A Molecular Target for Suppression of the Evolution of Antibiotic Resistance: Inhibition of the *Escherichia coli* RecA Protein by N^6-(1-Naphthyl)-ADP

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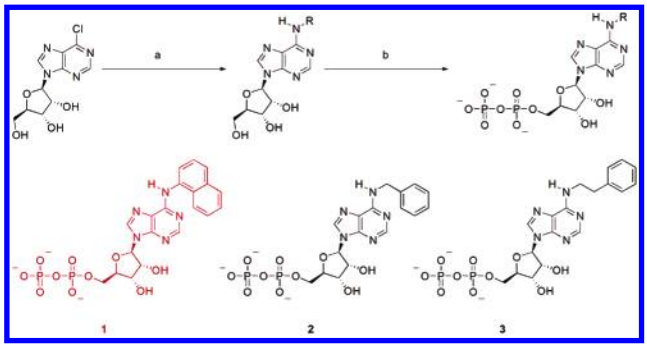
Received February 5, 2005

Abstract: We report that N^6-(1-naphthyl)-ADP (**1**) inhibits the *Escherichia coli* RecA protein in vitro. A novel rapid screen identified **1** as a potent inhibitor of RecA nucleoprotein filament formation, and further characterization established **1** as an ATP-competitive inhibitor of RecA-catalyzed ATP hydrolysis. **1** and other inhibitors of RecA activities represent a new approach for understanding the molecular targets and pathways involved in the evolution of antibiotic resistance in bacteria.

Antibiotic resistance in pathogenic bacteria has enormous human and economic consequences worldwide. The rapid rate at which bacteria develop drug resistance is due in large part to mutations arising during stress-induced DNA repair[1] and during the lateral transfer of genes between organisms.[2] The bacterial RecA protein is essential to both of these processes. In addition, RecA function is required for aspects of pathogenicity, including antibiotic-induced responses to ciprofloxacin[3] and β-lactams,[4] antigenic variation in *Neisseriae*,[5] and the induction of shiga toxin production.[6] All RecA functions require formation of an active nucleoprotein filament (NPF) comprising multiple RecA monomers, ATP, and single-stranded DNA (ssDNA). Hence, the discovery of small molecules that suppress the formation of such NPFs would be an important step in the development of inhibitors for the suppression of the evolution and transmission of antibiotic resistance. On the basis of our recent investigations of RecA mechanism,[7] we designed putative small-molecule inhibitors of RecA and a rapid microplate screening assay for its inhibition. Herein, we report the synthesis of N^6-(1-naphthyl)-ADP (**1**, Scheme 1) and demonstrate that it inhibits formation of the RecA−ssDNA complex that controls bacterial recombination and stress-induced mutagenesis.

Like most ATPases, RecA can be inhibited in vitro by ADP and a variety of nonhydrolyzable derivatives of ATP.[9,10] Our rationale for selective inhibitor design was inspired by the Steitz laboratory's initial observation that the orientation of ADP bound to protein in RecA crystals was different from those of nucleotides bound to related NTP-binding proteins.[11] To extend this investigation, we comparatively analyzed the RecA crystal structure with those of homologous motor proteins, such as F_1-ATPase, T7 helicase, and the rho transcription terminator, as well as other P-loop NTPases and kinases. As a result of ADP's unusual relative orientation, the adenosine moiety in RecA is located in a wide crevice near the surface of the protein (Figure 1A). In contrast, the ATP-binding sites of other NTPases and kinases envelop adenine in a hydrophobic pocket and maintain close contact with the edge and both faces of the purine moiety (Figure 1B), mitigating their potential interactions with N^6-substituted adenosine nucleotides.[12,13] Hence, we reasoned that the ATP-binding site of RecA could uniquely accommodate a sterically demanding substituent at N^6 of adenine. Our initial approach to inhibition of RecA thereby relies on the principles of negative design: the achievement of specificity involves the introduction of substituents that prevent the formation of nonproductive alternative complexes with nontargeted enzymes.

Scheme 1[a]



[a] Reagents: (a) RNH_2, EtOH, reflux, 59%; (b) (1) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 4 °C, (2) $^nBu_3NH$, H_2PO_4, pyridine, 0 °C, 13%.

We chose to synthesize N^6-substituted ADP analogues including those depicted in Scheme 1. Of the compounds in this class we have investigated, we selected **1**−**3** because they bear the largest N^6-substituents and because the 5′-*O*-triphosphates related to **2** and **3** have been shown to be poor substrates of protein kinases, myosin, and kinesin.[13] The syntheses began with the amination of 6-chloropurine ribonucleoside.[14] The resulting nucleosides were readily converted to the 5′-*O*-diphosphates (see Supporting Information).

In conjunction with the synthesis of potential inhibitors, we developed a novel high-throughput screening compatible assay to take advantage of the fact that binding of ADP can induce the release of RecA from ssDNA. In the context of mechanistic studies, we recently demonstrated that binding of ATP or ADP to a preformed RecA−ssDNA complex results in the release of the bound protein molecules, followed by reassociation of the ternary complex in the active conformation.[7] This discovery led us to consider whether RecA could be prevented from binding to ssDNA immobilized on streptavidin paramagnetic particles (SA-PMP).

Capturing biotinylated-$(dT)_{36}$ on SA-PMP allowed us to determine whether RecA is prevented from forming an NPF in the presence of a putative inhibitor by measuring the amount of RecA in the supernatant using a Bradford assay. In practice, the assay is conducted by incubating the nucleotide with RecA and $(dT)_{36}$, and then immobilizing the ssDNA and any bound RecA. This allows us to take advantage of the fact that binding of

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10.1021/jm050113z CCC: $30.25
Published on Web 07/29/2005

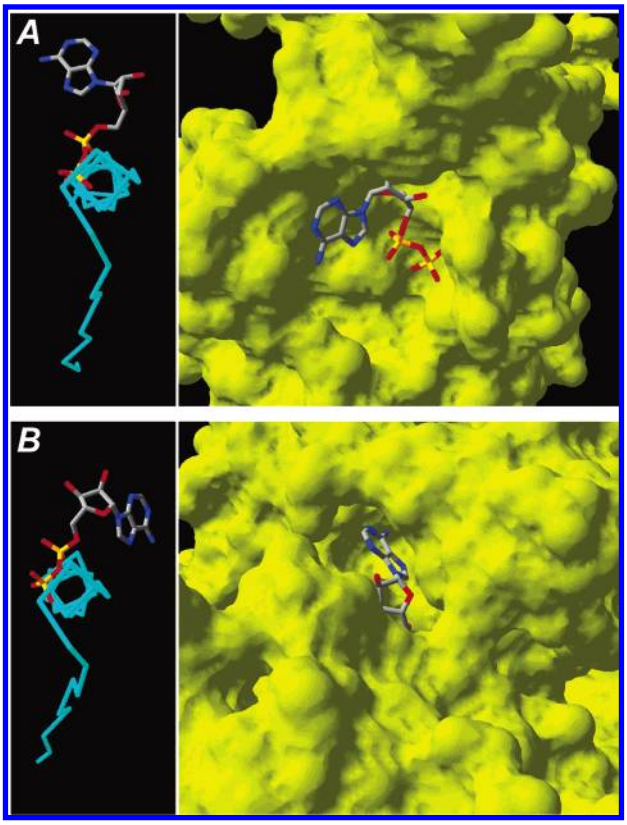

Figure 1. Comparison of the ATP-binding sites of RecA (A) and smooth muscle myosin (B). (Left panels) The structures were aligned with SwissPDB Viewer[8] using the C_α atoms of the residues in the phosphate-binding loop (P-loop) and the α-helix and β-strand adjacent to each (cyan). The ADP molecules are shown with atoms colored by elemental identity. (Right panels) The Connolly surfaces of the respective proteins and the bound ADP molecules (stick models) are shown following identical rotations to emphasize the different relative orientations of the nucleotides within the binding clefts.

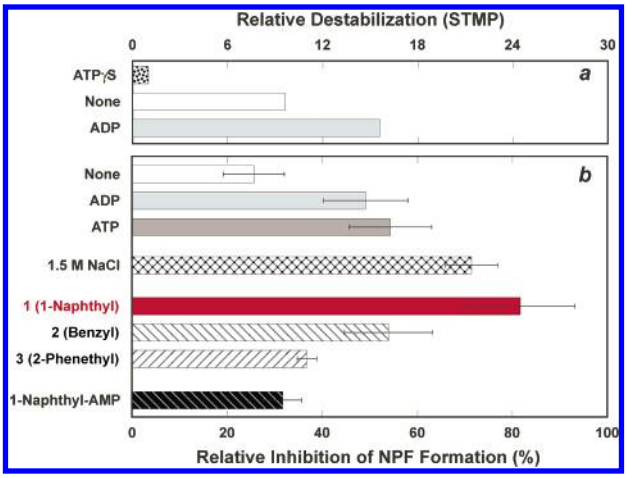

Figure 2. (a) Relative stabilities of NPFs formed in the presence or absence of nucleotide as measured by Menetski and Kowalczykowski using the salt titration midpoint (STMP, ref 10). (b) Relative inhibition of NPF formation in the presence of various nucleoside di- and triphosphates (100 μM each).

ADP to RecA reduces the apparent stability of the RecA−ssDNA complex (Figure 2a).[10]

Using the inhibition of NPF formation assay, we screened the 16 canonical nucleoside di- and triphosphates (both ribo- and 2′-deoxyribonucleotides) for RecA inhibition. The results clearly recapitulated what is known about the ability of various nucleotides to bind and activate (or deactivate) the RecA nucleoprotein filament. Indeed, the assay results (Figure 2b) demonstrate the expected trend in the relative NPF stabilities: RecA−ADP < RecA-only. Moreover, ATP has the same apparent effect on NPF stability as does ADP, consistent with the hydrolysis of ATP to ADP during the experiment. The fact that the amount of RecA protein released in the presence or absence of ADP/ATP is significantly less than that released by the addition of NaCl (final concentration of 1.5 M) verifies that substantial protein is coating the immobilized $(dT)_{36}$. The results for the nucleotides that are known to be RecA substrates were qualitatively similar to those for ADP/ATP.[15]

Using this assay, we investigated the release of RecA induced by three synthetic ADP analogues, N^6-(1-naphthyl)-, N^6-(benzyl)-, and N^6-(2-phenethyl)-ADP (**1**, **2**, and **3**, respectively; Figure 2b). Compound **1** (100 μM) inhibited NPF formation to the greatest extent of any agent examined to date. As a control, we observed that the N^6-(1-naphthyl)-adenosine *monophosphate* did not inhibit NPF formation, the extent of formation being the same within error as that observed in the absence of added nucleotide. We conclude that N^6-(1-naphthyl)-AMP does not bind RecA under these conditions. Importantly, the efficacy of **1** relative to its monophosphate for inhibition of NPF formation parallels that known for ADP and AMP.[16] The observation that the inhibitory effect of **1** responds to the phosphorylation state of the nucleoside suggests that its action does not result exclusively from interactions with the naphthyl moiety.

In contrast to those results for **1**, 100 μM **2** or **3** inhibited NPF formation to the same or lesser extent as 100 μM ADP. In this context, we tentatively conclude that **2** and **3** bind RecA similarly to ADP. Nucleotides **2** and **3** have been successfully used by the Shokat[13a,b] and Mitchison[13c] laboratories to control the functions of bioengineered (mutant) myosins, protein kinases, and kinesins.[17] The superiority of **1** for the inhibition of wild-type RecA likely results from its spacious surface crevice in which the ADP analogues are accommodated.

The formation of an active NPF in the presence of ATP results in ATP hydrolysis. By monitoring the steady-state kinetics of ATP hydrolysis by RecA in a poly(dT)-dependent ATPase assay,[18] we found that **1** exhibited fully competitive inhibition with respect to ATP and noncompetitive inhibition with respect to ssDNA (see Supporting Information). These results indicated that **1** binds in or near the RecA ATP-binding site, confirming our expectation that the ATP-binding cleft is large enough to accommodate the sterically demanding naphthyl moiety. Although relatively high, the apparent inhibition constant for **1** ($K_{ic} = 46 \pm 6$ μM) is essentially identical to the $S_{0.5}$ value for ATP[19,20] and within the range of K_d and K_i values for ADP.[9b] The observation that the affinities of **1**, ADP, and ATP are similar recapitulates the principles of negative design used to guide our choice of potential inhibitors.

We then conducted preliminary tests of the specificity of **1** for inhibition of RecA. In particular, we evaluated the inhibition of the ATPase activities of rabbit muscle pyruvate kinase, *E. coli* rho transcription terminator,

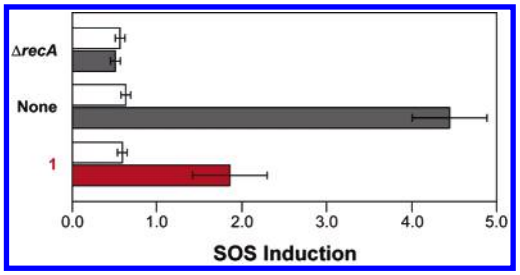

Figure 3. SOS induction in the absence and presence of **1** (100 μM). β-Galactosidase activities in permeabilized GY7313 bacteria were measured 2.5 h after treatment with mitomycin C (MMC, 0.5 μg/mL) as described in the Supporting Information. The gray and red bars represent activities measured after SOS induction by MMC, while the white bars represent basal activities measured without added MMC. The "Δ*recA*" data were obtained using GY7313 cells transformed by pTrc99A without a *recA* gene.

and chicken muscle myosin. Pyruvate kinase is an important glycolytic enzyme, the ATP binding site of rho is a close structural homologue of the RecA site, and the ATPase active site of myosin is known to tolerate a variety of base-substituted ATP analogues.[21,13b] Taken together, these three enzymes represent several classes of ATPases and provide a reasonable test of the promiscuity of **1**. At concentrations up to 300 μM, **1** led to no reduction in the rates of the three enzyme-catalyzed reactions. These results provide initial evidence that **1** is a specific inhibitor of RecA and validate the principles used to guide the design of **1**.

As an initial test of the biological activity of **1**, we tested its ability to inhibit the induction of the SOS response in permeabilized *E. coli* cells. As described above, the RecA protein stimulates DNA repair and mutagenesis processes by initiating the SOS response. In the presence of ATP and Mg^{2+}, the RecA protein bound to ssDNA activates the LexA repressor for autoproteolytic destruction and derepresses the genes controlled by LexA-repressible promoters. To evaluate the ability of **1** to modulate the SOS response to the DNA-damaging agent mitomycin C, we measured β-galactosidase activity in *E. coli* strain GY7313 harboring a *lacZ* reporter gene fused to the LexA-regulated *sfi* promoter. RecA-activated autoproteolysis of LexA derepresses *lacZ*, and the β-galactosidase produced can be quantitatively measured by an established colorimetric assay.[18]

For the investigation of ADP derivatives, the *E. coli* cells were made permeable to nucleotides with a hypotonic Tris-EDTA buffer at 4 °C. This treatment rendered the cells permeable to propidium iodide,[22] but importantly, they remained fully viable as judged by the ability to form colonies following recovery from permeabilization (see Supporting Information for details). Following permeabilization, β-galactosidase induction in Δ*recA* GY7313 harboring a plasmid with the WT *recA* gene expressed from the *trc* promoter was 7-fold greater than the basal level 2.5 h after treatment of the transformants with mitomycin C (Figure 3). Control experiments using GY7313 cells harboring the plasmid without a *recA* gene verify that the response is RecA-dependent. When 100 μM **1** was added just prior to treatment with mitomycin C, the level of induction was reduced more than 55%. The significant inhibition of β-galactosidase induction by **1** in this system is a particularly rigorous test of its biological activity because the RecA protein is constantly overexpressed from the *trc* promoter, whereas the normal basal expression level for RecA is low and it is overexpressed only *after* DNA damage induces the SOS response.[23] Control experiments performed using **1** in the absence of mitomycin C revealed no effect of **1** on the viability of permeabilized GY7313 cells. Moreover, a preliminary confirmation of a correlation between in vivo and in vitro activities was provided by the observation that treatment of permeabilized cells with **2** or **3** (100 μM) resulted in no significant reduction in SOS (β-galactosidase) induction (data not shown).

The observation that **1** mitigates the induction of SOS in permeabilized *E. coli* cells provides powerful proof that RecA activities, including its role in processes leading to the development and transfer of antibiotic resistance genes, may be selectively controlled by small molecules in living bacteria. Diphosphates such as **1** are, however, likely to be of little therapeutic utility because of membrane impermeability caused by the negative charges on the 5′-diphosphate moiety at physiological pH. Nevertheless, substantial progress has been made in the development of effective strategies for the intracellular delivery of nucleotide prodrugs (pronucleotides), particularly for use as anticancer and antiviral therapeutics.[24] Experiments to test the biological activities of pronucleotides related to **1** are under way.

In summary, we have reported the discovery that N^6-(1-naphthyl)-ADP prevents formation of the RecA−DNA filament that is essential for all RecA-associated functions. The fact that **1** is an inhibitor of RecA activities (IRA) provides essential proof of the principle that RecA functions can be selectively controlled by synthetic small molecules. Importantly, we have developed a novel assay for nucleotide analogue-dependent inhibition of NPF formation that is suitable for high-throughput screening of synthetic inhibitor libraries. IRAs that are active inside living bacteria will allow for the design of future chemical biology experiments to tease apart long-standing questions about the prokaryotic response to genome-wide DNA damage.[25] Moreover, we anticipate that these studies will provoke the development of lead compounds for inhibiting RecA-dependent processes leading to the development and transfer of antibiotic resistance.

Acknowledgment. This work was supported by a grant from the National Institutes of Health to S.F.S. (Grant GM58114). The authors thank Dr. Harold Kohn and Mr. Andrew Brogan for carrying out the rho ATPase assays, and Mr. Timothy Wigle and Mr. William Horton for technical assistance.

Supporting Information Available: Experimental procedures (including syntheses) and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hersh, M. N.; Ponder, R. G.; Hastings, P. J.; Rosenberg, S. M. Adaptive mutation and amplification in *Escherichia coli*: two pathways of genome adaptation under stress. *Res. Microbiol.* **2004**, *155*, 352−359. Matic, I.; Taddei, F.; Radman, M. Survival versus maintenance of genetic stability: a conflict of priorities during stress. *Res. Microbiol.* **2004**, *155*, 337−341. Foster, P. L. Stress responses and genetic variation in bacteria. *Mutat. Res.* **2005**, *569*, 3−11.

(2) Beaber, J. W.; Hochhut, B.; Waldor, M. K. SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **2004**, *427*, 72−74. Hastings, P. J.; Rosenberg, S. M.; Slack, A. Antibiotic-induced lateral transfer of antibiotic resistance. *Trends Microbiol.* **2004**, *12*, 401−404.
(3) Bisognano, C.; Kelley, W. L.; Estoppey, T.; Francois, P.; Schrenzel, J.; et al. A RecA−LexA-dependent pathway mediates ciprofloxacin-induced fibronectin binding in *Staphylococcus aureus*. *J. Biol. Chem.* **2004**, *279*, 9064−9071.
(4) Miller, C.; Thomsen, L. E.; Gaggero, C.; Mosseri, R.; Ingmer, H.; et al. SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science* **2004**, *305*, 1629−1631.
(5) Kline, K. A.; Sechman, E. V.; Skaar, E. P.; Seifert, H. S. Recombination, repair and replication in the pathogenic Neisseriae: the 3 R's of molecular genetics of two human-specific bacterial pathogens. *Mol. Microbiol.* **2003**, *50*, 3−13.
(6) Casjens, S. Prophages and bacterial genomics: what have we learned so far? *Mol. Microbiol.* **2003**, *49*, 277−300.
(7) Roca, A. I.; Singleton, S. F. Direct evaluation of a mechanism for activation of the RecA nucleoprotein filament. *J. Am. Chem. Soc.* **2003**, *125*, 15366−15375.
(8) Guex, N.; Peitsch, M. C. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **1997**, *18*, 2714−2723.
(9) For examples, see the following and references therein: (a) Moreau, P. L.; Carlier, M. F. RecA protein-promoted cleavage of LexA repressor in the presence of ADP and structural analogues of inorganic phosphate, the fluoride complexes of aluminum and beryllium. *J. Biol. Chem.* **1989**, *264*, 2302−2306. (b) Lee, J. W.; Cox, M. M. Inhibition of recA protein promoted ATP hydrolysis. 1. ATPγS and ADP are antagonistic inhibitors. *Biochemistry* **1990**, *29*, 7666−7676.
(10) Menetski, J. P.; Kowalczykowski, S. C. Interaction of recA protein with single-stranded DNA. Quantitative aspects of binding affinity modulation by nucleotide cofactors. *J. Mol. Biol.* **1985**, *181*, 281−295.
(11) Story, R. M.; Steitz, T. A. Structure of the recA protein−ADP complex. *Nature* **1992**, *355*, 374−376.
(12) Chene, P. ATPases as drug targets: learning from their structure. *Nat. Rev. Drug Discovery* **2002**, *1*, 665−673. Mao, L.; Wang, Y.; Liu, Y.; Hu, X. Molecular determinants for ATP-binding in proteins: a data mining and quantum chemical analysis. *J. Mol. Biol.* **2004**, *336*, 787−807.
(13) For examples concerning kinases, myosin, and kinesin, see: (a) Shah, K.; Liu, Y.; Deirmengian, C.; Shokat, K. M. Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3565−3570. (b) Gillespie, P. G.; Gillespie, S. K.; Mercer, J. A.; Shah, K.; Shokat, K. M. Engineering of the myosin-ibeta nucleotide-binding pocket to create selective sensitivity to N(6)-modified ADP analogs. *J. Biol. Chem.* **1999**, *274*, 31373-31381. (c) Kapoor, T. M.; Mitchison, T. J. Allele-specific activators and inhibitors for kinesin. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9106−9111.0
(14) Kikugawa, K.; Iizuka, K.; Ichino, M. Platelet aggregation inhibitors. 4. N6 -substituted adenosines. *J. Med. Chem.* **1973**, *16*, 358−364.
(15) For purposes of clarity, the data for these nucleotides and a variety of synthetic analogues are not shown; a full account of the structural basis for nucleotide selectivity will be published elsewhere.
(16) Watanabe, R.; Masui, R.; Mikawa, T.; Takamatsu, S.; Kato, R.; et al. Interaction of *Escherichia coli* RecA protein with ATP and its analogues. *J. Biochem. (Tokyo)* **1994**, *116*, 960−966.
(17) For a recent review, see: Specht, K. M.; Shokat, K. M. The emerging power of chemical genetics. *Curr. Opin. Cell Biol.* **2002**, *14*, 155−159.
(18) Berger, M. D.; Lee, A. M.; Simonette, R. A.; Jackson, B. E.; Roca, A. I.; et al. Design and evaluation of a tryptophanless RecA protein with wild type activity. *Biochem. Biophys. Res. Commun.* **2001**, *286*, 1195−1203.
(19) Stole, E.; Bryant, F. R. Reengineering the nucleotide cofactor specificity of the RecA protein by mutation of aspartic acid 100. *J. Biol. Chem.* **1996**, *271*, 18326−18328.
(20) The $S_{0.5}$ and K_d values reported for ATP binding range from 2.5 to 200 μM. See ref 9 and the following: Kowalczykowski, S. C. Interaction of recA protein with a photoaffinity analogue of ATP, 8-azido-ATP: determination of nucleotide cofactor binding parameters and of the relationship between ATP binding and ATP hydrolysis. *Biochemistry* **1986**, *25*, 5872−5881.
(21) Tonomura, Y.; Imamura, K.; Ikehara, M.; Uno, H.; Harada, F. Interaction between synthetic ATP analogues and actomyosin systems. IV. *J. Biochem. (Tokyo)* **1967**, *61*, 460−472. Cremo, C. R. Fluorescent nucleotides: synthesis and characterization. *Methods Enzymol.* **2003**, *360*, 128−177.
(22) Bunthof, C. J.; van Schalkwijk, S.; Meijer, W.; Abee, T.; Hugenholtz, J. Fluorescent method for monitoring cheese starter permeabilization and lysis. *Appl. Environ. Microbiol.* **2001**, *67*, 4264−4271.
(23) Sedgwick, S. G.; Levine, A.; Bailone, A. Induction of recA+-protein synthesis in *Escherichia coli*. *Mol. Gen. Genet.* **1978**, *160*, 267−276. Casaregola, S.; D'Ari, R.; Huisman, O. Quantitative evaluation of recA gene expression in *Escherichia coli*. *Mol. Gen. Genet.* **1982**, *185*, 430−439.
(24) Krise, J. P.; Stella, V. J. Prodrugs of phosphates, phosphonates, and phosphinates. *Adv. Drug Delivery Rev.* **1996**, *19*, 287−310. Schultz, C. Prodrugs of biologically active phosphate esters. *Bioorg. Med. Chem.* **2003**, *11*, 885−898. Meier, C.; Ruppel, M. F.; Vukadinovic, D.; Balzarini, J. "Lock-in"-cycloSal-pronucleotides−a new generation of chemical Trojan horses? *Mini-Rev. Med. Chem.* **2004**, *4*, 383−394. Peyrottes, S.; Egron, D.; Lefebvre, I.; Gosselin, G.; Imbach, J. L.; et al. SATE pronucleotide approaches: an overview. *Mini-Rev. Med. Chem.* **2004**, *4*, 395−408. Drontle, D. P.; Wagner, C. R. Designing a pronucleotide stratagem: lessons from amino acid phosphoramidates of anticancer and antiviral pyrimidines. *Mini-Rev Med Chem* **2004**, *4*, 409−419.
(25) Cox, M. M. Recombinational DNA repair of damaged replication forks in *Escherichia coli*: questions. *Annu. Rev. Genet.* **2001**, *35*, 53−82.

JM050113Z