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Ring substitution influences oxidative cyclisation and reactive metabolite formation of nordihydroguaiaretic acid analogues

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ABSTRACT

Nordihydroguaiaretic acid (NDGA) is a natural polyphenol with a broad spectrum of pharmacological properties. However, its usefulness is hindered by the lack of understanding of its pharmacological and toxicological pathways. Previously we showed that oxidative cyclisation of NDGA at physiological pH forms a dibenzocyclooctadiene that may have therapeutic benefits whilst oxidation to an *ortho*-quinone likely mediates toxicological properties. NDGA analogues with higher propensity to cyclise under physiologically relevant conditions might have pharmacological implications, which motivated this study. We synthesized a series of NDGA analogues which were designed to investigate the structural features which influence the intramolecular cyclisation process and help to understand the mechanism of NDGA's autoxidative conversion to a dibenzocyclooctadiene lignan. We determined the ability of the NDGA analogues investigated to form dibenzocyclooctadienes and evaluated the oxidative stability at pH 7.4 of the analogues and the stability of any dibenzocyclooctadienes formed from the NDGA analogues. We found among our group of analogues the catechols were less stable than phenols, a single catechol-substituted ring is insufficient to form a dibenzocyclooctadiene lignan, and only compounds possessing a di-catechol could form dibenzocyclooctadienes. This suggests that quinone formation may not be necessary for cyclisation to occur and the intramolecular cyclisation likely involves a radical-mediated rather than an electrophilic substitution process. We also determined that the catechol dibenzocyclooctadienes autoxidised at comparable rates to the parent catechol. This suggests that assigning in vitro biological activity to the NDGA dibenzocyclooctadiene is premature and requires additional study.

1. Introduction

The leaves of the plant creosote bush were used commonly in traditional medicines among the Native Americans for diverse beneficial effects.[1–3] The aqueous extract of this shrub, commonly referred to as Chaparral tea, was listed in the American pharmacopeia as an ethnobotanical used to treat tuberculosis, arthritis and cancer.[1] Documented traditional applications of the plant extract include treatment for infertility, rheumatism, arthritis, diabetes, gallbladder and kidney stones, pain and inflammation among many others.[2,3] Creosote bush is rich in lignans, particularly nordihydroguaiaretic acid (NDGA) (up to 15% d.w.).[2] NDGA is generally accepted as responsible for both beneficial and adverse effects associated with this shrub.[2–6] NDGA shows promise in the treatment of multiple diseases, including cardiovascular diseases,[7,8] neurological disorders[9–13] and cancers.[3,14–20] It also potently inhibits viruses such as human immunodeficiency virus (HIV-1), herpes simplex virus (HSV), human papilloma virus (HPV) and influenza virus.[2,21] The radical scavenging[22,23] and antioxidant effects[24] as well anti-inflammatory[25,26] and anti-proliferative properties may be of relevance in different diseases. Despite its broad pharmacological activities, NDGA use is associated with toxicity especially when ingested at higher doses. Hepato- and nephrotoxicity has been associated with NDGA use[24,27–30] and is likely linked to NDGA bioactivation to a reactive *ortho*-quinone.[31–33]

The origin of the dichotomous biological activity observed for NDGA remains unknown. Traditionally creosote bush-based products are prepared by boiling the leaves in water.[3] We previously demonstrated that incubation of NDGA at pH 7.4 gave a

Abbreviations: NDGA, nordihydroguaiaretic acid; cNDGA, NDGA cyclolignan; DMPA, 3,4-dimethoxyphenylacetone; ESI-MS, electrospray ionization-mass spectrometry; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; HIV-1, human immunodeficiency virus; HSV, herpes simplex virus; HPV, human papilloma virus; NADP(H), reduced nicotine adenine dinucleotide phosphate; TFA, trifluoroacetic acid.

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schisandrin-like dibenzocyclooctadiene lignan,[34] however in the presence of glutathione (GSH) a ring adduct is observed, indicating that NDGA can form an *ortho*-quinone.[31] Biological evaluations of NDGA are commonly conducted under aerobic conditions at pH 7.4 or higher, which may confound the results as biological activity could be the result of NDGA, its *ortho*-quinone, dibenzocyclooctadiene or a combination.[5,35] Traditional conditions of preparation favour oxidation and we suggested that the NDGA dibenzocyclooctadiene may make a contribution to the beneficial effects of NDGA, and that NDGA analogues with a higher propensity to cyclise under physiologically relevant conditions might have pharmacological implications. There are numerous reports of biological activity for naturally-occurring and synthetic dibenzocyclooctadiene lignans,[36–39] including a report that for a series of dibenzylbutanediols the dibenzocyclooctadiene structure enhanced anti-tumor activity and inhibition of MDA-MB-435 breast cancer cells.[40] Pharmacological activity from the uncyclised lignan cannot be ruled out however, as anti-viral properties have been reported for tetramethyl and tetraacetyl NDGA which would not be anticipated to cyclise under these conditions.[21]

The precise mechanism of the intramolecular cyclisation is unknown although it possibly follows one of two pathways (Scheme 1).[34,41–43] We[34] and others[22] have hypothesized that cyclisation occurs through a di-radical mechanism, whereas there are numerous examples where intermolecular coupling of catechols is proposed to occur via electrophilic substitution through an *ortho*-quinone intermediate.[42,44] We therefore propose to identify the structural features which control oxidative metabolism of NDGA-like lignans, including cyclisation. In order to accomplish these goals we plan to: investigate the oxidative stability of NDGA lignan and dibenzocyclooctadiene analogues at pH 7.4 and determine what structural features are necessary for formation of dibenzocyclooctadienes from the NDGA lignan analogues. We synthesized seven NDGA analogues (Fig. 1) and evaluated their stability including lignan half-life at pH 7.4, and product studies to determine their ability to form dibenzocyclooctadienes using our previously established conditions.[34] We were also interested in establishing the oxidative stability of the prepared analogues as stability of a compound in a test medium is an important parameter when investigating biological activity.[45,46]

The analogues were designed to help us understand the structural features which influence the intramolecular cyclisation process. This will be useful in understanding the mechanisms of NDGA autoxidative conversion to dibenzocyclooctadiene lignans and will allow us to control intramolecular cyclisation in the preparation of additional lignan analogues. We anticipate that these studies will provide information on the contribution that dibenzocyclooctadienes play in the in vitro pharmacological activity of NDGA.

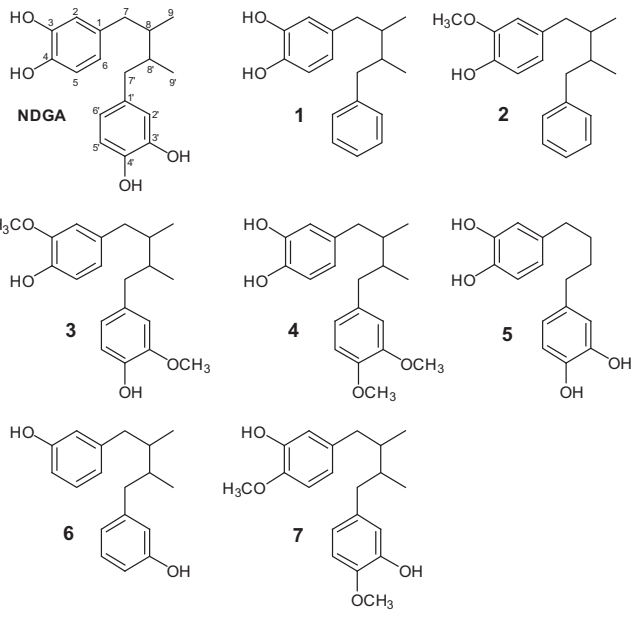
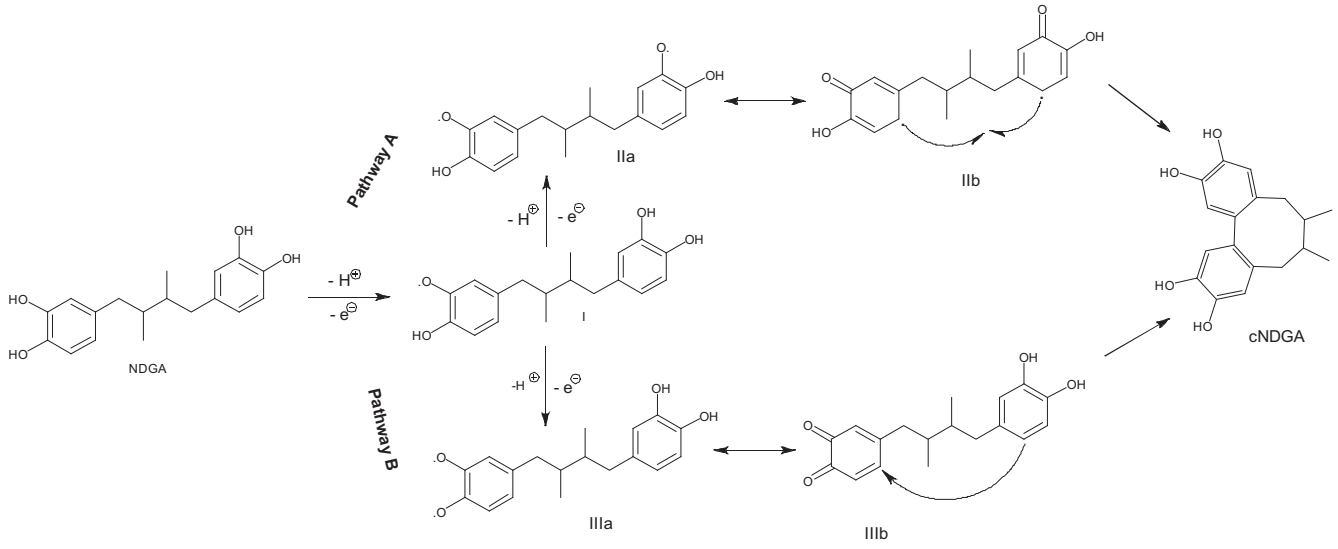


Figure 1. Structures for NDGA and its structural analogues synthesised for this study.

2. Materials and methods

2.1. Materials

Caution: *The following chemicals are hazardous and should be handled carefully:* ***meso***-Nordihydroguaiaretic acid (NDGA, 97%) from *Larrea tridentata*, reduced glutathione (GSH), 3,4-dimethoxyphenylacetone (DMPA), Tyrosinase from mushroom (EC1.14.18.1),



Scheme 1. Intramolecular conversion of NDGA to dibenzocyclooctadiene lignan (cNDGA) via radical-mediated process (pathway A) or electrophilic substitution mechanism (pathway B). Both pathways involve a 2 proton, 2 electron loss followed by isomerisation of a di-radical intermediate IIa–IIb or an *ortho*-quinone IIIa–IIIb and subsequent radical coupling or electrophilic substitution, respectively.

dimethylsulfoxide (Me_2SO), NADP(H), $AgNO_3$, K_2HPO_4 and $MgSO_4$ were purchased from Sigma–Aldrich Co. (Madison, WI). Citric acid and HCl were purchased from Fisher Scientific (Ottawa, ON). Formic acid was purchased from BDH. All solvents were HPLC grade. Water was purified via a Millipore (Mississauga, ON) Milli-Q system with a Quantum EX Cartridge. NDGA analogues were prepared by modifications of literature procedures.

2.2. Instrumentation

2.2.1. HPLC-UV diode array analysis

The HPLC system consisted of a Waters 2690 separation module equipped with a Waters 996 photodiode array detector and Masslynx V4.1 software (Waters Corp., Milford, MA). 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 90% A for 2 min, decreased to 60% A over 8 min, then 10% A over 12 min, held isocratic for 10 min, and finally increased to 90% A over 1 min and held isocratic at 90% A for 6 min to equilibrate the column.

2.2.2. ESI-MS analysis

All MS experiments were conducted using AB SCIEX 4000 QTRAP (AB SCIEX instruments) quadrupole linear ion trap mass spectrometer. Samples were infused directly at 10 μl/min. Data acquisition and analyses were performed using Analyst 1.5.1 software from AB SCIEX.

2.2.3. NMR analysis

All NMR experiments were performed on a Bruker AVANCE DPX-500 spectrometer and data processed by X-WIN NMR 3.5 software or TopSpin 3.2. All compounds were named using ACD/ChemSketch.

2.3. Methods

2.3.1. Synthesis and characterisation of NDGA analogues

The basic 18-carbon lignan skeleton was constructed using consecutive Stobbe condensation[47,48] or a Stobbe condensation followed by alkylation.[40,49] We followed literature procedures to synthesise NDGA analogues **1**–**7** and characterised them by the ^1H and ^{13}C NMR spectroscopy as well as ESI-MS methods. Yields reported are overall yields.

2.3.2. 4-(2,3-Dimethyl-4-phenylbutyl)benzene-1,2-diol (1)

Analogue **1** was obtained as dark purple oil (yield 8–15%). 96.7% pure by HPLC; [^1H] NMR (500 MHz, $CDCl_3$): δ (ppm) 0.84–0.87 (6H, 2 overlapping d, J = 4.3, H9, 9′), 1.74–1.84 (2H, m, H8, 8′), 2.32–2.36 (1H, dd, J = 8.5, 13.4, H7α), 2.43–2.48 (1H, dd, J = 8.6, 13.4, H7α′), 2.55–2.59 (1H, dd, J = 6.4, 13.6, H7β), 2.66–2.70 (1H, dd, J = 6.2, 13.6, H7β′), 5.78 (2H, bs, *o*, *p*-ArO*H*), 6.55–6.57 (1H, dd, J = 1.9, 8.1, H6), 6.64 (1H, d, J = 1.9, H2), 6.79 (1H, d, J = 7.9, H5), 7.14 (2H, d, J = 7.2, H2′, 6′), 7.21 (1H, t, J = 7.4, H4′), 7.30 (2H, t, H3′, 5′). [^{13}C] NMR (500 MHz, $CDCl_3$): δ (ppm) 13.97, 16.24, 38.10, 39.33, 40.67, 41.45, 115.18, 116.08, 121.55, 125.64, 128.17, 128.23, 129.13, 129.17, 134.89, 141.28, 141.78, 143.25. ESI-MS (m/z) = 269.1 $[M-H]^-$, found; 269.15 Da calcd.

2.3.3. 4-(2,3-Dimethyl-4-phenylbutyl)-2-methoxyphenol (2)

Compound **2** was obtained as yellow oil (yield 20–30%). >99% pure by HPLC; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.86–0.88 (6H, 2 overlapping d, H9, 9′), 1.77–1.85 (2H, m, H8, 8′), 2.38–2.49 (2H, m, H7α, 7α′), 2.57–2.68 (2H, m, H7β, H7β′), 3.84 (3H, s, OCH_3), 5.51 (1H, bs, *p*-ArO*H*), 6.58 (s, 1H), 6.62–6.65 (1H, m,), 6.84–6.88 (1H, m,), 7.13 (d, J = 7.2, 1H), 7.18–7.23 (m, 2H), 7.23–7.33 (m, 2H). ^{13}C NMR (500 MHz, $CDCl_3$): δ (ppm) 13.95, 16.11, 37.81, 38.94, 41.09, 41.45, 55.84, 111.34, 113.97, 121.67, 125.64, 128.15, 129.08, 133.57, 141.73, 143.55, 146.29. ESI-MS (m/z) = 283.2 $[M-H]^-$ found; 283.17 Da calcd.

2.3.4. 4,4′-(2,3-Dimethylbutane-1,4-diyl)bis(2-methoxyphenol) (3)

Compound (**3**) was obtained as off-white solid (yield 20–30%). >99% pure by HPLC; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.83–0.86 (6H, 2 overlapping d, J = 6.4, H9, 9′), 1.72–1.76 (2H, m, H8, 8′), 2.26–2.32 (1H, dd, J = ,H7α), 2.37–2.41 (1H, dd, J = 7.4, 13.4, H7α′), 2.51–2.55 (1H, dd, J = 7.4, 13.7, H7β), 2.72–2.76 (1H, dd, J = 4.1, 12.9, H7β′), 3.82 (3H, s, *p*-OCH_3), 3.87 (3H, s, *o*-OCH_3), 5.58 (2H, bs, *p*-ArO*H*), 6.55 (1H, s, H2), 6.60 (1H d, J = 7.8, H6), 6.64 (1H, s, H2′), 6.68 (1H, d, J = 7.8, H6′), 6.83 (1H, d, J = 8.0, H5), 6.85 (1H, d, J = 8.0, H5′). ^{13}C NMR (500 MHz, $CDCl_3$) δ (ppm) 14.04, 16.40, 21.25, 37.62, 39.07, 39.36, 41.26, 55.95, 56.03, 60.62, 111.46, 111.61, 114.04, 114.15, 121.81, 121.89, 133.77, 133.97, 143.67, 143.73, 146.42, 146.50. ESI-MS m/z: 329.1 $[M-H]^-$ found; 329.18 Da calcd.

2.3.5. 4[4-(3,4-Dimethoxyphenyl)-2,3-dimethylbutyl]benzene-1,2-diol (4)

Compound **4** was obtained as dark purple solid (yield 20–30%). >98% pure by HPLC; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.81–0.83 (6H, 2 overlapping d, J = 4.5, H9, 9′), 1.69–1.76 (2H, m, H8, 8′), 2.31–2.33 (2H, dd, J = 7.8, 13.4, H7α), 2.38–2.42 (1H, dd, J = 7.7, 13.4, 7α′), 2.46–2.51 (1H, dd, J = 7.0, 13.3, H7β), 2.51–2.55 (1H, dd, J = 7.3, 13.5, 7β′), 3.82 (3H, s, *p*-OCH_3), 3.86 (3H, s, *o*-OCH_3), 6.50 (1H, d, J = 6.2), 6.56 (1H, s), 6.58 (1H, s), 6.77–6.78 (m, 2H), ^{13}C NMR (500 MHz, MeOD): δ (ppm) 13.82, 13.89, 37.31, 37.44, 40.69, 40.98, 55.89, 55.92, 110.99, 112.28, 115.04, 115.96, 121.16, 121.49, 134.40, 134.69, 141.52, 143.29, 146.96, 148.55. ESI-MS (m/z) = 329.1 $[M-H]^-$ found; 269.18 Da calcd.

2.3.6. 4,4′-Butane-1,4-diyldibenzene-1,2-diol (5)

Compound **5** was obtained as brown solid (yield 40–45%). >98% pure by HPLC; ^1H NMR (500 MHz, CD_3OD): δ (ppm) 1.52 (4H, s, H8, 8′), 2.43 (4H, s, H7, 7′), 6.44 (2H, d, J = 8.0, H6, 6′), 6.56 (2H, s, H2, 2′), 6.63 (2H, d, J = 8.0, H5, 5′). ^{13}C NMR (500 MHz, CD_3OD): δ (ppm) 32.58 (C8, 8′), 36.26 (C7, 7′), 116.31 (C6, 6′), 116.64 (C5, 5′), 120.78 (C2, 2′), 135.75 (C1, 1′), 144.20 (C4, 4′), 146.13 (C3, 3′). ESI-MS (m/z) = 273.03 $[M-H]^-$ found; 273.11 Da calcd.

2.3.7. 3,3′-(2,3-Dimethylbutane-1,4-diyl)diphenol (6)

Analogue **6** was obtained as a brown oil (yield 20%). >96% pure by HPLC; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.87–0.89 (6H, 2 overlapping d, J = 6.5, H9, 9′), 1.61–1.69 (2H, m, H8, 8′), 2.32–2.36 (2H, dd, J = 8.3, 13.3, H7α, H7α′), 2.59–2.63 (2H, dd, J = 6.3, 13.4, H7β, H7β′), 5.56 (2H, bs, ArO*H*), 6.68–6.60 (2H, s), 6.70 (1H, d, J = 1.6), 6.76 (2H, d, J = 7.3), 6.83–6.86 (1H, m), 7.14–7.17 (2H, m). ESI-MS (m/z) = 269.1 $[M-H]^-$, found; 269.15 Da calcd.

2.3.8. 3,3′-(2,3-Dimethylbutane-1,4-diyl)bis(6-methoxyphenol) (7)

Compound **7** was obtained as off white solid (yield 20–30%). >98% pure by HPLC; ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.80–0.84 (6H, 2 overlapping d, J = 6.7, H9, 9′), 1.74–1.78 (2H, m, H8, 8′), 2.22–2.27 (1H, dd, J = 9.6, 13.3, H7α), 2.31–2.35 (1H, dd, J = 7.9, 12.8, H7α′), 2.54–2.58 (1H, dd, J = 5.8, 13.5, H7β), 2.70–2.74 (1H, dd, J = 4.5, 13.4, H7β′), 3.89 (6H, s, 3,3′ OCH_3), 5.58 (2H, bs, ArO*H*), 6.58–6.60 (1H, dd, J = 1.6, 8.1), 6.63–6.65 (1H, dd, J = 1.6, 8.1), 6.71 (1H, d, J = 1.6), 6.75–6.79 (3H, m). ESI-MS (m/z) = 329.13 $[M-H^+]^-$ obsd; 269.18 Da calcd.

2.3.9. Autoxidation study

The prepared analogues were evaluated for their potential to undergo intramolecular conversion to corresponding dibenzocyclooctadiene lignans using a method previously developed by our group.[34] Briefly, the substrate in CH_3CN (20 mM) is added to phosphate–citric acid buffer (0.5 M, pH 7.4) pre-equilibrated to 37 °C to give a final substrate concentration of 0.1 mM. The reaction mixture is stirred at the same temperature for 90 min and monitored by HPLC. The reaction was stopped by acidification to pH 1.5 with HCl. The reaction mixture is extracted with ethyl acetate, dried over $MgSO_4$ and solvent evaporated in vacuo. The oxidation product was purified over a C-18 column (RedSep®Rf GOLD, Teledyne Isco) on a Tris Pump connected to UA-6 UV/Vis Detector (Teledyne Isco) separation system using 70:30 H_2O/CH_3CN (v/v) containing 0.1% FA. The product was analysed by HPLC, MS and NMR techniques. Standard NDGA was used as positive control.

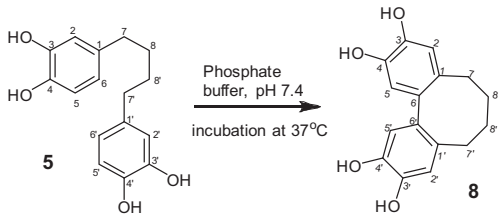
Autoxidation of **5** afforded compound **8** (Scheme 2) as a brown solid following purification by solid phase extraction. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 1.37 (2H, m), 1.94 (2H, m), 2.00 (2H, m), 2.47 (2H, m), 6.54 (2H, s, H2, 2′), 6.60 (2H, s, H5, 5′). ^{13}C NMR (500 MHz, CD_3OD): δ (ppm) 31.33 (C8, 8′), 33.36 (C7, 7′), 117.02 (C2, 5, 2′, 5′), 133.76 (C1, 1′), 135.63 (C6, 6′), 143.87 (C4, 4′), 145.71 (C3, 3′). The loss of C6, 6′ protons and the coupling of C7, 7′ as well as C8, 8′ protons are consistent with cyclisation and are also in agreement with our previous findings for the NDGA-derived dibenzocyclooctadiene lignan.[34] High-resolution mass spectroscopic data (see Supporting information) further confirmed cyclisation.

2.3.10. Chemical stability and reaction kinetics

The test compound in CH_3CN (20 mM) and internal standard (DMPA) were added to a phosphate–citric acid buffer (0.5 M, pH 7.4) pre-equilibrated to 37 °C to give final concentrations of 0.5 mM each. The mixture was incubated at 37 °C in incubating Orbital Shaker (VWR). Aliquots were taken at time intervals and the reactions quenched by acidification to pH 1.5 with HCl. The samples were analysed directly by HPLC. Test analogues were verified by the elution time of standards and quantitated by their corresponding standard curves (R^2 = 0.97–1.0). All calibration curves were developed by plotting peak area ratios of substrate versus internal standard as a function of concentration. The kinetics of the loss of test analogues were established from the concentration that remained in solution over time[46] in pH 7.4 buffer at 37 °C. The loss of NDGA in buffer at pH 7.4 follows apparent first-order kinetics.[31] Thus, the disappearance rate of the analogues can be described by:

$$\ln\frac{C}{C_o} = -kt$$

where C_o and C are initial concentration and concentration at time t, respectively; k is the reaction rate constant and t is time. k was obtained as the gradient from a plot of $\ln\left(\frac{C}{C_o}\right)$ as a function of time t.



Scheme 2. Intramolecular cyclisation of **5** to its dibenzocyclooctadiene derivative **8**.

2.3.11. Assessment of the effect of nucleophilic trapping agent on the intramolecular reaction

The test compound and internal standard (DMPA) at a final concentration of 0.5 mM in phosphate–citric acid buffer (0.5 M, pH 7.4) were incubated at 37 °C. In the first experiment, the incubation was carried out in either the absence or presence (2 fold excess) of glutathione (GSH) Aliquots were taken at various time intervals and acidified to pH 1.5. In the second experiment, GSH was added at various time intervals and similarly acidified to pH 1.5. A third incubation was conducted in the presence of a 20-fold excess of GSH at 37 °C for 6 h to further investigate the observed delay in the intramolecular cyclisation process. All samples were analysed directly by HPLC and further by ESI-MS.

3. Results and discussion

The main objectives of this study were to determine how functional groups on the aromatic rings of NDGA lignan analogues influence their stability and the formation of dibenzocyclooctadienes in phosphate buffer (pH 7.4) at 37 °C. From this we hoped to develop an understanding of the mechanism of autoxidative intramolecular cyclisation of NDGA and NDGA analogues, with the goal of controlling dibenzocyclooctadiene formation in the design of pharmacologically active lignans. We prepared seven NDGA analogues and investigated their oxidative stability at pH 7.4, their ability to form dibenzocyclooctadienes, and the stability of any dibenzocyclooctadienes using HPLC-UV, MS and NMR.

The dibenzocyclooctadiene family of lignans exhibit numerous pharmacological activities[36–38] including antiviral,[50–52] anticancer,[51] anti-inflammatory[52] and hepatoprotective effects.[53] Our group previously reported a unique schisandrin-like dibenzocyclooctadiene lignan derived from NDGA via autoxidation under physiologically relevant conditions.[34] Wagner and Lewis [54] had reported earlier that NDGA converts to 'activated' NDGA in the presence of molecular oxygen, although the structure of the oxidation product was not elucidated. The authors suggested that any biological action by NDGA treatment could result from either NDGA itself or its oxidation products[54] since many antimicrobial and antineoplastic agents are known to function by interacting with DNA and subsequently affecting nucleic acid metabolism.[55,56] Given the important biological activities of dibenzocyclooctadiene lignans,[38,39] and the fact that many bioassay conditions for NDGA[5,35] as well as methods of Chaparral tea preparation[24,34] favour autoxidation, we speculated that the intramolecular cyclisation product contributes to the broad spectrum of beneficial properties reported for NDGA. There is growing evidence in the literature that toxicity of NDGA can be reduced through structural modification, combined with enhanced therapeutic effects, indicating that derivatives of NDGA are potential drug targets.[6]

3.1. Oxidative stability of NDGA lignan analogues

We evaluated the stability of the NDGA analogues in 0.5 M phosphate buffer (pH 7.4) at 37 °C. These conditions were used previously by us[34] as they are representative of conditions comparable to those used in in vitro biological activity studies. We followed the loss of starting material (initial concentration 0.5 mM) via HPLC-UV and determined that the lignans followed pseudo-first order kinetics. The phenolic analogues were the most stable, displaying half-lives of 5.49 h (**2**), 6.70 h (**3**) and 4.68 h (**7**); a half-life was not determined for phenol analogue **6** as we observed less than 5% loss of starting material over 48 h. Conversely, NDGA and the catechol analogues were all determined to be less stable with half-lives of 3.94 h (NDGA), 4.22 h (**1**), 4.62 h (**4**) and 1.47 h (**5**) (Table 1) (see Figs. 2 and 3).

Table 1
[a]Rate of autoxidative degradation of NDGA and its analogues in 0.5 M phosphate–citric acid buffer (pH 7.4) at 37 °C

Compound	[a]Rate constant k (s^{-1})	Half-life $t_{1/2}$ (h)	R^2
NDGA	4.88×10^{-5}	3.94	0.9936
1	4.56×10^{-5}	4.22	0.9116
2	3.50×10^{-5}	5.49	0.9473
3	2.87×10^{-5}	6.70	0.9512
4	4.16×10^{-5}	4.62	0.9413
5	13.1×10^{-5}	1.47	0.9598
6	§		
7	4.11×10^{-5}	4.68	0.888

§ Compound **6** was stable over 48 h under these conditions (less than 5% loss of starting material).
[a] Lignan autoxidation was determined by following the loss of parent lignan (0.5 mM) via HPLC as described in the Experimental Procedures.

We also incubated NDGA and the seven analogues (0.1 mM) in 0.5 M phosphate buffer (pH 7.4) at 37 °C for 90 min and monitored the formation of dibenzocyclooctadiene products by HPLC. Dibenzocyclooctadienes typically elute earlier than the parent lignan[41] and HPLC peaks consistent with dibenzocyclooctadiene lignans were isolated and further characterised by MS and NMR methods. As shown in Scheme 2, analogue **5** underwent intramolecular cyclisation to form a dibenzocyclooctadiene derivative **8**. A 2 min decrease in retention time and a 3.5 nm shift to a higher absorbance observed by HPLC is consistent with previous[34] results obtained for NDGA under the same conditions. ESI-MS in negative ionization mode gave consistent molecular ions at m/z 299.1 and 271.1 Da for the dibenzocyclooctadiene derivatives of NDGA and analogue **5**, respectively, (Fig. 4). Both products were two mass units less than their acyclic lignan precursors at m/z 301.1 and 273.2, respectively, consistent with intramolecular cyclisation. ^{1}H and ^{13}C NMR and high-resolution mass spectroscopic data (see Supporting information S23) for the dibenzocyclooctadiene product **8** derived from analogue **5**, confirmed that intramolecular cyclisation occurred under oxidative conditions consistent with our previous report for NDGA.[34] We were unable to identify products consistent with dibenzocyclooctadienes from any of our other analogues.

We originally hypothesized that NDGA autoxidation occurred through a radical-mediated process in which the slightly lower pK_a of the *meta* hydroxyl group results in a preferential deprotonation/oxidation, followed by cyclisation of the carbon-centered resonance forms (Scheme 1 pathway A). Our proposed pathway has subsequently been supported through physical and theoretical studies.[22] The biosynthesis of dibenzocyclooctadiene lignans in plants is known to involve enzymatic intramolecular oxidative coupling of phenolic precursors via a radical cation intermediate,[57] which provides additional support for this mechanism. This approach has been exploited in synthetic strategies to construct the biaryl linkage of the dibenzocyclooctadiene lignan core structure.[38,41,57] Lignans belonging to the dibenzylbutane class have served as precursors to dibenzocyclooctadiene derivatives via intramolecular coupling reactions using coupling reagents such as RuO_2 for phenols[57] or 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for non-phenols.[38] No similar report exists in the literature involving a catechol containing substrate such as NDGA.

Oxidation of polyphenols by molecular oxygen has resulted in intermolecular reaction products,[42,44] and oxidation of polyphenols present in tea have resulted in dimers and other oligomers, proposed to occur via intermolecular nucleophilic attack of an un-oxidised ring on an *ortho*-quinone.[42,44] Thus an alternative mechanism for NDGA dibenzocyclooctadiene formation could be envisioned proceeding through initial *ortho*-quinone formation followed by nucleophilic attack by the un-oxidised aromatic ring. This alternative mechanism would imply that only one catechol ring would be necessary for autoxidative lignan cyclisation to a dibenzocyclooctadiene, provided that the second aromatic ring was sufficiently nucleophilic. A potentially useful tool to assess relative nucleophilicity of the un-oxidised aromatic ring is Hammett σ constants for the ring substituents.[58] We designed our lignan analogues such that our catechol analogues possessed substituents on the second ring which rendered the ring less nucleophilic (analogue **1**, H = 0.0) or similar (analogue **4**, *m*-OCH_3 = 0.12, *p*-OCH_3 = −0.27) compared to NDGA (*m*-OH = 0.12, *p*-OH = −0.37). We predicted that if *ortho*-quinone formation was necessary for dibenzocyclooctadiene formation, both analogues **1** and **4** should

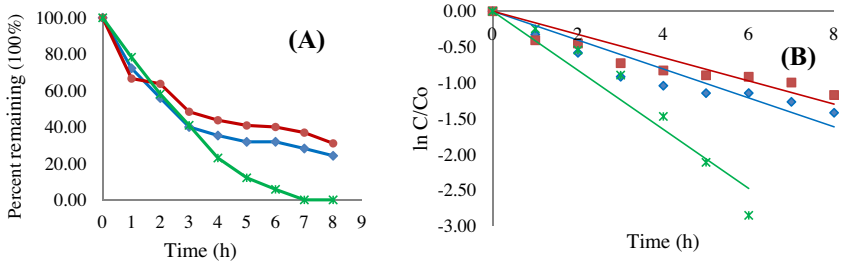


Figure 2. Typical degradation profile (panel A) and first-order regression lines (panel B) for the catechol analogues 1 (blue), 4 (red) and 5 (green) in 0.5 M phosphate–citric acid buffer (pH 7.4) at 37 °C.

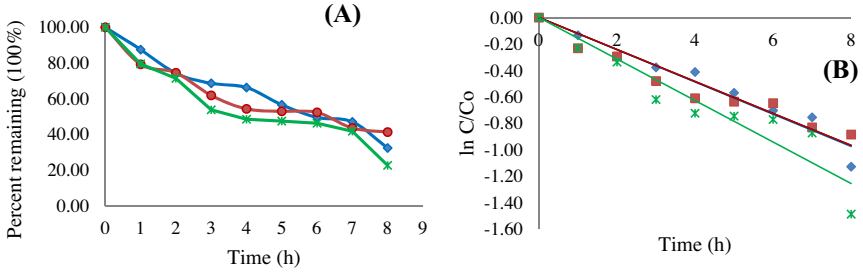


Figure 3. Typical degradation profile (panel A) and first-order regression lines (panel B) for the phenol analogues 2 (blue), 3 (red) and 7 (green) in 0.5 M phosphate–citric acid buffer (pH 7.4) at 37 °C.

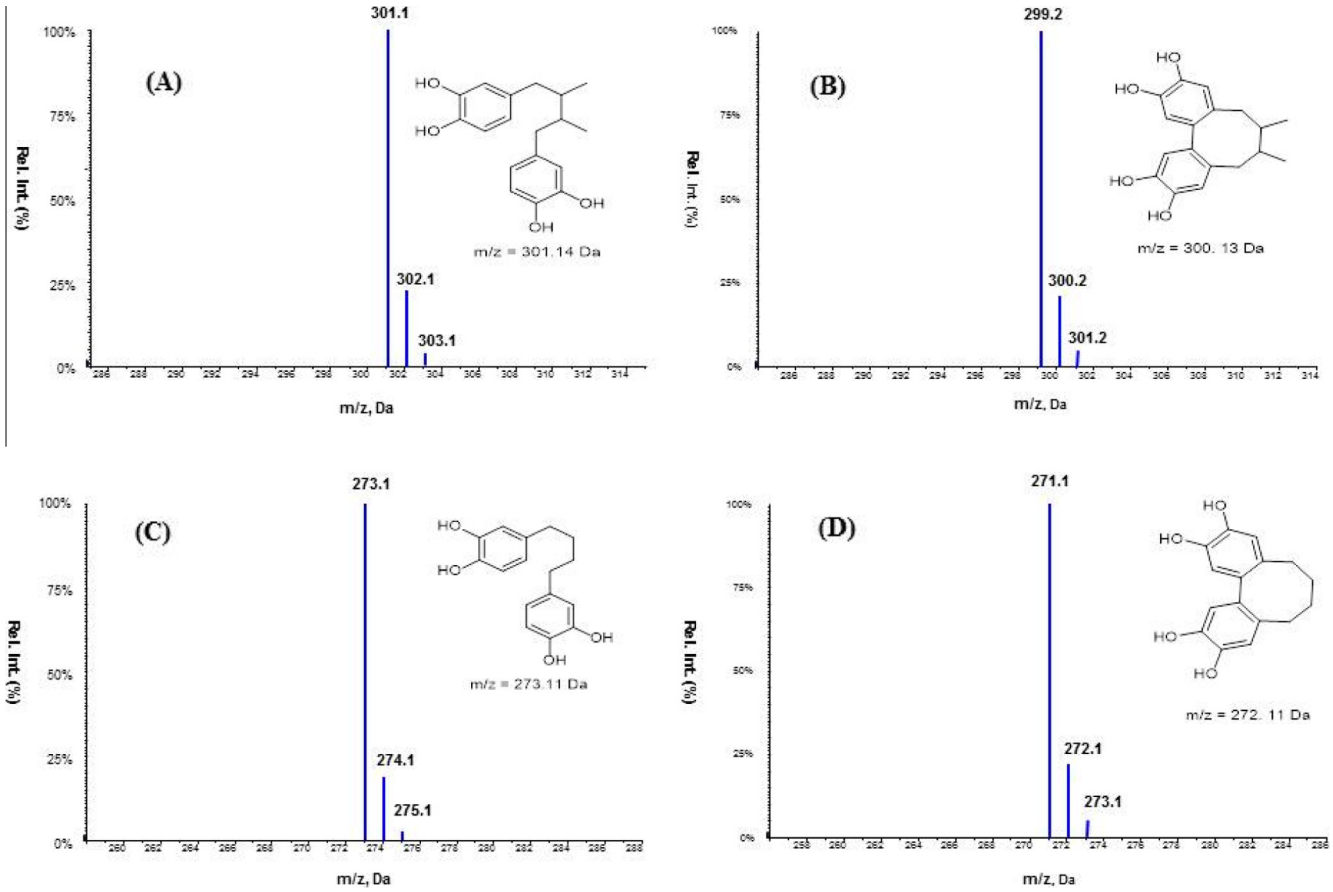


Figure 4. Enhanced resolution ESI-MS spectrum for NDGA and compound **5**: Panels (A) and (B) show NDGA before and after incubation; Panels (C) and (D) show compound **5** before and after incubation in a phosphate buffer (0.5 M pH 7.4) at 37 °C for 90 min. The decrease of 2 mass units after incubation is consistent with intramolecular cyclisation.

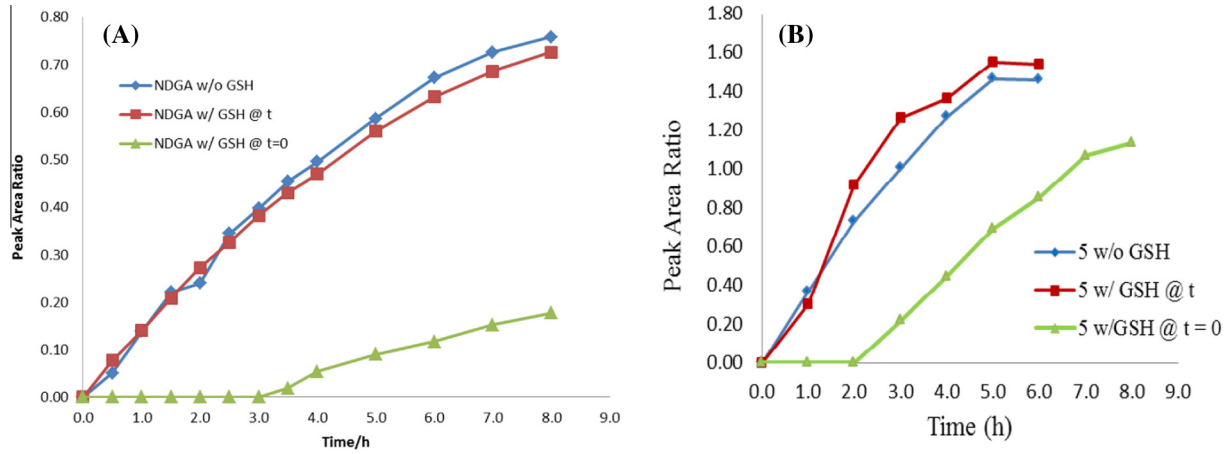


Figure 5. Formation of dibenzocyclooctadiene derivatives for NDGA (panel A) and **5** (panel B) over time in the absence of GSH (blue), GSH added at time *t* (red) and GSH added at time *t* = 0 h (green). The graphs show that the presence of GSH in the incubation mixture significantly affected the amount of dibenzocyclooctadiene formed over time.

form dibenzocyclooctadienes, and **1** would cyclise at a greatly reduced rate in comparison to NDGA while the cyclisation rate for **4** would be comparable to NDGA. We observed no evidence of oxidative cyclisation for the mono-catechol analogues **1** or **4**, although both compounds autoxidised at comparable rates to NDGA ($t_{1/2}$ NDGA: 3.94 h; **1**: 4.22 h; **4**: 4.62 h) forming *ortho*-quinones (manuscript in preparation). As predicted, the phenolic analogues **2** and **3** did not cyclise at pH 7.4. The absence of dibenzocyclooctadiene cyclisation products for mono-catechols **1** and **4** suggests that *ortho*-quinone formation on one lignan ring followed by nucleophilic attack by the unoxidised ring may be insufficient for dibenzocyclooctadiene formation, and implies that a radical-mediated process more accurately describes NDGA oxidative cyclisation.

Table 2
Relative composition of **5** or NDGA and their dibenzocyclooctadiene derivatives following 6 h incubation in phosphate–citrate buffer (pH 7.4) at 37 °C in the absence or presence of a 20 fold excess of glutathione

Compound	Incubation for 6 h at 37 °C in the presence of GSH (%)			Incubation for 6 h at 37 °C in the absence of GSH (%)		
	SM	CycloL	Adduct	SM	CycloL	Adduct
5	95.46	4.54	—	8.52	91.47	—
NDGA	87.08	—	12.91	66.90	33.09	—

SM = starting material; CycloL = dibenzocyclooctadiene derivative.

Table 3
[a]The rate of formation of dibenzocyclooctadiene derivatives cNDGA and **8** in phosphate buffer (pH 7.4) at 37 °C. [b]The rate of degradation of dibenzocyclooctadiene derivatives cNDGA and **8** in phosphate buffer (pH 7.4) at 37 °C

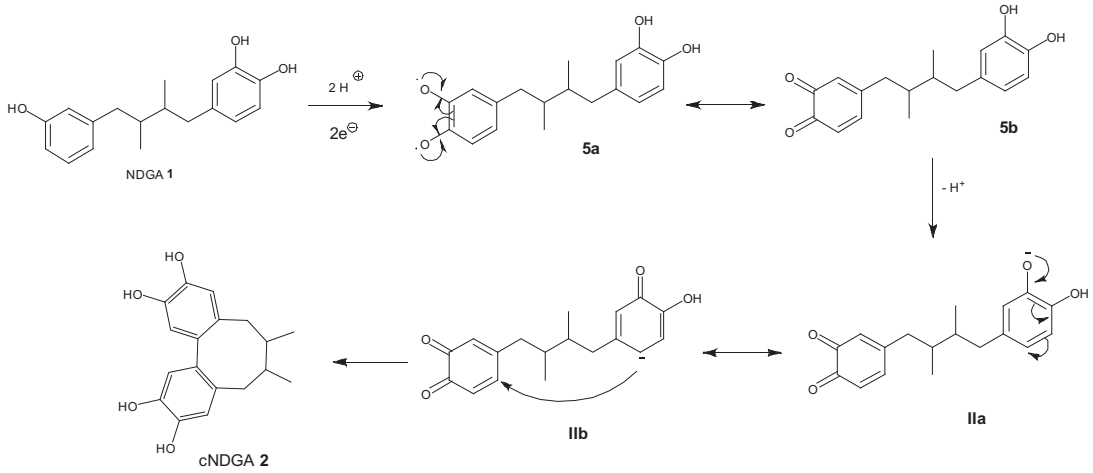
Compound	[a]$k \times 10^{-5}$ s^{-1}	[a]Rel rate	[b]$k \times 10^{-5}$ s^{-1}	[b]Rel rate
cNDGA	2.78	1	0.47	1
8	8.70	3.1	1.75	3.7

3.2. Effect of a nucleophilic trapping agent on the rate of intramolecular cyclisation

The use of GSH as a nucleophilic trapping agent is common when investigating quinone formation from catechols and phenols.[31] When we added GSH to pH 7.4 incubations of NDGA or analogue **5** at various time points, we observed a combination of GSH adducts and dibenzocyclooctadiene products (manuscript in preparation). If GSH was present at the beginning of the reaction however, we instead observed that the intramolecular reaction of NDGA or analogue **5** was inhibited (Fig. 5). The dibenzocyclooctadiene derivatives were formed within 30 min for both NDGA and **5** in the absence of GSH or when GSH was added at various time points. When GSH was present in the incubation mixture however, no intramolecular cyclisation occurred until after 3 h (NDGA) or 2 h (**5**). We saw no significant difference between the amounts of dibenzocyclooctadiene formed in the absence of or with GSH added at different time points, but product formation for both NDGA and **5** were significantly different when GSH was incubated together with the substrates. These observations triggered further investigations into whether the intramolecular reaction will be inhibited completely at a higher concentration of GSH. In the presence of 20 fold excess GSH, barely 5% of **5** converted to its dibenzocyclooctadiene derivative within a 6 h incubation compared with over 91% conversion in the absence of GSH (Table 2). We observed a similar trend for NDGA with no intramolecular reaction occurring in the presence of 20 fold excess GSH compared with 33% conversion in the absence of GSH. We did not assay for oxidised glutathione (GSSG), although it is likely that GSH acts as a radical scavenger in this reaction. This result is consistent with a radical-mediated cyclisation pathway.

3.3. *meta*-Phenol lignan analogues as probes of radical-mediated cyclisation

In order to further test our hypothesis that NDGA cyclisation proceeds through a di-radical intermediate, we synthesized analogue **6**. We incubated analogue **6** at pH 7.4 as this *meta*-phenol lignan can form the comparable di-radical species as predicted for NDGA, but cannot form an *ortho*-quinone. However, we were unable to detect the formation of a dibenzocyclooctadiene from **6** and speculated that the stability of a radical or di-radical intermediate might be important for cyclisation.[57] We based this assumption on the observation that enterolactone, a dibenzylbutanolide lignan, bearing *meta*-substituted phenols treated under oxidative coupling conditions, did not undergo intramolecular cyclisation although other phenolic natural dibenzylbutanolide lignans yielded dibenzocyclooctadiene derivatives under the same conditions.[57] In addition, our observation was similar to a result reported by Robin and Landais, where RuO_2 was employed for cyclisation of an *ortho*-phenol substituted lignan.[57] Robin observed that additional methoxy substituents on the aromatic ring facilitated cyclisation, suggesting that the electron-donating methoxy substituents may be critical to cyclisation, possibly due to enhanced stability of a radical intermediate. We investigated this hypothesis with analogue **7** where the *para*-methoxy substituents were expected to stabilize the intermediate radical formed at the *meta*-hydroxy groups to allow for cyclisation. In spite of the presence of these methoxy groups on both aromatic rings, incubation of **7** at pH 7.4 gave no intramolecular cyclisation product. It is possible that formation of the intermediate radical of **7** is inhibited by the electron-donating effect of the adjacent methoxy group, although the difference between the σ values for OH (−0.37) and OCH_3 (−0.27) is small[58] and we would anticipate this to slow, but not prevent, radical formation. Alternatively, any radical formed may be too stable to react, although given the observations of others this seems unlikely.[44] It is more likely that the intramolecular cyclisation reaction we observe under these conditions follows a di-radical coupling mechanism as proposed by us[34] and others,[22] but requires a di-catechol system.



Scheme 3. Modification to the proposed mechanism of intramolecular cyclisation of dibenzylbutane lignans to dibenzocyclooctadiene derivatives.

3.4. Rate of formation and degradation of the dibenzocyclooctadiene derivatives

We had previously hypothesized that the NDGA dibenzocyclooctadiene lignan may contribute to the biological activities reported for NDGA and therefore stability in commonly used conditions of biological evaluation may be relevant for inferring activity. We determined that the dibenzocyclooctadiene derivatives cNDGA and **8** (derived from NDGA and analogue **5**, respectively) are unstable in pH 7.4 phosphate buffer. The rate of loss of starting material was determined using the same conditions as for analogues **1**–**7** and we found that compound **8** (0.5 mM) degraded at a rate of $1.75 \times 10^{-5}\ s^{-1}$ in comparison with $4.70 \times 10^{-6}\ s^{-1}$ for cNDGA (Table 3). Given that the dibenzocyclooctadiene derivatives were unstable in phosphate buffer at pH 7.4, we inferred the rates of formation from the loss of their respective substrates since they were the only product peak by HPLC. Although the rate of cyclisation was faster for analogue **5** than NDGA (Fig. 5), the resulting dibenzocyclooctadiene lignan **8** also degraded faster than cNDGA. The steady rise in concentration of the dibenzocyclooctadiene derivatives fell precipitously after 6 or 9 h for **8** and cNDGA, respectively.

4. Conclusions

In conclusion we have determined that cyclisation of NDGA lignan analogues to a dibenzocyclooctadiene does not readily occur for mono-catechol lignan analogues, suggesting that catechol oxidation to an *ortho*-quinone followed by nucleophilic attack by the un-oxidised ring is an unlikely pathway. Our results lend further support to a di-radical coupling mechanism as previously suggested.[22,34] We were unable to show that *meta*-substituted phenols can cyclise in this manner, suggesting that the di-radical pathway describes the intramolecular cyclisation process for catechols, but not phenols. An intriguing possibility remains, which we have outlined in Scheme 3, involving nucleophilic attack of an *ortho*-quinone by an ionised ring. NDGA has a pK_a of 9.30[59] and the intramolecular cyclisation of NDGA is a base-catalysed process,[31] suggesting that this mechanism would be favoured at more alkaline pH. We are currently investigating this pathway using a mixed catechol–phenol lignan. The loss of the methyl substituents on the aliphatic side-chain of NDGA enhances the rate of dibenzocyclooctadiene formation, likely as a result of diminished steric interactions during cyclisation. Finally, we determined that the stability of the dibenzocyclooctadienes is comparable to that of their precursors, suggesting that any activity associated with the cyclised lignans may be related to their oxidised species. We are currently studying the oxidation products of cNDGA and will report on this in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.09.039.

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