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DNA Damaging Activity of Ellagic Acid Derivatives

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Abstract—A strain of yeast rendered repair deficient by the conditional expression of the RAD52 locus was used to search for natural products capable of damaging DNA. Four ellagic acid derivatives, namely 3,3′-dimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**1**), 3,3′,4-trimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**2**), 3′-methyl-3,4-*O*,*O*-methylidene ellagic acid (**3**) and 3′-methyl-3,4-*O*,*O*-methylidene-4′-*O*-β-D-glucopyranosyl ellagic acid (**4**), were identified by this assay as DNA damaging natural principles from several plants, including *Alangium javanicum, Anisophyllea apetala, Crypteronia paniculata, Mouririi* sp. and *Scholtzia parviflora*. Although none of the isolated principles mediated frank strand scission of DNA in vitro, all of them potently inhibited the growth of yeast in the absence of expression of RAD52.

Introduction

DNA damaging agents of interest for use in cancer chemotherapy include those which mediate frank strand scission, such as bleomycin[1] and the enediyne antibiotics,[2] modification of DNA (e.g., cisplatin),[3] or which interfere with the utilization of DNA by enzymes such as topoisomerases I[4] and II.[4,5] Recently, we have described our efforts to identify novel agents that act in the same fashion as such classes of DNA damaging agents by the use of the COMPARE algorithm to screen natural products extracts.[6,7]

Another strategy that can be employed to identify DNA interactive agents is the use of yeast strains rendered conditionally deficient in a specific locus for DNA repair. A few naturally occurring compounds such as furanonaphthoquinones,[8] ergosterol derivatives[9] and stilbene derivatives[10] have been identified in this fashion as putative DNA damaging agents. In the present study a yeast assay was employed to detect agents believed to induce double-strand DNA damage. This assay employed an RAD52 knock-out strain of *Sacchromyces cerevisiae*. This gene is responsible for the homologous recombination pathway for DNA double-strand break repair.[11–13] The resulting yeast strain was then transformed with a plasmid carrying the human RAD52 gene under the control of a galactose promoter.

A survey of crude plant extracts identified a number that inhibited the yeast tester strain grown on glucose, but not the same strain grown on galactose, i.e., putatively containing DNA damaging agents whose repair requires homologous recombination.

This survey indicated that extracts prepared from *Alangium javanicum, Anisophyllea apetala, Cryteronia paniculata, Mouririi* sp. and *Scholtzia parviflora* exhibited strong DNA damaging activity. Subsequent bioassay-guided fractionation of these extracts resulted in the isolation of 3,3′-dimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**1**)[14] from *C. paniculata*, 3,3′,4-trimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**2**)[15] from *A. javanicum*, 3′-methyl-3,4-*O*,*O*-methylidene ellagic acid (**3**)[16] from *A. apetala, Mouririi* sp. and *S. parviflora,* and 3′-methyl-3,4-*O*,*O*-methylidene-4′-*O*-β-D-glucopyranosyl ellagic acid (**4**)[17] from *A. apetala, C. paniculata,* and *A. javanicum.* Reported herein are the isolation and characterization of these principles as DNA damaging agents.

Results and Discussion

The goal of this study was the identification of novel, naturally occurring agents that damage DNA. These agents were detected in a survey of plant extracts using a

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strain of *S. cerevisiae* that lacked the homologous RAD52 locus, but harbored a plasmid containing human RAD52 under the control of a galactose promoter. The extracts were tested for differential effects when the yeast strain was grown in the presence of glucose or galactose, i.e., under repair deficient and proficient conditions, respectively. A control strain (YCp50) was prepared from the same yeast strain by transformation with the same plasmid lacking the ability to express RAD52; the testing of extracts containing putative DNA damaging agents on this strain in galactose permitted the identification of those differential effects due only to differences in carbon metabolism.

A number of crude plant extracts exhibited quite good differential activity in this assay system; several of these are summarized in Table 1 and included 1:1 CH_2Cl_2–MeOH extracts prepared from *Alangium javanicum, Anisophyllea apetala, Crypteronia paniculata, Mouririi* sp. and *Scholtzia parviflora*. All of these extracts were strongly cytotoxic to the yeast tester strain grown on a glucose medium, but much less toxic when the same strain was grown on galactose.

Bioassay-guided fractionation was carried out on each of the extracts listed in Table 1. The initial fractionation on a polyamide 6S column effected the removal of polyphenols. Chromatography on Sephadex LH-20, and then on one or more reversed phase resins, afforded the purified principles.

All of the isolated principles from these extracts proved to be one of four known ellagic acid derivatives. These included 3,3′-dimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**1**),[14] which was isolated from *C. paniculata*, 3,3′,4-trimethyl-4′-*O*-β-D-glucopyranosyl ellagic acid (**2**),[15] isolated from *A. javanicum*, and 3′-3,4-*O*,*O*-methylidene ellagic acid (**3**),[16] which was isolated from *Mouririi* sp., *S. parviflora* and *A. apetala*. Finally, 3′-methyl-3,4-*O*,*O*-methylidene-4′-*O*-β-D-glucopyranosyl ellagic acid (**4**) was isolated from *C. paniculata, A. javanicum,* and *A. apetala*.

The activities of the four purified ellagic acid derivatives in the yeast assay system are summarized in Table 2. A comparison of compounds **1** and **2** reveals that the presence of a 4-*O*-methyl, rather than OH, group enhanced the potency of DNA damage more than 10-fold. The most potent compound was ellagic acid **3**, which lacked the saccharide moiety.

Table 1. DNA damaging activities of crude extracts

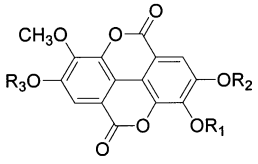
Source of extract	DNA damaging activity (IC_{50}, μg/mL)		
	RAD52 (glucose)	RAD52 (galactose)	YCp50 (galactose)
A. javanicum	0.09	1.63	0.14
A. apetala	0.66	3.0	1.67
C. paniculata	38.7	>500	21.9
Mouririi sp.	0.735	22.9	0.756
S. parviflora	6.66	>500	3.54

Table 2. DNA damaging activities of four ellagic acid derivatives (**1**–**4**)

Compd	DNA damaging activity (IC_{50}, μM)		
	RAD52 (glucose)	RAD52 (galactose)	YCp50 (galactose)
1	5.7	>500	3.2
2	0.36	>500	0.34
3	0.171	110	0.073
4	1.12	14.4	0.341

Ellagic acid and its derivatives exist widely in the plant kingdom. Their biological activities have been studied extensively. Besides their effects as anti-oxidants and antimutagens, ellagic acids strongly inhibit a number of important enzymes such as DNA gyrase[18] from *E. coli*, glucosyltransferase[19] from mutans *Streptococci*, aldose reductase[20] from rat lens, HIV-1 reverse transcriptase and polymerases α and β,[21] as well as human DNA topoisomerases I and II.[22] It is known that ellagic acid can inhibit the mutagenic effects of many carcinogens such as *N*-nitrosodimethylamine,[23] aflatoxin B-1[24] and *N*-methyl-*N*-nitrosourea[25] through an ellagic acid–duplex DNA binding mechanism. Therefore, the four ellagic acid derivatives studied here may contribute to DNA damage in a similar manner.

Although compounds **1**–**4** did not effect the relaxation of supercoiled plasmid DNA in a cell free system, either alone or in the presence of Cu^{2+} or Fe^{2+}, the yeast assay employed here involves an incubation in a whole cell system for 41 h. Clearly, the conversion of compounds **1**–**4** to one or more metabolites is possible under such conditions and may contribute importantly to the observed activity.



	R_1	R_2	R_3
1	CH_3	H	β-D-Glc
2	CH_3	CH_3	β-D-Glc
3	-CH_2-		H
4	-CH_2-		β-D-Glc

Experimental

General methods

Polyamide 6S (pour density 0.25 g/mL, a product of Riedel-de Haen, Germany) was obtained from Crescent Chemical Co. Lipophilic Sephadex LH-20 (Pharmacia, 40 μm) and TSK gel Toyopearl HW-40F (Tosoh Corp., 45 μm) were purchased from Sigma Chemicals. Baker-bond C_{18} (40 μm) and C_8 (40 μm) silica gels were obtained from ICN Biomedical Research Products. Lichroprep Diol (40–63 μm) is a product from EM Industries, Inc. A Kromasil C_{18} reversed-phase column (250×10 mm, 5 μm) for HPLC was obtained from Higgins Analytical Inc. ^{1}H NMR spectra were measured on General Electric

QE 300 or GN-300 NMR spectrometers. Mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer. RAD52 and YCp50 yeast strains were provided by Glaxo SmithKline. 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 obtained from Sigma Chemicals. Costar tissue culture 96-well flat-bottom plates were purchased from Fisher Scientific. All operations for the yeast assay were carried out with sterilized, deionized water.

Preparation of plant crude extracts

Leaves of *Alangium javanicum* were collected in Sabah in October 1987 (voucher specimen Q66O5281). Branches of *Anisophyllea apetala* were collected in Sabah in June 1992 (voucher speciment U44Z5091). Flowers, leaves and twigs of *Crypteronia paniculata* were collected in Palawan (Philippines) in January 1991 (voucher specimen U44Z2520). Roots of *Mouririi* sp. were collected in Tanzania in January, 1989 (voucher specimen Q66T1075). Stem bark and stem wood of *Scholtzia parviflora* were collected in Western Australia in September 1981 (voucher specimen SPJ7088). All of these voucher specimens are stored at the US National Aboretum, Herbarium, Washington, DC. The dried plant materials were soaked at room temperature with 1:1 CH_2Cl_2–CH_3OH, and the solvent was concentrated under diminished pressure to afford the extracts.

Bioassay-guided fractionation and isolation of compounds 1–4

The five crude extracts of *Alangium javanicum, Anisophyllea apetala, Crypteronia paniculata, Mouririi* sp. and *Scholtzia parviflora* exhibited strong activity in the yeast bioassay for DNA damaging agents (Table 1). Fractionation and isolation were guided by the DNA damage assay; typical examples are described here.

Scholtzia parviflora. The crude extract (825 mg, IC_{50}: 6.66 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 3.54 μg/mL for YCp50 in galactose) was applied to a polyamide 6S column which was washed successively with H_2O, 1:1 H_2O–MeOH, 9:1 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH and 9:1 MeOH–NH_4OH. The 9:1 CH_2Cl_2–MeOH fraction, which had the strongest DNA damaging activity (253 mg, IC_{50}: 7.98 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 4.18 μg/mL for YCp50 in galactose), was fractionated further on a Sephadex LH-20 column. The column was washed with hexanes, 1:1 hexanes–CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2–acetone, acetone and MeOH. The CH_2Cl_2 fraction (10.5 mg) which showed the strongest activity was fractionated further on a Lichroprep Diol open column using CH_2Cl_2–MeOH (1:0→0:1) to give the compound **3** as a pale yellow powder (2.0 mg). Its ^1H NMR data were consistent with those reported for 3′-methyl-3,4-*O,O*-methylidene ellagic acid.[16]

Crypteronia paniculata. The crude extract (410 mg, IC_{50}: 38.7 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 21.9 μg/mL for YCp50 in galactose) was applied to a polyamide 6S column, which was washed successively with H_2O, 1:1 H_2O–MeOH, 4:1 MeOH–CH_2Cl_2, 1:1 CH_2Cl_2–MeOH and 9:1 MeOH–NH_4OH. The 1:1 H_2O–MeOH fraction (40.5 mg, IC_{50}: 24.1 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 21.1 μg/mL for YCp50 in galactose) was fractionated further on a C_8 reversed-phase open column using H_2O–MeOH (10:0, 8:2, 6:4, 4:6, 2:8 and then 0:10). The 2:8 H_2O–MeOH fraction (5.0 mg, IC_{50}: 8.8 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 16.5 μg/mL for YCp50 in galactose) was fractionated further on a C_{18} reversed-phase HPLC column (250×10 mm, 5 μm), eluting with CH_3CN–H_2O, to afford **1** (0.4 mg) and **4** (0.4 mg). Their physicochemical and spectral data were the same as those reported previously.[14,17]

Alangium javanicum. The crude extract (1.5 g, IC_{50}: 0.09 μg/mL for RAD52 in glucose, 1.63 μg/mL for RAD52 in galactose, 0.14 μg/mL for YCp50 in galactose) was applied to a polyamide 6S column which was washed successively with H_2O, 1:1 H_2O–MeOH, 4:1 MeOH–CH_2Cl_2, 1:1 CH_2Cl_2–MeOH and 9:1 MeOH–NH_4OH. The 50% MeOH fractions were combined and concentrated to a small volume, affording a white precipitate (25 mg, IC_{50}: 0.16 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 0.01 μg/mL for YCp50 in galactose). The precipitate was fractionated further on a C_{18} reversed-phase HPLC column (250×10 mm, 5 μm), eluting with MeOH–H_2O, to afford **2** (10 mg) and **4** (10 mg). Their physicochemical and spectral data were the same as those reported in the literature.[15,17]

Mouririi sp. The crude extract (600 mg, IC_{50}: 0.735 μg/mL for RAD52 in glucose, 22.9 μg/mL for RAD52 in galactose, 0.756 μg/mL for YCp50 in galactose) was applied to a polyamide 6S column which was eluted successively with H_2O, 1:1 H_2O–MeOH, 4:1 MeOH–CH_2Cl_2, 1:1 CH_2Cl_2–MeOH and 9:1 MeOH–NH_4OH. The 1:1 H_2O–MeOH fractions were combined and fractionated further on a C_8 reversed-phase open column using H_2O–MeOH (10:0, 8:2, 6:4, 4:6, 2:8 and then 0:10). The 6:4, 4:6 and 2:8 H_2O–MeOH fractions showed strong DNA damaging activity and were combined and fractionated on a C_{18} reversed-phase open column, eluting with H_2O–MeOH (10:0, 8:2, 6:4, 4:6, 2:8 and then 0:10). The 4:6 and 2:8 H_2O–MeOH fractions were combined and purified further by chromatography on a C_{18} reversed-phase open column, again using H_2O–MeOH mixtures for elution (60:40→0:100). The 45:55 H_2O–MeOH fraction was fractionated on a C_{18} reversed-phase HPLC column (250×10 mm, 5 μm), eluting with CH_3CN–H_2O, to afford **3** (2 mg). Its physicochemical and spectral data were the same as those reported previously.[16]

Anisophyllea apetala. The crude extract (362.5 mg, IC_{50}: 0.66 μg/mL for RAD52 in glucose, 3.0 μg/mL for RAD52 in galactose, 1.67 μg/mL for YCp50 in galactose) was

applied to a polyamide 6S column which was washed successively with H_2O, 1:1 H_2O–MeOH, 9:1 CH_2Cl_2–MeOH, 1:1 CH_2Cl_2–MeOH and 9:1 MeOH–NH_4OH. After concentration, the 50% MeOH fraction gave a white precipitate (1.2 mg), which was confirmed to be 3′-methoxy-3,4-*O*,*O*-methylidene-4′-β-D-glucopyranosyl ellagic acid (**4**)[17] by comparison of spectral data and TLC behavior with an authentic sample. The 9:1 CH_2Cl_2–MeOH fraction (90.2, IC_{50}: 4.20 μg/mL for RAD52 in glucose, >500 μg/mL for RAD52 in galactose, 4.25 μg/mL for YCp50 in galactose) was fractionated on a Lichchroprep Diol open column using CH_2Cl_2–MeOH (1:0→0:1) to give a pale yellow powder (1.5 mg). This was shown to be 3′-methyl-3,4-*O*,*O*-methylidene ellagic acid (**3**)[16] through comparison of its TLC behavior and spectral data with those of an authentic sample.

Yeast assay for DNA damage

Yeast strains were grown from frozen glycerol stocks in 2% glucose minimal medium (0.67% YNB; 0.025 mg/mL, adenine and histidine) for 2 days. Cultures were transferred to new glucose media and grown overnight. These cultures were then transferred to fresh minimal media, now containing 2% raffinose as the carbon source, and again grown overnight. These final cultures were again transferred to fresh 2% raffinose and grown overnight, to a final OD_{595} between 1 and 3. These were stored at 4 °C. Samples were dissolved at a concentration of 5 mg/mL in DMSO and diluted to 1000, 500, 100, 50, and 10 μg/mL in 20% DMSO. Streptonigrin (final concentration 0.01 μg/mL) was used as a positive control for DNA damage.

To perform the yeast assay, yeast strains were each diluted in 2% minimal medium (RAD52 in glucose and in galactose, YCp50 in galactose) to an OD_{595} of 0.01. Each of three blank assay wells were filled with 10 μL of 20% DMSO and 90 μL minimal medium. Yeast growth controls contained 90 μL yeast in the appropriate minimal medium. Positive controls for DNA damage contained 10 μL streptonigrin (0.1 μg/mL) and 90 μL of the appropriate yeast culture in glucose or galactose. Test wells contained 10 μL of sample and 90 μL of the appropriate yeast dilution. Plates were read for t_0 OD_{595} and incubated for 41 h at 30 °C. After incubation, well OD_{595} values were again determined, and used to calculate IC_{50} values for each of the samples tested.

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