

Synthesis and Characterization of *N*-Demethylated Metabolites of Malachite Green and Leucomalachite Green

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Received November 27, 2002

Malachite green (MG), a triphenylmethane dye used to treat fungal and protozoan infections in fish, undergoes sequential oxidation to produce various *N*-demethylated derivatives (monodes-, dides(sym)-, dides(unsym)-, trides-, and tetra-des-) both before and after reduction to leucomalachite green (LMG). The close structure resemblance of the metabolites with aromatic amine carcinogens implicates a potential genotoxicity from exposure to MG. The availability of the synthetic standards is important for metabolic and DNA adduct studies of MG. This paper describes a simple and versatile method for the synthesis of MG, LMG, and their *N*-demethylated metabolites. The synthesis involves a coupling of 4-(dimethylamino)-benzophenone or 4-nitrobenzophenone with the aryllithium reagents derived from appropriately substituted 4-bromoaniline derivatives, followed by treatment with HCl in methanol. The resulting cationic MG and their leuco analogues showed systematic UV/vis spectral and tandem mass fragmentation patterns consistent with sequential *N*-demethylation. The extensive ¹H and ¹³C spectral assignments of the metabolites were aided by the availability of ¹³C₇-labeled MG and LMG. The results indicate the existence of a resonance structure with the cationic charge located in the central methane carbon (C₇). The synthetic procedure is general in scope so that it can be extended to the preparation of *N*-demethylated metabolites of other structurally related *N*-methylated triphenylmethane dyes.

Introduction

MG¹ (Figure 1), a member of the *N*-methylated triphenylmethane class of dyes, has long been used to control fungal and protozoan infections in fish (1, 2). Although prohibited in the U.S., the antifungal use of MG in commercial fisheries has continued worldwide due to its ready accessibility and low cost (3).

After treatment, MG is readily reduced in the tissues of fish to the corresponding base, LMG (Figure 1) (3). Because of its lipophilic nature, LMG excretes very slowly (*t*_{1/2} ~ 40 days) in certain fatty fish tissues. A similarly persistent accumulation of LMG has been documented in mammalian systems. MG and LMG are cytotoxic to both bacterial and mammalian cells but showed no significant mutagenic and clastogenic activities regardless of the presence of S9 activation (4, 5). MG has been reported to be a tumor promoter, but very little is known about the carcinogenicity of MG (6), although a structural

analogue, gentian violet, has been shown to induce liver tumors in mice (7). Thyroid peroxidase-catalyzed oxidation of LMG results in the formation of various *N*-demethylated primary and secondary aromatic amines, whose structures are similar to aromatic amine carcinogens (8). These facts, together with our recent findings of DNA adducts in MG-treated rats, imply a potential genotoxicity from exposure to MG (9). Because of concerns regarding its use in commercial fisheries, MG was designated by the National Toxicology Program as a priority chemical for carcinogenicity testing, and results from a 2 year feeding study are currently being evaluated.

The metabolic fates of MG and LMG have been studied both in vivo and in vitro. In rats and mice, MG undergoes systematic sequential oxidation to produce *N*-demethylated metabolites both before and after reduction to LMG (Figure 1) (9). Small amounts of the *N*-oxides derivatives of MG and monodes-MG have also been detected. Similar metabolic profiles have been observed in incubations of MG and LMG with the microsomal fractions of intestinal microflora (10) and the fungus *Cunninghamella elegans* (11) as well as thyroid peroxidase-catalyzed reactions (8) of LMG. The availability of these metabolites as standards is necessary for future DNA adduct studies and for use as biomarkers in metabolism studies.

We have previously reported the preparation of the 4'-amino and 4'-nitro derivatives of *N,N*-dimethylamino-triphenylmethane as precursors for presumed DNA ad-

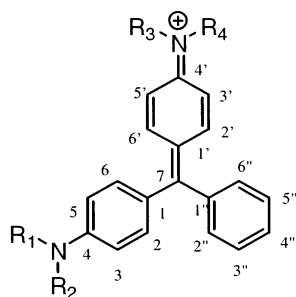
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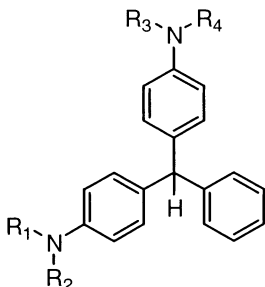
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¹ Abbreviations: APCI, atmospheric pressure chemical ionization; COSY, correlation spectroscopy; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; HRMS, high-resolution mass spectrometry; LMG, leucomalachite green; MG, malachite green; NOE, nuclear Overhauser effect.



	\underline{R}_1	\underline{R}_1	\underline{R}_3	\underline{R}_4
MG	CH ₃	CH ₃	CH ₃	CH ₃
Monodes-MG	CH ₃	CH ₃	CH ₃	H
Dides(sym)-MG	CH ₃	H	CH ₃	H
Dides(unsym)-MG	CH ₃	CH ₃	H	H
Trides-MG	CH ₃	H	H	H
Tetrades-MG	H	H	H	H



	\underline{R}_1	\underline{R}_1	\underline{R}_3	\underline{R}_4
LMG	CH ₃	CH ₃	CH ₃	CH ₃
Monodes-LMG	CH ₃	CH ₃	CH ₃	H
Dides(sym)-LMG	CH ₃	H	CH ₃	H
Dides(unsym)-LMG	CH ₃	CH ₃	H	H
Trides-LMG	CH ₃	H	H	H
Tetrades-LMG	H	H	H	H

Figure 1. Structures of MG, LMG, and their *N*-methylated derivatives.

ducts of MG (12). Here, we describe a simple and versatile synthesis of MG and LMG and their *N*-demethylated metabolites (Figure 1). We also provide a detailed spectral characterization of all of the compounds.

Experimental Procedures

Materials and Methods. THF was distilled fresh from sodium/benzophenone prior to use. ¹³C-labeled benzaldehyde was purchased from Isotec (Miamisburg, OH). All reagents and solvents were purchased from Aldrich, Inc. (Milwaukee, WI). Melting points were determined on a Thermoelectrical melting point apparatus and are uncorrected. Elemental analyses were obtained from M-H-W Laboratories, Phoenix, AZ. HRMS were obtained in the University of Illinois Mass Spectrometry Laboratory, School of Chemical Science, Urbana-Champaign, IL. ¹H and ¹³C NMR spectra were recorded on a Bruker AM500 spectrometer operating at 500 and 126 MHz, respectively. Chemical shifts are expressed in ppm with respect to the internal standard tetramethylsilane. Analytical HPLC analyses were conducted with a photodiode array detector using a Spherisorb column (CN, 4.6 mm × 250 mm, 5 μm) with a 30

min gradient, 10–90% acetonitrile/100 mM ammonium acetate, pH 4.5, 1 mL/min.

LC/MS. The HPLC separation for mass spectrometry was performed using a Prodigy ODS-3 column (5 μm, 4.6 mm × 250 mm, Phenomenex Co., Torrance, CA) at a flow rate of 1.0 mL/min, with a mobile phase gradient consisting of 50% acetonitrile and 50% aqueous ammonium acetate (20 mM, pH 4.5) to 100% acetonitrile over a 10 min linear ramp period delivered by a GP40 pump (Dionex Co., Sunnyvale, CA). The entire mobile phase flow was introduced into the mass spectrometer. For direct infusion studies, the solvent was delivered at ca. 10 μL/min using a syringe pump (KDS-100, KD Scientific Co., Boston, MA).

A Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an APCI interface with heated nebulizer probe at 450 °C was used with an ion source temperature of 130 °C. For MS/MS measurements, direct infusion of pure analytes, or LC/MS, experiments were performed using a collision cell gas pressure (Ar) of 1.8 × 10⁻³ mbar and a collision energy of 30 eV. The mass spectrometer was calibrated over the mass range *m/z* 85–1200 using a solution of poly(ethylene glycol).

General Procedure for Pathway A. (1) Synthesis of MG and LMG. Butyllithium (4.8 mL, 2.5 M in hexane, 12 mmol) was added via a syringe to a solution of 4-bromo-*N,N*-dimethylaniline (3.15 g, 10 mmol) in dry THF (100 mL) at -78 °C under an argon atmosphere. The reaction mixture was stirred at the same temperature for 2 h, and then, a solution of 4-(dimethylamino)benzophenone (4.5 g, 10 mmol) in 80 mL of THF was added dropwise via a dropping funnel. The mixture was warmed slowly to room temperature, and stirring was continued overnight. The reaction was quenched by addition of H₂O, and the residual THF was evaporated. The aqueous residue was then extracted with ether, and the combined ether extracts were washed with H₂O and dried over anhydrous MgSO₄. Concentration by a rotoevaporator gave 4.26 g of a blue-greenish viscous residue containing the carbinolic intermediate. A small portion of the residue was purified by column chromatography on silica to give pure MG carbinol, whose NMR characteristics match those of the standard. ¹H NMR (DMSO-*d*₆): δ 2.83 (s, 12 H, 2 -N(CH₃)₂), 5.87 (s, 1H, D₂O exchangeable OH), 6.60 (d, 4H, H_{3,5,3',5'}, *J* = 8.9 Hz), 6.96 (d, 4H, H_{2,6,2',6'}, *J* = 8.9 Hz), 7.15 (m, 1H, H_{4''}), 7.18 (m, 2H, H_{3'',5''}), 7.25 (m, 2H, H_{2'',6''}). ¹³C NMR (DMSO-*d*₆): δ 42.84 (-N(CH₃)₂), 82.52 (C₇), 113.93 (C_{3,5,3',5'}), 131.06 (C_{2,6,2',6'}). The remaining carbinolic residue was transferred to a flask containing 100 mL of methanol and 1 mL of concentrated HCl. The light green solution turned dark blue immediately upon contact with the acid solution. The mixture was refluxed for 1 h. After it was cooled, the solution was concentrated in vacuo to give a viscous residue that was subsequently partitioned between H₂O and ether. The aqueous layer was washed repeatedly with ether until TLC of the ether washings showed no organic residues. The green aqueous layer was concentrated overnight to dryness using a Speedvac concentrator. The residue was purified by column chromatography on silica using a 9:1 ratio of chloroform:methanol, which afforded 1.94 g of MG (53%) as a dark green residue. The UV, MS, ¹H and ¹³C NMR data of this sample match those of the standard (see Tables 1–3).

MG (1.63 g, 4.8 mmol) was treated with NH₂NH₂·H₂O (800 μL, 15.5 mmol) and 10% Pd/C (250 mg) in absolute ethanol (150 mL), and the mixture was heated to reflux. The green solution turned colorless upon refluxing for about 10 min. The refluxing was continued for 1 h, and the solution was cooled and filtered through a Celite bed in a Buchner funnel to remove the catalyst. The filtrate was concentrated and partitioned between H₂O and ether. The aqueous layer was extracted with ether several times, and the combined ether extracts were back-washed with water. The organic layer was dried over anhydrous MgSO₄, filtered, and evaporated. The resulting oily residue was applied to a silica gel column and eluted with ethyl acetate and hexane (1:4). Appropriate portions were pooled, concentrated, and recrystal-

lized from ethyl acetate and hexane to give 1.44 g (88%) of pure LMG (mp 86–88 °C) as a beige-colored solid. The UV, MS, ¹H and ¹³C NMR data of this sample match those of standard (see Tables 1 and 2).

(2) Synthesis of Monodes-MG and Monodes-LMG. (a) *N*-Methyl-4-bromoaniline. *N*-Methylaniline (1.07 g, 0.01 mol) in CH₂Cl₂ (20 mL) was cooled to –78 °C under an argon atmosphere. A solution of tetrabromo-2,5-cyclohexadienone (4.10 g, 0.01 mol) in CH₂Cl₂ (20 mL) was added dropwise (13). The solution underwent a series of color changes from colorless to dark gray, blue, and finally to dark blue. The mixture was allowed to warm to room temperature over a period of 2 h, at which time TLC indicated that the reaction was complete. The mixture was treated with 20 mL of 1 N NaOH and stirred for 10 min to remove the tribromophenol byproduct. The organic layer was washed with additional NaOH and H₂O and then dried over anhydrous MgSO₄. Evaporation of the solvent gave crude product as a dark purple liquid. Vacuum distillation (4 mmHg) gave 1.82 g (bp 85–93 °C, 99%) of pure product as a dark purple liquid; *R*_f = 0.50 (benzene:hexane:ether:triethylamine = 10:5:3:0.5). ¹H NMR (CDCl₃): δ 2.47 (s, –NCH₃), 3.38 (bs, 1 H, NH, D₂O exchangeable), 6.13 (d, 2H, H_{2,6}, *J* = 8.1 Hz), 6.86 (d, 2H, H_{3,5}, *J* = 8.1 Hz). Anal. calcd for C₇H₈NBr: C, 45.19; H, 4.33; N, 7.53. Found: C, 44.92; H, 4.16; N, 7.76.

(b) *N*-Methyl-*N*-trimethylsilyl-4-bromoaniline (2). Butyllithium (13 mL, 2.5 M in hexane, 32.4 mmol) was added to a solution of *N*-methyl-4-bromoaniline (5.06 g, 27 mmol) in dry THF (50 mL) at 0 °C under an argon atmosphere. The dark blue solution turned sequentially to brown, yellow brown, and finally a turbid colloidal solution upon the addition of butyllithium. The solution was warmed to room temperature over 1 h and then cooled in an ice bath, and chlorotrimethylsilane (3.52 g, 32.4 mmol) was added slowly via a syringe. The reaction, which was slightly exothermic, was stirred overnight at room temperature. The colloidal solution was filtered through a sintered glass to remove LiCl and centrifuged for 15 min. The supernatant was transferred to a distillation flask and distilled under vacuum to give 3.7 g (66%) of product as a colorless liquid; bp 60–62 °C (1 mmHg); *R*_f = 0.72 (benzene:hexane:ether:triethylamine = 10:15:3:0.5). ¹H NMR (acetone-*d*₆) (14): δ 0.15 (s, 9H, –Si(CH₃)₃), 2.75 (s, 3H, –NCH₃), 6.65 (d, 2H, H_{2,6}, *J* = 8.0 Hz), 7.18 (d, 2H, H_{3,5}, *J* = 8.0 Hz).

Monodes-MG and monodes-LMG were obtained via pathway A using *N*-methyl-*N*-trimethylsilyl-4-bromoaniline (2, 1.06 g, 5 mmol) and 4-(dimethylamino)benzophenone (1.13 g, 5 mmol). Monodes-MG (0.84 g, 59%): ¹H NMR (acetone-*d*₆): δ 3.09 (s, 3H, –NCH₃), 3.30 (s, 6H, –N(CH₃)₂), 7.01 (d, 2H, H_{3,5}, *J* = 9.3 Hz), 7.34 (d, 2H, H_{2,6}, *J* = 9.3 Hz), 7.34 (d, 2H, H_{3,5}), 7.38 (m, 4H, H_{2,6;2',6'}), 7.61 (m, 2H, H_{3',5'}), 7.72 (m, 1H, H_{4'}). Monodes-LMG: 0.374 g (44%) as a white solid; mp 68–69 °C (ethyl acetate and hexane). ¹H NMR (acetone-*d*₆): δ 2.74 (s, 3H, –NCH₃), 2.88 (s, 6H, –N(CH₃)₂), 4.72 (bs, 1H, –NH, D₂O exchangeable), 5.33 (s, 1H, H₇), 6.52 (d, 2H, H_{3,5}, *J* = 8.6 Hz), 6.66 (d, 2H, H_{2,6}, *J* = 8.6 Hz), 6.88 (d, 2H, H_{3,5}, *J* = 8.6 Hz), 6.95 (d, 2H, H_{2,6}, *J* = 8.6 Hz), 7.13 (m, 3H, H_{2',6',4'}), 7.24 (m, 2H, H_{3',5'}). ¹³C NMR (acetone-*d*₆): δ 30.63 (–NCH₃), 40.74 (–N(CH₃)₂), 56.00 (C₇), 112.53, 113.22, 126.44, 128.74, 130.03, 130.53, 130.58, 133.24, 133.65, 146.76, 149.17, 150.00. Anal. calcd for C₂₂H₂₄N₂: C, 83.50; H, 7.65; N, 8.85. Found: C, 83.57; H, 7.64; N, 8.78.

Dides(unsym)-MG and dides(unsym)-LMG were prepared in a similar manner using 4-bromo-*N,N*-bis(trimethylsilyl)aniline (3, 15) and 4-(dimethylamino)benzophenone as starting materials. Details of the synthesis and the spectroscopic characterizations were described previously (12).

General Procedure for Pathway B. (1) Synthesis of Dides(unsym)-LMG. Butyllithium (4.8 mL, 2.5 M in hexane, 12 mmol) was added via a syringe to a solution of *N,N*-(dimethylamino)-4-bromobenzene (2.40 g, 12 mmol) in dry THF (100 mL) at –78 °C under an argon atmosphere. The resulting turbid solution became clear after stirring at the same temperature for 2 h. A solution of 4-nitrobenzophenone (2.27 g, 10

mmol) in dry THF (100 mL) was added dropwise to the reaction mixture, which turned to orange initially and then dark brown. The mixture was warmed slowly to room temperature and stirred overnight. The reaction was quenched with H₂O and extracted with CH₂Cl₂. The combined extracts were evaporated, and the resulting residue was refluxed for 30 min with 50 mL of methanol containing 3 mL of concentrated HCl. The solution turned to blue immediately upon contact with the acid solution. The solvent was evaporated and partitioned between H₂O and ether. The aqueous layer was repeatedly washed with ether. The ether layer, which contained mostly the unreacted starting materials, was discarded. Concentration of the H₂O layer by Speedvac afforded ~1.5 g of a highly hygroscopic dark blue residue, whose TLC (chloroform:methanol = 9:1) exhibited a mixture of a major (*R*_f = 0.21) and a minor (*R*_f = 0.17) spot on silica TLC, which showed characteristic green and blue colors, respectively, upon standing. The crude cationic mixture was treated with excess NH₂NH₂·H₂O and 10% Pd/C in refluxing ethanol for 2 h. A usual work up with ether gave a mixture of dides(unsym)-LMG and 6, which was separated on silica using ethyl acetate and hexane as an eluting solvent system. The chromatographic and spectral properties of the polar fraction (*R*_f = 0.24, hexane:benzene:ether:triethylamine = 10:5:3:0.5) (50 mg, 1.7%) match those of dides(unsym)-LMG prepared via pathway A (12). The major nonpolar product 6 (*R*_f = 0.31) was recrystallized from ethyl acetate and hexane as fluffy needles (504 mg, 12.0%, mp 155–156 °C) and had the following properties: TLC: *R*_f = 0.31 (benzene:hexane:ether:triethylamine = 10:15:3:0.5), *R*_f = 0.75 (CHCl₃:methanol = 95:5). HPLC: *t*_R = 23.7 min (acetonitrile in 0.1 mM pH 4.5 ammonium acetate 10–90%, a 30 min gradient). UV (methanol): 297 nm (log 4.60), 263 nm (log 4.57). ¹H NMR (DMSO-*d*₆): δ 2.80 (s, 6H, –N(CH₃)₂), 2.83 (s, 6H, –N(CH₃)₂), 5.29 (s, 1H, methane proton), 6.64 (d, 2H, *J* = 8.8 Hz), 6.67 (d, 2H, *J* = 9.0 Hz), 6.77 (d, 2H, *J* = 8.6 Hz), 6.84 (d, 2H, *J* = 8.6 Hz), 6.90 (d, 2H, *J* = 8.7 Hz), 6.94 (d, 2H, *J* = 8.9 Hz), 7.08 (d, 2H, *J* = 7.2 Hz), 7.15 (dd, 1H, *J* = 7.3, 7.3 Hz), 7.26 (dd, 2H, *J* = 7.5, 7.6 Hz), 7.55 (s, 1H, –NH, D₂O exchangeable). ¹³C NMR (DMSO-*d*₆): δ 40.22 (–N(CH₃)₂), 40.85 (–N(CH₃)₂), 54.40 (methane carbon), 112.35, 113.85, 114.10, 120.87, 125.71, 128.04, 128.84, 129.38, 129.47, 132.11 (q), 132.11 (q), 133.03 (q), 133.64 (q), 143.89 (q), 145.27 (q), 145.86 (q), 148.74 (q). ESI-MS *m/z* 422 (M + 1)⁺. HRMS Calcd for C₂₉H₃₁N₃, 421.2510; found, 421.2518. Anal. calcd for C₂₉H₃₁N₃: C, 82.62; H, 7.41; N, 9.97. Found: C, 82.70; H, 7.66; N, 9.89.

General DDQ Oxidation Procedure. DDQ (51.3 mg, 0.226 mmol) was added to a solution of LMG (50 mg, 0.152 mmol) in benzene (2 mL). After it was stirred for 30 min at room temperature, the reaction mixture was poured onto a short column containing aluminum oxide. Elution with CH₂Cl₂:methanol (9:1) furnished a green residue. The residue was treated with 50 mL of methanol containing 3 drops of concentrated HCl and refluxed for 1 h. The solvent was evaporated by a rotoevaporator. The resulting dark residue was purified by column chromatography on silica using CH₂Cl₂:methanol (9:1) to give pure 45.3 mg (82%) of pure MG.

General PbO₂ Oxidation Procedure. LMG (50 mg, 0.152 mmol) in H₂O (1 mL) was treated with PbO₂ (50 mg, 0.193 mmol) and 5 drops of concentrated HCl, and the reaction was stirred at room temperature overnight. The mixture was diluted with methanol (30 mL) and filtrated through a Celite bed in a glass-fritted funnel, followed by chromatography on silica (CH₂Cl₂:methanol = 9:1), affording pure MG (46 mg, 99%).

Trides-LMG and Trides-MG. Pathway B, using 4-nitrobenzophenone (1.14 g, 5 mmol), *N*-methyl-*N*-trimethylsilyl-4-bromoaniline (2, 1.06 g, 5 mmol), and butyllithium (2.5 M in hexane, 1.91 mL, 5 mmol) gave 0.214 g of blue color residue. The crude mixture was reduced (NH₂NH₂·H₂O, 10% Pd/C) in the usual manner, and a portion was isolated using silica gel column chromatography (ethyl acetate:hexane = 1:4) to give trides-LMG (59 mg, 6% overall yield). ¹H NMR (acetone-*d*₆): δ 2.78 (s, 3H, –NCH₃), 4.43 (bs, 2H, –NH₂, D₂O exchangeable), 4.75 (bs, 1H, –NH, D₂O exchangeable), 5.27 (s, 1H, H₇), 6.50

(d, 2H, H_{3,5}, $J = 9.0$ Hz), 6.57 (d, 2H, H_{2,6}, $J = 9.0$ Hz), 6.79 (d, 2H, H_{3,5}, $J = 8.9$ Hz), 6.85 (d, 2H, H_{2,6}, $J = 8.9$ Hz), 7.13 (m, 3H, H_{2,6,4'}), 7.24 (m, 2H, H_{3,5'}). HRMS calcd for C₂₀H₂₀N₂, 288.1627; found, 288.1630. Anal. calcd for C₂₀H₂₀N₂: C, 83.50; H, 7.65; N, 8.85. Found: C, 83.57; H, 7.64; N, 8.78. Trides-MG was obtained by DDQ oxidation of trides-LMG. ¹H NMR (acetone-*d*₆): δ 3.15 (s, 3H, -NCH₃), 7.00 (d, 2H, H_{3,5}, $J = 9.0$ Hz), 7.08 (broad d, 2H, H_{3,5}, $J = 9.0$ Hz), 7.33 (d, 2H, H_{2,6}, $J = 9.0$ Hz), 7.42 (d, 2H, H_{2,6}, $J = 9.0$ Hz), 7.62 (m, 2H, H_{3,5'}), 7.77 (m, 1H, H_{4'}).

Tetradec-LMG and Tetradec-MG were prepared similarly via pathway B using 4-nitrobenzophenone and *N,N*-bis(trimethylsilyl)-4-bromoaniline (**3**). Tetradec-LMG (overall yield 4%): ¹H NMR (acetone-*d*₆): δ 4.43 (bs, 4H, 2 NH₂, D₂O exchangeable), 5.26 (s, 1H, H₇), 6.57 (d, 4H, H_{3,5,3',5'}, $J = 8.5$ Hz), 6.79 (d, 4H, H_{2,6,2',6'}, $J = 8.5$ Hz), 7.11 (d, 2H, H_{2,6'}, $J = 8.4$ Hz), 7.14 (m, 1H, H_{4'}), 7.23 (m, 2H, H_{3,5'}). HRMS calcd for C₁₉H₁₈N₂, 274.1470; found, 274.1470. Tetradec-MG was obtained by DDQ oxidation of Tetradec-LMG. ¹H NMR (acetone-*d*₆): δ 6.58 (d, 4H, H_{2,6,2',6'}, $J = 8.6$ Hz), 6.71 (d, 4H, H_{3,5,3',5'}, $J = 8.6$ Hz), 7.14 (m, 3H, H_{2,6,4'}), 7.25 (m, 2H, H_{3,5'}).

¹³C₇-LMG. ¹³C₇-LMG was synthesized following the literature procedure (16). A mixture of dimethylaniline (1.72 g, 14.2 mmol), ¹³CHO-benzaldehyde (492 mg, 4.6 mmol, Isotec), and concentrated HCl (1.0 mL) was refluxed overnight under an argon atmosphere. To the cooled greenish solution was added 10 mL of 1.0 N NaOH, and the mixture was extracted three times with ether. The combined ether extracts were washed with H₂O and dried over anhydrous MgSO₄. A yellow viscous residue resulted upon concentration. Silica column chromatography (ethyl acetate and hexane) followed by recrystallization from the same solvent system afforded 1.32 g (87%) of a white solid; mp 87–89 °C. UV: λ_{max} 261.9 nm. ESI-MS: 332.1 (M + H)⁺. See Tables 1 and 2 for ¹H and ¹³C NMR data.

¹³C₇-MG. ¹³C₇-LMG (0.25 g, 0.075 mmol) was suspended in a H₂O solution containing 0.25 mL of concentrated HCl. To the solution was added PbO₂ (0.18 g, 0.075 mmol) over a period of 10 min and stirred overnight at ambient temperature. The dark viscous residue was filtered through a Celite bed and concentrated and chromatographed using chloroform and methanol to give the cationic MG (100 mg). According to ¹H NMR, this product contains over 92% of MG and 7% of the leuco starting material. Repeated chromatography gave a pure MG (>98% purity). UV: λ_{max} 614.4 nm (methanol); see Tables 1 and 2 for ¹H and ¹³C NMR data. ESI-MS: 330 (M⁺). Anal. calcd for C₂₂¹³C₁H₂₆N₂: C, 83.64; H, 7.39; N, 8.45. Found: C, 83.88; H, 7.87; N, 8.67.

Dides(sym)-LMG. A mixture of methylaniline (4.3 g, 40 mmol), benzaldehyde (1.4 g, 13 mmol), and 30% HCl (5.0 mL) was refluxed under an argon atmosphere. The color of the solution changed from yellow to light green and finally dark green. After it was refluxed for 18 h, the condenser was removed and the mixture was heated for about 1 h to boil the excess *N*-methylaniline. The reaction mixture was cooled, 20 mL of 1.0 M NaOH was added, and the mixture was extracted with ether. The combined ether extracts were washed with H₂O and dried over anhydrous MgSO₄. Concentration of the organic solvent gave crude product as an oily residue. Silica gel column chromatography using ethyl acetate and hexane (1:4) as an eluant gave the product as a foamy and glassy residue (1.20 g, 31%). ¹H NMR (acetone-*d*₆): δ 2.74 (s, 6H, 2 NCH₃), 4.83 (bs, 2H, 2 NH, D₂O exchangeable), 5.29 (s, 1H, H₇), 6.51 (d, 4H, H_{3,5,3',5'}, $J = 8.6$ Hz), 6.86 (d, 4H, H_{2,6,2',6'}, $J = 8.5$ Hz), 7.12 (d, 2H, H_{2,6'}, $J = 8.9$ Hz), 7.13 (m, 1H, H_{4'}), 7.24 (m, 2H, H_{3,5'}). ¹³C NMR (acetone-*d*₆): δ 30.58 (-NCH₃), 56.11 (C₇), 112.50, 126.39, 128.70, 130.02, 130.67, 133.37, 146.88, 149.13. Anal. calcd for C₂₁H₂₂N₂: C, 83.40; H, 7.33; N, 9.26. Found: C, 83.38; H, 7.41; N, 9.09. The DDQ oxidation of dides(sym)-LMG gave dides(sym)-MG. ¹H NMR (acetone-*d*₆): δ 3.29 (s, 6H, 2 NCH₃), 6.56 (d, 4H, H_{3,5,3',5'}, $J = 8.5$ Hz), 6.87 (d, 2H, H_{2,6,2',6'}, $J = 8.5$ Hz), 7.10 (d, 2H, H_{2,6'}, $J = 7.2$ Hz), 7.12 (m, 1H, H_{4'}), 7.24 (m, 2H, H_{3,5'}).

Results and Discussion

The structures and the numbering system of MG and LMG and their *N*-demethylated derivatives are shown in Figure 1. The *N*-demethylated compounds are labeled to indicate the extent of *N*-demethylation (i.e., monodes, dides, trides, and tetradec). For the dides derivatives, two possibilities exist, symmetric or unsymmetric. The carbons in the molecules are numbered as shown for identification. The carbon in the center of the molecule is labeled as C₇. Because of the symmetric nature, the numberings of the substituted aromatic rings of MG, LMG, and their dides(sym) and tetradec derivatives are identical (i.e., C1 and C1').

Synthesis. Literature reports on the preparation of MG and LMG are scarce (1, 2), and no information is available on the systematic synthesis of the *N*-demethylated analogues of MG and LMG. The original synthesis of LMG entailed a condensation reaction of benzaldehyde with *N,N*-dimethylaniline (16). Aqueous PbO₂ oxidation of LMG gave a C₇-carbinolic intermediate, which was then subsequently treated with concentrated HCl to form the cationic MG as a chloride salt in quantitative yield. This procedure, although reported to be of good yield, suffers from a rather laborious salt-induced precipitation procedure and certain side reactions (16). The chloride salt can be converted to its oxalate salt by oxalic acid treatment of the carbinolic base, which is generated by reaction of the chloride salt with NaOH (17). Doerge et al. (18) have prepared ¹³C₆-MG as an internal mass spectral standard. The reaction involved an initial condensation of lithiated ¹³C₆-benzene derived from ¹³C₆-bromobenzene and Michler's ketone, followed by treatment with anhydrous HCl; however, no synthetic details were provided. DeFina and Dieckmann (19) have recently reported selectively ¹⁵N- or ¹³CH₃-labeled MG as model ligands for structural studies of RNA aptamer/ligand complexes. Their synthesis entails a coupling reaction of methylbenzoate with the Grignard reagent derived from an appropriately labeled 4-bromo-*N,N*-dimethylaniline.

Figure 2 shows our MG synthesis, which is general in scope and can be used to prepare *N*-demethylated derivatives. The overall synthetic strategy is similar to that of the preparation of gentian violet metabolites by McDonald and co-workers (20); however, major modifications, including the isolation procedure, have been made. MG was prepared via pathway A, which involves a coupling of 4-(dimethylamino)benzophenone with the aryllithium reagent derived from 4-bromo-*N,N*-dimethylaniline. The resulting crude carbinolic intermediate was subsequently treated with concentrated HCl in refluxing methanol. After the solvent was evaporated, the dark greenish residue was partitioned between ether and H₂O. The cationic MG was isolated from the aqueous layer and purified by column chromatography on silica using a 9:1 mixture of chloroform and methanol as an eluant. The overall yield of MG via this new procedure was in the range of 50–65%.

The new MG synthesis affords cleaner MG, in one pot, and less contamination from byproducts. This is a major improvement from the original condensation method, which involved extra steps and a laborious salt-induced precipitation (16). Conversion of the chromatic MG to the leuco base LMG took place smoothly with hydrazine in the presence of 10% Pd/C in refluxing ethanol. The products' identities were confirmed by chromatographic

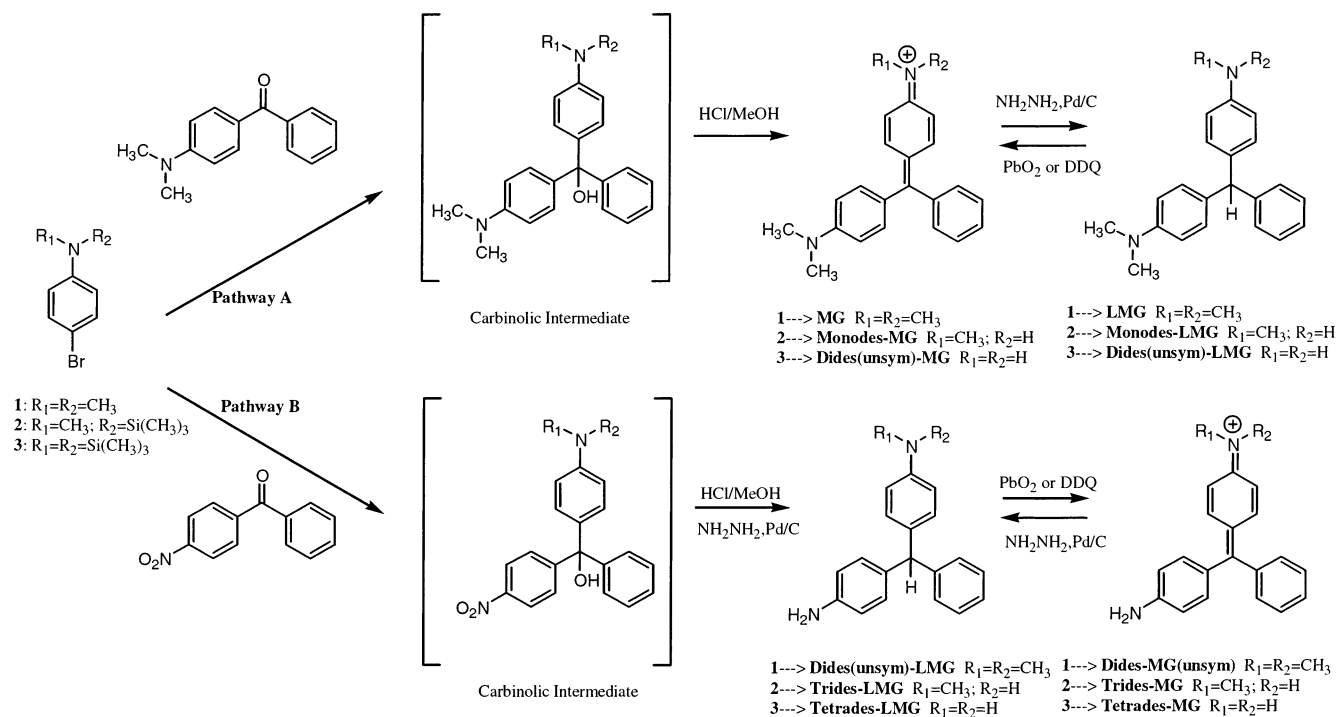


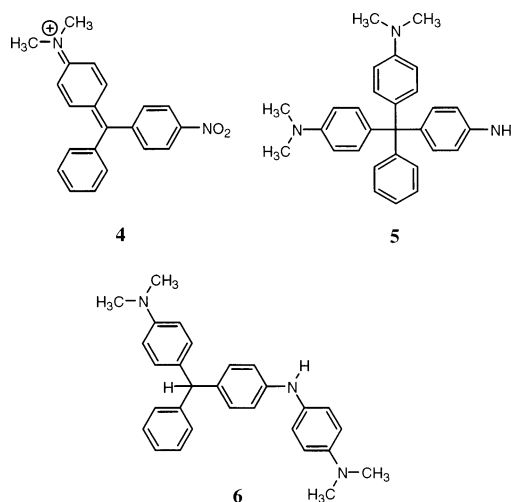
Figure 2. Synthesis of MG, LMG, and their *N*-demethylated derivatives.

and spectral comparison with those of available standards. Complete 1H and ^{13}C NMR spectral characterization of LMG and MG was made with the use of $^{13}C_7$ -labeled analogues (see Structure Characterization). Monodes- and dides(unsym)-MG and -LMG derivatives were prepared in a similar manner using **2** and **3** (Figure 2), respectively, as starting lithiating reagents (Figure 2).

An analogous sequence (pathway B), which involved the use of 4-nitrobenzophenone as a condensating agent (Figure 2), was devised for the trides and tetrades derivatives. We first attempted this procedure by reacting the lithiated **1** with 4-nitrobenzophenone, which in theory should give dides(unsym)-LMG as a product. The initial condensation, followed by a HCl treatment, afforded a highly hygroscopic inky residue, whose TLC (chloroform: methanol = 9:1) exhibited two spots ($R_f = 0.21$ and 0.17), with the former being the major one. Separation of the products proved to be difficult; so we proceeded to reduce the crude mixture directly (NH_2NH_2 , 10% Pd/C). The resulting leuco products were readily separated on a silica gel column using ethyl acetate and hexane. The minor product derived from the polar intermediate above was found to be the desired dides(unsym)-LMG, whose structure was confirmed by spectral comparison with the standard sample prepared by pathway A (12). This implied that the polar residue before reduction was the expected nitro intermediate (**4**).

The reduction product of the major nonpolar compound ($R_f = 0.21$) was obtained as fluffy needles from ethyl acetate and hexane and gave an elemental composition of $C_{29}H_{31}N_3$ (high-resolution mass and elemental analysis) and produced a protonated molecule at m/z 422 in an electrospray mass spectrum. The material was initially thought to be the tandem condensation product **5**; however, analysis of NMR data indicated the isomeric structure **6**. The 500 MHz 1H NMR spectrum in DMSO- d_6 of the major product exhibited a total of seven doublets (two protons each) and two doublets of doublets (one with

two protons and the other one with one proton), all of which were well-resolved. Additional features included a characteristic methane proton and carbon signals at 5.29 and 54.40 ppm, respectively, and a D_2O exchangeable proton at 7.55 ppm. The formation of **6** appears to be the consequence of a direct reaction between the aromatic nitro functionality and the lithiated 4-(*N,N*-dimethyl)aniline. Work is currently underway to elucidate the mechanism of this unusual reaction. The overall yields for dides(unsym)-LMG and the major unknown **6** from this three step reaction sequence were 1.7 and 12.0%, respectively.



Analogous condensation reactions using **2** and **3** (Figure 2) afforded trides- and tetrades-LMG as minor products (**6** and 4% overall yield), respectively. We did not characterize the major coupling adducts for trides and tetrades derivatives, which appear to be complex.

Pathway B afforded leuco bases as initial products, which required oxidation to the cationic MG derivatives using aqueous PbO_2 in concentrated HCl. Pure cationic

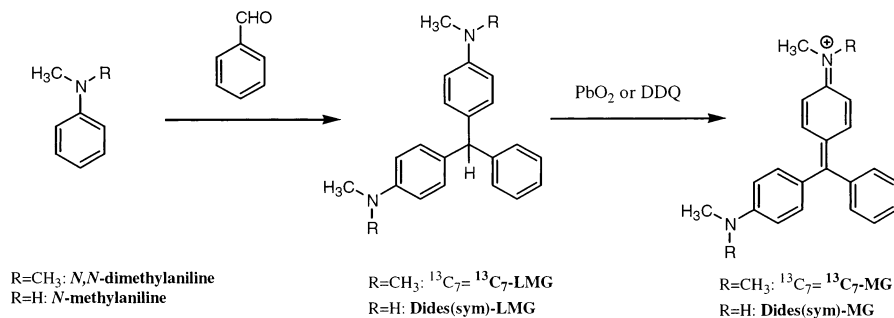


Figure 3. Synthesis of $^{13}\text{C}_7$ -MG, $^{13}\text{C}_7$ -LMG, and dides(sym)-MG and -LMG.

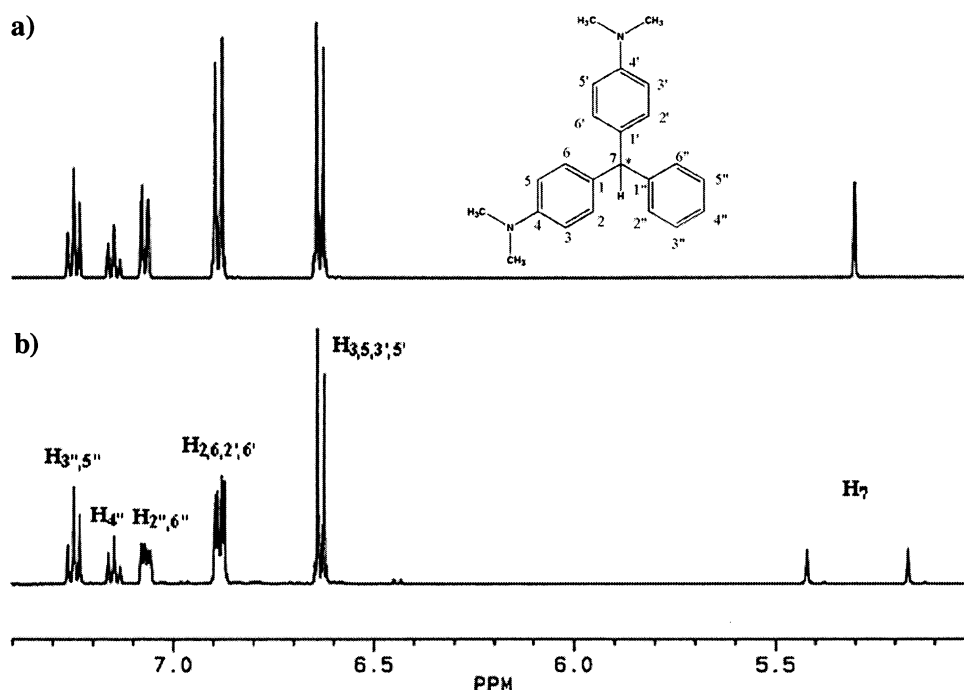


Figure 4. Expanded ^1H NMR spectra of (a) LMG and (b) $^{13}\text{C}_7$ -LMG in $\text{DMSO}-d_6$.

compounds were obtained by filtering through Celite, followed by silica column chromatography. A similar oxidation was achieved by treatment of the leuco derivatives with an excess amount of DDQ (21) for about 30 min at room temperature. Removal of excess DDQ via an aluminum oxide column and subsequent treatment with methanolic HCl gave pure cationic derivatives.

We prepared the symmetrical dides-MG and dides-LMG using *N*-methylaniline and benzaldehyde by a modification of the original MG synthesis procedure (Figure 3) (16). The same procedure was also employed for the preparation of $^{13}\text{C}_7$ -labeled LMG using ^{13}CHO -labeled benzaldehyde and *N,N*-dimethylaniline. Both dides(sym)-LMG and $^{13}\text{C}_7$ -LMG were converted to the cationic MG derivatives by DDQ or PbO_2 oxidation. As expected, $^{13}\text{C}_7$ -LMG and $^{13}\text{C}_7$ -MG exhibited a protonated molecule at m/z 332 and a cationic molecule at m/z 330, respectively. While $^{13}\text{C}_7$ -LMG showed an identical UV/vis λ_{max} (261.9 nm) as LMG, the corresponding cationic $^{13}\text{C}_7$ -MG exhibited a λ_{max} at 614.4 nm, which represented a sizable hypsochromic shift (-3.6 nm) from the nonlabeled MG (618.0 nm). The labeled compounds were used extensively to aid the ^1H and ^{13}C spectral assignments of MG and LMG, and their *N*-demethylated metabolites (see below).

NMR Structure Characterization. All of the *N*-demethylated LMG and some of the corresponding MG

derivatives listed in Figure 1 have previously been observed in biological systems and their structures have been examined primarily by mass spectrometry (8, 9, 11, 12, 22–24). Now, with the synthetic standards in hand, we were able to analyze their chromatographic (TLC, HPLC) and spectral (UV/vis, mass, ^1H and ^{13}C NMR) data in more detail and found them to be consistent with the previously assigned structures. We used $^{13}\text{C}_7$ -LMG and $^{13}\text{C}_7$ -MG to make spectral assignments for LMG and MG, whose full characterizations were necessary in order to elucidate the structures of their *N*-demethylated analogues. Wherever appropriate, ^1H and ^{13}C NMR spectral assignments were aided by “off-resonance” decoupling and one-dimensional NOE experiments, considerations of relative signal intensities and molecular symmetry.

The aromatic region of 500 MHz ^1H NMR spectrum of $^{13}\text{C}_7$ -LMG in $\text{DMSO}-d_6$ was virtually identical to that of the unlabeled LMG except for the multiplets at 7.07, 6.88, and 5.29 ppm, which arise from the couplings with $^{13}\text{C}_7$ (Figure 4b). The 5.29 ppm doublet was readily recognized as that of H_7 due to a large one bond coupling ($^1J_{\text{C-H}} = 125.98$ Hz) with $^{13}\text{C}_7$. The remaining signals at 6.88 and 7.07 were assigned to $\text{H}_{2,6,2',6'}$ (four protons) and $\text{H}_{2',6''}$ (two protons), respectively, which are three bonds away from $^{13}\text{C}_7$. Because of the symmetry, all 13 aromatic protons of the molecule appeared in five groups of signal in a 2:1:2:4:4 ratio. The most upfield high intensity doublet at

Table 1. ^1H NMR Chemical Shifts (ppm) and Coupling Constants (Hz) of $^{13}\text{C}_7\text{-LMG}$ and $^{13}\text{C}_7\text{-MG}^a$

	$\text{H}_{2,6,2',6'}$	$\text{H}_{3,5,3',5'}$	$\text{H}_{2'',6''}$	$\text{H}_{3'',5''}$	$\text{H}_{4''}$	H_7	$\text{N}(\text{CH}_3)_2$
$^{13}\text{C}_7\text{-LMG}$	6.88 ($J_{\text{C}_7\text{-H}_2} = 2.0$) ($J_{\text{H}_2\text{-H}_3} = 9.2$)	6.63 ($J_{\text{H}_3\text{-H}_2} = 9.2$)	7.07 ($J_{\text{H}_2''\text{-H}_3''} = 7.6$) ($J_{\text{C}_7\text{-H}_2''} = 4.1$) ($J_{\text{H}_2''\text{-H}_4''} = 1.4$)	7.25 ($J_{\text{H}_3''\text{-H}_2''} = 7.6$) ($J_{\text{H}_3''\text{-H}_4''} = 7.4$)	7.15 ($J_{\text{H}_4''\text{-H}_3''} = 7.4$)	5.29 ($J_{\text{C}_7\text{-H}_7} = 125.98$)	2.82
$^{13}\text{C}_7\text{-MG}^b$	7.36 ($J_{\text{C}_7\text{-H}_2} = 3.1$) ($J_{\text{H}_2\text{-H}_3} = 9.4$)	7.09 ($J_{\text{H}_3\text{-H}_2} = 9.4$)	7.36 ^c	7.62 ($J_{\text{H}_2''\text{-H}_3''} = 7.4$) ($J_{\text{H}_3''\text{-H}_4''} = 7.4$)	7.75 ($J_{\text{H}_4''\text{-H}_3''} = 7.4$) ($J_{\text{H}_4''\text{-H}_2''} = 1.2$)		3.28
$^{13}\text{C}_7\text{-MG}^d$	7.46 ($J_{\text{C}_7\text{-H}_2} = 3.2$) ($J_{\text{H}_2\text{-H}_3} = 9.3$)	7.14 ($J_{\text{H}_3\text{-H}_2} = 9.3$)	7.42 ($J_{\text{H}_2''\text{-H}_3''} = 7.9$) ($J_{\text{C}_7\text{-H}_2''} = 3.7$) ($J_{\text{H}_2''\text{-H}_4''} = 1.3$)	7.65 ($J_{\text{H}_3''\text{-H}_2''} = 7.9$) ($J_{\text{H}_3''\text{-H}_4''} = 7.4$)	7.79 ($J_{\text{H}_4''\text{-H}_3''} = 7.4$)		3.42

^a See Figure 1 for numbering system. Wherever symmetry is involved, only one relevant coupling constant is listed. ^b Measured in DMSO- d_6 . ^c Complete overlap with $\text{H}_{2,6,2',6'}$. No coupling constants could be measured. ^d Measured in acetone- d_6 .

Table 2. ^{13}C NMR Chemical Shifts (ppm) and Coupling Constants (Hz) of $^{13}\text{C}_7\text{-LMG}$ and $^{13}\text{C}_7\text{-MG}^a$ in DMSO- d_6

	$\text{C}_{1,1'}$	$\text{C}_{2,6,2',6'}$	$\text{C}_{3,5,3',5'}$	$\text{C}_{4,4'}$	$\text{C}_{1''}$
$^{13}\text{C}_7\text{-LMG}$	145.33 ($J_{\text{C}_7\text{-C}_1} = 42.6$) ^a	129.34 ($J_{\text{C}_7\text{-C}_2} = 2.2$)	112.38 ($J_{\text{C}_7\text{-C}_3} = 3.2$)	148.71	132.16 ($J_{\text{C}_7\text{-C}_{1''}} = 45.1$)
$^{13}\text{C}_7\text{-MG}$	126.33 ($J_{\text{C}_7\text{-C}_1} = 60.1$)	139.96 ($J_{\text{C}_7\text{-C}_2} = 1.6$)	114.05 ($J_{\text{C}_7\text{-C}_3} = 4.3$)	156.37	139.15 ($J_{\text{C}_7\text{-C}_{1''}} = 51.7$)
	$\text{C}_{2'',6''}$	$\text{C}_{3'',5''}$	$\text{C}_{4''}$	$^{13}\text{C}_7$	$\text{N}(\text{CH}_3)_2$
$^{13}\text{C}_7\text{-LMG}$	128.00 ($J_{\text{C}_7\text{-C}_{2''}} = 2.5$)	128.82 ($J_{\text{C}_7\text{-C}_{3''}} = 3.0$)	125.66	54.19 ($J_{\text{C}_7\text{-H}_7} = 126.6$)	41.33
$^{13}\text{C}_7\text{-MG}$	128.62 ($J_{\text{C}_7\text{-C}_{2''}} = 3.7$)	134.11 ($J_{\text{C}_7\text{-C}_{3''}} = 4.3$)	132.79	174.92	40.62

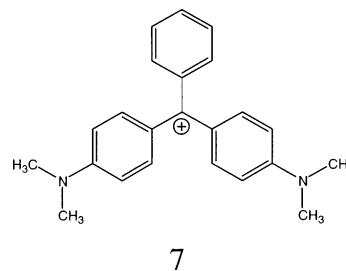
^a See Figure 1 for numbering system. Wherever symmetry is involved, only one relevant coupling constant is listed.

6.63 ppm was narrowed upon irradiation of the *N,N*-dimethyl signals at 2.82 ppm and was thus readily identified as $\text{H}_{3,5,3',5'}$. Additional decoupling and COSY experiments established the connectivities for the AX ($\text{H}_{2,3,5,6}$; $\text{H}_{2',3',5',6'}$) and AMX networks ($\text{H}_{2'',3'',4'',5'',6''}$), thus completing the ^1H assignments. The ^1H NMR data of $^{13}\text{C}_7\text{-MG}$ and $^{13}\text{C}_7\text{-LMG}$ are summarized in Table 1.

The symmetric nature of $^{13}\text{C}_7\text{-LMG}$ was also apparent in the ^1H -decoupled ^{13}C NMR spectrum that exhibited only five protonated and three quaternary aromatic carbons in addition to the singlet methyl signal at 40.71 ppm (not shown). The assignments are presented in Table 2. As expected, the $^{13}\text{C}_7$ -methane carbon at 54.19 ppm exhibited a large one bond C–H coupling ($^1J_{\text{C-H}} = 126.6$ Hz) in the ^1H -coupled ^{13}C NMR spectrum. The most downfield signal at 148.71 ppm was affected upon irradiation of the methyl protons and, thus, was assigned to $\text{C}_{4,4'}$. The two signals at 145.33 and 132.16 ppm were assigned to $\text{C}_{1,1'}$ and $\text{C}_{1''}$, respectively, on the basis of their one bond ($^1J_{^{13}\text{C}_7\text{-C}_{1,1'}} = 42.6$ Hz and $J_{^{13}\text{C}_7\text{-C}_{1''}} = 45.1$ Hz) couplings with $^{13}\text{C}_7$. The remaining aromatic carbon signals, except for the 125.66 ppm signal, showed small couplings ranging from 2.1 to 3.2 Hz (Table 2). Irradiation of the methyl protons collapsed the most shielded doublet at 112.32 ppm, which was assigned to $\text{C}_{3,5,3',5'}$. The observed coupling ($J = 3.2$ Hz), therefore, must be due to a three bond $^{13}\text{C}_7\text{-C}_{3,5,3',5'}$ coupling. The 128.82 ppm signal, which showed a similar three bond $^{13}\text{C}_7\text{-C}_{3'',5''}$ coupling (3.0 Hz), was then assigned to $\text{C}_{3'',5''}$. The signals at 129.34 and 128.00 ppm showed smaller couplings (2.2 and 2.5 Hz, respectively), with greater intensity for the former, and were assigned to $\text{C}_{2,6,2',6'}$ and $\text{C}_{2'',6''}$, respectively, because two bond C–C couplings are typically smaller than three bond ones. The remaining low intensity signal at 125.66 ppm was not split and, therefore, was attributed to $\text{C}_{4''}$. The signals in the ^{13}C NMR spectrum of $^{13}\text{C}_7\text{-MG}$ were assigned in a similar fashion

by decoupling experiments, and the results are summarized in Table 2.

Essentially, all of the carbon signals, except for $\text{C}_{1,1'}$, were shifted downfield going from the neutral LMG to the cationic MG (Table 2). The ^{13}C chemical shifts in the triphenylmethyl cations were interpreted in terms of charge densities (25–27). The deshielding effect was largest (–120.73 ppm) on C_7 , as expected from the behavior of para-substituted triphenyl cation systems (25–27). A substantial shielding (+19 ppm) on the aromatic point of attachment ($\text{C}_{1,1'}$) indicates a buildup of negative charge, which is a general feature predicted by theoretical calculations (Table 1) (25, 27). A similar trend, but more general deshielding effect, was observed throughout the molecule in the ^1H NMR spectrum (Table 1). These results indicate the existence of a resonance delocalization in the substituted aromatic rings with the cationic charge density primarily located in the central C_7 as depicted in structure 7.



The NMR assignments of LMG and MG helped in the elucidation of the structures of their *N*-demethylated derivatives. The spectral patterns of the metabolites were classified into two groups based on their molecular symmetry. For example, the symmetric dides(sym) and tetrares derivatives showed simple spectral patterns, similar to those of MG and LMG (i.e., five magnetically

equivalent signals for a total of 13 aromatic protons and five carbon signals for a total of five equivalent protonated aromatic carbons). The unsymmetric monodes, dides(unsym), and trides derivatives exhibited seven sets of proton signals for 13 aromatic protons and seven signals for the seven protonated aromatic carbons. In all cases, numbers of the methyl and exchangeable protons were in good agreement with the assigned structures. For example, the ^1H and ^{13}C NMR spectral patterns of dides(sym)-LMG were similar to those of LMG, except for the presence of a D_2O exchangeable singlet at 4.83 ppm and a methyl singlet at 2.74 ppm, which integrated as two and six protons, respectively. The ^1H NMR spectrum of monodes-LMG, however, showed two separate methyl signals at 2.74 and 2.88 ppm in a ratio of 1:2 and a broad singlet D_2O exchangeable signal at 4.72 ppm for the NH proton. The mono- and dimethyl signals appeared separately at 30.63 and 40.74 ppm in the ^{13}C NMR spectrum. Trides-LMG showed the presence of two broad D_2O exchangeable signals at 4.75 and 4.43 ppm in a ratio of 1:2 for the NH and NH_2 protons, respectively. As expected, tetra-LMG showed a broad singlet at 4.43 ppm that represented four protons. The structures of the cationic *N*-demethylated MG derivatives were elucidated similarly using the symmetry arguments.

Mass Spectral Analysis. The synthesis and characterization of the entire series of *N*-demethylated leuco and cationic derivatives of MG permitted a systematic examination of fragmentation patterns using tandem MS that was not previously possible using single quadrupole instrumentation. Figure 5 shows the positive APCI daughter ion mass spectra for the leuco (a) and cationic (b) series, all obtained at a common collision energy (30 eV). The fragmentation of the leuco species was dominated by consecutive losses of methyl and phenyl moieties. The isomeric dides-LMG species showed identical mass spectra at all collision energies (18). The mass spectra for cationic species show individual losses of all pendant groups; for example, MG lost methyl (m/z 313), benzene (m/z 251), and *N,N*-dimethylaniline (m/z 208) moieties. With the *N*-demethylated cationic derivatives, losses of each possible substituted aniline moiety were also observed; for example, monodes-MG lost the *N*-methylaniline and *N,N*-dimethylaniline moieties to give m/z 208 and m/z 194, respectively (Figure 5b). Because of this property, it was possible to assign unambiguously the structures for isomeric dides-MG. The unsymmetrical isomer dides(unsym)-MG lost *N,N*-dimethylaniline to yield m/z 180 and aniline to yield m/z 208 whereas the symmetrical isomer lost only *N*-methylaniline to yield m/z 194. The APCI mass spectra of the cationic species thus provided more structural information than the corresponding leuco species. Therefore, the use of on-line postcolumn oxidation of chromatographically separated leuco species with the PbO_2 postcolumn (see below) in conjunction with APCI/MS can provide unambiguous identification of all MG-derived products.

UV/vis Spectral Characteristics. The chromatographic and UV/vis absorption characteristics of LMG and MG derivatives are in good agreement with sequential *N*-demethylation, and the details are summarized in Table 3. Figure 6 shows the normalized on-line UV/vis spectral overlays of all six MG and LMG derivatives (Figure 6a,b, respectively). On-line spectra of the cationic MG derivatives were generated by injecting the corresponding LMG derivatives into a HPLC system, which

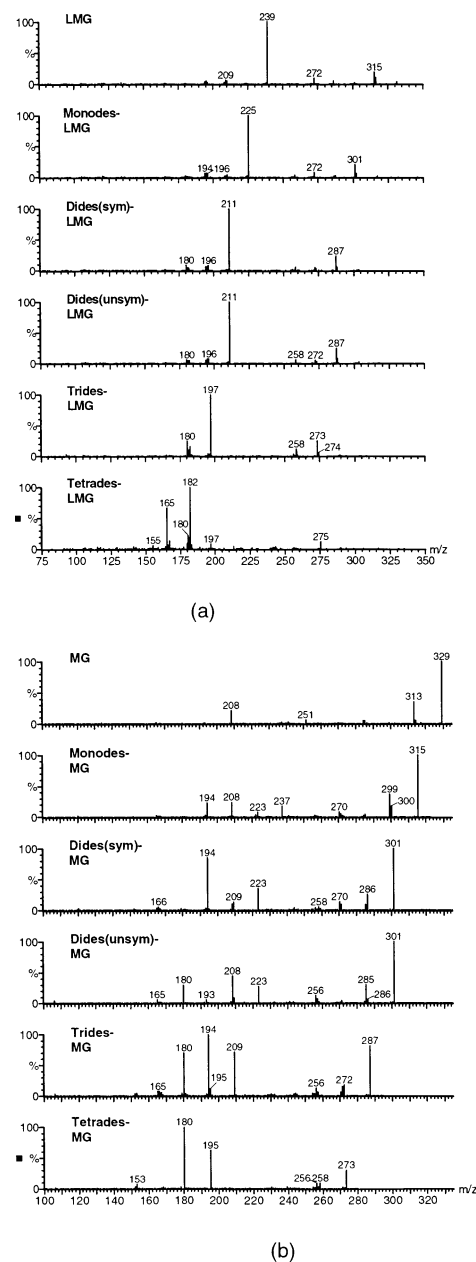


Figure 5. APCI mass spectra of (a) LMG, (b) MG, and their *N*-demethylated derivatives at collision energy 30 eV.

was attached to a column reactor containing 10% PbO_2 on Celite. This postcolumn technique, which was first developed by Allen and Meinertz (28), has been used widely since LMG and MG can be analyzed simultaneously with increased specificity (3, 29).

The overall absorption characteristics of the *N*-methylated LMG and MG derivatives are very similar to those of the parent compounds but showed a systematic hypsochromic shift upon sequential *N*-demethylation. The effect appears to follow the general additivity rules (30); for example, a hypsochromic shift was observed going from *N,N*-dimethylaniline (251 nm) to *N*-methylaniline (245 nm) and to aniline (233 nm) in methanol (not shown). A similar systematic, although small, hypsochromic shift (3.5–4.8 nm per methyl) was observed going from LMG to the fully *N*-demethylated tetra-LMG (Figure 6b). The effect was much greater for the cationic MG derivatives (Figure 6a, 12.2–13.5 nm per methyl), indicating the importance of resonance contributions to

Table 3. Summary of Chromatographic and Spectral Properties of LMG and MG and Their *N*-Demethylated Derivatives^a

compd	TLC <i>R_f</i> values ^b	HPLC <i>t_R</i> (min) ^c	λ_{\max} for LMG (nm)	λ_{\max} for MG (nm)	ESI-MS ^d (<i>m/z</i>)
LMG (MG)	0.70 [0.53, green]	22.17 [24.54] ^c	261.9, 308.0(sh)	618.0, 424.6, 316.6	331 [329]
monodes-LMG (-MG)	0.44 [0.47, light green]	21.08 [23.04]	258.4, 298.7(sh)	605.8, 418.4, 314.2	317 [315]
dides(sym)-LMG (-MG)	0.29 [0.37, dark purple]	19.85 [21.36]	253.6, 299.9(sh)	593.6, 412.3, 310.6	303 [301]
dides(unsym)-LMG (-MG)	0.24 [0.37, purple]	19.81 [21.55]	252.5, 298.0(sh)	592.3, 409.9, 310.6	303 [301]
trides-LMG (-MG)	0.11 [0.34, dark pink]	18.44 [19.71]	248.9, 288.6(sh)	578.9, 405.1, 307.1	289 [287]
tetrads-LMG (-MG)	0.05 [0.32, pink]	16.87 [17.96]	244.2, 289.2(sh)	566.7, 403.9, 305.9	276 [274]

^a The values in brackets indicate those of the cationic MG derivatives. ^b TLC solvent system = hexane:benzene:ether:triethylamine (10:10:3:0.5) for LMG derivatives; dichloromethane:methanol (9:1) for MG derivatives (colors appeared upon standing on the TLC plate). ^c HPLC conditions: a 30 min gradient 10–95% acetonitrile and ammonium acetate (pH 4.5), 1.0 mL/min. Spherisorb CN, 5 μ m, 4.6 mm \times 250 mm. A postcolumn containing 10% PbO₂ in Celite was used for the cationic MG derivatives. ^d (M + H)⁺ for LMG derivatives; M⁺ for MG derivatives.

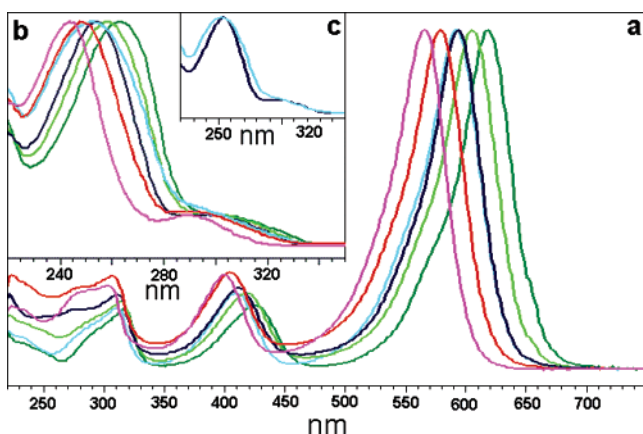


Figure 6. On-line HPLC–UV/vis spectral overlays of (a) MG, (b) LMG, and their *N*-demethylated metabolites: MG and LMG (dark green), monodes- (green), dides(unsym)- (cyan), dides(sym)- (black), trides- (red), and tetrads- (magenta). The inset in panel c shows the UV spectra of dides(sym)-LMG and dides(unsym)-LMG. HPLC separation of the cationic MG derivatives was obtained with a photodiode array detector using a Spherisorb column (CN, 4.6 mm \times 250 mm, 4.6 mm \times 250 mm, 5 μ m) with a 30 min gradient, 10–90% acetonitrile/100 mM ammonium acetate, pH 4.5, 1.0 mL/min.

the cationic MG structure (see above). It is well-known that the interaction between the nonbonding and the π -orbitals is most effective in the para-substituted aniline ring system (31).

While the HPLC retention times of the isomeric dides-MG and -LMG derivatives (sym and unsym) were very similar (Table 3), their UV/vis absorption characteristics were different; for example, the λ_{\max} of the symmetric dides-MG was slightly more red-shifted (1.3 nm) as compared to the unsymmetric one (Figure 6a). The shift difference was smaller (<1.1 nm) for the corresponding leuco derivatives (Figure 6b). The dides(unsym)-LMG isomer also exhibited a flattened absorption maximum, which was noticeably different from the sym one (Figure 6c) and other derivatives. As discussed above, the APCI mass fragmentation could differentiate the isomeric dides-MG derivatives but not the corresponding leuco ones.

In summary, we have described convenient synthetic procedures for preparing MG and LMG and their *N*-demethylated derivatives. The availability of the synthetic standards and their thorough spectral characterization are important for future metabolic and DNA adduct studies. The synthetic procedure is general in scope so that it can be extended to the preparation of *N*-demethylated metabolites of other structurally related triphenylmethane dyes.

Acknowledgment. We thank D. R. Doerge for valuable comments and reading the manuscript. This research was supported in part by an appointment of B.P.C. to the Faculty Research Participation Program at the National Center for Toxicological Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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