Peptidyl Transferase Substrate Specificity with Nonaromatic Aminoacyl Analogues of Puromycin

Kei-Lai L. Fong and Robert Vince*

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455.
Received January 27, 1978

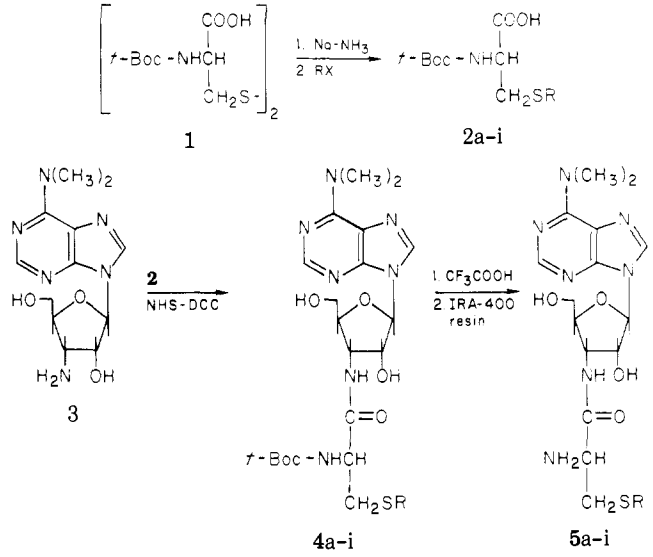
A series of puromycin analogues, 3′-*N*-(S-substituted L-cysteinyl)puromycin aminonuleosides, has been prepared and examined as substrates for ribosomal peptidyl transferase. S-Substituted *N*-*tert*-butyloxycarbonyl-L-cysteines were coupled with puromycin aminonucleoside using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. Removal of the *t*-Boc blocking group with anhydrous trifluoroacetic acid gave the desired puromycin analogues. Kinetic studies indicate that the nonaromatic aminoacyl analogues of puromycin are effective substrates for the peptidyl transferase reaction. In addition, the discovery of the existence of hydrophilic character beyond the region normally occupied by hydrophobic amino acid R groups of the aminoacyladenyl termini of tRNA molecules, and the proper exploitation of this information, has provided the first active purmoycin analogue possessing a hydrophilic amino acid.

The peptide chains of proteins are synthesized on ribosomes by a series of reactions culminating in peptide bond formation by a transfer reaction catalyzed by peptidyl transferaze [peptidyl-tRNA:aminoacyl-tRNA (O→N)-peptidyl transferase].[1,2] The transfer seems to involve a nucleophilic attack of the α-amino group of aminoacyl-tRNA on the carboxyl ester link of peptidyl-tRNA with subsequent cleavage of the ester and formation of the peptide bond.[3-5] Although relatively little is known about the precise mechanism of catalysis, current models of the active center of the ribosomal peptidyl transferase invoke a P site which binds the CCA terminus of peptidyl-tRNA and an A site which binds the CCA terminus of aminoacyl-tRNA.[6] The antitumor antibiotic, puromycin, inhibits protein synthesis as a consequence of its striking resemblence to the aminoacyladenyl terminus of aminoacyl-tRNA. It has been demonstrated that puromycin competes with aminoacyl-tRNA at the A site and subsequently interacts with the peptidyl-tRNA at the P site, causing premature release of the polypeptide chains from the ribosome.[4,7] For this reason, puromycin has been used in the investigation of the peptidyl transferase reaction.

Nathans and Neidle[8] were the first to observe that the nature of the amino acid of puromycin derivatives played an important role in the ability of these compounds to bind to the ribosome. Symons et al.[9] and Harris et al.[10] have recently confirmed previous reports[11-13] that the substitution of the amino acid moiety of puromycin with other amino acids produced derivatives with high activity in releasing peptide from procaryotic and eucaryotic ribosomes only when the amino acid contained an aromatic ring. Harris and Symons have proposed a model of the peptidyl transferase center which includes a hydrophobic site which is relatively specific for the binding of the aromatic aminoacyl R groups of puromycin derivatives.[14] In this communication, we explore the requirement for an aromatic amino acid in the puromycin molecule. The puromycin analogue, 3′-*N*-cysteinylpuromycin aminonucleoside (PAN-L-Cys), was used to provide a variety of *S*-alkyl and *S*-aryl derivatives for this purpose. These compounds were used to examine the region extending beyond the hydrophobic region of the aminoacyl binding area of the A site for possible hydrophilic character. The subsequent placement of a single hydroxyl group into such a hydrophilic pocket may impart sufficient binding to enhance the formation of a complex with the ribosome.

Synthesis. The synthetic route for the preparation of *S*-aryl- and *S*-alkyl-L-cysteinyl analogues of puromycin is outlined in Scheme I. Thus, reduction of *N*-*tert*-butyloxycarbonyl-L-cystine (1) with sodium and liquid ammonia followed by addition of alkyl halide gave the S-substituted

Scheme I



a, R = $(CH_2)_2CH_3$
b, R = $(CH_2)_3CH_3$
c, R = $(CH_2)_4CH_3$
d, R = $(CH_2)_5CH_3$
e, R = $(CH_2)_7CH_3$
f, R = $(CH_2)_5CH_2OH$
g, R = $CH_2C_6H_5$
h, R = $CH(C_6H_5)_2$
i, R = $CH(C_2H_5)_2$
t-Boc = *tert*-butyloxycarbonyl
NHS = *N*-hydroxysuccinimide
DCC = dicyclohexylcarbodiimide

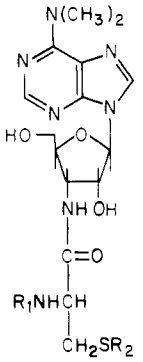
N-*tert*-butyloxycarbonyl-L-cysteines **2**. The blocked amino acids **2** were coupled with puromycin aminonucleoside (PAN, **3**) using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide by a method previously described.[15] Removal of the *tert*-butyloxycarbonyl blocking group from **4** with anhydrous trifluoroacetic acid gave the desired puromycin analogues **5** (Table I).

Results and Discussion

Puromycin analogues in which the *p*-methoxyphenylalanyl group was replaced by *S*-alkyl- and *S*-arylcysteinyl moieties were evaluated as substrates for the peptidyl transferase reaction. In this assay, the puromycin analogue was incubated with a preformed complex consisting of *Escherichia coli* ribosomes, poly(U), and *N*-acetyl[^{14}C]-phenylalanyl-tRNA as illustrated in Figure 1. Orientation of the analogue into the A site would allow the *S*-alkyl or *S*-aryl moiety (R) to interact with the hydrophobic area normally occupied by the aromatic ring of puromycin.

0022-2623/78/1821-0792$01.00/0 © 1978 American Chemical Society

Table I. Physical Data



compd	R_1	R_2	% yield	mp, °C	mol formula
4a	*t*-Boc	$-(CH_2)_2CH_3$	72	173-175	$C_{23}H_{37}N_7O_6S$
5a	H	$-(CH_2)_2CH_3$	69	148-150	$C_{18}H_{29}N_7O_4S$
4b	*t*-Boc	$-(CH_2)_3CH_3$	82	169-170	$C_{24}H_{39}N_7O_6S$
5b	H	$-(CH_2)_3CH_3$	77	153-154	$C_{19}H_{31}N_7O_4S$
4c	*t*-Boc	$-(CH_2)_4CH_3$	66	165-167	$C_{25}H_{41}N_7O_6S$
5c	H	$-(CH_2)_4CH_3$	69	148-149	$C_{20}H_{33}N_7O_4S$
4d	*t*-Boc	$-(CH_2)_5CH_3$	76	165-166	$C_{26}H_{43}N_7O_6S$
5d	H	$-(CH_2)_5CH_3$	78	154-155	$C_{21}H_{35}N_7O_4S$
4e	*t*-Boc	$-(CH_2)_7CH_3$	73	169-170	$C_{28}H_{47}N_7O_6S$
5e	H	$-(CH_2)_7CH_3$	68	140-141	$C_{23}H_{39}N_7O_4S$
4f	*t*-Boc	$-(CH_2)_5CH_2OH$	65		$C_{26}H_{43}N_7O_7S$
5f	H	$-(CH_2)_5CH_2OH$	67	167-168	$C_{21}H_{35}N_7O_5S$
4g	*t*-Boc	$-CH_2C_6H_5$	89	155-156	$C_{27}H_{37}N_7O_6S$
5g	H	$-CH_2C_6H_5$	63	156-160	$C_{22}H_{29}N_7O_4S$
4h	*t*-Boc	$-CH(C_6H_5)_2$	78		$C_{33}H_{41}N_7O_6S$
5h	H	$-CH(C_6H_5)_2$	72	192-194	$C_{28}H_{33}N_7O_4S$
4i	*t*-Boc	$-CH(C_2H_5)_2$	67	173-175	$C_{25}H_{41}N_7O_6S$
5i	H	$-CH(C_2H_5)_2$	61	159-160	$C_{20}H_{33}N_7O_4S$

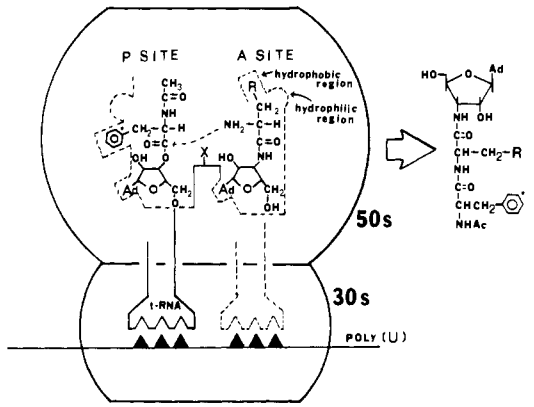
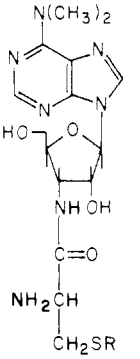


Figure 1. Schematic representation of the peptidyl transferase reaction involving transfer of *N*-acetyl[^{14}C]phenylalanine from the P site to a puromycin analogue located at the A site. Peptide bond formation results from attack of the α-amino group of aminoacyl-tRNA (or puromycin derivatives) on the carboxyl terminal ester of peptidyl-tRNA. It is equally possible that a ribosome–peptide intermediate (via X) may first occur. The dotted lines overlapping the two subunits indicate the site normally occupied by an aminoacyl-tRNA. Ad is used to represent an adenine or *N*,*N*-dimethyladenine in this diagram.

Since the A site must accommodate all amino acids under normal conditions, an adjacent hydrophilic region is also postulated in Figure 1 to accommodate the corresponding hydrophilic side chains. The length of the R groups in the present study provided the first opportunity to explore the binding character of the ribosome beyond the hydrophobic region occupied by the R groups of the natural hydrophobic amino acids. Double reciprocal plots of initial velocities of product formation vs. substrate concentrations were used to obtain the kinetic parameters, K_m and V_{max}, for each analogue. The ratio V_{max}/K_m appears as a rate constant in the Michaelis–Menton equation at low substrate concentrations. Thus, V_{max}/K_m was used as a measure of substrate efficiency for each analogue.

Kinetic studies demonstrate that the PAN-*S*-alkyl-L-Cys derivatives **5a–e** are capable of binding at the A site and can accept a donor from the P site (Table II) in a manner identical with the mechanism of action of puromycin. The data in Table II also reveal that a parallel increase in substrate efficiency accompanies the additional hydrophobic character of the side chain as R is extended from four carbons (**5b**) to six carbons (**5d**). However, the resultant drop in substrate efficiency as the side chain is extended to eight carbons (**5e**) suggested the possibility of contact with a hydrophilic region. To test this hypothesis, 3′-*N*-[*S*-(6-hydroxyhexyl)-L-cysteinyl]puromycin aminonucleoside (**5f**) was prepared in which a hydroxy group was used to impart hydrophilic character beyond C-6 of the alkyl chain. As expected, a dramatic increase in substrate efficiency was noted when **5f** was compared with **5e** in the peptidyl transferase reaction. Equally interesting is the observation that the previously reported PAN-*S*-Bzl-L-Cys[9,10,16] (**5g**) exhibited a lower substrate efficiency than any of the nonaromatic analogues. The branched chain compounds, **5h** and **5i**, were used to evaluate the bulk tolerance of the ribosome at the aminoacyl binding site. The inability of **5h** and **5i** to act as acceptors in the transpeptidation reaction suggested that the increased bulk of the R group prevented the binding of these analogues to the ribosome. An alternate possibility for the lack of product formation could also be envisaged by invoking the formation of a nonproductive reversible ribosome–analogue complex. This possibility was eliminated by competitive inhibition studies in which it was observed that the branched-chain analogues were unable to compete with puromycin in the peptidyl transferase reaction.

Table II. Kinetic Constants and Substrate Efficiencies for Puromycin Analogues in the Peptidyl Transferase Reaction[a]



compd	R	K_m, mM	V_{max}, pmol/min	V_{max}/K_m	substrate efficiency (% of puromycin)[b]
5a	$(CH_2)_2CH_3$	0.374	2.86	7.65	44.8
5b	$(CH_2)_3CH_3$	0.395	2.43	6.16	36.2
5c	$(CH_2)_4CH_3$	0.255	2.36	9.26	54.3
5d	$(CH_2)_5CH_3$	0.215	2.46	11.4	67.5
5e	$(CH_2)_7CH_3$	0.177	1.38	7.79	45.6
5f	$(CH_2)_5CH_2OH$	0.166	2.71	16.3	95.6
5g	$CH_2C_6H_5$	0.460	1.36	2.95	17.4
5h	$CH(C_6H_5)_2$	Inactive[c]			
5i	$CH(C_2H_5)_2$	Inactive[c]			

[a] The Ac[^{14}C]Phe-tRNA was bound to the ribosomes in a reaction mixture containing 100 mM Tris-Cl (pH 7.5), 100 mM NH_4Cl (pH 7.6), 15 mM $Mg(OAc)_2$, 0.65 mM dithiothreitol, 2.78 A_{260} units of washed *E. coli* ribosomes, 1.2 mM GTP, 63 μg of FWR, 0.35 A_{260} units of poly(U), and 21 pmol of Ac[^{14}C]Phe-tRNA (464 pCi/pmol). The binding mixture was incubated at 28 °C for 8 min, and the peptidyl transferase reaction was intiated by the addition of 80 μL of incubation cocktail to 20 μL of puromycin analogue. Reactions were incubated for a specified time and product formation was measured as described in ref 16. K_m and V_{max} values were determined by double reciprocal plots. [b] The K_m and V_{max} values for puromycin in this assay were 0.234 mM and 3.99 pmol of product per minute, respectively. [c] No significant product formation was detected at the highest concentration tested (2×10^{-4} M).

Earlier studies to determine the structural requirements for puromycin activity have led to the general conclusion that there is an absolute requirement for an aromatic amino acid in the puromycin molecule. Nathans and Neidle[8] showed that PAN-L-Tyr and PAN-L-Phe were active in an *E. coli* system whereas L-Leu and L-Gly derivatives were inactive. Symons and Harris et al.[9,10,14,16] studied the requirements for aromatic amino acids in the inhibition of protein synthesis by puromycin analogues and obtained results similar to those of Nathans and Neidle.[8] Attempts to explain the function of the aromatic group have included the following: a stacking of the aromatic ring with purine rings of puromycin and the adenylic acid of tRNA;[17] the fortuitous binding of the aromatic ring with the binding site normally occupied by the cytosine ring of the pentultimate base of tRNA;[9] the optimal fit into a hydrophobic pocket of the ribosome;[14] and the creation of a favorable conformation of the acceptor by the aromatic amino acid at the expense of thermodynamic parameters.[18]

Although the postulated (Figure 1) hydrophobic and hydrophilic areas are required at the A site to accommodate the R groups of all 20 amino acids, it is most likely that the increased interaction between the R group of the aromatic amino acids and the corresponding binding site are of sufficient force to stabilize the otherwise weak binding of the puromycin molecule. Thus, the contribution of the phenyl ring may be a simple hydrophobic interaction at the A site. This idea is confirmed by the recent report by Ariatti and Hawtrey of an active puromycin analogue in which a cyclohexyl ring replaces the phenyl ring of the nucleoside antibiotic.[19] The present study also confirms the existence of a hydrophobic binding site and extends the exploration of the ribosome beyond the area normally occupied by the R groups of the aminoacyladenyl termini of tRNA molecules. The discovery of the existence of hydrophilic character beyond the normal binding region, and the proper exploitation of this information, has led to the development of the first active puromycin analogue possessing a hydrophilic amino acid R group.

Experimental Section

Puromycin dihydrochloride and PAN were obtained from ICN Pharmaceuticals, Inc., [^{14}C]-L-phenylalanine was obtained from New England Nuclear, and *E. coli* cell paste (B, mid log) was purchased from General Biochemicals. The polynucleotides were obtained from Miles Laboratories, and ATP, GTP, phosphoenolpyruvate, and pyruvate kinase were purchased from Sigma. Preparation of ribosomes, S-100, factors washable from ribosomes (FWR), and Ac[^{14}C]-L-Phe-tRNA was as previously described.[20]

Elemental analyses were performed by M-H-W Laboratories, Garden City, Mich. Melting points were determined on a Mel-Temp and are corrected. Nuclear magnetic resonance was obtained with a Varian T-60A spectrometer, infrared with a Perkin-Elmer 237B spectrophotometer, and ultraviolet with a Beckman 25 recording spectrophotometer. Compounds were purified by preparative thin-layer chromatography on glass plates (20 × 20 cm) coated with 2 mm of silica gel 254 (E. Merck, Darmstadt) or with column chromatography on silica gel 60, 70–230 mesh (E. Merck, Darmstadt). Since several compounds were prepared by similar procedures, only one representative example is described in the Experimental Section. Satisfactory elemental analyses (±0.4% of calculated values) were obtained for each compound listed in Scheme I.

S-Alkylated Cysteines. *N*-*tert*-Butyloxycarbonyl-*S*-propyl-L-cysteine (2a). To a solution of 1[21] (1.0 g, 2.27 mmol) in liquid ammonia (40 mL) was added metallic sodium until a blue color persisted for 15 min. Ammonium chloride was added to discharge the color and 1-bromopropane (0.58 g, 4.76 mmol) was added. The reaction mixture was stirred at room temperature and the ammonia was allowed to evaporate. The last traces of ammonia were removed in vacuo leaving a white residue. The residue was dissolved in ice water (30 mL) and acidified with cold 1 N hydrochloric acid. The semisolid precipitate was extracted

with ethyl acetate (3 × 30 mL). The combined extracts were washed with half-saturated sodium chloride (30 mL), dried (Na_2SO_4), and evaporated and gave 1.21 g of **2a** as a colorless oil. The crude product was purified by preparative thin-layer chromatography using chloroform–methanol–glacial acetic acid (90:5:3) as the developing solvent. The pure product (R_f 0.47) was extracted from the silica gel with ethyl acetate and methanol (4:1) and gave 789 mg (66%) of pure **2a** as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 1.0 (t, 3, J = 7.0 Hz, $-SCH_2CH_2CH_3$), 1.2–1.68 (m, 2, $-SCH_2CH_2CH_3$) overlapping 1.43 (s, 9, $C(CH_3)_3$], 2.5 (t, 2, J = 7.0 Hz, $-SCH_2CH_2CH_3$), 2.93 (**br**, 2, CH_2), 4.38 (br, 1, CH), 5.62 (br, 1, NH), 10.9 (s, 1, COOH). Anal. ($C_{11}H_{21}NO_4S$) C, H, N.

All remaining *N-tert*-butyloxycarbonyl-S-substituted L-cysteines were prepared in the same way and were isolated as colorless oils with the exception of **2h** (mp 90–92 °C) which was recrystallized from ethyl acetate–petroleum ether.

6-(Dimethylamino)-9-[3′-(*N*-*tert*-butyloxycarbonyl-*S*-propyl-L-cysteinylamino)-3′-deoxy-β-D-ribofuranosyl]purine (4a). To a solution of **2a** (234 mg, 0.890 mmol), PAN (250 mg, 0.850 mmol), and *N*-hydroxysuccinimide (105 mg, 0.890 mmol) in dry dimethylformamide (8 mL) was added freshly subliminated dicyclohexylcarbodiimide (184 mg, 0.890 mmol). The solution was stirred at ambient temperature for 20 h under anhydrous conditions. The precipitated dicyclohexylurea was removed by filtration and washed with ethyl acetate (25 mL). The combined filtrate and wash were evaporated in vacuo and the residue was dissolved in hot ethyl acetate (10 mL), chilled, and filtered. The filtrate was evaporated to dryness and the residue was dissolved in a small amount of chloroform and passed through a silica gel column (60 g) eluted with 3% methanol–chloroform. The eluent containing **4a** was collected and evaporated to a white solid product: 331 mg (73%); mp 173–175 °C; IR (KBr) 3420 (br, OH, NH), 1690 (carbamate), 1660 (amide), 1600 cm^{-1} (purine); ^{1}H NMR ($CDCl_3$) δ 0.97 (t, 3, J = 7.0 Hz, $-SCH_2CH_2CH_3$), 1.27–1.73 (m, 2, $-SCH_2CH_2CH_3$) overlapping with 1.43 [s, 9, $C(CH_3)_3$], 2.53 (t, 2, J = 7 Hz, $-SCH_2CH_2CH_3$), 2.88 (d, 2, J = 6 Hz, CH_2) 3.4 [s, 6, $N(CH_3)_2$], 3.9 (br, 2, H-5′), 4.1–4.93 (m, 4, α-CH, H-2′, H-3′, H-4′), 5.74 (s, 1, H-1′) overlapped by 5.33–6.17 (m, 3 exchangeable H), 7.38 (br, 1, NH), 8.0 (s, 2, H-8, H-2). Anal. ($C_{23}H_{37}N_7O_6S$) C, H, N.

6-(Dimethylamino)-9-[3′-(*S*-propyl-L-cysteinylamino)-3′-deoxy-β-D-ribofuranosyl]purine (5a). A solution of 255 mg (0.47 mmol) of **4a** in anhydrous trifluoroacetic acid (6 mL) was incubated at ambient temperature for 8 min. The trifluoroacetic acid was removed in vacuo (25 °C, 1 mm) by azeotroping with freshly dried acetonitrile (8 × 3 mL). The white solid foam residue was dissolved in methanol and passed through a column of IRA-400 (OH^-) resin (20 mL) packed in methanol. The basic eluent was collected and evaporated to dryness and gave the crude product. The product was applied to a silica-gel column (35 g) packed in 3% methanol in chloroform. The pure material was removed from the column using 6.5% methanol in chloroform as the eluent and 143 mg (69%) of **5a** was obtained as a white solid: mp 148–149 °C; UV max in nm ($\epsilon \times 10^{-3}$) (pH 1) 269 (18.3), (pH 7) 276 (18.6), (pH 13) 276 (18.5); IR (KBr) 3420 (br, OH, NH, NH_2), 1655 (amide), 1601 (C=N); NMR ($CDCl_3$) δ 0.98 (t, 3, J = 7 Hz, $-SCH_2CH_2CH_3$), 1.57 (sextet, 2, J = 7 Hz, $-SCH_2CH_2CH_3$), 2.47 (t, 2, J = 7 Hz, $-SCH_2CH_2CH_3$) overlapping with 1 exchangeable H, 2.97–2.7 (m, 2, $C_\beta H_2$), 5.9 (d, 1, $J_{1',2'}$ = 3.0 Hz, H-1′), 8.07 (s, 2, H-2, H-8) overlapping by 1 exchangeable H, 3.4 (s, NMe_2). Anal. ($C_{18}H_{29}N_7O_4S$) C, H, N.

Acknowledgment. We thank Jay Brownell for competent assistance with the biological assays. This investigation was supported by Grants CA 13592 and CA 16623 and Research Career Development Award (to R.V.) CA 25258 from the National Cancer Institute, Department of Health, Education and Welfare.

References and Notes

(1) R. E. Monro, *J. Mol. Biol.*, **26**, 147 (1967).
(2) B. E. H. Maden, R. R. Traut, and R. E. Monro, *J. Mol. Biol.*, **35**, 333 (1968).
(3) D. W. Allen and P. C. Zamecnik, *Biochim. Biophys. Acta*, **55**, 865 (1962).
(4) D. Nathans, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **23**, 984 (1964).
(5) D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.*, **51**, 585 (1964).
(6) R. E. Monro, T. Staehelin, T. Celma, and D. Vazquez, *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 357 (1969).
(7) R. R. Traut and R. E. Monro, *J. Mol. Biol.*, **10**, 63 (1964).
(8) D. Nathans and A. Neidle, *Nature (London)*, **197**, 1076 (1963).
(9) R. H. Symons, R. J. Harris, L. P. Clarke, J. F. Wheldrake, and W. H. Elliott, *Biochim. Biophys. Acta*, **179**, 248 (1969).
(10) R. J. Harris, J. E. Hanlon, and R. H. Symons, *Biochim. Biophys. Acta*, **240**, 244 (1971).
(11) J. P. Waller, T. Erdos, R. Lemoine, S. Guttmann, and E. Sandrin, *Biochim. Biophys. Acta*, **119**, 566 (1966).
(12) J. Cerna, S. Chladek, I. Rychlik, and J. Zemlicka, *Biochim. Biophys. Act*, **199**, 291 (1970).
(13) J. Cerna, I. Rychlik, J. Zemlicka, and S. Chladek, *Biochim. Biophys. Acta*, **204**, 203 (1970).
(14) R. J. Harris and R. H. Symons, *Bioorg. Chem.*, **2**, 286 (1973).
(15) S. Daluge and R. Vince, *J. Med. Chem.*, **15**, 171 (1972).
(16) D. J. Eckermann, P. Greenwell, and R. H. Symons, *Eur. J. Biochem.*, **41**, 547 (1974).
(17) I. D. Raacke, *Biochem. Biophys. Res. Commun.*, **43**, 168 (1971).
(18) A. A. Krayevsky, M. K. Kukhanova, and B. P. Gottikh, *Nucleic Acid Res.*, **2**, 2223 (1975).
(19) M. Ariatti and A. O. Hawtrey, *Biochem. J.*, **145**, 169 (1975).
(20) P. H. Duquette, C. L. Ritter, and R. Vince, *Biochemistry*, **13**, 4855 (1974).
(21) I. Photaki, *J. Am. Chem. Soc.*, **88**, 2290 (1966).