Use of COMPARE Analysis to Discover Functional Analogues of Bleomycin

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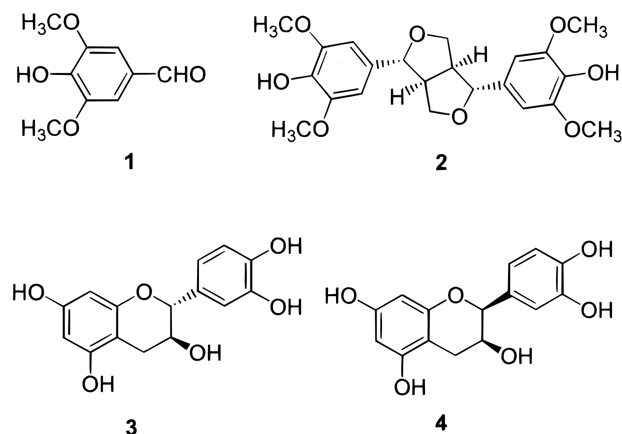
Bleomycin was used as the reference compound in a COMPARE analysis to identify extracts in the National Cancer Institute's Natural Products Repository exhibiting cytotoxicity profiles similar to this antitumor agent. One of the extracts so identified was a CH_2Cl_2−methanol extract prepared from *Gymnosporia trigyna*, which effected relaxation of supercoiled pSP64 DNA in the presence of Cu^{2+}. Bioassay-guided fractionation using DNA strand-scission activity as an end point resulted in the isolation of four active principles. These were identified as syringaldehyde (**1**), (−)-syringaresinol (**2**), (+)-catechin (**3**), and (+)-epicatechin (**4**). Compounds **1** and **2** represent a new type of DNA strand-scission agent.

The search for new anticancer agents from natural sources continues to be a productive strategy for the identification of new clinical candidates.[1] Presently used strategies emphasize mechanism-based approaches involving discrete biochemical or cellular targets.[2]

The antitumor antibiotic bleomycin has been of interest to us for a number of years,[3] due in no small measure to its unique mechanism of action, which is believed to involve the sequence-selective cleavage of DNA, and possibly also of RNA, in a metal-ion- and oxygen-dependent fashion.[4] In an effort to identify structurally novel agents that may work in the same fashion as bleomycin, we have previously focused on the isolation of natural products that mediate DNA strand scission in the presence of metal ions.[5] We, and others, have reported naturally occurring flavanoid,[5,6] aurone,[6d] 5-alkylresorcinol,[5,7] pterocarpan,[8] and biphenyl[9] derivatives that mediate DNA strand scission in the presence of metal ions.

Because not all agents that mediate DNA strand scission in a cell-free assay system will prove to have antitumor activity, or even to exhibit cytotoxicity in mammalian cell culture, we have adopted a modified approach based on a COMPARE analysis[10] of bleomycin with cytotoxic extracts in the NCI Natural Products Repository. Forty-three extracts in the NCI Repository had COMPARE profiles similar to that of bleomycin, and an extract prepared from *Gymnosporia trigyna* (Celastraceae) was found to mediate DNA strand scission in the presence of Cu^{2+} in analogy with bleomycin itself.[11] Presently, we describe the isolation and structure determination of four natural principles (**1**−**4**), all of which are capable of mediating Cu^{2+}-dependent DNA strand scission.



Results and Discussion

The COMPARE algorithm was developed at NCI,[10] and its potential in the drug discovery process has been described.[12] By comparing the mean graph cytotoxicity profile in a 60-cell-line panel[13] for some antitumor agent having a known mechanism of action with those of single compounds not previously characterized, it is possible to identify new agents whose cytotoxicity profiles are similar to those of the "seed", and which, therefore, putatively share the same mechanism of action. This approach has been illustrated at NCI by identifying novel DNA topoisomerase II inhibitors from among a set of synthetic compounds,[12] as well as for the identification of novel agents that target tubulin.[14] However, the present study represents the first report of the use of the COMPARE algorithm to identify functional analogues of bleomycin in natural product extracts.

Analysis of >20 000 extracts in the NCI Natural Products Repository identified 43 having mean graph cytotoxicity profiles similar to bleomycin (i.e., having a correlation coefficient of at least 0.65). The analysis was carried out using a sample of bleomycin tested in June 1997 (experiment 9706MD48-12) in a 48-hour assay at bleomycin test concentrations ranging from 10^{-5} to 10^{-9} M, according to established protocols.[12] The range of correlation coefficients for the 43 extracts was 0.65−0.86. A CH_2Cl_2−methanol extract was found to have a correlation coefficient of 0.705, and was also shown to mediate Cu^{2+}-dependent relaxation of supercoiled pSP64 plasmid DNA when tested at a 100-μg/mL concentration. Accordingly, this extract was chosen for bioassay-guided fractionation to identify the principle(s) responsible for the observed activity.

Initially, the crude extract was applied to a Sephadex LH-20 column, which was washed with hexanes and then successively with solvents of increasing polarity. The acetone and methanol fractions, which showed the strongest DNA cleavage activity at 50 μg/mL, were combined and fractionated further on a C_8 reversed-phase open column using mixtures of methanol and water for elution.

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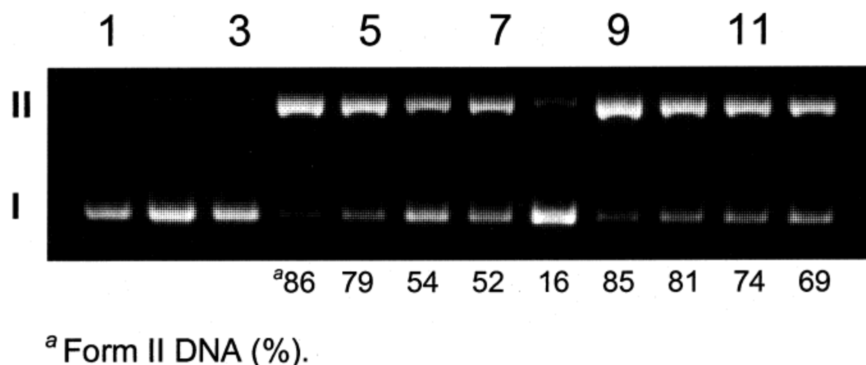

Figure 1. DNA strand scission by compounds **1** and **2**, measured after agarose gel electrophoresis. Lane 1, pSP64 plasmid DNA alone; lane 2, DNA + 20 μM Cu^{2+}; lane 3, 150 μM **1**; lane 4, 150 μM **1** + 20 μM Cu^{2+}; lane 5, 100 μM **1** + 20 μM Cu^{2+}; lane 6, 50 μM **1** + 20 μM Cu^{2+}; lane 7, 10 μM **1** + 20 μM Cu^{2+}; lane 8, 50 μM **2**; lane 9, 50 μM **2** + 20 μM Cu^{2+}; lane 10, 25 μM **2** + 20 μM Cu^{2+}; lane 11, 10 μM **2** + 20 μM Cu^{2+}; lane 12, 1 μM **2** + 20 μM Cu^{2+}. The percent Form II DNA present is shown below each lane.

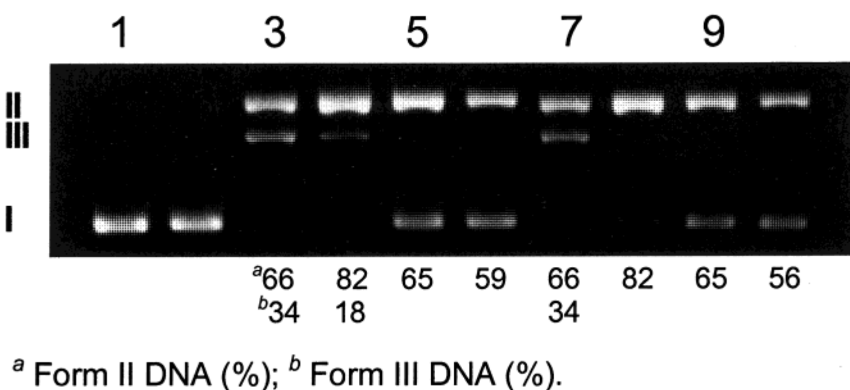

Figure 2. DNA strand scission by compounds **3** and **4**, measured after agarose gel electrophoresis. Lane 1, pSP64 plasmid DNA alone; lane 2, DNA + 20 μM Cu^{2+}; lane 3, 10 μM **3** + 20 μM Cu^{2+}; lane 4, 5 μM **3** + 20 μM Cu^{2}; lane 5, 1 μM **3** + 20 μM Cu^{2+}; lane 6, 0.5 μM **3** + 20 μM Cu^{2+}; lane 7, 10 μM **4** + 20 μM Cu^{2+}; lane 8, 5 μM **4** + 20 μM Cu^{2+}; lane 9, 1 μM **4** + 20 μM Cu^{2+}; lane 10, 0.5 μM **4** + 20 μM Cu^{2+}. The percent Form II (III) DNA present is shown below each lane. No DNA cleavage was observed with **3** or **4** in the absence of Cu^{2+}.

The 1:9 and 2:8 MeOH−H_2O fractions from the C_8 column had the strongest activity and were each applied to a C_{18} reversed-phase HPLC column for further fractionation; these afforded four active fractions having the greatest potency as DNA cleaving agents. Purification of the four active fractions using the same C_{18} reversed-phase HPLC column provided four purified compounds capable of DNA cleavage, denoted **1**, **2**, **3**, and **4**, respectively.

Active principles **1**−**4** were identified as syringaldehyde,[15,16] (−)-syringaresinol,[17] (+)-catechin, and (+)-epicatechin,[18] respectively, by comparison with literature values (mp, $[\alpha]_D$, ^1H and ^{13}C NMR, and MS data). Compounds **1**−**4** were tested for their ability to relax pSP64 plasmid DNA, a supercoiled, covalently closed, circular DNA, in the absence and presence of Cu^{2+}, using a cell-free DNA cleavage assay.[7,19] Dose-dependent relaxation of supercoiled pSP64 plasmid DNA was observed for compounds **1**−**4** in the presence of Cu^{2+} (Figures 1 and 2). In common with the crude extracts from which they were derived, compounds **1**−**4** exhibited little, if any, DNA cleavage in the presence of Fe^{2+} or in the absence of added metal ion; the limited cleavage noted in lane 8 of Figure 1 is attributed to adventitious metal ions present in the assay mixture.

As regards their properties as DNA cleaving agents, benzaldehyde derivative **1** and lignan **2** were found to be reasonably potent DNA cleaving agents; greater than 50% conversion to Form II (nicked) DNA was observed at 10 μM and 1.0 μM concentrations of **1** and **2**, respectively (Figure 1). In other experiments DNA relaxation by these compounds was readily apparent at concentrations of **1** and **2** as low as 2.5 μM and 0.25 μM, respectively, when 20 μM Cu^{2+} was also present (not shown). As is apparent in Figure 1, compounds **1** and **2** effected the conversion of Form I → Form II DNA efficiently, but produced no detectable Form III (linear duplex) DNA under any tested condition. Interestingly, compounds **1** and **2** and certain analogues have been reported to exhibit various biological activities, including antifungal activity,[16] inhibition of adenosine cyclic 3′,5′-monophosphate phosphodiesterase,[15,20] antileukemic and cytotoxic activities,[17c,21] and piscicidal properties.[22] This is the first report of the ability of a benzaldehyde derivative and lignan derivative to effect DNA cleavage.

It is interesting to consider the mechanism of DNA cleavage by compounds **1** and **2**. Previously, we have demonstrated that DNA cleavage by 5-alkylresorcinols involves oxygenation of the aromatic nucleus, affording catecholic moieties that were postulated to coordinate Cu^{2+} and subsequently effect the reduction of dioxygen to reactive species with concomitant oxidation of the catechol moiety via the coordinated Cu^{2+} ion.[23] In support of the generality of this mechanism, it may be noted that a number of other natural products that cleave DNA also contain catechol moieties or analogous functionalities[6,8,9] and that closely related structural analogues lacking this functionality have been found to lack activity as DNA-cleaving agents.[6a,8]

In this context, it is interesting that neither compound **1** nor **2** contains a catechol moiety, nor is it clear how an oxidizable functionality could be formed in situ. In fact, when **1** and **2** were subjected to the conditions[23] employed previously to oxygenate 5-alkylresorcinols, and thereby

activate them for DNA cleavage, no enhancement of cleavage was observed. Thus, it appears that compounds **1** and **2** may mediate DNA cleavage by a novel mechanism.

As reflected in Figure 2, flavans **3** and **4** both mediated DNA cleavage with good efficiency; greater than 50% conversion of Form I to Form II DNA was apparent when **3** or **4** was employed at a 500-nM concentration in the presence of 20 μM Cu^{2+}. Further, when employed at the 10-μM concentration, **3** and **4** produced Form III (linear duplex) DNA, in addition to nicked circular (Form II) DNA, again underscoring the differences in cleavage relative to that achieved with compounds **1** and **2**. It is interesting that (−)-epicatechin has previously been reported to mediate Cu^{2+}-dependent DNA cleavage at concentrations comparable to those noted for **3** and **4**,[6a,24] but without producing Form III DNA. Therefore, the mechanism of DNA cleavage by these agents may involve processes beyond the presumed Cu-catalyzed oxidation of the catechol moieties[23] in **3** and **4**; because the optical antipodes of epicatechin differ in the patterns of cleavage they produce from supercoiled plasmid DNA, it is logical to conclude that differences in spatial orientation are critical to the production of Form III DNA. If this conclusion is correct, it argues for the actual association of **3** and **4** with DNA as part of the process by which Form III DNA is produced, rather than a process that is solely dependent on the diffusion of oxygen free radicals.

As regards the strategy employed here for the identification of extracts putatively containing bleomycin-like species, it may be noted that the 43 extracts identified in the initial comparison were tested in a number of assays intended to monitor polynucleotide damage. The majority of extracts were found to mediate relaxation of supercoiled plasmid DNA in a metal-ion-dependent fashion, in analogy with the results provided in the present report. Virtually all of the 43 extracts had activity in at least one of the assay systems employed. This finding underscores both the validity of the strategy employed here, as well as the need to utilize one or more appropriate assay systems to permit identification of the active principles involved.

Experimental Section

General Experimental Procedures. Sephadex LH-20 (40 μm) was purchased from Sigma Chemicals. Reversed-phase C_8 resin (32−60 μm) was obtained from ICN Biomedicals. A Kromasil C_{18} reversed-phase column (250 × 10 mm, 5 μm) for HPLC was obtained from Higgins Analytical Inc. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are not corrected. Optical rotations were measured on a Perkin-Elmer 243 B polarimeter. ^1H and ^{13}C NMR were recorded on General Electric GN-300 or QE−300 NMR spectrometers. Low-resolution chemical ionization (CI) and electron impact (EI) mass spectra were recorded on a Finnigan MAT4600 mass spectrometer. Supercoiled pSP64 plasmid DNA was a gift from Shelley R. Starck. Agarose gels were quantified for percent DNA cleavage utilizing Molecular Dynamics ImageQuant Version 5.0 software.

Plant Materials. *G. trigyna* was collected in the Malagasy Republic by the Missouri Botanical Garden acting as the NCI Collection Contractor for Africa. A voucher specimen is deposited at both the Missouri Botanical Garden under #Q66V3305 and also in the Museum of Natural History at the Smithsonian Institution under the same number.

Extraction and Fractionation. The dried roots of *G. trigyna* were ground and extracted in an equal volume of CH_2Cl_2−MeOH (1:1) at room temperature overnight. Following draining and a wash with MeOH (ca. 10% column volume), the combined organic fractions were concentrated to dryness on a rotary evaporator (bath temperature <40 °C). The resulting syrup was dried under vacuum overnight at room temperature, yielding 4.81 g of a brown-black residue from 574 g of milled root powder. The CH_2Cl_2−MeOH (1:1) crude extract was found to be capable of mediating pSP64 plasmid DNA relaxation efficiently in the presence of Cu^{2+} at 100 μg/mL concentration, but there was no significant DNA cleavage activity in the presence of Fe^{2+}, nor in the absence of added metal ion. The crude extract was, therefore, chosen for bioassay-guided fractionation, using an assay that detected DNA relaxation activity in the presence of Cu^{2+}. A total of 1.524 g of the crude extract of *G. trigyna* was used for fractionation, and a typical set of experiments is described below. The crude extract (528 mg) was applied to a 20-g Sephadex LH-20 column, which was washed successively with hexanes; hexanes−CH_2Cl_2 (1:1); CH_2Cl_2; CH_2Cl_2−acetone (1:1); acetone; and MeOH. The acetone and methanol fractions (188 mg), which displayed the strongest DNA relaxation activity at 50 μg/mL, were combined and applied to a 15-g C_8 reversed-phase open column. This column was washed with MeOH−H_2O (0:10, 1:9, 2:8, 4:6, 6:4, and finally 10:0). The MeOH−H_2O fractions (1:9, 6 mg; 2:8, 4 mg) had the strongest activity in the DNA relaxation assay. Fractionation of the MeOH−H_2O (1:9) fraction on a C_{18} reversed-phased HPLC column (250 × 10 mm, 5 μm), employing a linear gradient elution with a solvent system ranging from 95% aqueous CH_3CN to 75% aqueous CH_3CN over a period of 30 min at a flow rate of 2.0 mL/min (detection at 235 nm) afforded **1** (0.4 mg) and **2** (1.0 mg). Fractionation of the MeOH−H_2O (2:8) fraction from the C_8 open column using the same HPLC column (elution with 85% aqueous CH_3CN → 75% aqueous CH_3CN over a period of 40 min at a flow rate of 2.0 mL/min, UV detection at 235 nm), provided two fractions having strong DNA relaxation activity in the presence of Cu^{2+}. Purification of these two fractions by HPLC, employing the same conditions described above, afforded **3** (0.3 mg) and **4** (1.2 mg).

Syringaldehyde (1): $C_9H_{10}O_4$; pale yellow needles (MeOH); mp 113−114 °C; EIMS *m/z* 182 $[M]^+$. The mp and ^1H and ^{13}C NMR data for this compound were consistent with those described for syringaldehyde.[15,16]

(−)- Syringaresinol (2): $C_{22}H_{26}O_8$; colorless needles (MeOH); mp 198−199 °C; $[\alpha]^{22}_D$ −7° (*c* 0.05, MeOH); EIMS *m/z* 418 $[M]^+$; structure assigned by direct comparison with mp, $[\alpha]_D$, ^1H and ^{13}C NMR, and MS data reported previously.[17]

(+)-Catechin (3): $C_{15}H_{14}O_6$; $[\alpha]^{22}_D$ +14° (*c* 0.08, acetone); CIMS *m/z* 419 $[M + 1]^+$; structure identified by direct comparison with $[\alpha]_D$, ^1H and ^{13}C NMR, and MS data reported previously.[18]

(+)-Epicatechin (4): $C_{15}H_{14}O_6$; $[\alpha]^{22}_D$ +54° (*c* 0.11, acetone); CIMS *m/z* 419 $[M + 1]^+$; structure identified by direct comparison with $[\alpha]_D$, ^1H and ^{13}C NMR, and MS data reported previously.[18]

DNA Cleavage Assay. Extracts, fractions, or compounds were dissolved in DMSO−MeOH (1:1); 1 μL of each of the samples was added to a 25-μL reaction mixture (total volume) 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 1 h, terminated by addition of 5 μL of 0.125% bromophenol blue in 30% glycerol, and applied to a 1% agarose gel containing 0.7 μg/mL ethidium bromide. The gel was run in 89 mM Tris containing 8.9 mM boric acid and 2.0 mM Na_2−EDTA at 130 V for 3 h, then visualized by UV irradiation.

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