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Facile Synthesis of a Fluorescent Deoxycytidine Analogue Suitable for Probing the RecA Nucleoprotein Filament

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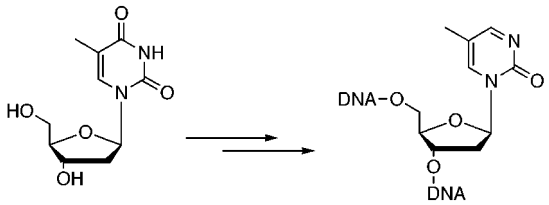
ABSTRACT



We report the synthesis of the fluorescent 2′-deoxycytidine analogue 5-methylpyrimidin-2-one nucleoside, its incorporation at three specified sites in a single 60-nucleotide DNA molecule, and the use of its total and polarized intrinsic fluorescence to characterize RecA–DNA complexes.

The repair of damaged DNA is essential to the maintenance of heritable genetic information. The *Escherichia coli* RecA protein plays indispensable roles in the detection of and response to genomic DNA damage, and its study has provided a paradigm for understanding recombinational DNA repair in all phylogenetic kingdoms.[1] The initiating signal in vivo is single-stranded DNA (ssDNA) which is the substrate upon which a RecA filament forms (Figure 1). A combination of in vivo and in vitro studies suggests that this nucleoprotein filament is essential for directing subsequent DNA repair. To address fundamental issues concerning the initiation of DNA repair processes, the key RecA−ssDNA intermediate must be understood.

We report the synthesis and characterization of oligodeoxyribonucleotides (ODNs) containing an intrinsically fluorescent analogue of 2′-deoxycytidine (5-methylpyrimdin-2-one nucleoside, **m^{5}K**) incoroporated at multiple positions. The total and polarized fluorescence emission signals of these ODNs allow RecA−single-stranded-DNA (RecA-ssDNA) filaments to be probed directly. To our knowledge, this is the first demonstration that such intrinsically fluorescent

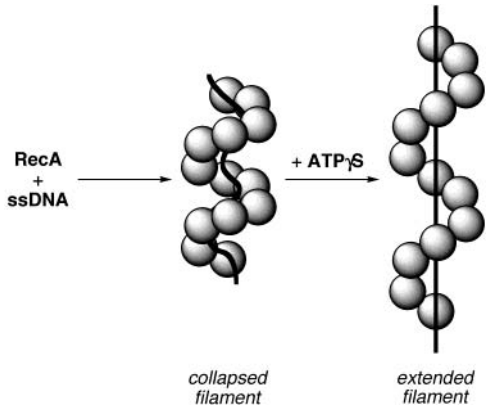


Figure 1. Cartoons depicting the collapsed and extended RecA−ssDNA complexes formed in the absence and presence of ATPγS, respectively.

(1) Roca, A. I.; Cox, M. M. *Prog. Nucleic Acid Res. Mol. Biol.* **1997**, *56*, 129−223.

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synthetic DNAs can be used to characterize definitively the assembly of the RecA−ssDNA complexes that initiate DNA repair responses.

A wealth of low-resolution structural information indicates that the helical RecA nucleoprotein filament in the absence of a cofactor (or with ADP) is "collapsed" (Figure 1), while an "extended" filament (50% increase in the helical pitch) is observed with nonhydrolyzable analogues of ATP.[2] In addition, the RecA protein filament comprises at least two distinct DNA binding sites, each of which can accommodate 3−4 nucleotides (nts) per RecA monomer. The active, repair-initiating filament contains ssDNA bound at only one site (site I) with an overall DNA−protein stoichiometry of 3 nts per monomer. At a minimum, any solution-phase method to analyze the filaments must recapitulate these observations. Biochemical assays such as those based on the RecA ATP hydrolysis activity have been traditionally used to study the RecA·ATP·DNA filament. However, it is not possible to study the collapsed filament by such a method. Thus, there are few definitive structural and functional studies which directly compare the collapsed and extended RecA filaments.

Spectrofluorometry provides a powerful method for monitoring RecA−DNA filaments in real time. Unfortunately, the natural DNA bases are only minimally luminescent[3] and the RecA tryptophans do not serve as reporters for DNA binding.[4] The use of extrinsic fluorophores such as fluorescein is not optimal because of the potential for the reporter moiety to influence the interactions under study,[5] leading to an abnormal DNA−protein stoichiometry.[6] Moreover, polynucleotides containing 1,N^6-ethenoadenine (ϵA), one of the most commonly used fluorsecent base replacements, show a stoichiometry of 1:6 resulting from two DNA molecules binding RecA.[7] However, the natural bases of DNA can be replaced with isomorphic fluorescent analogues. In particular, the adenine analogue 2-aminopurine (2AP) has been used in an extensive variety of biochemical experiments. Nevertheless, DNA-containing single-site 2AP substitutions was not suited to the quanitative characterization of RecA−DNA complexes.[8,9] When attempting to overcome this problem by using 2AP at multiple positions within a single ODN, we have observed the significant accumulation of non-full-length oligomers during both synthesis and storage, presumably as a result of the hydrolytic proclivity of the 2AP heterocycle.[10]

The report[11] of ODNs comprised exclusively of pyrimidin-2-one nucleotides led us to consider the properties and synthesis of **m^{5}K**.

Nucleoside **m^{5}K** possesses high intrinsic fluorescence[12] and is, formally, a conservatively modified derivative of 2′-deoxycytidine (Scheme 1). The fluorescence properties of the 5-methylpyrimdin-2-one heterocycle are sensitive to its microenvironment and can serve as as a reporter for DNA structural dynamics.[12] On the basis of emission changes previously described for ϵA-labeled DNA,[13] we anticipated that RecA binding to ssDNA containing **m^{5}K** would produce an emission increase. Importantly, this nucleoside analogue is synthetically accessible from thymidine (dT).

Scheme 1. Structures of **m^{5}K** and ODNs

5′-(N)$_m$-TAGCT**x**CTTCGGTCCTC**y**GATCGTTGT**x**AG-(N)$_m$-3′

ODN **30a** (*m* = 0; **x** = **C**; **y** = **m^{5}K**)	ODN **30b** (*m* = 0; **x**, **y** = C)
ODN **60a** (*m* = 15; **x**, **y** = **m^{5}K**)	ODN **60b** (*m* = 15; **x**, **y** = C)

The first syntheses of pyrimindin-2-one nucleosides were effected in low yield by direct reduction of the corresponding thymidine or uridine derivative with sodium amalgam.[14] The approach was later improved, and the synthesis of **m^{5}K** was achieved via oxidation of 4-hydrazinopyrimidinone nucleosides and rearrangement (with extrusion of dinitrogen) of the intervening diazene.[15] In our hands, the adaptation of the latter approach to larger scale provided several difficulties stemming from the need for robust protecting groups during the oxidation of 4-hydrazinopyrimidinone by Ag_2O in refluxing aqueous dioxane (or EtOH). Thus, we were led to investigate alternative routes inspired by the mild, homogeneous oxidation of monosubstituted hydrazines.[16] The opportunity for further improvement was afforded by the reported ability of 4-triazolopyrimidinone nucleosides related

(2) Egelman, E. H. *J. Mol. Biol.* **2001**, *309*, 539−542.
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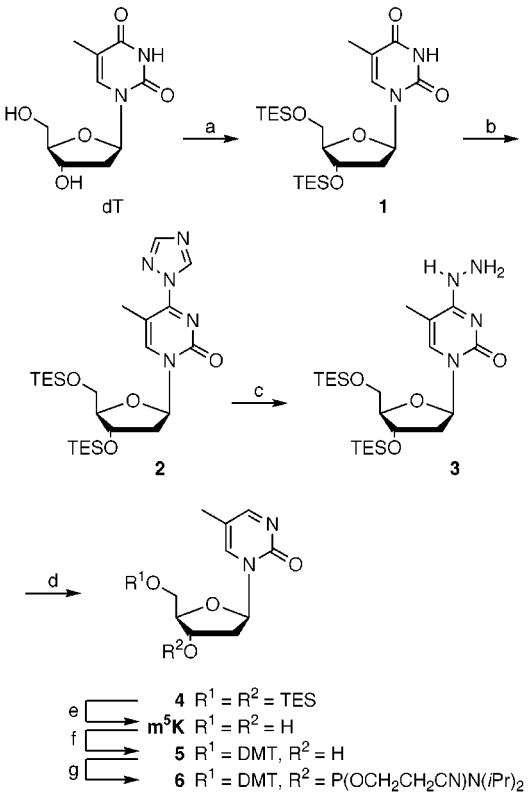
(7) Menetski, J. P.; Kowalczykowski, S. C. *J. Mol. Biol.* **1985**, *181*, 281−295. Zlotnick, A.; Mitchell, R. S.; Steed, R. K.; Brenner, S. L. *J. Biol. Chem.* **1993**, *268*, 22525−22530.
(8) 2AP is characterized by a relatively low emission quantum yield and excitation and emission spectra that substantially overlap those of RecA. These limitations are exacerbated by the fact that RecA protein also causes a significant background problem from light scattering.
(9) A single 2AP substitution in dsDNA *can* cause local structural and dynamic perturbations (Nordlund, T. M.; Xu, D.; Andersson, S.; Nilsson, L.; Rigler, R.; Gräslund, A.; McLaughlin, L. W.; Gildea, B. *Proc. SPIE* **1990**, *1204*, 344−353).
(10) Fujimoto, J.; Nuesca, Z.; Mazurek, M.; Sowers, L. C. *Nucleic Acids Res.* **1996**, *24*, 754−759.
(11) Zhou, Y.; Ts'o, P. O. P. *Nucleic Acids Res.* **1996**, *24*, 2652−2659.
(12) Wu, P.; Nordlund, T. M.; Gildea, B.; McLaughlin, L. W. *Biochemistry* **1990**, *29*, 6508−6514.
(13) ϵA-Labeled DNA shows the following trend in emission intensities: free DNA < RecA-DNA < RecA-ATPγS-DNA (Cazenave, C.; Toulmé, J. J.; Hélène, C. *EMBO J.* **1983**, *2*, 2247−2251).
(14) Laland, S. G.; Hanssen, G. S. *Biochem. J.* **1964**, *90*, 76−81. Helgeland, L.; Tipson, R. S. *Biochim. Biophys. Acta* **1964**, *87*, 353−355.
(15) (a) Gildea, B.; McLaughlin, L. W. *Nucleic Acids Res.* **1989**, *17*, 2261−2281. (b) Connolly, B. A.; Newman, P. C. *Nucleic Acids Res.* **1989**, *17*, 4957−4974.
(16) See: Myers, A. G.; Finney, N. S.; Kuo, E. Y. *Tetrahedron Lett.* **1989**, *30*, 5747−5750, and references therein.

Scheme 2[a]



[a] (a) TESCl, pyridine; (b) 1,2,4-triazole, Et_3N, $POCl_3$, CH_3CN; (c) NH_2NH_2, 1,4-dioxane; (d) PTAD, 1,4-dioxane; (e) AcOH/THF/H_2O (4/4/1, v/v/v); (f) DMTCl, pyridine; (g) β-cyanoethyldiisopropylphosphorochloridite, DIPEA, CH_2Cl_2.

to **2** (Scheme 2) to undergo facile nucleophilic addition−elimination reactions.[17] Thus, we envisioned that an improved synthesis of **m^{5}K** would proceed via the intermediacy of **2**, followed by the displacement of triazole with hydrazine and mild, homogeneous oxidation of the resulting 4-hydrazinopyrimidinone nucleoside **3**.

The requisite 4-triazolopyrimidinone nucleoside (**2**), appropriately protected as the di-*O*-triethylsilyl ether, is available in 65% yield from dT following etherification of the dT sugar hydoxyls and subsequent transformation at *O*4.[17] The triazole group of **2** was directly substituted by hydrazine under mild conditions, and the intermediate (**3**) was oxidized using *N*-phenyltriazolinedione (PTAD) without purification to give the protected nucleoside **4** in greater than 50% yield in two steps from **2**. The triethylsilyl groups were readily removed by AcOH in aqueous THF to afford fluorescent nucleoside **m^{5}K** in 81% yield. This synthetic approach provides an efficient method for large-scale production of **m^{5}K**. Importantly, the key intermediacy of **2** opens up the possibility that other milder oxidizing agents might be employed. Ultimately, ODNs containing 4-triazolopyrimidinone could be converted to 5-methyl-2-pyrimidinone on the solid support following automated solid-phase synthesis by using mild displacement−oxidation conditions.[18]

(17) Xu, Y.-Z.; Zheng, Q.; Swann, P. F. *J. Org. Chem.* **1992**, *57*, 3839−3845.

Synthesis of a monomer suitable for automated, solid-phase DNA synthesis was initiated by protection of the 5′-hydroxyl of **m^{5}K** as the 4,4′-dimethoxytrityl (DMT) ether. **5** was treated with 2-cyanoethyldiisopropylphosphorochloridite in the presence of Hünig's base in dry methylene chloride to give the desired final phosphoramidite **6**. This monomer was incorporated into ODNs **30a** and **60a** using standard automated DNA synthesis protocols in quantitative yield. Following deprotection and purification, analysis of nucleoside composition, capillary electropherograms, and MALDI-TOF mass spectrometric data confirmed that bona fide **m^{5}K** was present in the desired position(s) within each ODN.

The RecA−DNA complexes described here involve a 60mer ODN containing nucleoside **m^{5}K** at three positions (ODN **60a**, Scheme 1).[19] The emission of the fluorophore within the ODN is quenched ca. 80% relative to the free nucleoside,[20] presumably due to base stacking interactions.[12] An enhancement of the emission is observed upon RecA addition to ODN **60a** in the absence of ATPγS (Figure 2),

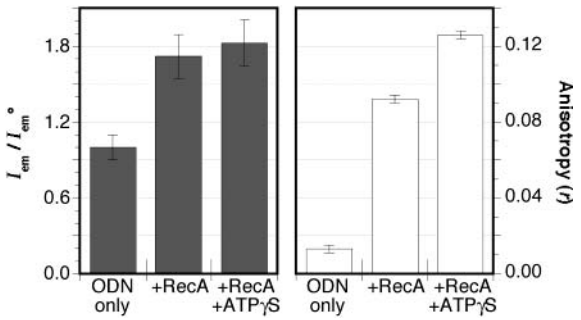


Figure 2. Normalized emission intensities ($I_{em}/I_{em}°$) and fluorescence anisotropies ($r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$) collected for **60a** alone (ODN) or in the presence of either RecA (+ RecA) or RecA and ATPγS (+ RecA + ATPγS). Fluorescence data were collected using 50 nM **60a** and excitation and emission wavelengths of 315 and 385 nm, respectively, with spectral bandwidths of 4 nm. See Supporting Information for details.

providing a direct spectroscopic measure of RecA−DNA complex formation. In contrast, there is no signal change when RecA binds nonfluorsecent ODN **60b**, establishing the absence of interference from the tryptophan fluorescence background. Although we anticipated that the DNA fluorescence would change upon association with the RecA·ATPγS filament, the emission intensities of the complexes

(18) For examples of this "convertible nucleoside" approach, see ref 17 as well as the following: MacMillan, A. M.; Verdine, G. L. *J. Org. Chem.* **1990**, *55*, 5931−5933. Gao, H.; Fathi, R.; Gaffney, B. L.; Goswami, B.; Kung, P.-P.; Rhee, Y.; Jin, R.; Jones, R. A. *J. Org. Chem.* **1992**, *57*, 6954−6959. Kim, S. J.; Stone, M. P.; Harris, C. M.; Harris, T. M. *J. Am. Chem. Soc.* **1992**, *114*, 5480−5481.

(19) The ODN sequences used here were derived from those capable of exhibiting RecA−DNA pairing activitiy in the presence of ATPγS (Podyminogin, M. A.; Meyer, R. B.; Gamper, H. B. *Biochemistry* **1995**, *34*, 13098−13108). As a control we performed a spectroscopic ATP hydrolysis assay to show that these ODNs were competent for activitating the RecA protein (details are available in the Supporting Information).

(20) Details and graphics are available in the Supporting Information.

in the presence of ATPγS are not significantly different from those of the complexes formed in its absence. Thus, for these particular DNA sequences, the steady-state fluorescence signals of **m^{5}K** display only modest differences between the collapsed and extended RecA−DNA complexes. The lack of a diagnostic difference in fluorescence intensity between the collapsed and extended RecA−DNA complexes led us to explore other fluorescence observables. The binding of RecA protein to the ODNs results in an increase in the polarized emission[21] from **m^{5}K** (Figure 2). Importantly, the anisotropy data serves as a good discriminator of the different RecA−DNA filaments, suggesting that anisotropy may prove useful for future time-dependent studies of RecA−DNA complex assembly.

Monitoring total DNA emission as a function of increasing protein concentration provided evidence that the signal changes are saturatable (Figure 3). Moreover, the saturation

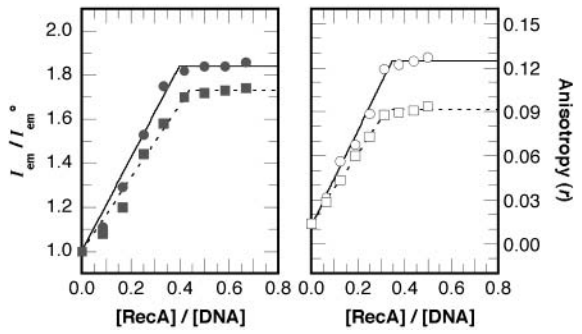


Figure 3. Total (left) and polarized (right) fluorescence titrations of ODN **60a** with RecA in either the absence (squares) or presence (circles) of ATPγS.

behavior allowed the stoichiometry of the RecA−DNA complexes to be determined. An equivalence point analysis[22] of the DNA binding isotherms in Figure 3 yielded RecA monomer to DNA nucleotide ratios of 1:2.5 ± 0.4 in the absence of cofactor for ODN **60a**. In the presence of ATPγS, the value was 1:2.7 ± 0.4. The observation that the anisotropy changes saturate at the same points provides strong corroborative evidence that the signal changes are monitoring complex formation directly.

Importantly, the titration results of Figure 3 show that **m^{5}K** does not disturb RecA−DNA interactions. Specifically, these RecA−DNA complexes have the canonical 3:1 nts to RecA monomer stoichiometry determined by other techniques.[1] This is the first demonstration of properly assembled RecA−DNA filaments using intrinsic DNA fluorescence. By comparison, changes in the DNA structure caused by ϵA[7,13,22] and fluorescein[5,6] labeling results in the binding of two ssDNA molecules and a stoichiometry of 1:6. Hence, the use of ODNs containing the intrinsic DNA fluorophore **m^{5}K** offer the unique opportunity to compare directly DNA binding at site I in the collapsed and extended RecA filaments.

We have presented proof of principle for monitoring the assembly of RecA nucleoprotein filaments using intrinsic DNA fluorescence. Emission from **m^{5}K** uniquely characterizes collapsed and extended RecA−ssDNA filaments with appropriate stoichiometries. In the future, the construction of a kinetic scheme describing RecA−DNA complex formation will form the basis for elucidating the detection of and response to DNA damage directed by RecA. Such use of instrinsic fluorescence from chemically synthesized nucleosides to study the thermodynamics and kinetics of recombination molecular motors promises to play a prominent role in a new era of biochemistry for RecA and related DNA motor proteins.

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Supporting Information Available: Protein and DNA preparation procedures, detailed fluorescence spectral data, and steady-state ATP hydrolysis kinetics for RecA−ODN complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

OL0167863

(21) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Plenum Publishing: New York, 1999; Chapter 10.

(22) Silver, M. S.; Fersht, A. R. *Biochemistry* **1982**, *21*, 6066−6072.