Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3261–3266

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF A FLUORESCENT MICROGONOTROPEN (FMGT-1) AND ITS INTERACTIONS WITH THE DODECAMER d(CCGGAATTCCGG)

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Received 2 August 1999; accepted 7 September 1999

Abstract: A new type of microgonotropen that fluoresces upon binding to dsDNA has been synthesized. FMGT-1, an analogue of the minor groove binder Hoechst 33258, is functionalized with a polyamine chain capable of interacting with the phosphate backbone of DNA. Binding studies indicate that FMGT-1 binds more tightly to dsDNA than the parent compound Hoechst 33258.

Reagents capable of sequence-selective recognition of DNA, particularly small organic compounds capable of binding to the minor groove of B-DNA, have drawn tremendous attention due to their known biological activity and diverse medicinal uses.[1-10] Although the mechanism of minor groove binders (MGBs) biological activity is still under investigation, recent in vitro studies indicate that MGBs may influence the regulation of gene expression as a result of affecting the binding of regulatory proteins to their consensus binding sites.[11-18]

Microgonotropens (MGTs) are a novel class of minor groove binding ligands that consist of an A +T - selective DNA minor groove binding tripyrrole peptide and polyamine chains attached to the central pyrrole that extend drug contact into the DNA major groove (Chart 1).[12,14,19,20] Interaction of the MGTs polyamine moiety with the phosphate backbone leads to extremely tight binding and results in bending of the DNA. MGTs are extraordinarily effective inhibitors of the association of the transcription factor E2 factor 1 (E2F1), to its DNA promoter element.[12] The most active MGT, MGT-6a, was three orders of magnitude more effective than distamycin. These studies demonstrate that the sequence and groove preference of MGBs and transcription factors (TF) are important determinants for inhibition of TF–DNA complex formation.[12,13]

Our goal is to design a new class of MGTs to further investigate the structure–activity relationships between DNA binding strengths, TF inhibition, and phosphate backbone interactions. A minor groove binding vehicle was designed that can be readily functionalized and was based upon the widely used DNA fluorophore Hoechst 33258 (Ht), which is known to bind A + T rich sequences of dsDNA.[19-25] Its fluorescent properties allows for straight forward determination of binding stoichiometry and DNA–Drug association constants through fluorescence spectroscopy. This report describes the synthesis of the first fluorescent microgonotropen (FMGT-1) and its interactions with the annealed duplex of the self-complimentary dodecamer (5'-CCGGAATTCCGG) (**12**).

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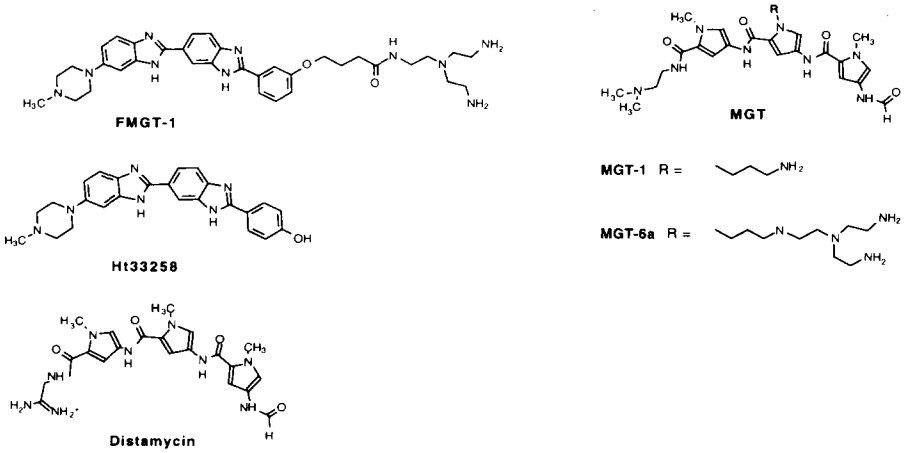
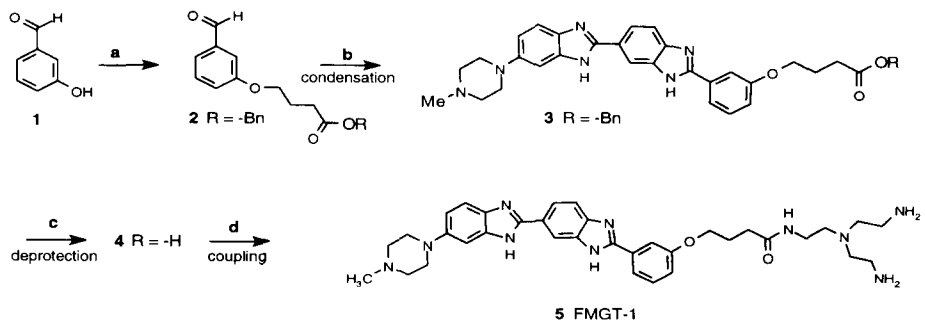
PII: S0960-894X(99)00599-5

CHART 1.



Synthesis. Scheme 1 outlines the synthesis of FMGT-1. Mitsunobu[26] type reaction of 3-hydroxybenzaldehyde (**1**) with 4-hydroxybutyrate[27] gave **2**. Condensation of the appropriate *ortho* diamine with **2** gave **3**, which following deprotection of the carboxylic acid derivative by base hydrolysis yielded **4**.[28] The free acid derivative (**4**) was designed to be a synthetic building block for making any number of FMGTs. It is similar in structure to Ht but with a short phenol ether arm at the *meta* position that terminates with a free carboxylic acid, which may be functionalized through a peptide coupling reaction. Compound **4** was substituted at the *meta* position to help ensure that the polyamine moiety would be able to interact with the phosphate backbone of dsDNA.[29] Tren amine, found to make the most effective polyamine moiety for the previous class of tripyrrole peptide microgonotropens (MGT-6a), was then coupled to **4** using Py-Bop® to give FMGT-1.[30,31]

Scheme 1.



(a) 4-hydroxybutyrate, $(Ph)_3P$, DEAD, DCM; (b) 2-(3,4-Diaminophenyl)-6-(4-methyl-1-piperazinyl)benzimidazole, nitrobenzene, 120 °C; (c) K_2CO_3, DMF, H_2O, 90 °C; (d) PyBOP, tris(2-aminoethyl)amine, DMF.

Binding. Fluorescence spectroscopy was used to compare both the stoichiometry and the equilibrium binding constants for the association of FMGT-1 and Ht to **12**.[32] Stoichiometry of binding was found to be 1:1 (ligand:**12**) for both FMGT-1 and Ht.[33] Stoichiometry was determined by titrating 50 to 100 nM initial concentrations of **12** such that fluorescence intensity increased linearly with the addition of ligand. After saturation of binding sites fluorescence intensity was found to remain constant.

Association constants were determined by monitoring fluorescence intensity upon titration of 1 to 3 nM of FMGT-1 or Ht with **12** (Figure 1).[34] It was found that at these concentrations a linear relationship did not occur between fluorescence signal and addition of titrant. Association constants (K_a) for FMGT-1 and Ht were then calculated by curve fitting analysis of the isothermal binding curves using Equations 1 and 2.[35]

Eq. 1 $$F = \sum \Phi_f \left(\frac{K_a[DNA]}{1 + K_a[DNA]} \right)$$, where $[DNA]$ is defined by Eq. 2

Eq. 2 $$[DNA] = 0.5 \left(-b + \sqrt{b^2 + \frac{4[DNA]_t}{K_a}} \right)$$, where $b = [ligand]_t - [DNA]_t + 1/K_a$

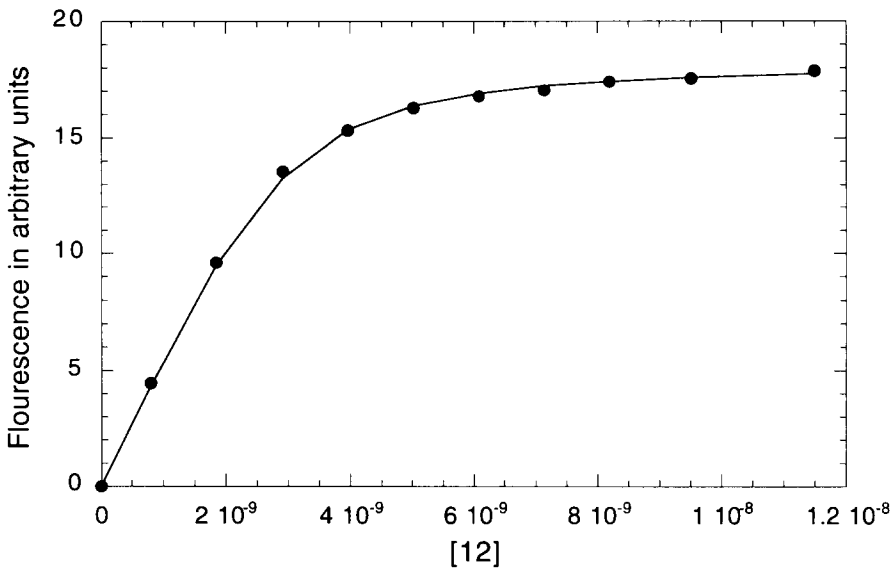


Figure 1. Isothermal binding curve for the titration of 3 nM FMGT-1 with **12**. Curve fitting and determination of binding constants was carried out using nonlinear least-squares procedures.

Table 1 lists the calculated association constants (K_a) for FMGT-1 and Ht and thermal melting (ΔT_m) values for FMGT-1, Ht, and MGT-6a with the **12**.[36,37] Binding constants suggest a five fold increase in binding affinity for FMGT-1 relative to Ht. Thermal melting studies also indicate extremely strong binding by FMGT-1 with a ΔT_m value 7 °C greater than for Ht and equal to that of the strongest binding tripyrrole peptide MGT-6a. Together, K_a and ΔT_m values show FMGT-1 to bind **12** more tightly than Ht and as tightly as MGT-6a.

Table 1. Ligand-**12** association constants (K_a) and ΔT_m values.

Drug	K_a (M^{-1})[a,b]	ΔT_m [c]
FMGT-1	$4.8 \times 10^9 \pm 7 \times 10^8$	30 °C
Ht[35]	$1.3 \times 10^9 \pm 3 \times 10^8$	23 °C
MGT-6a	–	30 °C

[a]The association constants given are the average of six trials. [b]Error values were determined by calculating the standard deviations for the six trials. [c]The melting temperature of **12** was found to be 39 °C.

Conclusions. In previously published reports, seven MGTs based upon the tripyrrole peptide motif with polyamine arms differing in overall size, branching, and number of positive charges, were tested for their ability to inhibit the transcription factor E2F1.[12] All the MGTs tested in those studies were more effective at inhibiting E2F1 binding than distamycin. Even MGT-1 which carries only a single positive charge in its polyamine chain had a relative inhibitory reactivity five times greater than distamycin. The most effective microgonotropen, MGT-6a, which carries 4 positive charges in its polyamine chain, showed a relative reactivity 4470 times greater than distamycin. Those results showed a direct correlation between the DNA binding constant and relative inhibitory reactivity. Due to the large DNA binding strength, FMGT-1 appears to have great potential as an effective TF inhibitor. Other FMGTs are currently being developed in this laboratory towards the goal of further investigating the relationship between polyamine structure and the ability to interact with the phosphate backbone.

Acknowledgment: This project was supported by the National Institute of health (3 R37 DK09171-3451).

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31. ^{1}H NMR (D_2O) for FMGT-1: ∂ = 2.02 (m, 2H, -CH_2-), 2.45 (t, J = 7.34 Hz, 2H, -CH_2-CONHR), 2.74 (broad singlet, 2H, -C-CH_2-NR_2), 2.90 (broad singlet, 4 H, -RN(CH_2-C-N)$_2$), 3.00 (singlet, CH_3-N-), 3.0–3.1 (m, (-CH_2)$_2$-NMe, piperazine), 3.13 (broad singlet, -RN(C-CH_2-N)$_2$), 3.36 (triplet, J = 6.1 Hz, 2H, O=C-N-CH_2-), 3.6–3.8 (m, 4H, -(CH_2)$_2$N-Ar, piperazine), 3.82 (broad singlet, 2H, AR-O-CH_2-), integration of all 6 peaks between 6.74 and 7.55 gives 11 protons, this accounts for 1, -(O=C)NH-, and 10, Ar H, protons, 6.74 (broad singlet), 6.90 (broad singlet), 7.07 (broad multiplet), 7.28 (broad singlet), 7.41 (broad singlet), 7.55 (broad multiplet) ppm; HRMS (FAB) *m/e* 639.388346 (calcd for C_{37} H_{49} N_7 O_3 (M + H^+) 639.390093)

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33. The stoichiometry of binding for Ht to **12** has been previously found to be 1:1 (refs 23 and 24).

34. Isothermal binding curves were performed using fixed concentrations of ligand ranging from 1 to 3 nM and titrating with **12**. In some instances a fixed concentration of **12** was titrated with Ht. This was found to have no significant effect on the calculated binding constant. In all cases the ligand was excited at 354 nm and emission intensity was monitored at 350 nm and 375 nm for Ht and FMGT-1, respectively. All buffers used were 0.01 M potassium phosphate and 0.01 M NaCl, pH values were either 6.95 or 7.02. All measurements were carried out at 24 °C. Ht and FMGT-1 concentrations were quantified by NMR spectroscopy with an internal standard. Concentrations of **12** were measured spectroscopically using ϵ_{260} = 1.9×10^5.

35. Where $\sum\Phi_f$ is the maximum fluorescence intensity, $[Ligand]_t$ is the concentration of FMGT-1 or Ht, and $[DNA]_t$ is the total concentration of **12** added at each titration point.

36. The equilibrium association constant for the binding of Ht to dsDNA given here is slightly larger than previously reported by other laboratories using **12**, 3.2×10^8 M(duplex)$^{-1}$ and 3.15×10^8 M (duplex)$^{-1}$ (refs 23 and 24, respectively). These differences may be explained by different experimental conditions, particularly differences in ionic strength.

37. Thermal denaturations were carried out by monitoring the absorption at 260 nm between 15 °C and 95 °C at a scan rate of 0.2 deg. C minute^{-1} using 0.01 M potassium phosphate and 0.01 M NaCl, pH = 6.95 buffer. Ligand to **12** ratios were 1.2 in all cases. All melting temperatures were determined using the first derivative of the thermal denaturation spectra.