Isolation and Biochemical Characterization of a New Topoisomerase I Inhibitor from *Ocotea leucoxylon*

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In a continuation of our search for potential tumor inhibitors from plants, we found that a crude extract from *Ocotea leucoxylon* showed selective activity typical of inhibitors of the enzyme topoisomerase I in a yeast assay for DNA-damaging agents. Using a bioassay-directed fractionation approach, the major bioactive compound was isolated and identified as the known aporphine alkaloid dicentrinone (**4**); the inactive alkaloid dicentrine (**3**) was also isolated. Compound **4** showed selective bioactivity against the rad52 repair-deficient yeast strain RS322 (IC_{12} 49 μg/mL) and was inactive against the rad52- and topo1-deficient strain RS321 (IC_{12} > 2000 μg/mL) and against the repair-proficient strain RJ03 (IC_{12} > 2000 μg/mL). Biochemical studies with recombinant human topoisomerase I indicated that dicentrinone (**4**) is an inhibitor of the human enzyme. Colony formation studies suggest that it is weakly cytotoxic, but that its mechanism of toxicity differs from that of camptothecin and its derivatives.

The important anticancer natural product camptothecin (**1**) was isolated from *Camptotheca acuminata* by Wall and Wani and their collaborators in 1966.[1] It showed strong activity in a number of assays, but it was very insoluble in water and was thus subjected to clinical testing in the 1970s as the soluble sodium salt of the ring-opened lactone. Unfortunately, this salt proved to have unacceptable toxicity and minimal efficacy at its maximally tolerated dose, and the clinical trials were abandoned. Subsequently, one of us studied the mechanism of action of camptothecin and found that it acts as a potent inhibitor of the enzyme DNA topoisomerase I.[2] Topoisomerase I acts by uncoiling natural supercoiled DNA; its molecular mechanism involves transiently breaking one of the two strands of DNA. Because chromosomal DNA is supercoiled, the importance of topoisomerase I to cell viability is readily evident. The finding that camptothecin is an inhibitor of topoisomerase I, and the fact that it was the first potent inhibitor to be discovered, gave new impetus to its development as an anticancer drug. After extensive studies a team that included two of us succeeded in developing the active water-soluble derivative topotecan (**2**);[3] the water-soluble prodrug irinotecan was also developed by other workers.[4] Both topotecan and irinotecan are currently in clinical use for the treatment of a variety of cancers; they are particularly attractive agents because of their broad spectrum of activity.[5]

The success of camptothecin and its analogues as anticancer agents has spurred a search for additional agents acting by inhibition of topoisomerase I; to date several new classes of inhibitors have been described. Thus, terbenzimidazoles act as topoisomerase I poisons, as do certain indolocarbazoles;[7] natural sources have also provided new topoisomerase I inhibitors.[8] We have been searching for additional new inhibitors for several years,[9] in part by using a yeast-based assay as the primary tool. This assay depends on the fact that yeast strains lacking the gene for the rad52 DNA repair pathway (designated RS322 or *rad52*) are sensitive to agents that damage DNA in a way that would normally be repaired by this pathway. Yeast strains that additionally lack the gene for topoisomerase I (designated RS321 or *rad52.top1*) and thus depend entirely on topoisomerase II for topological changes in their DNA, overproduce topoisomerase II and are hypersensitive to its inhibitors. A compound that selectively inhibits the RS322 strain and not the RS321 strain is therefore a putative inhibitor of yeast topoisomerase I due to the fact that the enzyme is converted into a DNA-damaging agent by interaction with drugs such as camptothecin that selectively inhibit the religation reaction. Despite the extensive homology between eukaryotic topoisomerases I,[10] it has been reported recently by Goldman et al.[11] that human and *Aspergillus* topoisomerases I responded differently to bibenzimidazoles and terbenzimidazoles. Therefore, inhibitors of yeast topoisomerase I also need to be characterized as inhibitors of the human topoisomerase I before proceeding further with their evaluation as potential therapeutic agents. In addition, it is of interest to know whether a novel topoisomerase I inhibitor likely exerts its cytotoxic effect by stabilizing complexes containing strand-cleaved DNA, as does camptothecin. This information can be inferred from a comparison of its cytotoxic potencies in wild type and camptothecin-resistant mammalian cell cultures.[12]

As a part of our systematic search for potential anticancer agents from natural sources, we received a crude extract of *Ocotea leucoxylon* (Sw.) de Lanessan (Lauraceae) from the National Cancer Institute (NCI). The selection of this plant, known as "malde blanco" among the Awá peoples, was made based on ethnobotanically driven selection guidelines. Local Awá community members use this plant for construction because of the decay-resistant properties of the wood. The extremely wet site conditions that exist in these pluvial rainforests of northwest Ecuador have forced the Awá to discover and depend on such decay

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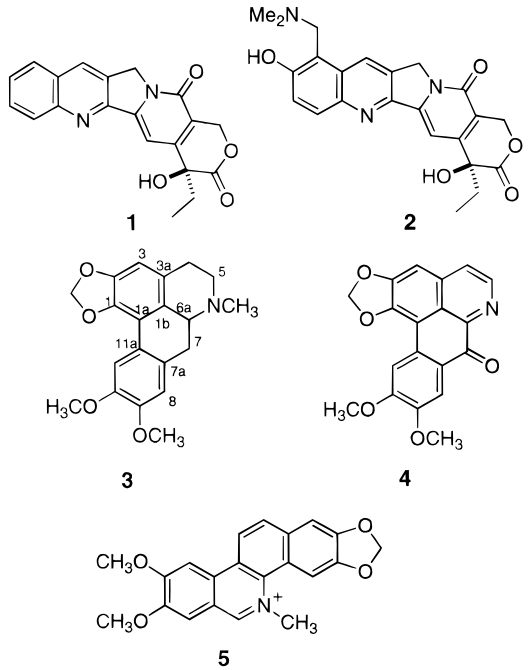
(fungal and termite)-resistant materials for their rural agrarian lifestyles.

The extract of *O. leucoxylon* showed DNA-damaging activity in our yeast assay, and so we undertook the isolation of its bioactive constituent(s) by a bioassay-directed fractionation approach. It has been reported in the literature that *Ocotea* species contain aporphine, isoquinoline, and bisisoquinoline alkaloids.[14] Among these compounds, the aporphine alkaloid dicentrine (**3**) has been reported to have antineoplastic, analgesic, sedative, and antibacteral bioactivities,[15] and ocoteine has been demonstrated to be an antitussive, hypotensive, spasmolytic, and adrenolytic agent in experimental animals.[16] It has also been reported that the aporphine alkaloid leucoxylonine has been obtained from the leaves and stems of *Ocotea leucoxylon*,[14,17] but there are no reports on its biological activity.



Results and Discussion

Chemistry. Bioassay of the extract from *Ocotea leucoxylon* was carried out as described previously[18] in the RS322, RS321, and RAD$^+$ mutant yeast strains; activities in this assay are recorded as IC_{12} values, which are the concentration (in μg/mL) required to give an inhibition zone 12 mm in diameter around a 100-μL well in a 4-mm agar layer plated with the yeast strain. The crude extract gave an IC_{12} value of 880 μg/mL against the RS322 yeast strain and showed minimal activity against RS321 (IC_{12} 10 000 μg/mL) and the repair-proficient yeast RAD$^+$ strains (IC_{12} >8000 μg/mL), a pattern that suggested the presence of an inhibitor of topoisomerase I. After partition between various organic solvents and aqueous MeOH, 2.30 g of the crude extract gave an active CH_2Cl_2 fraction (1.12 g, IC_{12} 310 μg/mL on RS322). After column chromatography on Si gel, eluting with 50:1 CH_2Cl_2−MeOH, fraction 6 (332 mg) showed bioactivity against RS322 with an IC_{12} of 79 μg/mL. Preparative TLC of this fraction on Si gel (50:1 CH_2Cl_2−MeOH) yielded compound **3** (184 mg, inactive in the yeast assay) from the less polar zone and compound **4** (68 mg) from the more polar zone. Compound **4** was active in the RS322 yeast strain (IC_{12} 49 μg/mL), but had little activity in the RS321 (IC_{12} >2000 μg/mL) or RAD$^+$ strains (IC_{12} >2000 μg/mL).

Table 1. ^{1}H and ^{13}C NMR Data for Dicentrinone (δ in ppm, J in Hz) in $CDCl_3$−CD_3OD at 45 °C

position	δ_H	δ_C	HMBC (H to C)	NOESY
1		152.17[a]		
1a		107.52		
1b		122.17		
2		147.58[a]		
3	6.14 (1H, s)	102.48	C-1, C-2, C-4, C-1b	-O-CH_2-O-
3a		136.15		
4	7.54 (1H, d, J = 5.2)	124.28	C-1b, C-3	H-3
5	8.48 (1H, d, J = 5.2)	142.91	C-6a, C-3a, C-4	
6a		143.74		
7		180.36		
7a		124.99[b]		
8	7.58 (1H, s)	109.07	C-10, C-9, C-7a, C-1a	
9		149.38		
10		154.12		
11	7.59 (1H, s)	108.78		
11a		127.71[b]		
9-OCH3	3.74 (3H, s)	55.62	C-9	H-8
10-OCH3	3.81 (3H, s)	55.71	C-10	H-11
−O-CH2-O−	6.14 (2H, s)	102.67	C-1, C-2	

[a,b] Signals carrying the same superscript may be interchanged.

Compound **4** was obtained as fine yellow needles from CH_2Cl_2−EtOH, and its molecular formula was established as $C_{19}H_{13}NO_5$, by HREIMS. Its NMR spectra indicated the presence of two CH_3O− groups [δ_H 3.74 and δ_C 55.62; δ_H 3.81 and δ_C 55.71], one methylenedioxy group [δ_H 6.14 (2H,s) and δ_C 102.67], three isolated aromatic protons [δ_H 6.85 (1H, s) and δ_C 102.48; δ_H 7.58 (1H, s) and δ_C 109.07; δ_H 7.59 (1H, s) and δ_C 108.78], and two *ortho*-coupled aromatic protons [δ_H 7.54 (1H, d, 5.2) and δ_C 124.28; δ_H 8.48 (1H, d, 5.2) and δ_C 142.91]. In addition, one carbonyl group [δ_C 180.36] and 10 quaternary carbons were present in the molecule. These data, together with comparison with data from the literature, established the structure of compound **4** as that of the known aporphine alkaloid dicentrinone.[15c,19] Because its NMR assignments have not previously been reported in the literature, we assigned its ^{1}H and ^{13}C NMR spectra unambiguously by the long-range correlations in its HMBC, HMQC, and NOESY spectra, as shown in Table 1.

Compound **3** was found to be the known alkaloid dicentrine by comparison of its UV and ^{1}H and ^{13}C NMR spectra with the data in the literature for authentic **3**.[15b,c,20]

Biochemical Studies. The ability of dicentrinone (**4**) to stabilize the formation of the topoisomerase I−DNA covalent binary complex was studied using purified recombinant human topoisomerase I as described in the Experimental Section. The known topoisomerase I inhibitor camptothecin (**1**) was employed as a control. The binary complex was converted to nicked, circular (Form II) DNA after enzyme denaturation and degradation with SDS−proteinase K. Densitometric analysis of the agarose gel (Table 2) demonstrated that human topoisomerase I-dependent DNA breakage increased from 22% to 29% in the presence of 100 μM dicentrinone (**4**). Under these conditions, there was no indication that dicentrinone (**4**) alone cleaved DNA. However, camptothecin (**1**), at a lower inhibitory concentration (50 μM), stabilized the topoisomerase I−DNA binary complex to a much greater extent (90% Form II DNA) than dicentrinone (**4**).

The effects of dicentrinone (**4**) on human topoisomerase I-mediated DNA relaxation were examined as well (Figure 1). As reported previously using calf thymus DNA topoisomerase I,[21] nitidine (**5**) completely inhibited the relaxation of supercoiled pBR322 plasmid DNA catalyzed by the human enzyme. In comparison, dicentrinone (**4**) showed no

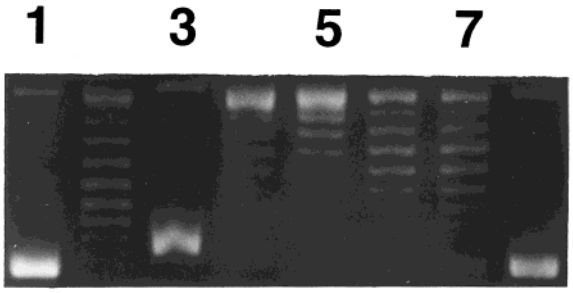


Figure 1. Effects of dicentrinone (**4**) on human DNA topoisomerase I-mediated relaxation of pBR322 plasmid DNA. Lane 1, DNA alone; lane 2, DNA + topoisomerase I; lane 3, DNA + topoisomerase I + 20 μM nitidine (**5**); lanes 4−7, DNA + topoisomerase I + 200, 100, 50, and 20 μM dicentrinone (**4**), respectively; lane 8, DNA + 200 μM dicentrinone (**4**).

Table 2. Stabilization of Human Topoisomerase I−DNA Covalent Complex by Dicentrinone[a]

inhibitor	topoisomerase I	covalent binary complex[b]
none	−	18
none	+	22
camptothecin (**1**)	+	90
dicentrinone (**4**)	−	18
dicentrinone (**4**)	+	29

[a] Cleavage reactions were carried out as described in the Experimental Section. Camptothecin (**1**) was employed at 50 μM final concentration; dicentrinone (**4**) at 100 μM concentration. [b] DNA present in Form II (nicked, circular) as a percentage of all DNA.

inhibition of human topoisomerase I relaxation activity at any inhibitor concentration tested, although there was a topoisomerase I-dependent alteration of the distribution of DNA topoisomers.

The foregoing biochemical studies indicated that dicentrinone (**4**) is a weak inhibitor of human topoisomerase I in vitro, acting through the stabilization of the enzyme−DNA covalent binary complex. To date, there have been a number of compounds reported to inhibit topoisomerase I function in very different fashions.[22] For example, the alkaloid camptothecin stabilized the topoisomerase I−DNA covalent binary complex efficiently but had only a limited effect on DNA relaxation.[2a,23] In comparison, fagaronine inhibited DNA relaxation more effectively than camptothecin, but was not active in a topoisomerase I-induced DNA nicking assay.[21] Likewise, corilagin and chebulagic acid inhibited topoisomerase I function only at the levels of DNA nicking and relaxation.[22d] The fact that dicentrinone substantially inhibited the growth of yeast in a topoisomerase-dependent fashion and yet exhibited negligible effects on DNA relaxation by human DNA topoisomerase I and only weak stabilization of the topoisomerase−DNA covalent binary complex suggests that either topoisomerase I−DNA interaction is not the sole locus of action of this compound or else that the compound is a specific inhibitor of the yeast topoisomerase I. It seems possible that dicentrinone may also affect topoisomerase I function in other ways. The recently noted ability of topoisomerase I to phosphorylate SR proteins involved in splicing[24] may be of interest in this regard. In fact, camptothecin has been noted to inhibit this kinase activity of topoisomerase I.

Biological Studies. To evaluate the mechanistic similarity between dicentrinone and camptothecin in mammalian cells, its toxic potency was determined against wild-type and camptothecin-resistant P-388 mouse leukemia cells using a soft-agar colony-formation experiment.[12] These cells are resistant by virtue of depletion of topoisomerase I mRNA and protein. The small amount of topoisomerase I present retains sensitivity to inhibition by camptothecin. Thus, this cell line should be cross-resistant to all topoisomerase I poisons. IC_{50} values (i.e., the concentration of compound inhibiting colony formation by 50%) for these two cell lines have been found to differ by approximately 3 logs in the case of camptothecin and its analogues. The potency of camptothecin in the wild-type line is in the nanomolar range.[12,25] Thus, a topoisomerase I inhibitor behaving as camptothecin in mammalian cells would be expected to exhibit far more potent toxicity toward wild-type P-388 cells than toward the camptothecin-resistant line. Dicentrinone (**4**), however, was found to be only weakly toxic toward both wild-type and camptothecin-resistant P-388 cells, with no difference in IC_{50} (ca. 100 μM in both cell lines). Three other mammalian cultured cell lines (murine lines B16F10 melanoma and Lewis lung, and human Colo-205 colon tumor-derived cells) were assayed for dicentrinone toxicity by standard growth inhibition protocols[26] with no cytotoxicity seen up to 30 μM. These results are consistent with the diminished potency of dicentrinone relative to camptothecin in the biochemical studies, and with its likely divergence from camptothecin in its mechanism(s) of inhibition of topoisomerase I.

Experimental Section

General Experimental Procedures. Optical rotations were recorded with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded in $CDCl_3$ for dicentrine (**3**) and in $CDCl_3$−CD_3OD at 45 °C for dicentrinone (**4**) on a Varian Unity 400 NMR instrument at 399.951 MHz for ^1H and 100.578 MHz for ^{13}C, using standard Varian pulse sequences programs. UV spectra were measured on a Shimadzu UV 1201 UV spectrophotometer.

Biological Assays. The yeast bioassay was carried out by determining growth inhibition against *Saccharomyces cerevisiae* RS322 (*rad52*), RS321 (*rad52.top1*), and RAD^+ engineered yeast strains. The yeast was grown on agar plates containing Yeast Morphology Agar (YMA, Difco) with a soft agar overlay of Difco-Bacto agar seeded individually with RS322, RS321, and RAD^+ yeast strains. Samples were dissolved in 100 μL of 1:1 DMSO−MeOH and placed in 7-mm wells cut in the agar. The plates were incubated at 28 °C for 48 h, and the zones of inhibition were measured in millimeters. Activity was determined from a dose−response curve, and reported as an IC_{12} value, which is the dose (in μg/mL) required to produce a zone of inhibition 12 mm in diameter. Camptothecin at 2 μg/mL, streptonigrin at 4 μg/mL, and camptothecin at 50 μg/mL were used as positive controls for RS322, RS321, and RJ03(RAD^+), respectively. Standard soft-agar colony-formation assays, XTT growth-inhibition assays, and routine cell cultures were performed as described in previous publications.[12,26] Camptothecin and dicentrinone were administered to cells as 10-mM stock solutions in dimethyl sulfoxide (DMSO).

Recombinant Human Topoisomerase I-Mediated DNA Cleavage and Relaxation. Recombinant human DNA topoisomerase I was expressed in a baculovirus system and purified by FPLC heparin chromatography as described.[27] Topoisomerase I-mediated DNA cleavage was assayed in 20

μL (total volume) of 40 mM Tris-HCl, pH 7.5, containing 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 30 μg/mL of bovine serum albumin, 250 ng of supercoiled pBR322 plasmid DNA (New England Biolabs), and 36 ng of human topoisomerase I in the presence or absence of potential inhibitors (final concentration as indicated in Table 2). The reaction mixture was incubated at 37 °C for 60 min and then terminated by proteinase K treatment (1 mg/mL having 1% SDS, 37 °C, 60 min). The reaction mixture was analyzed on 1% agarose gel containing 0.6 μg/mL of ethidium bromide. The amount of Form II (nicked, circular) DNA was quantified by using a Molecular Dynamics densitometer.

The assay for human topoisomerase I-mediated relaxation of supercoiled plasmid DNA was adapted from Wang et al.[21] The incubation mixtures (20 μL total volume) contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 50 μg/mL bovine serum albumin, 250 ng of supercoiled pBR322 plasmid DNA (New England Biolabs), and 1.8 ng of human topoisomerase I. The final concentrations of potential inhibitors included were varied from 20 μM to 200 μM. Reactions were carried out at 37 °C for 30 min and then quenched by the addition of 5 μL of a gel loading solution containing 2.5% SDS, 30% glycerol, and 0.125% bromophenol blue. The reaction mixtures were resolved by electrophoresis on 1% agarose gels and stained with 0.5 μg/mL ethidium bromide solution.

Plant Material. The plant material of *Ocotea leucoxylon* (Sw.) de Lanessan (Lauraceae) was collected on 19 November 1995 in Ecuador, Carchi Province, Awá Indigenous Forest Territory, community of Gualpi Alto, in very humid premontane primary forest, altitude 825 m, 01° 01′ N, 78° 18′ W. Herbarium vouchers are deposited at The New York Botanical Gardens and at QCNE in Ecuador. Extraction of *O. leucoxylon* was carried out at the NCI by soaking the plant material in MeOH.

Isolation of Dicentrine (3) and Dicentrinone (4). The crude extract N094109 (2.3 g, IC_{12} 880 μg/mL in the RS322 assay, 8000 μg/mL in the RS321 assay, and >10 000 μg/mL in the RAD^+ assay) was partitioned between *n*-hexane and 60% aqueous MeOH. The aqueous MeOH fraction was then diluted to 50% aqueous MeOH and partitioned with CH_2Cl_2 to give a bioactive CH_2Cl_2 fraction (1.12 g 49.7%, IC_{12} 310 μg/mL against RS322, 5400 μg/mL against RS321, and >5500 μg/mL against RAD^+). The CH_2Cl_2 fraction was then subjected to column chromatography on Si gel with elution by 50:1 CH_2Cl_2−MeOH, to give 10 fractions, after combination of similar fractions as judged by TLC. Bioactivity was detected in fraction 6 (332 mg, IC_{12} 79 μg/mL against RS322). Preparative TLC of fraction 6 on Si gel (50:1 CH_2Cl_2−MeOH) yielded compound **3** (184 mg, inactive in the yeast assay) from a less polar zone and compound **4** (68 mg) from a more polar zone. Compound **4** was active against the RS322 yeast strains with IC_{12} 49 μg/mL, but was essentially inactive against RS321 (IC_{12} > 2000 μg/mL) and RAD^+ (IC_{12} > 2000 μg/mL).

Compound 3: amorphous powder; $[\alpha]^{25}_D$ +60.7° (*c* 0.43, CHCl3); UV λ_{max} (MeOH, log ϵ) 221 (4.40), 281 (4.07), and 306 (4.08); ^{1}H NMR ($CDCl_3$) δ 7.66 (1H, s, H-11), 6.51 (1H, s, H-3), 5.92 and 6.07 (1H each, d, J = 1.5 Hz), 4.77 (1H, s, H-8), 3.92 (3H, s, $-OCH_3$), 3.91 (3H, s, $-OCH_3$), 3.19 (1H, dd, J = 14.3, 4.42 Hz), 3.10 (1H, m), 3.08 (1H, m), 2.66 (1H, dd, J = 14.3, 14.3 Hz), 2.63 (2H, m), 2.56 (3H, s, $-N-CH_3$), 2.54 (1H, 1H, dd, J = 11.5, 11.9 Hz); ^{13}C NMR ($CDCl_3$) δ 28.98, 34.06, 43.67, 53.47, 55.86, 56.08, 62.31, 100.63, 106.75, 110.45, 111.21, 116.59, 123.47, 126.30, 126.49, 128.19, 141.80, 146.66, 147.66, 148.23; these values are the same as those reported for dicentrine;[8c] HREIMS *m/z* 339.1463; calcd for $C_{20}H_{21}NO_4$, 339.1470.

Compound 4: fine yellow needles from CH_2Cl_2−EtOH, mp 300 °C (dec); UV (EtOH) λ_{max} (log ϵ) 213 (4.59), 254 (4.75), 271 (4.47), 311 (4.00), 349 (4.04), 400 (3.97); EIMS *m/z*: 335 (M^+), 276, 261, 246, 231, 218, 191, 163, 69(100%); ^{1}H and ^{13}C NMR, HMBC, and NOESY data, see Table 1; HREIMS *m/z* 335.0794; calcd for $C_{19}H_{13}NO_5$, 335.0794.

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