A Coumarin from *Mallotus resinosus* that Mediates DNA Cleavage

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A crude extract prepared from roots of *Mallotus resinosus* exhibited significant Cu^{2+}-dependent DNA strand scission activity and was thus selected for bioassay-guided fractionation. Scopoletin (**1**), a simple coumarin, was identified as the active principle responsible for DNA cleavage activity of the crude extract. The DNA strand scission activity of **1**, as well as that of three structural analogues, is reported.

The discovery of the bleomycins as potent DNA strand scission agents[1,2] and the demonstration that they are efficacious as antitumor agents[1,3] has prompted efforts to identify other naturally occurring molecules that are capable of mediating DNA strand scission. Such molecules could potentially form the basis for new classes of clinically useful antitumor agents. Several types of natural products have been reported to cleave DNA in the absence or presence of discrete metal ions.[4,5]

As part of our efforts to identify novel DNA cleaving agents, a crude extract prepared from roots of *Mallotus resinosus* Merr. (Euphorbiaceae) was found to mediate DNA strand scission in the presence of Cu^{2+}. Bioassay-guided fractionation of this extract, monitored by the use of an in vitro DNA cleavage assay, led to the isolation of a known coumarin, scopoletin (**1**), as a Cu^{2+}-dependent DNA strand scission agent having moderate potency. The isolation of scopoletin (**1**) and the Cu^{2+}-dependent DNA cleavage activities of **1** and three analogues, one of which proved to be significantly more potent than **1**, are described.

A crude organic extract prepared from the dried roots of *M. resinosus* was first fractionated on a Sephadex LH-20 column; elution was carried out successively with hexane, 1:1 hexane−CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2−acetone, acetone, and MeOH. The acetone and MeOH fractions exhibited significant Cu^{2+}-dependent DNA cleavage activity; these were combined and fractionated further on a C_{18} reversed-phase column using MeOH−H_2O mixtures for elution. The 1:1 MeOH−H_2O fraction exhibited the most potent DNA strand scission activity and was subsequently applied to a C_8 reversed-phase column, which was washed with MeOH−H_2O mixtures. The 3:7 MeOH−H_2O fraction exhibited the strongest DNA strand breakage in the presence of Cu^{2+}. C_{18} reversed-phase HPLC purification of this fraction resulted in the isolation of the pure, active compound **1**. The presence of a coumarin moiety seemed probable on the basis of the NMR data, and the isolate was confirmed as scopoletin by the comparison of its NMR and MS with published data.[6]

This represents the first report of the isolation of a coumarin from *M. resinosus*. It is also the first time that scopoletin (**1**) has been found to induce DNA strand breakage in the presence of Cu^{2+}, although **1** has been reported to exhibit a number of interesting biological activities including cytotoxicity toward P-388 and KB cells,[7,8] inhibitory activity toward rat basophilic leukemia 5-lipoxygenase,[9] in vitro inhibition of cAMP phosphodiesterase,[10] and inhibition of rat lens aldose reductase.[11]

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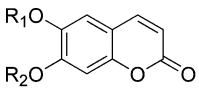
While no study of the secondary metabolites of *Mallotus resinosus* has been reported previously, other species in this genus have been investigated. Chemical constituents reported have included benzopyran, phloroglucinol, and di- and triterpenoids, as well as tannins and polyphenols.[12]

Using supercoiled pBR322 plasmid DNA as a substrate, an in vitro DNA strand scission assay was employed to evaluate the activity of **1**, along with three structurally related analogues, namely, isoscopoletin (**2**), esculetin (**3**), and 6,7-dimethoxycoumarin (**4**), the latter three of which were commercially available. In the presence of Cu^{2+}, dose-dependent single-strand DNA breakage of the supercoiled pBR322 plasmid DNA was observed for compounds **1**−**3** (Table 1). None of these compounds displayed DNA strand scission activity in the presence of Fe^{2+} or in the absence of added metal ion.



1 $R_1 = CH_3$ $R_2 = H$
2 $R_1 = H$ $R_2 = CH_3$
3 $R_1 = H$ $R_2 = H$
4 $R_1 = CH_3$ $R_2 = CH_3$

In the presence of 20 μM Cu^{2+}, compounds **1** and **2** clearly induced single-strand DNA breakage with similar potencies. About 36% and 42% conversion of form I (supercoiled) to form II (nicked) DNA was observed, respectively, when **1** and **2** were employed at 1 mM concentration. When **1** and **2** were employed at 250 μM concentration, the percent conversion to form II DNA diminished to about 15% and 14%, respectively. As shown in Table 1, esculetin (**3**), which has two hydroxyl groups, displayed the most potent DNA cleavage activity. About 76% conversion to form II DNA resulted when 250 μM **3** was incubated simultaneously with Cu^{2+} and DNA. In contrast, no DNA cleavage was observed when **4** was tested at 1 mM concentration. Considering the structures of these compounds, it is clear that the number of phenolic hydroxyl groups in the structure is crucial to the ability of these compounds to induce Cu^{2+}-dependent DNA strand breakage, while the position of the OH group had little effect.

Previously, detailed studies were reported on the mechanism of DNA strand scission by 5-alkylresorcinols in the presence of Cu^{2+} ion.[13] The mechanism involved oxygenation of the aromatic nucleus, affording catecholic moieties that were proposed to coordinate Cu^{2+}. The Cu complexes reduce dioxygen to reactive oxygen species with concomitant oxidation of the catechol moiety via the coordinated Cu^{2+} ion.[13] It seems likely that reductive activation

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Table 1. Cu^{2+}-Dependent DNA Strand Scission by Compounds **1**−**4**

compound	Cu^{2+} (20 μM)	form II DNA (%)
none	−	2
none	+	2
scopoletin (**1**, 1000 μM)	−	3
scopoletin (**1**, 1000 μM)	+	36
scopoletin (**1**, 500 μM)	+	22
scopoletin (**1**, 250 μM)	+	15
isoscopoletin (**2**, 1000 μM)	−	2
isoscopoletin (**2**, 1000 μM)	+	42
isoscopoletin (**2**, 500 μM)	+	26
isoscopoletin (**2**, 250 μM)	+	14
esculetin (**3**, 250 μM)	−	2
esculetin (**3**, 250 μM)	+	76
esculetin (**3**, 100 μM)	+	64
esculetin (**3**, 50 μM)	+	55
6,7-dimethylcoumarin (**4**, 1000 μM)	−	2
6,7-dimethylcoumarin (**4**, 1000 μM)	+	3

of Cu(II)·**1**−**3** in the presence of O_2 is also responsible for the observed cleavage activity in the present case. However, since preincubation of **1** with Cu^{2+} ion and alkali actually *decreased* subsequent DNA cleavage, it is logical to conclude that the Cu^{2+}-dependent DNA cleavage activity of these compounds does not involve initial oxygenation of the aromatic nucleus. This is fully consistent with the observed order of activities, i.e., **3** > **1** = **2** > **4**. While the potency of DNA cleavage by these agents is not exceptional, the simplicity of the structure involved argues for the potential utility of this structural motif in the design of more complex DNA-damaging agents intended to inflict damage at specific DNA loci.

Experimental Section

General Experimental Procedures. Sephadex LH-20 was purchased from Sigma Chemicals. Silica C_{18} (40 μm) was obtained from J. T. Baker Chemicals. A Higgins Kromasil 100 C_{18} reversed-phase column (250 × 10 mm, 5 μm) was used for HPLC separations. ^1H and ^{13}C NMR spectroscopic experiments were performed on a Varian Unity Inova 300 spectrometer. Low-resolution chemical ionization mass spectra were recorded on a Finnigan MAT 4600 mass spectrometer. Esculetin, ethidium bromide, bromophenol blue, and Trizma base were purchased from Sigma Chemicals. Isoscopoletin and 6,7-dimethoxycoumarin were obtained from Indofine Chemical Company Inc. Boric acid was obtained from EM Sciences. (Ethylenedinitrilo)tetraacetic acid (EDTA), disodium salt was purchased from J. T. Baker. Cupric chloride and glycerol were obtained from Mallinckrodt, Inc. Ultrapure agarose was obtained from Bethesda Research Laboratories. Supercoiled pBR322 plasmid DNA was purchased from New England Biolabs. Pierce microdialysis cassettes were used to remove EDTA from the pBR322 plasmid DNA from New England Biolabs.

Plant Material. Roots of *M. resinosus* were collected on Palawan Island, Republic of the Philippines, on March 21, 1990, by D. D. Soejarto, E. Reynoso, E. Sagcal, and R. Edrada. The taxonomy was carried out by W. Meijer. A voucher specimen (U44Z1604) is stored at the U.S. National Arboretum, Herbarium, Washington, DC.

Extraction and Isolation. Dried roots of *M. resinosus* were steeped in 1:1 methylene chloride−methanol overnight at room temperature, then drained and washed with methanol. The combined organic solution was concentrated under diminished pressure. The crude extract of *M. resinosus* displayed significant Cu^{2+}-dependent DNA cleavage activity. In a typical fractionation experiment, the crude extract (605 mg) was first applied to a Sephadex LH-20 column, which was eluted successively with hexane, 1:1 hexane−CH_2Cl_2, CH_2Cl_2, 1:1 CH_2Cl_2−acetone, acetone, and MeOH. The acetone and MeOH fractions (27 mg and 412 mg, respectively) induced potent DNA strand scission at 100 and 50 μg/mL concentrations in the presence of Cu^{2+}. These two fractions were combined and fractionated further on a C_{18} reversed-phase column using MeOH−H_2O mixtures for elution. The 1:1 MeOH−H_2O fraction (306 mg) exhibited strong Cu^{2+}-dependent DNA cleavage and was applied to a C_8 reversed-phase column, which was washed with MeOH−H_2O mixtures. The 3:7 MeOH−H_2O fraction (22 mg) exhibited strong DNA cleavage activity and was fractionated further on a C_{18} reversed-phase HPLC column (250 × 10 mm, 5 μm). Elution of the column was effected with a linear gradient of 1:19 → 2:3 CH_3CN−H_2O over a period of 40 min at a flow rate of 3.5 mL/min (UV monitoring at 215 nm). One particularly active fraction (2.5 mg) was obtained from the reversed-phase HPLC fractionation. Purification of the active fraction, employing the same HPLC conditions, afforded the active compound **1** (1.2 mg).

DNA Strand Scission Assay. DNA strand scission was assayed using 500 ng of supercoiled pBR322 plasmid DNA in the absence or presence of 20 μM Cu^{2+} in 25 μL (total volume) of 10 mM Tris-HCl buffer (pH 8.0) containing crude extracts, fractions, or pure compounds (dissolved in DMSO, with a final DMSO concentration of no more than 5% in the 25 μL reaction solution). Each set of experiments included one blank control (DNA alone) and one metal control (DNA + Cu^{2+}). After incubation at 37 °C for 60 min, the reaction product was mixed with 5 μL of 30% glycerol−0.01% bromophenol blue and was analyzed by electrophoresis in a 1.0% agarose gel containing 0.7 μg/mL ethidium bromide. Electrophoresis was carried out in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 110−120 V for 2−3 h. Following electrophoresis, the gel was photographed under ultraviolet light.

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