Notes

Nordihydroguaiaretic Acid Autoxidation Produces a Schisandrin-like Dibenzocyclooctadiene Lignan

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Received March 4, 2008

The lignan *meso*-nordihydroguaiaretic acid is known to undergo spontaneous oxidation in alkaline solution. In the presence of the trapping agent glutathione, the major oxidation products are consistent with the formation of a *meso*-nordihydroguaiaretic acid *ortho*-quinone. In the absence of a trapping agent however, the major oxidation product of *meso*-nordihydroguaiaretic acid in aqueous solution is a unique, stable schisandrin-like dibenzocyclooctadiene lignan that may be responsible for some of the biological effects of nordihydroguaiaretic acid.

Nordihydroguaiaretic acid (NDGA, masoprocol, **1**) (Figure 1) is a naturally occurring lignan from the creosote bush (*Larrea tridentata*). NDGA has been utilized in traditional healing practices for a wide range of ailments[1,2] and was licensed for use as a topical treatment for actinic keratosis (Actinex, Chemex Pharmaceuticals, Denver, CO). Use of NDGA for therapeutic purposes is currently limited due to reports of contact hypersensitivity,[3] nephrotoxicity,[4] hepatotoxicity,[5,6] and cytotoxicity.[5,7,8] It has been suggested that the toxicity is the result of oxidation to a reactive *ortho*-quinone species,[4] the presence of which we recently confirmed via trapping of NDGA *ortho*-quinones as glutathione (GSH) adducts.[9] NDGA has been shown to increase oxidized glutathione (GSSG) and lipid peroxidation levels in murine hematopoietic cells,[10] suggesting an increase in oxidative stress consistent with the formation of an *ortho*-quinone. There are numerous reports that NDGA can be unstable in aqueous media,[4,11−13] especially at elevated pH,[11,13] with a half-life of 3.1 h at pH 7.4.[9] NDGA, in the presence of O_2, has been reported to undergo conversion to an *ortho*-quinone,[12] or to an unspecified "activated NDGA", with a λ_{max} 286nm.[11] In this study we have isolated and identified the major non-GSH reactive product of NDGA autoxidation in aqueous solution and determined that NDGA forms a unique, stable nonquinone compound with a λ_{max} of 286 nm, suggesting it is the "activated NDGA" previously reported.[11]

NDGA was incubated at pH 7.4 in the absence of GSH, and HPLC was used to monitor the reaction. A mixture of products was observed and the reaction mixture was further analyzed by LC-MS (Figure 2). One of the LC-MS peaks was consistent with an *ortho*-quinone of NDGA that has undergone hydroxylation (t_R = 29 min, **3** ES-MS *m*/*z* 315 (100%) [M − H]$^-$). The ES-MS results for the 22 and 25 min peaks were consistent with either an *ortho*-quinone or *para*-quinone methide (ES-MS *m*/*z* 299 (100%) [M − H]$^-$), although previous studies suggested that *para*-quinone methide formation is unlikely.[9] Addition of GSH to the incubation mixture resulted in disappearance of both the 25 min and the 29 min peaks and the appearance of NDGA-SG adducts (data not shown). The major product, the 22 min peak (**4**), however was unaffected by the addition of GSH, suggesting it was not a quinone species. The peak at 34 min was confirmed as being NDGA (**1**) by MS (ES-MS *m*/*z* 301 (100%) [M − H]$^-$) and comparison with an authentic standard.

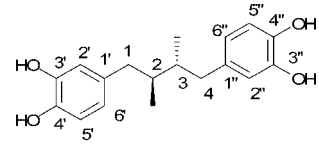

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Figure 1. Nordihydroguaiaretic acid (NDGA).

Compound **4** was isolated and purified via reversed-phase flash column chromatography and was found to have an identical UV absorbance maximum (286 nm) to the previously unidentified autoxidation product of NDGA.[11] NDGA possesses a plane of symmetry, so that only half of the protons and carbons appear in the NMR spectrum. ^{1}H and ^{13}C NMR spectroscopic data for compound **4** indicate that distinct resonances are observed for all of the alkyl and aromatic protons. There are several pieces of NMR evidence to suggest that **4** is an intramolecular cyclization product of NDGA (Figure 3). Four singlets in the aromatic region corresponding to one proton each were observed (HMQC), suggesting that two of the aromatic protons of NDGA have been substituted due to the formation of a new carbon−carbon bond. A coupling reaction involving C-2′, C-2″, C-5′, or C-5″ of NDGA would result in splitting of the aromatic ^{1}H NMR signals. Our results suggest that coupling has occurred between the C-6′ and C-6″ positions of NDGA, which have previously been shown to be highly reactive.[9] A second piece of evidence comes from a COSY NMR experiment for **4**, where it was determined that only one of the CH_2 protons at C-7 is coupled to the adjacent CH proton at C-8 (this is also the case for the protons on C-7′ and C-8′), suggesting that one of the CH_2 protons is held at a 90° dihedral angle to the CH, a conformation that may be attained in an eight-membered ring. A compound that has cyclized between the aromatic C-6′ and C-6″ carbons of NDGA would result in the dibenzocyclooctadiene lignan **4**, as represented in Figure 4. As a result of the nonplanar eight-membered ring that is formed, compound **4** has no plane of symmetry and the NMR chemical shifts for all of the protons and carbon atoms of **4** are distinguishable. The aromatic protons at C-1 and C-9 of compound **4** (the numbering in NDGA **1** differs from that for the NDGA-derived dibenzocyclooctadiene lignan **4**; refer

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10.1021/np8001354 CCC: $40.75
Published on Web 08/02/2008

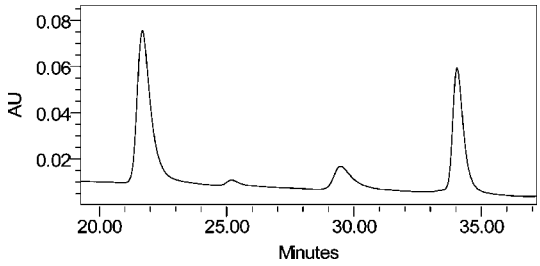

Figure 2. LC-MS-ES(−) (microbore column, see Experimental Section) for NDGA autoxidation products, pH 7.4, 37 °C. t_R = 22 min, *m/z* 299 $[M - H]^-$ (**4**); t_R = 25 min, *m/z* 299 $[M - H]^-$ (**2**); t_R = 29 min, *m/z* 315 $[M - H]^-$ (**3**); t_R = 34 min, *m/z* 301 $[M - H]^-$ (NDGA **1**).

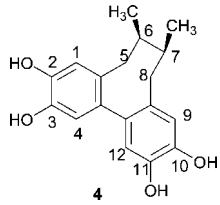

Figure 3. Structure of the NDGA-derived dibenzocyclooctadiene lignan **4**, the major NDGA autoxidation product at pH 7.4, 37 °C.

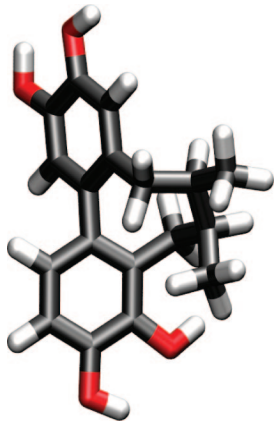

Figure 4. Representation of NDGA-derived dibenzocyclooctadiene lignan **4** in the *R*-biphenyl configuration from *meso*-NDGA **1**. This representation is based on NMR and CD data, after energy minimization in the MMFF94 force field using Spartan '06 (Wavefunction Inc., Irvine, CA). Image generated using VMD (Urbana, IL).

to Figures 1 and 3) were tentatively assigned from observed NOEs with H-5 and H-8, respectively. The ^1H and ^{13}C NMR results for the alkyl region were comparable to those of other dibenzocyclooctadiene lignans including gomisin J[14] and the (*R*,*R*)-tetramethoxy analogue of **4**.[15,16] A crystal structure reported for the (*R*,*R*)-tetramethoxy analogue of **4** indicates the phenyl rings are twisted about the biphenyl bond by an angle of 117.8°.[16] Compound **4**, determined by CD to be in the *R*-biphenyl configuration, displays a similar out-of-plane twist.

Similar coupling products have been prepared via oxidation of 4-methylcatechol with phenoloxidase,[17] and numerous synthetic dibenzocyclooctadiene lignans have been prepared from intramolecular cyclization reactions of matairesinol derivatives.[18−20] One possible mechanism for formation of dibenzocyclooctadiene lignan **4** from NDGA via a radical intermediate is suggested in Figure 5, although other radical and nonradical processes cannot be ruled out. This mechanism requires initial formation of phenoxy radicals at the 3′-OH and 3″-OH of NDGA. This diphenoxy radical of NDGA would undergo radical coupling of the carbon atoms *para* to the 3′- and 3″-OH of NDGA via the carbon-centered resonance forms (Figure 5).

A group of dibenzocyclooctadiene lignans known as schisandrins, isolated from *Schisandra chinensis*, have been studied for their potential therapeutic properties including the ability to protect against oxidative stress as antioxidants[21] or via enhancement of GSH synthesis;[22,23] the ability to inhibit P-glycoprotein[24] and multidrug resistance-associated protein 1;[25] and enhancement of apoptosis through caspase-9 activation.[26] In addition, schisandrin B is a substrate for murine CYP2E1,[27] and several schisandrins have been found to be CYP3A4 inhibitors.[28] The NDGA-derived dibenzocyclooctadiene lignan **4** is an analogue of the schisandrins and thus may also display similar biological activity. It is conceivable that the NDGA-derived dibenzocyclooctadiene lignan is also responsible for a portion of the biological activity of NDGA, especially if NDGA undergoing biological study is maintained under aerobic conditions for extended periods of time at pH 7.4 or higher.

We have observed that in the absence of GSH at pH 8.0, 37 °C, 50% of the NDGA is mainly converted to **4** after a 1 h incubation, with smaller amounts present as the *ortho*-quinone and hydroxylated *ortho*-quinone. In our microsomal incubations (pH 7.4, 37 °C) NDGA is preincubated for 5 min followed by a 60 min incubation, which is sufficient for measurable NDGA autoxidation to occur. NDGA is a known antioxidant[29] and a lipoxygenase inhibitor[2] and has been shown to inhibit P450[30] and suppress growth of breast cancer cells.[31] All of these systems incubate NDGA at or near pH 7.4, suggesting that at least some of the observed activity may be the result of the NDGA-derived dibenzocyclooctadiene lignan or an NDGA *ortho*-quinone. An *ortho*-quinone of NDGA would be expected to be involved in redox cycling or adduct formation with biological macromolecules,[9] whereas the dibenzocyclooctadiene lignan appears to be more stable and may be responsible for mediating biological processes associated with NDGA that are not necessarily associated with toxicity.

Extracts from Creosote bush containing NDGA used in traditional healing practices are typically prepared by boiling in water and then being either applied topically as a salve or paste or consumed as a tea (T. D. Johnson, personal communication). It is possible that the traditional extraction process may result in a significant conversion of NDGA- to NDGA-derived dibenzocyclooctadiene; however, this will require further investigation. An NDGA-derived dibenzocyclooctadiene lignan, in which the stereochemistry was not specified, has been screened by the National Cancer Institute (NSC 669349) in the United States and displays LD_{50} values in the μM range. In order to determine whether the NDGA-derived dibenzocyclooctadiene lignan **4** is responsible for the effects in the NCI study or any other biological activity associated with NDGA, it will be necessary to test the NDGA-derived dibenzocyclooctadiene lignan derived from *meso*-NDGA in a variety of *in vitro* systems. Of perhaps greater issue is that due to the rapid formation of the NDGA-derived dibenzocyclooctadiene lignan under conditions used for many *in vitro* studies, interlaboratory differences in incubation conditions for NDGA-dependent experiments could lead to significantly different results depending on the actual amount of NDGA- or NDGA-derived dibenzocyclooctadiene lignan present.

Experimental Section

General Experimental Procedures. *Caution*: *NDGA is hazardous and should be handled carefully.* *meso*-Nordihydroguiairetic acid (NDGA) from *Larrea tridentata* was purchased from Sigma-Aldrich Co. (Madison, WI). Citric acid and K_2HPO_4 were purchased from Fisher Scientific (Ottawa, ON). HCl was purchased from BDH (Toronto, ON). All solvents were HPLC grade. Water was purified via a Millipore Milli-Q system (Mississauga, ON) with a Quantum EX cartridge.

UV spectra were recorded on an Agilent 8453 using Chem Station software. Negative electrospray mass spectra were obtained using an API Qstar XL with an Agilent 1100 HPLC (Saskatchewan Structural

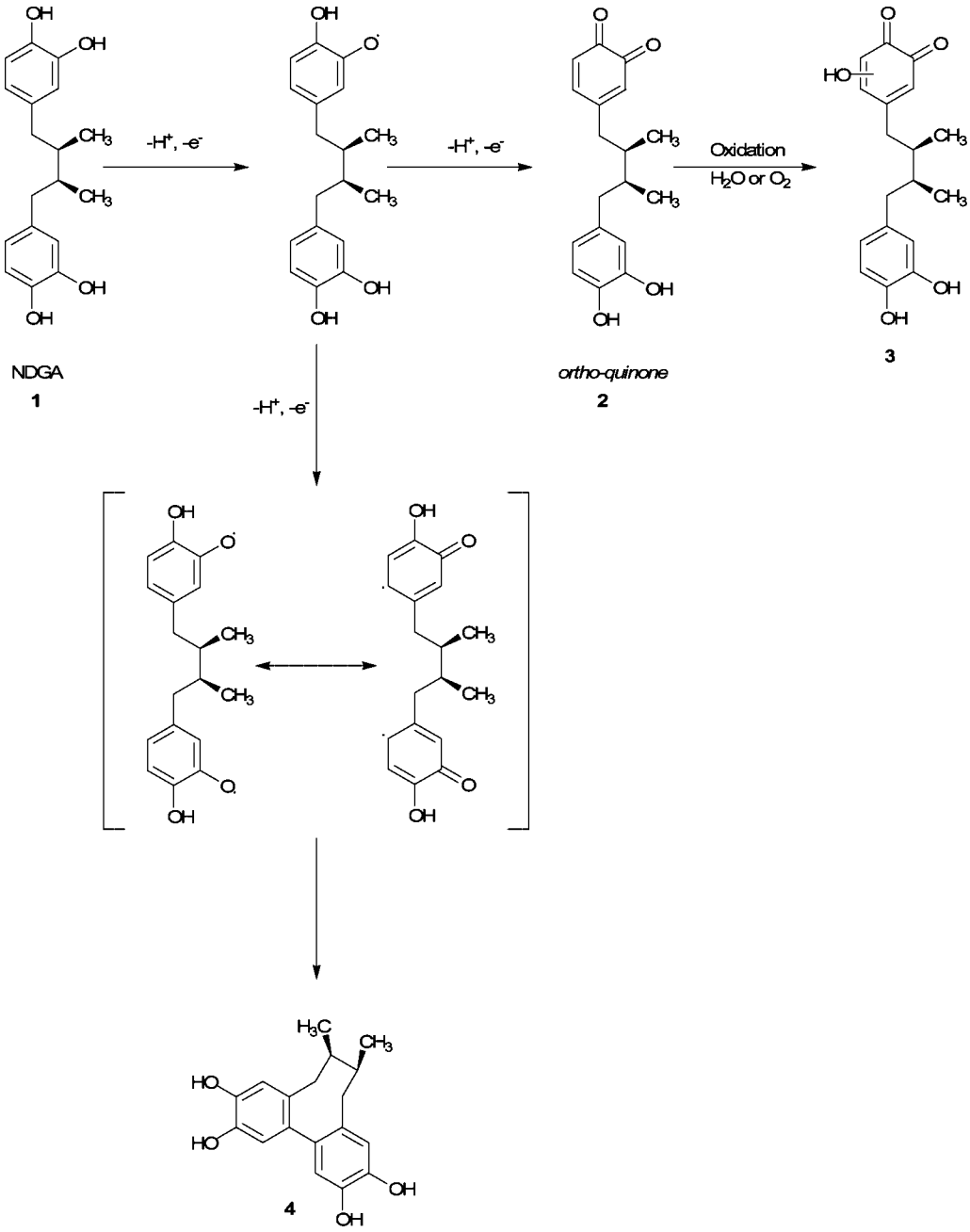

Figure 5. Possible reaction pathway for the formation of NDGA autoxidation products **3** and **4** in the absence of GSH.

Sciences Centre). NMR data were obtained on a Bruker AVANCE DPX-500 (Dept of Chemistry, University of Saskatchewan) operating at 500 and 125 MHz for proton and carbon, respectively, and the data analyzed using XWIN-NMR version 3.0. Residual signals from CD_3OD [3.30 ppm (^1H) and 49.0 ppm (^{13}C)] served as an internal standard. Programs for 2D experiments were available from the software package XWINNMR, provided by Bruker. The DEPT experiment together with 2D NMR experiments including COSY, HMQC, and NOESY experiments were performed with gradient pulses. CD data were obtained on an Applied Photophysics PiStar-180 spectrometer.

Autoxidation Incubation for ES-MS Analysis. A solution of NDGA in CH_3CN (20 mM) was added to 0.5 M pH 7.4 phosphate-citric acid buffer pre-equilibrated to 37 °C to give a final NDGA concentration of 0.1 mM. The reaction mixture was placed in a shaking water bath for 60 min, during which time the colorless solution became pink. The reaction was stopped by acidification to pH 1.5 with HCl. Autoxidation was not effected by light, as no difference was observed for dark control reactions. Aliquots of the supernatant (50 μL) were analyzed directly by electrospray LC-MS operating in the negative mode. An Allsphere ODS-2 microbore column (3 μm, 150 × 2.1 mm) operating at a flow rate of 0.2 mL/min was used to run a gradient elution. Solvent A: 0.1% formic acid/H_2O, solvent B: 0.1% formic acid/CH_3CN. An initial isocratic phase of 80% A for 2 min, decreased to 65% A over 38 min, isocratic for 1 min, and finally increased to 80% A over 1 min.

Synthesis of the NDGA Autoxidation Product. A solution of NDGA in CH_3CN (20 mM) was added to 0.5 M pH 8.5 phosphate-citric acid buffer pre-equilibrated to 37 °C to give a final NDGA concentration of 0.1 mM. The reaction mixture was placed in a shaking water bath for 90 min, during which time the colorless solution became pink. The reaction was stopped by acidification to pH 1.5 with HCl. The reaction mixture was concentrated *in vacuo* to yield a purple solid. The oxidation product was purified by C-18 flash column chromatography, using 70:30 H_2O/CH_3CN (v/v) containing 0.1%TFA, yielding an off-white solid.

(6*R*,7*S*)-2,3,10,11-Tetrahydroxy-6,7-dimethyl-5,6,7,8-tetrahydrodibenzo[*a*,*c*]cyclooctene (4): CD (*c* 0.62, CH_3OH) $[\theta]_{286}$ +15158.7; UV (CH_3CN) λ_{max} (log ϵ) 286 nm (3.87); ^1H NMR (CD_3OD, 500 MHz) δ 0.78 (3H, d, J = 6.9 Hz, CH_3-6), 0.99 (3H, d, J = 7.0 Hz, CH_3-7), 1.79 (1H,m, CH-7), 1.88 (1H, m, CH-6), 1.93 (1H, d, J = 13.2 Hz, CH_2-8b), 2.21 (1H, dd, J = 9.6, 13.2 Hz, CH_2-8a), 2.39 (1H,d, J =

13.5 Hz, CH_2-5b), 2.54 (1H, dd, $J = 7.5$, 13.5 Hz, CH_2-5a), 6.54 (1H, s, ArCH-9), 6.57 (1H, s, ArCH-1), 6.61 (1H, s), 6.63 (1H, s); ^{13}C NMR (CD_3OD, 125 MHz) δ 12.7 (CH_3-6), 22.2 (CH_3-7), 35.1 (C-6), 35.9 (C-8), 39.7 (C-5), 42.2 (C-7), 116.7, 117.3 (ArC-9), 117.6 (ArC-1), 119.9, 130.3, 133.7, 134.7, 135.8, 143.9, 144.1, 144.5, 145.6; ES-MS $t_R = 22$ min, negative ion electrospray-MS (80% CH_3CN with 0.1% formic acid), *m/z* 299 (100%) $[M - H]^-$); yield 16.2 mg, 54%.

Acknowledgment. This work was funded by HSURC, NSERC, and CFI. J.L.B. was a recipient of an NSERC PGS award. The authors wish to thank Dr. D. R. J. Palmer for help with generating the dibenzocyclooctadiene lignan image.

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NP8001354