Nucleic Acids Research, Vol. 19, No. 23 **6465–6468**

Stereochemistry of methyl transfer catalyzed by tRNA (m^5U54)-methyltransferase—evidence for a single displacement mechanism

James T.Kealey, Sungsook Lee[1], Heinz G.Floss[1] and Daniel V.Santi*
Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics, University of California, San Francisco, CA 94143 and [1]Department of Chemistry, University of Washington, Seattle, WA 98195, USA

Received September 4, 1991; Revised and Accepted October 25, 1991

ABSTRACT

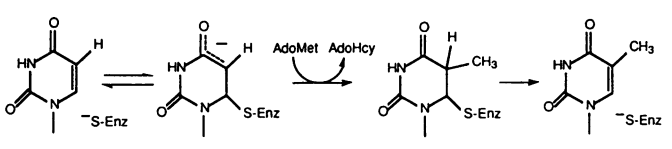
tRNA (m^5U54)-methyltransferase (RUMT) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the 5-carbon of uridine 54 of tRNA. We have determined the steric course of methyl transfer, using (*methyl*-R)- and (*methyl*-S)-[*methyl*-$^2H_1$,^3H]-AdoMet as the chiral methyl donors, and tRNA lacking the 5-methyl group at position 54 as the acceptor. Following methyl transfer, ribothymidine was isolated and degraded to chiral acetic acid for configurational analysis. Transfer of the chiral methyl group to U54 proceeded with inversion of configuration of the chiral methyl group, suggesting that RUMT catalyzed methyl transfer occurs by a single SN2 displacement mechanism.

INTRODUCTION

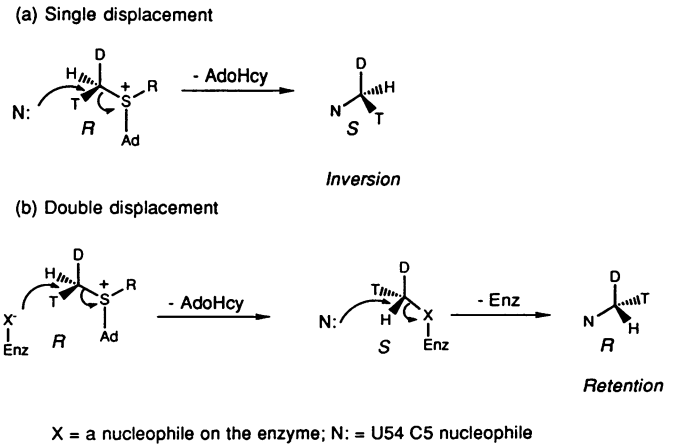
tRNA (m^5U54)-methyltransferase (RUMT, EC 2.1.1.35) catalyzes the methylation of uridine 54 (U54) of tRNA by S-adenosyl-L-methionine (AdoMet). The product of the reaction is the modified nucleoside, ribothymidine, which is invariant in the TΨC-loop of most eubacterial and eukaryotic tRNAs (1). As currently understood, the mechanism of RUMT-catalyzed transmethylation is shown in Scheme I. In the first step the thiol group of cysteine 324 reacts with C6 of U54 to produce a nucleophilic center (enol or enolate) at C5 (2)(3). Next, the methyl group from AdoMet is transferred to C5 of U54, and then β-elimination produces the methylated tRNA product and free enzyme. As shown in Scheme II, the methyl transfer proceeds by either (a) direct displacement of the methyl group of AdoMet by C5 of the U54 covalent adduct, or (b) by a double displacement reaction, whereby the methyl group is first transferred to a nucleophile on the enzyme, and subsequently to C5 (Scheme II). These two mechanisms can be distinguished by performing the methylation reaction with chiral methyl AdoMet ([*methyl*-$^2H_1$, ^3H]-AdoMet) as the cofactor and monitoring the stereochemical fate of the methyl group. The single displacement mechanism predicts inversion of configuration of the chiral methyl group, whereas the double displacement mechanism would proceed by double inversion and predict overall retention of configuration of the chiral methyl group (Scheme II).

In this paper we show that the RUMT catalyzed methyl transfer proceeds with inversion of configuration of the chiral methyl group derived from AdoMet; therefore, the reaction must occur by a single displacement mechanism.

Scheme I



Scheme II



* To whom correspondence should be addressed

EXPERIMENTAL

General Materials and Methods

Escherichia coli GB1−5-39, a strain deficient in tRNA (m^5U54)-methyltransferase, was a gift from Glenn Björk of the University of Umeå, Umeå, Sweden. Calf intestinal alkaline phosphatase was purchased from Pharmacia, P-1 Nuclease from Boehringer Mannheim, [^{3}H]-methyl-AdoMet (70 Ci/mmol) from Amersham, and DEAE-cellulose was DE-52 obtained from Whatman. Tri-N-octyl-amine and Freon-TF (1,1,2-trichlorotrifluoroethane) were purchased from Aldrich. One unit of RUMT is the amount of enzyme that methylates one pmol of U54 methyl-deficient tRNA per min at 30°C under standard assay conditions (2). (*Methyl*-R)- and (*Methyl*-S)-[*methyl*-$^2H_1$,^{3}H]AdoMet (80 mCi/mmol, 91% and 86% ee, respectively) were prepared as described (4, 5).

Preparation of m^5U54-deficient tRNA

A single colony of tRNA (m^5U54) methyltransferase-deficient *E. coli* GB1−5-39 was grown in 30 mL of Luria broth (6) at 37°C overnight. Three liters of Luria broth were inoculated with the overnight culture and the cells were grown until the A_{590} reached 3.4. The cells were harvested by centrifugation and frozen at −20°C. Frozen cells were resuspended in 50 mL of 0.1M Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and bulk methyl-deficient tRNA was purified as described (7).

Preparation of RUMT

RUMT was prepared from *E. coli* HB101/pMTXR1 as described (8) with the following modifications. To concentrate the enzyme, a pooled protein fraction from phosphocellulose chromatography containing RUMT (100 ml at 0.33 mg/ml; 610 units/ml; ca. 50% pure), was adsorbed to a 25 ml DEAE-cellulose column (2.5×12.5 cm) previously equilibrated with Buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, and 20% glycerol). After washing the column with 50 ml of Buffer A containing 50 mM KCl, RUMT was eluted at 1.5 ml/min in Buffer A containing 0.33 M KCl, and 3 ml fractions were collected. Based on protein assay (9), fractions 7−11 were pooled, yielding a final concentration of 1.8 mg protein/ml.

Methyl Transfer from [*methyl*-$^2H_1$,^{3}H]AdoMet to Methyl-deficient tRNA and Purification of the Methylated tRNA

A 3.1 ml reaction mixture containing chiral *methyl-R* AdoMet (100 nmoles, 10 μCi), 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM DTT, 22 μM RUMT (1750 units) and 82 μM methyl-deficient tRNA was kept at room temperature for 45 minutes. A second aliquot of RUMT (21 nmoles, 550 units) was added and the reaction was allowed to continue for 45 minutes, then terminated by freezing at −20°C.

The methylated tRNA was adsorbed to a 17 mL DEAE-cellulose column (2.5×12.5 cm), equilibrated with 20 mM Tris-HCl, pH 7.6, 0.2 mM EDTA (Buffer B), containing 50 mM KCl. The column was washed with 65 mL equilibration buffer to remove unreacted AdoMet, and then washed with 60 mL Buffer B containing 250 mM KCl to remove protein. The tRNA was eluted in Buffer B in 1 M KCl, and 2.3 ml fractions were collected. Fractions containing radioactivity (7−9) were pooled, and the RNA was precipitated with 2.5 volumes of cold 95% ethanol. The tRNA precipitate was collected by centrifugation, washed with 70% cold ethanol, and dried under vacuum. The yield of methylated tRNA derived from *methyl-R* AdoMet following DEAE-cellulose chromatography was 29 nmoles. Methyl transfer from *methyl-S* AdoMet (98 nmoles, 7.85 μCi) to methyl-deficient tRNA and purification of the methylated tRNA were performed as described for the *methyl-R* AdoMet. The yield of methylated tRNA following DEAE-cellulose chromatography was 28 nmoles.

Nuclease Digestion of tRNA and Isolation of [*methyl*-$^2H_1$,^{3}H] Ribothymidine

The pellets of chiral methyl tRNA, derived from either (*methyl-R*)- or (*methyl-S*)-AdoMet, were each dissolved in 400 μl of water, heated at 90°C for 2 min, and cooled on ice. To these solutions were added 40 μl of 100 mM $ZnCl_2$, 50 μl of 100 mM NH_4OAc, pH 6.2, and 100 μg of P-1 nuclease (at 1 mg/ml). After 2 hours incubation at 37°C, a second 50 μg aliquot of P-1 nuclease was added, and digestion was continued for 1 hour. The resulting solutions were adjusted to 0.2 M Tris (pH 8.9) with 1 M Tris-HCl, and 20 units of alkaline phosphatase (1 u/μl) were added to each sample. After 2 hours of incubation at 37°C, 10 more units of alkaline phosphatase were added and incubation was continued for 45 min. Conversion of tRNA to nucleosides was verified by analytical DEAE-cellulose chromatography. Nucleosides eluted in Buffer B containing 50 mM KCl, mononucleotides eluted in Buffer B containing 250 mM KCl, and tRNA eluted in Buffer B containing 1 M KCl.

To each solution of nucleosides, one-tenth volume of 50% w/v TCA was added and the samples were kept at 4°C for 30 min. The precipitated protein was removed by centrifugation, and the supernatants were extracted with an equal volume of 0.5 M tri-N-octyl-amine/ 78% v/v Freon-TF (1,1,2-trichlorotrifluoroethane) to remove TCA (10)(11). The aqueous phase was clarified by centrifugation and applied to HPLC.

Reverse Phase HPLC was performed on a Rainin Rabbit *HP* system, equipped with a Vydak C18 column (4.6 mm×25 cm), using the following conditions: Solvent A, 0.25 M ammonium acetate, pH 6; Solvent B, 40/60 (v/v) acetonitrile/water; gradient: 0−3 min 0% B, 3−10 min 0−5% B, 10−25 min 5−25% B, 25−30 min 25−50% B, 30−34 min 50−75% B, 34−37 min 75% B, 37−45 min 75−100% B; flow rate, 1.0 ml/min (7). Prior to chromatography of the [*methyl*-$^2H_1$,^{3}H]ribothymidine samples, the system was calibrated with a mixture of standard ribonucleosides, which exhibited the following retention times: Cyd, 5.1 min; Urd, 6.0 min; ribothymidine, 13.2 min; Guo, 14.5 min; and Ado, 19.2 min. This system afforded base-line separation of ribothymidine from Guo, its closest neighbor. For preparative isolation of [*methyl*-$^2H_1$,^{3}H]ribothymidine, each nucleoside solution was loaded separately to the HPLC, and the [^{3}H]-labeled ribothymidine peak from each run was collected, and dried under vacuum. The yields of [*methyl*-$^2H_1$,^{3}H]ribothymidine from (*methyl-R*)- and (*methyl-S*)-[*methyl*-$^2H_1$, ^{3}H]AdoMet were 17.6 and 13.2 nmoles, respectively.

Degradation of [*methyl*-$^2H_1$,^{3}H]Ribothymidine

Individual reaction mixtures (2.1 mL) containing 2 mg (15.8 μmol) thymine, 300 mg (1.28 mmol) potassium periodate, 0.8 mg (5.7 μmol) potassium permanganate and 0.58 μCi (*methyl-R*)- or 0.68 μCi (*methyl-S*)-[*methyl*-$^2H_1$, ^{3}H]ribothymidine were stirred at room temperature for 2.5 hour, then filtered. The filtrate was diluted with 30 ml water and transferred to a 100 ml round bottom flask. After addition of 6 ml of 6 N NaOH the solution was concentrated to 3 mL by distillation (oil bath at 140°C). The residue was acidified with 10 ml of 5 N H_2SO_4 and distilled to

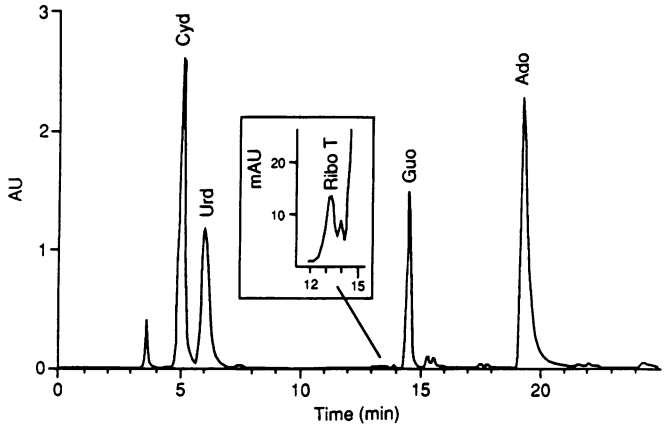


Figure 1. Analytical HPLC of nucleosides derived from tRNA methylated at U54 with (*methyl-R*)-[*methyl*-$^2H_1$,^3H] AdoMet. The inset shows an expanded region of the chromatogram corresponding to the elution position of [^3H]-labeled Ribo T. The Ribo T elution position was verified by co-injection of an authentic Ribo T standard (data not shown).

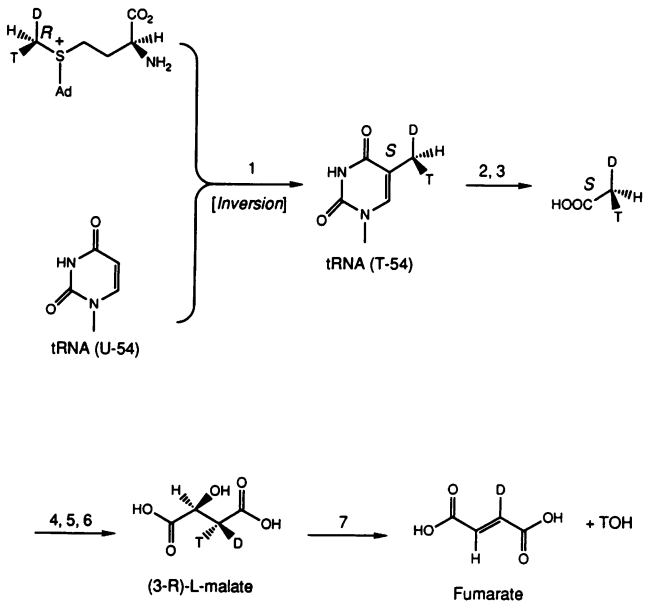


Figure 2. Methylation of tRNA with (*methyl-R*)-[*methyl*-$^2H_1$,^3H] AdoMet, degradation to chiral acetate, and synthesis of malate. 1, tRNA (m^5U54)-Methyltransferase; 2, nuclease, phosphatase, HPLC; 3, KIO_4, $KMnO_4$; 4, Acetate kinase, ATP; 5, Phosphotransacetylase, CoASH; 6, Malate synthase, glyoxylate; 7, Fumarase.

obtain chiral acetic acid. The distillate was brought to pH 10 with 0.1 N NaOH, evaporated to dryness *in vacuo*, and the [*methyl*-$^2H_1$, ^3H]acetate was subjected to configurational analysis as described (12).

In separate experiments, transfer by lyophilization was used to recover the chiral acetates. After filtering insoluble salts from the reaction mixture, the filtrate was acidified with 50 μl of 5N sulfuric acid. The solution was placed in a round bottom flask, which was connected to a receiving flask immersed in a dry ice/isopropanol bath. The sample flask was frozen in a dry ice/isopropanol bath, the bath was removed, and the system was evacuated to about 500 Torr to cause transfer of volatiles to the cooled receiving flask. The transferred material was thawed, adjusted to pH 10 with 0.1 N NaOH to convert acetic acid to sodium acetate, and then lyophilized to dryness. The chiral acetate residue was dissolved in 1 ml of water, lyophilized, and subjected to configurational analysis as described (12).

RESULTS AND DISCUSSION

The U54 methyl-deficient tRNA used in this study was derived from a strain of *E. coli* that did not contain active methylase. This non-conditional mutant displays a 4% reduction in growth rate compared to wild type *E coli*, but is fully viable (13). In a control experiment, it was shown that over 95% of the methyl-deficient tRNA was methylated by [^3H]-methyl-AdoMet and RUMT. The chiral methyl group from (*methyl-R*)- or (*methyl-S*)-[*methyl*-$^2H_1$, ^3H]AdoMet was transferred to U54 of the methyl-deficient tRNA using reagent amounts of RUMT, and the labeled tRNAs were isolated in 23 to 28% overall yield. The tRNAs were extensively digested with P-1 nuclease and alkaline phosphatase to give a 99% conversion to nucleosides as determined by DEAE-cellulose chromatography. The labeled ribothymidine was then purified by reverse-phase HPLC using a system which separates all nucleosides of tRNA (7) (Fig 1.). The labeled ribothymidine product was isolated in 13 to 14% overall yield.

The samples of [*methyl*-$^2H_1$,3H] ribothymidine were diluted with carrier thymine and degraded by a Lemieux-von Rudloff oxidation with potassium periodate and a catalytic amount of potassium permanganate (14). The resulting [*methyl*-$^2H_1$,3H] acetic acid products were recovered by distillation or lyophilization, and subjected to configurational analysis (15, 16), using a method routinely employed in one of our laboratories (12). In this procedure (Figure 2), the chiral acetate (excess R or S enantiomer) is phosphorylated, converted to acetyl-coenzyme A with phosphotransacetylase, and subsequently condensed with glyoxylate to form malate in the presence of malate synthase. The latter reaction involves abstraction of a proton from the methyl group of acetyl-Coenzyme A, and due to a large primary kinetic isotope effect (k_H/k_D = 3.8)(17), results in an uneven distribution of tritium between the two methylene hydrogens of the resulting L-malate. Since the methyl group of acetyl-coenzyme A is converted into the methylene of malate with inversion of configuration, (3-R)-L-malate is produced from S-acetate. The L-malate is then converted to fumarate with fumarase, which stereoselectively equilibrates the *pro-3R* hydrogen of L-malate with solvent protons. If tritium is present in the *pro-3R* position of L-malate, it is released into the solvent by fumarase; if it is present in the *pro-3S* position, it is retained

Table 1. Stereochemical Analysis of the tRNA (m^5U54)-methyltransferase Reaction.

Substrate	F Value[e] (acetate)	% ee
(*methyl-R*)-[*methyl*-$^2H_1$,^3H] AdoMet[c]	25.9[a]	83
	23.8[b]	90
(*methyl-S*)-[*methyl*-$^2H_1$,^3H] AdoMet[d]	73.8[a]	82
	80.1[b]	100

[a] Acetate as recovered by distillation.
[b] Acetate as recovered by lyophilization.
[c] 91% ee.
[d] 86% ee.
[e] F values are accurate to about ± 2 under the assay conditions used

in fumarate. The enantiomeric excess (ee) is the percent excess of one enantiomer of the chiral methyl group in the product, as described by equation 1.

$$ee = \text{enantiomeric excess} = \frac{|[methyl\text{-}R] - [methyl\text{-}S]|}{[methyl\text{-}R] + [methyl\text{-}S]} \times 100\ (\%) \quad (1)$$

The percentage of tritium retention in fumarate is called the F value and, by calibration of the assay procedure, has been shown to be related to the ee by equation 2. For this assay, stereochemical purity (i.e. 100% ee) is defined as a deviation of 29 from the 50% (racemic) value (16).

$$ee(\%) = [|50 - F| \times 100]/29 \quad (2)$$

Hence, chirally pure R acetate will give an F value of 79 and chirally pure S-acetate will give an F value of 21. The results of the chirality analyses are shown in Table 1. Isolation of acetate by lyophilization rather than distillation gave slightly higher ee values, probably because heating in strong base causes some exchange of the chiral acetate samples (18).

The data in Table 1 clearly show that the methyl group transfer catalyzed by tRNA (m^5U54)-methyltransferase proceeds with inversion of configuration of the methyl group. This is consistent with a mechanism of methyl transfer that involves S_N2 attack of the C5 of U54 on the methyl group of AdoMet. The results rule out a mechanism which might occur by two sequential displacements at the methyl group, first by a nucleophile of the enzyme, and then by the U54, since this should give overall retention of configuration of the methyl group (Scheme II). The data also argue against formation of a stable carbonium ion since an exposed carbonium ion would have resulted in racemization; however, a carbonium ion stabilized by the enzyme as an ion pair, *albeit* unlikely, cannot be ruled out.

Although the tRNA substrate used in this study lacked ribothymidine, there is good evidence that its structural features are homologous to those of yeast $tRNA^{Phe}$. First, unmodified and fully processed yeast $tRNA^{Phe}$ have similar tertiary interactions (19). Notably, both the U54-A58 and U55-G18 tertiary contacts are preserved in the unmodified tRNA. Second, since Ψ55 is present in the methyl deficient tRNA (20), the local base stacking arrangement in this RNA is probably the same as that observed in yeast $tRNA^{Phe}$. In the crystal structure of yeast $tRNA^{Phe}$ U54 stacks between adjacent bases G53 and Ψ55, and as a result C5 and C6 of U54 are buried. Solvent accessibility calculations have shown that C5 is 95% buried and C6 is completely solvent inaccessible. We have previously concluded that in order to form the covalent adduct between C6 of U54 and Cys 324, RUMT must facilitate opening of the T-loop of tRNA (3). The results of this study suggest additional structural constraints on reactive intermediates. That is, the C5 anion of the U54 adduct of tRNA must be positioned to accommodate an S_N2 transition state in which the *p*-orbital of C5 of the U54 adduct, the methyl group, and the sulfur of AdoMet are co-linear for S_N2 displacement. (21).

A number of methyltransferases have been examined for the steric course of the methyl group transfer. In a compilation of thirteen such reactions, eleven proceeded by inversion of configuration and two with retention (18). The two enzymes studied which catalyze N- or C-methylation of DNA bases both proceed with inversion. RUMT is the first RNA modification enzyme for which the steric course of methyl transfer has been determined. Our results clearly show that the steric course conforms to the majority of the methyltransferases thus far studied in that methyl transfer occurs with inversion of configuration.

ACKNOWLEDGEMENTS

This work was supported by USPHS grant CA14394 to D.V.S. and GM 32333 to H.G.F.

REFERENCES

1. M. Sprinzl, J. Moll, F. Meissner and T. Hartmann, *Nucleic Acids Res.* **13**, r1–r49 (1985).
2. D. V. Santi and L. W. Hardy, *Biochemistry* **26**, 8599–8606 (1987).
3. J. T. Kealey and D. V. Santi, *Biochemistry* **30**, 9724–9728 (1991).
4. R. W. Woodard, L. Mascaro, R. Horhammer, S. Eisenstein and H. G. Floss, *J. Amer. Chem Soc.* **102**, 6314–6318 (1980).
5. R. W. Woodard, M.-D. Tsai, H. G. Floss, P. A. Crooks and J. K. Coward, *J. Biol. Chem* **255**, 9124–9127 (1980).
6. J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning, A laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989).
7. M. Buck, M. Connick and B. N. Ames, *Anal. Biochem.* **129**, 1–13 (1983).
8. X. Gu and D. V. Santi, *DNA and Cell Biology* **9**, 273–278 (1990).
9. M. M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976).
10. J. Y. Khym, *Clin. Chem.* **21**, 1245–1252 (1975).
11. A. L. Pogolotti and D. V. Santi, *Anal. Biochem.* **126**, 335–345 (1982).
12. H. G. Floss and M.-D. Tsai, *Adv. Enzymol.* **50**, 243–302 (1979).
13. G. R. Bjork and F.C. Neidhardt, *J. Bacteriol.* **124**, 99–111 (1975).
14. R. U. Lemieux and E. von Rudloff, *Canad. J. Chem.* **33**, 1701–1709 (1955).
15. J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel and C. Gutschow, *Nature (London)* **221**, 1212–1213 (1969).
16. J. Lüthy, J. Retey and D. Arigoni, *Nature (London)* **221**, 1213–1215 (1969).
17. H. Lenz and H. Eggerer, *Eur. J. Biochem.* **65**, 237–246 (1976).
18. D. K. Ho, J. C. Wu, D. V. Santi and H. G. Floss, *Arch. Biochem. and Biophys.* **284**, 264–269 (1991).
19. K.B. Hall, J.R. Sampson, O.C. Uhlenbeck, and A.G. Redfield, *Biochemistry* **28**, 5794–5801 (1989)
20. S. Yang, E.R. Reinitz, and M.L. Gefter, *Arch. Biochem. and Biophys* 157, 55–62 (1973).
21. J. March, *Advanced Organic Chemistry* (John Wiley & Sons, Inc., 1985), p. 258.