DNA-Damaging Agents from *Crypteronia paniculata*

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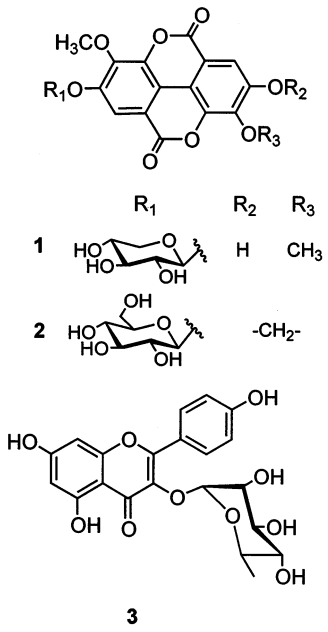
A survey of crude plant extracts using a new yeast strain designed to identify DNA-damaging agents resulted in the identification of an extract prepared from *Crypteronia paniculata*. Bioassay-guided fractionation resulted in the isolation of three active compounds. Two of these were ellagic acid derivatives, namely, 3,3′-di-*O*-methylellagic acid 4′-*O*-*β*-D-xylopyranoside (**1**) and 3′-*O*-methyl-3,4-methylenedioxyellagic acid 4′-*O*-*β*-D-glucopyranoside (**2**). The third was identified as kaempferol-3-*O*-α-L-rhamnoside (**3**). The three principles exhibited strong, selective cytotoxity toward the RAD52 repair-deficient yeast strain.

In recent years, the search for new anticancer agents has involved increased emphasis on the discovery of selective agents through the use of improved screening methods.[1] An assay for DNA-damaging agents based on the differential response of DNA repair-deficient and -proficient yeast strains to the test samples has been used for the detection of potentially novel agents.[2−5] The use of a eukaryotic assay system may also confer some advantage relative to those involving bacteria. RAD52, the locus of a major DNA repair pathway in yeast, forms the basis for homologous recombination which is associated with repair of double-strand breaks.[6−8] This pathway was selectively deleted in *S. cerevisiae*, yielding yeast incapable of effecting this type of DNA repair. The resulting strain of yeast was then transformed with a multicopy plasmid containing the yeast RAD52 gene under the control of the GAL1 promotor. This afforded a single screening strain that would be rad52 protein-negative when grown on glucose (which strongly suppresses the GAL1 promotor) but would express rad52 protein when grown on galactose and thus would be DNA damage repair-proficient.

Crude plant extracts were surveyed for their ability to inhibit the growth of the yeast tester strain maintained on glucose versus galactose. An extract prepared from *Crypteronia paniculata* (Crypterniaceae) was found to be cytotoxic toward the yeast tester strain maintained on glucose (IC_{50} 39 μg/mL), but much less cytotoxic when the yeast strain was maintained on galactose (IC_{50} >500 μg/mL). Accordingly, this extract was chosen for fractionation. Bioassay-guided fractionation employing the same yeast assay led to the identification of three potent DNA-damaging agents in two different structural classes. The isolated agents included two ellagic acid derivatives (**1** and **2**), as well as a flavonol glycoside (**3**). Presently, we describe the isolation of these three natural principles and their potency as DNA-damaging agents.

To detect natural principles capable of inducing double-strand DNA breaks, the yeast strain harboring pRAD52 was incubated in glucose or galactose medium in the presence of each of the extracts under study. A yeast strain containing control plasmid YCp50 was also treated with the same extracts in the presence of galactose to detect toxicity related to the growth medium employed. Extracts having the desired activity gave low IC_{50} values in yeast harboring pRAD52 that were grown on glucose or YCp50 grown on galactose. However, little or no toxicity was apparent when yeast harboring pRAD52 was grown on galactose, presumably reflecting repair-proficiency. The criterion used to select extracts in the primary screen was no less than a 3-fold difference in cytotoxicity when the yeast was grown on glucose versus galactose.



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A crude 1:1 MeOH−CH_2Cl_2 extract prepared from *Crypteronia paniculata* showed significant selective toxicity toward the yeast tester strain (IC_{50} 39 μg/mL when grown on glucose; IC_{50} >500 μg/mL on galactose); the control strain harboring plasmid YCp50 was inhibited with an IC_{50} value of 22 μg/mL when grown on galactose. To permit isolation and characterization of the principles responsible for the DNA damage, this crude extract was subjected to bioassay-guided fractionation using the same assay. Initially, the extract was fractionated on a polyamide 6S column which was washed successively with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and then 9:1 MeOH−NH_4OH. The 1:1 MeOH−H_2O fraction showed the strongest DNA-damaging activity. Further fractionation employing a reversed-phase C_8 open column provided an 8:2 MeOH−H_2O fraction, which had the strongest DNA-damaging activity. The active fraction was applied to a C_{18} reversed-phase HPLC column, which was washed with an acetonitrile−H_2O gradient. Three active

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Table 1. IC_{50} Values for Compounds **1**, **2**, and **3**

	IC_{50} (μM)		
compound	RAD52 (glu)	RAD52 (gal)	YCp50 (gal)
1	3.1	>1000	23.1
2	1.12	14.4	0.341
3	7.4	>1000	9.3

constituents were separated, and these were purified by further fractionation on the same HPLC column, affording potent DNA-damaging principles **1**, **2**, and **3**.

On the basis of their mass spectra, ^{1}H and ^{13}C NMR spectra, and $[\alpha]_D$ data, and comparison with data that have appeared in the literature, compounds **1** and **2** were found to be known ellagic acid derivatives. The structure of compound **1** was established as 3,3′-di-*O*-methylellagic acid 4′-*O*-*β*-D-xylopyranoside,[9] while that of compound **2** was determined to be 3′-*O*-methyl-3,4-methylenedioxyellagic acid 4′-*O*-*β*-D-glucopyranoside.[9a] By comparison with physicochemical and spectral data in the literature, compound **3** was shown to be kaempferol-3-*O*-α-L-rhamnoside.[10] It may be noted that to date this assay has identified only a limited number of DNA-damaging agents such as alkaloids[3,4] and stilbene derivatives;[5] no report has appeared describing the DNA-damaging activity of ellagic acid derivatives or flavonol glycosides.

The purified principles were characterized for their DNA-damaging properties in yeast- and cell-free systems. As shown in Table 1, ellagic acid derivative **1** was more inhibitory to the yeast tester strain grown on glucose (IC_{50} 3.1 μM) than on galactose (IC_{50} >1000 μM). The same trend was also observed for derivative **2**, which had an IC_{50} value of 1.12 μM when the yeast strain harboring pRAD52 was grown on glucose. Toxicity was diminished to an IC_{50} of 14.4 μM when the same strain was grown on galactose. For compounds **1** and **2**, the cytotoxicity to yeast harboring plasmid YCp50, grown in the presence of galactose, was similar to that observed when yeast harboring pRAD52 was grown on glucose. This supports the interpretation that DNA damage represents the molecular locus at which the cytotoxic response was mediated.

A similar pattern was observed when the yeast strains were treated with kaempferol-3-*O*-α-L-rhamnoside (**3**). The strain harboring pRAD52 was inhibited more strongly when grown on glucose (IC_{50} 7.4 μM) than when grown on galactose (IC_{50} >1000 μM), and the control strain exhibited an IC_{50} value of 9.3 μM when grown on galactose.

Ellagic acid is widely distributed in higher plants. It has been reported to have some biological activities, such as stimulation of the formation of glycosaminoglycan chains,[9b] inhibition of yeast strain Sc-7,[11] inhibition of *N*-acetyltransferase,[12] and DNA gyrase activities,[13] as well as antimutagenic effects,[14] and other activities.[15] In the present yeast DNA damage assay, ellagic acid derivatives **1** and **2** showed potent activity and large differential values when grown on glucose versus galactose. Compounds **1** and **2** differ structurally only in the substituents at positions 3 and 4 and in the sugar moiety at position 4′; however, their absolute cytotoxic potencies differed substantially. This argues that among ellagic acid derivatives the substituents on positions 3 and 4 and the sugar moiety must play important roles in contributing to the potency of DNA damage in yeast.

Compounds **1**−**3** were also tested for their ability to cleave DNA in a cell-free assay involving relaxation of supercoiled plasmid DNA.[16] None of the compounds was observed to cleave DNA, even when tested at high concentrations (e.g., >1 mM for **3**) or in the presence of Cu^{2+}.[17]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer 243B polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded using tetramethylsilane (TMS) as an internal standard on a Varian-Unity Inova 500/51 or QE-300 spectrometer at 500/300 and 125 MHz, respectively. Low-resolution chemical ionization (CI) and fast atom bombardment (FAB) mass spectra were obtained on Finnigan MAT 4600 and VG ZAB-SE mass spectrometers, respectively. All reagents were of the best available commercial quality and were used without further purification. Polyamide 6S (a product of Riedel-de Haen, Germany, pour density 0.25 g/mL) for column chromatography was purchased from Crescent Chemical Co., Inc. Lipophilic Sephadex LH-20 (a product of Pharmacia Inc., Sweden, bead size 25−100 μm) was from Sigma Chemicals. C_8 (32−63 μm) resin was obtained from ICN Pharmaceuticals. A Kromasil reversed-phase C_{18} HPLC column (250 × 10 mm, 5 μm) was from Higgins Analytical Inc. Distilled, deionized water from a Milli-Q system was used for all aqueous manipulations.

For the yeast assay, Yeast Nitrogen Base without amino acids, D-(+)-galactose containing <0.01% glucose, anhydrous D-(+)-glucose, D-(+)-raffinose pentahydrate, adenine hemisulfate salt, L-histidine monohydrochloride, and streptonigrin were purchased from Sigma Chemicals. The assays were carried out in 96-well plates and monitored at 595 nm using a microplate reader.

Plant Materials. Flowers, leaves, and twigs of *Crypteronia paniculata* were collected in January 1991 in Palawan (Philippines). A voucher specimen (U44Z2520) is stored at the U.S. National Arboretum, Herbarium, Washington, DC.

Extraction and Isolation. A crude 1:1 MeOH−CH_2Cl_2 extract prepared from flowers, leaves, and twigs of *C. paniculata* was found to exhibit significant selective cytotoxicity toward a yeast strain harboring pRAD52 under the control of a galactose promotor (IC_{50} 39 μg/mL) in the presence of glucose (>500 μg/mL in the presence of galactose). Accordingly, this crude extract was subjected to bioassay-guided fractionation using the yeast assay. A typical set of experiments is described below. The extract (410 mg) was applied initially to a 10 g polyamide 6S column; this column was washed successively with H_2O, 1:1 MeOH−H_2O, 4:1 MeOH−CH_2Cl_2, 1:1 MeOH−CH_2Cl_2, and 9:1 MeOH−NH_4OH. The 1:1 MeOH−H_2O fraction (41 mg) exhibited the strongest DNA-damaging activity [(IC_{50} 24 μg/mL) (glucose); IC_{50} >500 μg/mL (galactose)]. This fraction was applied to an 8 g C_8 reversed-phase open column and washed successively with 0:10, 2:8, 4:6, 6:4, 8:2, and then 10:0 MeOH−H_2O. The 8:2 MeOH−H_2O fraction (2.0 mg) was found to have the strongest activity [(IC_{50} 8.8 μg/mL (glucose); IC_{50} >500 μg/mL (galactose)] and was fractionated further by C_{18} reversed-phase HPLC using a linear gradient from 3:17 to 11:9 MeCN−H_2O over a period of 60 min at a flow rate of 3.0 mL/min (detection at 265 nm), which gave three peaks of active material (0.5, 0.4, and 0.6 mg, in order of elution from the HPLC column). Further purification of the three most active peak fractions using the same HPLC column and conditions afforded **1** (t_R 16.4 min; 0.4 mg), **2** (t_R 23.3 min; 0.2 mg), and **3** (t_R 23.9 min; 0.4 mg), respectively.

3,3′-Di-*O*-methylellagic acid 4′-*O*-*β*-D-xylopyranoside (1): crystals from methanol; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.74 (1H, s, H-5′), 7.51 (1H, s, H-5), 5.15 (1H, d, J = 7.2 Hz, H-1″), 4.05 (3H, s, 3′-OCH_3), 4.02 (3H, s, 3-OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 158.2 (C-7′), 158.1 (C-7), 101.5 (C-1″); positive ion CIMS m/z 463 $[M + H]^+$, 331. The structure was identified by the ^{1}H and ^{13}C NMR and MS data and by comparison with literature data.[9]

3′-*O*-Methyl-3,4-methylenedioxyellagic acid 4′-*O*-*β*-D-glucopyranoside (2): crystals from methanol; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.75 (1H, s, H-5′), 7.28 (1H, br s, H-5), 5.14 (1H, d, J = 6.2 Hz, H-1″), 4.12 (3H, s, 3′-OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 157.5 (C-7′), 157.1 (C-7), 101.4 (C-1″); negative ion CIMS m/z 525 $[M + Cl]^-$, 327. The structure was

identified by the ^{1}H and ^{13}C NMR and MS data and by comparison with literature data.[9a]

Kaempferol-3-*O*-α-L-rhamnoside (3): yellow powder; $[\alpha]_D^{20}$ −180° (*c* 0.08, MeOH); ^{1}H NMR (CD_3OD, 300 MHz) δ 7.68 (2H, d, J = 8.2 Hz, H-2′, 6′), 6.85 (2H, d, J = 8.2 Hz; H-3′, 5′), 6.26 (1H, d, J = 2.2 Hz, H-8), 6.08 (1H, d, J = 2.2 Hz, H-6), 5.13 (1H, d, J = 2.0 Hz, H-1″), 0.82 (3H, d, J = 6.5 Hz, 6″-CH_3); ^{13}C NMR (CD_3OD, 125 MHz) δ 178.5 (C-4), 103.5 (C-1″), 100.5 (C-6), 95.0 (C-8), 17.6 (6′-CH_3); positive CIMS *m*/*z* 433 [M + H]$^+$, 287. The structure was identified from the ^{1}H and ^{13}C NMR and MS data and by comparison with literature data.[10]

Yeast Strain Growth. Transformed strains of *S. cerevisiae*, RS321NpRAD52 and RS321NYCp50 (referred to here as RAD52 and YCp50, respectively), had the following genotypes.

RS321NphRAD52 Mat a ade2-1 his3-1 leu3,112 trp1-1 ura3-1 can1-100 erg6 rad52::TRP1 top1-8::LEU2 pRAD52::URA

RS321NYCp50 Mat a ade2-1 his3-1 leu3,112 trp1-1 ura3-1 can1-100 erg6 rad52::TRP1 top1-8::LEU2 YCp50::URA

Yeast strains were grown from a 15% glycerol stock to an optical density (595 nm) between 1 and 3 in minimal medium consisting of 0.67% YNB, 2.0% glucose, 0.025 mg/mL adenine, and 0.025 mg/mL histidine. The yeast were then transferred to the same minimal medium containing raffinose, a neutral carbon source, instead of glucose. Cultures were then grown to an optical density (595 nm) of 1−3.

Yeast Assay for DNA Damage. Yeast strains were diluted to an optical density (595 nm) of 0.01 in glucose minimal medium (RAD52) or galactose minimal medium (RAD52 and YCp50). Samples to be assayed were dissolved in DMSO to 5 μg/mL, then diluted to 1, 0.5, 0.1, 0.05, and 0.01 μg/mL at final DMSO concentrations of 20%. Each of three blank wells contained 10 μL of 20% DMSO and 90 μL of either glucose- or galactose-containing minimal medium. Two control wells (no growth inhibition) were prepared for each strain with 10 μL of H_2O and 90 μL of diluted yeast strain. Streptonigrin was used as a positive control for DNA-damaging activity, at a final concentration of 0.01 μg/mL. Test wells (and positive controls) contained 10 μL of the sample to be tested and 90 μL of the desired yeast strain. Plates were incubated in a humidity chamber at 30 °C for 41 h. The optical density (595 nm) of each well was determined using a microplate reader. Data points were plotted as OD_{595} versus natural log of concentration. This provided a linear plot for easy analysis of IC_{50} values, defined for our purposes as the concentration of a compound at which yeast growth was inhibited by 50%.

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