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A Microgonotropen Branched Decaaza Decabutylamine and its DNA and DNA/Transcription Factor Interactions

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Abstract—The central pyrrole of a site-selective DNA minor groove binding tripyrrole peptide **1** has been attached to a branched decaaza decabutylamine via a -$(CH_2)_2$-NHCO-$(CH_2)_3$- linker to provide the decaaza-microgonotropen (**8**). The decaaza decabutylamine moiety of **8** was designed to have a much greater affinity to the phosphodiester linkages of the backbone of DNA. Employing Hoechst 33258 (**Ht**) as a fluorescent titrant, the equilibrium constants for the binding of **8** to the hexadecameric duplex d(GGCGCA$_2$T$_2$GGCGG)/d(CCGCCA$_2$T$_2$GCGCC) and to calf thymus DNA were determined. The log of the product of equilibrium constants (log $K_{11}K_{12}$) for 1:1 and 1:2 complexes formation at A_3T_3 is 17 (35 °C). Results of studies of the inhibition of the binding of several proteins to target DNA are discussed. Binding of the E2F1 transcription factor to its DNA target is 50% inhibited at ~2 nM concentration of **8**. © 1997 Elsevier Science Ltd.

Introduction

There has been considerable interest in reagents capable of sequence-selective nonintercalative complexation to DNA. Small organic compounds able to complex to the minor groove of B-DNA[1] have drawn profound interest in recent years. A crescent shape to match the natural curvature of the minor groove of B-DNA is a common motif of each of these molecules.[2] New among the minor groove binding molecules are the microgonotropens **1**, **5**, **6**, and **7** (Chart 1).[3–5] Like distamycin, the characteristic shape of microgonotropens is dictated by the peptide linkages between three 1-methyl-4-aminopyrrole-2-carboxylic acid residues.[2a] The present microgonotropen design is based on the concept of two essential parts: (1) a recognition unit (the selective DNA minor groove binding molecule)[1] that serves as a carrier, and (2) a polyamine moiety reaching out of the minor groove and capable of firmly grasping the phosphodiester backbone. In so doing, the microgonotropens are able to increase the binding affinities to DNA and alter its conformation.[5,6]

In a previous synthesis[4] microgonotropen **1** was prepared with a -$(CH_2)_3NH_2$ linker arm on the central pyrrole nitrogen. Different agents capable of modifying or cleaving DNA can then be conveniently attached covalently to the tripyrrole carrier through a coupling reaction with the anchoring group. We report here the synthesis of the decaaza microgonotropen **8**. In addition, the equilibrium association constants to DNA have been evaluated and compared to the constants of other tripyrrole peptides. Results of studies of the ability of **8** to inhibit formation of transcription factor/DNA complexes are provided.

Results and Discussion

Synthesis

A convergent synthetic strategy was envisioned in which **8** was synthesized by reacting microgonotropen **1**[4] with a properly functionalized branched polyamine. The synthesis of the branched polyamine **7** was based on S_N2 reaction of an appropriate tosylate of *N*-Boc-pentaazapentabutyl amine (**II**) with *N*-Boc-pentaazapentabutyl amine (**I**) (Scheme 1).[5] The amino group of pentaazapentabutyl amine (**I**) was protected by a *tert*-butoxycarbonyl (Boc) group and the terminal hydroxyl group was tosylated to give compound **II** in 32% yield. For consistency microgonotropens were numbered as in the previous publications[3] and synthetic intermediates were numbered with roman numerals. A substitution reaction was performed on the tosylated polyamine **II** with the polyamine **I** in DMF and K_2CO_3 at 90 °C to give the branched polyamine **III** in 78% yield. The branched polyamine **III** was subsequently transformed to the corresponding carboxylic acid (**IV**) using pyridinium dichromate in DMF[7] in 40% yield (Scheme 1). The carboxylic acid **IV** was attached to the *N*-propyamino linker of **1** by forming an amide linkage. The carboxylic acid **IV** was reacted[8] with the tripyrrole peptide **1** in presence of DCC and HOBT in DMF at room temperature (Scheme 2) to afford **V** in 46% yield. The carbobenzyloxy protecting groups in **V** were removed by catalytic hydrogenation (10% Pd-C) and the Boc protecting groups were removed using 40% TFA in CH_2Cl_2 (Scheme 2). Finally, **8** was generated in 95% yield from its TFA-salt by treatment with Dowex 1 × 8–100 ion exchange resin (OH form) in methanol.

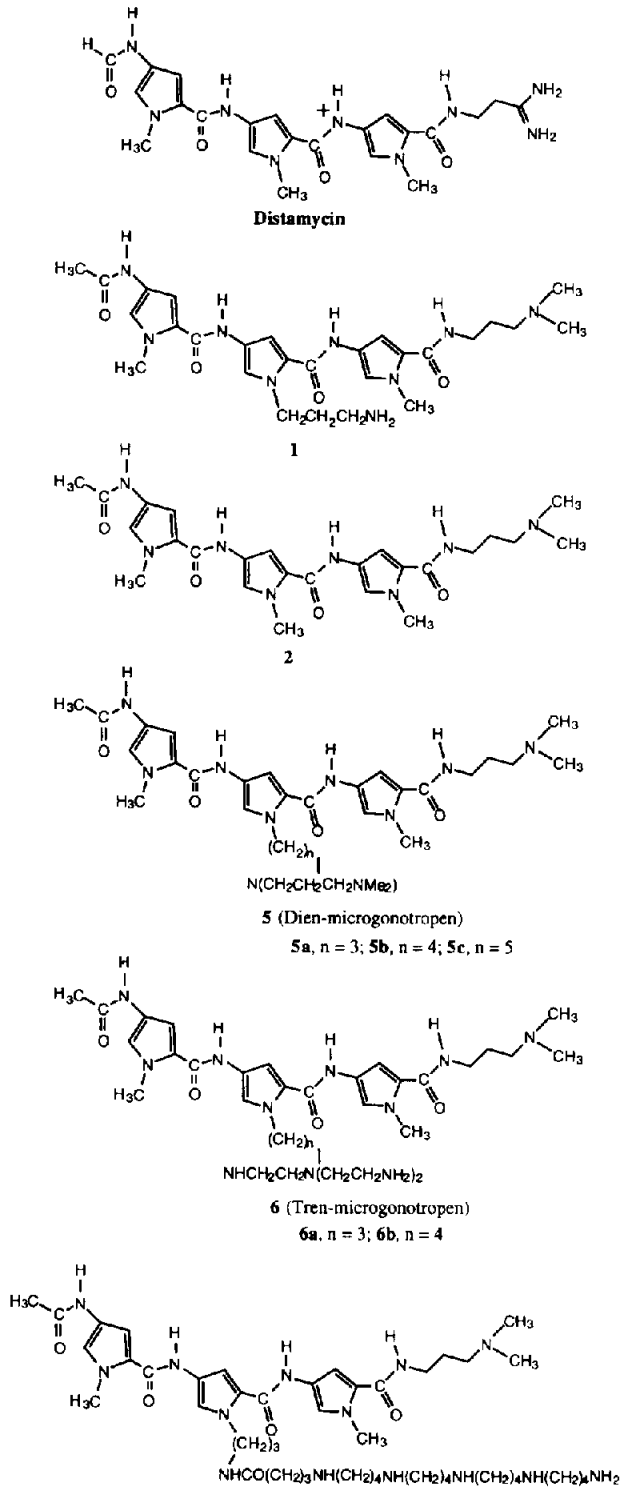


Chart 1.

The ^{1}H signals of **7** were assigned from a COSY experiment (Table 1). In DMSO-d_6 the polyamino substituent's methylene protons are heavily overlapped at 1.4 and 2.5 ppm. However, we observed separate resonances for CH_2^{R4}(1,1′) and CH_2^{R4}(2,2′,3,3′) from the heavily overlapped CH_2 resonances α to the nitrogens and middle CH_2 resonances in the close proximity to the quaternary nitrogen, respectively. The NH resonances from the polyamine substituent are partially exchanged due to the water traces present in DMSO-d_6.

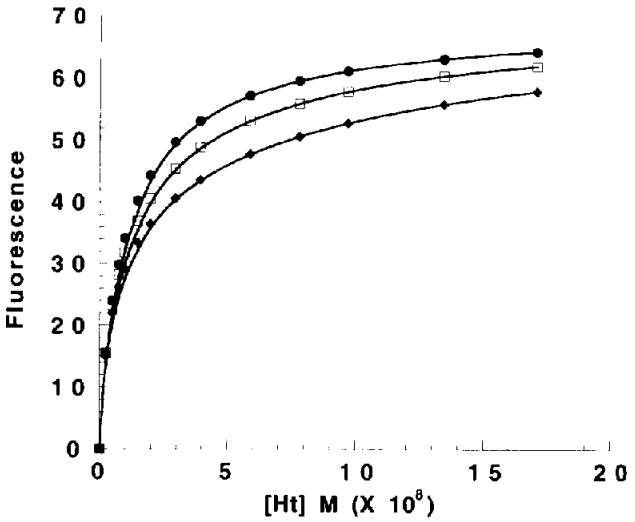


Figure 1. Representative plots of fluorescence (F, in arbitrary units) vs total Hoechst 33,258 (**Ht**) concentration at pH 7.0 and 35 °C for **7** at 5.0×10^{-9} M (●), 7.5×10^{-9} M (□) and 1.0×10^{-8} M (◆) in the presence of 5.0×10^{-9} M d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC). The theoretical curves which fit the points were computer generated by optimizing the constants in eq 1.

The assignments of all other signals are consistent with the earlier assignments of the microgonotropens.[9]

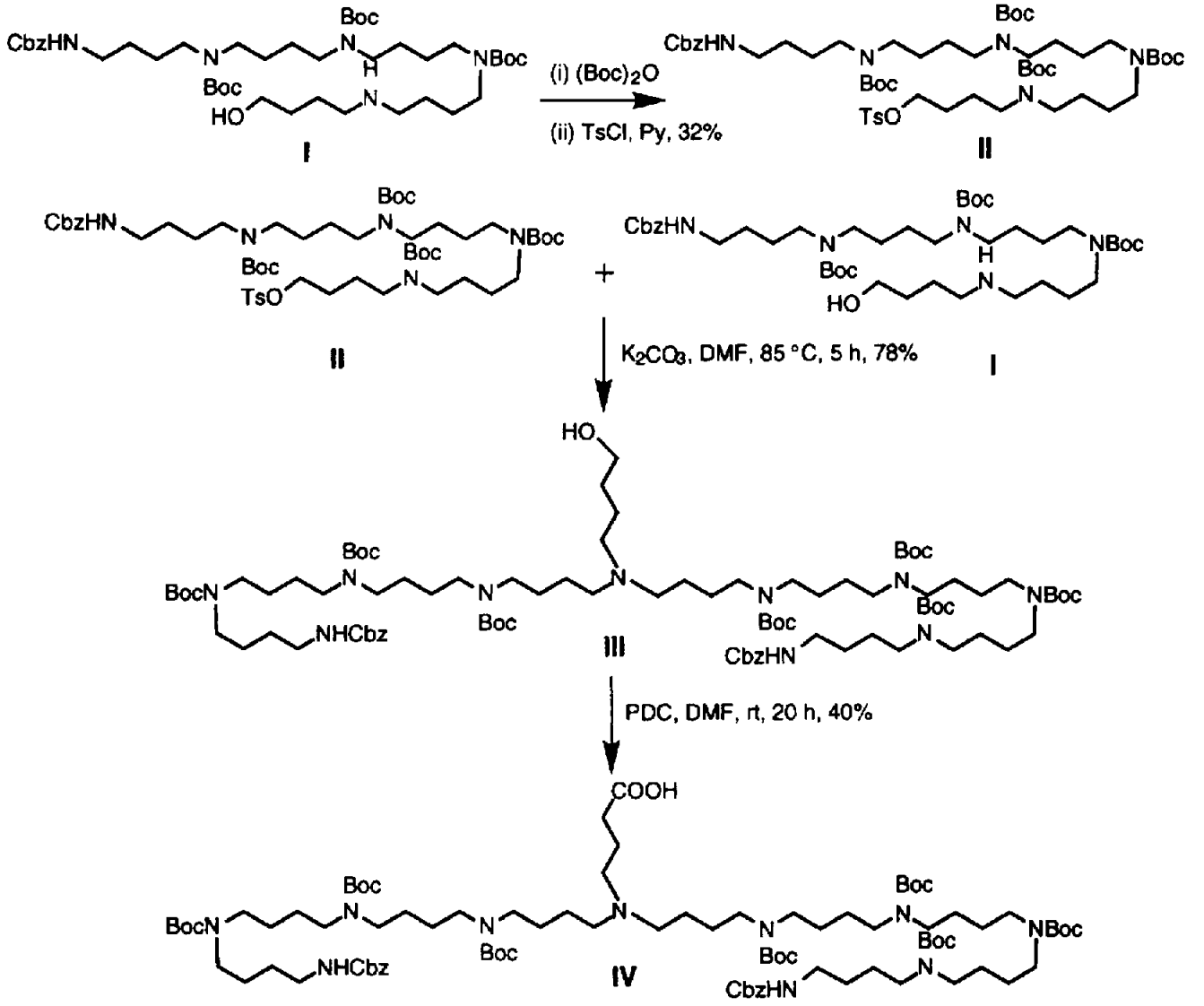
Equilibrium constants

Equilibrium constants for the association of **8** with d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC) were determined by complexing **8** (designated as **L**) to the duplex hexadecameric DNA in aqueous solutions at 35 °C (2.8 mL solutions containing 0.01 M phosphate buffer, pH 7.0, and 0.01 M KCl). The concentration of **8** was confirmed by peak integration of ^{1}H NMR resonances with resonances of an approximately equivalent but known concentration of 2,4,6-trimethyl benzoate (potassium salt). The extent of complex formation was determined by titration of d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC) with Hoechst 33258 and the increase in fluorescence due to formation of dsDNA:**Ht** complexes[10] as **Ht** competes for a common A$_3$T$_3$ minor groove binding site.

Equation 1, derived from Scheme 3, relates the fluorescence (F) to each of the equilibrium binding constants, the total fluorescence ($\sum\Phi$), the total concentrations of **8** [**L**], and Hoechst 33258 [**Ht**]. The derivation of eq 1 has been described in considerable detail.[11] The values of $K_{Ht1} = 3.75 \times 10^7$ M^{-1} and $K_{Ht2} = 1.45 \times 10^9$ M^{-1} used in this study were determined and reported previously.[3d] The equilibrium association constants calculated as best fits to the experimental data points for **8** with eq 1 are presented in Table 2. Q′ represents the internal quenching constant by **8** of fluorescence of **Ht** in the complex DNA:**Ht**:**8**. Plots of fluorescence (F) vs [**Ht**] using these constants at 5.0×10^{-9}, 7.5×10^{-9}, and 1.0×10^{-8} M **7** in the presence of 5.0×10^{-9} M d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC) are shown in Figure 1.

$$F = \frac{\sum \Phi K_{Ht1}[Ht] + (0.5K_{Ht2}[Ht_2] + 0.5K_{HtL}[L]Q')}{1 + K_{Ht1}[Ht] + K_{Ht1}K_{Ht2}[Ht]^2 + K_{Ht1}K_{HtL}[Ht][L] + K_{L1}[L] + K_{L1}K_{L2}[L]^2} \quad (1)$$

Several conclusions can be made from a comparison of the equilibrium association constants found in Table 2. Substituting the amino terminal formyl group of distamycin with an acetamido and the carboxy terminal amidine with $-CH_2CH_2CH_2N(CH_3)_2$ yields compound **2** (Chart 1) along with a 3 order of magnitude decrease in binding affinity ($K_{L1}K_{L2}$) compared to distamycin (1.0×10^{16} M^{-2}). Changing the *N*-methyl group on the central pyrrole of **2** to provide the *N*-pentaaza pentabutylamine **7** more than compensates for **2**'s decrease in binding affinity (it increases by ca. 25,000-fold). The protonated *N*-propylamine of the central pyrrole is able to electrostatically interact with the DNA's phosphates' oxyanions while an *N*-methyl group of **2** only provides van der Waals interactions with the walls of the minor groove. Elaboration of **1** by substituting the $-NH_2$ substituent by a tren-ligand provides the tren-microgonotropen-a (**6a**, Chart 1) and an increase in $K_{L1}K_{L2}$ of two orders of magnitude. Addition of a $-CH_2-$ moiety to the linker of **6a** provides **6b** and an increase in $K_{L1}K_{L2}$ over that seen in **1** by 15-fold. Adding the branched decaaza decabutylamine to the central pyrrole nitrogen of **1** provides an increase in $K_{L1}K_{L2}$ over that seen in **1** of 7-fold. The polyamines are protonated under neutral conditions and intereact with negatively charged phosphate backbone by electrostatic bonding.[12] The values of $K_{L1}K_{L2}$ for **6a** and **6b** exceed that for **8** by 13- and 8-fold, respectively. The tris(2-aminoethyl) amino moiety of **6a** and **6b** sequesters two of DNA's phosphodiester groups.[3d] The solution



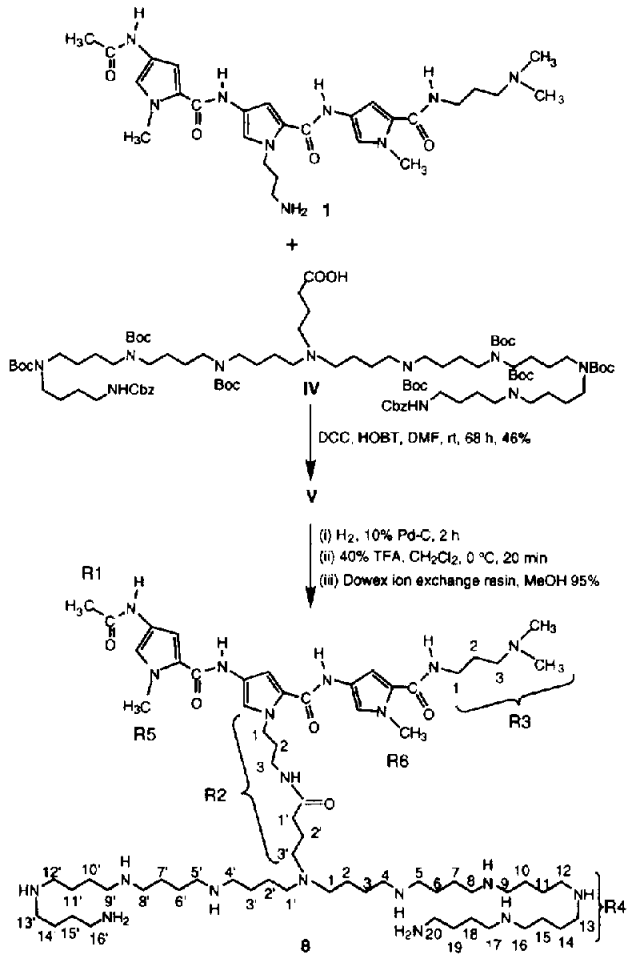
Scheme 1.

Table 1. ^{1}H Chemical shifts of **8** in DMSO-d_6 at 25 °C[a]

		R1	R2	R3	R4	R5	R6
CH_3		1.97		2.13		3.80	3.83
CH_2	(1)		4.30	3.20	3.33		
	(2)		1.80	1.62	1.20		
	(3)		3.04	2.24	1.20		
	(4)				2.50[b]		
	(6,7)				1.37[c]		
	(1′)		1.61		3.33		
	(2′)		1.23		1.20		
	(3′)		3.30		1.20		
	(4′)				2.50[b]		
	(16′,20)				1.57		
NH		9.84	7.85	8.07		9.92	9.92
NH	(1,1′)				5.00		
NH	(2,2′)				5.30		
NH	(3,3′,4)				5.60		
Pyrrole:			H3 7.33			H3 7.15	H3 7.18
			H5 7.03			H5 6.82	H5 6.82

[a]In ppm relative to TMS.
[b]The same value for CH_2 (5, 5′, 8, 8′, 9, 9′, 12, 12′, 13, 13′, 16, 17).
[c]The same value for CH_2 (6′, 7′, 10, 10′, 11, 11′, 14, 14′, 15, 15′, 18, 19).

structure of the 1:1 complex of dsDNA with **6b** has been determined earlier by 2-D NMR spectroscopy (NOESY).[9b]



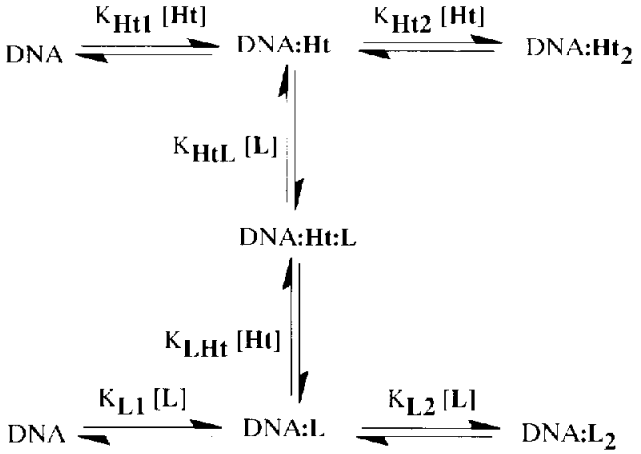
Scheme 2.

Equilibrium constants for the association of **8** with calf thymus DNA (ctDNA) were determined in aqueous solutions at 35 °C (2.8 mL solutions containing 0.01 M phosphate buffer, pH 7.0 and 0.01 M KCl). The extent of complex formation was determined by titration of a solution of ctDNA containing a known concentration of **8** with Hoechst 33258. Increase in fluorescence is due to formation of ctDNA:**Ht**, ctDNA:**Ht2**, and ctDNA:**Ht:L** complexes as **Ht** competes with **8** for a common A_3T_3 minor groove binding site. The equilibrium association constants calculated as best fits to the experimental data points when L represents **8** as in eq 1 are presented in Table 2. Plots of fluorescence (F) vs [**Ht**] using these

Table 2. Equilibrium association constants (Scheme 3) for **1**, **2**, **7**, **6a**, **6b**, **8**, and distamycin to d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC) and **8** to ctDNA

Ligand (L)	log K_{L1}	log K_{L2}	log $K_{L1}K_{L2}$	log K_{HtL}
d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC)				
2[a,b]	6.8	6.2	13.0	−1.2
7[c,d]	8.6	8.8	17.4	10.7
8[c,d]	8.2	8.8	17.1	9.9
1[c,e]	8.4	8.0	16.4	9.5
6a[a,b]	9.2	9.2	18.4	10.7
6b[a,b]	8.9	9.0	17.9	10.3
Distamycin[a,b]	7.6	8.4	16.0	8.8
		ctDNA		
8[a,f]	9.3	7.9	17.3	10.1

[a]Reactions were performed in H_2O, 0.01 M phosphate buffer, pH 7.0, and 0.01 M NaCl at 35 °C.
[b]Ref 3d.
[c]Reactions were performed in H_2O, 0.01 M phosphate buffer, pH 7.0, and 0.01 M KCl at 35 °C.
[d]Mean values are the result of experiments at 5.0×10^{-9}, 7.5×10^{-9}, 1.0×10^{-8} M in **8**.
[e]Ref 3.
[f]Mean values are the result of experiments at 3.0×10^{-8}, 9.0×10^{-8}, 1.8×10^{-7} M in **8**.



Scheme 3.

constants at 3.0×10^{-8}, 9.0×10^{-8} and 1.8×10^{-7} M **8** in the presence of 1×10^{-6} M ctDNA are shown in Figure 2. Considering the association of **8** with ctDNA, log K_{L1} is 11-fold greater than observed in the binding of **8** to hexadecameric DNA. The second association constant log K_{L2} for **8** with ctDNA is 9-fold less than that observed with hexadecameric DNA.

Biological assays

Microgonotropen **8** was assayed for its ability to inhibit formation of transcription factor/DNA complexes under cell-free conditions. Three transcription factors, TATA binding protein (TBP),[13,14] early growth response protein (EGR1)[15] and E2 factor (E2F1)[16,17] were evaluated for their relative sensitivity to **8**. Representative gel mobility shift assays shown in Figure 3 demonstrate the potential of **8** to inhibit protein/DNA complexes for each of the transcription factors (TFs). Estimates of the relative potency of **8** against each of the TFs is shown in Table 3. As we have seen (Table 2),

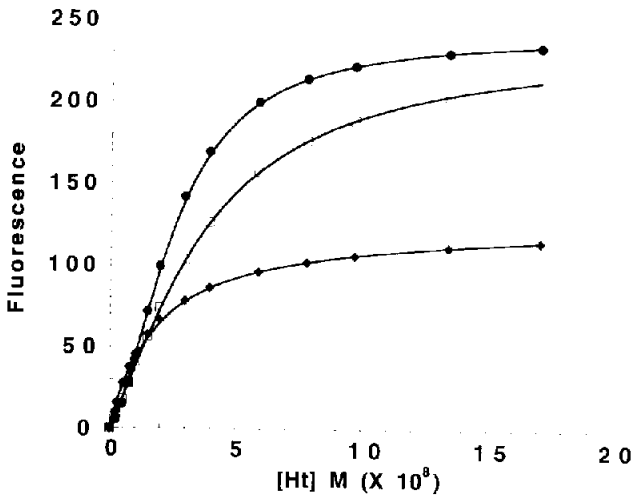


Figure 2. Representative plots of Fluorescence (F, in arbitrary units) vs total Hoechst 33,258 (**Ht**) concentration at pH 7.0 and 35 °C for **7** at 3.0×10^{-8} M (●), 9.0×10^{-8} M (○) and 18.0×10^{-8} M (◆) in the presence of 1.0×10^{-6} M ctDNA. The theoretical curves which fit the points were computer generated by optimizing the constants in eq 1.

Table 3. Inhibition by **8** of transcription factor/DNA complex formation

Transcription factor	DNA binding motifs	Relative potency[a]
TBP	AT rich	++
EGR1	GC rich	+
E2F1	AT and GC rich	+++

[a]The symbols +++, ++, and + indicate 50% inhibition of complex formation at a [**8**] = <2 nM, 2–10 nM, and ≥100 nM, respectively.

8 binds tightly to AT-rich sequences within the DNA minor groove and much of the strength of binding comes from the interaction of protonated amino functions with the phosphate backbone on the major groove site. The ability of **8** to prevent TBP complex formation with DNA (herpes simplex virus latency promoter) occurs by the competition of promoter and **8** binding in the DNA minor groove at the 5′-ATATA$_3$ consensus sequence.[16] Significant inhibition (>50%) was observed with 10 nM **8** (Fig. 3A, lane 2; Table 3). Inhibition is comparable to that obtained with the starting microgonotropen **7**, which contains the trypyrrole peptide function but lacks one of polyamine chains. In comparison the DNA minor groove binding drug distamycin required a 160 nM concentration to inhibit by 50% TBP/DNA complexes with the adenovirus major late promoter.[18]

EGR1, a zinc finger protein which binds to a GC-rich region within the DNA major groove was also tested for its sensitivity to **8**. Since EGR1/DNA interactions occur only within the major groove, inhibition of complex formation would be likely to occur via the polyamine portion of the drug which interacts with GC-rich regions of the DNA major groove. To assist binding the tripyrrole portion of the drug to the GC-rich EGR1 DNA binding site, an oligonucleotide derived from the HSVL promoter was used that contained a four base A rich flanking sequence 5′ to the GC-rich transcription factor binding site (5′-A$_4$GCGCGCGG).[19] There was no effect on EGR1/DNA complexes at drug concentrations that inhibited DNA complex formation with TBP (Fig. 3B, lane 4). However, at higher drug levels from 100 to 1000 nM, there was a loss of the normal EGR1/DNA complex and the appearance of a new lower mobility complex which is likely to be a ternary complex of drug/EGR1/DNA (Fig. 3B, lane 2 and 3; Table 3).

E2F1 is a helix loop helix transcription factor that binds to both an AT-rich sequence and to the GC-rich region (5′-T$_3$CGCGCC). E2F1 was evaluated for its sensitivity to microgonotropen **8**. The binding of E2F1 to DNA was the most sensitive to the drug as 50% inhibition of the DNA/complex was observed at ~2 nM **8** (Fig. 3C, lane 3; Table 3). It would appear that a transcription factor that shares similar features of the microgonotropen DNA binding motif is particularly vulnerable to disruption of their association with their DNA binding site.

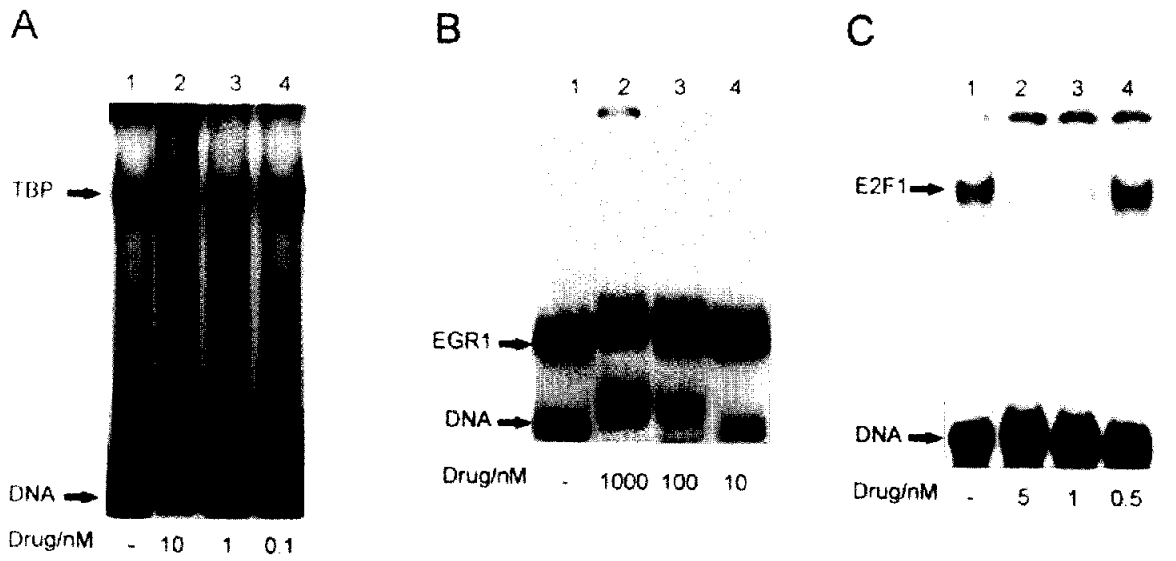


Figure 3. Representative gel mobility shift assay in the presence of **8**. The activity of **8** to inhibit DNA complex formation of transcription factors was demonstrated by gel mobility shift assays. Each of individual transcription factors, TBP (A), EGR1 (B), E2F1 (C), was added to the reaction following treatment with **8** at the indicated concentrations. The upper arrow indicates transcription factor/DNA complex and the lower arrow indicates free DNA.

Experimental

Materials and methods

Reagent grade chemicals were used without further purification unless otherwise stated. Pyridine was dried over KOH and distilled. Dichloromethane was distilled over P_2O_5. Anhydrous DMF, 4-amino-1-butanol, benzyl chloroformate, di-*tert*-butyl dicarbonate, tosyl chloride, DCC, pyridinium dichromate, 1-hydroxybenzotriazole (HOBT) were purchased from Aldrich. After treatment with 0.5 M KOH, the ion-exchange resin (Aldrich) was washed with distilled water and methanol before using. IR spectra were obtained neat on a Matteson Genesis Series FT IR. ^{1}H NMR Spectra were recorded on a Gemini-200 (Varian) or a Varian-500 spectrometer using $CDCl_3$ or DMSO-d_6. Chemical shifts are reported relative to TMS (0 ppm), coupling constants are reported in Hertz. DQ-COSY spectra were recorded on a Varian-500 spectrometer. Low-resolution mass spectra (EI and low resolution FAB (using NBA matrix)) were recorded on a VG-70 E double focusing mass spectrometer with VG-Opus/Cios data system interface. High-resolution mass spectrometry was performed at the Mass Spectrometry Facility, Department of Chemistry and Biochemistry, University of California, Los Angeles, using FAB technique and NBA matrix. Chromatographic silica gel (ICN Silica 32-60, 60 A) was used for flash chromatography and glass-backed plates of 0.25 mm silica gel 60-F_{254} (Merck) were used for thin layer chromatography. Atmospheric Presure Chemical Ionization mass spectra (APCI) were recorded at the Mass Spectrometry Facility, Department of Chemistry and Biochemistry, University of California, Los Angeles.

DNA Binding studies

The complementary hexadecameric oligonucleotides d(GGCGCA$_3$T$_3$GGCGG) and d(CCGCCA$_3$T$_3$GCGCC) were synthesized at UCSF's Biomolecular Resource Facility. Annealing and characterization procedures for the duplex hexadecamer have been described.[11,3d] Calf thymus DNA (Pharmacia) and Hoechst 33258 (Aldrich) were used without further purification. The duplex hexadecamer was maintained in a stock solution containing 0.01 M potassium phosphate buffer, pH 7.0, 0.01 M NaCl. The ctDNA was maintained in a stock solution containing 0.02 M potassium phosphate buffer, pH 7.0, 0.02 M KCl. All other stock solutions were in distilled deionized water. Stock solutions were stored on ice for the duration of a given experiment and maintained at −20 °C between experiments. In all fluorescence titrations, solutions were buffered with 0.01 M potassium phosphate buffer, pH 7.0, 0.01 M KCl (filtered through a sterile 0.2 micron Nalgene disposable filter). The final concentration of duplex hexadecamer was 5.0×10^{-9} M and the final concentration of ctDNA was 1×10^{-6} M. The concentrations of **8** used were 5.0×10^{-9}, 7.5×10^{-9}, and 1.0×10^{-8} M for experiment with hexadecamer DNA and 3×10^{-8}, 9×10^{-8}, and 1.8×10^{-7} for experiment with ctDNA. Buffered solutions (2.8 mL) containing dsDNA with/without **8** were titrated with a 3.5×10^{-6} M solution of Hoechst 33258 (**Ht**) until a final concentration of 1.8×10^{-7} M was reached. All titration volumes were measured with Gilson Pipetman microliter pipettes and disposable pipette tips. The solutions were excited at 354 nm and fluorescence emissions were measured at 450 nm using the mean value of triplicate data collections with a thermostated (35 °C) Perkin–Elmer LS-50 fluorescence spectrophotometer. The samples were continuously stirred in matched quartz cuvettes (1-cm

path length) and allowed at least 2 min to equilibrate between each titrant addition and fluorescence recording. The cuvettes were washed exhaustively with 10% HNO_3 and rinsed at least five times with distilled deionized H_2O before drying and subsequent use. Background fluorescence intensity {buffered solution of d(GGCGCA$_3$T$_3$GGCGG)/d(CCGCCA$_3$T$_3$GCGCC) and ctDNA before the addition of any **Ht**} was subtracted from each titration point to provide the corrected fluorescence intensity, F. These corrected fluorescence intensity data points were fit to theoretical curves based on eq 1 with SigmaPlot 4.1.4 (Jandel Scientific) on a Macintosh Quadra 800 computer. Since the total fluorescence ($\sum\Phi$) varied slightly from one experiment to the next, this value was determined for each experiment from a titration of the dsDNA with **Ht** in the absence of any added ligand.

Inhibition of protein binding to DNA

A mobility shift assay was used to measure the ability of microgonotropen **8** to prevent complex formation between purified transcription factors and oligonucleotides containing their DNA binding sites. Purified transcription factors, TBP and EGR1 were generously supplied by Dr Frank Rauscher, Wistar Institute, while E2F1 was provided by Dr Jane Azizkhan, Roswell Park Cancer Institute. The mobility shift assay has been described previously.[18–20] Essentially an oligonucleotide containing the consensus DNA binding site of each transcription factor was end-labeled with alpha [^{32}P]-ATP, incubated for 30 min at 30 °C followed by the addition of the appropriate transcription factor, and incubated for an additional 30 min. Subsequently, the mixture was electrophoresed in a native polyacrylamide gel. The intensities of radioisotope-labeled transcription factor/DNA complex and free DNA were measured using a Molecular Dynamics densitometer.

5,10,15,20-Pentaaza-5,10,15,20-tetratertbutyloxycarbonyl-25-benzyloxycarbonyl-icosonol *p*-toluenesulfonate (II). To a solution of **I**[5] (608 mg, 0.75 mmol) in CH_2Cl_2 (6 mL) was added dropwise di-*tert*-butyl dicarbonate (180 mg, 0.82 mmol) at 0–5 °C (ice bath). The mixture was stirred for 30 min and solvent was removed under reduced pressure. The residue was dissolved in pyridine (5 mL) and *p*-toluenesulfonyl chloride (214 mg, 1.1 mmol) was added at 0–5 °C. The mixture was stirred for 3 h at the same temperature, poured in ice water (10 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layer was dried (Na_2SO_4) and solvent removed under reduced pressure. The residue was purified by chromatography (silica, 15% EtOAc:CH_2Cl_2) to give **II** (254 mg, 32%) as a viscous liquid. ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 36H, -C(CH_3)$_3$), 1.41–1.60 (m, 20H, -CH_2-CH_2-), 2.44 (s, 3H, Ar-CH_3), 3.14 (m, 16H, -CH_2-N(Boc)-), 4.02 (t, J = 6.0 Hz, 2H, -CH_2-OTs), 5.09 (s, 2H, -OCH_2-Ar), 7.32–7.35 (m, 7H, ArH), 7.78 (d, J = 8.4 Hz, 2H, ArH); IR (film) 2968, 1685, 1475, 1420, 1363, 1270, 1172, 734 cm^{-1}; LRMS (FAB) m/z 1062(M+H$^+$).

Compound 7. To a solution of **II** (193 mg, 0.18 mmol) in DMF (1.0 mL) was added I[5] (220 mg, 0.27 mmol) and K_2CO_3 (50 mg, 0.36 mmol). The mixture was heated at 90 °C for 5 h under argon atmosphere, cooled to the room temperature and poured into ice water (5 mL). The solution was made alkaline (pH 12) by adding 5% aqueous KOH solution and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layer was washed with brine (1 × 3 mL), dried (Na_2SO_4) and solvent removed under reduced pressure. The residue was purified by chromatography (silica, 15% EtOAc:CH_2Cl_2, then CH_2Cl_2:MeOH:Et_3N, 17:2:1) to give **III** (362 mg, 78%) as a clear viscous oil. ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 63H, -C(CH_3)$_3$), 1.35–1.7 (m, 40H, -CH_2-CH_2-), 3.13 (m, 34H, -CH_2-N(Boc)- and N-CH_2), 3.54 (t, J = 6.3, 2H, -CH_2OH), 5.07 (s, 4H, -OCH_2-Ar), 7.33-7.35 (m, 10H, ArH); IR (film) 3347, 2933, 1685, 1533, 1419, 1369, 1247, 1166, 738 cm^{-1}; LRMS (FAB) m/z 1699 (M+H$^+$).

Compound IV. To a solution of **III** (362 mg, 0.21 mmol) in DMF (3 mL) was added pyridinium dichromate (489 mg, 1.3 mmol). The mixture was stirred at room temperature for 19 h, poured into ice water (10 mL) and extracted with ether (3 × 5 mL). The combined organic layer was washed with brine (1 × 5 mL), dried (Na_2SO_4), and solvent removed under reduced pressure. The residue was purified by chromatography (silica, 7% MeOH:CH_2Cl_2) to give **IV** (133 mg, 40% yield). ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 63H, -C(CH_3)$_3$), 1.34–1.65 (m, 40H, -CH_2-CH_2-), 3.14 (m, 34H, -CH_2-N(Boc)- and N-CH_2), 5.07 (s, 4H, -OCH_2-Ar), 7.32–7.35 (m, 10H, ArH); IR (film) 3345, 2933, 1692, 1469, 1419, 1369, 1247, 1166, 875 cm^{-1}; LRMS (FAB) m/z 1712(M+H$^+$).

Compound V. To a solution of **IV** (108 mg, 0.06 mmol) in DMF (900 μL) was added **1** (45 mg, 0.08 mmol), HOBT (13 mg, 0.09 mmol) followed by DCC (17 mg, 0.08 mmol). The mixture was stirred at room temperature for 67 h and solvent removed under reduced pressure. The residue was purified by chromatography (silica, CH_2Cl_2:MeOH:Et_3N, 18:1.5:0.5 then, CH_2Cl_2:MeOH:Et_3N, 17:2:1) to give **6** (65 mg, 46% yield). ^{1}H NMR (DMSO-d_6) δ 1.35 (s, 63H, -C(CH_3)$_3$), 1.34 (m, 40H, -CH_2-CH_2-), 1.65 (m, 2H, -CH_2CH_2CONH), 1.80 (m, 2H, -CH_2NHCO), 1.97 (s, 3H, -$COCH_3$), 2.16 (s, 6H, -N(CH_3)$_2$), 2.99 (m, 4H, -CH_2NHCbz), 3.08 (m, 32H, -CH_2N(Boc)-CH_2-), 3.18 (m, 2H, -CONHCH_2), 3.79, 3.82 (2s, 6H, pyrrole -NCH_3), 4.30 (t, J = 2.6 Hz, pyrrole N-CH_2), 4.94 (s, 4H, -OCH_2Ar), 6.82 (d, J = 0.8 Hz, 1H, pyrrole ArH), 6.86 (s, 1H, pyrrole ArH), 7.02 (d, J = 0.9 Hz, 1H, pyrrole ArH), 7.14 (s, 1H, pyrrole ArH), 7.18 (s, 1H, pyrrole ArH), 7.27–7.32 (m, 11H, ArH and pyrrole ArH), 7.80 (t, J = 2.2 Hz, 1H, -NHCO), 8.08 (t, J = 2.2 Hz, 1H, -NHCO), 9.83 (s, 1H, Pyr-CONH-Pyr), 9.92 (s, 2H, Pyr-CONH-Pyr); IR (film) 2923, 2854, 1629, 1579, 1456, 1413, 1255, 1159, 833 cm^{-1}; LRMS (FAB) m/z 2248 (M+H$^+$).

Compound 8. To a solution of **V** (46 mg) in methanol (1.2 mL) was added two drops of Et_3N and stirred in

hydrogen atmosphere for 3 h with 10% Pd-C catalyst (12 mg). The slurry was filtered through celite and solvent removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (1.8 mL), cooled to 0–5 °C, and TFA (1.2 mL) was added dropwise. The mixture was stirred for 20 min and solvent removed under reduced pressure. The residue was dissolved in methanol (30 mL), stirred with Dowex 1 × 8–100 ion-exchange resin OH form at room temperature for 30 min. The resin was removed by filtration, and the filtrate was evaporated to give **8** (25 mg, 95% yield; purity by ^{1}H NMR ~98%). ^{1}H NMR refer to Table 1; IR (film) 3405, 2354, 1565, 1471, 1417, 1165, 1047, 862, 745 cm^{-1}; APCI(MS) *m/z* 1279.

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