Umbelactonyl Cinnamate Derivatives from *Crypteronia paniculata* that Mediate DNA Strand Scission

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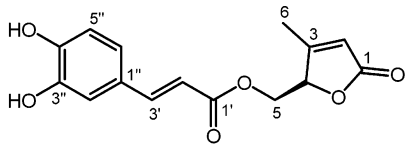
As part of an initiative to discover functional natural product analogues of bleomycin guided by the use of the COMPARE algorithm, a CH_2Cl_2−MeOH extract prepared from *Crypteronia paniculata* was found to exhibit relaxation of supercoiled pSP64 DNA in the presence of Cu^{2+}. Bioassay-guided fractionation employing a DNA strand scission assay resulted in the isolation of three novel DNA cleavage agents. Their structures were elucidated as umbelactonyl cinnamate derivatives **1**−**3** through their NMR and MS spectral data analyses. This is the first example of the isolation and structural characterization of naturally occurring umbelactonyl cinnamate derivatives. Compound **1** exhibited strong Cu^{2+}-dependent relaxation of supercoiled pSP64 DNA, while compounds **2** and **3** had only weak DNA cleavage activity.

The COMPARE algorithm[1] can be used to identify extracts in the National Cancer Institute Natural Products Repository that exhibit cytotoxicity profiles similar to existing antitumor agents. We have adopted a related approach,[2] using COMPARE analysis to discover functional analogues of bleomycin. This antitumor antibiotic has a mechanism of action involving the cleavage of DNA, and possibly also RNA, in a metal ion- and oxygen-dependent fashion.[3] This approach has been illustrated previously through the identification of four natural principles capable of mediating Cu^{2+}-dependent DNA strand scission.[2] This was the first report of the use of the COMPARE algorithm to identify functional analogues of bleomycin in natural product extracts.

As noted previously, 43 extracts in the NCI Natural Products Repository were found to have COMPARE profiles similar to that of bleomycin.[2] A CH_2Cl_2−MeOH extract prepared from one of these extracts, *Crypteronia paniculata* Kurz (Crypteroniaceae) (Tropicos), was found to mediate Cu^{2+}-dependent relaxation of supercoiled pSP64 plasmid DNA in analogy with bleomycin itself.[4] Accordingly, this extract was subjected to bioassay-guided fractionation. This fractionation, employing a cell-free DNA cleavage assay to detect species capable of mediating relaxation of supercoiled pSP64 plasmid DNA in the presence of Cu^{2+}, led to the isolation of three novel compounds, namely **1**, **2**, and **3**. Compound **1** displayed potent Cu^{2+}-dependent DNA cleavage activity, while compounds **2** and **3** mediated DNA strand scission weakly. Previously, we isolated three DNA-damaging agents[5] in two different structural classes from the same extract of *C. paniculata* using a yeast strain harboring the RAD52 gene on a multicopy plasmid under the control of the GAL1 promoter. By monitoring differential growth inhibition when the yeast was grown on glucose versus galactose, it was possible to identify cytotoxic agents whose DNA damage required expression of the RAD52 locus for repair. None of these isolated principles were observed to mediate frank DNA strand scission. Herein, we describe the isolation and structure determination of three new umbelactonyl cinnamate derivatives



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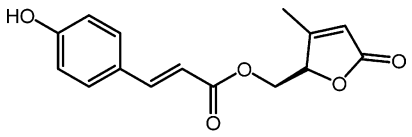

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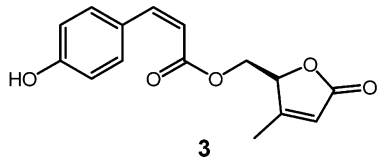

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(**1**, **2**, and **3**) and their potency as frank DNA cleavage agents.

In an effort to identify extracts containing functional analogues of bleomycin, >20 000 cytotoxic extracts in the NCI Natural Products Repository were analyzed to identify those having mean graph cytotoxicity profiles similar to that of bleomycin. Forty-three extracts were found to have the desired properties, including a CH_2Cl_2−MeOH extract prepared from *C. paniculata.* This extract was also found to mediate relaxation of supercoiled pSP64 plasmid DNA in the presence of 20 μM Cu^{2+} when tested at a concentration of 100 μg/mL. Isolation and characterization of the principle(s) responsible for the supercoiled DNA cleavage led to the identification of **1**, **2**, and **3**. A 410 mg sample of the extract upon purification afforded 1.0, 0.4, and 0.3 mg of three new compounds, **1**, **2**, and **3**, respectively, as described in the Experimental Section.

Compound **1** was isolated as a slightly blue viscous mass. The molecular formula $C_{15}H_{14}O_6$ was obtained through analysis of the high-resolution ESI/FTMS spectrum of **1**. The ^{1}H NMR spectrum of **1** showed signals characteristic of a 1,3,4-trisubstituted benzene at δ_H 6.77 (1 H, d, J = 8.0 Hz), 6.93 (1 H, dd, J = 8.0, 1.8 Hz), and 7.02 (1 H, d, J = 1.8 Hz), and an AB system at δ_H 6.20 (1 H, d, J = 16.0 Hz) and 7.52 (1 H, d, J = 16.0 Hz). Combined analysis of

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Table 1. NMR Data for Compounds **1**, **2**, and **3** (CD_3OD, 500 MHz for ^1H and 125 MHz for ^{13}C)

	1		**2**		**3**	
position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1		175.28		175.26		176.32
2	5.96 br s	118.75	5.96 br s	118.76	5.91 br s	118.60
3		168.86		168.86		168.81
4	5.23 br s	84.44	5.26 br s	84.42	5.21 br s	84.47
5	4.47 dd, J = 12.5, 3.5 Hz 4.60 dd, J = 12.5, 2.5 Hz	62.41	4.47 dd, J = 12.5, 3.5 Hz 4.60 dd, J = 12.5, 2.5 Hz	62.41	4.41 dd, J = 12.5, 3.5 Hz 4.58 dd, J = 12.5, 2.5 Hz	62.39
6	2.16 br s	13.86	2.16 br s	13.85	2.16 br s	13.84
1′		168.28		168.23		168.30
2′	6.20 d, J = 16.0 Hz	113.97	6.28 d, J = 16.0 Hz	114.06	5.71 d, J = 12.0 Hz	115.7
3′	7.52 d, J = 16.0 Hz	147.81	7.60 d, J = 16.0 Hz	147.42	6.90 d, J = 12.0 Hz	146.56
1″		127.49		126.91		127.50
2″	7.02 d, J = 1.8 Hz	115.16	7.45 d, J = 8.0 Hz	131.30	7.45 d, J = 8.0 Hz	133.72
3″		146.81	6.80 d, J = 8.0 Hz	116.82	6.80 d, J = 8.0 Hz	116.01
4″		149.81		161.47		160.31
5″	6.77 d, J = 8.0 Hz	116.49	6.80 d, J = 8.0 Hz	116.82	6.80 d, J = 8.0 Hz	116.01
6″	6.93 dd, J = 8.0, 1.8 Hz	123.14	7.45 d, J = 8.0 Hz	131.30	7.45 d, J = 8.0 Hz	133.72

the MS fragment ion peak at *m/z* 163 (base peak) and a carbonyl signal at δ_C 168.86 (s) suggested the presence of a caffeic acid moiety in **1**. An ABX pattern appeared in the ^1H NMR spectrum at δ_H 4.47 (1 H, dd, J = 12.5, 3.5 Hz), 4.60 (1 H, dd, J = 12.5, 2.5 Hz), and 5.23 (1 H, br s), along with an olefinic proton signal at δ_H 5.96 (1 H, br s), and a vinylic methyl at δ_H 2.16 (3 H, br s). An α,β-unsaturated lactone system could be deduced from the ^1H and ^{13}C NMR spectra (Table 1). Subsequently, an umbelactone substructure[6] in **1** could be assigned. The cross-peaks between CH_3-6 (δ 2.16) and H-2 (δ 5.96), and CH_3-6 and H-4 (δ 5.23), in the NOESY spectrum further supported this assignment. The correlations between H-5 (δ 4.47 and 4.60) and C-1′ (δ 175.28) were observed in an HMBC experiment. Therefore, the structure of **1** was established as umbelactonyl caffeate. All assignments of **1** were confirmed further by the $^1H-^1H$ COSY, HMQC, HMBC, and NOESY spectra. Although the absolute stereochemistry of C-4 in **1** could not be determined by the NMR data, it was presumed to be the *R*-configuration on the basis of its positive optical rotation, which was consistent with that of naturally occurring (*R*)-(+)-umbelactone.[6]

The ^1H and ^{13}C NMR spectra of **2** and **3** were similar to those of **1** except for the signals corresponding to the 1,4-disubstituted benzene moiety. A *p*-hydroxycinnamic acid moiety in **2** and **3** could be deduced from the combined analyses of their ^1H NMR [at δ 7.45 (2 H, d, J = 8.0 Hz) and 6.80 (2 H, d, J = 8.0 Hz)] (Table 1) and MS spectra (*m/z* 147). The coupling constants [J = 16.0 (δ 7.60 with δ 6.28) in **2**, and J = 12.0 Hz (δ 6.90 with δ 5.71) in **3**] of the AB system signals in their ^1H NMR spectra indicated the presence of a *trans-p*-hydroxycinnamic acid moiety in **2** and a *cis-p*-hydroxycinnamic acid moiety in **3**, respectively. Accordingly, the structures of **2** and **3** were determined as umbelactonyl *trans-p*-hydroxycinnamate and umbelactonyl *cis-p*-hydroxycinnamate, respectively. Also, all NMR assignments of **2** and **3** were confirmed further by their $^1H-^1H$ COSY, HMQC, HMBC, and NOESY spectra. We cannot exclude the possibility that **3** was formed from **2** (e.g., by the action of light) during extract storage or fractionation. It may be noted that this is the first example of isolation and structural characterization of umbelactonyl cinnamates from a natural source.

Compounds **1**, **2**, and **3** were tested for their ability to cleave pSP64 plasmid DNA, a supercoiled, covalently closed, circular DNA, in the absence and presence of Cu^{2+}.[2,7] Relaxation of the DNA was observed for compound **1**, but only in the presence of Cu^{2+} (Figure 1). Compound **1** displayed dose-dependent cleavage producing 92, 88, 68, 32, and 16% nicked (Form II) DNA at 10, 5, 1, 0.1, and 0.01 μM concentrations, respectively, when 20 μM Cu^{2+} was employed. As shown in Figure 1, compound **1** effected the conversion of Form I to Form II (nicked circular) DNA efficiently, but no Form III (linear duplex) DNA was observed. Compounds **2** and **3** exhibited only very weak Cu^{2+}-dependent relaxation of the plasmid DNA even at 250 μM concentration.

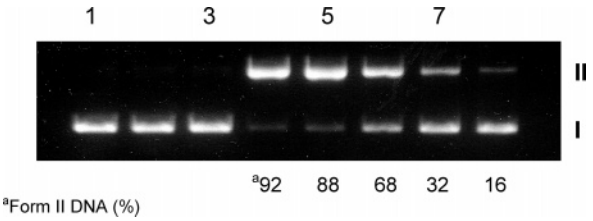

Figure 1. DNA strand scission by compound **1**, analyzed by agarose gel electrophoresis. Lane 1, pSP64 plasmid DNA alone; lane 2, pSP64 plasmid DNA + 20 μM Cu^{2+}; lane 3, 10 μM **1**; lane 4, 10 μM **1** + 20 μM Cu^{2+}; lane 5, 5 μM **1** + 20 μM Cu^{2+}; lane 6, 1 μM **1** + 20 μM Cu^{2+}; lane 7, 0.1 μM **1** + 20 μM Cu^{2+}; lane 8, 0.01 μM **1** + 20 μM Cu^{2+}. The percent Form II DNA present is shown below each lane.

A number of natural products have now been shown to mediate DNA cleavage, and the most potent cleavage appears to be associated with compounds having catechol functionalities, or which can be converted to catechols in situ.[8−10] Compounds **1**, **2**, and **3** are closely related in structure, the differences being the number of phenolic hydroxyl groups in the hydroxycinnamic acid moiety, or the geometry of the double bond (in **2** versus **3**). The relative DNA cleavage activities of **1**−**3** are generally quite consistent with the results obtained for other phenolic species,[8−10] although it is not presently known whether this is due to a common mechanism of action. It should be noted that umbelactone is a naturally occurring γ-(hydroxymethyl)-α,β-butenolide that was isolated from *Memycelon umbelatum*.[11] The crude extract of *M. umbelatum* has been reported to exhibit activity against Ranikhe disease virus and to show spasmolytic and antiamphetamine activity.[6b,11] The finding of the DNA cleavage activity and putatively associated cytotoxicity of umbelactonyl cinnamates may be of utility in designing novel classes of compounds of interest as potential antineoplastic agents.

Experimental Section

General Experimental Procedures. 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. C_8 reversed-phase (32−63 μm) and C_{18}

reversed-phase (32−63 μm) resins were obtained from ICN Pharmaceuticals. A Kromasil C_{18} reversed-phase HPLC column (250 × 10 mm, 5 μm) was purchased from Higgins Analytical Inc. Optical rotations were measured on a Perkin-Elmer 243 B polarimeter. The ^1H and ^{13}C NMR spectra were recorded using CD_3OD as an internal standard on a Varian-UnityInova 500/51 spectrometer at 500 MHz (for ^1H) and 125 MHz (for ^{13}C), respectively. The low-resolution chemical ionization (CI, methane) mass spectrum was obtained on a Finnigan MAT 4600, and the high-resolution ESI/FT mass spectrum was recorded on a New Star T70 FT/MS spectrometer at Glaxo SmithKline. Agarose gels were quantified for percent DNA cleavage utilizing Molecular Dynamics ImageQuant version 5.0 software.

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

Extraction and Isolation. A crude 1:1 MeOH−CH_2Cl_2 extract of leaves, twigs, and flowers of *C. paniculata* was found to be capable of mediating supercoiled pSP64 plasmid DNA relaxation efficiently in the presence of 20 μM Cu^{2+} at 100 μg/mL concentration, but no significant DNA relaxation was observed in the presence of Fe^{2+}, nor in the absence of added metal ion. Accordingly, this crude extract was subjected to bioassay-guided fractionation using a Cu^{2+}-dependent DNA cleavage assay to detect the DNA relaxation activity. 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_4-OH. The 1:1 MeOH−H_2O fraction (41 mg) exhibited the strongest DNA cleavage activity. This fraction was fractionated further on an 8-g C_8 reversed-phase open column. The column was washed successively with 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0 MeOH−H_2O. The 2:8 MeOH−H_2O fraction (19 mg) was found to show the greatest DNA cleavage activity and was fractionated further on a C_{18} reversed-phase open column, which was washed successively with 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0 MeOH−H_2O. The 6:4 MeOH−H_2O fraction (4.0 mg) had the strongest DNA cleaving activity. This fraction was applied to a C_{18} reversed-phase HPLC column for purification, using a linear gradient from 2:18 to 9:11 CH_3CN−H_2O over a period of 60 min at a flow rate of 3.0 mL/min (detection at 265 nm). This HPLC column separation afforded **1** (1.0 mg), **2** (0.4 mg), and **3** (0.3 mg) in that order of elution.

Compound 1: slight blue viscous mass; $[\alpha]^{20}_D$ +8.5° (*c* 0.2, MeOH); ^1H and ^{13}C NMR, see Table 1; CIMS (methane) *m/z* 581 (100) $[2M + 1]^+$, 291 (8) $[M + 1]^+$, 163 (100); HRESI/FTMS *m/z* 581.1648 $[2M + 1]^+$ (calcd for $C_{30}H_{29}O_{12}$, 581.1659).

Compound 2: viscous mass; $[\alpha]^{20}_D$ +6.5° (*c* 0.09, MeOH); ^1H and ^{13}C NMR, see Table 1; CIMS (methane) *m/z* 549 (100) $[2M + 1]^+$, 275 (6) $[M + 1]^+$, 147 (100).

Compound 3: viscous mass; $[\alpha]^{20}_D$ +5.5° (*c* 0.04, MeOH); ^1H and ^{13}C NMR, see Table 1; CIMS (methane) *m/z* 549 (100) $[2M + 1]^+$, 275 (5) $[M + 1]^+$, 147 (80).

DNA Cleavage Assay. Test samples (extract, fractions, or pure compounds) were dissolved in 1:1 DMSO−MeOH; 1 μL of each of the sample was added to a 25 μL (total volume) reaction mixture containing 600 ng of pSP64 plasmid DNA and 20 μM $CuCl_2$ in 10 mM Tris-HCl, pH 7.2. The reactions were incubated at 37 °C for 60 min, terminated by the addition of 5 μL of 0.125% bromophenol blue in 30% glycerol, and applied to a 1.0% agarose gel containing 0.7 μg/mL ethidium bromide. The gel was run in 89 mM Tris with 8.9 mM boric acid and 2.0 mM Na_2-EDTA at 125 V for 2.5 h, then visualized by UV irradiation.

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