Galloyl Depsides of Tyrosine from Young Leaves of *Inga laurina*

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In addition to the free protein amino acid L-tyrosine, the expanding young leaves of *Inga laurina* accumulate high concentrations of three new depsides, galloyl, *m*-digalloyl, and *m*-trigalloyl L-tyrosine (**1**, **2**, and **3**). The structures of these compounds were determined on the basis of their spectroscopic properties and through degradation and derivatization experiments. They occur in young leaves at the following dry-weight mass percentages: tyrosine, 10.4%; **1**, 3.1%; **2**, 5.0%; **3**, 1.3%. These concentrations are most consistent with chemical defense during the vulnerable expansion stage of leaf development. Neither free tyrosine nor its galloyl depsides are present in mature leaves.

The young leaves of tropical forest trees typically experience far higher rates of herbivory than the mature leaves of the same plant.[1,2] This is because young leaves, unlike mature leaves, cannot be defended by inelastic structural tissues during the expansion phase. As a result, the energy and nutrient content of young leaves is high relative to mature leaves, and they are a preferred food source for many herbivores. In the absence of toughness, young leaves are principally defended by allelochemicals. Selection on leaf defense chemistry, therefore, is strongest during the expansion phase, and innovations in defense chemical expression are more likely to be seen in young rather than mature leaves.

As part of a study of young leaf defense chemistry in tropical forest plants, we are surveying the full range of metabolites with possible defense function in species of the genus *Inga* (Fabaceae: Mimosoideae). *Inga* is a large neotropical taxon, most of whose approximately 300 species are lowland moist-forest trees. We have found highly age-specific modes of chemical defense in *Inga*. For example, the young, expanding leaves of *Inga umbellifera* (Vahl.) Steud. accumulate the protein amino acid L-tyrosine to an average of 10% of leaf dry weight,[3] yet free tyrosine is virtually absent from mature *I. umbellifera* leaves. Such high concentrations of tyrosine in young leaves are most consistent with defense function. In bioassays with larvae of the noctuid moth *Heliothis virescens*, 10% dietary tyrosine reduced larval growth to 2% of controls.[3]

In this paper, we report the structures of a series of L-tyrosine-derived defense chemicals in the young leaves of another *Inga* species. *I. laurina* (L.) Willd., like *I. umbellifera*, accumulates free tyrosine to very high concentrations in young leaves. In addition, it synthesizes mono-, di-, and trigalloyl depsides of tyrosine (**1**, **2**, and **3**). Depsides, in the strict sense, are compounds comprised of two or more aromatic rings bound by a phenolic oxygen−ester linkage. In lichens, a group known for its diverse depside chemistry, the source of these aromatic rings is orsellinic acid. In higher plants, a group with a considerably less elaborate depside chemistry, the most common source of depsidically linked aromatic rings is gallic acid. Polygalloyl depsides are relatively common among plants that synthesize gallotannins having a hexose core. However, in the rare cases where the gallotannin core is aromatic, other depside forms are observed. Examples include salidroside gallates from Fagaceae,[4] 2,4,6-trihydroxyphenylacetic acid gallates from Ericaceae,[5] Papaveraceae,[6] and Myricaceae,[7] and now L-tyrosine gallates from Fabaceae.

In the young leaves of *I. laurina*, tyrosine and its galloyl depsides comprise the great majority of phenolic metabolites. Neither proanthocyanidins, a class of compound that is widespread in *Inga*, nor gallotannins are present. The only other phenolic metabolite that was observed to accumulate to substantial concentration was the flavonoid myricetin 3-*O*-α-(2″-*O*-galloyl)rhamnoside, a compound known from several plant families.[8−11]

For the present study, young leaves of *I. laurina* were collected from understory saplings growing in the Barro Colorado Nature Monument (79°50′ W, 9°10′ N) in the Republic of Panama. Leaves were dried fresh under high vacuum and pulverized before extraction. The tyrosine depsides were found to be unstable in alcoholic solutions, slowly depolymerizing to give tyrosine and alcohol esters of gallic acid. Although extraction in EDTA-saturated alcoholic solutions prevented this degradation, 50% aqueous DMSO was both a more efficient extractor and far less reactive than alcoholic solvents.[12] Separation of the individual depsides was possible only by HPLC using an ODS solid phase with spherical particles. **1** and **2** were isolated as white powders and **3** as a pale pink powder.
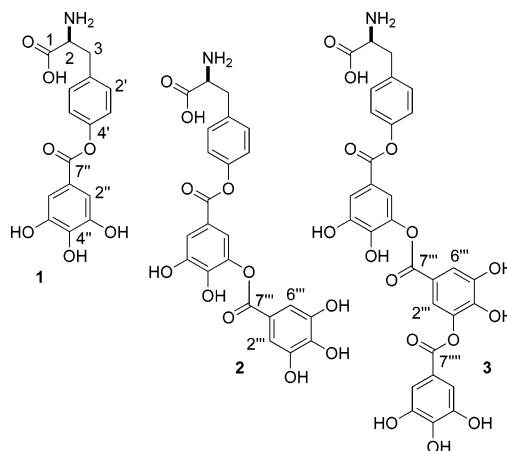
The HRMALDITOF mass spectrum of **1** gave an [M + H]+ ion at *m/z* 334.0921, indicating a molecular formula of $C_{16}H_{15}NO_7$. The ^{1}H NMR spectrum showed three resonances in the aromatic region, a pair of 8.0 Hz two-proton doublets (δ 7.39 and 7.15) and a two-proton singlet (δ 7.15). This indicated that the compound contained two symmetrical aromatic ring systems, one with one pair and one with two pairs of equivalent protons. The ^{1}H NMR spectrum additionally showed a spin system comprised of one sp[3] methine and two sp[3] methylene protons. Analysis of HSQC and HMBC experiments showed that all three sp[3] protons correlated with a

carboxyl carbon (δ 174.0) and a quaternary aromatic carbon (δ 135.2). The latter carbon had correlations with the aromatic doublet at δ 7.15. The sum of these data was consistent with the presence of a substituted tyrosine moiety. The substituent then had an implied molecular formula of $C_7H_5O_4$. The symmetrical ring requirement meant this moiety was either a phloroglucinol carboxylic acid or a gallic acid. The determination was made by dissolving a sample of **1** in MeOH and allowing it to degrade at room temperature. The degradation resulted in a brownish supernatant over a white precipitate. The precipitate was removed by centrifugation and the supernatant was dried at reduced pressure to give a brown powder. Both solids were analyzed by NMR and MS. The precipitate was identified as tyrosine by its LRESI mass spectrum (m/z 180.1 [M − H]$^-$) and by comparison of its ^{1}H and ^{13}C NMR spectra to those of a commercial standard (Aldrich). The remaining soluble component was identified as the methyl ester of gallic acid by its LRESI mass spectrum (m/z 182.8 [M − H]$^-$) and by comparison of its ^{1}H and ^{13}C NMR spectra to a commercial standard (Aldrich). Collectively, these data showed that **1** was the galloyl ester of tyrosine.

The HRMALDITOF mass spectra of compounds **2** and **3** gave [M + H]$^+$ ions at m/z 486.1047 and 638.1178, indicating molecular formulas of $C_{23}H_{19}NO_{11}$ and $C_{30}H_{23}NO_{15}$, respectively. Analysis of the ^{1}H NMR spectra of both **2** and **3** showed the characteristic three-proton sp^3 spin system and two-proton aromatic doublets indicative of a tyrosine moiety. The empirical formulas, however, as well as the 152 Da mass increments, suggested the presence of an additional one and two gallic acid moieties, respectively, in comparison to **1**. The monomeric components of the compounds were identified through degradation experiments in MeOH, which again gave only tyrosine and methyl gallate. Analysis of 1D ^{1}H and ^{13}C NMR spectra showed the presence of only one symmetrical pyrogallol ring in each compound. On the other hand, HMBC showed the presence of one and two asymmetrical pyrogallols in **2** and **3**, respectively. This indicated that the compounds were di- and trimeric *meta*-linked esters of gallic acid, respectively, conjugated with tyrosine.

Owing to the low density of nonexchangeable protons in **2** and **3**, indirect detection NMR methods were of little use in identifying the phenolic carbon through which the *m*-galloyl linkages occurred. Instead, this determination was made by exploiting the β-isotope effect[13,14] on carbons that are bound to elements with exchangeable protons. A small but measureable change in the ^{13}C NMR shift is observed when one β-hydrogen isotope is substituted for another. If no substitution is possible, i.e., where an ester or other linkage occupies the exchange site, little or no change in shift occurs when the hydrogen isotopes are switched. To make this observation, a second 1D ^{13}C NMR spectrum was acquired under experimental conditions identical with the first except that the solvent employed was CH$_3$OH in place of CD$_3$OD. For compound **2**, $\Delta\delta$ ^{13}C at C″-3, 4, and 5 was 0, −0.42, and +0.10 ppm, respectively. From these data, we concluded that the ester linkage between gallate moieties occurs at C″-3. $\Delta\delta$ ^{13}C at the tyrosine phenolic carbon was also 0 ppm, indicating that the galloyl linkage to tyrosine is an ester involving the phenolic carbon. For compound **3**, assignments of the β-ester phenolic carbons were made by comparison to compound **2**, in which the phenolic ^{13}C NMR resonances were all closely analogous.

The enantiomeric composition of *I. laurina* leaf tyrosine was determined by HPLC following conversion of the metabolite to Marfey diastereomers.[15] As with the leaf tyrosine isolated from *I. umbellifera*, *I. laurina* leaf tyrosine was comprised entirely of the L-enantiomer.

The average mass percentage of tyrosine metabolites in young, shade *I. laurina* leaves was determined using HPLC. Detector response curves for tyrosine and its three depside forms were generated with standard solutions prepared from the purified metabolites. Analysis of a combined sample (young leaves, 20−

90% of full expansion from 13 different trees) gave the following mean dry-weight mass contents of tyrosine and depsides: tyrosine, 10.4%; **1**, 3.1%; **2**, 5.0%; **3**, 1.3%. When corrected for gallic acid mass content, the mean dry-weight mass percentage of tyrosine in the young leaves of *I. laurina* is 14.4. This analysis shows that the young leaves of *I. laurina* understory trees accumulate approximately the same concentration of free tyrosine as do the young leaves of *I. umbellifera*. However, the total average amount of tyrosine present is ca. 4% higher due to the quantity of conjugated tyrosine. One possible explanation for this is that the derived tyrosine metabolites have evolved as a mechanism to increase the total leaf tyrosine concentration, with the result that herbivore deterrence is increased. HPLC analysis of mature leaf material from understory *I. laurina* plants was unable to detect tyrosine or the tyrosine depsides. Thus, like its congener *I. umbellifera*, tyrosine hyperproduction in *I. laurina* is only active during the leaf expansion phase. The same is true for the other major phenolic metabolite, myricetin 3-*O*-α-(2″-*O*-galloyl)rhamnoside, which is also absent in mature leaves. This work provides another example of tight phenological control of defense chemistry. To date, we have investigated the young leaf chemistry of 11 *Inga* species from lowland Panama. Only two of these species accumulate tyrosine and/or tyrosine gallates. They are the first reported examples of a hyperproduced protein amino acid (or derivative) with putative defense function.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. UV absorbance was measured on a Cary Conc 50 UV−vis spectrometer (Varian, Walnut Creek, CA). IR spectra were acquired on a Nicolet 560 FTIR instrument. HPLC analyses were carried out on a Hitachi LaChrom Elite system (Hitachi, San Jose, CA) with a diode array detector. Depside leaf content and tyrosine chirality assays were run on Varian Omnisphere and Varian Microsorb 5 μm ODS analytical (4.6×250 mm) columns, respectively. Compound purifications were carried out using an Omnisphere 5 μm ODS semipreparative (10×250 mm) column. NMR spectra were acquired using a Varian Inova 500 MHz instrument with the probe temperature maintained at −30 °C. Samples were dissolved in CD$_3$-OD (CH$_3$OH for the β-isotope experiment), and signals were referenced to TMS (0.0 ppm). HRMALDITOF mass spectra were obtained on a Voyager DE-STR instrument (MDS Sciex, Concord, ON) in acetonitrile/CHCA.

Plant Material. *Inga laurina* leaves were collected at Barro Colorado Nature Monument, Republic of Panama, a field station run by the Smithsonian Tropical Research Institute (STRI). Plant identification was made by one of the authors (P.D.C.). Voucher specimens are maintained at the STRI Herbarium (#6405/7584). Young, shade leaves, 20−90% of full expansion, were harvested from 13 individual understory saplings. Leaves were dried under high vacuum without freezing and then returned to the University of Utah for analysis.

Leaf Extraction and Depside Isolation. Young leaves were combined and pulverized *en masse* using a Retsch MM 200 mill (Retsch GmbH & Co., Haan, Germany). Then 50 mg portions of powdered leaf tissue were extracted with 2 mL of 50% aqueous DMSO at 80 °C for 15 min and injected directly onto the semipreparative column. The mobile phase consisted of MeOH (A) in H$_2$O (B) with a constant flow rate of 3.25 mL/min. The following set of linear gradients was used: 0 min, 22% A in B; 12 min, 35% A in B; 18 min, 60% A in B; followed by a return to initial conditions. Tyrosine, **1**, **2**, **3**, and myricetin 3-*O*-α-(2″-*O*-galloyl)rhamnoside eluted at 4.4, 12.4, 17.4, 19.9, and 23.2 min, respectively. The depside fractions were stored on dry ice until the MeOH was removed under reduced pressure. The fractions were then frozen and lyophilized. The extraction process was repeated until sufficient mass for spectroscopic analysis and other experiments was obtained.

Tyrosine Metabolite Quantification. Portions of the purified tyrosine metabolites were used to generate calibration curves. Four-point curves were prepared over a concentration range of 0.0625−0.25 mg/mL for tyrosine and **3** and 0.25−1.0 mg/mL for **1** and **2**. Data points were based on peak areas of the 275 nm trace which was

extracted from the diode array. The r^2 values were ≥ 0.998 in each case. For purposes of quantifying free leaf tyrosine, three separate 5.0 mg portions of the ground material described in the previous section were extracted with 2.0 mL of H_2O at 80 °C for 10 min. For the depside quantifications, the same extraction was repeated with 20.0 mg portions of powdered leaf and 2.0 mL of 50% aqueous DMSO. Repeat extractions showed that, in each case, these procedures removed >98% of the metabolites of interest in a single extraction. Leaf content of each metabolite was determined by reference to the standard curves.

Tyrosine Chirality Analysis. The configuration of *I. laurina* leaf tyrosine was determined following treatment with Marfey's reagent.[15] An MeOH extraction of leaf tissue was allowed to degrade to tyrosine and the methyl gallate. Tyrosine was removed by centrifugation. The extracted plant tyrosine as well as reagent L- and DL-tyrosine (Sigma-Aldrich) were converted to their respective diastereomers and analyzed by HPLC. The derivatives were separated using a mobile phase of 0.1% v/v TFA/H_2O (A) and acetonitrile (B) at 1 mL/min. A linear gradient elution of 90−40% A in B over 40 min resolved the L- and D-tyrosine derivatives, which eluted at 38.5 and 39.8 min, respectively.

Galloyltyrosine (1): white powder; $[\alpha]^{22}_D$ −18.9 (*c* 0.003, MeOH); UV (MeOH) λ_{max} (log ϵ) 216 (sh) (3.72), 286 (3.26) nm; IR (KBr) ν_{max} 3433, 1710, 1623, 1509, 1452, 1400, 1348, 1314, 1219, 1200 cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) δ 7.39 (2H, d, *J* = 8.5 Hz, H-2′, H-6′), 7.15 (2H, d, *J* = 8.5 Hz, H-3′, H-5′), 7.15 (2H, s, H-2″, H-6″), 3.79 (1H, dd, *J* = 9.5, 3.9 Hz, H-2), 3.35 (1H, dd, *J* = 15.0, 3.9 Hz, H-3), 3.01 (1H, dd, *J* = 15.0, 9.5 Hz, H-3); ^{13}C NMR (CD$_3$OD, 125 MHz) δ 173.8 (COO, C-1), 167.3 (COO, C-7″), 151.6 (C, C-4′), 146.6 (2C, C-3″, C-5″), 140.5 (C, C-4″), 135.0 (C, C-1′), 131.6 (2CH, C-2′, C-6′), 123.6 (2CH, C-3′, C-5′), 119.9 (C, C-1″), 110.1 (2CH, C-2″, C-6″), 57.4 (CH, C-2), 37.5 (CH$_2$, C-3); HRMALDITOF *m/z* 334.0921 [M + H]$^+$ (calcd for C$_{16}$H$_{16}$NO$_7$, 334.0927).

Digalloyltyrosine (2): white powder; $[\alpha]^{22}_D$ −28.6 (*c* 0.002, MeOH); UV (MeOH) λ_{max} (log ϵ) 217 (sh) (3.80), 277 (3.32), 298 (sh) (3.20) nm; IR (KBr) ν_{max} 3433, 1719, 1623, 1514, 1443, 1385, 1362, 1195 cm^{-1}; ^{1}H NMR (CD$_3$OD, 500 MHz) δ 7.49 (1H, br s, H-2″), 7.41 (1H, d, *J* = 1.9 Hz, H-6″), 7.39 (2H, br s, H-2′, H-6′), 7.20 (2H, m, H-3′, H-5′), 7.19 (2H, m, H-2‴, H-6‴), 3.80 (1H, m, H-2), 3.35 (1H, m, H-3), 3.01 (1H, m, H-3); ^{13}C NMR (CD$_3$OD, 125 MHz) δ 173.9 (COO, C-1), 166.6 (COO, C-7″), 166.3 (COO, C-7‴), 151.6 (C, C-4′), 147.8 (C, C-5″), 146.5 (2C, C-3‴, C-5‴), 145.7 (C, C-4‴), 140.3 (C, C-4″), 140.0 (C, C-3″), 135.0 (C, C-1′), 131.6 (2CH, C-2′, C-6′), 123.6 (2CH, C-3′, C-5′), 119.9 (C, C-1‴), 119.5 (C, C-1″), 117.9 (CH, C-2″), 114.9 (CH, C-6″), 110.2 (2CH, C-2‴, C-6‴) 57.4 (CH, C-2), 37.5 (CH$_2$, C-3); HRMALDITOF *m/z* 486.1047 [M + H]$^+$ (calcd for C$_{23}$H$_{20}$NO$_{11}$, 486.1037).

Trigalloyltyrosine (3): pink powder; ^{1}H NMR (CD$_3$OD, 500 MHz) δ 7.54 (1H, d, *J* = 2.0 Hz, H-6‴), 7.50 (1H, d, *J* = 2.0 Hz, H-6″), 7.44 (1H, d, *J* = 2.0 Hz, H-2″), 7.43 (1H, d, *J* = 2.0 Hz, H-2″), 7.38 (2H, d, *J* = 8.5 Hz, H-2′, H-6′), 7.20 (2H, m, H-3′, H-5′), 7.19 (2H, m, H-2‴, H-6‴), 3.79 (1H, m, H-2), 3.38 (1H, dd, *J* = 15.0, 3.1 Hz, H-3), 3.01 (1H, dd, *J* = 15.0, 10.0 Hz, H-3); ^{13}C NMR (CD$_3$OD, 125 MHz) δ 173.8 (COO, C-1), 166.5 (COO, C-7″), 166.4 (COO, C-7‴), 165.6 (COO, C-7‴), 151.7 (C, C-4′), 147.8 (C, C-5‴), 147.6 (C, C-5″), 146.5 (2C, C-3‴, C-5‴), 145.2 (C, C-4‴), 145.2 (C, C-4″), 140.4 (C, C-4‴), 140.0 (C, C-3‴), 139.9 (C, C-3″), 135.0 (C, C-1′), 131.6 (2CH, C-2′, C-6′), 123.6 (2CH, C-3′, C-5′), 120.0 (C, C-1‴), 120.0 (C, C-1‴), 120.0 (C, C-1″), 117.9 (CH, C-2‴), 117.9 (CH, C-2″), 115.0 (CH, C-6″), 114.9 (CH, C-6‴), 110.3 (2CH, C-2‴, C-6‴), 57.4 (CH, C-2), 37.5 (CH$_2$, C-3); HRMALDITOF *m/z* 638.1178 [M + H]$^+$ (calcd for C$_{30}$H$_{24}$NO$_{15}$, 638.1147).

References and Notes

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