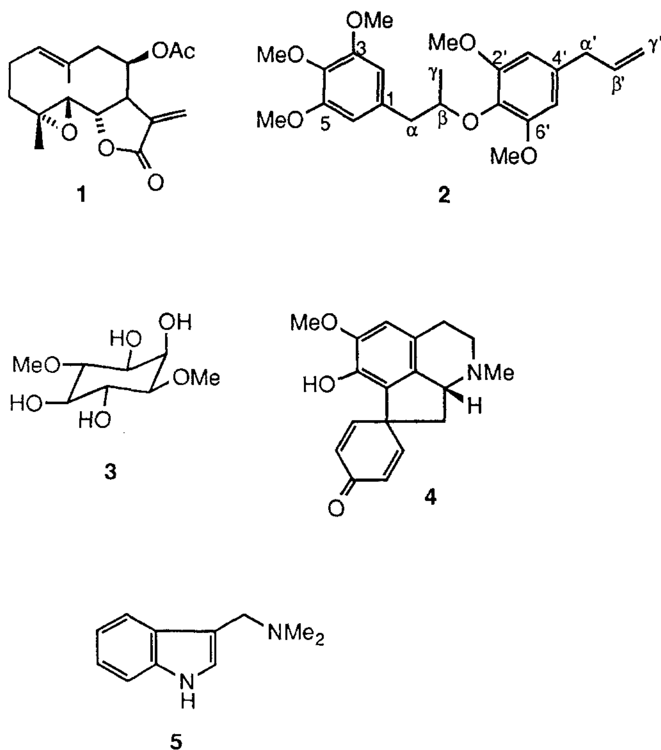


FIG. 1. Components of *Liriodendron tulipifera*: **1** (lipiferolide), **2** [1-(3,4,5-trimethoxyphenyl)-2-(4-allyl-2,6-dimethoxy)propane], **3** (liriodendritol), **4** (*N*-methylcrotsparine); and *Acer rubrum*: **5** (gramine).

The methanol was removed from aqueous methanol layer, and the aqueous solution remaining was extracted with ethyl acetate and then 1-butanol. An additional 491 mg of liriodendritol was recovered from the water and butanol layers. The ethyl acetate layer was evaporated to dryness to leave 2.9 g of residue, which was chromatographed (silica gel, chloroform with increasing amounts of methanol) to yield 158 mg of yellow gum (10% methanol in chloroform fractions) identified as lipiferolide, **1** (Figure 1), by comparison of [$^1$H]- and [$^{13}$C]NMR spectral and optical rotation with those previously reported (Doskotch et al., 1975) and with a standard sample. Additional amounts of the bisphenylpropanoid, **2** (Figure 1), were detected in early fractions from the chromatography. A recent report has described the isolation of this compound from seeds of *L. tulipifera* (Muhammad and Hufford, 1989).

L. tulipifera *Alkaloids.* From the same bulk sample of dried June leaves,

we isolated the proaporphine alkaloid (−)-*N*-methylcrotsparine, **4** (Figure 1), as the major alkaloid. Identification was by optical rotation, mass spectrum, and [$^{1}$H]- and [$^{13}$C]NMR spectra in comparison to values in the literature. Smaller amounts of other alkaloids could be detected by heavy spotting of TLC samples but were not further investigated. The total alkaloid yield varied from 0.01 to 0.08% of the dry weight, depending upon the acid used in the isolation procedure. Use of 0.5 M HCl and rapid extraction seemed to give the best yield, which also occurred with small leaf samples. When large samples were extracted, the procedure was slower, and alkaloids remained in acid for a longer time. Proaporphine alkaloids are acid-sensitive and losses may occur depending upon the acid strength used in the isolation procedure. This might account for our higher yield than that of 0.003% reported by Lindroth et al. (1986), where a somewhat different extraction scheme was used. On the other hand, our results were much lower than the 0.1–0.3% total alkaloids reported by Ziyaev et al. (1975) from leaves at various growth stages. The lability and low concentration of **4** (Figure 1) did not permit our gravimetric analysis method to resolve differences among methods of leaf preservation, samples taken over time, or among trees of a species.

Semiquantitative analyses of the 5-g subsamples from the 1987 sampling scheme (using TLC spot size in comparison to a standard) indicated that (−)-*N*-methylcrotsparine was present during the entire sampling period. This method showed little difference in concentration between air- and freeze-dried samples.

Finally, *L. tulipifera* foliage was found to contain HCN. Tulip tree leaves rapidly caused six test strips to change color, ranging from blue to deep purple [see Methuen Handbook of Colour (Kornerup et al., 1984), p. 18: C8, D8, and E8; and p. 23: C7 and D8], indicating the presence of HCN. The positive control, potassium cyanide, turned deep violet (Kornerup et al., 1984, p. 18: E8). The negative controls, water, and catalpa leaves failed to produce a change in test strips.

*Acer rubrum.* From 2 kg of fresh leaf material, collected in September 1987, we isolated 4.4 mg (less than 0.001% based on dry weight) of gramine, **5** (Figure 1). This is significantly less than the 0.03% reported from fruits of *A. rubrum* (Pachter, 1959) and far less than the 100–200 mg/kg wet weight reported for barley seedlings (Zuniga et al., 1985). No gramine could be detected from any of the weekly leaf subsamples (i.e., the 5-g samples) collected from May to June of 1987, even after TLC application of the entire alkaloid extract. This failure can be attributed to the low concentrations present, which were well below the minimum detectable limits of the TLC technique used.

*Cornus florida.* Although Willaman and Schubert (1961) reported the presence of an unknown alkaloid in leaf and stem tissues, no alkaloids were detected in our large-scale leaf extracts.

## DISCUSSION

*Liriodendron tulipifera.* Although over 40 secondary metabolites have been reported from this tree (Scriber et al., 1987; Ziyaev, 1987), we were able to identify one more, a bisphenylpropanoid, **2** (Figure 1), not previously reported from *L. tulipifera*. As far as we are aware, it has been found previously only in nutmeg (or mace), i.e., in the seeds of *Myristica fragrans* Houtt (Isogai et al., 1973; Forrest et al., 1974). Nutmeg was reported to inhibit growth of silkworm larvae, but it contains many other secondary metabolites in addition to numerous bisphenylpropanoids (Isogai et al., 1973; Hattori et al., 1987; Nakamura et al., 1988).

The compound that we found in large quantity, liriodendritol, was recently shown to be absent in young leaves and synthesized only in fully expanded leaves (Dittrich and Schilling, 1988). Tulip trees break buds early in the eastern United States, and leaves are fully expanded by June. The majority of feeding by gypsy moth larvae is accomplished by the ultimate and penultimate instars, which usually occur in June.

Although we did not quantify HCN content, Hegnauer (1958) reported a concentration of 225–248 mg/kg of "June" leaves and Mirande (1913), 490 mg/kg of "May" leaves. The high cyanide content, at a time when gypsy moth larvae are present, could act as an antifeedant or antibiotic, but this has not been tested (Doskotch et al., 1981; Scriber et al., 1987).

The literature contains many reports on alkaloids of *L. tulipifera*, but nearly all deal with analysis of plant parts other than leaves, in particular of outer wood and heart wood. The main exception is that of Ziyaev et al. (1975), who reported leaf alkaloid content of 27- to 30-year-old *L. tulipifera* at the Botanical Garden of the Uzbek SSR Academy of Science (Tashkent, U.S.S.R.), at the beginning of flowering (0.32%), the end of flowering (0.30%), time of fruiting (0.23%), and yellowing (0.11%). Only the first two sampling periods correspond to the times of our leaf collections, and individual alkaloid isolation was not reported by Ziyaev for these early growth periods, only for the latter two cases. At the time of fruiting, only aporphine alkaloids were identified, whereas yellow leaves contained aporphines along with the *N*-methylcrotsparine reported in this study. The alkaloid glaucine, which was reported by William and Liu (1970), was not reported by Ziyaev et al. (1975), nor did we find it in our study. It is probably only a wood alkaloid and hence of no importance to gypsy moth larval feeding. In one of the few other reports on leaf alkaloids of *L. tulipifera*, Tomita and Furukawa (1962) suggested that glaucine was present in trace amounts, but this was based only on a paper chromatographic test, and the spot was not identified further. Using March leaves, Tomita and Furukawa (1962) also noted a small amount of an unknown phenolic base that was too unstable to isolate.

*N*-Methylcrotsparine is undoubtedly the biosynthetic precursor of most of

the leaf aporphines that occur at later growth stages, and our finding of this alkaloid as the major component in early season leaves is therefore expected. *N*-Methylcrotsparine has been reported to have hypotensive activity (Ishiwatari et al., 1974), and its enantiomer, glaziovine, is reported to have potential as a tranquilizer (Casagrande and Canonica, 1974a,b). Potent antibacterial activity against both gram-positive and gram-negative bacteria was recently reported with an extract of *L. tulipifera* leaves, but the active ingredient was not isolated (Bae and Byun, 1987).

Acer rubrum *and* Cornus florida. Although gramine has been implicated as a deterrent to insect feeding for some plants (Zuniga et al., 1985; Zuniga and Corcuera, 1987), the extremely low level of gramine found in leaves of *A. rubrum* may make it an unlikely deterrent to gypsy moth feeding.

*C. florida* was reported to contain alkaloids in a compilation of literature reports for 3671 species (Willaman and Schubert, 1961). The positive result was part of a survey of over 900 species for the presence of alkaloids (Wall et al., 1959). Individual data from large surveys of this sort nearly always need confirmation before their results can be properly assessed. Alkaloid screening tests that employ $NH_3$ in the procedure can create alkaloids from iridoids artifactually, and this could be the source of false alkaloid tests reported for *C. florida*.

*Cornus florida* has been reported (Hostettmann et al., 1978) to contain molluscicidal saponins. Since the association between the presence of saponins and the unfavorableness of host plants to the gypsy moth approached statistical significance (Barbosa and Krischik, 1987), it is possible that it is the saponin content of *C. florida* that is deterrent. One also should note, however, that the allelochemical category "iridoids" was not included in the Barbosa and Krischik (1987) survey, and the unfavored families Bignoniaceae, Caprifoliaceae, Cornaceae, Ericaceae and Oleaceae are all iridoid-containing. Iridoid glucosides have been found in *C. florida* (Jensen et al., 1975). In addition, in a study similar to that of Barbosa and Krischik (1987), Hanson and Miller (unpublished data) noted that iridoids as well as alkaloids correlated positively with unfavorableness of host plants of the gypsy moth in the western United States.

These results suggest, as a first approximation, that using data on secondary metabolites (such as alkaloids) at the generic level to suggest their presence in any species of the genus (Barbosa and Krischik, 1987) may have some validity, but it is subject to a high margin of error. In general, although alkaloids have been reported to occur in the three species examined, alkaloids were either absent in the leaves of red maple and flowering dogwood or occurred in very low concentrations. Only one major alkaloid was found in early season tulip tree foliage, and the previously reported occurrence of the antifeedant lipiferolide was confirmed. In addition, a new foliage metabolite, the bisphenylpropanoid, **2** (Figure 1), was identified. Future feeding experiments incorporating

some of these potentially important allelochemicals into diets will elucidate their biological activity for the gypsy moth and help determine the relative importance of qualitative defenses in apparent plants.

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