Bisindolylmaleimides Linked to DNA Minor Groove Binding Lexitropsins: Synthesis, Inhibitory Activity against Topoisomerase I, and Biological Evaluation

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The synthesis, characterization, inhibitory activity against topoisomerase I, and biological evaluation of a series of oligopeptide-substituted bisindolylmaleimides **7**−**12** are described. Compounds **7**−**9**, which contain a basic C-terminus function such as (dimethylamino)propyl and bind to DNA with C_{50} values of 200, 160, and 135 μM, respectively, exhibited inhibition of topoisomerase I in a concentration dependent manner. Also, the relative order of observed topoisomerase I inhibition is **9** > **8** > **7** at ≤100 μM concentration, corresponding to the increase of the number of pyrrole units in the oligopeptide moiety. Compounds **10**−**12**, which contain an electrostatically neutral moiety, such as methyl ester, did not bind to DNA templates nor inhibit topoisomerase I. However, the cytotoxicity activities of these compounds were 1.5 times greater than those of compounds **7**−**9**.

Introduction

DNA topoisomerases are ubiquitous enzymes that perform essential cellular functions involved in replication, recombination, and packaging and unfolding of DNA in chromatin.[1] In addition to their normal cellular functions, both topoisomerase I and topoisomerase II have recently emerged as important cellular targets for chemical intervention in the development of anticancer agents. Eukaryotic topoisomerase I is the target for the antitumor plant alkaloid camptothecin[2] and its synthetic derivatives such as CPT-11[3] and topotecans.[4] Recently certain synthetic derivatives of indolocarbazole antibiotics have been found to exhibit anticancer activity *in vivo*, and this activity correlates with their topoisomerase I inhibition activity.[5] The studies on several new camptothecin and indolocarbazole derivatives indicate that the antitumor activity is correlated with their abilities to induce topoisomerase I mediated DNA cleavage *in vitro*.[6] Thus the identification of new drugs which induce cleavable complex formation with topoisomerase I is now viewed as a promising approach to develop clinically effective antitumor agents.

Indolocarbazoles KT6006 and KT6528 (Figure 1) represent a distinct class of *in vivo* active mammalian DNA topoisomerase I inhibitory antitumor drugs.[5] In contrast to camptothecin and VP-16-based agents that do not interact with the DNA template,[7] the indolocarbazoles intercalate.[5] This property implies that the indolocarbazoles may interact with the template significantly in the process of inhibiting topoisomerase I. Thus functionalizing the indolocarbazoles so as to promote their DNA binding and sequence selectivity appears to be a worthwhile objective.

A number of minor groove binding drugs (distamycin, netropsin, Hoechst 33258, and DAPI) have proven to inhibit the catalytic activity of isolated topoisomerases (both I and II), while at low concentrations distamycin and netropsin are also able to stimulate enzymatic activity.[7] Minor groove binding effects on isolated enzymes parallel the influence of such agents on induction of cleavable complex formation in nuclei by topoisomerase inhibitors.[7,8] Therefore, we reasoned that the attachment of an effector of the enzyme−DNA complex to a minor groove vector should selectively deliver the effector to the minor groove of unique DNA sequences and thereby possibly influence the potency and selectivity of the antitumor agent.

To achieve this goal, bisindolylmaleimide, a derivative of indolocarbazole, has been conjugated to novel DNA minor groove binders, lexitropsins (*i.e.*, information-reading analogues of netropsin and distamycin). This paper describes the synthesis and topoisomerase I inhibitory function of a series of these lexitropsin containing bisindolylmaleimides. This study reveals the markedly high potencies of some new compounds and also examines the effects of changing certain structural features of the lexitropsin moieties. The first is the consequence of increasing the inhibitory activity of the drugs by increasing the number of pyrrole units. The second structural feature is the type of C-terminus group chosen. The lexitropsin C-terminus, either methyl ester or dimethyl amino propyl amide, differ in two respects. The methyl ester has a neutral function, while dimethylamino would be protonated at physiological pH of 7.4 to provide favorable electrostatic attraction to the overall negative electrostatic potential of the DNA. These and other factors possibly affecting cytotoxicity are discussed.

Synthesis

The target conjugates **7**−**12** were synthesized by condensation of the bisindolylmaleimide moiety, as its carboxylic acid, with the amino function of distamycin analogues (Figure 2). As a typical reaction sequence, the synthesis of compound **7** is outlined in Scheme 1. We recently established an efficient synthetic route to the staurosporine aglycon,[9] in which compound **13** was

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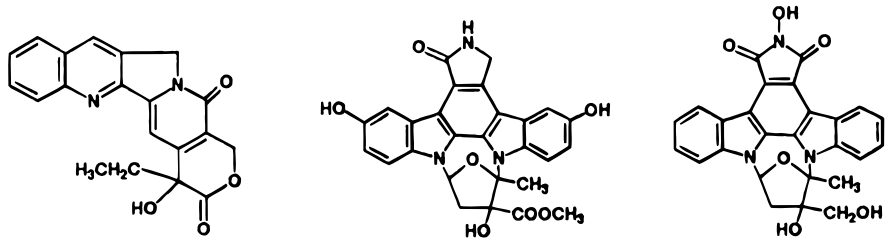


Figure 1. Structures of camptothecin and staurosporine analogues.

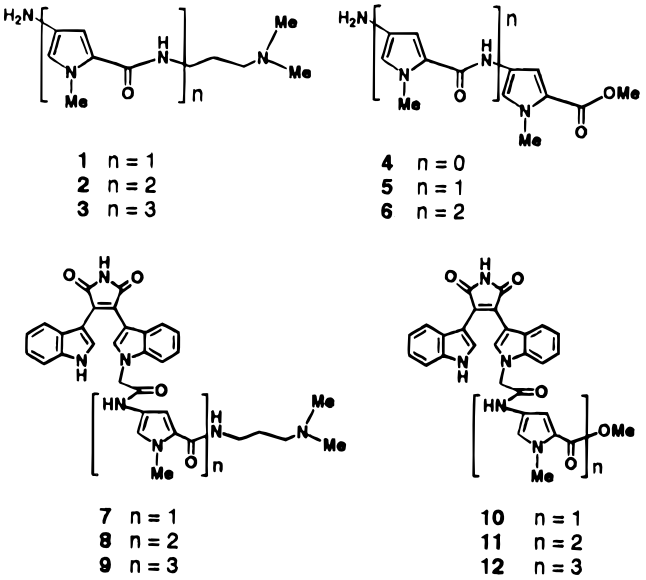


Figure 2. Structures of lexitropsin carriers (**1**−**6**) used in this study and the corresponding bisindolylmaleimide−lexitropsin conjugates (**7**−**12**).

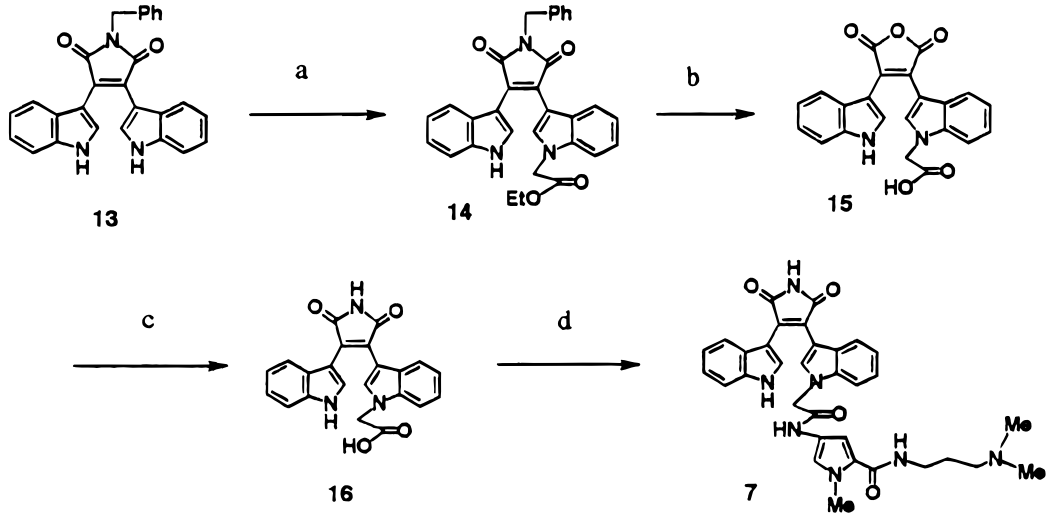
conveniently prepared in two steps from the easily available dibromomaleic acid. In the present study, we took **13** as the starting material and treated it with sodium hydride, followed by addition of ethyl bromoacetate, to obtain **14** in a moderate yield. Since the deprotection of the *N*-benzyl group has not proved to be possible,[9,10] we utilized an indirect method.[9] Thus, treatment of **14** with strong base, 5 M potassium hydroxide, readily afforded the anhydride **15** in high yield, and simultaneously, the ester group of **14** was converted to the desired carboxylic acid function. Subsequently, conversion of the anhydride to the imide was achieved by heating with ammonium acetate overnight, yielding the key intermediate **16** in good yield. Finally, **16** was coupled with the amine-containing oligopeptide **1**, prepared from *N*-methylpyrrole, according to the method previously developed in this laboratory,[11] in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) to give the target compound **7** in a reasonable yield. Similarly, coupling of **16** with other oligopeptides **2**−**6** afforded the products **8**−**12** in moderate to good yields.

Results and Discussion

The inhibitory activity of these novel conjugates toward the topoisomerase I relaxation reaction was evaluated by both agarose gel electrophoresis (Table 1) and an ethidium bromide fluorescence end point assay (Table 2).[12]

The ability of these compounds to inhibit topoisomerase I was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing concentration of the ligands (Figure 3a). Supercoiled DNA (Figure 3a, lanes 1 and 2) was converted to relaxed DNA in the presence of topoisomerase I (Figure 3a, lanes 3 and 4). For comparison, camptothecin, a recognized inhibitor of topoisomerase I, was included. As expected, the supercoiled DNA remaining in the assay system increased with the increasing concentration of camptothecin. The percentage of supercoiled DNA remaining was determined by densitometry (Figure 3b). Also, it was observed that

Scheme 1. Synthetic Route to Representative Bisindolylmaleimide−Lexitropsin Conjugates[a]



[a] Reaction condition: (a) NaH in THF then $BrCH_2CO_2Et$; (b) 5 M KOH; (c) NH_4OAc, 140 °C; (d) **1**, DCC, DMAP.

Table 1. Effects of the Bisindolylmaleimides on Topoisomerase I DNA Relaxation Activity, Estimated from Agarose Gel Electrophoresis

compound	concn, μM	topo I inhibn[a]
camptothecin	50	+
	100	+
7	25	0
	50	0
	100	+/−
	250	+
8	25	+/−
	50	+
	100	++
	250	+++
9	25	+
	50	++
	100	+++
	250	++++
10	25	0
	50	0
	100	0
	250	0
11	25	0
	50	0
	100	0
	250	0
12	25	0
	50	0
	100	0
	250	0

[a] Topoisomerase I inhibition was estimated from the agarose gel electrophoresis results. The scoring system is as follows: <10%, 10−25%, 25−50%, 50−75%, and 75−100% for +/−, +, ++, +++, and ++++, respectively.

ligands having an *N*,*N*-dimethylpropyldiamine side chain exhibited topoisomerase I inhibition which increased with the number of *N*-methyl-2-formamido-4-pyrrolamides in the molecule. Thus the relative order of the observed topoisomerase I inhibition is **9** > **8** > **7** at ≤100 μM concentration corresponding to the increase in the number of pyrrole units in the oligopeptide units. It should be noted, however, that at much higher extreme concentrations (250 μM) anomalous behavior was observed (Figure 3b). Most likely these compounds which were shown to bind to DNA (*vide infra*) inhibit the binding of topoisomerase I to DNA. Further confirmation for the lack of binding of the agents **10**, **11**, and **12** (*vide infra*) with DNA was observed in control samples (Figure 3a, lanes 36−40), whereby in the absence of topoisomerase I, the percentage of supercoiled DNA remained unaltered.

Since a topoisomerase inhibitor can cause a reduction in topoisomerase−DNA/ethidium bromide fluorescence, the ethidium bromide fluorescence end point assay is a useful method for quantitative determination of topoisomerase inhibition. These novel conjugates caused a significant reduction in DNA/ethidium bromide fluorescence, below control levels (both in the presence and absence of topoisomerase I; Table 2) in a manner which was concentration dependent and proportional to the number of *N*-methyl-2-formamido-4-pyrrolamides in the molecule. The maximum decrease in fluorescence was about 30% below control levels for compound **9** at a concentration of 25 μM and remained unaltered at higher concentrations tested. Since these compounds were incubated together with the DNA for 30 min prior to the addition of ethidium bromide solution, it could be expected that their binding to the DNA minor groove interferes with ethidium bromide intercalation, resulting in the concentration dependent fluorescence reduction. The fluorescence values returned to approximate

Table 2. Effects of the Bisindolylmaleimides on Topoisomerase I DNA Relaxation Activity, Estimated from Ethidium Bromide Assay

		% change[a]			
		Topo I (+)		Topo I (−)	
compound	concn, μM	BH	AH	BH	AH
camptothecin	25	−6	+7	−	−
	50	+12	+7	−	−
	75	+9	+9	−	−
	100	+3	+3	−	−
7	25	+3	+6	+1	+12
	50	+5	0	−1	+7
	100	−6	0	−6	−3
	250	−10	+3	−18	+2
8	25	−7	+3	−2	+5
	50	−10	−4	−14	−3
	100	−18	−15	−21	−12
	250	−14	−9	−32	−14
9	25	−11	−3	−28	−6
	50	−15	−7	−26	−15
	100	−20	−15	−24	−13
	250	−14	+13	−28	−4
10	25	+1	+18	−2	+15
	50	−1	+18	−3	+12
	100	+3	+12	+6	+14
	250	+1	+9	+2	+17
11	25	−3	+13	+5	+4
	50	+2	+7	+3	+15
	100	+1	+12	+2	+15
	250	+24	+3	+33	+10
12	25	+1	+6	+11	+14
	50	+9	+9	+25	+17
	100	+15	0	+33	+17
	250	+36	+3	+52	+20

[a] Percent change: BH, before heat denaturation of pBR322 DNA; AH, after heat denaturation of pBR322 DNA. (−) and (+) values indicate decrease and increase in fluorescence, respectively. Topo I (+) indicates the presence of enzyme in the reaction mixture. Topo I (−) indicates absence of enzyme, *i.e.*, only DNA and compounds in the reaction mixture. Excitation and emission wavelengths were 525 and 600 nm, respectively.

Table 3. Cytotoxicities to KB (ATCC CCL 17) Cells in Culture

compound	TD_{50}[a], μg/mL	compound	TD_{50}[a], μg/mL
7	84.0 ± 0.4	**11**	58.0 ± 0.3
8	92.3 ± 0.5	**12**	77.3 ± 0.4
9	>100 ± 0.5	camptothecin	0.01
10	53.2 ± 0.3		

[a] Concentration required to reduce KB cell population by 50%. Values given are an average of three of more determinations.

control levels after heat denaturation of the DNA treated with the topoisomerase I inhibitors. This decrease in fluorescence is due to irreversible heat denaturation of open circular DNA (OC DNA), because the alkaline pH of ethidium bromide assay medium (pH 12) prevents self-annealing of OC DNA so that it is only the covalently closed circular DNA (CC DNA) which readily renatures upon cooling. This net loss of duplex DNA is observed as a decrease in fluorescence. We, however, observed a significant increase in fluorescence. It is reasonable to conclude that the heat-denatured DNA released the minor groove binders, and upon cooling and renaturation of the CCC DNA, more ethidium bromide intercalated the DNA, resulting in an increased fluorescence. Among the compounds with the neutral carbomethoxy side chain (**10**−**12**), **10** and **11** had little effect on DNA fluorescence other than a slight increase after heating, consistent with no binding. Compound **12** caused an increase in fluorescence prior to heating. This increased fluorescence was substantially reduced after heat denaturation. Agarose gel

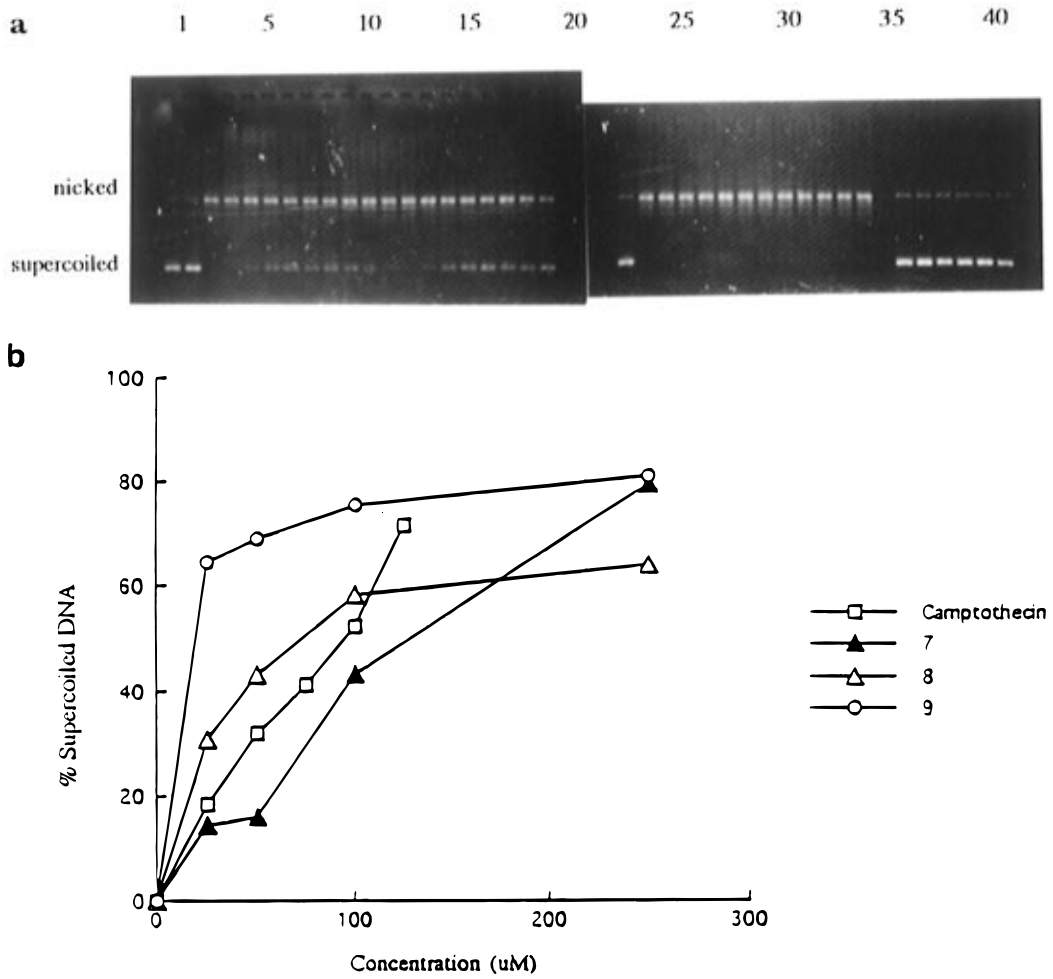


Figure 3. (a) Topoisomerase I relaxation assays as described in methodology; 0.5 unit of topoisomerase I was used in each assay. Lane 1 and 2: supercoiled pBR322 DNA, no enzyme, no drug. Lanes 3 and 4: no drug. Lanes 5−9: 25, 50, 75, 100, and 125 μM camptothecin, respectively. Lanes 10−13: 250, 100, 50, and 25 μM of **7**, respectively. Lanes 14−17: 25, 50, 100, and 250 μM of **8**, respectively. Lanes 18−21: 25, 50, 100, and 250 μM of **9**, respectively. Lanes 22−33: 25, 50, 100, and 250 mM of **10**, **11**, and **12**, respectively. Lanes 35−40: no enzyme, 250 μM of **7**, **8**, **9**, **10**, **11**, and **12**, respectively. (b) Densitometric analysis of percent supercoiled DNA remaining as a function of increased concentration of the ligands.

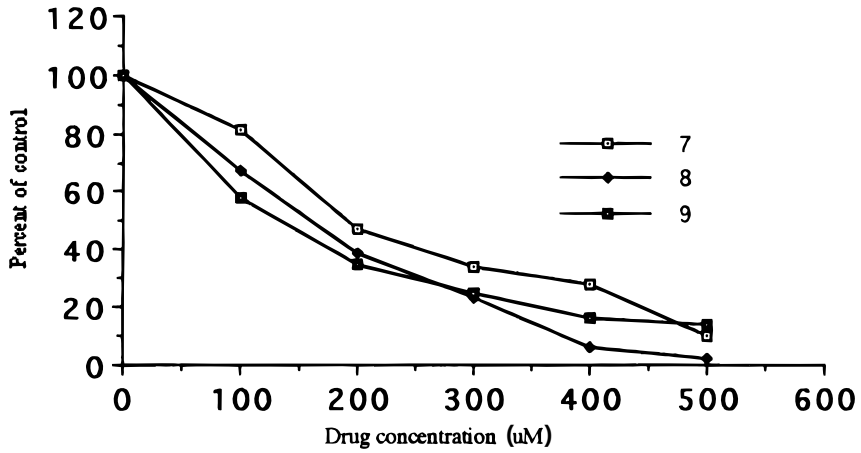


Figure 4. Fluorescence values of ethidium and lambda DNA (0.7 at A_{260}) complex in the presence of compounds **7**−**9** at concentrations indicated. The fluorescence was measured at 605 nm with excitation at 540 nm.

electrophoresis results confirmed that there was no DNA nicking by these compounds.

The next step was to determine the comparative binding ability of compounds **7**−**12**. This was accomplished by determining the micromolar drug concentration necessary to reduce the fluorescence of initially DNA-bound ethidium by 50% (*i.e.*, C_{50} value[17]) (Figure 4). The lower the C_{50} value, the greater the binding ability of the drug under the assay conditions. The percent decrease in fluorescence seen with added drug concentrations permitted the calculation of C_{50} values for compounds **7**−**9** as 200, 160, and 135 μM, respectively. The increase in observed DNA binding from **7** to **9** is in accord with the increasing number of *N*-methylpyrrole units in the lexitropsin carrier from 1 to 3. While ethidium quenching is often a significant factor in drug−DNA binding studies, minor groove binding drugs show low levels of quenching in relation

to ethidium displacement that quenching can be ignored.[17] Compounds **10**, **11**, and **12** showed no evidence of DNA binding.

The inability of the ligands having methyl esters (**10**−**12**) to interfere with the action of topoisomerase I resulted in complete relaxation of the supercoiled DNA by the enzyme (Figure 3a, lanes 22−33). Overall, these results indicate that the interaction between the binding moiety of ligands (**7**−**9**) and DNA interferes with the action of topoisomerase I. The significance of structure−activity relationship could be deduced by a recent study[13] indicating that tyrphostin derivatives not only block protein kinase but also bind to topoisomerase I. It has also been shown previously that genistein, which blocks tyrosine kinase, also blocks topoisomerase II.[14] However, these studies could not elaborate whether the antiproliferative effect of these derivatives is due to their effect on either kinase or topoisomerase inhibition or both. Our recent communication[15] and the present study indicate that while compounds of this type, containing a template-binding dimethylamine moiety as a peptide terminus, inhibit either kinase or topoisomerase I, compounds **10**−**12** containing nonbinding methyl ester inhibit only protein kinase activity. The anticancer cytotoxic potency of the compounds **10**−**12** exhibited approximately 1.5 times greater activity than the former group (**7**−**9**). This is perhaps expected because compounds with a neutral side chain (**10**−**12**) can penetrate the cellular membrane more easily. While more potent kinase inhibitors are evidently more effective against the particular KB cancer cell line, the ability of compounds **7**−**9** to act in parallel on cellular targets inhibiting both kinase and topoisomerase I activity may serve for the development of more selective chemotherapeutic agents. Such studies are in progress and will be reported in due course.

Experimental Section

Chemistry. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The IR spectra were recorded on a Nicolet 7199 FT spectrophotometer, and only the principal bands are reported. The ^{1}H NMR spectra were recorded on Bruker WH-300 and WH-400 spectrometers in $CDCl_3$ with tetramethylsilane as an internal standard or in DMSO-d_6. FAB (fast atom bombardment) mass spectra were determined on an Associated Electrical Industries (AEI) MS-9 and MS-50 focusing high-resolution mass spectrometers. Kieselgel 60 (230−400 mesh) of E. Merck was used for TLC, with the solvent system indicated in the procedure. TLC plates were visualized by using UV light or 2.5% phosphmolybdic acid in methanol with heating.

All reagents obtained commercially were used without further purification unless otherwise stated. Ethanol and methanol were distilled from magnesium turnings, tetrahydrofuran (THF) was distilled from sodium/benzophenone under an atmosphere of dry argon, and dimethylformamide (DMF) was distilled from barium oxide and stored over molecular sieves (3A). Hydrogenation reactions were usually conducted under atmospheric pressure at room temperature over a palladium catalyst (10% on charcoal) unless otherwise specified. In atmospheric hydrogenation, a hydrogen balloon was used to furnish a constant source of hydrogen. In high-pressure hydrogenation experiments, a Parr shaker or high-pressure autoclave was used. As a necessary criterion of homogeneity and purity, it was determined for each new compound by TLC analysis prior to the determination of accurate molecular mass by high-resolution spectrometry.

3-[1-[(Ethoxycarbonyl)methyl]-3-indolyl]-4-(3-indolyl)-1-(phenylmethyl)pyrrole-2,5-dione (14). A solution of maleimide **13** (326 mg, 0.78 mmol) in 5 mL of THF was added to a stirred suspension of sodium hydride (57 mg, 1.92 mmol) in THF (2 mL) with exclusion of moisture (argon). After 30 min, ethyl bromoacetate (137.4 mg, 0.78 mmol) was added at 0 °C, the mixture was stirred at room temperature for 2 h under argon, and then concentrated to dryness. The residue material was taken up in ethyl acetate. After washing with brine, drying (Na_2SO_4), and removal of the solvent, purification of the crude product by column chromatography, eluting with $CHCl_3$−EtOAc−Et_3N (97:2:1), gave 199 mg (51% yield) of the product **14** as a red solid: mp 94−95 °C; ^{1}H NMR ($CDCl_3$) δ 1.25 (3 H, d, J = 7.5 Hz), 4.25 (2 H, q, J = 7.5 Hz), 4.87 (2 H, s), 4.89 (2 H, s), 6.68−6.75 (13 H, m), 7.72 (1 H, d, J = 3.0 Hz), 7.75 (1 H, s), 8.50 (1 H, br); HRMS (EI) calcd for $C_{31}H_{23}O_4N_3$ 501.168 85, found 501.168 65.

3-[1-(Carboxymethyl)-3-indolyl]-4-(3-indololy)-2,5-furandione (15). To a solution of ester **14** (98 mg, 0.195 mmol) in 5 mL of EtOH at 0 °C was added 2 mL of 5 M KOH. The resulting solution was stirred at room temperature for 2 h, cooled, and acidified with 2 N HCl. The red precipitate was collected by filtration and purified by flash chromatography, eluting with EtOAc−CHCl−MeOH (1:1:0.8) to give 65 mg (86% yield) of **15** as a red solid: mp 143−144 °C; ^{1}H NMR δ 5.22 (2 H, s), 6.70 (2 H, t, J = 7.5 Hz), 6.88−7.10 (4 H, m), 7.45 (1 H, s), 7.46 (1 H, s), 7.97 (2 H, d, J = 1.5 Hz), 11.02 (1 H, br); HRMS (EI) calcd for $C_{22}H_{14}O_5N_2$ 386.090 27, found 386.090 71.

3-[1-(Carboxymethyl)-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (16). The maleic anhydride **15** (56 mg, 0.145 mmol) was heated with ammonium acetate (2 g) at 145 °C (bath temperature) for 12 h. The mixture was allowed to cool, water (10 mL) was added, and the mixture was extracted with ethyl acetate. The extracts were combined, washed with water, dried (Na_2SO_4), and evaporated *in vacuo* to give a dark red residue which was purified by flash chromatography, eluting with EtOAc−$CHCl_3$−MeOH (1:1:1) to yield 46 mg (81% yield) of **16** as a red solid: mp 155−157 °C; ^{1}H NMR δ 5.18 (2 H, s), 6.60−6.68 (2 H, m), 6.85−7.08 (4 H, m), 7.35−7.40 (2 H, m), 7.85 (2 H, s), 9.70 (1 H, br), 10.80 (1 H, br); HRMS (EI) calcd for $C_{22}H_{15}O_4N_3$ 385.106 26, found 385.106 27.

General Procedure for Coupling Reactions between Acid 16 and Oligopeptides 1−6: Preparation of 3-[1-[[[[1-Methyl-2-[[[3-(*N*,*N*-dimethylamino)propyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (7). A solution of the nitro ester, the precursor of amino ester **1** (19 mg, 0.074 mmol), was hydrogenated over 15 mg of 10% Pd−C in 2 mL of MeOH and 1 mL of THF at atmospheric pressure. When TLC indicated completion of the reduction, the catalyst was filtered off and the solvent was evaporated *in vacuo*. The resulting amine was dissolved in 2 mL of THF and cooled to 5 °C. To this solution was added the acid **16** (26 mg, 0.06 mmol) followed by EDCI (14.2 mg, 0.074 mmol). The mixture was stirred at room temperature for 4 h. Five milliliters of water was added to the solution, and the mixture was extracted with EtOAc (2 × 2 mL). The organic layer was removed, dried on Na_2SO_4, and concentrated to give a residue which was purified by flash chromatography, eluting with EtOAc−hexane−MeOH (3:6:1), to yield 23 mg (65% yield) of **7** as a light red solid: mp 112 °C dec; IR (CH_2Cl_2−MeOH) 2940, 1705, 1638, 1578, 1530, 1465, 1387, 1339, 745 cm^{-1}; ^{1}H NMR δ 1.60 (2 H, m), 2.18 (6 H, s), 2.23 (2 H, t, J = 7.5 Hz), 3.15 (2 H, q, J = 7.5 Hz), 5.08 (2 H, s), 6.55−6.70 (4 H, m), 6.89−7.04 (3 H, m), 7.10 (1 H, d, J = 2.0 Hz), 7.35 (1 H, s), 7.37 (1 H, s), 7.68 (1 H, d, J = 2.0 Hz), 7.90 (1 H, s), 8.08 (1 H, t, J = 6.0 Hz), 10.30 (1 H, s), 10.90 (1 H, s), 11.15 (1 H, s); HRMS (FAB) calcd for $C_{33}H_{33}O_4N_7H$ (MH^+) 592.2672, found 592.2652.

Spectroscopic and Analytic Data on Bisindolylmaleimides. 3-[1-[[[[1-methyl-2-[[[1-methyl-2-[[[1-methyl-2-[[[3-(*N*,*N*-dimethylamino)propyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]-methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (8). Prepared from **2** (32.5 mg, 0.086 mmol) and **16** (28 mg, 0.072 mmol) as a red solid in 58% yield as described above for **7**: mp 138 °C dec; IR (CH_2Cl_2−MeOH) 3253, 3054, 2979, 2938, 1707, 1645, 1577, 1465, 1401, 1339, 1264, 1107, 744 cm^{-1}; ^{1}H NMR δ 1.65 (2 H, p, J = 7.5 Hz), 2.23 (6 H, s), 2.39 (2 H, t, J = 7.5 Hz), 3.20 (2 H, q, J = 7.5 Hz), 3.78 (3 H, s), 3.84 (3 H, s), 5.08 (2 H, s), 6.58−6.70 (3 H, m), 6.80 (1 H, s), 6.85−7.04 (4 H, m), 7.18−7.20 (2 H, m), 7.70 (1 H, d, J = 2.0 Hz), 7.90 (1 H, s),

8.08 (1 H, t, J = 7.5 Hz), 9.88 (1 H, s), 10.38 (1 H, s), 10.92 (1 H, s), 11.15 (1 H, s); HRMS (FAB) calcd for $C_{39}H_{39}O_5N_9H$ (MH^+) 714.3152, found 714.3148.

3-[1-[[[[1-Methyl-2-[[[1-methyl-2-[[[1-methyl-2-[[[3-(*N*,*N*-dimethylamino)propyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (9). Prepared from **3** (37 mg, 0.075 mmol) and **16** (25 mg, 0.065 mmol) as a red solid in 52% yield as described above for **7**: mp 154 °C dec; IR (CH_2Cl_2−MeOH) 3245, 1706, 1643, 1578, 1465, 1403, 1124, 1063 cm^{-1}; ^1H NMR δ 1.64 (2 H, p, J = 7.5 Hz), 2.18 (6 H, s), 2.28 (2 H, t, J = 7.5 Hz), 3.20 (2 H, q, J = 7.5 Hz), 3.80−3.91 (9 H, m), 5.12 (2 H, s), 6.60−6.72 (3 H, m), 6.81 (1 H, s), 6.92−7.05 (5 H, m), 7.15−7.40 (5 H, m), 7.72 (1 H, d, J = 2.0 Hz), 7.92 (1 H, s), 8.10 (1 H, t, J = 7.5 Hz), 9.90 (1 H, s), 9.94 (1 H, s), 10.48 (1 H, s), 10.95 (1 H, s), 11.18 (1 H, s); HRMS (FAB) calcd for $C_{45}H_{45}O_6N_{11}H$ (MH^+) 836.3632, found 836.3629.

3-[1-[[[[1-Methyl-2-(methoxycarbonyl)-4-pyrrolyl]amino]carbonyl]methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (10). Prepared from **4** (22 mg, 0.12 mmol) and **16** (40.5 mg, 0.105 mmol) as a red solid in 69% yield as described above for **7**: mp 91−92 °C; IR (CH_2Cl_2−MeOH) 3304, 1705, 1577, 1532, 1449, 1406, 1340, 1258, 1141, 1062, 794 cm^{-1}; ^1H NMR δ 3.25 (3 H, s), 3.80 (3 H, s), 5.08 (2 H, s), 6.60−6.58 (3 H, m), 6.75 (1 H, d, J = 2.0 Hz), 6.90−7.05 (3 H, m), 7.71 (1 H, d, J = 2.0 Hz), 7.89 (1 H, s), 10.38 (1 H, s), 10.90 (1 H, br), 11.65 (1 H, s); HRMS (EI) calcd for $C_{29}H_{23}O_5N_5$ 521.169 92, found 521.169 37.

3-[1-[[[[1-Methyl-2-[[[1-methyl-2-(methoxycarbonyl)-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (11). Prepared from **5** (37 mg, 0.12 mmol) and **16** (38.5 mg, 0.10 mmol) as a red solid in 53% yield as described above for **7**: mp 142 °C dec; IR (CH_2Cl_2−MeOH) 3204, 2948, 1758, 1632, 1548, 1423, 1104, 978 cm^{-1}; ^1H NMR δ 3.72 (3 H, s), 3.83 (3 H, s), 3.84 (3 H, s), 5.10 (2 H, s), 6.58−6.70 (3 H, m), 6.85 (1 H, d, J = 2.0 Hz), 6.88−7.00 (5 H, m), 7.15 (1 H, d, J = 2.0 Hz), 7.35−7.40 (2 H, m), 7.45 (1 H, d, J = 2.0 Hz), 7.69 (1 H, d, J = 2.0 Hz), 7.90 (1 H, s), 9.92 (1 H, s), 10.38 (1 H, s), 10.90 (1 H, br), 11.65 (1 H, s); HRMS (FAB) calcd for $C_{35}H_{29}O_6N_7H$ 644.2257, found 644.2231.

3-[1-[[[[1-Methyl-2-[[[1-methyl-2-[[[1-methyl-2-(methoxycarbonyl)-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]-4-pyrrolyl]amino]carbonyl]methyl]-3-indolyl]-4-(3-indolyl)-1*H*-pyrrole-2,5-dione (12). Prepared from **6** (32 mg, 0.074 mmol) and **16** (25.7 mg, 0.06 mmol) as a red solid in 48% yield as described above for **7**: mp 198 °C dec; IR (CH_2Cl_2−MeOH) 3279, 1703, 1652, 1615, 1580, 1532, 1465, 1341, 1256, 1191, 1059 cm^{-1}; ^1H NMR δ 3.71 (3 H, s), 3.83 (9 H, s), 5.10 (2 H, s), 6.56−6.70 (3 H, m), 6.90−7.08 (6 H, m), 7.18 (1 H, d, J = 2.0 Hz), 7.24 (1 H, d, J = 2.0 Hz), 7.32−7.40 (2 H, m), 7.46 (1 H, d, J = 2.0 Hz), 7.60 (1 H, d, J = 2.0 Hz), 7.91 (1 H, s), 9.95 (2 H, s), 10.48 (1 H, s), 10.92 (1 H, s), 11.15 (1 H, s); HRMS (FAB) calcd for $C_{41}H_{35}O_7N_9H$ (MH^+) 766.2737, found 766.2700.

Topoisomerase I Relaxation Assay and Fluorescence Assay by Ethidium Bromide. The assays were done as described previously.[12,16] In brief, 0.25 μg of pBR322 DNA and 0.5 unit of topoisomerase I were incubated for 30 min at 37 °C in the presence of the ligands in a final volume of 10 μL. Following the incubation, an equal volume of agarose gel loading buffer[5] (2 × TBA, 0.1% bromophenol blue, 0.2% SDS and 20% glycerol) was added and incubated for another 30 min at 37 °C prior to loading. The gel was run overnight and stained in 0.5 μg/mL ethidium bromide solution, and the DNA was visualized using 300 nm wavelength transiluminator and photographed with Polaroid film. The negative was scanned on an LKB ultroscan XL laser densitometer.

For fluorescence studies, 0.25 μg of pBR322 DNA was incubated with ligands in the presence and absence of topoisomerase I (0.5 unit) for 30 min at 37 °C. Following the incubation, 1.2 mL of ethidium bromide buffer (0.5 μg/mL ethidium bromide, 20 mM K_3PO_4, and 0.5 mM EDTA, pH 12) was added for fluorometry. The fluorescence was recorded on a Perkin-Elmer Model 650-40 fluorescence spectrophotometer with excitation and emission wavelengths of 525 and 600 nm, respectively. Fluorescence was recorded both before (BH) and after (AH) heat denaturation of DNA.

Drug−DNA Binding. C_{50} values of drugs were determined as described.[17] The assay was performed at pH 5.0 (9.3 mM NaCl, 2 mM NaOAc buffer, pH 5.0, 0.1 mM EDTA, and 1.26 μM ethidium bromide) so that the added drugs exist in cationic form.

Cell Culture Cytotoxicity Assay. *In vitro* cytotoxicity assay of compounds was performed using KB cancer cell line (ATCC CCL 17).[18] KB cells were cultivated in Eagle's minimum essential medium supplemented with 10% calf serum and incubated in a humidified 5% CO_2 atmosphere at 37 °C. Cells were counted on a Neubauer hemocytometer and seeded at 100 μL of 3 × 10^3 cells per mL per well and allowed to culture for 24 h. Test compounds were added in triplicate at different concentrations. Control wells were identical except that the test compound was absent. After 3 days, the cells were fixed in 25% glutaraldehyde, washed with water, dried, and then stained with 100 μL of 0.05% crystal violet. The wells were eluted with 0.05 M NaH_2PO_4−ethanol (1:1 v/v) and read at OD_{450} on a multiscan spectrophotometer. TD_{50} values were determined as the concentrations required to reduce KB cell count by 50%.

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