Synthesis of Ribonucleoside 5′-Triphosphates Derived from 2-Thiocytidine, 4-Thiouridine, and 5-Bromocytidine

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Dedicated to Professor K. Antoš, in honour of his 70th birthday

The synthesis of 5′-triphosphates of 4-thiouridine, 2-thiocytidine, 5-bromocytidine, and [6-^{15}N]adenosine is described. All compounds are characterized by chromatographic and spectral data. Phosphorylation of corresponding nucleotides was performed using phosphoryl chloride and condensation with pyrophosphate. Intermediates were not isolated. The final products were purified by chromatography and characterized by ^{31}P NMR spectroscopy. The nucleoside triphosphates are substrates for RNA polymerase from bacteriophage T7 and were used for enzymatic synthesis of modified and isotopically labelled RNA.

5′-Triphosphates of modified nucleosides are of interest to us for three main reasons. First, they serve as substrates for enzymatic preparation of modified RNA transcripts suitable for crosslink reactions with different protein molecules [1]. Second, according to the previously described procedure, enzymatic incorporation of s^2C into position 75 in the CCA terminus of tRNA allows modification of spectroscopic labels [2—8]. Third, introduction of [6-^{15}N]-labelled adenosine into position 76 of the CCA end of tRNAs provides the possibility for ^{15}N NMR investigations.

Early methods of chemical synthesis of nucleoside 5′-triphosphates involved condensation of an activated nucleotide derivative, mostly 5′-phosphoromorpholidate [9] or 5′-phosphoroimidazolidate [10—12] with inorganic pyrophosphate [9]. The procedure of choice is presently the "one-pot" phosphorylation reaction of nonprotected nucleosides with phosphoryl chloride in trialkyl phosphates. This procedure provides predominantly nucleoside 5′-phosphorodichloridates [13] which are transformed in a good yield to nucleoside 5′-triphosphates using an excess of $(Bu_3NH)_2H_2P_2O_7$ in *N,N*-dimethylformamide (DMF) under anhydrous conditions, followed by neutral hydrolysis [14—16]. The reaction may proceed *via* a highly reactive imidoyl phosphate intermediate formed by condensation of phosphorodichloridate with DMF [16]. We have applied this "one-pot" procedure for preparation of 5′-triphosphates of photolabile and relatively unstable modified nucleosides. Here we provide a detailed isolation and purification procedure as well as spectral and chromatographic data for the isolated products.

EXPERIMENTAL

Solvents were purchased from Merck and then additionally dried as follows: pyridine and DMF were stored for 24 h over dry molecular sieves (0.3 nm); toluene was distilled over sodium. Diethyl ether and HPLC grade methanol were used as purchased. Triethylamine for triethylammonium bicarbonate buffer (TEAB) preparation was distilled before use. Reagents were purchased from Aldrich. Tributylamine and trimethyl phosphate were used directly, and phosphoryl chloride was freshly distilled before use. $(Bu_3NH)_2H_2P_2O_7$ (0.5 M-DMF solution) was prepared according to the described procedure [9].

2-Thiocytidine (s^2C) was purchased from Aldrich (Steinheim, Germany). 4-Thiouridine (s^4U) was synthesized by thionation of 2′,3′,5′-tri-*O*-benzoyluridine and its subsequent deprotection [17]. 5-Bromocytidine (br^5C) was prepared by direct bromination of cytidine [18]. [6-^{15}N]Adenosine was a kind gift of Professor *Ishido* of Tokyo University (Japan). Nucleosides were dried prior to use by coevaporation 3 times with anhydrous pyridine and coevaporation 5 times with anhydrous toluene.

Uridine derivatives br^5UTP, i^5UTP, and s^4UDP and the cytidine derivative i^5CTP were purchased from Sigma (Deisenhofen, Germany). For purifica-

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tion of triphosphates a DEAE-Sephadex A-25 ion-exchange column (40 mm×10 mm) (Pharmacia, Heidelberg, Germany) was used. Elution was done at 4 °C in a TEAB buffer (pH 7.5) gradient with the flow rate 2 $cm^3 min^{-1}$. Evaporation of pooled fractions was done under water pump pressure.

Thin-layer chromatography was performed on precoated TLC plates Cel 300-10 UV254 (0.1 mm) (Macherey—Nagel, Düren, Germany) in a 1-propanol—ammonium hydroxide—water (φ_r = 11:7:2) solvent system.

HPLC analysis was performed on a Beckman System Gold instrument (San Roman, CA, USA). SupercosilTM LC-18 column (4.6 mm × 25 cm) (Supleco, Bellefonte, PA, USA) was applied for reversed-phase ion-paired (RP—IP) analysis of triphosphates in isocratic conditions: potassium phosphate solution (c = 0.050 mol dm^{-3}, pH 6.7), tetrabutylammonium hydrogen sulfate (TBHAS) solution (c = 0.010 mol dm^{-3})—acetonitrile (φ_r = 9:1) with the exception of s^4UTP and UTP where φ_r of the solvents was 3:1. Conditions were as follows: temperature 35 °C, flow rate 1 cm^3 min^{-1}, detection at 252 nm.

^{31}P NMR spectra were recorded at 202.45 MHz on a Bruker AM spectrometer (Bruker, Karlsruhe, Germany). Samples with a concentration of 0.02 mol dm^{-3} in a D_2O—methanol-d_4(φ_r = 9:1) solvent system were measured at 277 K. 85 % phosphoric acid was used as an external reference.

Ultraviolet spectra were recorded on a UV VIS recording spectrophotometer Shimadzu UV-160A (Shimadzu, Kyoto, Japan) under the conditions indicated.

General Phosphorylation Procedure

A nucleoside (0.1 mmol) was dissolved in trimethyl phosphate (0.25 cm^3) and cooled to 0 °C, then phosphoryl chloride (0.13 mmol, 12.1 mm^3) was added dropwise under stirring and the reaction mixture was left for 3 h at 0 °C. A cold mixture of DMF solution of $(Bu_3NH)_2H_2P_2O_7$ (0.5 mmol, 1 cm^3) and tributylamine (0.1 cm^3) was added with shaking to the reaction mixture. After 3 min of shaking (except br^5C, for which 5 min shaking was necessary) at 0 °C, cold 1 M-TEAB buffer of pH 7.5 (5 cm^3) was added to stop the reaction. The reaction mixture was then extracted once with diethyl ether (5 cm^3) and the aqueous layer was applied onto DEAE-Sephadex A-25 column equilibrated in TEAB buffer. The product was eluted with a gradient of 50—500 mM-TEAB buffer of pH 7.5 (1 dm^3 each), with the exception of s^2CTP, where a gradient of 50—800 mM-TEAB buffer of pH 7.5 was applied. Fractions containing the triphosphate were combined, evaporated under reduced pressure and coevaporated several times with methanol for removing traces of triethylammonium bicarbonate. Triphosphates were obtained as sodium salts by precipitation from concentrated methanol solutions with 1 % sodium perchlorate in acetone solution (0.5 cm^3), centrifugation and washing with acetone (4 × 0.2 cm^3 portions) and dried over phosphorus pentoxide under vacuum. Yields were 79 % for s^4UTP (*Va*), 15 % for s^2CTP (*Vb*), 30 % for br^5CTP (*Vc*), and 70 % for [6-^{15}N]ATP (*Vd*).

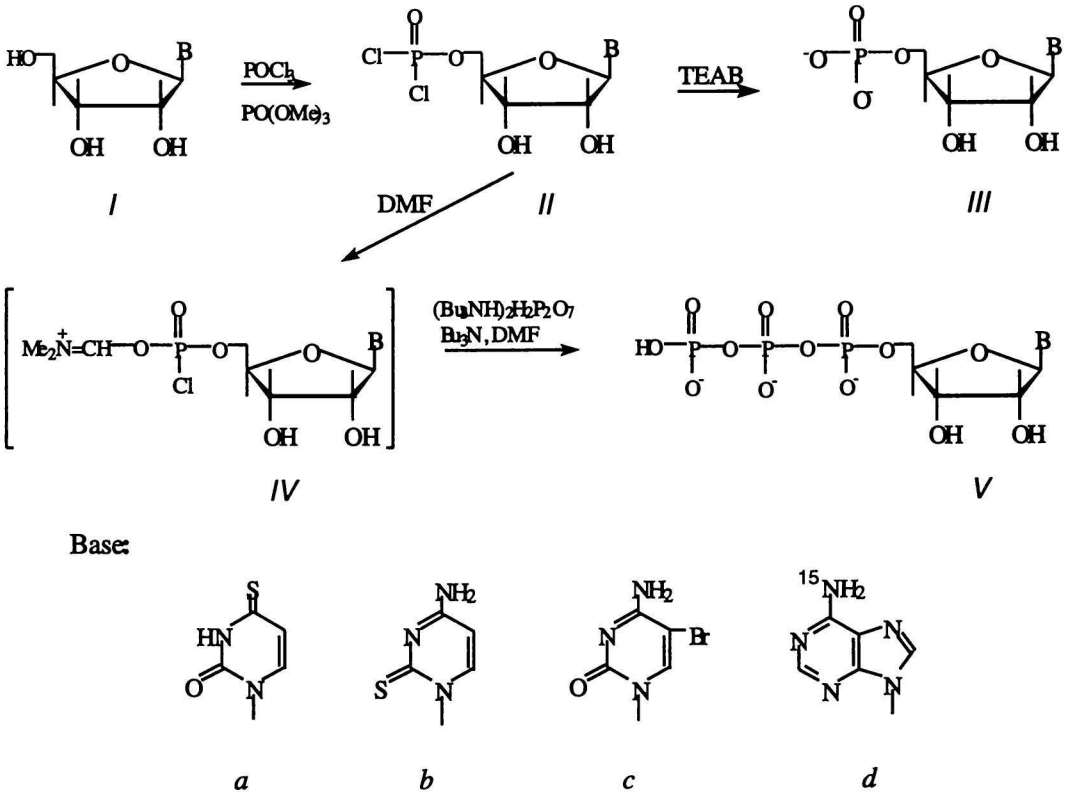
UV spectrum, λ_{max}/nm (log{ε}): for *Va* (in H_2O, pH 6) 331 (4.31), *Vb* (in H_2O, pH 6) 250 (4.35), *Vc* (in CH_3OH, pH 2) 302 (3.94), *Vd* (in H_2O, pH 7) 259 (4.19).

RNA was transcribed from pT7TthF plasmid DNA, which contains the complete synthetic gene corresponding to the *Thermus thermophilus* $tRNA^{Phe}$ flanked by the consensus promotor (−17 to −1) of T7 RNA polymerase [19] and a *Bst*NI restriction site. The transcription reactions were typically carried out in 400 mm^3reaction mixtures containing 40 mM-Tris-HCl, pH 8.1, 1 mM-Spermidine, 0.01 vol. % Triton X-100, 8 vol. % PEG (M_r = 6000), 5 mM-DTT, 24 mM-$MgCl_2$, 0.1 g dm^{-3} *Bst*NI-digested pT7TthF plasmid, 16 mM-GMP, each of the usual NTPs (c = 0.004 mol dm^{-3}), [α-^{32}P]GTP (740 MBq $mmol^{-1}$) and T7 RNA polymerase solution prepared according to [20] (c = 5 units mm^{-3}). Photoreactive cytidine or uridine nucleoside incorporations into $tRNA^{Phe}$ transcripts were made by replacing CTP or UTP in the reaction mixture with (c_i = 0.004 mol dm^3) br^5CTP, i^5CTP, s^2CTP or with br^5UTP, i^5UTP, s^4UTP, respectively. After incubation of the reaction mixtures for 2 h at 37 °C, EDTA was added to a final concentration of 0.050 mol dm^{-3}, and the reaction mixtures were precipitated with ethanol. The products of transcription were separated to single nucleotide resolution by vertical gel electrophoresis on a denaturing 12 % polyacrylamide gel (40 cm × 20 cm × 0.2 cm) containing 8 M-urea. The bands were located and their radioactivity counted in a Canberra Packard Instant Imager 2024 (Packard, Frankfurt, Germany) to determine the yield.

RESULTS AND DISCUSSION

Application of a "one-pot" synthesis of nucleoside 5′-triphosphates enabled us to obtain 5′-triphosphates of the following modified nucleosides: 4-thiouridine (s^4U) *Ia*, 2-thiocytidine (s^2C) *Ib*, 5-bromocytidine (br^5C) *Ic*, and [6-^{15}N]adenosine [6-^{15}N]A *Id*. The synthesis (Scheme 1) was done according to the previously described method of *Ludwig* [16] with a slight modification in the reaction time and isolation of products.

Generally, the first step of the synthesis is based on the selective phosphorylation of the 5′-OH group of a nonprotected nucleoside with phosphoryl chloride in trimethyl phosphate under anhydrous conditions [13]. The reaction was done for 3 h at 0 °C to achieve sufficient yield. The progress of the reaction was monitored by TLC of reaction aliquots



Scheme 1

Table 1. Chromatographic Data of Nucleosides and Their 5′-Mono- and 5-Triphosphates

Compound		TLC R_f	HPLC[a] R_t/min		Purity/%
s^4U	*Ia*	0.60			
s^2C	*Ib*	0.65			
br^5C	*Ic*	0.77			
[6-^{15}N]A	*Id*	0.61			
s^4UMP	*IIIa*	0.35			
s^2CMP	*IIIb*	0.27			
br^5CMP	*IIIc*	0.26			
[6-^{15}N]AMP	*IIId*	0.31			
s^4UTP	*Va*	0.13	8.90[b]		80.5
s^2CTP	*Vb*	0.10	29.98		89.0
br^5CTP	*Vc*	0.11	17.31		98.0
[6-^{15}N]ATP	*Vd*	0.12	25.58		99.0
UTP		0.10	18.95	(6.15[b])	
ATP		0.12	25.13		
GTP		0.12	25.13		
CTP		0.11	14.63		

a) 50 mM potassium phosphate (pH 6.7), 10 mM-TBHAS—acetonitrile (φ_r = 9 : 1); *b*) 50 mM potassium phosphate (pH 6.7), 10 mM-TBHAS—acetonitrile (φ_r = 3 : 1).

(2 mm^3) decomposed with an excess of 1 M-TEAB. The relative mobilities (R_f) of the nucleoside 5′-monophosphates are listed in Table 1. The condensation of 5′-phosphorodichloridates *IIa—IId* with an excess (5 mol) of 1/2$(Bu_3NH)_2H_2P_2O_7$ in DMF under anhydrous conditions, followed by neutral hydrolysis with TEAB buffer of pH 7.5, yielded nucleoside 5′-triphosphates. The reaction is catalyzed by formation of highly reactive imidoyl phosphate intermediate *IVa—IVd,* synthesized by condensation of phosphorodichloridate with DMF, which is the reaction solvent [16]. The reaction time, which is crucial for this step, usually is not longer than 3 min (twice as long as the reaction time used for the synthesis of ATP or 2′-dATP [16], except for the case of br^5C, for which a longer reaction time was necessary). In the most extreme cases, for hypermodified 5-substituted uridine and cytidine derivatives, the reaction time was prolonged up to 2—3 h [15]. The excess of $(Bu_3NH)_2H_2P_2O_7$ and the prolonged reaction time can lead, however, to appreciable formation of 1,4-dinucleotide tetrapyrophosphates, which decompose in neutral conditions to 5′-nucleoside diphosphates, contaminating the desired product [9].

Isolation of the desired 5′-triphosphates, as well as their purification, was achieved by DEAE-Sephadex A-25 column chromatography in triethylammonium form (pH 7.5) at 4 °C. Elution of products was conducted by a gradient solvent system from 50 mmol dm^{-3} to 500 mmol dm^{-3} (800 mmol dm^{-3} for s^2CTP) triethylammonium buffer, pH 7.5. Fractions eluted with the buffer concentration in the range 250—380 mmol dm^{-3} were collected. Minor contaminants in the crude reaction product, nucleoside 5′-mono- and diphosphates, were removed during this purification step. Final purification of 5′-triphosphates was

Table 2. ^{31}P NMR Data for Nucleoside 5′-Triphosphates *Va*—*Vd* in D_2O—Methanol-d_4 ($\varphi_r = 9 : 1$), $T = 277$ K, $c = 0.02$ mol dm^{-3}

Compound	P-α $\delta(J/\mathrm{Hz})$	P-β $\delta(J/\mathrm{Hz})$	P-γ $\delta(J/\mathrm{Hz})$
Va	−8.245 (19.8)	−20.003 (19.89)	−7.501 (19.4)
Vb	−8.402 (18.48)	−20.099 (br s)	−7.769 (18.87)
Vc	−8.373 (15.46)	−20.000 (br s)	−7.528 (17.29)
Vd	−8.212 (19.55)	−20.006 (br s)	−7.0464 (19.59)

achieved by their precipitation with sodium salt from 1 % acetone solution of sodium perchlorate. The yield of the nucleoside 5′-triphosphates *Va*—*Vd* varied from 15 % to 79 %, depending on the nature of the nucleobase. The phosphorylation of 2-thiocytidine provided the lowest yield. Here the reaction of $POCl_3$ with the relative 2-thio function led probably to decomposition of the nucleoside [21]. Chromatographic characterization of the products was performed by TLC and HPLC (Table 1).

Reverse-phase ion-paired (RP—IP) analysis of nucleoside 5′-triphosphates on a C-18 column was achieved by application of the retaining agent tetrabutylammonium hydrogen sulfate in an aqueous acetonitrile solvent system [22]. Chromatographic analysis of the cytidine derivatives br^{5}CTP and s^{2}CTP, as well as the uridine derivative s^{4}UTP showed a prolongation of the retention times in comparison with the retention times of parent nucleoside 5′-triphosphates due to their increased hydrophobicity. In the case of the most lipophilic s^{4}UTP, a less polar solvent system had to be used for elution. All compounds were homogeneous as shown by RP HPLC analysis.

^{31}P NMR analysis showed spectra typical for nucleoside 5′-triphosphates: three signals corresponding to α-, β-, and γ-phosphorus atoms. The most upfield shifted δ signal (triplet or broad singlet) at −20 belongs to the resonance of the β-phosphorus atom, which is shielded by two adjacent electronegative phosphorus atoms. Both remaining phosphorus atoms, α and γ appear at lower magnetic field giving rise to doublets of δ in the ranges −8.2 to −8.4 and −7.0 to −7.7, respectively (Table 2).

The synthesized nucleoside 5′-triphosphates were tested as substrates for DNA-dependent RNA polymerase from bacteriophage T7. The s^{2}CTP, s^{4}UTP, and br^{5}CTP as well as the commercially available br^{5}UTP, i^{5}UTP, and i^{5}CTP were incorporated into the corresponding position of the *T. thermophilus* tRNA^{Phe} by *in vitro* transcription. In each transcription experiment one nucleotide derivative was introduced in place of the parent nucleotide. The nucleotide analogues were accepted as substrates for T7 RNA polymerase at distinctly different degrees (Table 3). In the case of the unmodified tRNA transcript, 500 to 600 tRNA molecules per mole of DNA template were synthesized, thus milligram quantities of transcript were prepared. The yields of the syntheses of

Table 3. The Yields (n) of Synthesized tRNA^{Phe} Transcripts per mol of Plasmid

	tRNA^{Phe} transcripts containing						
	natural NTPs	s^{4}U	s^{2}C	i^{5}C	i^{5}U	br^{5}C	br^{5}U
$\frac{n(\mathrm{tRNA^{Phe}})}{n(\mathrm{template})}$	583	435	0	112	136	429	432

br^{5}C-, br^{5}U-, and s^{4}U-substituted tRNA transcripts were slightly lower. For 5-iodonucleotide-substituted tRNA transcripts we observed a significant decrease of the yield probably due to the slightly larger van der Waals radius of iodine as compared to bromine. The lack of incorporation of 2-thiocytidine is surprising. This polymerase must therefore be especially sensitive to modification of cytidine in position 2. Other polymerizing enzymes, such as polynucleotide phosphorylase [23] or ATP (CTP) tRNA nucleotidyltransferase [24], accept 2-thiocytidine. Possibly, the highly specific template-dependent RNA polymerase, which requires correct base-pairing between elongating rNTP and template, cannot read a Watson—Crick s^{2}C—G base pair. In this base pair the hydrogen bonds are widened by about 0.06 nm in comparison to C—G base pair. This results in weaker hydrogen bonds or in a distortion of the base pair geometry [25].

Previously s^{4}UTP was randomly incorporated into RNA by T7 RNA polymerase and the resulting RNA was used for crosslinking studies [26]. In this case the photoreactive nucleotide was obtained enzymatically using nucleoside 5′-diphosphate kinase, which converts s^{4}UDP into s^{4}UTP [27]. Our attempts to apply the enzymatically synthesized s^{4}UTP which was prepared according to [27] without chromatographic purification were also successful, but complete replacement of U by s^{4}U could not be achieved. The latter transcription assay was successful when a chemically synthesized s^{4}UTP derivative was employed. The use of chemically synthesized s^{4}UTP provided RNA in which uridines were completely replaced by 4-thiouridines. The low activity of enzymatically prepared substrates is probably due to contamination. We found the chemical synthesis of nucleoside 5-triphosphates more convenient for preparative purposes than the alternative enzymatic method.

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REFERENCES

1. Wower, J., Rosen, K. V., Hixson, S. S., and Zimmermann, R. A., *Biochimie 76,* 1235 (1994).
2. Sprinzl, M., Scheit, K. H., and Cramer, F., *Eur. J. Biochem. 34,* 306 (1973).
3. Sprinzl, M., Krämer, E., and Stehlik, D., *Eur. J. Biochem. 49,* 595 (1974).
4. Sprinzl, M., Siboska, G. E., and Pedersen, J. A., *Nucleic Acids Res. 5,* 861 (1978).
5. Kruse, T. A., Siboska, G. E., Sprinzl, M., and Clark, B. F. C., *Eur. J. Biochem. 107,* 1 (1980).
6. Joshi, R. L., Faulhammer, H. G., Haenni, A. L., and Sprinzl, M., *FEBS Lett. 208,* 189 (1986).
7. Ott, G., Faulhammer, H. G., and Sprinzl, M., *Eur. J. Biochem. 184,* 345 (1989).
8. Hainfeld, J. F., Sprinzl, M., Mandiyan, V., Tumminia, S. J., and Boublik, M., *J. Struct. Biol. 107,* 1 (1991).
9. Moffatt, J. G., *Can. J. Chem. 42,* 599 (1964).
10. Scheit, K.-H., *Chem. Ber. 101,* 1141 (1968).
11. Hoard, D. E. and Ott, D. G., *J. Am. Chem. Soc. 87,* 1785 (1965).
12. Scheit, K.-H. and Faerber, P., in *Nucleic Acid Chemistry II.* (Townsend, L. B. and Tipson, R. S., Editors.) P. 793. Wiley, New York, 1978.
13. Yoshikawa, M., Kato, T., and Takenishi, T., *Bull. Chem. Soc. Jpn. 42,* 3505 (1969).
14. Seela, F. and Mersmann, K., *Helv. Chim. Acta 75,* 1885 (1992).
15. Ruth, J. L. and Cheng, Y.-C., *Mol. Pharmacol. 20,* 415 (1981).
16. Ludwig, J., *Acta Biochim. Biophys. Hung. 16,* 131 (1981).
17. Kochetkov, N. K., Budovskii, E. I., Shibaev, V. N., and Grachev, M. A., *Izv. Akad. Nauk SSSR, Ser. Khim. 9,* 1592 (1963).
18. Russell, A. F., Prystasz, M., Hamamura, E. K., Verheyden, J. P. H., and Moffatt, J. G., *J. Org. Chem. 39,* 2182 (1974).
19. Dunn, J. J. and Studier, F. W., *J. Mol. Biol. 166,* 477 (1983).
20. Zawadzki, V. and Gross, H. J., *Nucleic Acids Res. 19,* 1948 (1991).
21. Kröger, M. and Cramer, F., *Chem. Ber. 110,* 361 (1977).
22. Perrone, P. A. and Brown, P. R., *J. Chromatogr. 317,* 301 (1984).
23. Scheit, K.-H. and Faerber, P., *Eur. J. Biochem. 24,* 385 (1971).
24. Sprinzl, M., Scheit, K.-H., and Cramer, F., *Eur. J. Biochem. 34,* 306 (1973).
25. Bugg, C. E. and Thewalt, U., *J. Am. Chem. Soc. 92,* 7441 (1970).
26. Brimacombe, R., Mitchell, P., Osswald, M., Stade, K., and Bochkariov, D., *FASEB J. 7,* 161 (1993).
27. Stade, K., Rinke-Appel, J., and Brimacombe, R., *Nucleic Acids Res. 17,* 9889 (1989).