2′(3′)-O-Glycyl Oligoribonucleotides with Sequences of the 3′-Terminus of Glycyl-tRNA: Chemical Synthesis and Properties in Partial Reactions of Protein Biosynthesis†

Claudia Scalfi-Happ,‡ Erwin Happ,‡ Sandhya Ghag,§ and Stanislav Chládek*

Department of Chemistry, Michigan Cancer Foundation, Detroit, Michigan 48201

Received December 30, 1986; Revised Manuscript Received April 6, 1987

ABSTRACT: Specific syntheses of 2′(3′)-*O*-aminoacyl oligoribonucleotides C-C-A-Gly (**12**), C-C-A(AcGly) (**7**), U-C-C-A-Gly (**17**), and C-U-C-C-A-Gly (**19**), which are the 3′-terminal sequences of *Escherichia coli* Gly-tRNA (or AcGly-tRNA, respectively) are described. Compounds **12**, **17**, and **19** were synthesized by employing the benzotriazolyl phosphotriester approach with the following protection groups on the components: benzoyl for the heterocyclic amino groups, 2-chlorophenyl group for internucleotide phosphate protection, dimethoxytrityl and levulinoyl groups for blocking of the 5′-hydroxyl, methoxytetrahydropyranyl group for protection of the 2′-hydroxyl functions, and *N*-(benzyloxycarbonyl)orthoglycinate as the masked aminoacyl group simultaneously protecting the 2′,3′-cis diol group of the 3′-terminal adenosine moiety. The fully protected tri-, tetra-, and pentanucleotides were obtained via 5′-extension of di- and trinucleotide blocks after prior selective removal of the 5′-*O*-levulinoyl group with hydrazine. The blocked derivatives **11**, **16**, and **18** were totally deprotected by reactions with NH_4OH, H^+, and H_2/Pd to yield the target compounds **12**, **17**, and **19** in good yields. C-C-A(AcGly) (**7**) was synthesized according to a stepwise procedure via activation of preformed diesters with (mesitylenesulfonyl)tetrazole. C-C-A-Gly (**12**), U-C-C-A-Gly (**17**), and C-U-C-C-A-Gly (**19**) were all acceptor substrates in the peptidyltransferase reaction with the Ac-Phe-tRNA–70S ribosome–poly(U) system. All three models also promoted EF-Tu–70S ribosome GTP hydrolysis. Since activities of compounds **12**, **17**, and **19** in both systems are quite similar, addition of nucleotides next to the common C-C-A sequence of tRNA does not appear to enhance binding of these models to either peptidyltransferase A site or EF-Tu. The GTPase-promoting activity of C-C-A-Gly (**12**) in the EF-Tu–70S ribosome system is greatly stimulated by presence of EF-Ts. C-C-A(AcGly) (**7**) was completely inactive as a donor in the peptidyltransferase reaction with Phe-tRNA in the 70S ribosome system under fragment reaction conditions.

It is well established that 2′(3′)-*O*-aminoacyl oligoribonucleotides may mimic the role of the aa-tRNA[1] 3′-terminus in various subreactions of the protein biosynthesis elongation cycle. Specifically, 2′(3′)-*O*-aminoacyl oligoribonucleotides with a sequence related to the 3′-end of aa-tRNA interact with EF-Tu and ribosomal A and P sites and can serve as acceptors or donors in the peptidyltransferase reaction. Thus, these compounds become important tools for studying the role of the tRNA 3′-end in protein biosynthesis (Chládek & Sprinzl, 1985). The development of specific chemical syntheses for the 2′(3′)-*O*-aminoacyl oligoribonucleotides is thus of considerable importance, since the enzymic degradation of aa-tRNA may provide only compounds with the natural sequence of a given tRNA and in very limited quantities. Several years ago, we reported a specific synthesis of these compounds, using triester methodology (Kumar et al., 1982). Our ultimate goal in this field is to develop specific and efficient syntheses of longer 2′(3′)-*O*-aminoacyl oligoribonucleotides, which could be base-paired with a complementary oligonucleotide strand, thus mimicking the structure of the entire aa-tRNA aminoacyl stem. Accordingly, we have been studying methods for efficient routes to longer 2′(3′)-*O*-aminoacyl oligoribonucleotides. In this communication we report on the synthesis of 2′(3′)-*O*-glycyl tri-, tetra-, and pentanucleotides, the latter being the longest 2′(3′)-*O*-aminoacyl oligoribonucleotide prepared by purely chemical means to date. We have also investigated the properties of the synthetic products in several partial reactions of protein biosynthesis.

Materials and Methods

General Methods. Chromatography. Thin-layer chromatography (TLC) was performed on silica gel coated aluminum foils 60-F_{254} (EM Laboratories) in the following systems: S_1,

† This investigation was supported, in part, by U.S. Public Health Service Research Grant GM-19111 from the National Institutes of Health and by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit. This is paper 42 in the series Aminoacyl Derivatives of Nucleosides, Nucleotides, and Polynucleotides. For the preceding paper in this series (41), see Bhuta and Zemlicka (1985). A part of this work was presented at the 7th International Round Table: Nucleosides, Nucleotides, and Their Biological Applications, Konstanz, West Germany, 1986 (Scalfi-Happ et al., 1987).

‡ Present address: Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany.

§ Present address: Ely Lilly Co., Indianapolis, IN 46205.

[1] Abbreviations: Ac, acetyl; aa-tRNA, aminoacyl transfer ribonucleic acid; A-Gly, 2′(3′)-*O*-glycyladenosine (similar abbreviations are used for oligonucleotide derivatives); Bz, benzoyl; BT, benzotriazolyl; 2-ClPh, 2-chlorophenyl; DCC, dicyclohexylcarbodiimide; DMT, 4,4′-dimethoxytrityl; DTT, dithiothreitol; EF-Ts, elongation factor Ts; EF-Tu, elongation factor Tu; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; Lev, levulinyl; MMT, 4-methoxytrityl; MST, (mesitylenesulfonyl)tetrazole; MTHP, 4-methoxytetrahydropyran-4-yl; Z, benzyloxycarbonyl; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; A_{260} unit, a quantity of material contained in 1 mL of solution that has an absorbance of 1.00 at 260 nm when measured in a 1-cm path length cell. Standard abbreviations for nucleotides are according to CBN recommendations (Sober, 1970).

0006-2960/87/0426-4682$01.50/0

Table I

	UV data[a]		NMR[b] (ppm)					
	λ_{max} (nm) [ϵ ($\times 10^{-3}$)]	λ_{min} (nm)	solvent	H-8 or H-2, H-6 or H-5	aromatic protons	H-1′	OCH_3 (MTHP)	other signals
1b	236 (32.04), 307 (10.07), 262 (25.14)	292, 246, 222	$CDCl_3$		8.25–6.8 (m, 20)	6.27 (d, 1, J = 4.4)	3.2 (s, 3)	8.81 (aromatic OCH_3) (s, 6)
1c	306 (5.08), 261 (13.56)	290, 238	$CDCl_3$		8.13–7.27 (m, 7)	6.14 (d, 1, J = 3.8)	3.19 (s, 3)	2.21 (CH_3CO) (s, 3)
2c	280 (13.09)	248	acetone-d_6		8.75–7.36 (m, 7)	6.50 (2 d, 1, J = 6.6)		2.05 (CH_3CO) (s, 3)
3b	260 (11.64), 235 (23.94)	256, 222	$CDCl_3$		7.84–6.78 (m, 15)	6.19 (d, 1, J = 6.4)	3.19 (s, 3)	3.79 (aromatic OCH_3) (s, 6)
4b	279 (20.04), 234 (29.78)	258, 223	$CDCl_3$	8.72 (s, 1), 8.21 (s, 1)	8.07–6.76 (m, 14)	6.23 (d, 1, J = 6.7)	2.82 (s, 3)	3.78 (aromatic OCH_3) (s, 6)

[a] In 95% ethanol. [b] J values are given in hertz.

CH_2Cl_2–1% CH_3OH; S_2, CH_2Cl_2–5% CH_3OH; S_3, CH_2Cl_2–10% CH_3OH; S_4, 1-propanol–water–NH_4OH (6:3:1). TLC was also performed on cellulose plates (Avicel F Uniplate, Analtech, Inc.) in the following systems: S_5, 1-butanol–water–acetic acid (5:3:2); S_6, 2-propanol–water–NH_4OH (7:2:1). Column chromatography was performed on silica gel columns (EM reagents; 70–230 mesh) with a step gradient of methanol in CH_2Cl_2. The 2′(3′)-*O*-aminoacyl oligonucleotides were also purified on Baker's 10 SPE octadecyl columns (J. T. Baker Chemical Co.) with a step gradient of CH_3CN in ammonium acetate buffer (5 mM, pH 4.5). The products were usually eluted with 10–20% CH_3CN. High-performance liquid chromatography (HPLC) was performed on an Altex-Spectraphysics instrument with a Kratos Spectroflow 773 absorbance detector (at 254 nm).

Paper electrophoresis was conducted on a Savant flat plate with 1 M acetic acid on Whatman no. 1 paper at 40 V/cm for 2 h.

Spectra. UV spectra were obtained by using a Perkin-Elmer Lambda 5 UV/vis spectrophotometer. Yields of oligonucleotides were determined spectrophotometrically at pH 2.0 (0.01 N HCl) by using the following molar extinction coefficients: for C-C-A, $\epsilon_{260} = 27.9 \times 10^3$; for U-C-C-A, $\epsilon_{260} = 37.8 \times 10^3$; for C-U-C-C-A, $\epsilon_{260} = 44.6 \times 10^3$ (neglecting hypochromicity). NMR spectra were recorded on a JEOL-FX 100 instrument with tetramethylsilane as the internal standard. The pertinent spectral data of new compounds are given in Table I.

Enzymic Digestions. Pancreatic ribonuclease (RNase A) degradation was performed with ca. 0.5–2 A_{260} units of 2′-(3′)-*O*-aminoacyl oligonucleotide that was first treated with concentrated NH_4OH (10 μL; 5 min), and after evaporation and freeze-drying, the sample was dissolved in the enzyme solution [25 μL; 1 mg of RNase A (Sigma Co.) in 1 mL of 0.1 N Tris·HCl, pH 7.8] and incubated for 3 h at 37 °C. The reaction was stopped by heating at 100 °C for 1 min, and the mixture was analyzed by HPLC on a μBondapak C_{18} analytical column (Waters Associates) with a gradient of methanol (1–5%) in 50 mM ammonium acetate, pH 4.5. The peaks of digestion products were identified by comparison with authentic samples, and the ratio was obtained by integration. Quantitations of nucleoside and nucleotide peaks was done by using appropriate extinction coefficients (van Boom & de Rooy, (1977). Snake venom phosphodiesterase degradation was performed with ca. 0.5–2.0 A_{260} units of oligonucleotide samples pretreated with ammonia. The samples were incubated in 0.1 M Tris–acetate, pH 8.8 (25 μL), 0.3 M magnesium acetate (5 μL), and the enzyme solution [10 μL; containing 1 unit of snake venom phosphodiesterase (Cooper Biochemicals) in 0.1 M Tris–acetate, pH 8.8] for 3 h at 37 °C, treated for 1 min at 100 °C, and analyzed by HPLC as described in the previous paragraph.

Other general methods for chemical synthesis were the same as those described in previous papers in this series (Chládek et al., 1974; Ryu et al., 1977; Kumar et al., 1982). Starting nucleosides and reagents were commercial preparations (Sigma and Aldrich). The sources of biochemicals, enzymes, and ribosomes were the same as in previous papers (Chládek et al., 1974; Bhuta et al., 1982a).

Assay of Peptidyltransferase Activity. Peptidyltransferase activity was measured essentially as described before (Chládek et al., 1974). Each reaction mixture of 0.1 mL contained 50 mM Tris·HCl (pH 7.4), 100 mM NH_4Cl, 10 mM $MgCl_2$, 4.0 A_{260} units of NH_4Cl-washed 70S ribosomes, 10 μg of poly(U), 0.3 A_{260} unit of N-Ac[^{14}C]Phe-tRNA (prepared from unfractionated tRNA (*Escherichia coli*), 13 000 cpm/A_{260} units; concentration of Ac[^{14}C]Phe-tRNA was ca. 0.2 μM), and the acceptors in the given concentration. The reaction mixtures were incubated for 30 min at 37 °C and processed by precipitation with 2.5% trichloroacetic acid. The results are reported in Figure 1. The products of reaction after saponification (N-Ac[^{14}C]Phe-Gly) were identified by paper electrophoresis as described previously by Bhuta et al. (1981) (results not shown).

The peptidyltransferase activity of C-C-A(AcGly) (**7**) as a potential donor was measured in the fragment reaction system with [^{14}C]Phe-tRNA as an acceptor as described previously (Quiggle et al., 1981). This compound was found to be inactive in the wide range of concentrations tested (data not shown).

The EF-Tu–70S ribosome dependent GTP hydrolysis assay was carried out essentially as described in previous papers (Bhuta et al., 1982a; Takahashi et al., 1986) by mixing of the EF-Tu·GTP aminoacyl oligonucleotide complex and the ribosomal pool. The EF-Tu·GTP aminoacyl oligonucleotide complex contained the following in 0.05 mL: Tris·HCl, 60 mM, pH 7.8; NH_4Cl, 150 mM; $MgCl_2$, 10 mM; DTT, 1 mM; phosphoenolpyruvate, 3 mM; pyruvate kinase, 7 μg; [γ-^{32}P]-GTP, 0.01 mM, specific activity 1 Ci/mmol; EF-Tu·GDP, 150 pmol; and the test compounds at the indicated concentrations. In the experiments where the effect of EF-Ts on GTPase was investigated, EF-Ts was also added (0.11 pmol), and the amount of EF-Tu·GDP was 10 pmol. The EF-Tu pool was preincubated at 32 °C for 10 min prior to addition of [γ-^{32}P]GTP and then for another 5 min at 37 °C. The ribosomal pool contained the following in final volume of 0.05 mL: Tris·HCl, 60 mM, pH 7.8; NH_4Cl, 150 mM; DTT, 1 mM; 70S ribosome, 2.00 A_{260} units; $tRNA^{Phe}_{yeast}$, 20 μg; poly(U), 10 μg. The ribosomal pool was incubated for 10 min at 37 °C, the GTPase reaction was initiated by mixing of both pools, and the combined reaction mixture was incubated at 30 °C for 5 min. The reactions were terminated by silicotungstic

acid and worked up as described in Takahashi et al. (1986). The results are reported in Figures 2 and 3.

5′-O-(Dimethoxytrityl)-2′-O-(4-methoxytetrahydropyran-4-yl) Nucleosides (**1b**, **3b**, *and* **4b**). The title compounds were prepared, starting from **1a**, **3a**, and **4a** (Kumar et al., 1982; Reese et al., 1970) (0.5 mmol) and dimethoxytrityl chloride (0.19 g, 0.55 mmol) in the presence of a catalytic amount of 4-(dimethylamino)pyridine in pyridine solution (5 mL). After the reactions were shown to be complete by TLC (S_3), they were quenched with cold 5% ammonium bicarbonate (5 mL) with cooling; the mixture was extracted with CH_2Cl_2 and the organic layer concentrated in vacuo after drying with $MgSO_4$. The residue was coevaporated with toluene and precipitated with petroleum ether (20 mL). The crude reaction product was purified on a column (3 × 5 cm) of silica gel with a CH_2Cl_2–CH_3OH step gradient (1–4%). The fractions containing the pure products **1b**, **3b**, and **4b** were pooled and evaporated in vacuo. The title compounds were obtained in solid form after trituration with CH_2Cl_2 and petroleum ether. The yields of **1b**, **3b**, and **4b** were in the 80–90% range. Anal. Calcd for **1b** ($C_{43}H_{45}N_3O_{10}$): C, 67.61; H, 5.94; N, 5.5. Found: C, 67.7; H, 5.9; N, 5.61. Anal. Calcd for **3b** ($C_{36}H_{40}N_2O_{10}$): C, 65.44; H, 6.10; N, 4.24. Found: C, 65.65; H, 6.37; N, 4.13. Anal. Calcd for **4b** ($C_{44}H_{45}N_5O_9$): C, 67.08; H, 5.76; N, 8.89. Found: C, 67.31; H, 5.86; N, 8.82.

N^4-Benzoyl-5′-O-levulinoyl-2′-O-(4-methoxytetrahydropyran-4-yl)cytidine (**1c**). This compound was prepared according to the procedure of van Boom and Burgers (1978), starting from **1a** (Kumar et al., 1982) (0.46 g, 1 mmol), which was treated at 0 °C in dry dioxane (5 mL) with 1,2-dimethylimidazole (0.5 mmol, 0.05 mL), freshly distilled levulinic acid (0.307 mL, 3 mmol), and 2,6-lutidine (0.3 mL). The solution of DCC (0.62 g, 3 mmol) in dioxane (2.5 mL) was slowly added through a septum, and the suspension was stirred for 2.5 h. Dicyclohexylurea was filtered off, the solution was diluted with CH_2Cl_2 and quenched with 5% ammonium bicarbonate (20 mL) at 0 °C, the product was extracted with CH_2Cl_2, and the organic layer was dried over $MgSO_4$. The crude reation product was purified on a silica gel column (2.5 × 13.5 cm) with the step gradient of CH_3OH in CH_2Cl_2; the pure product was eluted with ca. 3% CH_3OH. Compound **1c** was obtained after the pooling and evaporation of the appropriate fractions and freeze-drying from benzene; the yield was 0.31 g (55%). Anal. Calcd for $C_{27}H_{33}N_3O_{10}$: C, 57.96; H, 5.90; N, 7.51. Found: C, 57.71; H, 5.82; N, 7.36.

2′,3′-O-[[(Acetylamino)methyl]ethoxymethylene]-N^6-benzoyladenosine (**2c**). Compound **2a** (Kumar et al., 1982) (0.41 g, 0.69 mmol) was dissolved in methanol (40 mL) and hydrogenated by bubbling a stream of hydrogen through the solution at 0 °C in the presence of boric acid (0.55 g) and 10% PdO/$BaSO_4$ (0.46 g; Engelhardt) until TLC in system S_3 showed virtually complete disappearance of the starting material. The catalyst was filtered off through the Celite bed, and the filtrate was evaporated in vacuo. The residue was coevaporated in vacuo with methanol until disappearance of boric acid (flame test). The intermediate **2b** [see Zemlicka and Chládek (1965)] was converted directly to its *N*-acetyl derivative **2c** according to Chládek (1972); it was dissolved in dimethylformamide (DMF) (7 mL) and treated with 8-(acetyloxy)-5-chloroquinoline (0.6 g, 2.76 mmol) overnight at room temperature. The reaction mixture was evaporated and the residue purified on a silica gel column (3 × 5 × 27 cm) with a linear gradient of CH_3OH (0–6%) in CH_2Cl_2. Fractions containing a mixture of diastereoisomers of **2c** were pooled and evaporated to yield 0.28 g (86%) of chromatographically uniform white foam.

P,P′-Bis(2-chlorophenyl) N^4-Benzoyl-2′-O-(4-methoxytetrahydropyran-4-yl)-5′-O-(methoxytrityl)cytidylyl(3′–5′)-N^4-benzoyl-2′-O-(4-methoxytetrahydropyran-4-yl)cytidylyl(3′–5′)-2′,3′-O-[[(acetylamino)methyl]ethoxymethylene]-N^6-benzoyladenosine (**6**). The trinucleotide **6** was prepared by coupling of dinucleotide **5** (0.166 g, 0.1 mmol) with nucleoside **2c** (0.075 g, 0.15 mmol) in pyridine (8 mL) in the presence of (mesitylenesulfonyl)tetrazole (0.065 g, 0.25 mmol) as described previously (Kumar et al., 1982). After a usual workup, the product **6** was isolated by preparative TLC on silica gel plate in system S_3 to obtain 0.14 g (65%) of the chromatographically uniform product **6**: UV (95% ethanol) λ_{max} 263 (ϵ 52.2 × 10^3), λ_{min} 243 (ϵ 36.5 × 10^3).

Cytidylyl(3′–5′)cytidylyl(3′–5′)-2′(3′)-O-(N-acetylglycyl)-adenosine (**7**). Derivative **6** (0.05 g, 23.5 μmol) was dissolved in dioxane–aqueous ammonia (1:1; 8 mL), and the mixture was heated with stirring to 60 °C for 24 h. The solution was concentrated to a small volume, the residue extracted with ether, and the water layer freeze-dried. The residue was dissolved in 0.1 N HCl–dioxane (10 mL; 1:1), left at room temperature for 16 h, freeze-dried, and co-freeze-dried with dioxane. The residue was dissolved in the methanol–acetic acid mixture and applied on one cellulose plate (Avicel) which was developed in system S_5. The strong band of product was eluted with the system, and the eluate was evaporated and coevaporated with water. The reaction product was purified by preparative paper electrophoresis in 1 M acetic acid (Kumar et al., 1982). The band of product was eluted with diluted acetic acid and the solution freeze-dried to yield 7.8 μmol (33%) of **7**, which is chromatographically and electrophoretically uniform and gives C-C-A as the sole UV-detectable product by alkaline hydrolysis.

General Method for Preparation of Fully Protected Oligoribonucleotides. The modified procedure of Wreesmann et al. (1983) was used. The component with free 3′-OH (**1b**, **1c**, **3b**, or **15**; 0.11 mmol) was dried by coevaporation with absolute pyridine until the formation of a foam. Pyridine was introduced without opening the apparatus, which was bled with dry N_2 prior to the operation. The agent **8** (Wreesmann et al., 1983) was added (0.12 mmol) in dry tetrahydrofuran (0.6 mL) via syringe and septum, and the reaction mixture was left for 30 min at room temperature. Subsequent TLC in system S_3 usually showed the formation of the base line material. The component with free 5′-OH (**2a**, **3a**, **10**, or **14**; 0.1 mmol) was predried by coevaporation with pyridine, dissolved (under the exclusion of atmospheric air) in dry tetrahydrofuran (0.6 mL), and transferred into the solution of the diester, and *N*-methylimidazole (38.8 μL, 0.5 mmol) was added. The TLC of the reaction mixture (system S_3) after ca. 30 min to several hours showed the almost quantitative formation of a new fast-moving product. The reaction was quenched at 0 °C (with 5% NH_4HCO_3 (2–3 mL), the mixture extracted with CH_2Cl_2, and the organic layer dried with $MgSO_4$. The residue after the concentration in vacuo was charged to a silica gel column (3 × 5 cm) which was developed by a step gradient of CH_3OH in CH_2Cl_2. The fractions of the desired oligonucleotide were pooled and evaporated to recover pure products (listed in Table II) which were used in the subsequent steps.

Cleavage of the 5′-O-Levulinoyl Group from Protected Oligonucleotides **9** *and* **13**. Oligonucleotide **9** or **13** (ca. 0.1 mmol) was dissolved in $N_2H_4 \cdot H_2O$ (0.5 M) in pyridine–acetic acid solution (3:2; 1 mL) and the solution kept for 4 min at 0 °C. Freshly distilled acetylacetone (1 mmol, 0.1 mL) was added, and the mixture was diluted with CH_2Cl_2 and extracted

Table II: Synthesis of Protected Oligoribonucleotides

3′-OH component	5′-OH component	product	yield (%)
1c	**2a**	**9**	83.2
1b	**3a**	**15**	73.0
1c	**10**	**13**	85.0
1b	**10**	**11**	62.5
3b	**14**	**16**	66
15	**14**	**18**	59

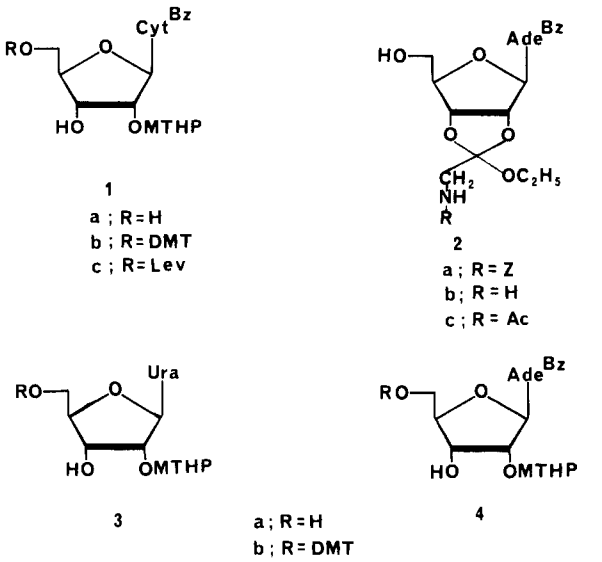
with water. The organic layer was dried with $MgSO_4$ and the reaction product recovered by precipitation with petroleum ether. The yields were in the 55–60% range of chromatographically uniform materials **10** or **14**, which are not distinguishable by TLC from the starting material. The test phosphorylation of **10** or **14** with reagent **8** shows the quantitative formation of diesters, thus proving the total absence of materials with the 5′-*O*-levulinoyl group.

2′(3′)-O-Glycyl Oligoribonucleotides. General Deblocking Procedure. The protected derivatives **11**, **16**, and **18** (ca. 20 μmol) were stirred with saturated methanolic ammonia (5 mL) for 36 h at room temperature. After that time TLC in system S_3 has shown the complete conversion to base line material. The residue after evaporation in vacuo was taken up to redistilled water (10 mL), and the suspension was extracted with ether. The water solution was carefully concentrated in vacuo (temperature of bath below 30 °C) and the residue treated with cold 80% formic acid (5 mL) under stirring for 20 min. The solution was diluted with redistilled water (50 mL) and freeze-dried. The residue was dissolved in 20% acetic acid and extracted with ether. The acetic acid solution was applied on two plates of cellulose (Avicel) that were developed with system S_5. The oligonucleotide band was eluted with the system, the solution evaporated in vacuo, and the remaining solution clarified by centrifugation from remainders of cellulose. The resulting material was directly used for the next step. The hydrogenolysis of the *N*-benzyloxycarbonyl group was performed as described previously (Kumar et al., 1982) in the acetic acid solution in the presence of 5% $PdO/BaSO_4$ catalyst, except that an extended hydrogenation time (up to 4 h) was necessary to achieve complete removal of the *N*-benzyloxycarbonyl group. The products of hydrogenation were further purified on a Baker 10 SPE C_{18} column with a step gradient (0, 5, 10, 20, 50, and 100%) of CH_3CN in 5 mM ammonium acetate, pH 4.5. The fractions were evaporated in vacuo, freeze-dried, and analyzed by TLC in system S_5. The oligonucleotides **12**, **17**, and **19** were recovered quantitatively from the column. The total yields of combined deblocking procedures were in the 50–60% range. The final products **12**, **17**, and **19** were chromatographically and electrophoretically uniform, and their characterization is given in Table III.

Results and Discussion

Synthesis. We have chosen to investigate the synthesis of longer 2′(3′)-*O*-glycyl oligoribonucleotides for a number of reasons. First, ethyl *N*-(benzyloxycarbonyl)orthoglycinate is readily available [unlike the protected ortho esters of other amino acids; see Zemlicka and Murata (1976)] and reacts smoothly with unprotected nucleosides to form 2′,3′-*O*-cyclic ortho esters (Zemlicka & Chládek, 1966). These ortho esters are fully stable in an alkaline medium that is used for the removal of benzoyl groups from aglycons as well as 2-chlorophenyl groups from phosphotriesters (Kumar et al., 1982). Thus glycine derivatives provide convenient models for elaborating the synthesis of longer 2′(3′)-*O*-aminoacyl oligoribonucleotides.

Chart I



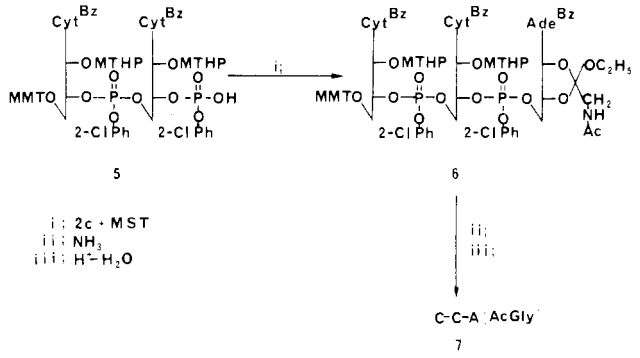
In this paper, the simple nucleoside building blocks **1b**, **3b**, and **4b** were prepared in high yields via dimethoxytritylation of corresponding starting nucleosides **1a**, **3a**, and **4a** (Kumar et al., 1982) (Chart I). The synthon **1c** was obtained in ca. 55% yield by levulinylation of the 5′-hydroxyl group of **1a** according to the procedure of van Boom and Burgers (1978). The relatively lower yield of this compound reflects the fact that under reaction conditions employed the formation of the 3′,5′-*O*-dilevulinoyl ester took place, and thus no 3′-*O*-monolevulinoyl derivative contaminates the desired product **1c**. Compound **2a** (Kumar et al., 1982) served as the 3′-terminal unit for the synthesis of 2′(3′)-*O*-glycyl oligoribonucleotides. At the same time it was also employed as the starting material for the synthesis of **2c**, which was to be used for the synthesis of C-C-A(AcGly) (**7**). Thus, **2a** was hydrogenated over palladium catalyst in the presence of boric acid. The latter accelerates the hydrogenolysis without affecting the acid sensitive 2′,3′-*O*-ortho ester grouping and is, moreover, easily removable from the reaction mixture by coevaporation with methanol. The intermediate **2b** (with a free amino group) was then easily converted to its *N*-acetyl derivative **2c** via a specific acetylation under the aegis of 8-(acetoxy)-5-chloroquinoline

Table III: 2′(3′)-*O*-Glycyl Oligoribonucleotides

compd		UV spectra (0.01 N HCl soln) λ_{max} (nm)	250/260	280/260	290/260	2′–5′ isomer[a] (%)	nucleoside/nucleotide	3′/3′ or 3′–2′ isomer[b]	products[b]
C-C-A(AcGly)	**7**	265	0.81	0.99	0.77	0	Cp:A = 1.72:1	0	C + pC + pA
C-C-A-Gly	**12**	268	0.72	0.94	0.62	0	Cp:A = 1.92:1	0	C + pC +pA
U-C-C-A-Gly	**17**	264	0.73	0.71	0.41	0	(2Cp + Up):A = 1:1.08[c] Cp:Up = 2.04:1	0	U + pC + pA
C-U-C-C-A-Gly	**19**	268	0.70	0.9	0.58	0	(3Cp + Up):A = 1.03:1[c]	0	C + pU + pC + pA

[a] Determined by the pancreatic ribonuclease digestion. [b] Determined by the snake venom phosphodiesterase digestion. [c] Presence of Up and Cp verified by paper electrophoresis, since these nucleotides did not separate on the HPLC column.

Scheme I



(Chládek, 1972). The synthesis of the donor model C-C-A-(AcGly) (**7**) (Scheme I) followed a previously published procedure (Kumar et al., 1982). The interaction of dinucleotide **5** with nucleoside **2c** in the presence of (mesitylenesulfonyl)tetrazole led, in high yield, to fully protected trinucleoside diphosphate **6**. This trinucleotide was converted in a two-step deblocking procedure, using treatment with ammonia and diluted hydrochloric acid, to C-C-A(AcGly) (**7**), which was isolated by preparative TLC and paper electrophoresis.

The 5′-*O*-levulinoyl group can easily be removed from oligonucleotides, whereas other protecting groups may be expected to remain intact following brief treatment with hydrazine (van Boom & Burgers, 1978). Thus, synthon **1c** is a convenient starting material for synthesis of the oligonucleotide intermediates **10** and **14** (Scheme II), which possesses a free 5′-OH group required for the further extension of the oligonucleotide chain in the 5′ direction. Compound **1c** was quantitatively phosphorylated with 2-chlorophenyl bis(1-benzotriazolyl) phosphate (**8**) (Wreesmann et al., 1983), and the intermediate diester was converted in situ to the dinucleoside phosphate **9** via reaction with nucleoside component **2a**. Brief treatment with hydrazine converted **9** into compound **10**. Analogously, intermediate **10** was phosphorylated with reagent **8** and converted to trinucleoside bisphosphates **11** and **13** by reaction with nucleosides **1b** and **1c**. In the latter case, the 5′-OH group was again regenerated by brief treatment with hydrazine acetate to give intermediate **14**. Extension of nucleotide chain of compound **14** was affected with nucleoside **3b** or dinucleotide block **15**[2] after the prior phosphorylation with reagent **8** in order to generate fully protected tetra- and pentanucleotides **16** and **18**, respectively. This set of reactions thus led to C-C-A-Gly (**12**), identical with the product prepared previously by a different route (Kumar et al., 1982). The oligonucleotides **11**, **16**, and **18** were deprotected by the following three-step procedure: (i) ammonia treatment removed *N*-benzoyl groups from aglycons and 2-chlorophenyl groups from phosphorus; (ii) 80% formic acid removed both the 5′-*O*-dimethoxytrityl and 2′-*O*-methoxytetrahydropyranyl groups and hydrolyzed the 2′,3′-*O*-ortho ester grouping; (iii) hydrogenolysis over palladium catalyst finally removed the *N*-benzyloxycarbonyl group from the glycine moiety. As the deblocking of all intermediates proceeded via the same pathway, the procedures for isolation of the final products **12**, **17**, and **19** were identical: the 2′(3′)-*O*-*N*-(benzyloxycarbonyl)-glycyl derivatives of C-C-A, U-C-C-A, and C-U-C-C-A were purified by TLC on cellulose plates in acidic medium, whereas the target compounds **12**, **17**, and **19** were isolated by chromatography on Baker's C_{18} reverse-phase column to afford the chromatographically and electrophoretically uniform compounds. Compounds **12**, **17**, and **19** were further characterized by UV spectra and hydrolysis to parent oligonucleotides and glycine. The digestion of **12**, **17**, and **19** with RNase A and snake venom phosphodiesterase gave the expected products in correct ratios without any appreciable residue of the enzyme-resistant materials as shown by analysis usuing HPLC. Thus, the final products **12**, **17**, and **19** contain only correct 3′–5′ phosphodiester linkages.

[2] This dinucleotide was synthesized as shown in Scheme II, via coupling of nucleosides **1b** and **3b** in the presence of reagent **8**.

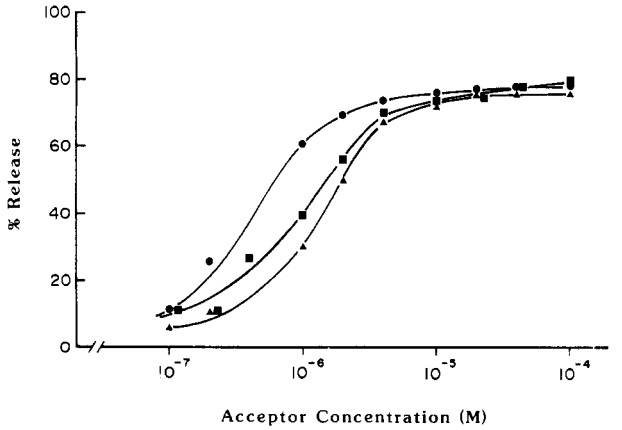


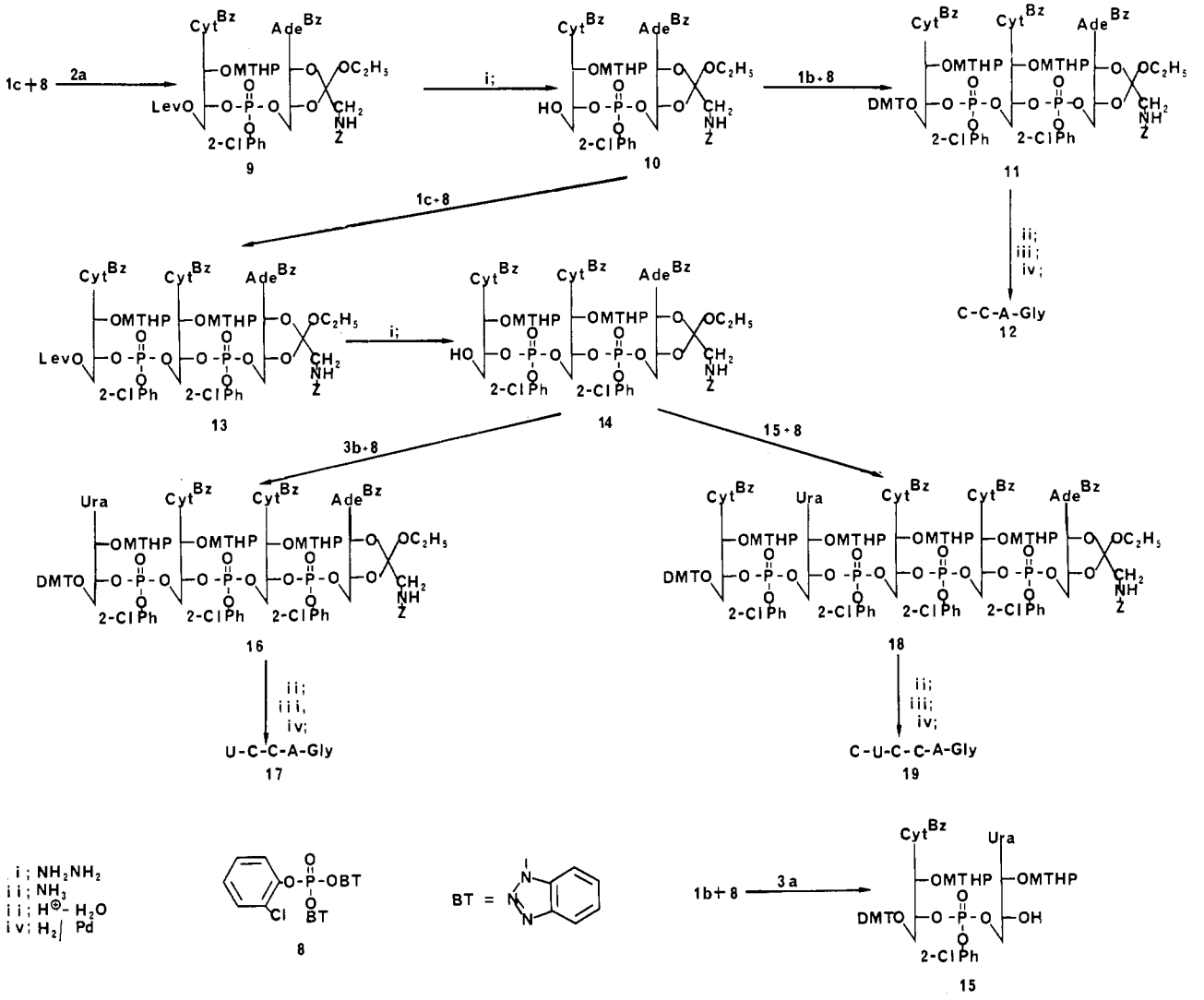
FIGURE 1: 2′(3′)-*O*-Glycyl oligonucleotide dependent release of Ac-[^{14}C]Phe residue from Ac[^{14}C]Phe-tRNA–70S ribosome–poly(U)–tRNA^{Phe} complex. Percent release represents the acceptor-dependent decrease in CCl_3COOH-precipitated counts trapped by Millipore membranes. For other details, see Materials and Methods, Chládek et al. (1974), and Bhuta et al. (1981). (●) C-C-A-Gly (**12**); (▲) U-C-C-A-Gly (**17**); (■) C-U-C-C-A-Gly (**19**).

The yields of coupling reactions using the hydroxybenzotriazolyl method averaged about 70% after isolation of products on silica gel columns, and it would seem to be the method of choice for the preparation of higher oligomers. The improved isolation procedures developed in this work have made the target compounds available in good yields, high purity, and sufficient quantities for biochemical investigations.

The combination of protecting groups used in this work is feasible only for preparation of the 2′(3′)-*O*-glycyl derivatives. Therefore, other blocking schemes are necessary for the syntheses of aminoacyl oligonucleotides derived from other amino acids wherein the alkali-stable ortho esters are not easily accessible. Additionally, we have noted here that removal of *N*-benzyloxycarbonyl group by hydrogenolysis is rather slow on the longer oligomers. Accordingly, alternative blocking groups would be required for an equally efficient, specific, and general method of synthesis of 2′(3′)-*O*-aminoacyl oligonucleotides. These problems are being currently addressed in another facet of our work (Scalfi-Happ et al., 1987).

Biochemical Results. Figure 1 shows the acceptor activity of C-C-A-Gly (**12**), U-C-C-A-Gly (**17**), and C-U-C-C-A-Gly (**19**) in the peptidyltransferase reaction with AcPhe-tRNA–poly(U)–70S ribosome (*E. coli*) complex. It is apparent that the activities of all three compounds are quite similar. Thus the extension of the oligonucleotide chain beyond the third (Cp residue 74) nucleotide does not result in an increase of activity. This is in an agreement with previous findings that the binding constant of C-C-A-Phe to the ribosomal A site is approximately the same as that of C-A-C-C-A-Phe (Lessard & Pestka, 1972; Bhuta et al., 1982b). On the other hand, it is clear that the acceptor activity increases in order A-Gly < C-A-Gly < C-C-A-Gly, pointing to a special role of the last three terminal nucleotides of tRNA in binding to the ribosomal acceptor site [Bhuta et al., 1982b; see also Chládek and Sprinzl

Scheme II



(1985)]. It should be mentioned, however, that these findings may not be, in principle, in variance with a proposed base-pairing scheme of the aa-tRNA 3′-terminus with a stretch of 23S RNA, involving nucleotide 73 (Chládek & Sprinzl, 1985). It is likely that a similar pairing scheme may not be easily attainable with 2′(3′)-*O*-aminoacyl oligonucleotide models in the absence of a more orderly structure which would be expected in the tRNA aminoacyl stem.

The trinucleotide derivative C-C-A(AcGly) (**7**) is inactive as the donor substrate in the peptidyltransferase reaction with Phe-tRNA (results not shown). This is in accord with findings of Monro et al. (1968) that *N*-acylglycyl oligoribonucleotides are very poor donors in the peptidyltransferase reaction using puromycin as the acceptor substrate.

The EF-Tu–70S ribosome (*E. coli*) promoting GTPase activities of models **12**, **17**, and **19** are similar, as documented in Figure 2. This finding again seems to indicate the lack of an effect of nucleotide residues beyond nucleotide 74 on binding to EF-Tu. It has been well documented that EF-Tu·GTPase promoting activity increases in order of, e.g., A-Gly < C-A-Gly < C-C-A-Gly (Bhuta et al., 1982a), but on the other hand, some role of tRNA nucleotide 73 in interaction with EF-Tu seems to be indicated (Kruse et al., 1980).

Figure 3 provides a comparison of the C-C-A-Gly (**12**) EF-Tu·GTPase promoting activity in the absence and presence of EF-Ts. It is apparent that EF-Ts greatly stimulates the GTPase activity, even in the presence of regenerating pyruvate

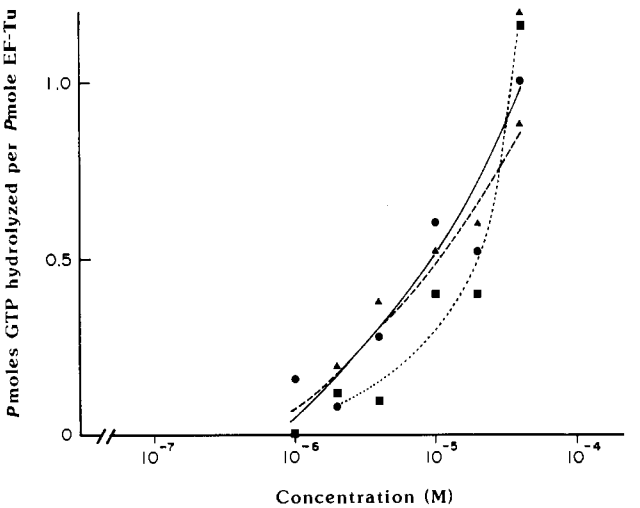


FIGURE 2: Effect of 2′(3′)-*O*-glycyl oligonucleotides on EF-Tu–70S ribosome GTP hydrolysis. For details see Materials and Methods and Bhuta et al. (1982a,b). (●, —) C-C-A-Gly (**12**); (▲, - - -) U-C-C-A-Gly (**17**); (■, ···) C-U-C-C-A-Gly (**19**).

kinase–phosphoenolpyruvate system. This finding provides reasons for the rather low levels of the EF-Tu·GTPase promoted by various aa-tRNA 3′-terminal fragments in systems lacking EF-Ts (Bhuta et al., 1982a), which are due to the apparent inability of the pyruvate kinase system to recycle

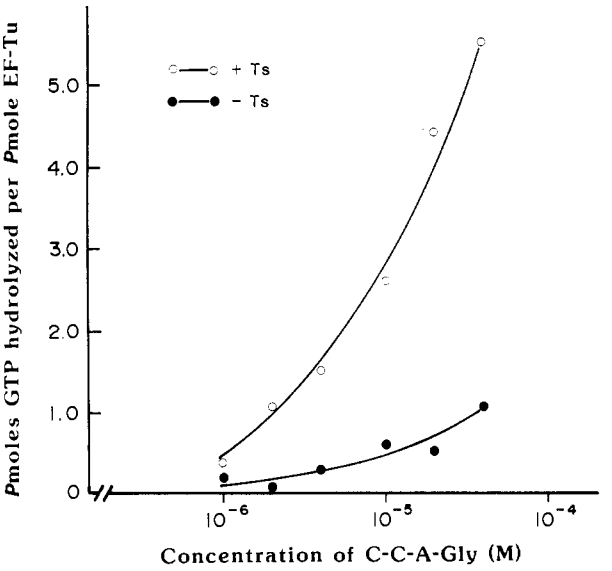


FIGURE 3: Effect of EF-Ts on EF-Tu–70S ribosome GTP hydrolysis promoted by C-C-A-Gly (**12**). See Materials and Methods for details. (○) +EF-Ts; (●) –EF-Ts.

EF-Tu [see also Parlato et al. (1983)]. Thus, it appears that in the presence of EF-Ts the only limiting factor in the EF-Tu·GTPase reaction is the concentration of GTP.

ACKNOWLEDGMENTS

The synthesis of some intermediates used in this work was initiated in this laboratory by Drs. G. Kumar and L. Celewicz. We are indebted to Dr. J. P. Horwitz, Michigan Cancer Foundation, for critical reading of the manuscript.

REFERENCES

Bhuta, A., Quiggle, K., Ott, T., Ringer, D., & Chládek, S. (1981) *Biochemistry 20*, 8–15.

Bhuta, P., & Zemlicka, J. (1985) *Biochim. Biophys. Acta 841*, 145–150.

Bhuta, P., Kumar, G., & Chládek, S. (1982a) *Biochemistry 21*, 899–905.

Bhuta, P., Kumar, G., & Chládek, S. (1982b) *Biochim. Biophys. Acta 696*, 208–211.

Chládek, S. (1972) *J. Org. Chem. 37*, 2863–2867.

Chládek, S., & Sprinzl, M. (1985) *Angew. Chem., Int. Ed. Engl. 24*, 371–391.

Chládek, S., Ringer, D., & Quiggle, K. (1974) *Biochemistry 13*, 2727–2735.

Kruse, T. A., Clark, B. F. C., Appel, B., & Erdmann, V. A. (1980) *FEBS Lett. 117*, 315–317.

Kumar, G., Celewicz, L., & Chládek, S. (1982) *J. Org. Chem. 47*, 634–644.

Lessard, J. L., & Pestka, S. (1972) *J. Biol. Chem. 247*, 6901–6908.

Monro, R. R., Cerna, J., & Marcker, K. A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 1042–1049.

Parlato, G., Pizzano, R., Picone, D., Guesnet, J., Fasano, D., & Parmeggiani, A. (1983) *J. Biol. Chem. 258*, 995–1000.

Quiggle, K., Kumar, K. G., Ott, T. W., Ryu, E. K., & Chládek, S. (1981) *Biochemistry 20*, 3480–3485.

Reese, C. B., Saffhill, R., & Sulston, J. E. (1970) *Tetrahedron 26*, 1023–1030.

Ryu, E. K., Quiggle, K., & Chládek, S. (1977) *J. Carbohydr. Nucleosides, Nucleotides 4*, 387–408.

Scalfi-Happ, C., Happ, E., & Chládek, S. (1987) *Nucleosides Nucleotides 6*, 345–348.

Sober, H. A., (1970) *Handbook of Biochemistry*, CRC Press, Cleveland, OH.

Takahashi, K., Ghag, S., & Chládek, S. (1986) *Biochemistry 25*, 8330–8336.

van Boom, J. H., & De Rooy, J. F. M. (1977) *J. Chromatogr. 131*, 169–177.

van Boom, J. H., & Burgers, P. M. J. (1978) *Recl. Trav. Chim. Pays-Bas 97*, 73–80.

Wreesmann, C. T. J., Fidder, A., van der Marel, G. A., & van Boom, J. H. (1983) *Nucleic Acids Res. 11*, 8389–8405.

Zemlicka, J., & Chládek, S. (1965) *Tetrahedron Lett.*, 3057–3063.

Zemlicka, J., & Chládek, S. (1966) *Collect. Czech. Chem. Commun. 31*, 3775–3789.

Zemlicka, J., & Murata, M. (1976) *J. Org. Chem. 41*, 3317–3321.