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Aurine Tricarboxylic Acid, a Potent Metal-Chelating Inhibitor of NFκB–DNA Binding

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Abstract—The metal-interaction of aurine tricarboxylic acid (ATA) and its inhibitory effect on the DNA binding of NFκB were studied. Chemical speciation and spectroscopic studies have shown the strong interaction of ATA with metal ions present in the biological systems. EPR, FTIR and electronic spectral studies indicated the square planar structure of the metal-binding carboxylic and hydroxyl groups of ATA indicating the ground state $^2B_{1g}$. Electrophoretic mobility shift assay using NFκB and ^{32}P labeled DNA has shown that ATA was inhibitory against the DNA-NFκB binding at 30 μM. This activity was the strongest among the metal-chelating inhibitors of NFκB-DNA binding reported so far.

Introduction

The development of new potential therapeutic agents for the treatment of acquired immunodeffciency syndrome (AIDS) is an urgent problem that medicinal chemists are now facing. Although the mutidrug therapy using inhibitors of HlV-1 reverse transcriptase and protease achieved a remarkable success in delaying the progression of AIDS, this cocktail therapy came across a serious difficulty mainly due to the acquirement of drug resistance.[1–3] Thus, there is a clear need of new antiviral agents that affect unique targets but do not have cross resistance with existing drugs.

The frequently observed mutation of HIV-1 is known to be a cause of the drug resistance. This difficulty could be circumvented by targeting the host proteins such as a transcription factor NFκB that is utilized by HIV-1 for the replication. NFκB is an inducible human transcription factor that binds to the κB site present in various cellular genes critical for immune or inflammatory responses.[4–6] Of particular importance is that a tandem sequence of κB site is found in the long terminal repeat of HIV provirus[7–9] and the expression of HIV-1 provirus is governed by NFκB.[10,11]

Zabel et al. have reported *o*-phenanthroline is inhibitory against the DNA binding of NFκB.[12] We previously reported synthetic ligands comprising a pyridine and histidine and found their inhibitory activity against the DNA binding of NFκB.[13–15] Recent interest in aurine tricarboxylic acid (ATA, Figure 1) has resulted from its activity against all HIV strains including that resistant to azidothymidine (AZT), a HIV reverse transcriptase inhibitor.[16] It has been shown that ATA blocks the cytopathic effect of HIV in cell cultures with low cytotoxicity,[17] and thus ATA is promising as an anti-AIDS remedy with lower side effects.[18] Herein we examined the effect of ATA on the DNA binding of NFκB and found that ATA is the strongest inhibitor of NFκB among the molecules tested so far. Since the above inhibitors of NFκB possess metal-binding properties, we also investigated the metal chelating behavior of ATA focusing on copper chelation chemistry mainly due to the convenience in the physicochemical measurement.

Results

Chemistry

The interaction of ATA with metal ion was firstly studied. pH-Metric technique is a robust and versatile way of measuring ionization, lipophilicity, distribution of drugs in biological fluids and assessing their interaction with

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metal ion.[17] Figures 2 and 3 show pH titration curve for ATA-Cu_2 interaction and species distribution plot of Cu_2ATA, respectively. Proton dissociation constants of the ammonium salt of ATA, having two dissociable phenolic protons, are found to be 10.50 and 8.77 respectively. The formation constants and species distribution were determined using the program BEST[19] and SPE.[19] Species distribution plot indicated the exclusive formation of Cu_2ATA complex at physiological pH region. The formation constant of Cu_2ATA ($\beta_{M2L} = [M_2L]/[M]^2[L]$) calculated to be 19.56 also indicated the formation of M_2L type of complex at biological pH value. The formation constant of Zn_2ATA was 18.68 showing that both the metal ions are having similar binding capability and the stability of Cu_2ATA is a little greater than Zn_2ATA. Species distribution curves also indicated the formation of M_2L type of complex at biological pH value. Using these studies Cu_2L complex was isolated in the solid state and the presence/percentage of copper was proved by means of atomic absorption spectrometry (Atomic absorption study indicated that 5 ppm solution of the complex [00228 g complex/l] contains 4.617 ppm of copper [0.004617 g copper/l], indicating 20.25% of copper in complex. Calculated 20.50%.). Concerning the metal-binding sites of ATA, FTIR spectra indicated the involvement of the carboxyl and phenolic oxygens. The C=O and C–O stretching frequencies which appear at 1650 and 1350 cm^{-1} in the free ligand are shifted to 1670 and 1280 cm^{-1} in the complex. The νOH mode of the phenolic group in the free ligand at 3150 cm^{-1} disappeared in the complex, indicating the metal binding through phenolic oxygen. The visible spectra showed absorption band at 19,200 cm^{-1} assignable to transition $^2E_g \leftarrow {}^2B_{1g}$ of a square planar structure. The coordination modes are further supported by the EPR measurement (Fig. 4). The value of $g_{//}$ and $g_{\perp}$ were found to be 2.439 and 2.285 respectively. The relationship $g_{//} > g_{\perp} > 2.0023$ indicated that the unpaired electron most likely resides in the $d_x2\text{-}_y2$ orbitals implying $^2B_{1g}$ ground state. The lipophilicity parameter (log P, partition coefficient in octanol/water) of ATA was determined to be 1.43, indicating ATA to be sufficiently lipophilic to be absorbed in the body (Fig. 5). Figure 6 shows a proposed structure of Cu_2ATA complex obtained by molecular mechanics on the basis of the above results.

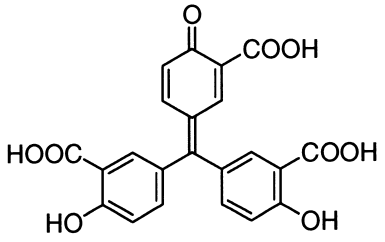

Figure 1. Aurine tricarboxylic acid (ATA).

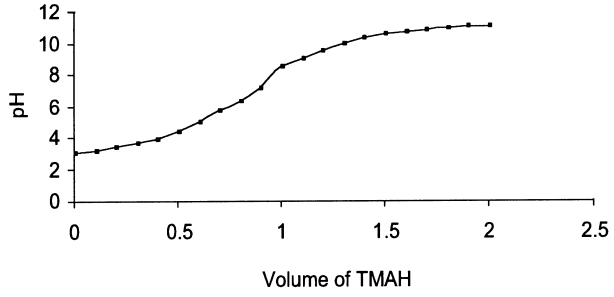

Figure 2. pH titration curve for ATA-Cu_2 interaction.

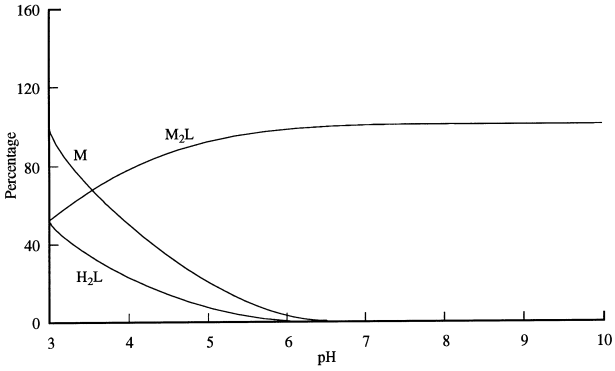

Figure 3. Species distribution curve for Cu_2ATA complex.

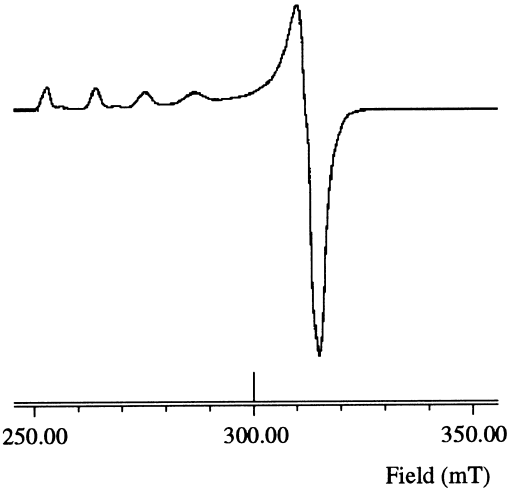

Figure 4. EPR Spectra of Cu_2ATA complex.

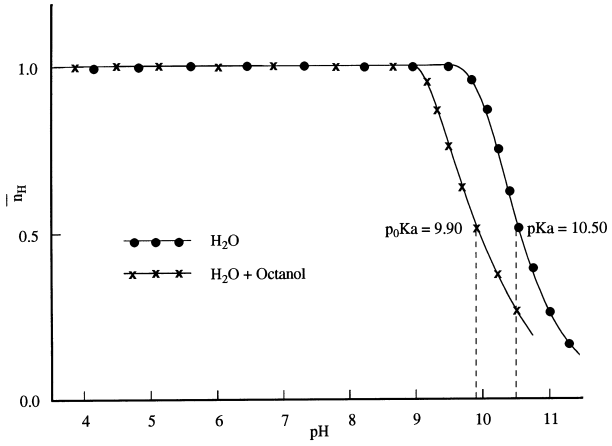

Figure 5. Difference curve for the determination of lipophilicity of ATA.

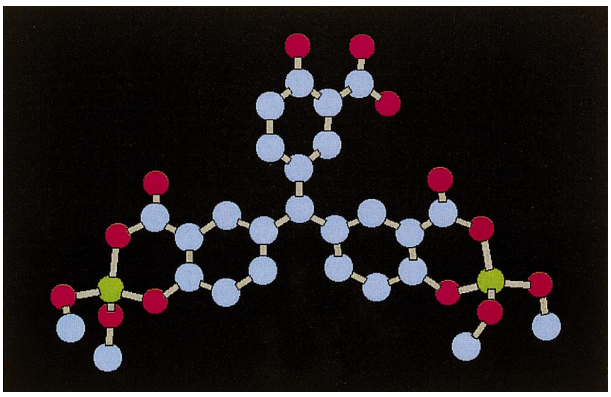

Figure 6. Proposed energy minimized structure of the Cu_2ATA complex.

Inhibition of the DNA binding of NFκB

Figure 7 shows the inhibitory effect of various concentrations of ATA on the DNA binding of NFκB as demonstrated by electrophoretic mobility shift assay. Nuclear extract of Jurkat T cells stimulated with TNF-α contained NFκB (lane 7, Figure 7). ATA was found to inhibit the DNA binding of NFκB at 30 μM concentration (lane 5, Fig. 7). Higher concentration of ATA resulted in complete inhibition (data not shown). Inhibition of the NFκB-DNA binding by ATA was shown to be partly blocked by adding Zn^{++} (Figure 8). ATA at 30 μM concentration reproducibly inhibited the DNA-NFκB binding (lane 3, Figure 8). Partial but evident recovery of the DNA binding property of NFκB was observed when 2 equivalents of Zn^{++} was introduced before (lane 5) or after (lane 4) the addition of 30 μM concentration of ATA. Addition of large excess of Zn^{++} gave virtually the same results (lane 6 and 7, Figure 8).

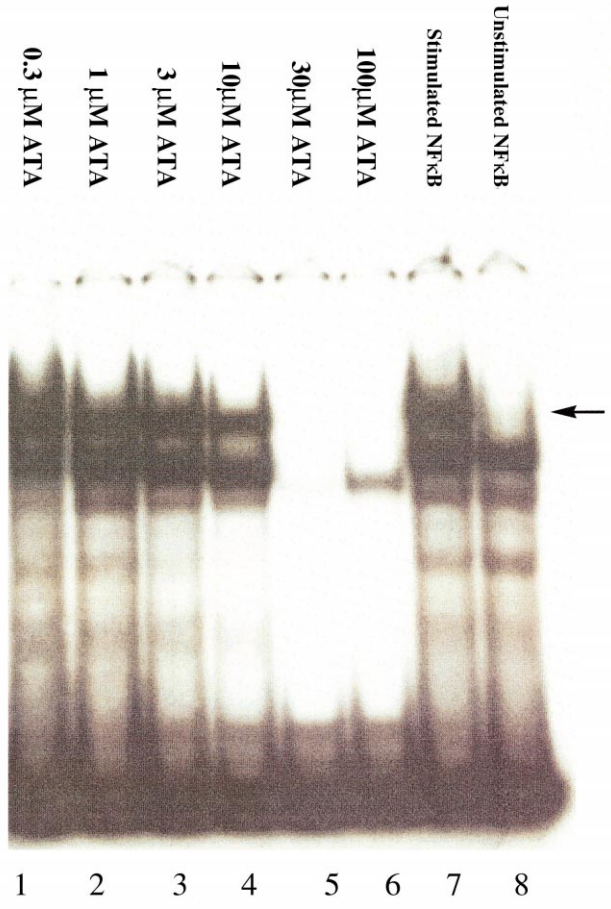

Figure 7. Effect of ATA on the DNA binding of NFκB. After stimulated NFκB was incubated with ATA in the presence of poly(dI-dC) at room temperature, a radioactive DNA probe containing a κB site from the Mouse κ light-chain enhancer was added. Sample was loaded onto a polyacrylamide band shift gel, and the gel electrophoresis was run.

Discussion

It has been reported that ATA inhibits various nucleotide-processing proteins including DNA polymerase,[20–22] RNA polymerase,[22–26] reverse transcriptase,[27,28] aminoacyl-^{t}RNA synthetase,[29] ribonucleotide reductase,[30] and ribonuclease.[31–35] The present finding of NFκB inhibition constitutes a new entry in the proteins that ATA targets. It is noteworthy that ATA is the most potent inhibitors of the DNA–NFκB binding among the compounds reported

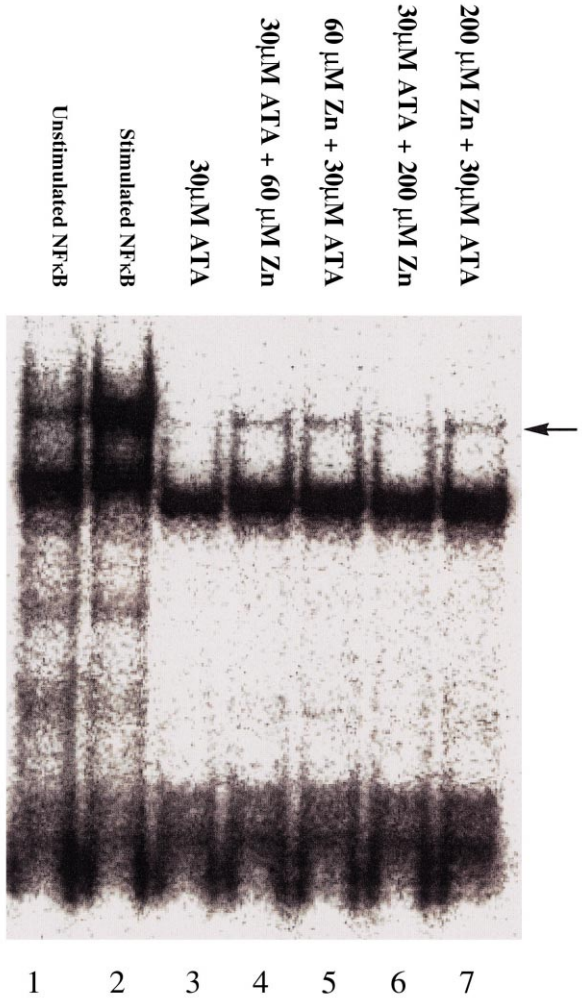

Figure 8. Effect of zinc in the inhibition of the DNA binding of NFκB by ATA (30 μM). Zinc was introduced before (lanes 5 and 7) or after (lanes 4 and 6) the addition of ATA.

so far. Zabel et al. found that 2 mM concentration of *o*-phenanthroline shows inhibitory effect on the DNA–NFκB binding.[12] We previously reported that synthetic metal chelators consisting of dimethylaminopyridine and histidine also inhibit the DNA–NFκB binding at lower concentrations (500∼1000 μM).[11] We now demonstrated that ATA inhibited the DNA–NFκB binding at the more lower concentration, 30 μM. We found that addition of Zn^{++} (2 equiv) partially restored the DNA-binding property of NFκB. This observation is in consistent with Zabel's finding that addition of Zn^{++} blocked the *o*-phenanthroline inhibition of DNA–NFκB binding.[12]

However, recent X-ray crystallographic analyses of NFκB–DNA complexes[36–39] and NFκB–IκB[40,41] revealed that no Zn is contained in NFκB or IκB. According to the X-ray structure of NFκB–IκB complex,[41] the mechanism of inhibition of NFκB–DNA binding by IκB could be discussed based on the interaction between the acidic carboxy-terminal Pro-Glu-Ser-Thr sequence of IκB and the basic DNA-contacting amino acids from the bottom of the p50 dimerization domain of NFκB. We hypothesize that the carboxyl and hydroxyl groups of ATA, analogous to the Pro-Glu-Ser-Thr sequence of IκB, could neutralize the basic DNA-binding amino acid residues of NFκB. This may be a possible mechanism of the inhibition of DNA–NFκB binding by ATA. The DNA-binding capability of NFκB was partially recovered by addition of zinc as mentioned above. This could be explained if we assume that zinc bound to the carboxyl-hydroxyl groups of ATA and the zinc-chelated ATA could not neutralize the basic amino acids of NFκB as efficiently as the metal-free ATA. Inhibitory effect of ATA on other proteins[20–35] may be explained by the same mechanism.[18] Advanced molecular design of ATA to attain the NFκB-specific inhibition is our next objective.

Experimental

General procedures

All the materials used were of reagent grade and were used without purification unless notified. The ammonium salt of aurine tricarboxylic acid was procured from BDH England. A Shimadzu AA-640-13 atomic absorption spectrometer was used for the determination of copper in the complex. Chemical speciation studies were carried out using a digital Radiometer (Copenhagen) PHM83 with a combined glass electrode, the glass electrode was calibrated before the titrations as described by Martell and Motekaitis.[19] The AUTOCAL pH-meter was calibrated over the pH range 0–14, before performing the titrations, to read the pH directly. To ensure constant ionic strength (0.1 M) during the titrations, an inert electrolyte sodium perchlorate (Fluka) was added in requisite amounts. A solution of tetramethylammonium hydroxide (TMAH, E. Merck) in water has been used as the titrant. TMAH solution was standardized by standard solution of oxalic acid. The titrations were performed in a covered glass-jacketed titration cell under a stream of presaturated nitrogen. All measurements were made in water medium at 25 °C temperature (+0.5 °C) maintained constant by using Julabo VC type thermostat. Stepwise protonation constants were calculated by fitting the pH data with the help of the program PKAS.[19] Formation constants and species distribution curves were obtained using program BEST[19] and SPE[19] respectively. UV–vis spectra were recorded on a PC based Beckman DU-64 spectrophotometer. Infrared spectra were recorded on a JEOL-6599W FTIR spectrophotometer as KBr pellets or film. EPR spectra of the frozen solution at 77 K were recorded on a JEOL TE-200 spectrometer. Molecular mechanics calculations were performed using HyperChem Professional 5.1 Modeling Systems, Hypercube Inc, USA.

Copper complex of ATA

Two mmol of copper perchlorate and 1 mmol of ammonium salt of ATA were dissolved in methanol in separate flasks. Metal solution was added dropwise into ATA solution and pH of the solution was raised to 8.0. The mixture was stirred for 24 h and then it was left for cooling after reducing the volume of the solvent. The brown coloured powder thus obtained was filtered, washed with methanol and dried in vacuum over P_2O_5. IR (KBr) 3237, 1670, 1580, 1430, 1380, 1280, 360, 380 cm^{-1}. EPR $g_{//}$ 2.439, $g_{\perp}$ 2.285. UV–vis 19,200 cm^{-1}. pH-Metric log βM_2L l9.56. Lipophilicity log P = 1.43.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotide containing a κB site from the mouse immunoglobulin κ light chain enhancer

5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′
3′-AGTCTCCCCTGAAAGGCTCTCCAGCT-5′

was phosphorylated with polynucleotide kinase in the presence of [γ-^{32}P]ATP (Amersham > 5000 Ci/m mol) and purified by a G-50 Sephadex spin column. Nuclear extract from Jurkat human T cell line either unstimulated or stimulated with TNF-α (10 ng/mL) for 20 min was prepared. Five ng of nuclear extract was used for EMSA. After incubation of each reaction mixture containing binding buffer (15 mM Tris·HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 1.5 mM dithiothreitol, 7.5% glycerol, 0.3% NP-40, 1 mg/mL BSA), 0.5 μg of poly dI-dC, nuclear extract and each compound at room temperature for 5 min, labeled DNA probe (30,000 cpm) was added and the mixture was further incubated at room temperamre for 15 min. The sample in a volume of 20 μL was loaded onto 4% poly(acrylamide) gels and electrophoresed at 150 CV.

Acknowledgements

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References

1. Cohen, J. *Science* **1997**, *277*, 32.
2. Wong, J. K.; Hezareh, M.; Gunthard, H. F.; Havlir, D. V.; Ignacio, C. C.; Spina, C. A.; Richman, D. D. *Science* **1997**, *278*, 1291.
3. Finzi, D.; Hermankova, M.; Pierson, T.; Carruth, L. M.; Buck, C.; Chaisson, R. E.; Quinn, T. C.; Chadwick, K.; Margolick, J.; Brookmeyer, R.; Gallant, J.; Markowitz, M.; Ho, D. D.; Richman, D. D.; Siliciano, R. F. *Science* **1997**, *278*, 1295.
4. Lenardo, M. J.; Baltimore, D. *Cell* **1989**, *58*, 227.
5. Baeuerle, P. A. *Biochim. Biophys. Acta* **1991**, *1072*, 63.
6. Grimm, S.; Baeuerle, P. A. *Biochem. J.* **1993**, *290*, 297.
7. Varishnav, Y. N.; Wong-Staal, F. *Ann. Rev. Biochem.* **1991**, *60*, 577.
8. Gaynor, R. *AIDS* **1992**, *6*, 347.
9. Jones, K. A.; Peterlin, B. M. *Ann. Rev. Biochem.* **1994**, *63*, 717.
10. Osborn, L.; Kunkel, S.; Nabel, G. J. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 2336.
11. Griffin, G. E.; Leung, K.; Folks, T. M.; Kunkel, S.; Nabel, G. J. *Nature* **1989**, *339*, 70.
12. Zabel, U.; Schreck, R.; Baeuerle, P. A. *J. Biol. Chem.* **1991**, *266*, 252.
13. Fujita, M.; Otsuka, M.; Sugiura, Y. *J. Med. Chem.* **1996**, *39*, 50.
14. Otsuka, M.; Fujita, M.; Aoki, T.; Ishii, S.; Sugiura, Y.; Yamamoto, T.; Inoue, J. *J. Med. Chem.* **1995**, *38*, 3264.
15. Otsuka, M.; Fujita, M.; Sugiura, Y.; Ishii, S.; Aoki, T.; Yamamoto, T.; Inoue, J. *J. Med. Chem.* **1994**, *37*, 4267.
16. *Chem. Br.*, **1994**, *30*, 90.
17. Balzarini, J.; Mitsuya, H.; De Clercq, E.; Broder, S. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 64.
18. Cushman, M.; Kanamathareddy, S.; Clercq, E. D.; Schols, D.; Goldman, M. E.; Bowen, J. A. *J. Med. Chem.* **1991**, *34*, 337.
19. Martell, A. E.; Motekaitis, R. J. *Determination and Use of Stability Constants*; VCH Publishers, New York. 1992.
20. Seki, S.; Tsutsui, K.; Oda, T. *Biochem. Biophys. Res. Commun.* **1977**, *79*, 179.
21. Tsutsui, K.; Seki, S.; Tsutsui, K.; Oda, T. *Biochim. Biophys. Acta* **1978**, *517*, 14.
22. Nakane, H.; Balzarini, J.; De Clercq, E.; Ono, K. *Eur. J. Biochem.* **1988**, *177*, 91.
23. Blumenthal, T.; Landers, T. A. *Biochem. Biophys. Res. Commun.* **1973**, *55*, 680.
24. Liao, L.-L.; Horwitz, S. B.; Huang, M.-T.; Grollman, A. P.; Steward, D.; Martin, J. *J. Med. Chem.* **1975**, *18*, 117.
25. Schulz-Harder, B.; Tata, J. R. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 903.
26. Iapaluccu-Espinoza, S.; Haim, L.; Franze-Fernández, M. *Mol. Cell Biochem.* **1983**, *55*, 41.
27. Givens, J. F.; Manly, K. F. *Nucleic Acids Res.* **1976**, *3*, 405.
28. Baba, M.; Schols, D.; Pauwels, R.; Balzarini, J.; De Clercq, E. *Biochem. Biophys. Res. Commun.* **1988**, *155*, 1404.
29. Igarashi, S. J.; Zmean, J. A. *Can. J. Biochem.* **1975**, *53*, 120.
30. Baumann, H.; Hofmann, R.; Lammers, M.; Schimpff-Weiland, G.; Follmann, H. *Z. Naturforsch.* **1983**, *39C*, 276.
31. Apirion, D.; Dohonar, D. *Antibiotics* (NY) **1975**, *3*, 327.
32. Zaug, A. J.; Cech, T. R. *Cell* **1980**, *19*, 331.
33. González, G.; Haxo, R. S.; Schleich, T. *Biochemistry* **1980**, *19*, 4299.
34. Ramón, D.; Ferrer, S.; Vicente, E.; Uruburu, F. *FEBS Microbiol. Lett.* **1986**, *36*, 9.
35. Kumar, A.; Ali, R. *Indian J. Exp. Biol.* **1986**, *24*, 79.
36. Ghosh, G.; Van Duyne, G.; Ghosh, S.; Sigler, P. B. *Nature* **1995**, *373*, 303.
37. Müller, C. W.; Rey, F. A.; Sodeoka, M.; Verdine, G. L.; Harrison, S. C. *Nature* **1995**, *373*, 311.
38. Chen, F. E.; Huang, D. B.; Chen, Y. Q.; Ghosh, G. *Nature* **1998**, *391*, 410.
39. Chen, Y. Q.; Ghosh, S.; Ghosh, G. *Nature Str. Biol.* **1998**, *5*, 67.
40. Jacobs, M. D.; Harrison, S. C. *Cell* **1998**, *95*, 749.
41. Huxford, T.; Huang, D.-B.; Malek, S.; Ghosh, G. *Cell* **1998**, *95*, 759.