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Mechanism of the Superoxide Scavenging Activity of Neoandrographolide − A Natural Product from *Andrographis paniculata* Nees

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It was hypothesized that neoandrographolide might scavenge free radicals by donating the allylic hydrogen of the unsaturated lactone ring. It was found that the stoichiometry of the reaction between neoandrographolide and superoxide radical generated from KO_2 in DMSO was 2 to 1. One major reaction product was isolated and determined to be a diacid formed by the opening of the lactone ring. It was concluded that the antiradical activity of neoandrographolide proceeded by hydrogen abstraction from carbon C-15. A reaction mechanism was proposed.

KEYWORDS: ***Andrographis paniculata;*** **neoandrographolide; superoxide radical; antiradical activity; diterpene lactone**

INTRODUCTION

Neoandrographolide is one of the bioactive diterpene lactones from the medicinal plant *Andrographis paniculata* Nees (*1*). The plant is found in many Asian countries where it is used in traditional medicine against a variety of diseases including cold, fever, snakebite, diarrhea, and malaria (*2*, *3*). Andrographis extract and some of its diterpene lactones are claimed to be antidiabetic (*4*), antiinflammatory, immunostimulant (*5*), hepatoprotective (*6*), antimalarial (*7*), analgesic, antipyretic, and antiulcerogenic (*8*).

In an earlier pharmacological study, Kapil et al. (*9*) demonstrated that andrographolide (**1**), andrographoside (**2**), and neoandrographolide (**3**) (**Figure 1**) protected rats' liver against the hepatotoxins carbon tetrachloride (CCl_4) and *tert*-butyl hydroperoxide (tBHP) by reducing the levels of the lipid oxidation product malondialdehyde (MDA) and by maintaining high levels of the reduced form of glutathione (GSH). They suggested that inhibition of malondialdehyde formation revealed the free radical scavenging properties of diterpene lactones. On the basis of the vast amount of literature now available on the antioxidant mechanism of polyphenols it is hard to believe that the glucosidic moiety would account for free radical scavenging of a compound. Arora et al. (*10*) demonstrated that the flavonol aglycon quercetin was more effective than its glycoside rutin against lipid oxidation in liposomes. Sanbongi et al. (*11*) found that quercetin was more effective an antioxidant than 3-glucosylquercetin and 3-arabinosylquercetin in hydrophilic environment. We thought that the antiradical mechanism of neoandrographolide might originate from its aglycon, especially the unsaturated lactone ring.

Neoandrographolide does not possess the common features of traditional nutritional antioxidants (aromatic ring or extensively conjugated double bond); its antiradical mechanism might be very unique and might offer an interesting alternative model. From a structural perspective, neoandrographolide is a simple α,β unsaturated lactone with allylic hydrogen on carbon C-12 and C-15. In this study we hypothesized that neoandrographolide scavenged free radicals by donating the hydrogen atoms at the allylic position of the α,β unsaturated lactone either by homolytic cleavage or by deprotonation−oxidation mechanisms. The resulting intermediate is stabilized by resonance and also by steric hindrance around the reactive site. We followed the kinetics of the reaction between neoandrographolide and superoxide radical ($O_2^{-\bullet}$) generated from KO_2 in DMSO. We isolated and determined the structure of one major reaction product from the superoxide system that led to proposing a reaction mechanism.

MATERIALS AND METHODS

Instrumentation, Chromatography Supplies, and Chemicals. The HPLC apparatus used for analytical work was an HP 1090 LC with a reverse-phase column Supelcosil LC-18 (Supelco). The solvent was a gradient of water/methanol at 0.2 mL/min flow rate, and detection was done by a UV−Vis absorbance detector (Spectroflow 757). The gradient consisted of 0 to 100% MeOH in 15 min, followed by a 10 min holding time, and a decrease to 0% MeOH in 5 min. Silica gel was obtained from Sorbent Technology, Inc. (Atlanta, GA) as standard grade 60 Å, 32−63 μm. Thin-layer chromatography was performed on TLC plates (250 μm thick, 2−25 μm particle size) from Fisher Scientific (Suwanee, GA). Spectrophotometric measurements were done on a Milton Roy Spectronic 301 spectrophotometer. Hexane, chloroform, methanol, ethyl acetate, butanol, ethanol, water, sodium chloride, potassium chloride, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from Fisher Scientific. Dimethyl sulfoxide, potassium superoxide (KO_2), soluble starch, nitro blue tetrazolium (NBT^{2+}), 1,4,7,-

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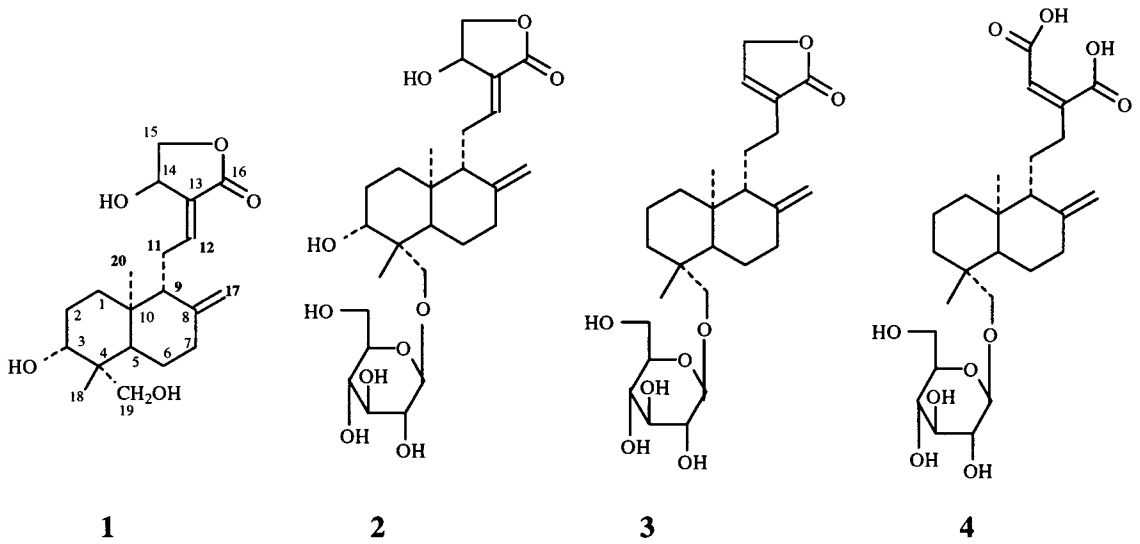

Figure 1. Chemical structures of andrographolide (**1**), andrographoside (**2**), neoandrographolide (**3**), and neoandrographic diacid (**4**).

10,13,16-hexaoxacyclooctadecane (18-crown-6), and deuterated solvents ($(CD_3)_2SO$, CCl_3D, CD_3OD, and D_2O) were purchased from Sigma-Aldrich.

Isolation of Neoandrographolide. The extract of *Andrographis paniculata* was a gift from America Home Products Corporation (Richmond, VA). A crude extract (380 g) of the plant was chromatographed over silica gel with a gradient of hexane/EtOAC in a chromatographic column 60 cm × 7.5 cm i.d. The fractions eluted with hexane/EtOAC (1:1 and 0:1) were accumulated and rechromatographed over silica gel with a mixture of $CHCl_3$/MeOH (100:6) to obtain 10 g of neoandrographolide (yield 2.63%). The isolation was monitored by TLC with a mixture of $CHCl_3$/MeOH (10:1) as migration solvent. Its purity as determined by HPLC was 95% at $\lambda = 228$ nm. The molecular weight of neoandrographolide was determined by atmospheric pressure chemical ionization mass spectrometry (APCI-MS) on a Fisons/VG Platform II mass spectrometer (Micromass, Beverly, MA) which showed a pseudo-molecular ion $[M - H]^-$ at $m/z = 479$. The identity of neoandrographolide was further confirmed by comparing its ^{1}HNMR spectrum recorded in CD_3OD on a Varian 600 Spectrometer to published data (*1*, *2*).

Compound **3** was obtained as a white powder. ^{1}H NMR (CD_3OD, 600 MHz): 7.38 (1H, s, H-14), 4.87 (1H, brs, H-17a), 4.85 (2H, brs, H-15), 4.64 (1H, brs, H-17b), 4.17 (1H, d, $J = 7.8$ Hz, H-1 of Glc), 4.09 (1H, d, $J = 9.6$ Hz, H-19a), 1.04 (3H, s, H-18), 0.71 (3H, s, H-20).

Scavenging of Superoxide Radical by Neoandrographolide. Superoxide was generated from KO_2 solution in DMSO prepared by the method of Valentine et al. (*12*) with slight modification. Briefly, a stock solution of KO_2 (0.15 M) was prepared in DMSO containing 0.3 M 18-crown-6. The working solution was prepared immediately before use by diluting the stock solution to a concentration of 3.12 mM in DMSO. The test consisted of adding 2 mL of KO_2 solution to 3 mL of neoandrographolide solution in DMSO at different concentrations. Samples in triplicate were incubated at 37 °C for 15 min then the amount of $O_2^{-\bullet}$ scavenged was determined by reacting 0.5 mL of sample with 1.5 mL of NBT^{2+} solution (0.11 mM) in phosphate buffer (pH 7.4), and the absorbance at 560 nm was measured within 1 min. The percentage of $O_2^{-\bullet}$ scavenged was calculated using the following formula:

$$\%\ O_2^{-\bullet}\ \text{scavenged} = \frac{\text{absorbance blank} - \text{absorbance sample}}{\text{absorbance blank}} \times 100$$

γ-Butyrolactone and starch were used as negative control.

Isolation of the Major Reaction Product of Neoandrographolide with Superoxide. It was determined by HPLC that at all concentrations there was one major reaction product (**4**) with retention time of 10 min compared to 15 min for neoandrographolide. This reaction product had strong UV absorption at 228 and 254 nm in contrast to neoandrographolide which absorbs at 228 nm only. To isolate this compound (**4**), 1 g of neoandrographolide was reacted with 0.05 g of KO_2 in DMSO at 37 °C for 15 min. DMSO was removed under vacuum, and the total reaction product was recovered in methanol and chromatographed over a reversed-phase chromatographic column (60 cm × 2.5 cm i.d.) using water to obtain 40 mg of (**4**) (yield 4%). The FAB-MS of (**4**) was run on a JEOL HX-110 double-focusing mass spectrometer (Michigan State University, Lansing, MI). The ^{1}H and ^{13}CNMR in D_2O were obtained on a Bruker AC-200 FT NMR Spectrometer. The ^{1}HNMR in CD_3OD was run on a Varian 600 Spectrometer.

Compound **4** was isolated as a pale amorphous substance. ^{1}H NMR (CD_3OD, 600 MHz): 5.56 (1H, s, H-14), 4.85 (1H, brs, H-17a), 4.63 (1H, brs, H-17b), 4.20 (1H, d, $J = 7.8$ Hz, H-1 of Glc), 4.13 (1H, d, $J = 9.6$ Hz, H-19a), 1.06 (3H, s, H-18), 0.74 (3H, s, H-20). ^{1}H NMR (D_2O, 200 MHz): 5.47 (1H, s, H-14), 4.89 (1H, brs, H-17a), 4.63 (1H, brs, H-17b), 4.35 (1H, d, $J = 7.8$ Hz, H-1 of Glc), 4.13 (1H, d, $J = 10.0$ Hz, H-19a), 1.01 (3H, s, H-18), 0.66 (3H, s, H-20). ^{13}C NMR (CD_3OD, 150 MHz): 181.7 (s, C-16), 177.4 (s, C-15), 155.3 (s, C-8), 152.7 (d, C-14), 122.1 (s, C-13), 108.7 (t, C-17), 106.3 (d, C-1 of Glc), 78.6 (d, C-3 and C-5 of Glc), 76.4 (t, C-19), 76.1 (d, C-2 of Glc), 72.5 (d, C-4 of Glc), 63.5 (t, C-6 of Glc), 58.9 (d, C-9), 58.3 (d, C-5), 42.0 (s, C-4), 41.0 (t, C-1), 40.8 (t, C-12), 40.6 (s, C-10), 38.4 (t, C-7), 36.6 (t, C-3), 29.7 (q, C-18), 27.0 (t, C-6), 24.6 (t, C-11), 21.3 (t, C-2), 17.6 (q, C-20). Positive HRFAB-MS m/z 551.2240 $[M + K]^+$ (calcd for $C_{26}H_{40}O_{10}K$, 551.2258).

RESULTS

Antiradical Activity of Neoandrographolide. Superoxide system generated from KO_2 in DMSO was used to study the antiradical activity of neoandrographolide. The stoichiometry of the reaction was approximately 2 molecules of neoandrographolide for 1 molecule of superoxide, as shown in **Figure 2**. Neoandrographolide scavenged 45% of $O_2^{-\bullet}$ when the concentration ratio $R = [\text{neoandrographolide}]/[KO_2] = 1$, but doubling this ratio resulted in 90% scavenging. Under the same experimental conditions γ-butyrolactone scavenged less than 10% of $O_2^{-\bullet}$. We can rule out the possibility for a saturated lactone to scavenge $O_2^{-\bullet}$ because γ-butyrolactone was relatively unreactive. It is well-known that the nucleophilic properties of $O_2^{-\bullet}$ predominates in aprotic environment (*13*), so it is conceivable that the reaction of $O_2^{-\bullet}$ with neoandrographolide might proceed through a deprotonation of an active C−H bond followed by oxidation. To determine the reaction site we purified and elucidated the structure of the major reaction product (**4**) with KO_2.

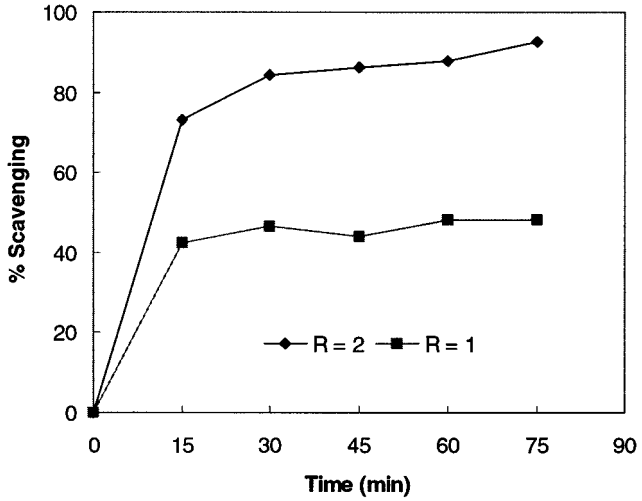

Figure 2. Scavenging of $O_2^{-\bullet}$ by neoandrographolide (R = [neoandrographolide]/[KO_2]).

Identification of the Major Reaction Product. The reaction product (**4**) was more polar than neoandrographolide (**3**) with a retention time of 10 min on HPLC, and it was water soluble, in contrast to neoandrographolide which is very hydrophobic. Compound **4** was assigned the molecular formula of $C_{26}H_{40}O_{10}$ determined by positive-ion HRFAB-MS as well as from its ^{13}C NMR data. The molecular formula indicated seven degrees of unsaturation, which showed that **4** had the same unsaturation as **3**. The ^1H NMR (CD_3OD) spectrum of **4** showed the presence of a broad singlet signal at δ 5.56 for H-14 proton, the H-17 protons of the exomethylene group as two broad singlets at δ 4.85 and 4.63, the anomeric doublet at δ 4.20 (d, J = 7.8 Hz), and two singlets signals due to tertiary methyl groups at δ 0.66 and 1.01 ppm (H-20 and H-18). Thus, the significant difference in the ^1H NMR spectrum of **4** compared to that of **3** is the loss of protons on carbon C-15 at δ 4.85 ppm. In addition, a comparison of the ^{13}CNMR data of **4** with the data of **3** (*14*) indicated that **4** had two carbonyl groups at δ 177.4 and δ 181.7 ppm, which is one more than **3**, while the signal for C-15 in **3** at δ 70.6 disappeared in **4**. As we mentioned above **4** and **3** had the same unsaturation. All of these mean that **4** was the diacid derivative of **3** (**Figure 1**). This was in agreement with the upfield shift for H-14 in the ^1H NMR (CD_3OD) spectrum of **4** by 1.82 ppm lower than that in **3**. This can be explained by the fact that H-14 of compound **4** is in the shield area of the carbonyl group at position 15. Thus, the structure of **4** was elucidated as shown (**Figure 1**) and named neoandrographic diacid.

DISCUSSION

We proposed that the formation of neoandrographic diacid resulted from a sequence of reactions that begins with deprotonation of carbon 15 to form andrographolide anion and hydroperoxyl radical ($HO_2^{\bullet}$). The deprotonation of organic compounds with active C−H bonds by superoxide radical was proposed earlier by Frimer et al. (*15*). Andrieux et al. (*16*) demonstrated that $HO_2^{\bullet}$ could accept one electron in an electron-transfer process. On the basis of these models, we suggested that the attack of $O_2^{-\bullet}$ on C-15 of neoandrographolide will result in formation of neoandrographolide radical. In general, a carbon center radical can fix one molecular oxygen to form a peroxyl radical, which in turn abstracts hydrogen from a donor to become a peroxide. In this case neoandrographolide peroxide

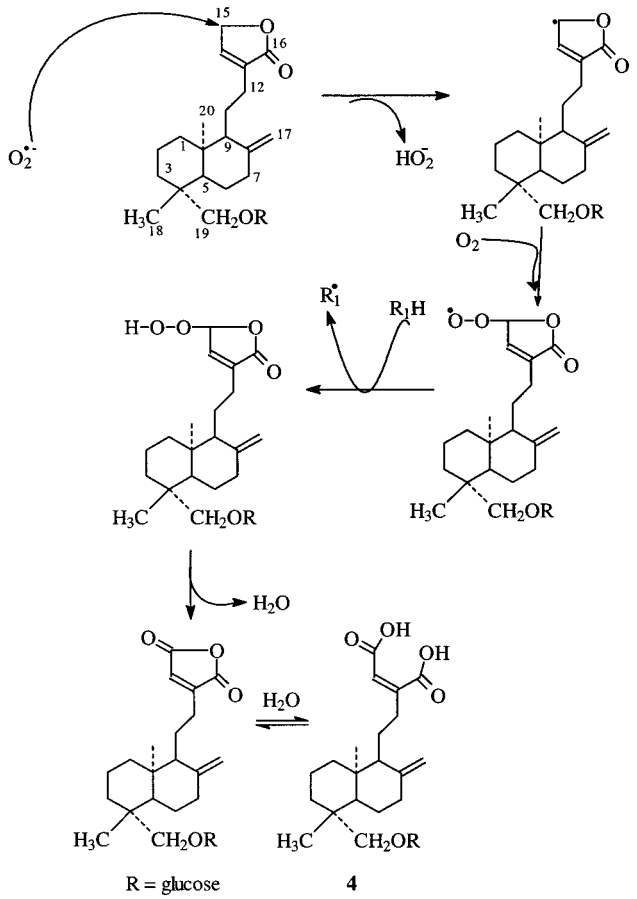

Figure 3. Proposed reaction mechanism between neoandrographolide and superoxide.

spontaneously hydrolyzed to form an unstable dicarbonyl which hydrated into a water soluble diacid (**4**). The steps of this sequence of reaction are summarized in **Figure 3**. The ultimate reason neoandrographolide would be an effective scavenger of small radicals in vivo lies in the steric hindrance around carbon C-13, which reduces the reactivity of the resonant neoandrographolide radical.

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