

TOBRAMYCIN-EDTA CONJUGATE: A NONINNOCENT AFFINITY-CLEAVING REAGENT

Hai Wang and Yitzhak Tor*

*Department of Chemistry and Biochemistry
University of California, San Diego, La Jolla, CA 92093-0358, U.S.A.*

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Abstract: The design, synthesis, and ribozyme inhibitory activity of a novel EDTA-aminoglycoside conjugate are reported. This affinity cleaving reagent is a noninnocent RNA binder: its RNA affinity, judged by its ability to inhibit the hammerhead ribozyme HH16, is different than the parent natural product and is markedly dependent on the oxidation state of the chelated metal ion. © 1998 Elsevier Science Ltd. All rights reserved.

The affinity cleavage method has emerged as a powerful tool for mapping nucleic acids structure and complexes.¹ The key feature of this technique is the conjugation of a nonspecific cleaving molecule, such as EDTA-Fe(II), to a specific binder.² Following the formation of a ligand–host complex, the cleavage chemistry is initiated resulting in strand scissions near the binding site. The cleavage patterns revealed by high-resolution polyacrylamide gel electrophoresis contain information regarding the size, location and orientation of the binding site. A fundamental requirement is that the conjugation of the cleaving moiety should not alter the binding characteristics of the molecule under investigation.

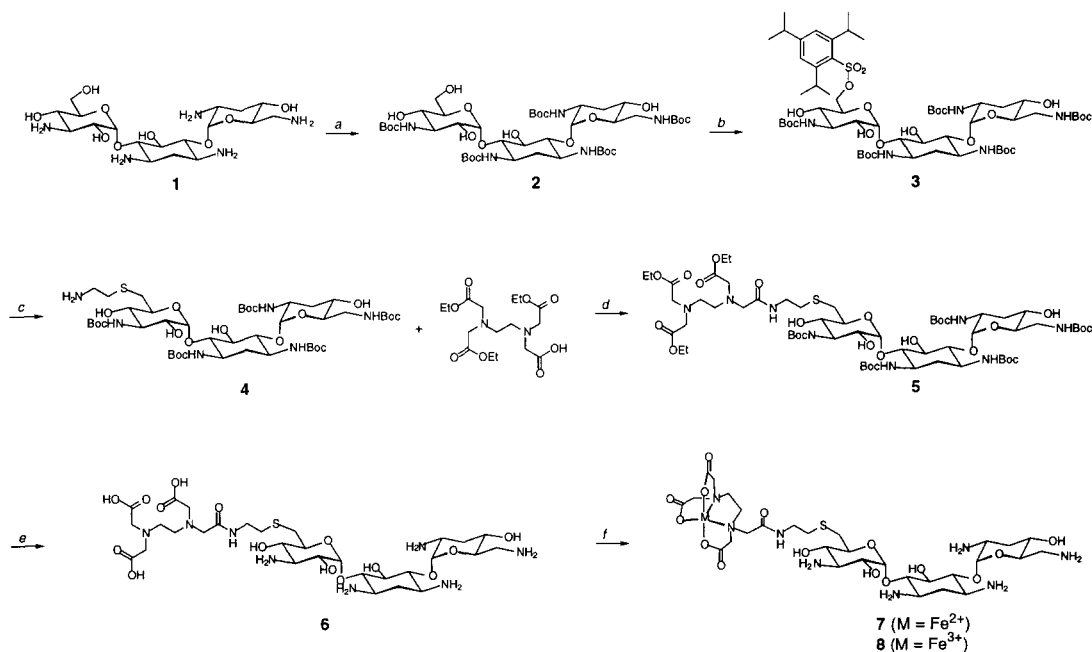
While well-established for DNA-recognizing molecules,^{2,3} less attention has been given to the affinity cleavage of RNA molecules.⁴ This may be due to our limited knowledge of the interactions between small molecules and RNA biomolecules.⁵ Recently, aminoglycoside antibiotics have emerged as an intriguing family of specific RNA binders. These natural products have been shown to bind to ribosomal RNA,⁶ to inhibit self-splicing group I introns⁷ and the hammerhead ribozyme,⁸ and to interfere with protein–RNA interactions.⁹ Aminoglycosides have become a useful entry into the largely unexplored area of RNA-small molecules recognition.¹⁰

Aminoglycosides, 2-deoxystreptamine-containing pseudo oligosaccharides with a large number of amino groups, are highly charged at physiological pH.¹¹ Electrostatic interactions have been demonstrated to play a major role in their binding to various RNA structures.^{12,13} Our recent studies have focused on investigating the inhibition of the hammerhead ribozyme by modified aminoglycosides. The results obtained with deoxygenated tobramycin derivatives,¹² amino-aminoglycosides¹⁴ and dimeric aminoglycosides,¹⁵ have led us to propose a general model for RNA-aminoglycoside recognition.¹⁴ This model emphasizes a three-dimensional electrostatic complementarity and is in agreement with recent Molecular Dynamics Simulations.^{16,17}

Although high-resolution NMR structures for two different RNA-aminoglycoside complexes have been recently reported,¹⁸ a generalized recognition motif has not yet emerged. Furthermore, it appears that aminoglycoside binding to RNA can be a rather dynamic process with multiple binding modes,^{14,16} and fast *off* rates.¹⁹ These issues complicate the application of high-resolution structural as well as footprinting techniques for the determination of aminoglycosides' binding sites. We have sought to apply the affinity cleavage method to locate the aminoglycoside binding sites(s) on catalytically-active RNA molecules. Here we present the

design, synthesis and ribozyme inhibitory activity of a novel EDTA–tobramycin conjugate and its metal complexes. We report that this affinity cleaving reagent is a “noninnocent” binder. The implication of this unprecedented observation on the utilization of the affinity cleavage method for the study of functional RNA molecules is discussed.

In designing aminoglycoside-containing affinity-cleaving reagents, a major factor to be considered is the tethering position. A priori, the EDTA moiety can be conjugated to an amino or hydroxyl group. Our earlier studies have revealed the critical role of electrostatic interactions in aminoglycoside–RNA binding.^{12,14} We have therefore excluded modifying any of the amino groups, because such a modification reduces the overall positive charge of the aminoglycoside and lowers its RNA affinity. Our studies with deoxygenated aminoglycosides have indicated that 6''-deoxytobramycin is comparable, yet slightly inferior, to tobramycin in its affinity to the hammerhead ribozyme.¹² This observation suggests that the primary 6''-OH is not essential for RNA binding. The EDTA moiety was therefore tethered to the 6''-position of tobramycin via a short bifunctional linker.



Scheme 1. Synthesis of EDTA–tobramycin conjugate **6** and its metal complexes. *Reagents and conditions:* (a) (*t*-BuOCO)₂O, DMSO, H₂O, 94%; (b) 2,4,6-triisopropylbenzenesulfonyl chloride, pyridine, 65%; (c) HSCH₂CH₂NH₃Cl, NaOEt, EtOH, 90%; (d) HOBt, EDCI, DMF, 80%; (e) (i) LiOH, H₂O, (ii) CF₃COOH, 80% (two steps); (f) (NH₄)₂Fe(SO₄)₂ for **7**; (NH₄)Fe(SO₄)₂ for **8**.

Scheme 1 illustrates the synthesis of the EDTA–tobramycin conjugate **6**. All the amino groups in tobramycin **1** were first protected as their *N*-Boc derivative to give **2**. Selective activation of the primary 6''-hydroxyl is achieved by treating **2** with 2,4,6-triisopropylbenzenesulfonyl chloride to furnish **3**. The fully Boc-

protected monosulfonate **3** is treated with a large excess of 2-aminoethanethiol·HCl in NaOEt/EtOH to afford the desired aminoethyl tobramycin derivative **4**. Ethylenediaminetetraacetic acid triethyl ester² is condensed with the free amine **4** in the presence of *N*-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl (EDCI) in DMF to furnish the fully-protected conjugate **5**. Hydrolysis of the ethyl esters by lithium hydroxide followed by acidic removal of the Boc groups afforded the desired conjugate **6**, shown in Scheme 1 in its uncharged form.²⁰ Metal complexation, affording Fe(II)-EDTA–tobramycin **7** or Fe(III)-EDTA–tobramycin **8**, has been carried out prior to the evaluation of RNA binding.

We have selected the hammerhead ribozyme HH16, a well-studied small RNA enzyme,²¹ as our RNA binding assay (Figure 1). Uhlenbeck and his coworkers have demonstrated that, for the HH16, cleavage is strongly favored over substrate dissociation and ligation, ensuring efficient conversion of E16·S16 to E16·P₁·P₂.²² In addition, product release is energetically disfavored and nearly all cleaved substrate remains bound in the E16·P₁·P₂ complex. When the reaction is conducted under single turn over conditions, the appearance of cleaved product directly reflects the rate of ribozyme-mediated cleavage. Since the cleavage reaction happens within the hammerhead complex, it can be treated as a first order intramolecular reaction; hence, the reaction rate constant k_2 can be easily extracted from measurements of the ribozyme's initial kinetics.

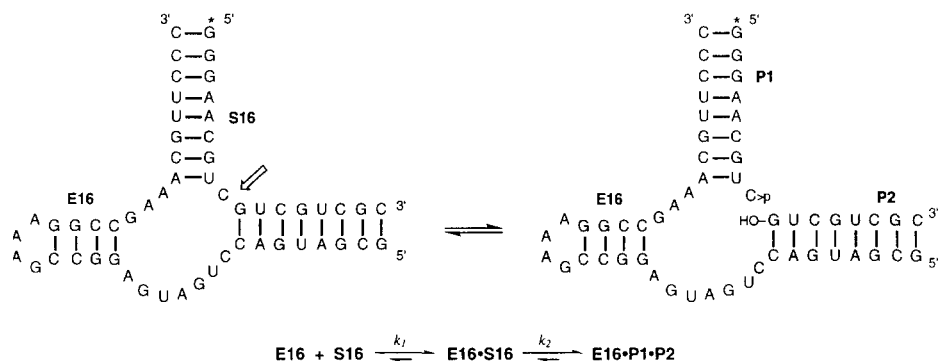


Figure 1. Top: secondary structure and reaction of the hammerhead ribozyme (HH16),⁸ showing the enzyme E16, the ³²P 5'-labeled substrate S16 (left), and the cleavage products P1 and P2 (right). The arrow shows the cleavage site. Bottom: the relevant kinetic scheme of the HH16 cleavage.

Tobramycin (**1**), the EDTA conjugate (**6**), and its metal complexes (**7** and **8**) have been tested for their ability to inhibit the hammerhead ribozyme HH16. Their effect on the S16 cleavage rate has been investigated at pH 7.3 under single turn over conditions in the presence of subsaturating Mg²⁺ and ribozyme concentrations.²³ Under these conditions, the ribozyme cleaves its substrate with a pseudo first-order rate constant of 0.17 min⁻¹. At 100 μM tobramycin decreases the cleavage rate sixfold to 0.028 min⁻¹. On the other hand, Fe(II)-EDTA–tobramycin (**7**) at 100 μM completely abolishes ribozyme cleavage (Figure 2). At 10 μM **7** shows a similar cleavage rate to that observed in the presence of 100 μM tobramycin **1**. These results indicate that incorporating a Fe(II)-EDTA moiety into an aminoglycoside can convert a moderate ribozyme inhibitor into a powerful one.

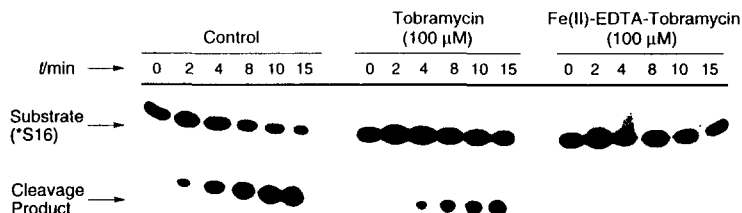


Figure 2. An autoradiogram of a 20% polyacrylamide gel used to separate the products of the time-dependent hammerhead ribozyme cleavage reactions at pH 7.3 in the absence of aminoglycosides (control) and the presence of tobramycin **1** (middle) and Fe(II)-EDTA-tobramycin **7** (right).

To illustrate the dramatic differences between the aminoglycoside derivatives in their ability to inhibit the ribozyme HH16, a single time-point assay was conducted. In this experiment, the total amount of ribozyme-mediated substrate cleavage is evaluated after 30 min (Figure 3). Under our standard conditions, the ribozyme cleaves 97% of its substrate after 30 min (lane 2). In the presence of a 100 μ M tobramycin **1** (lane 3), the amount of cleavage is reduced to approximately 60%, illustrating a relatively weak inhibitory effect of tobramycin at this concentration. Similarly, the metal-free EDTA-tobramycin conjugate **6** at 100 μ M appears to be a rather poor inhibitor, allowing 78% of the substrate to be cleaved (lane 4). In a marked contrast, 100 μ M Fe(II)-EDTA-tobramycin (**7**) completely abolishes ribozyme activity leading to less than 2% substrate cleavage (lane 5). Surprisingly, 100 μ M Fe(III)-EDTA-tobramycin (**8**) is a much weaker ribozyme inhibitor (lane 6) leading to only 62% substrate cleavage, a value comparable to that observed in the presence of the parent natural product **1**. Exposing the ribozyme Fe(II)-EDTA-tobramycin mixture to 5 mM DTT, thus initiating the interconversion of Fe(II) and Fe(III) via Fenton Chemistry,²⁴ results in little inhibition, where 75% of the substrate is cleaved after 30 min (lane 7).

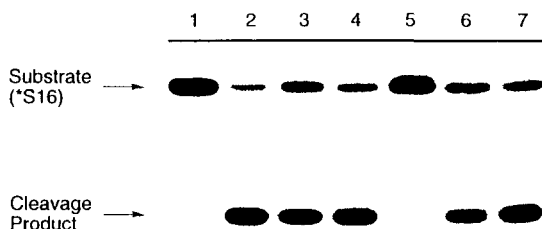


Figure 3. An autoradiogram of a 20% polyacrylamide gel showing the pure substrate *S16 (lane 1) and the 30 min HH16 ribozyme-mediated cleavage in the absence (lane 2) and the presence of 100 μ M tobramycin **1** (lane 3), 100 μ M EDTA-tobramycin **6** (lane 4), 100 μ M Fe(II)-EDTA-tobramycin **7** (lane 5), 100 μ M Fe(III)-EDTA-tobramycin **8** (lane 6), and 100 μ M Fe(II)-EDTA-tobramycin **7** plus 5 mM DTT (lane 7).

How can the potent inhibitory activity of the Fe(II)-EDTA-tobramycin derivative **7** be explained? According to our current working hypothesis, the positively charged aminoglycosides exert their inhibitory behavior by competing with RNA-bound Mg^{2+} ion(s).^{12,14,16,17} The removal or dislocation of a structurally or

functionally essential Mg^{2+} ion from its native site can handicap the ribozyme.¹⁷ While the conjugated EDTA complex has a net zero charge in the Fe(III)-EDTA–tobramycin (**8**), the conjugated Fe(II)-EDTA moiety in Fe(II)-EDTA–tobramycin (**7**) still possesses a single negative charge. The conjugated Fe(II)-EDTA fragment is capable of coordinating a positively charged ion such as Mg^{2+} , a function that is missing in the overall neutral Fe(III)-EDTA moiety. Indeed, Fe(III)-EDTA–tobramycin **8** shows an inhibitory activity very similar to the parent tobramycin **1**. On the other hand, the metal-free EDTA conjugate **6**, that is likely to have a different RNA binding mode due to its potential zwitterionic structure, is a weaker inhibitor. The unanticipated drop in inhibitory activity of **7** after addition of DTT might result from chemical degradation of the EDTA-moiety and/or the linked aminoglycoside, as previously reported for other affinity cleaving agents.^{3a,25} We conclude that the conjugated Fe(II)-EDTA fragment is a noninnocent functionality and is capable of interfering with the hammerhead ribozyme function.

Is there a precedent for RNA binders that contain negatively charged residues? Wong has reported a combinatorial synthesis of aminoglycoside mimetics.²⁶ Using the Ugi multi-component condensation, a library of RNA binders functionalized with amino acid residues was synthesized with neamine as a core.²⁶ These derivatives were screened for their ability to interfere with the binding of the HIV-1 Rev protein to its RNA target, the Rev-Response-Element (RRE). The most active derivatives contained at least two carboxylic acid residues.²⁶ Protecting one of the carboxylates as the corresponding methyl ester resulted in much lower inhibitory activity. Although these results remain unexplained, it is important to point out the novelty of RNA binders that contain negatively charged residues. We speculate that these aminoglycoside mimetics bind RNA in a similar fashion to our Fe(II)-EDTA-functionalized tobramycin, where negatively charged residues coordinate an essential metal ion. Although there is no structural evidence for the involvement of Mg^{2+} ions in stabilizing the folded RRE structure, molecular modeling studies suggest that Na^+ ions play such a role.²⁷

Our observations have important implications on the design of RNA binders and the utilization of affinity-cleaving methodology to the study of functional RNA molecules. Strong RNA binders are likely to possess sufficient positive charges to electrostatically bind to the RNA target site.^{10,17} Efficient competition with bound metal ions, in particular Mg^{2+} , can be achieved by a strategic incorporation of ammonium groups with predetermined three-dimensional projection. Surprisingly, it appears that the incorporation of negatively charged functional groups may also enhance RNA binding. These residues may exert their effect by coordinating bound metal ion(s), leading to a conformational change or to dislocation of an essential cleavage component in catalytically active RNA molecules. In the case of the hammerhead ribozyme HH16, the Fe(II)-EDTA fragment cannot be considered an inert moiety; although, upon oxidation to the Fe(III)-EDTA, this neutral fragment may cause less interference. Our observations imply that the binding characteristics of affinity-cleaving reagents developed for the study of functional RNA biomolecules must be carefully evaluated and critically compared to their parent molecules.

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References and Notes

1. Dervan, P. B. *Science* **1986**, 232, 464. Sigman, D. S.; Mazumder, A.; Perrin, D. M. *Chem. Rev.* **1993**, 93, 2295.
2. Schultz, P. G.; Taylor, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1982**, 104, 6861.
3. For selected examples, see: (a) Dreyer, G. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, 82, 968. (b) Moser, H. E.; Dervan, P. B. *Science* **1987**, 238, 645. (c) Mack, D. P.; Sluka, J. P.; Shin, J. A.; Griffin, J. H.; Simon, M. I.; Dervan, P. B. *Biochemistry* **1990**, 29, 6561. (d) Wade, W. S.; Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, 114, 8783. (e) Ebright, Y. W.; Chen, Y.; Pendergrast, P. S.; Ebright, R. H. *Biochemistry* **1992**, 31, 2829. (f) Chen, C.-H. B.; Mazumder, A.; Constant, J.-F.; Sigman, D. S. *Bioconjugate Chem.* **1993**, 4, 69.
4. Wang, J.-F.; Cech, T. R. *Science* **1992**, 256, 526. For a tobramycin-EDTA derivative (conjugated via the 6'-NH₂), see: Wang, Y.; Killian, J.; Hamasaki, K.; Rando, R. R. *Biochemistry* **1996**, 35, 12338.
5. For a review on RNA-ligand interactions: Chow, C. S.; Bogdan, F. M. *Chem. Rev.* **1997**, 97, 1489.
6. Moazed, D.; Noller, H. F. *Nature, (London)* **1987**, 327, 389.
7. von Ahsen, U.; Davies, J.; Schroeder, R. *Nature, (London)* **1991**, 353, 368. von Ahsen, U.; Davies, J.; Schroeder, R. *J. Mol. Biol.* **1992**, 226, 935.
8. Stage, T. K.; Hertel, K. J.; Uhlenbeck, O. C. *RNA* **1995**, 1, 95.
9. Zapp, M. L.; Stern, S.; Green, M. R. *Cell* **1993**, 74, 969. Mei, H.-Y.; Galan, A. A.; Halim, N. S.; Mack, D. P.; Moreland, D. W.; Sanders, K. B.; Truong, H. N.; Czarnik, A. W. *Bioorg. Med. Chem. Lett.* **1995**, 5, 2755.
10. Michael, K.; Tor, Y. *Chem. Eur. J.* **1998**, 4, 2091.
11. Dorman, D. E.; Paschal, J. W.; Merkel, K. E. *J. Am. Chem. Soc.* **1976**, 98, 6885. Botto, R. E.; Coxon, B. J. *Am. Chem. Soc.* **1983**, 105, 1021. Szilágyi, L.; Pusztahelyi, Z. S.; Jakab, S.; Kovács, I.; *Carbohydr. Res.* **1993**, 247, 99.
12. Wang, H.; Tor, Y. *J. Am. Chem. Soc.* **1997**, 119, 8734.
13. Clouet-d'Orval, B.; Stage, T. K.; Uhlenbeck, O. C. *Biochemistry* **1995**, 34, 11186.
14. Wang, H.; Tor, Y. *Angew. Chem. Int. Ed.* **1998**, 37, 109.
15. Wang, H.; Tor, Y. *Bioorg. Med. Chem. Lett.* **1997**, 7, 1951.
16. Hermann, T.; Westhof, E. *J. Mol. Biol.* **1998**, 276, 903.
17. Tor, Y.; Hermann, T.; Westhof, E. *Chem. Biol.* **1998**, 5, R277.
18. Fourmy, D.; Recht, M. I.; Blanchard, S. C.; Puglisi, J. D. *Science* **1996**, 274, 1367. Jiang, L.; Suri, A. K.; Fiala, R.; Patel, D. J. *Chem. Biol.* **1997**, 4, 35.
19. Hendrix, M.; Priestley, E. S.; Joyce, G. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, 119, 3641.
20. All new compounds showed satisfactory analytical and spectral data. Selected data for EDTA-tobramycin conjugate **6**: $R_f = 0.25$ (Silicagel 60, dichloromethane/methanol/acetic acid/water 1/1/1/2); ESI MS (CH₃CN, H₂O) m/z calcd for C₃₀H₅₇N₈O₁₅S [M + H]⁺ 801.9, found 801.5; ¹H NMR (500 MHz, D₂O) δ 5.54 (d, 1H, $J = 4.0$ Hz), 5.12 (d, 1H, $J = 3.5$ Hz), 4.10 (m, 2H), 3.94–4.00 (m, 8H), 3.72–3.84 (m, 7H), 3.54–3.66 (m, 7H), 3.38–3.46 (m, 3H), 3.28 (dd, 1H, $J_1 = 12.5$ Hz, $J_2 = 6.0$ Hz), 3.15 (dd, 1H, $J_1 = 15.0$ Hz, $J_2 = 3.0$ Hz), 2.84 (m, 2H), 2.73 (m, 1H), 2.55 (m, 1H), 2.34 (m, 1H), 2.04 (m, 2H); ¹³C NMR (125 MHz, D₂O) δ 172.3, 171.0, 168.6, 100.6, 95.0, 93.6, 78.6, 74.2, 72.4, 70.3, 68.1, 67.7, 64.5, 57.2, 56.8, 56.4, 54.8, 52.2, 51.3, 49.7, 48.1, 39.8, 38.1, 31.7, 31.6, 29.4, 27.7.
21. Fedor, M. J.; Uhlenbeck, O. C. *Biochemistry* **1992**, 31, 12042.
22. Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, 33, 3374.
23. Reaction conditions: 20 nM E16, trace ³²P 5'-labeled S16 (≤ 1 nM), 7.5 mM MgCl₂, 50 mM Tris-HCl, pH 7.3, with the indicated concentrations of aminoglycosides. Reactions were initiated by mixing equal volumes of preincubated solutions of the enzyme E16 and the labeled substrate S16. Aliquots were removed at various times and quenched by adding excess stop solution/loading buffer (8 M urea, 50 mM Na₂-EDTA, pH 7.3, bromophenol blue and xylene cyanol). The substrate and product were resolved on 20% polyacrylamide/7 M urea gels, and the fraction cleaved was quantified using a Phosphorimager. Plotting $\log(1-S/S_0)$ vs. time (min), gives a straight line with a slope of $-k_2/2.303$.
24. Walling, C. *Acc. Chem. Res.* **1975**, 8, 125.
25. Lin et al. reported the rapid degradation of the EDTA moiety in conjugated oligodeoxynucleotide methylphosphonates upon initiation of Fenton chemistry with DTT. See: Lin, S.-B.; Blake, K. R.; Miller, P. S.; Ts'o, P. O. P. *Biochemistry* **1989**, 28, 1054–1061.
26. Park, W. K. C.; Auer, M.; Jaksche, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1996**, 118, 10150.
27. Le, S.-Y.; Pattabiraman, N.; Maizel, J. V., Jr. *Nucleic Acids Res.* **1994**, 22, 3966.