Novel (S)-(-)- and R-(+)-*seco*-iso-cyclopropylfurano[*e*]indoline-5,6,7-trimethoxyindole-2-carboxamide (iso-CFI) Analogs of Duocarmycin C2: Synthesis and Biological Evaluation

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Abstract: Racemic *seco*-iso-CFI (cyclopropylfurano[*e*]indoline) analogs of the duocarmycins and CC-1065 have recently been reported by our group. These compounds covalently react with AT-rich sequences of DNA, and they exhibit potent cytotoxicity against cancer cells but are less toxic to normal bone marrow cells. This article details the synthesis of enantiomerically pure (S)-(-)- and R-(+)-*seco*-iso-CFI (cyclopropylfurano[*e*]indoline)-5,6,7-trimethoxyindole-2-carboxamide analogs, (S)-(-)-**1** and (R)-(+)-**1**, respectively. The covalent DNA binding properties and cytotoxicity of both enantiomers against L1210 murine leukemia and B16 murine melanoma cells grown in culture are reported and compared to racemate (±)-**1**. The natural (S)-(-)-enantiomer of **1** is more reactive with DNA and more cytotoxic than its unnatural mirror image and the racemic mixture.

Key Words: Duocarmycins, CC1065, DNA alkylation, cytotoxicity, sequence specificity.

INTRODUCTION

(+)-CC1065 [1] and the duocarmycins, such as (+)-duocarmycin A [2], (+)-duocarmycin SA [3], and (+)-duocarmycin C2 [4], Fig. (**1**), are natural occurring compounds isolated from *Streptomyces sp*. Through their ability to covalently react with purine-N3 atoms within the minor groove at AT-rich sequences of DNA, such as 5'-AAAAA and 5'-PuNTTA [5], these agents possess extremely potent cytotoxicity against the growth of cancer cells in culture. Their IC_{50} values following 3-4 day drug exposure are within the range of 10-40 pM [1-4]. Cells treated with CC-1065 were induced to undergo apoptosis, leading to cell death [6].

Even though the naturally occurring compounds are highly active in killing cancer cells, their development into clinical agents have been hampered by their severe toxicity, particularly to the bone marrow and liver [7]. As a result, there has been significant interest in the design of novel analogs with favorable biological profiles. Several compounds have been designed and assessed in the clinics for cancer treatment. Three compounds, adozelesin [8], carzelesin [9], and KW2189 [10], have not proceeded past phase II clinical studies due to toxicity to the bone marrow. Currently only bizelesin, which produces DNA interstrand cross links, remains in phase II clinical trials [11]. Concurrent with the development of these compounds, a significant effort was underway to establish a structure-activity relationship for this class of compounds. It has been demonstrated that the cytotoxicity exhibited by analogs of CC-1065 and the duocarmycins is directly related to their stability in aqueous cell culture media [1,2]. Analogs, such as cyclopropylbenzo[*e*]indolone (CBI, **2**) [2,12], cyclopropylpyrazolo[*e*]indolone (CPzI, **3**) [13], and cyclopropylfurano[*e*]indolone (CFI, **4**) [14], Fig. **2**, have long half-lives in aqueous solutions and display potent cytoxicities.

As part of a program focused on the design of novel duocarmycin analogs that might have reduced undesired toxicity, whilst maintaining the potent anticancer activity, our laboratory has recently reported a class of duocarmycins analogs that contain the *seco*-iso-cyclopropylfurano[*e*] indolone (iso-CFI, **1**) DNA reactive subunit (Fig. (**2**)) [15]. The *seco*-iso-CFI functionality eliminates HCl to produce the cyclopropane-containing iso-CFI analog, which ultimately reacts with DNA. The iso-CFI analog was designed on the premise that the furano[*e*] moiety stabilizes the cyclopropylcyclohexadienone moiety through resonance effects from the non-bonded electrons of the furan-oxygen atom. Results from solvolysis studies on iso-CFI and CBI compounds showed the former to be more stable in a methanol solution at pH of 3, with half lives of 410 hours versus 235 hours, respectively [15]. The racemic *seco*-iso-CFI-TMI, (±)-**1**, was found to have significant cytotoxicity against the growth of cancer cells in culture, with IC_{50} value against L1210 cells following three-day continuous exposure of 15 nM [15]. Moreover, racemic (±)-**1** demonstrated similar covalent sequence selectivity for the 5'-AAAAA site to adozelesin, and it displayed low toxicity against murine bone marrow cells at a dose that was extremely toxic for *seco*-CBI-TMI **2**. With such interesting biochemical and biological properties of (±)-**1**, our laboratory has undertaken a project to prepare both enantiomers of compound **1**, and to assess their DNA and cytotoxicity.

RESULTS AND DISCUSSION

The synthesis of both enantiomers of *seco*-iso-CFI-TMI **1** is depicted in Scheme (**1**), and it followed the same strategy

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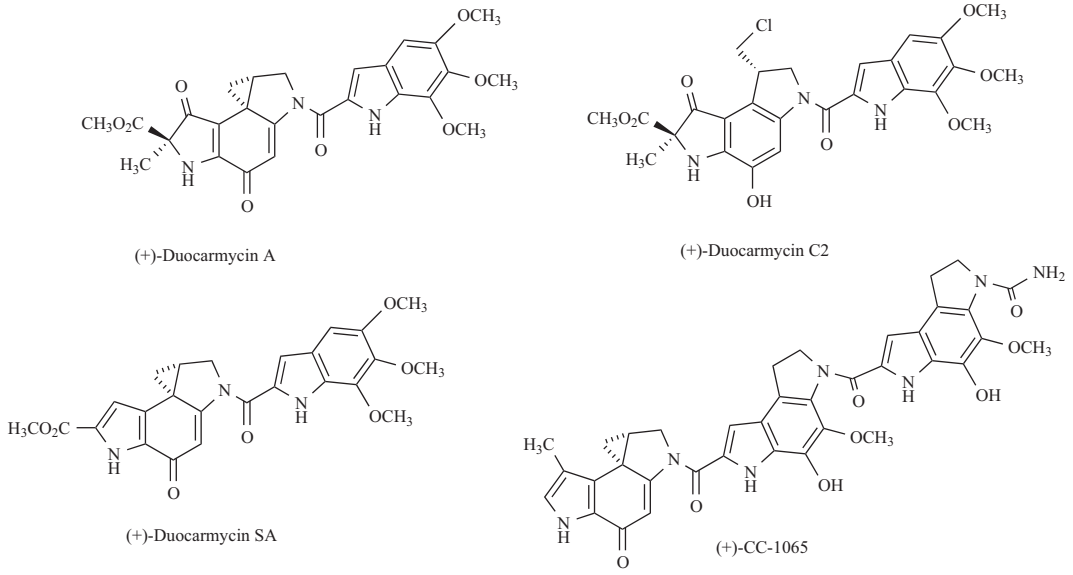


Fig. (1). Structures of (+)-duocarmycin A, (+)-duocarmycin SA, (+)-duocarmycin C2, (+)-CC-1065.

that was used in the reported synthesis of the racemic mixture of compound **1** [15]. The racemic mixture of O-benzyl and N-BOC protected *seco*-iso-CFI intermediate **5** was produced from free radical promoted cyclization of a mixture of E and Z-alkenes **6** in about 35% yield. Agent **5** was resolved by HPLC, using a chiral phase column (Diacel Chiralcel OD) and a procedure previously reported for the resolution of O-Bn-N-BOC-*seco*-CCBI [16] and O-Bn-N-BOC-*seco*-CFI [14]. As depicted in Fig. (**3**), both enantiomers of intermediate **5** were effectively resolved, using a 7% isopropanol/hexane solvent and a flow rate of 1 mL/min. The faster eluting enantiomer (retention time of 20.3 min) that gave a specific rotation of +14.8° was assigned the (R)-enantiomer, while the slower eluting isomer (retention time of 22.3 min) which gave a specific rotation of -14.6° was assigned the (S)-isomer. As demonstrated in Fig. (**3**), both enantiomers of compound **5** were isolated in high purity (>95%). Assignment of the absolute configuration of the chiral intermediates of compound **5** was made by comparing their specific rotations and their retention times to the previously reported compounds, O-Bn-N-BOC-*seco*-CCBI [16] and O-Bn-N-BOC-*seco*-CFI [14a]. For example, using an identical HPLC protocol, (R)-(+)-O-Bn-N-BOC-CCBI eluted earlier at 20.7 min compared to its (S)-(-)-mirror image, which eluted more slowly at 28.5 min [16]. Moreover, the (R)-enantiomer of O-Bn-N-BOC-*seco*-CFI gave a positive optical rotation, while the (S)-enantiomer produced a negative specific rotation [14a].

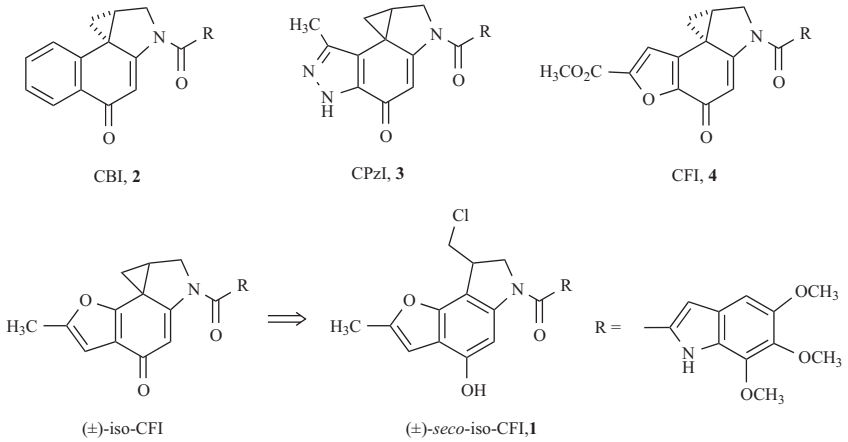
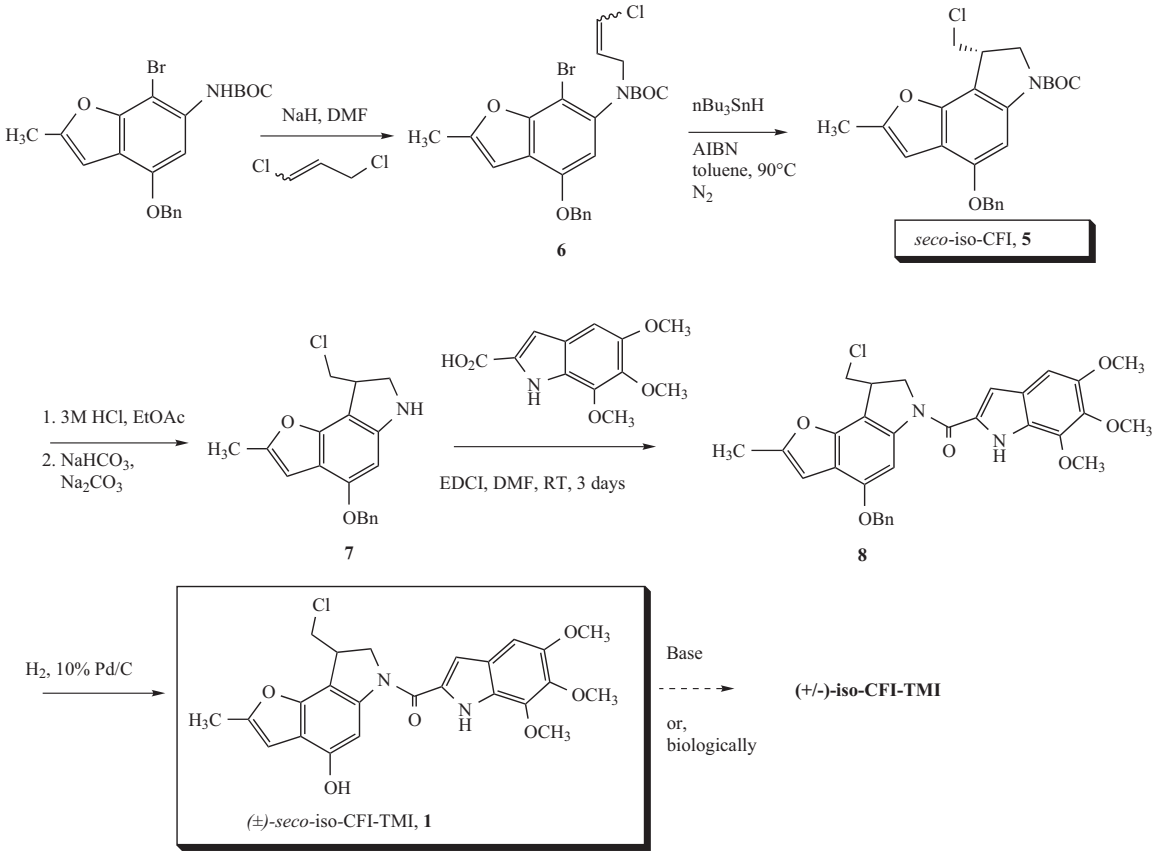


Fig. (2). Examples of known DNA alkylating subunits of the duocarmycins and CC-1065. CBI = cyclopropylbenzo[*e*]indolone, CPzI = cyclopropylpyrazolo[*e*]indolone, CFI = cyclopropylfurano[*e*]indolone, iso-CFI = iso-cyclopropylfurano[*e*]indolone, and R = TMI = 5,6,7-trimethoxyindole-2-carboxylate.



Scheme (1). Synthesis of (±)-*seco*-iso-CFI-TMI (**1**).

Following the synthetic strategy outlined in Scheme (**2**), both enantiomers of intermediate **5** were converted to the respective *seco*-iso-CFI-TMI products. The BOC protecting group was removed by treatment of compound **5** with 3M hydrochloric acid in ethyl acetate, and the resulting amine **7** was coupled to 5,6,7-trimethoxyindole-2-carboxylic acid in presence of EDCI. The (R)-and (S)-O-benzyl-*seco*-iso-CFI-TMI intermediates **8** were isolated in 77 and 66% yields, respectively. The 500 Hz ^{1}H-NMR and FT-IR data for these compounds were identical to those previously reported for the racemic compound [15]. The benzyl protecting was removed by catalytic hydrogenation over 10% palladium-on-carbon to produce the desired optically active product *seco*-iso-CFI-TMI (+)-(R)-**1** and (-)-(S)-**1**. The (R)-enantiomer was found to have a positive specific rotation (+18°), and the (S)-enantiomer gave a negative specific rotation (-19°). Reverse Phase HPLC analysis of (+)-(R)-**1** and (-)-(S)-**1** using a Zorbax eclipse XDB-CA column, using a 3/1 acetonitrile/water solvent) confirmed their homogeneity. Both enantiomers gave identical 500 Hz ^{1}H-NMR and FT-IR data, as well as HPLC retention times, to the data reported for racemic (±)-**1** [15]. The absolute configuration for the target compounds was verified by comparing the optical rotations of compound **1** to those for *seco*-CCBI-TMI [(+)-(R); (-)-(S)-] [16] and *seco*-CFI-TMI [(+)-(R); (-)-(S)] (Fig. (**4**)) [14b].

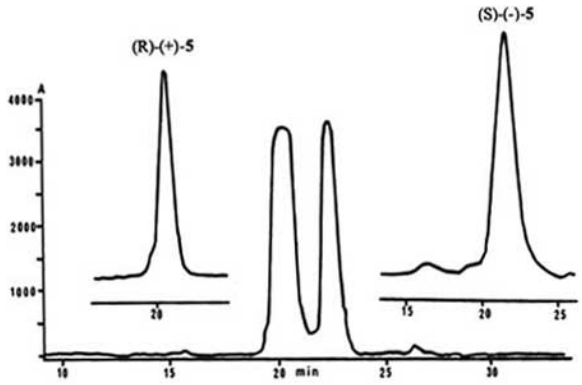
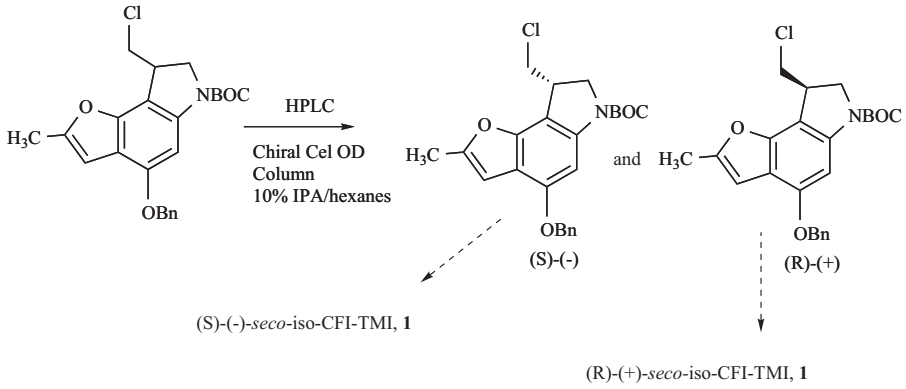


Fig. (3). HPLC chromatogram of compound **5**. The analysis was performed on a Diacel Chiralcel OD column, using a 10% isopropanol/hexanes solvent, and a flow rate of 1 mL/min.

The ability of both enantiomers of compound **1** and its racemic mixture to covalently interact with DNA was studied using a thermal induced DNA cleavage experiment [17]. In this study the thermally unstable covalent purine-N3 adducts were converted to strand breaks upon heating. Treatment of supercoiled pBR322 DNA with 6.6 μM of the



Scheme (2). Resolution and synthesis of both enantiomers of *seco*-iso-CFI-TMI (**1**).

compounds for 40 hours at 37 °C produced a significant degree of single strand breaks, as evidenced by the formation of Form II DNA. The results from this experiment are depicted in Fig. (**5**). While the control untreated pBR322 DNA spontaneously produced 58±3% of Form II DNA, the natural enantiomer (S)-(-)-**1** generated 87±3%, (R)-(+)-**1** produced 74±3%, and the racemic (±)-**1** gave 83±3% Form II DNA, respectively. These results are consistent with covalent reaction of compound **1** with purine-N3 groups in the minor groove of the DNA.

The thermally induced DNA sequence selective strand break assay is commonly used for determining the covalent sequence selectivity of compounds provided that the alkylation occurs with purine N3 atoms in the minor groove [1,18]. For this study, the probe sequence was obtained from

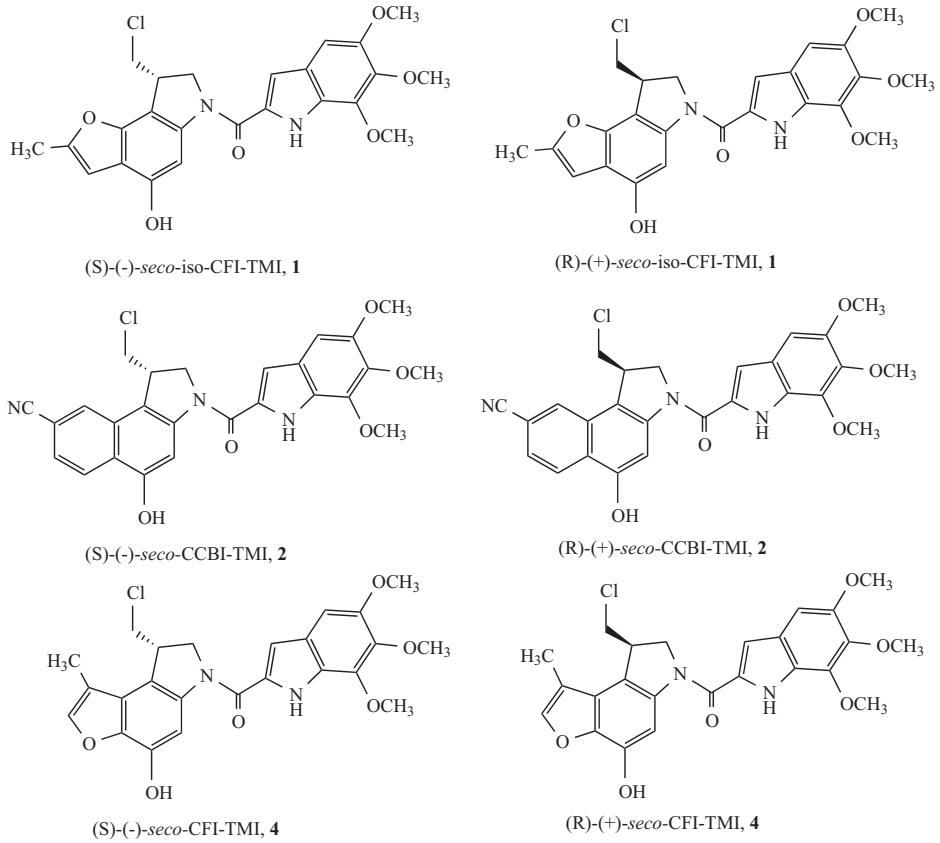


Fig. (4). Structure and absolute configurations of *seco*-iso-CFI-TMI **1**, *seco*-CCBI-TMI **2**, and *seco*-CFI-TMI **4**.

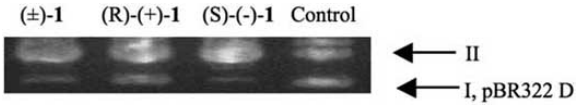


Fig. (5). Agarose gel depicting DNA (pBR322) strand breaks induced by compound **1**. The compound/DNA samples were incubated at 37 °C for 40 h. Form I is supercoiled and Form II is open circular DNA.

PCR amplification of base pairs 749-956 of plasmid pUC18 that was linearized with *Hind III*. In addition to a singly radiolabeled primer 5'-^{32}P-CTCACTCAAAGGCGGTAA TAC-3' primer 1, a second primer 2 (CTGTCGGGTTT) fragment was used as a forward primer so that the final DNA fragment was singly end labeled. Results from the thermal cleavage assay for both enantiomers and racemate **1** is shown in Fig. (**6**). The results confirmed the reaction of these agents with the N3 position of adenine residues, and they gave similar sequence selectivity for the sequence 3'-AAAAA-5' at position 865. The highest reactivity was observed for the natural (S)-(-) enantiomer, followed by the raemic mixture (±)-**1**, and the unnatural (R)-(+) enantiomer gave the weakest level of interactions. The racemic and (S)- enantiomer also recognized secondary sites, for example, at A764 and A772 within AT-rich sequences. These results are in agreement with those previously reported for racemic (±)-**1**, and are consistent with the an initial loss of HCl to generate an identical iso-CFI-TMI compound, followed by DNA alkylation. Moreover, the findings in this study agree with earlier reports that the natural (S)-enantiomers of the duocarmycins and CC-1065 are normally interact with DNA more effective with DNA than their unnatural (R)-counter-parts, including (S)-(-)*seco*-CBI-TMI [16] and (S)-(-)-*seco*-CFI-TMI [14b].

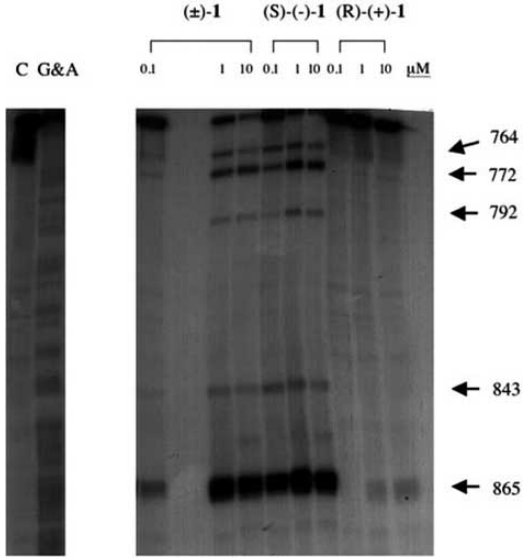


Fig. (6). Thermal cleavage gel showing purine-N3 lesions on the bottom strand of the mixed AT/GC region. C=control; G&A=purine line; (±)-**1**=0.1, 1 and 10 μM; (S)-(-)-**1**=0.1, 1 and 10 μM; (R)-(+)-**1**= 0.1,1 and 10 μM.

Although it is important to characterize the interaction of potential anticancer agents with their DNA target, our goal is to design compounds, which are capable of inhibiting the growth of tumor cells. Using an MTT based growth inhibition assay, the IC_{50} values for racemic (±)-**1**, and both enantiomers of compound **1** against murine L1210 leukemia and murine B16 melanoma cells were determined. The cells were continuously treated with the compounds for three days [15,19]. The racemic *seco*-CBI-TMI (racemic 2) was used as a reference compound for the cytotoxicity studies. The results given in Table (**1**) demonstrate that, among the iso-CFI compounds, the (S)-(-) enantiomer of compound **1** produced the highest cytotoxicity (IC_{50} in the nM range), followed by the racemic compound (about 10 fold less potent), and then the (R)-(+)-enantiomer, which was another 10-fold less active. The cytotoxicity of (S)-(-)-**1** was comparable to that for the highly active compound (±)-*seco*-CBI-TMI; however, the latter compound was highly toxic to murine bone marrow cells [15]. These results are consistent with the observed order of the reactivity with DNA, (S)-(-)-**1** > (±)-**1** > (R)-(+)-**1**, thereby providing indication that these agents derive their activity from covalent reaction with DNA. Analogs of the duocarmycins and CC-1065 including the racemic compound **1** [15], promote cancer cells to undergo apoptosis [6].

In conclusion, the *seco*-iso-CFI DNA alkylation subunit has been confirmed to possess potent cytotoxicity and DNA reactivity. The results further affirm that the natural (S)-(-) enantiomer of compound **1** has higher reactivity with purine-N3 in the minor groove, and it is significantly more cytotoxicity against cancerous cells than its enantiomer and the racemate. Current studies in our laboratory are focused on assessing the *in-vivo* anticancer activity and toxicity of the (S)-(-)-**1**, and the results will be reported in due course.

METHODS AND MATERIALS

Melting points were determined on a Mel-Temp apparatus and were uncorrected. The ^{1}H- NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer. The appropriate deuterated solvents are indicated in the procedure, with TMS as the internal standard and line positions are recorded in ppm from the reference signal. Infared spectra were recorded on a Perkin Elmer Paragon 500 FT-spectrophotometer and only the principle sharply defined bands were reported in cm^{-1}. The optical rotation measurements were performed on a DigiPol 781 automatic polarimeter. Mass spectra and accurate mass measurements were recorded at the University of South Carolina. Elemental analyses were performed at Midwest Microlabs, Indiana.

Commercial grade solvents and reagents were used without further purification with the following exceptions: triethylamine, acetonitrile, acetic anhydride, formic acid, $CDCl_3$, pyridine, methanol, and deuterated DMSO-d_6 were dried over molecular sieves 3Å. THF was dried by distillation over sodium and benzophenone. DMSO was distilled over NaOH under vacuum and stored over molecular sieves 3Å. DMF was distilled over BaO under vacuum and stored over molecular sieves 3Å.

Table 1. Cytotoxicity of the Racemic Mixture and Enantiomers of Compound 1. The IC_{50} Values, M, were Determined Following a Three Day Continuous Exposure of the Cells to the Compounds

Compound	L1210	B16
(±)-**1**	1.5×10^{-8}	1.5×10^{-8}
(S)-(-)-**1** (natural)	6.3×10^{-9}	9.1×10^{-9}
(R)-(+)-**1**	1.1×10^{-7}	4.1×10^{-7}
(±)-*seco*-CBI-TMI	8.4×10^{-9}	7.6×10^{-8}

3-(Chloromethyl)-6-hydroxy-N-(5,6,7-trimethoxyindole-2-carbonyl)-(5 methylfurano)[e]indoline, (±)-1 [15]

The racemic compound **1** was prepared following a procedure that was previously reported. It was isolated as a white powder (0.136 g, 0.289 mmol, 99% in the last hydrogenation step). TLC (5% MeOH/$CHCl_3$) R_f = 0.50. M.p. 263-267 °C. IR (Neat) 3433, 3100, 1963, 1927, 1634, 1586, 1524, 1488, 1457, 1391, 1311, 1262, 1220, 1195, 160, 1107, 1049, 800, 668. ^{1}H-NMR ($CDCl_3$) 9.45 (s, 1H), 9.42 (s, 1H), 7.82 (s, 1H), 7.64 (s, 1H), 6.98 (d, 2.0, 1H), 6.89 (s, 1H), 6.46 (d, 2.0, 1H), 4.75 (t, 10.0, 1H), 4.59 (dd, 4.5, 10.0, 1H), 4.19 (dd, 3.5, 11.0, 1H), 4.11 (s, 3H), 3.96 (s, 3H), 3.93 (s, 3H), 3.64 (t, 10.0, 1H), 2.46 (s, 3H). FAB-MS (NBA) (m/z rel. intensity) 471 (M+H, 4). Accurate mass (FAB-NBA) for $C_{24}H_{24}N_2O_6{}^{35}Cl$: calcd. 471.1323, obsd. 471.1307.

HPLC Resolution of O-Bn-N-BOC-seco-iso-CFI Intermediate 5

The racemic mixture of compound **5** (100 mg) was dissolved in acetonitrile (1 mL) and IPA (0.2 mL). A Diacel chiral OD column (10 µM, 2 x 25 cm) was used for the separation. The samples were injected at a volume of 100 µL, the solvent was 10% isopropanol/hexanes, and the flow rate of 1mL / min. The (R)-(+) enantiomer eluted at 20.3 min and was collected from multiple runs, and was concentrated to a give a white solid (31.4 mg). The (S)-(-)-enantiomer eluted at 22.3 min and was collected and concentrated as a white solid (31.9 mg). The 500 MHz ^{1}H-NMR and FT-IR spectra of these compounds were identical to those of the published racemic mixture [15]. The specific rotation of (R)-(+)-**5** was +14.75° (sodium D-line, room temperature, $CHCl_3$, c=0.0314 g/ 5 mL), and the specific rotation for the (S)-(-) enantiomer was14.58°($CHCl_3$, c=0.0319 g/ 5 mL), respectively. The purified enantiomers were reexamined on the Chiralcel OD column and were found to be enantiomerically homogeneous.

(S)-(-)-3-(Chloromethyl)-6-hydroxy-N-(5,6,7-trimethoxy-indole-2-carbonyl)-(5 methylfurano)[e]indoline, (S)-(-)-1

A sample of (S)-(-)-**5** (0.032 g, 0.077 mmol) was dissolved in dry ethyl acetate (0.75 mL) and kept under nitrogen. A solution of 3M HCl-ethyl acetate (0.75 mL) was added and the solution was stirred at room temperature for 1 h, at which time another aliquot of 3M HCl-EtOAc (0.75 mL) was added and the solution was stirred for 1.5 h at room temperature. Ethyl acetate (50 mL) was added and the solution was neutralized with 2.5 M Na_2CO_3 (3.5 mL). The ethyl acetate layer was collected and dried over sodium sulfate, filtered, concentrated to give amine **7** as an oily residue.

The crude amine **7** was combined with EDCI (0.059 g, 0.31 mmol) and 5,6,7-trimethoxyindole-2-carboxylic acid (0.031 g, 0.12 mmol) and dissolved in dry DMF (1 mL). The reaction mixture was kept under a nitrogen atmosphere and was stirred for 4 days at room temperature. The DMF was removed using a kugelrohr apparatus (0.1 mm Hg, 50 °C), and the residue was purified using silica gel column chromatography ($CHCl_3$). The desired fractions were collected and concentrated to give a white solid (S)-**8** (0.037 g, 66%). The 500 MHz ^{1}H-NMR spectrum of this compound was identical to that of the published racemic form [15].

The (S)-**8** intermediate (0.037 g, 0.067 mmol) was combined with 10% Pd/C (0.037 g) and suspended in chilled THF (10 mL). The mixture was purged with a vacuum and hydrogen (3 times) and the suspension was stirred overnight. The catalyst was removed from the reaction mixture by filtration over celite and the residue was washed with ethyl acetate. Concentration of the filtrate gave a residue, which was purified by silica gel column chromatography (0.5% MeOH/$CHCl_3$). The desired fractions were collected and concentrated to give (S)-(-)-**1** (0.018g, 62%) as an off-white solid. A 500 MHz ^{1}H-NMR spectrum of this compound was identical to that of the previously published racemic form [15]. The sample was confirmed to be homogeneous by HPLC analysis (reversed phase column Zorbax eclipse XDB-CA 4.6 mm X 15 cm), using a 3/1 acetonitrile/ water solvent. The flow rate was 2 mL/min and a 10 µL injection was made (retention time was 1.42 min). Specific rotation of (S)-(-)-*seco*-iso-CFI-TMI **1** was -19° ($CHCl_3$, c=0.0183 g/ 5 mL).

(R)-(+)-3-(Chloromethyl)-6-hydroxy-N-(5,6,7-trimethoxy-indole-2-carbonyl)-(5 methylfurano)[e]indoline, (R)-(+)-1

The (R)-(+)-**1** compound was prepared using a similar procedure as the synthesis of (S)-(-)-**1**, except (R)-(+)-**5** was used as starting material. (R)-(+)-**1** was isolated as an off-white powder (0.012g, 44%). A 500 MHz ^{1}H-NMR spectrum was identical to the racemic form of compound **1**. HPLC analysis confirmed its homogeniety (retention time was 1.44 min). Specific rotation of (R)-(+)-*seco*-iso-CFI-TMI **1** was +18° ($CHCl_3$, c=0.0118 g/ 5mL).

Cytotoxicity Studies

The racemic (±)-**1**, both enantiomers of **1**, and the stock racemic *seco*-CBI-TMI compounds were dissolved in

DMSO to obtain 1.75 x 10^{-2} M stock solutions. These solutions were diluted with DMSO to prepare standard solutions from concentrations of 1.75 x 10^{-3} M to 1.75 x 10^{-10} M. These solutions were then further diluted with DMEM (24 µL standard solution in 176 µL media). Addition of these solutions (5 µL) to wells containing 100 µL media/cell suspension resulted in final compound concentrations ranging from 1.0 x 10^{-12} M to 1.0 x 10^{-4} M.

The murine leukemia L1210 cells were obtained from American Type Tissue Culture Collection (ATCC). The murine melanoma B16-F_0 cell line was obtained from the Cancer Center of the Greenville Hospital System. The cell lines were grown in Delbecco's Modified Eagle Medium (DMEM, Atlanta Biochemicals) supplemented with 10% fetal bovine serum, Hepes Buffer (2 mM, Mediatech Cellgro, 25-060-Cl), L-Glutamine (2 mM, Mediatech Cellgro), and penicillin/streptomycin (50,000 units penicillin, 50,000 mg streptomycin, Atlanta Biologicals). Cells were maintained at 37ºC in a 5% humidified CO_2 atmosphere.

Cultured cells were counted using a hemocytometer and were resuspended in fresh DMEM at a concentration of 8 x 10^4 cells/mL. The cell suspension (100 µL) was added to 96 well flat -bottom cell culture plates. At this concentration, 8,000 cells were seeded in each well. The drug solutions diluted in DMEM (detailed above) were added to each well (5 µL/well). Quadruplicate wells were prepared for each drug concentration. The plates were incubated for 72 hr at 37°C in a 5% CO_2 atmosphere. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS (5 mg/ml). After the indicated cell incubation period, 10 µL stock MTT solution was added to each well, and the plates were further incubated for 4 h at 37 °C in a 5% CO_2 atmosphere. After this final incubation, 100 µL acidic isopropanol solution (16 µL 12.1 N HCl in 5 mL isopropanol) was added to each well. The contents of the wells were mixed thoroughly by pipetting the cell suspension up and down and the plates were allowed to sit at room temperature for 15 min in order to allow for full development of the purple color. The plates were then read on a Dynatech Plate Reader, utilizing Dynex Revelation 3.2 software, with a test wavelenth of 570 nm and a reference wavelength of 630 nm. The dose inhibiting the growth by 50% (IC_{50}) was extrapolated from curves generated based on the averages of the absorbance data (4 points/concentration).

DNA Alkylation – DNA Strand Breaks

Solutions of the enantiomers and racemic mixture of compound **1** were prepared by dissolving them in DMSO to obtain final concentrations of 100 µM. The pBR322 plasmid DNA (Sigma) was at an initial concentration of 304 µg/mL in buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The reactions were performed by combining DNA (16.5 µL), Tris-EDTA KCl buffer (11.5 µL) and different compounds (2 µL) in separate Ependorf tubes. The samples were incubated at 37ºC for 40 h. The samples were removed, and then centrifuged for one minute. To the samples were added 2 µL of 10% sodium dodecylsulfate, 5 µL of 50% glycerol, and 2 µL of bromophenol blue. The contents of the tubes were mixed and centrifuged. The samples were loaded onto an agarose gel and electrophorese at 80 V for ~4 h or until the bands traveled 3/4 of the way down the gel. The gel was removed from the chamber and stained with ethidium bromide for ~15 min. The dye was poured off of the stained gel, and the gel was washed with tap water. Pictures of the gels were obtained using an electronic U.V. Transilluminator camera (ULTRA-Lum) and the intensities of each band (Form I, II, and III) were determined using the Advanced American Biotechnology (AAB) 1-D Advanced Software.

Thermal Cleavage Experiment

All drug-DNA reactions were performed in 25 mM triethanolamine, 1 mM EDTA, pH 7.2, at 37 °C for 5 h. Following incubation, DNA was precipitated by addition of 1/10 volume of 3M sodium acetate and 3 volumes of 95% ethanol and washed with 70% ethanol. The resulting pellet was dried by lyophilization.

The region of the plasmid pUC18 containing the prominent sites of damage on the bottom strand was PCR amplified, using the pUC1 primer and the synthetic primer 5'-TGGTATCTTTATAGTCCTGTCG-3', 5' – end labeled and binding on the complementary (upper) strand at positions 956 – 935. The 166 base pairs singly end - labeled fragment generated, was purified by agarose gel electrophoresis and isolated using a Bio101 kit according to the manufacturer's instruction.

The dry DNA pellets from the drug – DNA incubations were resuspended in sodium citrate buffer (pH 7.2) and heated to 90 °C for 30 min to thermally cleave at sites of adenine – or guanine – N3 lesions as described by Reynolds[24]. Samples were chilled, precipitated and dried.

The samples were dissolved in formamide loading dye, heat-denatured for 3 min at 95 °C, cooled on ice and electrophoresed at 2000 V for ≈ 2 h on a 6% acrylamide denaturing gel (Sequagel, National Diagnostics). The gels were dried under vacuum at 80 °C and exposed to film (X-OMAT,Kodak). Densitometry was performed on a Bio-Rad GS-670 imaging densitometer.

ACKNOWLEDGEMENTS

Support (to M.L.) from the Dreyfus Foundation, Research Corporation, National Science Foundation-REU, Enzacta Ltd. (UK), and the National Cancer Institute are gratefully acknowledged. J.A.H. thanks the Cancer Research UK for support.

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Received: 26 August, 2005 Revised: 10 October, 2005 Accepted: 11 October, 2005