In Vitro Photoinduced Cytotoxicity and DNA Binding Properties of Psoralen and Coumarin Conjugates of Netropsin Analogues: DNA Sequence-Directed Alkylation and Cross-Link Formation

Moses Lee,* Mary C. Roldan, Margaret K. Haskell, Simon R. McAdam,† and John A. Hartley†

Department of Chemistry, Furman University, Greenville, South Carolina 29613, and Department of Oncology, University College London Medical School, London, U.K. WIP 8BT

Received August 20, 1993®

The synthesis, DNA binding and *in vitro* photoinduced cytotoxic properties of a number of minor groove and sequence-directed psoralen and coumarin conjugates of pyrrole- and imidazole-containing distamycin analogues **2–5** are described. Results from an ethidium displacement assay on calf thymus and T4 DNA suggest that like distamycin these agents bind strongly to the minor groove of DNA. The data show that these conjugates exhibit a lower AT preference than distamycin and the decrease is significantly greater for the imidazole-containing compounds. All of the compounds along with 8-methoxypsoralen, **1**, were relatively noncytotoxic in the dark with only the imidazole–psoralen compound **3** giving an IC_{50} value below 100 μM. Following UV activation, all compounds showed an increased potency with photoinduced dose modifications in the human chronic myeloid leukemia K562 cells of >333, 12, >1.3, and >2.5 for compounds **2–5**, respectively, under the UV irradiation conditions employed. The psoralen–pyrrole analogue **2** was over 300 times more active following UV activation than agent **1**, 250 times more potent than the corresponding coumarin conjugate **4**, and 15-fold more potent than its imidazole analogue **3**. Data from CD dilution (with DMF) studies show that upon irradiation with light at 366 nm, compounds **2–5** bind irreversibly to DNA. Furthermore, upon irradiation compound **2** produced interstrand cross-linked DNA in quantitative yield, with isolated DNA, at >300- and >3000-fold lower drug concentrations than the imidazole analogue **3** and 8-methoxypsoralen, respectively. As expected coumarin conjugates **4** and **5** did not produce any cross-linked DNA under any conditions. Since the psoralen conjugates are more phototoxic than their coumarin analogues, these results suggest that DNA interstrand cross-link formation may be an important mechanism by which they exert their biological activity in cells. In addition, the enhanced photocytotoxic potency of conjugate **2** over **3** may be related to its larger binding constant, more efficient DNA cross-linking ability, and possibly to its preference for AT-rich sequences.

Psoralens, such as 8-methoxypsoralen, **1**, are used as photoactive drugs for the treatment of psoriasis[1] and cutaneous T-cell lymphoma[2] and as probes for nucleic acid structure and function.[3,4] These molecules are planar bifunctional photoactivatable agents that are capable of intercalating into double stranded DNA. Upon irradiation with UVA, the 3,4 or 4′,5′ double bond of the pyrone or furan groups of 8-methoxypsoralen, respectively, can undergo a [2 + 2] cycloaddition reaction with the 5,6 double bond of thymine residues forming monoadducts.[5,6] If the psoralen has intercalated into a suitable site, e.g., 5′-TpA-3′, the furan-side monoadduct can undergo a further cycloaddition reaction with the other strand of the DNA duplex to produce an interstrand cross-link. Structural analysis of the photoadducts formed by 8-methoxypsoralen with DNA showed that the 8-methoxy group is located in the minor groove.[7] The photochemical cross-link formation mechanism has been attributed to the mode of action of psoralens in cells.[8,9] However, the ability of these compounds to recognize long, unique DNA sequences is limited. Therefore, there is an interest in the development of agents that can deliver psoralen molecules to specific DNA sequences with the intention of increasing their therapeutic value.[10,11]

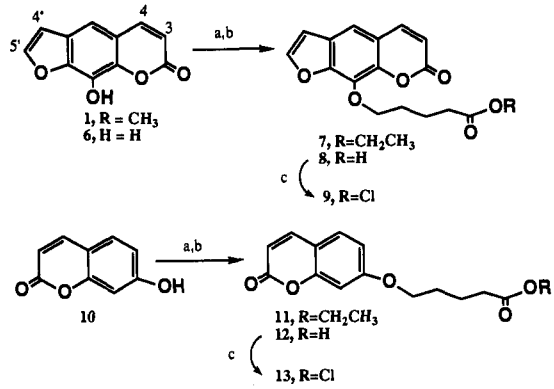
To achieve this goal, psoralen has been attached to oligonucleotides using either the antisense[11a–c] or triple helix[11d,e] approach to recognize specific DNA sequences. However, the use of oligonucleotides suffers from a number of problems, such as their poor cellular penetration, susceptibility to nuclease degradation, and syntheses as mixtures of diastereomers.[12] Therefore, a new mechanism for the delivery of psoralen to specific DNA sequences is sought.

The minor groove of DNA has proven to be a viable target for the interaction of a number of anticancer agents[13] such as the AT sequence selective benzoyl mustard derivative of distamycin FCE-24517,[14] CC1065,[15] and the GC site-selective agent, mitomycin C.[16] Therefore, we reason that the attachment of an 8-(alkyloxy)psoralen group to a minor groove vector may selectively deliver the photoreactive agent to the minor groove of unique DNA sequences. In this paper, we will demonstrate that the bifunctional and photoactivatable psoralen can be targeted to specific AT- and GC-rich sequences by attaching a psoralen derivative to a pyrrole-[13,17] and imidazole-containing[17,18] analogue of distamycin, respectively. GC-rich DNA sequences are of interest because they are commonly found in the genomes of mammals, and a functional role is suggested by their frequent occurrence in genes associated with proliferation, including a number of oncogenes.[19] The DNA sequence selectivity, photoinduced cross-link formation, and cytotoxicity of these compounds against the growth of K562 leukemia cells in culture are reported. In order to study the importance of

† University College London Medical School.
® Abstract published in *Advance ACS Abstracts*, March 15, 1994.

0022-2623/94/1837-1208$04.50/0

Scheme 1[a]



[a] (a) K_2CO_3, 2-butanone, reflux, ethyl 5-bromovalerate; (b) NaOH, THF/H_2O, reflux, 2 M HCl; (c) Oxalyl chloride, THF, reflux.

DNA cross-link formation for photoinduced cytotoxicity by the psoralen compounds **2** and **3**, the corresponding coumarin conjugates **4** and **5**, which can only form monoadducts, were also studied.

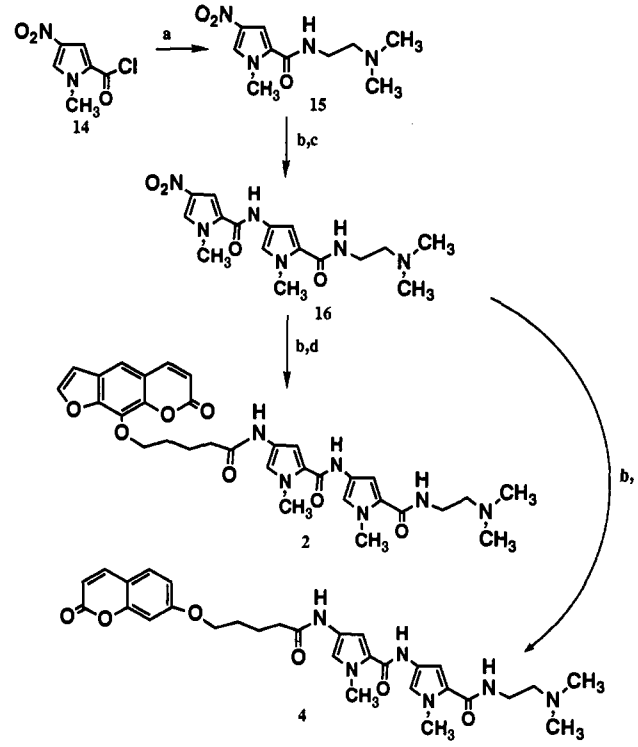
Results and Discussion

Synthesis. The target conjugates **2–5** were synthesized by condensation of the photoactivatable moieties, as their acid chlorides, with the amino function of the pyrrole- and imidazole-containing analogues of distamycin. In the synthesis of 8-substituted psoralen acid chloride **9** as depicted in Scheme 1, 8-hydroxypsoralen **6**[20] was reacted with ethyl 5-bromovalerate in the presence of K_2CO_3 to give ether **7** in 48% yield. Hydrolysis of the ester group of **7** followed by reaction of acid **8** with thionyl chloride afforded the desired acid chloride **9** in quantitative yield. The synthesis of 7-substituted coumarin acid chloride **13** followed a similar approach, except 7-hydroxycoumarin **10** was the starting material (see Scheme 1). Ether **11**, prepared in 78% yield from **10**, was hydrolyzed to acid **12**, which was converted to acid chloride **13** in good yield.

The syntheses of the pyrrole–psoralen and coumarin conjugates **2** and **4** are shown in Scheme 2. Pyrrole acid chloride **14**[21] was coupled with *N,N*-dimethylethylenediamine to give amide **15** in 83% yield. Reduction of the nitro group of **15** followed by condensation of the resulting amine with **14** gave the dipeptide **16** in 39% yield. The nitro function of **16** was reduced by catalytic hydrogenation, and then coupling with psoralen acid chloride **9** or coumarin acid chloride **13** gave the desired conjugates **2** and **4** in 28 and 19% yield, respectively. In preparation of the imidazole conjugates as shown in Scheme 3, the above acid chlorides were coupled with amine **17**.[22] The bifunctional psoralen conjugate **3** was obtained in 42% yield and the coumarin conjugate **5** was prepared in 30% yield. The syntheses of compounds **2–5** were conducted in dim conditions to avoid decomposition of the products.

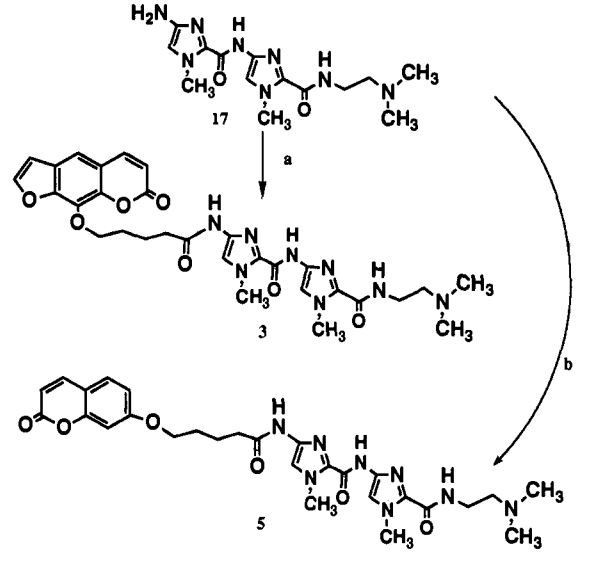
DNA Sequence and Groove Selectivity. The abilities of these compounds to bind to calf thymus and coliphage T4 DNAs were determined using an ethidium displacement assay.[23] The results given in Table 1 show that the pyrrole analogues **2** and **4** generally bind more strongly to the DNAs than their imidazole counterparts. The larger binding constants of compounds **2** and **3** to the DNAs than 8-MOP **1** suggest that both the oligopeptide and psoralen moieties are interacting with DNA. These data show that conjugates **2–5** exhibit lower AT preference than distamycin, and the decrease is significantly greater for the imidazole-containing compounds. Since these compounds bind to T4 DNA as strongly as distamycin, it suggests that they interact in the minor groove because

Scheme 2[a]



[a] (a) $H_2NCH_2CH_2N(CH_3)_2$, triethylamine, CH_2Cl_2; (b) H_2, 5% Pd–C, CH_3OH; (c) **14**, triethylamine, CH_2Cl_2; (d) **9**, triethylamine, CH_2Cl_2; (e) **13**, triethylamine, CH_2Cl_2.

Scheme 3[a]



[a] (a) **9**, triethylamine, CH_2Cl_2; (b) **13**, triethylamine, CH_2Cl_2.

Table 1. Apparent Binding Constants ($\pm 0.2 \pm 10^5$ M^{-1}) of Compounds **2–5** Determined by an Ethidium Displacement Assay

compound	T4	calf thymus	poly(dG-dC)	poly(dA-dT)
8-MOP, **1**	0.14 ± 0.02	0.48 ± 0.02	0.25 ± 0.02	0.17 ± 0.02
2	6.6 ± 0.2	5.5 ± 0.1	0.9 ± 0.1	2.9 ± 0.4
3	2.7 ± 0.1	0.4 ± 0.1	2.1 ± 0.4	0.9 ± 0.2
4	1.2 ± 0.2	1.7 ± 0.3	1.1 ± 0.3	35 ± 5
5	1.0 ± 0.2	1.0 ± 0.5	1.4 ± 0.1	1.1 ± 0.2
distamycin[18]	6.5	7.7	2.0	348

Table 2. Photoinduced DNA Cross-Link Formation on Isolated DNA and In Vitro Photocytotoxicity by Compounds 2–5

compound	XL_{50}[a] (μM)	cytotoxicity IC_{50} (μM) dark	irradiated[b]	photoinduced dose modification
1	18	>100	100	>1
2[b]	0.005	>100	0.3	>333
3	1.6	55	4.5	12
4	c	>100	75	>1.3
5	c	>100	40	>2.5

[a] Dose to produce 50 ± 5% cross-linked DNA following irradiation at 366 nm. [b] Irradiation at 366 nm. [c] No DNA cross-links were observed under any conditions.

the major groove of this nucleic acid is blocked by α-glycosylation of the hydroxymethyl group of cytidine residues.[24]

The DNA sequence selectivity of compounds 2 and 3 on the BamHI-saII fragment of pBR322 DNA and binding of 2 to $d(ATGCAT)_2$ were also determined by MPE-Fe(II) footprinting and ^{1}H-NMR studies, respectively, and the results are reported elsewhere.[25]

In Vitro Photoinduced Cytotoxicity. The cytotoxic properties of compounds 1–5 against human chronic myeloid leukemia cells grown in culture were determined under "dark" and "irradiated" (ca. 366 nm) conditions, and the IC_{50} values are given in Table 2. Under dark conditions only compound 3 showed any significant cytotoxicity, while the others were relatively nontoxic with IC_{50} values of >100 μM. Following UV illumination, all compounds showed an increased cytotoxicity, which was particularly striking for the psoralen-containing compounds 2 and 3 which gave photoinduced dose modifications of >333 and 12, respectively. Under the conditions employed, the UV exposure was completely nontoxic in the absence of drug. The psoralen–pyrrole conjugate 2 is significantly more photocytotoxic than 8-methoxypsoralen (1) by a factor of over 300. Compounds 2 and 3 are also significantly more potent following activation than the corresponding coumarin-containing analogues 4 and 5, with agent 2 being 250 times more potent than 4. The evidence of photoinduced cytotoxicity indicates that the biological activities of these compounds may be due to covalent interactions between the psoralen and coumarin groups in their excited states with the C5–C6 double bond of thymine residues.

DNA Monoadduct and Cross-Link Formation. The ability of these compounds to form covalent adducts with calf thymus DNA was determined by the reduction in the DNA-induced ligand CD band at ~290 nm of the preirradiated drug–DNA complex upon dilution with DMF.[14c] The DNA-induced ligand bands observed in these studies are presumably due to the $n \rightarrow \pi^*$ UV absorption band of the drug. Since the drugs do not produce any CD spectra by themselves, these results unambiguously confirm their binding to calf thymus DNA.[26] The CD alkylation experiment works on the premise that covalently bound ligands cannot be removed from DNA upon dilution with DMF; thus, the induced ligand band would persist. Upon irradiation of compounds 2–5 at 366 nm for 6–8 min the ellipticities of the induced ligand bands were close to half the original value before dilution. This suggests that (>95 ± 5)% of the compounds were irreversibly bound to the DNA, presumably by forming adducts with thymine residues. Under dark conditions only [(26–34) ± 5]% of irreversibly bound ligands were observed.

The DNA interstrand cross-linking ability of the compounds were determined by an agarose cross-link gel assay.[27] The data given in Table 2 show that, upon UV activation, conjugate 2 was highly efficient at producing DNA cross-links with (50 ± 5)% cross-linking occurring at 0.005 μM. At this drug concentration, and based on the K_{app} value of 2 with calf thymus DNA and concentration of the DNA used in the cross-link assay of 3.1×10^{-7} M bp, the percent of 2 bound reversibly to the DNA was calculated to be 17%. Upon UV irradiation the drugs that were bound to DNA would produce interstrand cross-links which, under our conditions, drove the overall reaction to produce 50% cross-linked DNA. Thus, the value of XL_{50} for 2 is reasonable for the K_{app} values observed for this compound. The cross-link assay is extremely sensitive in that the DNA substrate (4363 base pairs) will run as double stranded when containing a single cross-link, i.e., one lesion per 4363 base pairs. The XL_{50} is therefore a measure of the dose required for half the DNA molecules to contain one or more cross-links. Under identical conditions, the XL_{50} values for 8-methoxypsoralen (1) and conjugate 3 were 18 and 1.6 μM, respectively. Without UV activation, none of these compounds produced any DNA cross-links even at 1 mM drug concentration (data not shown), and no DNA damage was observed in the absence of drug. Furthermore, as expected, the coumarin-containing compounds did not produce any cross-links under any conditions. Since conjugates 2 and 3, which can form interstrand cross-links, are more photocytotoxic than 4 and 5 upon irradiation at 366 nm, these data suggest that interstrand cross-link formation may be responsible for the increased cytotoxicity. It is also worthy to note that the photocytotoxicity of the pyrrole conjugate 2 is significantly higher than that for the imidazole analogue 3 which may be related to its larger DNA binding constant, enhanced efficiency to produce DNA interstrand cross-links, and possibly to its increased AT sequence selectivity.

Experimental Section

The general experimental section is similar to that previously reported[21] with the following exceptions. The irradiation experiments were performed with either a Rayonet photoreactor or a clamped 366-nm UV lamp. The ^{1}H-NMR spectra of compounds 2–5 are given in the supplementary material.

Ethyl 5-(8-Psoralenyloxy)pentanoate (7). A solution of 8-hydroxypsoralen 6[20] (1.2 g, 6.0 mmol) in dry 2-butanone (175 mL) was combined with ethyl bromovalerate (0.97 mL, 6.1 mmol) and pulverized anhydrous K_2CO_3 (0.95 g, 6.9 mmol), and the mixture was heated to reflux under a drying tube overnight (17 h). The mixture was filtered and then concentrated on a Kugelrohr apparatus, yielding an oily residue which was purified by column chromatography using a stepwise gradient of 5% ethyl acetate in hexane as eluent (5% increment of ethyl acetate every 100 mL). The product was concentrated under reduced pressure to give 7 as an oil (2.81 g, 48%): TLC (7% $CH_3OH/CHCl_3$) R_f 0.50; ^{1}H NMR (300 MHz $CDCl_3$) δ 1.26 (t, 6.0, 3H), 1.92 (s br, 4H), 2.43 (m, 2H), 4.12 (q, 6.0, 2H), 4.51 (m, 2H), 6.42 (d, 9.9, 1H), 6.83 (d, 2.4, 1H), 7.36 (s, 1H), 7.68 (d, 2.4, 1H), 7.78 (d, 9.9, 1H); IR (neat) ν 2958, 1731, 1623, 1583, 1436, 1402, 1328, 1243, 1152, 1096, 1027, 988 cm^{-1}; MS (EI) m/z (rel intensity) 330 (M^+, 3), 285 ($M^+ - OCH_2CH_3$, 7), 202 (26), 129 (100). Anal. ($C_{18}H_{18}O_6$) C, H.

5-(8-Psoralenyloxy)pentanoic Acid (8). A solution of ester 7 (2.7 g, 8.1 mmol) in dry THF (5 mL), water (10 mL), and 2 M NaOH (4.5 mL) was heated to reflux for 25 min. After TLC analysis indicated that the reaction was complete, the solvent was removed under reduced pressure and the aqueous layer was washed with $CHCl_3$ (40 mL, twice). The aqueous layer was acidified with 2 M HCl (pH <3) and extracted with $CHCl_3$ (100

mL, twice). The organic layer was washed with brine and then dried with anhydrous Na_2SO_4. The product was concentrated in vacuo at room temperature to give **8** as an oil which crystallized to a tan solid upon standing in the freezer (2.66 g, 100%): mp 93–95 °C; ^{1}H NMR ($CDCl_3$) δ 1.83 (s br, 4H), 2.41 (m, 2H), 4.41 (m, 2H), 6.27 (d, 9.8, 1H), 6.72 (s, 1H), 7.15 (s, 1H), 7.60 (s, 1H), 7.68 (d, 9.8, 1H), 12.00 (s, 1H); IR ($CHCl_3$, cast) ν 3019, 2398, 2385, 1714, 1589, 1523, 1420, 1218, 924 cm^{-1}.

5-(8-Psoralenyloxy)pentanoyl Chloride (9). To a solution of acid **8** (0.170 g, 0.600 mmol) in dry THF (1.5 mL) was added a solution of oxalyl chloride (1.5 mL) in dry THF (1.5 mL). The reaction mixture was heated to reflux under a drying tube for 1 h. The reaction mixture was then concentrated under vacuum and coevaporated with dry CH_2Cl_2 (10 mL, twice) to give an oily residue which was used directly.

5-(7-Coumarinyloxy)pentanoyl Chloride (13). **13** was prepared in a similar three-step procedure used in the preparation of acid chloride **9**. Ester **11** was obtained as an off-white solid (6.54 g, 72.7%): mp 62–64 °C; TLC (20% $CH_3OH/CHCl_3$) R_f 0.60; ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 6.0, 3H), 1.85 (m, 4H), 2.50 (m, 2H), 4.05 (m, 2H), 4.14 (q, 6.0, 2H), 6.25 (d, 9.0, 1H), 6.82 (m, 2H), 7.35 (d, 8.7, 1H), 7.65 (d, 9.0, 1H); IR ($CHCl_3$, cast) ν 3022, 1723, 1610, 1507, 1216, 1124, 925, 768 cm^{-1}; MS (EI) m/z (rel intensity) 290 (M^+, 9), 129 (100). Anal. ($C_{16}H_{18}O_6$) C, H. Acid **12** was obtained as a pale yellow solid (6.29 g, 54.1%): mp 112–120 °C; ^{1}H NMR ($CDCl_3$) δ 1.65 (m, 2H), 1.75 (m, 2H), 2.25 (m, 2H), 4.07 (m, 2H), 6.28 (d, 10.2, 1H), 6.96 (m, 2H), 7.62 (d, 8.4, 1H), 8.00 (d, 10.2, 1H); IR ($CHCl_3$, cast) ν 3007, 1729, 1605, 1403, 1355, 1280, 1211, 1131 cm^{-1}.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-nitropyrrole-2-carboxamide (15).** A suspension of 1-methyl-4-nitropyrrole-2-carboxylic acid[21] (3.00 g, 17.6 mmol) and thionyl chloride (6.0 mL) was heated to reflux under a drying tube for 5 min. The excess thionyl chloride was removed under pressure, and the residue was coevaporated twice with dry CH_2Cl_2 (30 mL each), yielding acid chloride **14**[21] as a yellow powder.

To a cooled (−20 °C) and stirring solution of *N*,*N*-dimethylethylenediamine (4.8 mL, 45 mmol), dry triethylamine (6.3 mL, 45 mmol), and dry CH_2Cl_2 (30 mL) was added a solution of the above acid chloride dissolved in dry CH_2Cl_2 (30 mL). The mixture was kept at −20 °C for an additional 15 min and then warmed up to room temperature and stirred under a drying tube (2 h). Water (40 mL) was added and the product extracted with $CHCl_3$ (80 mL). The aqueous layer was extracted three more times with $CHCl_3$ (70 mL each). The organic layers were combined, dried (Na_2SO_4), and then concentrated under reduced pressure to give **15** an off white powder (3.52 g, 83%): mp 126–128 °C; TLC (5% $CH_3OH/CHCl_3$) R_f 0.25; ^{1}H NMR (5:1 $CDCl_3$–DMSO-d_6) δ 2.66 (s, 6H), 2.49 (t, 6.0, 2H), 3.43 (q, 6.0, 2H), 3.99 (s, 3H), 6.65 (t br, 6.0, 1H), 7.09 (d, 2.1, 1H), 7.55 (d, 2.1, 1H); IR (Nujol) ν 3273, 2980, 1657, 1557, 1310 cm^{-1}; MS (EI) m/z (rel intensity) 240 (M^+, 6), 197 ($M - (CH_2)_2N(CH_3)_2$, 100). Anal. ($C_{10}H_{26}N_4O_3$) C, H.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (16).** A solution of **15** (1.00 g, 4.16 mmol) in methanol (30 mL) was hydrogenated over 5% Pd on carbon (250 mg) at room temperature and atmospheric pressure for 2.5 h. The catalyst was removed by filtration. The filtrate was concentrated, and the residue was coevaporated with dry CH_2Cl_2 (15 mL, twice) to give the amine intermediate as a yellow oil, which was unstable and used directly in the next step.

To a cooled (−20 °C) and stirred solution of the above amine and dry triethylamine (0.60 mL, 4.3 mmol) in dry CH_2Cl_2 (25 mL) was added acid chloride **14** [prepared from 1-methyl-4-nitropyrrole-2-carboxylic acid (779 mg, 4.58 mmol) with 8.0 mL of thionyl chloride] in dry CH_2Cl_2 (16 mL). The mixture was kept at −20 °C for an additional 5 min and then allowed to warm to room temperature overnight (17 h). The reaction mixture was concentrated under reduced pressure to a solid which was dissolved in $CHCl_3$ (100 mL) and washed with 1 M NaOH (10 mL). Concentration of the organic layer gave a crude product which was purified by column chromatography using 5% CH_3OH in $CHCl_3$ as eluent. The product was precipitated from CH_2Cl_2/hexane, and the yellow powder was dried in vacuo at room temperature (0.510 g, 33.1%): mp 120–122 °C; TLC (10% $CH_3OH/CHCl_3$) R_f 0.32; ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 6H), 2.55 (t, 5.4, 2H), 3.48 (q, 5.4, 2H), 3.90 (s, 3H), 4.05 (s, 3H), 6.70 (s br, 1H), 6.75 (d, 0.5, 1H), 7.22 (d, 0.5, 1H), 7.60 (d, 0.5, 1H), 7.65 (d, 0.5, 1H), 9.80 (s, 1H); IR (Nujol) ν 3398, 3020, 1623, 1302, 1216, 1216, 1046 cm^{-1}; MS (EI) m/z (rel intensity) 362 (M^+, 9). Anal. ($C_{16}H_{22}N_6O_4$) C, H.

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[5-(8-psoralenyloxy)pentanoyl]amino]pyrrole-2-carboxamido]-pyrrole-2-carboxamide (2).** A solution of **16** (200 mg, 0.55 mmol) in methanol (35 mL) was hydrogenated over 5% Pd on carbon (100 mg) at room temperature and atmospheric pressure (3 h). The catalyst was removed by filtration over Celite. The filtrate was concentrated, and the residue was coevaporated with dry CH_2Cl_2 (10 mL, twice) to give a yellow oil which was dissolved in dry CH_2Cl_2 (20 mL) with dry triethylamine (0.11 mL, 0.70 mmol) and then chilled (0 °C).

A solution of 5-(8-psoralenyloxy)pentanoyl chloride (**9**) (150 mg, 0.50 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise to the above chilled solution of the amine. After 15 min at 0 °C the mixture was allowed to stir at room temperature overnight (17 h) and then concentrated under pressure. The tan solid was suspended in water (10 mL) and extracted in $CHCl_3$ (50 mL, 3 times). The organic layers were combined, dried (Na_2SO_4), and concentrated, and the resulting residue was purified by column chromatography (silica gel) with a stepwise gradient of 1% CH_3OH in $CHCl_3$ that increased by 1% of CH_3OH every 100 mL of eluent. The desired fractions were concentrated and dried in vacuo at room temperature to give **2** as an off-white foam (150 mg, 28.1%): mp 115–120 °C; TLC (10% CH_3OH–$CHCl_3$) R_f 0.35; ^{1}H NMR ($CDCl_3$) δ 2.00 (qn, 6.6, 2H), 2.15 (qn, 6.6, 2H), 2.32 (s, 6H), 2.54 (t, 6.3, 2H), 2.75 (t, 8.4, 2H), 3.48 (q, 6.3, 2H), 3.92 (s, 3H), 3.94 (s, 3H), 4.51 (t, 5.4, 2H), 6.49 (d, 9.3, 2H), 6.59 (t br, 1H), 6.63 (d, 1.8, 1H), 6.83 (d, 1.8, 1H), 6.85 (d, 2.4, 1H), 7.18 (d, 1.8, 1H), 7.27 (d, 1.8, 1H), 7.44 (s, 1H), 7.69 (s br, 1H), 7.72 (d, 2.1, 1H), 7.88 (d, 9.3, 1H), 8.80 (s br, 1H); IR ($CHCl_3$, cast) ν 3324, 2954, 1709, 1638, 1584, 1540, 1436, 1398, 1262, 1153 cm^{-1}; UV (H_2O) λ_{max} 298 nm (ϵ 4.52 × 10^4 cm^{-1} M^{-1}); MS (FAB, NBA) m/z (rel intensity) 617 ($M + H^+$, 5). HRMS (FAB, NBA) m/z 617.2727 ($C_{32}H_{37}N_6O_7$ requires 617.2726).

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[5-(7-coumarinyloxy)pentanoyl]amino]pyrrole-2-carboxamido]pyrrole-2-carboxamide (4).** Compound **4** was prepared using a similar procedure to that for **2** except acid chloride **13** was used. The compound was obtained as an off-white foam (150 mg, 19%): mp 96–98 °C; TLC (7% CH_3OH–$CHCl_3$) R_f 0.15; ^{1}H NMR ($CDCl_3$) δ 1.81 (m, 4H), 2.22 (s, 6H), 2.35 (m, 2H), 2.47 (t, 5.0, 2H), 3.40 (q, 5.0, 2H), 3.78 (s, 3H), 3.80 (s, 3H), 3.95 (m, 2H), 6.15 (d, 9.6, 1H), 6.47 (s, 1H), 6.55 (d, 1.5, 1H), 6.68 (m, 1H), 6.72 (dd, 2.4, 8.4, 1H), 6.78 (s br, 1H), 7.06 (d, 9.0, 1H), 7.10 (s, 1H), 7.26 (d, 8.4, 1H), 7.55 (d, 9.0, 1H), 7.76 (s br, 1H), 8.15 (s br, 1H); IR (Nujol) ν 3391, 2397, 2354, 1729, 1659, 1520, 1424, 1210 cm^{-1}; MS (FAB, NBA) m/z (rel intensity) 577 ($M + H^+$, 8); HRMS (FAB, NBA) m/z 577.2777 ($C_{30}H_{37}N_6O_6$ requires 577.2777).

***N*-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[5-(8-psoralenyloxy)pentanoyl]amino]imidazole-2-carboxamido]imidazole-2-carboxamide (3).** A solution of amine **17**[18] (239 mg, 0.71 mmol) was dissolved in dry CH_2Cl_2 (20 mL) with dry triethylamine (0.1 mL, 0.70 mmol) and chilled (0 °C). Then a solution of acid chloride **9** (184 mg, 0.66 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise. After 15 min at 0 °C the reaction mixture was allowed to stir at room temperature overnight (17 h). The reaction mixture was concentrated under pressure yielding a tan solid. The solid was then suspended in 2% NH_4OH (aqueous) (10 mL) and extracted in $CHCl_3$ (50 mL, three times). The organic layers were combined, dried (Na_2SO_4), and concentrated to give **3**. The resulting compound was purified by column chromatography (silica gel) with a stepwise gradient of 1% CH_3OH in $CHCl_3$ that increased by 1% CH_3OH every 100 mL of eluent. The desired fractions were concentrated and dried in vacuo at room temperature to give **3** as an off-white foam (154 mg, 42.2%): mp 74–75 °C; TLC (7% CH_3OH–$CHCl_3$) R_f 0.25; ^{1}H NMR ($CDCl_3$) δ 1.87 (qn, 5.7, 2H), 1.95 (qn, 5.7, 2H), 2.18 (s, 6H), 2.41 (t, 6.0, 2H), 2.52 (t, 7.2, 2H), 3.36 (q, 7.2, 2H), 3.92 (s, 6H), 4.40 (t, 5.7, 2H), 6.30 (d, 7.8, 1H), 6.74 (d, 1.7, 1H), 7.29 (s, 1H), 7.30 (s, 1H), 7.34 (s, 1H), 7.56 (t br, 1H), 7.60 (d, 1.7, 1H), 7.73 (d, 7.8, 1H), 9.24 (s, 1H), 9.34 (s, 1H); IR ($CHCl_3$, cast) ν

3022, 2397, 1729, 1654, 1526, 1216, 1034 cm^{-1}; UV (10% ethanol–H_2O) λ_{max} 248 (ϵ 2.26 × 10^3 cm^{-1} M^{-1}), 302 (ϵ 3.47 × 10^3 cm^{-1} M^{-1}); MS (FAB, NBA/TFA) m/z (rel intensity) 619 (M + H^+, 11), 419 (10); HRMS (FAB, NBA/TFA) m/z 619.2631 ($C_{30}H_{35}N_8O_7$ requires 619.2632).

N-[2-(Dimethylamino)ethyl]-1-methyl-4-[1-methyl-4-[[5-(7-coumarinyloxy)pentanoyl]amino]imidazole-2-carboxamido]imidazole-2-carboxamide (5). The synthesis of **5** followed the procedure employed for the **3** except acid chloride **13** was used. The compound was isolated as an off-white foam (162 mg, 30.1%): mp 80–83 °C; TLC (10% CH_3OH–$CHCl_3$) R_f 0.32; ^1H NMR ($CDCl_3$) δ 1.95 (m, 4H), 2.45 (s, 6H), 2.52 (t, 5.5, 4H), 3.50 (t, 5.7, 2H), 4.02 (s, 6H), 4.12 (m, 2H), 6.25 (d, 9.3, 1H), 6.83 (m, 2H), 7.36 (d, 8.4, 1H), 7.40 (s, 1H), 7.42 (s, 1H), 7.63 (d, 9.3, 1H), 7.74 (s, 1H), 8.07 (s, 1H), 9.25 (s, 1H); IR ($CHCl_3$, cast) ν 3370, 2942, 1734, 165, 1617, 1536, 1472, 1125, 1018 cm^{-1}; UV (H_2O) λ_{max} 218 (ϵ 721 cm^{-1} M^{-1}) 314 (ϵ 100 cm^{-1} M^{-1}); MS (FAB, NBA/TFA) m/z (rel intensity) 579 (M + H^+, 60); HRMS (FAB, NBA/TFA) m/z 579.2683 ($C_{28}H_{35}N_8O_6$ requires 579.2683).

Ethidium Displacement Assay. See ref 22.

CD Alkylation Studies.[14c] Into a 1-mm cell were added $2A_{260}$ DNA (140 μL, 1.4 × 10^{-4} M bp final concentration) in 10 mM sodium phosphate, 0.25 mM EDTA at pH 7.2, and 1 mM drug solution (8 μL, 5.4 × 10^{-5} M final concentration). The control sample was stored in the dark. The test sample was irradiated at 366 nm, 6–8 min. The CD spectra of both samples were recorded from 200–400 nm. Then 70 μL of each of the DNA–drug solutions was removed, replaced with DMF (70 μL), agitated, and the CD spectrum recorded. The percentage alkylated drug was calculated from the equation given below:

$$\% \text{ alkylation} = 2A^*/A \times 100$$

where A is the starting ellipticity of the DNA-induced ligand band and A^* is the ellipticity after dilution with DMF.

Agarose Gel Cross-Link Assay. pBR322 plasmid DNA was linearized by digestion with Hind III and dephosphorylated by treatment with bacterial alkaline phosphatase. The DNA was 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (5000Ci/mmol, Amersham). Following precipitation and removal of unincorporated ATP, the DNA was resuspended in sterile double-distilled water at 1 μg/μL. Approximately 10 ng of labeled DNA was used for each experimental point.

Reactions with drug were performed in 25 mM triethanolamine, 1 mM EDTA, pH 7.2, at 37 °C for 2 h in the dark, in a final volume of 50 μL. The final concentration of the DNA was 3.1 × 10^{-7} M bp. In these reactions, aliquots of stock solutions of the drugs (10 or 1.0 or 0.1 mM in DMSO) were added to the reaction mixture to produce final drug concentrations of 0.0001–100 μM. Where appropriate the reactions were irradiated for 2 min with 366-nm UV light. The reactions were terminated by addition of an equal volume of stop solution (0.6 M sodium acetate, 20 mM EDTA, 100 μg/mL tRNA), and the DNA was precipitated by the addition of 3 vol of 95% ethanol. Following centrifugation and removal of supernatant, the DNA pellet was dried by lyophilization.

Samples were dissolved in 10 μL of strand separation buffer (30% dimethyl sulfoxide, 1 mM EDTA, 0.04% bromphenol blue, 0.04% xylene cyanol), heated at 90 °C for 2 min, and chilled immediately in an ice–water bath prior to loading. Control undenatured samples were dissolved in 10 μL of 6% sucrose, 0.04% bromphenol blue, and loaded directly. Electrophoresis was performed on 20-cm-long 0.8% submerged horizontal agarose gels at 40 V for 16 h. The gel and running buffer was 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.1.

Gels were dried at 80 °C onto one layer of Whatman 3 MM and one layer of DE81 filter papers on a Bio-Rad Model 583 gel dried connected to a vacuum. Autoradiography was performed with Hyperfilm MP (Amersham) for 4 h at −70 °C using a DuPont-Cronex Lightening-plus intensifying screen. Sharper images were obtained by overnight exposure without the intensifying screen. The percentage cross-linked (double-stranded) DNA was determined by microdensitometry using an Ultrascan XL enhanced laser densitometer (LKB Instruments).

A representative copy of a crosslink gel for compound **2** and 8-MOP (**1**) is given in the supplementary material.

Cytotoxicity Studies. The K562 human chronic myeloid leukemia cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% CO_2 and were incubated with a specified dose of drug for 1 h at 37 °C in the dark. Drug-containing medium was removed by centrifugation and the cell pellet washed once in fresh medium. Where appropriate, cells were resuspended in phosphate buffer saline and irradiated for 2 min with a 366-nm lamp (UVP Inc., San Gabriel, CA, 50 Hz, 420 μW/cm^2) that was clamped 5 cm from the open Petri dishes (35-mm diameter) that contained 2 mL of solution. In these experiments no additional filters were used. Following centrifugation, the cells were resuspended in fresh medium.

Following the appropriate drug treatment, the cells were transferred to 96-well microtitre plates, 10^4 cells per well, 8 wells per sample. In these experiments, aliquots of stock solutions of the drugs (10 or 1.0 or 0.1 mM in DMSO) were added to the culture medium to give final drug concentrations of 0.01–100 μM. Plates were then kept in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. The assay is based on the ability of viable cells to reduce a yellow soluble tetrazolium salt, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) to an insoluble purple formazan precipitate.[28] Following incubation of the plates of 4–5 days (to allow control cells to increase in the number by 10-fold), 20 μL of a 5 mg/mL solution of MTT in phosphate-buffered saline was added to each well and the plates further incubated for 5 h. The plates were then centrifuged for 5 min at 300g and the bulk of the medium was pipetted from the cell pellet, leaving 10–20 μL per well. DMSO (200 μL) was added to each well, and the samples were agitated to ensure complete mixing. The optical density was then read at a wavelength of 550 nm on a Titertek Multiscan ELISA plate reader and the dose–response curve constructed. For each curve, an IC_{50} value was read as the dose required to reduce the final optical density to 50% of the control value.

Acknowledgment. The authors acknowledge the National Science Foundation (REU and ILI) and Research Corporation for financial support.

Supplementary Material Available: ^1H NMR spectra of **2–5** and a cross-link gel assay on **2** and **1** (5 pages). Ordering information is given on any current masthead page.

References

(1) Ben-Hur, E. The photochemistry and photobiology of furocoumarins (Psoralens). *Adv. Radiat. Biol.* 1984, *11*, 132–171.
(2) Edelson, R.; Berger, C.; Gasparro, F.; Jegassothy, B.; Heald, P.; Wintroub, B.; Vonderheid, E.; Knobler, R.; Wolff, K.; Plewig, G.; McKiernan, G.; Christiansen, I.; Oseter, R. N. M.; Honigsmann, H.; Wildford, H.; Kokoschka, E.; Rehle, T.; Perex, M.; Stingl, G.; Laroch, L. Treatment of cutaneous T-cell lymphoma by extracorpeal photochemotherapy: Preliminary Results. *N. Engl. J. Med.* 1987, *316*, 297–303.
(3) Cimino, G. D.; Gamper, H. B.; Isaacs, S. T.; Hearst, J. E. Psoralens as Photoactive Probes of Nucleic Acid Structure and Function: Organic Chemistry, Photochemistry, and Biochemistry. *Annu. Rev. Biochem.* 1985, *54*, 1151–1193.
(4) Shi, Y. B.; Lipson, S. E.; Chi, D. Y.; Spielman, H. P.; Monforte, J. A.; Hearst, F. E. Applications of Psoralens as Probes of Nucleic Acids Structure and Function. In *Bioorganic Photochemistry*; Morrison, H., Ed.; John Wiley & Sons: New York, 1989; Vol. 1, pp 341–378.
(5) (a) Hearst, J. E. Psoralen photochemistry. *Annu. Rev. Biophys. Bioeng.* 1981, *10*, 69–86. (b) Kumeresan, K. R.; Ramaswamy, M.; Yeung, A. T. Structure of the DNA Interstrand Cross-Link of 4,5′,8-Trimethylpsoralen. *Biochemistry* 1992, *31*, 6773–6783 and references therein.
(6) *Psoralen DNA Photobiology*; Gasparro, F. P., Ed.; CRC Press, Inc.: Boca Raton, FL, 1988; Vols. 1 and 2.
(7) Tomic, M. T.; Wemmer, D. E.; Kim, S.-H. Structure of a Psoralen Cross-linked DNA in Solution by Nuclear Magnetic Resonance. *Science* 1987, *238*, 1722–1725.
(8) (a) Nielsen, P. E.; Linnane, W. P. III. Differentiated Inhibition of DNA, RNA and Protein-Synthesis in L1210 Cells by 8-Methoxypsoralen. *Biochem. Biophys. Res. Commun.* 1983, *112*, 965. (b) Nielsen, P. E.; Bohr, V. Phototoxic Effects of Four Psoralen on L1210 Cells. The Correlation with DNA Interstrand Cross-Linking. *Photochem. Photobiol.* 1983, *38*, 653.

(9) Varga, J. M.; Weisehahn, G.; Bartholomew, J. C.; Hearst, J. E. Dose-Related Effects of Psoralen and UV-Light on the Cell Cycle of Murine Melanoma Cells. *Cancer Res.* **1982**, *42*, 2223.
(10) Woo, J.; Hopkins, P. B. Template-Directed Modification of Single-Stranded DNA by Psoralen-Tethered Oligonucleotides: Sites of Photoadduct Formation Analyzed by Sequence-Specific and Sequence-Random Cleavage. *J. Am. Chem. Soc.* **1991**, *113*, 5457–5459.
(11) (a) Miller, P. S.; Bhan, P.; Cushman, C. D.; Kean, J. M.; Levis, J. T. Antisense Oligonucleoside Methylphosphonates and their Derivatives. *Nucleosides Nucleotides* **1991**, *10*, 37–46. (b) Pieles, U.; Englisch, U. Psoralen Covalently Linked toOligodeoxyribonucleotides: Synthesis, Sequence Specific Recognition of DNA and Photo-Crosslinking to Pyrimidine Residues of DNA. *Nucleic Acids Res.* **1989**, *17*, 285–299. (c) Englisch, U.; Gauss, D. H. Chemically Modified Oligonucleotides as Probes and Inhibitors. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 613–629. (d) Takasugi, M.; Guendouz, A.; Chassignol, M.; Decout, J. L.; Lhomme, J.; Thuong, N. T.; Helene, C. Sequence-Specific Photo-Induced Cross-Linking of the Two Strands of Double-Helical DNA by Psoralen Covalently Linked to a Triple Helix Forming Oligonucleotide. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5602–5606. (e) Giovannangeli, C.; Thuong, N. T.; Helene, C. Oligodeoxynucleotide Directed Photoinduced Cross-linking of HIV Proviral DNA via Triple Helix Formation. *Nucleic Acids Res.* **1992**, 420, 4275–4281.
(12) Miller, P. S.; Tso, P. O. P. A New Approach to Chemotherapy based on Molecular Biology and Nucleic Acid Chemistry: Matagen (Masking Tape for Gene Expression). *Anti-Cancer Drug Des.* **1987**, *2*, 117–128.
(13) (a) Hurley, L. H.; Boyd, F. L. Approaches Toward the Design of Sequence Specific Drugs for DNA. *Annu. Rep. Med. Chem.* **1987**, *22*, 259–268 and references therein. (b) Lown, J. W. Lexitropsins: Rational Design of DNA Sequence Reading Agents as Novel Anticancer Agents and Potential Cellular Probes. *Anti-Cancer Drug Des.* **1988**, *3*, 25–40. (c) Thurston, D. E.; Thompson, A. S. The Molecular Recognition of DNA. *Chem. Br.* **1990**, 767–772.
(14) (a) Barbieri, B.; Giuliani, C.; Pezzoni, G.; Lazzari, E.; Arcamone, F.; Mongelli, N. In-vivo Antitumor Activity of FCE 24517, a Novel Distamycine A Derivative with Specificity for AT Rich Sequences of DNA. *Proc. Am. Cancer Soc.* **1989**, *29*, 330. (b) Broggini, M.; Ballinari, D.; Spinelli, L.; Geroni, C.; Spreafico, F.; D'Incalici, M. Studies on the Mode of Action of the New Distamycine Derivative FCE 24517. *Ibid.* **1990**, *31*, 348. (c) Arcamone, F.; et al. Synthesis, DNA Binding Properties, and Antitumor Activity of Novel Distamycin Derivatives. *J. Med. Chem.* **1989**, *32*, 774–778.
(15) (a) Warpehoski, M. A.; Gerhard, I.; Kelly, R. C.; Kruger, W. C.; Li, L. H.; McGovren, J. P.; Prairie, M. P.; Wicnienski, N.; Wierenga, W. Stereoelectronic Factors Influencing the Biological Activity and DNA Interaction of Synthetic Antitumor Agents Modeled on CC1065. *Ibid.* **1988**, *31*, 590–603. (b) Li, L. H.; Bhuyan, B. K.; Wallace, T. L. Comparison of Cytotoxicities of Agents on Monolayer and Spheroid Systems. *Proc. Am. Assoc. Cancer Res.* **1989**, *30*, 612.
(16) Verweig, J.; Pinedo, H. M. Mitomycin C: Mechanism of Action, Usefulness and Limitations. *Anticancer Drugs* **1989**, *1*, 5–13.
(17) Krowicki, K.; Lee, M.; Hartley, J. A.; Ward, B.; Kissinger, K.; Skorobogty, A.; Dabrowiak, J. C.; Lown, J. W. Molecular Recognition between Oligopeptides and Nucleic Acids - Rational Design of Sequence Specific DNA Binding Agents. In *Structure and Expression*; Sarma, R. H., Sarma, M. H., Eds.; Adenine Press: New York, 1988; Vol. 2, pp 251–271.
(18) Lee, M.; Rhodes, A. L.; Wyatt, M. D.; Forrow, S.; Hartley, J. A. GC Base Pair Recognition by Oligoimidazolecarboxamide and C-Terminus Modified Analogues of Distamycin Deduded from CD, ^{1}H-NMR and MPE Footprinting Studies. *Biochemistry* **1993**, *32*, 4237–4245.
(19) Barbacid, M. *ras* Genes. *Annu. Rev. Biochem.* **1987**, *56*, 779–872.
(20) Schonberg, A.; Sina, A. Experiments with Xanthotoxin and Imperatonin Obtained from the Fruits of *Ammi majus* (L.). *J. Am. Chem. Soc.* **1950**, *72*, 4826–4828.
(21) Lown, J. W.; Krowicki, K. Efficient Total Synthesis of the Oligopeptide Antibiotics Netropsin and Distamycin. *J. Org. Chem.* **1985**, *50*, 3774–3779.
(22) Lee, M.; Rhodes, A. R.; Wyatt, M. D.; Forrow, S.; Hartley, J. A. In-Vitro Cytotoxicity of GC Sequence Directed Alkylating Agents Related to Distamycin A. *J. Med. Chem.* **1993**, *36*, 863–870.
(23) Morgan, A. R.; Lee, J. S.; Pulleyblank, D. F.; Murray, N. L.; Evans, D. H. Ethidium Fluorescence Assays. Part 1. Physiochemical Studies. *Nucleic Acids Res.* **1979**, *7*, 547–569.
(24) Lown, J. W. New Approaches to the Study of the Mechanism of Action of Antitumor Antibiotics. *Acc. Chem. Res.* **1982**, *15*, 381–387.
(25) Hartley, J. A.; McAdam, S. R.; Das, S.; Roldan, M. C.; Haskell, M. K.; Lee, M. Molecular and Cellular Pharmacology of Novel Photoactive Psoralen and Coumarin Conjugates of Pyrrole- and Imidazole-Containing Analogues of Netropsin. *Anti-Cancer Drug Des.*, in press.
(26) (a) Zimmer, C.; Wahnert, U. Nonintercalating DNA Binding Ligands: Specificity of the Interaction and Their Use as Tools in Biophysical, Biochemical and Biological Investigations of the Genetic Material. *Prog. Biophys. Mol. Biol.* **1986**, *47*, 31–112. (b) Burckhardt, G.; Luck, G.; Strol, K.; Zimmer, C.; Lown, J. W. Binding to DNA of Selected Lexitropsins and Effects on Prokaryotic Topoisomerase Activity. *Biochim. Biophys. Acta* **1993**, *1173*, 262–272.
(27) Hartley, J. A.; Berardini, M. D.; Souhami, R. L. An Agarose Gel Method for the Determination of DNA Interstrand Crosslinking: Applicable to the Measurement of Total and "Second Arm" Crosslinking Reactions. *Anal. Biochem.* **1991**, *193*, 131–134.
(28) Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. Evaluation of a Tetrazolium Based Semi-automated Colorimetric Assay. I: Assessment of Chemosensitivity Testing. *Cancer Res.* **1987**, *47*, 936.