General Synthesis of 2′(3′)-O-Aminoacyl Oligoribonucleotides. The Protection of the Guanine Moiety[1]

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Received December 15, 1988

The chemical synthesis of 2′(3′)-O-aminoacyl oligoribonucleotides requires the development of a specific blocking scheme for all nucleoside components. Herein, we describe the synthesis of guanosine synthons protected at the O^6-position with the β-cyanoethyl group, at the N^2-position with the [(9-fluorenylmethyl)oxy]carbonyl group, at the 2′-hydroxy group with the 4-methoxytetrahydropyranyl group, and at the 5′-hydroxy group with the dimethoxytrityl group. Using these nucleoside synthons and previously developed derivatives of the other three nucleosides, the guanosine containing 2′(3′)-O-L-phenylalanyl trinucleotides were assembled by a stepwise phosphotriester approach in high yields and without formation of side products. Thus, this blocking scheme allows for the general synthesis of aa-tRNA fragments incorporationg any common nucleoside residue in an efficient and specific fashion.

The synthesis of 2′(3′)-O-aminoacyl oligoribonucleotides has been the subject of an abiding interest in our laboratory for the past several years. Besides the obvious chemical challenge, the main impetus for the synthesis of these compounds is the fact that 2′(3′)-O-aminoacyl oligoribonucleotides with a sequence identical with or resembling the natural sequence of the tRNA 3′-terminus (C-C-A) can mimic the role of the aa-tRNA in certain steps of protein biosynthesis.[2] Thus, 2′(3′)-O-aminoacyl oligoribonucleotides are important tools for investigating the mechanisms of protein synthesis in in vitro systems.

In previous papers of this series, we have developed a chemical synthesis of 2′(3′)-O-aminoacyl oligoribonucleotides by a stepwise triester method and a suitable combination of protecting groups for the components.[1a,3] This methodology allows for the removal of all protecting groups without cleavage of the sensitive 2′(3′)-O-aminoacyl ester bond.

In this paper, we extend the synthesis of the title compounds to the protection of guanosine, which has been problematical.[1a] By developing a synthesis of new guanosine synthons, protected on both the N^2- and O^6-positions, the synthesis of guanosine-containing oligonucleotides was improved, and better yields of the protected oligonucleotides were achieved.

Results and Discussion

It has been shown conclusively in several literature reports that both the N^2- and O^6-positions of the guanine moiety can react with the various activating and phosphorylating reagents commonly employed in the triester synthesis of oligonucleotides.[4] This also includes O,O-bis(1-benzotriazolyl)-2-chlorophenyl phosphate[4c] (1, Figure 1) originally suggested by van Boom et al.[5] as a condensing reagent for oligonucleotide synthesis. In fact, we have verified that reagent 1 indeed reacts with compound 2 under conditions of phosphorylation by detecting a slow-moving product, which is presumably generated by phosphorylation of 2 at its O^6-position.

Thus a rigorous approach to the synthesis of oligonucleotides requires protection of both the N^2- and O^6-positions of the guanine residue with suitable blocking groups.[6] Although several combinations of protecting groups for the guanine residue have been developed recently,[4a,b,6] the conditions for removing these groups are not compatible with preserving the 2′(3′)-O-aminoacyl function, which is unstable in alkaline or near neutral conditions. Thus, it is evident that none of these protecting groups is applicable to the synthesis of 2′(3′)-O-aminoacyl oligonucleotides. In previous work[1a] we have succeeded in synthesizing several guanosine containing 2′(3′)-O-aminoacyl oligonucleotides via a benzotriazolyl approach with only N^2-protection of the guanosine residue. Although we were unable to detect any side products (by analysis of the deprotected reaction mixture), yields of the guanosine-containing oligonucleotides were consistently low, and in order to be quantitatively phosphorylated on 3′-OH, the guanosine components required a large excess of the phosphorylating reagent 1. These results, together with the observation of the O^6-phosphorylation of model 2 with reagent 1 (vide supra), made the development of an appropriate blocking scheme for guanosine a mandatory task before the synthesis of larger aa-tRNA fragments could be undertaken.

Since it is known that the guanosine O^6-(3,5-dichlorophenyl) group is removable by reaction with

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(1) (a) This paper is No. 46 in the series Aminoacyl Derivatives of Nucleosides, Nucleotides, and Polynucleotides. For a preceding report in this series, see: Hagen, M. D.; Scalfi-Happ, C.; Happ, E; Chládek, S. *J. Org. Chem.* **1988**, *53*, 5040. (b) This investigation was supported, in part, by a training grant from U.S. Public Health Service T32-CA-09531 and by an institutional Grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit. This work was presented in part at the 8th International Round Table: Nucleosides, Nucleotides and Their Biological Applications, Orange Beach, AL. Hagen, M. D.; Chládek, S. *Nucleosides Nucleotides*, in press. (c) For abbreviations used, see: *Handbook of Biochemistry*; Sober, H. A., Ed.; CRC Press: Cleveland OH; Sections A and B. Other abbreviations: aa-tRNA, aminoacyl transfer ribonucleic acid; Ade^{Fmoc}, N^6-[[(9-fluorenylmethyl)oxy]carbonyl]adenin-9-yl; BPOC, 2-(4,4′-biphenylyl)-2-(propyloxycarbonyl); BT, benzotriazolyl; CNEt, 2-cyanoethyl; Cyt^{Fmoc}, N^4-[[(9-fluorenylmethyl)oxy]carbonyl]cytosin-1-yl; G-C-A-Phe, guanylyl-(3′–5′)cytidylyl-(3′–5′)-2′(3′)-O-(L-phenylalanyl)adenosine and similar abbreviations for other oligonucleotide derivatives; 2-Cl-Ph, 2-chlorophenyl; DMT, 4,4′-dimethoxytrityl; Fmoc, [(9-fluorenylmethyl)oxy]carbonyl; $Gua^{Fmoc,CNEt}$, N^2-[[(9-fluorenylmethyl)oxy]carbonyl]-O^6-(2-cyanoethyl)guanin-9-yl; MST, 1-(2-mesitylenesulfonyl)tetrazole; Mthp, 4-methoxytetrahydropyran-4-yl; $TIPDSiCl_2$, 1,1,3,3-dichlorotetraisopropyldisiloxane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; THF, tetrahydrofuran. A_{260} unit is a quantity of material contained in 1 mL of solution which has an absorbance of 1.00 at 260 nm when measured in a 1 cm path length cell.

(2) Chládek, S.; Sprinzl, M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 371.

(3) Happ, E.; Scalfi-Happ, C.; Chládek, S. *J. Org. Chem.* **1987**, *52*, 5387.

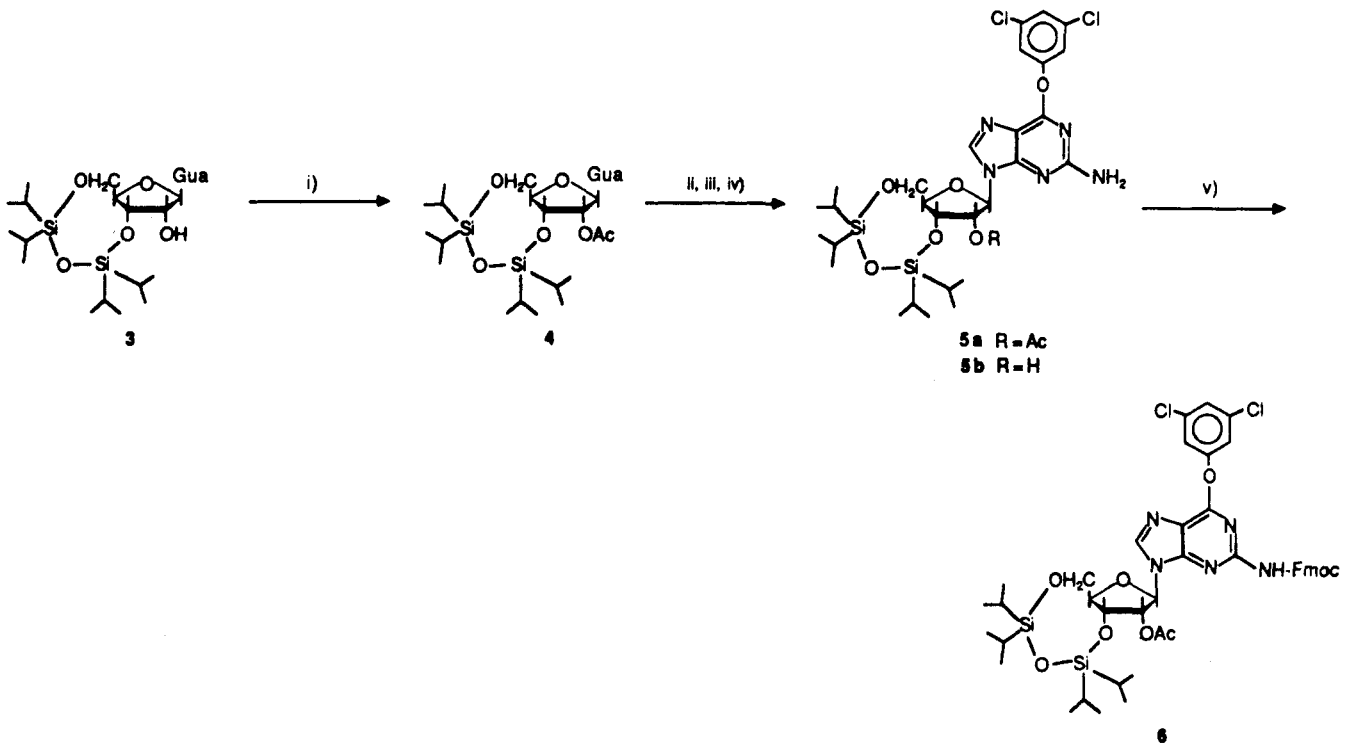
(4) (a) Himmelsbach, F.; Schulz, B. S.; Trichtinger, T.; Charubala, R.; Pfleiderer, W. *Tetrahedron* **1984**, *40*, 59. (b) Pon, R. T.; Usman, N.; Damha, M. J.; Ogilvie, K. K. *Nucleic Acids Res.* **1986**, *14*, 6453. (c) Reese, C. B.; Richards, K. H. *Tetrahedron Lett.* **1985**, 2245.

(5) (a) Wreesmann, C. T. J.; Fidder, A.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1983**, *11*, 8389. (b) de Vroom, E.; Fidder, A.; Marügg, J. E.; van der Marel, G. A.; van Boom, J. H. *Nucleic Acids Res.* **1986**, *14*, 5885.

(6) Reese, C. B.; Skone, P. A. *J. Chem. Soc., Perkin Trans. 1* **1984**, 1263.

0022-3263/89/1954-3189$01.50/0

Scheme I[a]



[a] Reagents: (i) Ac_2O + Et_3N; (ii) mesitylenesulfonyl chloride + Et_3N; (iii) *N*-methylpyrrolidine; 3,5-dichlorophenol; (iv) NH_3/MeOH; (v) FmocCl.

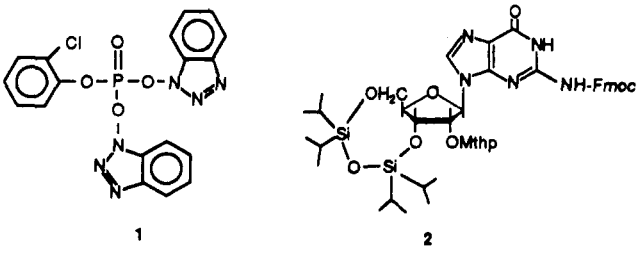


Figure 1.

N^1,N^1,N^3,N^3-tetramethylguanidinium 2-nitrobenzaldehyde oximate[6] and that the N-protected 2′(3′)-*O*-aminoacyl ester is stable to the oximate reagent,[3] the application of the 3,5-dichlorophenyl group appeared to be plausible. Thus, compound **3** (cf. ref 7, Scheme I) was converted into its 2′-*O*-acetyl derivative **4** via a brief treatment with acetic anhydride and triethylamine, which as shown by the NMR analysis leaves the N^2-amino group intact. Derivative **5a** was then generated by using a known sequence[6] of reactions: activation of the O^6-function with 2-mesitylenesulfonyl chloride, conversion to the *N*-methylpyrrolidinium salt, and displacement of this salt with 3,5-dichlorophenol. Acylation of the N^2-amino group of **5a** with 9-fluorenylmethyl chloroformate (FmocCl) proceeded smoothly and in high yield to obtain **6**. This was a sharp contrast to an analogous acylation of 2′,3′,5′-*O*-tris(trimethylsilyl)-guanosine, wherein the presence of the reactive 1-NH group leads to various side reactions.[1a] The increased reactivity of the N^2-amino group of **5a** relative to that of guanosine is remarkable and clearly demonstrates the deactivating effect of the 6-oxypurine residue. Once the O^6-carbonyl group is converted to an O^6-aryl ether, the electron-withdrawing effect of the O^6-carbonyl group disappears and the N^2-amino group becomes a stronger nucleophile.

Compound **6** served as a simple model for studying the oximate reaction. We found, however, that the O^6-(3,5-dichlorophenyl) group of **6** was stable toward the oximate reagent, and, as expected,[1a] the N^2-Fmoc group was cleaved instantly. After 1 h with the oximate/TMG reagent, compound **6** was converted only to **5a**. Prolonged treatment with the oximate reagent results in the cleavage of the 2′-acetate to afford compound **5b** and only a trace of the O^6-deprotected products **3** and **4**. This finding differs with the results of Reese and Skone,[6] who observed a smooth cleavage of the guanosine O^6-(3,5-dichlorophenyl) group with the oximate/TMG reagent when the N^2-amino group was protected with a phenylacetyl group, which is stable toward the oximate reagent. We have also observed that whereas O^6-(3,5-dichlorophen-1-yl)-N^2,O^2',O^3',O^5'-tetraacetylguanosine[8] undergoes rapid O^6-deprotection with the oximate reagent, only a trace of O^6-deprotection was observed for compound **5b** (prepared from **5a** by treatment with methanolic ammonia) after 24 h. Evidently, smooth removal of O^6-(3,5-dichlorophenyl) group with the oximate reagent is only possible if the N^2-amino group is acylated with a group that resists attack by this reagent. These results suggest that the free N^2-amino group, which is a stronger electron donor than the N^2-aminoacyl group, deactivates the ring toward nucleophilic attack by the oximate ion. Thus, it was clear that this route was not applicable to the synthesis of the target compounds.

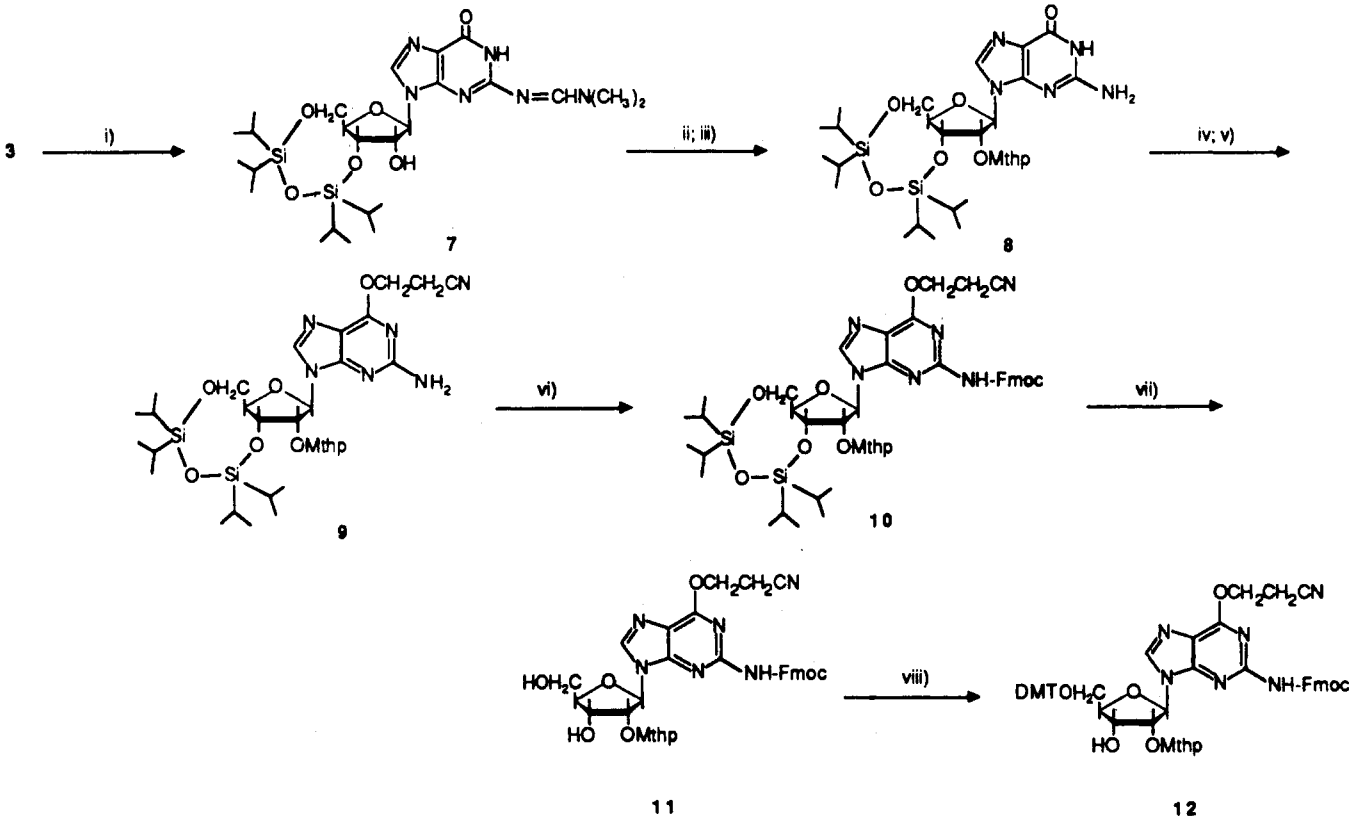
We then investigated the use of O^6-protecting groups, which could be removed via a β-elimination process. Using an available deoxyguanosine model, we observed that the β-cyanoethyl group[9] is readily cleaved with the oximate

(7) Markiewicz, W. T.; Wiewiorowski, M. In *Nucleic Acids Chemistry*; Townsend, L. B.; Tipson, R. S., Eds.; Wiley: New York, 1986; p 229.

(8) Happ, E., unpublished results.

(9) Gaffney, B. L.; Jones, R. A. *Tetrahedron Lett*, **1982**, 2257.

Scheme II[a]



[a] Reagents: (i) dimethylformamide dimethyl acetal; (ii) 4-methoxydihydropyran + H^+; (iii) NH_3/MeOH; (iv) mesitylenesulfonyl chloride + Et_3N; (v) *N*-methylpyrrolidine; β-cyanoethanol + DBU; (vi) FmocCl; (vii) *n*-Bu_4NF; (viii) DMTCl.

reagent; unfortunately, the O^6-deprotection of the more accessible O^6-[2-(4-nitrophenyl)ethyl]-2′-deoxyguanosine requires DBU,[4a] which would cleave the 2′(3′)-*O*-aminoacyl group. Therefore, we synthesized the required O^6-(β-cyanoethyl)guanosine synthons **11** and **12** as shown in Scheme II. The N^2-[(dimethylamino)methylene] derivative **7** was easily obtained from compound **3** (cf. ref 7) via reaction with *N*,*N*-dimethylformamide dimethyl acetal[10] and was used without purification. Derivative **8** was prepared from **7** by a two-step procedure:[3] the 2′-OH was protected by reaction with 4-methoxy-5,6-dihydro-2*H*-pyran under acid catalysis and then the temporary N^2-[(dimethylamino)methylene] group was removed with ammonia. Intermediate **8** was then converted to its O^6-(β-cyanoethyl) derivative **9** by using the two-step procedure of Reese and Skone[6] and Gaffney and Jones.[9] Due to the instability of the β-cyanoethyl group in the presence of DBU, which is used as a catalyst, the conditions for the displacement of the *N*-methylpyrrolidinium salt had to be carefully controlled.[9] Column chromatography afforded a mixture of compound **9** and 2-cyanoethanol. This mixture proved difficult to separate and was used without further purification. Acylation of this mixture with an excess of FmocCl afforded compound **10** in an overall yield of 60% from intermediate **8**.

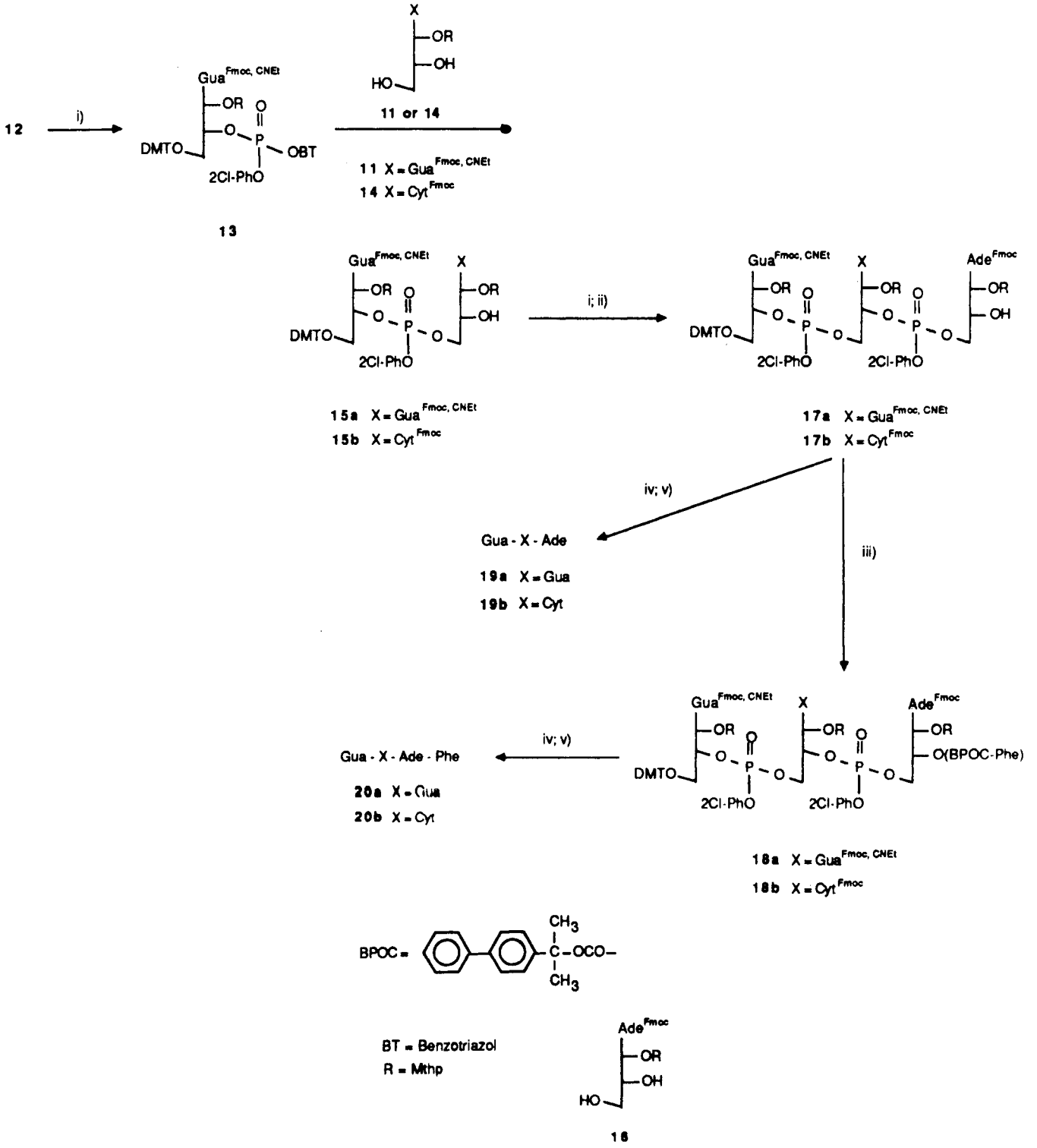
For this approach to be effective, the conversion of **10** to **11** was critical. We have observed, in agreement with the literature,[9] that anhydrous tetrabutylammonium fluoride (TBAF) removes not only the 3′,5′-*O*-bis(siloxane) moiety, but also the β-cyanoethyl group and the Fmoc group as well. Since acidic fluoride (TBAF/HF, cf. ref 9) cannot be used in presence of the 2′-*O*-methoxytetrahydropyranyl group, we resorted to a brief treatment with the equivalent of "deactivated" TBAF (in presence of pyridine and water, cf. ref 11) to remove the 3′,5′-*O*-bis(siloxane) group. This reagent neatly removes the 3′,5′-*O*-bis(siloxane) blocking group[1a] and leaves both the O^6-(β-cyanoethyl) and N^2-Fmoc groups intact to produce compound **11**. The dimethoxytrityl derivative **12** was then prepared by standard methodology[3] from **11**. The latter is the extension unit for the oligonucleotide synthesis and its derivative **12** is the 5′-terminal unit.

The nucleoside components were assembled in a stepwise fashion using the benzotriazolyl approach[3,5] as shown in Scheme III. The 5′-terminal component **12** was phosphorylated with reagent **1** to form the diester **13**, which was then condensed with component **11** or **14** to produce dinucleotide **15**. The reaction sequence was then repeated to incorporate the 3′-terminal component **16**. The yields of condensations were in the 60–70% range, a marked improvement over the yields of condensations with the N^2-protected guanosine components.[1a] Also, intermediates **12** or **15** did not consume any excess of reagent **1** during the phosphorylaion steps, as was the case with the N^2-protected guanosine synthons.[1a] Both trinucleotides **17** were aminoacylated with BPOC-Phe in the presence of 1-(2-mesitylenesulfonyl)tetrazole to furnish, in quantitative yields, the fully protected intermediates **18**. The deprotection of **18** to generate **20** (or **17** or provide **19**) was achieved by a routine, two-step treatment: (i) dry oximate/TMG reagent[12] to remove the Fmoc, CNEt, and 2-Cl-Ph groups and (ii) dilute formic acid at 0 °C to remove the remaining DMT, Mthp, and BPOC groups.[3] The pure 2′(3′)-*O*-(L-phenylalanyl) trinucleotides **20** were obtained

(10) Žemlička, J., Holý, A. *Collect. Czech. Chem. Commun.* **1967**, *32*, 3159.

(11) Kumar, G.; Celewicz, L.; Chládek, S. *J. Org. Chem.* **1982**, *47*, 634.
(12) Reese, C. B.; Titmas, R. C.; Yau, L. *Tetrahedron Lett.* **1978**, 2727.

Scheme III[a]



[a] Reagents: (i) 1; (ii) 16; (iii) BPOC-Phe + MST; (iv) oximate; (v) H^+.

in yields of about 25% by chromatography with Baker's 10 SPC C18 columns.

Compounds **20** were characterized by usual criteria including TLC, paper electrophoresis, UV spectroscopy, and alkaline hydrolysis to furnish the parent oligonucleotide **19** and phenylalanine. The target compounds **20** were also quantitatively cleaved with T_1 RNAse (or combination of T_1 RNAse and RNAse A for compond **20b**) and snake venom phosphodiesterase to the appropriate products in the correct ratios, without detecting significant amount of the unsplit materials. Accordingly the isomeric purity of compound **20** was established.

Thus, this study, together with previous work from our laboratory[1a,3] provides for a general approach to the synthesis of 2′(3′)-*O*-aminoacyl oligonucleotides with any sequence. The methodology is now available for the construction of longer fragments of aa-tRNA, which will be interesting models for biochemical studies.

Experimental Section

General Methods. The general methods were the same as those described in previous papers of this series.[1,3] Elemental

analyses were performed by MHW Laboratories.

Chromatography and Electrophoresis. Thin-layer chromatography (TLC) was performed on silica gel (60F-254) coated aluminum foils containing fluorescent indicator (E. M. Laboratories) and on cellulose plates (Avicel F Uniplate; Analtech). Preparative TLC was performed on silica gel GF (pre-coated thin-layer chromatography plates, 2000 μm; Analtech). The following chromatographic systems were used on silica gel: S_1, CH_2Cl_2–5% CH_3OH; S_2, CH_2Cl_2–10% CH_3OH; and on cellulose: S_3, 1-butanol–water–acetic acid (5:3:2). Paper electrophoresis was conducted on a Savant flat plate (at 4 °C) by using 0.05 M sodium hydrogen citrate (pH 3.5) on Whatman No. 1 paper at 50 V/cm for 2 h. Column chromatography was performed on silica gel (E. M. Reagent, 70–230 mesh) using a step gradient of methanol in CH_2Cl_2. The 2'(3')-O-aminoacyl oligonucleotides were purified on Baker's 10 SPE Octadecyl column (J. T. Baker Chemical Co.) using a step gradient of CH_3CN in ammonium acetate buffer (5 mM; pH 4.5). High-performance liquid chromatography (HPLC) was conducted on an Altex-Spectraphysics instrument with a Kratos Spectroflow 773 absorbance detector (at 254 nm).

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 $\epsilon_{260} = 32.9 \times 10^3$ for G-C-A and $\epsilon_{260} = 37.9 \times 10^3$ for G-G-A. ^1H NMR spectra were recorded on a QE-300 instrument (General Electric) at 300 MHz with tetramethylsilane as an internal standard.

Enzymic Digestions. The digestions with Ribonuclease A (Sigma), Ribonuclease T_1 (Sigma), and Snake venom phosphodiesterase from *Crotalus durissus* (Boehringer, Mannheim) were performed with ca. 0.5–2.0 A_{260} units of oligonucleotides as described previously.[1a,3] The digestion mixtures were analyzed by HPLC on a Synchropak RP C-18 analytical column (Altex) using an isocratic elution with 0.1 M KH_2PO_4 and 0.05 M NaCl (pH 4.5). The peaks of digestion products were identified by a comparison with authentic samples, and the ratio was obtained by using appropriate extinction coefficients.[13]

Starting Materials. Nucleosides, amino acids, and reagents were commercial preparations (Sigma, Aldrich, Fluka).

2'-O-Acetyl-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (4). To a suspension of **3** (cf. ref 7; 2.00 g, 3.81 mmol) in methylene chloride (20 mL) at room temperature was added triethylamine (4.00 mL, 28.8 mmol), 4-(dimethylamino)pyridine (0.02 g, 0.16 mmol), and acetic anhydride (0.50 mL, 5.30 mmol). After being stirred for 2 h, the reaction mixture was poured into cold saturated aqueous sodium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. Crystallization from methylene chloride and hexane afforded 1.84 g (85%) of **4**: ^1H NMR ($CDCl_3$) δ 0.98–1.22 (m, 28 H), 2.15, (s, 3 H), 4.01–4.12 (m, 3 H), 4.70–4.78 (m, 1 H), 5.72 (d, J = 5.0 Hz, 1 H), 5.88 (s, 1 H), 6.45 (br s, 2 H), 7.71 (s, 1 H), 12.04 (br s, 1 H). Anal. Calcd for $C_{24}H_{41}H_5O_7Si_2$: C, 50.77; H, 7.28; N, 12.33. Found: C, 50.64; H, 7.19; N, 12.11.

2'-O-Acetyl-6-O-(3,5-dichlorophen-1-yl)-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (5a). Compound **5a** was prepared according to the procedure of Reese and Skone.[6] To a solution of **4** (1.00 g, 1.76 mmol) in methylene chloride (6 mL) at room temperature was added triethylamine (0.85 mL, 6.16 mmol), 4-(dimethylamino)pyridine (0.02 g, 0.16 mmol), and 2-mesitylenesulfonyl chloride (0.767 g, 3.52 mmol). After being stirred for 4 h, the reaction mixture was poured into cold saturated aqueous sodium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The mesitylenesulfonyl intermediate was used without purification.

To a solution of the crude mesitylenesulfonyl intermediate (1.32 g) in methylene chloride (8 mL) at room temperature was added 1-methylpyrrolidine (1.20 mL, 1.5 mmol). After the mixture was stirred for 45 min, 3,5-dichlorophenol (0.481 g, 2.94 mmol) was added. The reaction mixture was stirred for 15 h at room temperature and was then poured into cold saturated aqueous sodium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (25 g) and a step gradient of methanol in methylene chloride (0–2%) to afford 0.92 g (73%) of **5a**: ^1H NMR ($CDCl_3$) δ 0.96–1.32 (m, 28 H), 2.18 (s, 3 H), 4.02–4.19 (m, 3 H), 4.80–4.85 (m, 1 H), 4.95 (br s, 2 H), 5.78 (d, J = 5.0 Hz, 1 H), 5.96 (s, 1 H), 6.79–7.25 (m, 3 H), 7.96 (s, 1 H). Anal. Calcd for $C_{30}H_{43}N_5O_7Si_2Cl_2$: C, 50.56; H, 6.08; N, 9.83; Cl, 9.95. Found: C, 50.76; H, 5.98; N, 9.93; Cl, 10.13.

6-O-(3,5-Dichlorophen-1-yl)-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (5b). To a solution of **5a** (0.213 g, 0.30 mmol) in methanol (10 mL) at room temperature was added a saturated solution of methanolic ammonia (5 mL). After being stirred for 4 h, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography using silica gel (25 g) and a 1% solution of methanol in methylene chloride to yield 0.157 g (78%) of product **5b**: ^1H NMR ($CDCl_3$) δ 1.07–1.15 (m, 28 H), 3.24 (br s, 1 H), 4.05–4.26 (m, 3 H), 4.52 (d, J = 5.6 Hz, 1 H), 4.80–4.86 (m, 1 H), 4.98 (br s, 2 H), 5.96 (s, 1 H), 7.18–7.29 (m, 3 H), 7.92 (s, 1 H). Anal. Calcd for $C_{28}H_{41}Cl_2N_5O_6Si_2$: C, 50.14; H, 6.16; N, 10.44; Cl, 10.57. Found: C, 50.27; H, 6.32; N, 10.12; Cl, 10.31.

2'-O-Acetyl-6-O-(3,5-dichlorophen-1-yl)-N^2-[[(9-fluorenylmethyl)oxy]carbonyl]-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (6). To a solution of **5a** (0.234 g, 0.33 mmol), predried by coevaporation with absolute pyridine, in methylene chloride (2 mL) at room temperature was added pyridine (0.10 mL, 1.24 mmol) and 9-fluorenylmethyl chloroformate (0.122 g, 0.47 mmol). After being stirred for 2 h, the reaction mixture was poured into cold 5% ammonium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (20 g) and a step gradient of methanol in methylene chloride (0–1%) to afford 0.287 g (93%) of **6**: ^1H NMR ($CDCl_3$) δ 0.98–1.07 (m, 28 H), 2.09 (s, 3 H), 4.05–4.09 (m, 2 H), 4.21–4.30 (m, 3 H), 4.54 (d, J = 6.8 Hz, 2 H), 4.85–4.91 (m, 1 H), 5.74 (d, J = 5.0 Hz, 1 H), 6.12 (s, 1 H), 7.27–7.65 (m, 9 H), 7.78 (d, J = 7.5 Hz, 2 H), 8.15 (s, 1 H). Anal. Calcd for $C_{45}H_{53}N_5O_9Si_2Cl_2$: C, 57.81; H, 5.71; N, 7.49; Cl, 7.58. Found: C, 58.02; H, 6.04; N, 6.94; Cl, 7.16.

2'-O-(4-Methoxytetrahydropyran-4-yl)-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (8). To a suspension of **3** (cf. ref 7; 3.95 g, 7.52 mmol) in DMF (20 mL) at room temperature was added *N*,*N*-dimethylformamide dimethyl acetal (15.0mL, 113 mmol). After being stirred for 20 h, the reaction mixture was concentrated in vacuo. The last traces of DMF were removed under high vacuum to afford **7** in quantitative yield.

Intermediate **7** was dissolved in anhydrous THF (20 mL). To this solution at 0 °C was added 5,6-dihydro-4-methoxy-2*H*-pyran (13.0 mL, 116 mmol) and 2-mesitylenesulfonic acid (4.40 g, 18.6 mmol). After being stirred for 3 h at room temperature, the reaction mixture was slowly poured into cold saturated aqueous sodium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo.

To a solution of the residue in methylene chloride (30 mL) was added a saturated solution of methanolic ammonia (300 mL). After being stirred for 24 h at room temperature, the reaction mixture was concentrated in vacuo. The crude product was purified by column chromatography using silica gel (60 g) and a step gradient of methanol in methylene chloride (0–5%). Crystallization from ethanol afforded 3.07 g (64%) of product **8**: ^1H NMR ($CDCl_3$) δ 0.95–1.31 (m, 28 H), 1.88–2.00 (m, 4 H), 3.31 (s, 3 H), 3.61–3.85 (m, 4 H), 4.01–4.32 (m, 3 H), 4.48–4.52 (m, 2 H), 5.84 (s, 1 H), 6.56–6.72 (br s, 2 H), 7.91 (s, 1 H), 11.90 (br s, 1 H). Anal. Calcd for $C_{28}H_{49}N_5O_8Si_2$: C, 52.56; H, 7.22; N, 10.94. Found: C, 52.73; H, 7.46; N, 11.05.

O^6-(2-Cyanoethyl)-2'-O-(4-methoxytetrahydropyran-4-yl)-3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (9). Compound **9** was prepared by a modified procedure of Gaffney and Jones.[9] To a solution of **8** (1.92 g, 3.00 mmol) in methylene chloride (15 mL) at room temperature was added triethylamine (1.60 mL, 11.5 mmol), 4-(dimethylamino)pyridine (0.07 g, 0.57 mmol), and 2-mesitylenesulfonyl chloride (1.45 g, 6.62 mmol). After being stirred for 3 h, the reaction mixture was poured into

(13) van Boom, J. H.; de Rooy, J. F. M. *J. Chromatogr.* **1977**, *131*, 169.

cold saturated aqueous sodium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (60 g) and a 1% solution of methanol in methylene chloride to yield 2.03 g (82%) of the mesitylenesulfonyl intermediate.

To a solution of the mesitylenesulfonyl intermediate (2.03 g, 2.47 mmol) in methylene chloride (12 mL) at room temperature was added 1-methylpyrrolidine (4.00 mL, 38.5 mmol). After stirring for 1 h, the solution was cooled to −25 °C, and 2-cyanoethanol (2.6 mL, 38.1 mmol) was added. 1,8-Diazabicyclo-[5.4.0]undec-7-ene (0.54 mL, 3.62 mmol) was then added dropwise. The reaction mixture was stirred for 20 min at −25 °C and then warmed to 0 °C. After being stirred for 1 h at 0 °C, the mixture was poured into cold 5% ammonium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (70 g) and a step gradient of methanol in methylene chloride (0–2%) to yield 1.81 g of product **9** as a viscous oil. This oil also contained 2-cyanoethanol (which was difficult to remove) and was used without any further purification. An analytical sample was obtained by prep TLC: ^{1}H NMR ($CDCl_3$) δ 0.97–1.15 (m, 28 H), 1.90–2.03 (m, 4 H), 2.94 (t, J = 6.8 Hz, 2 H), 3.34 (s, 3 H), 3.61–3.75 (m, 2 H), 3.83–3.91 (m, 2 H), 3.99–4.26 (m, 3 H), 4.54–4.60, (m, 2 H), 4.72 (t, J = 6.8 Hz, 2 H), 4.85 (br s, 2 H), 5.94 (s, 1 H), 8.04 (s, 1 H). Anal. Calcd for $C_{31}H_{52}N_6O_8Si_2$: C, 53.73; H, 7.56; N, 12.13. Found: C, 53.87; H, 7.62; N, 11.95.

O^6-(2-Cyanoethyl)-N^2-[[(9-fluorenylmethyl)oxy]carbonyl]-2′-O-(4-methoxytetrahydropyran-4-yl)-3′,5′-O-(tetraisopropyldisiloxane-1,3-diyl)guanosine (10). To a solution of semipure **9** (1.80 g), predried by coevaporation with absolute pyridine, in methylene chloride (15 mL) and anhydrous pyridine (2 mL) at room temperature was added excess 9-fluorenylmethyl chloroformate (2.00 g, 7.72 mmol). After being stirred for 1.5 h, the reaction mixture was poured into cold 5% ammonium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (70 g) and a step gradient of methanol in methylene chloride (0–2%) to afford 1.65 g (60% from **8**) of product **10**: ^{1}H NMR ($CDCl_3$) δ 0.97–1.20 (m, 28 H), 1.90–2.13 (m, 4 H), 3.01 (t, J = 6.6 Hz, 2 H), 3.33 (s, 3 H), 3.57–3.57 (m, 4 H), 4.04–4.05 (m, 1 H), 4.22–4.31 (m, 4 H), 4.52–4.60 (m, 4 H), 4.74–4.81 (m, 2 H), 6.09 (s, 1 H), 7.31–7.44 (m, 4 H), 7.63 (d, J = 7.4 Hz, 2 H), 7.78 (d, J = 7.4 Hz, 2 H), 8.34 (s, 1 H). Anal. Calcd for $C_{46}H_{62}N_6O_{10}Si_2$: C, 60.37; H, 6.83; N, 9.18. Found: C, 60.14; H, 7.02; N, 8.87.

O^6-(2-Cyanoethyl)-N^2-[[(9-fluorenylmethyl)oxy]carbonyl]-2′-O-(4-methoxytetrahydropyran-4-yl)guanosine (11). To a solution of **10** (1.50 g, 1.64 mmol) in THF (10 mL) at 0 °C was added a solution of tetrabutylammonium fluoride (7.0 mmol) in THF/water/pyridine (5 mL/5 mL/5 mL). After being stirred at room temperature for 2 h, the reaction was quenched with cold 5% ammonium bicarbonate and extracted with methylene chloride. The combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (50 g) and a 5% solution of methanol in methylene chloride to yield 0.850 g (77%) of **11**: ^{1}H NMR ($CDCl_3$) δ 1.64–1.83 (m, 4 H), 2.67 (s, 3 H), 3.01 (t, J = 6.5 Hz, 2 H), 3.34–3.82 (m, 7 H), 3.96 (d, J = 11.9 Hz, 1 H), 4.42 (d, J = 4.8 Hz, 1 H), 4.54 (d, J = 6.7 Hz, 2 H), 4.78 (t, J = 6.5 Hz, 2 H), 5.05 (br s, 1 H), 5.22–5.29 (m, 1 H), 5.91 (d, J = 7.5 Hz, 1 H), 7.33 (t, J = 7.3 Hz, 2 H), 7.42 (t, J = 7.3 Hz, 2 H), 7.64 (d, J = 7.4 Hz, 2 H), 7.78 (d, J = 7.4 Hz, 2 H), 7.97 (s, 1 H). Anal. Calcd for $C_{34}H_{36}N_6O_9$: C, 60.07; H, 5.39; N, 12.49. Found: C, 60.48; H, 5.52; N, 12.12.

O^6-(2-Cyanoethyl)-5′-O-(4,4′-dimethoxytrityl)-N^2-[[(9-fluorenylmethyl)oxy]carbonyl]-2′-O-(4-methoxytetrahydropyran-4-yl)guanosine (12). To a solution of **11** (0.149 g, 0.22 mmol), predried by coevaporation with absolute pyridine, in anhydrous pyridine (2 mL) at room temperature was added 4,4′-dimethoxytrityl chloride (0.112 g, 0.33 mmol) and 4-(dimethylamino)pyridine (0.022 g, 0.18 mmol). After being stirred for 2 h, the reaction mixture was poured into cold 5% ammonium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel (10 g) and a step gradient of methanol in methylene chloride (0–2%) to yield the product (0.196 g) as a yellow solid. Trituration with methylene chloride and hexane afforded 0.170 g (79%) of **12** as a white powder: ^{1}H NMR ($CDCl_3$) δ 1.68–1.90 (m, 4 H), 2.84 (s, 3 H), 3.04 (t, J = 6.5 Hz, 2 H), 3.41–3.63 (m, 6 H), 3.75 (s, 6 H), 4.21–4.47 (m, 5 H), 4.80 (t, J = 6.5 Hz, 2 H), 5.33–5.40 (m, 1 H), 6.13 (d, J = 6.9 Hz, 1 H) 6.78 (d, J = 8.7 Hz, 4 H), 7.22–7.46 (m, 13 H), 7.55 (d, J = 7.4 Hz, 2 H), 7.78 (d, J = 7.4 Hz, 2 H), 8.01 (s, 1 H). Anal. Calcd for $C_{55}H_{54}N_6O_{11}$: C, 67.75; H, 5.58; N, 8.62. Found: C, 67.54; H, 5.77; N, 8.41.

Reaction of 2-Chlorophenyl Bis(1-benzotriazolyl) Phosphate (1) with Nucleosides. To a solution of the model substrate **2** or **10** (0.005 mmol), predried by repetitive coevaporation with absolute pyridine, in anhydrous pyridine (0.05 mL) at room temperature was added the phosphorylating reagent **1** (cf. ref 5; 0.030 mmol) and 1-methylimidazole (0.01 mL). The reaction mixture was then monitored by TLC at time intervals of 30 min, 1, 2, 3, 4, 6, and 24 h. The following results were observed: (a) Compound **2** underwent complete decomposition within 4 h. (b) Compound **10** was unchanged after 24 h.

Reaction of N^1,N^1,N^3,N^3-Tetramethylguanidinium 2-Nitrobenzaldehyde Oximate with Nucleosides. To a solution of the substrate (0.02 mmol) in dry acetonitrile (0.5 mL) at room temperature was added a freshly prepared solution of N^1,N^3,N^3-tetramethylguanidine (0.33 M) and 2-nitrobenzaldoxime (0.38 M) in dry acetonitrile (0.5 mL). The reaction mixture was then monitored by TLC at time intervals of 10 and 30 min, 1, 3, and 24 h. The following results were observed. (a) Compound **5b**: After 24 h only a trace of the O^6-deprotected product **4** was formed. (b) Compound **6**: The Fmoc group was cleaved within 10 min to form **5a**, and after 1 h cleavage of the 2′-acetyl group was observed. After 24 h the acetyl group was extensively cleaved to afford **5b**, and only a trace of the O^6-deprotected products **3** and **4** were formed. (c) Compound **10**: The Fmoc and β-cyanoethyl groups were quantitatively cleaved to produce intermediate **8** within 30 min.

General Method for Preparation of Protected Oligoribonucleotides (17). The procedure was essentially the same as used in the previous reports in this series.[1a,3] All operations were performed under complete exclusion of atmospheric moisture. To a solution of the 5′-terminal component **12** (0.11 mmol), predried by repetitive coevaporation with absolute pyridine, in THF (1–2 mL) and anhydrous pyridine (0.10 mL) at room temperature was added phosphorylating reagent **1** ref 5; 0.12–0.15 mmol). The reaction mixture was stirred at room temperature until TLC (system S_2) showed a quantitative formation of the diester **13** (base-line material), and then a solution of a predried 5′-OH component **11** or **14** (0.1 mmol) and 1-methylimidazole (0.02 mL) in THF (ca 0.5 mL) was added. The reaction mixture was stirred for 3 h and was then quenched with 5% aqueous ammonium bicarbonate. The aqueous phase was extracted with methylene chloride, and the combined organic extracts were dried over magnesium sulfate and concentrated in vacuo. The residue was purified by column chromatography using silica gel and a step gradient of methanol chloride (0–3%). Products **17** were isolated as colorless solids in yields of 55–65%.

Aminoacylation of Protected Trinucleoside Diphosphates (18). The previously described procedure was used.[3,11] The protected trinucleotide (**17**; 0.015 mmol) and dicyclohexylammonium salt of BPOC-Phe (0.027 g, 0.045 mmol) were dried by coevaporation with anhydrous pyridine and dissolved in methylene chloride (1.0 mL). To this solution was added 1-(2-mesitylenesulfonyl)tetrazole (0.017 g, 0.06 mmol) and 1-methylimidazole (0.02mL). After 1–3 h at room temperature, the reaction mixture was diluted with methylene chloride and was directly purified by preparative TLC (system S_1). Products **18** were isolated in quantitative yields as chromatographically uniform amorphous solids.

2′(3′)-O-(L-Phenylalanyl) Trinucleotides (20). The two-step deprotection procedure was carried out essentially as described previously.[1a,3] The protected derivative **18** (0.01 mmol) was treated with a freshly prepared solution of N^1,N^1,N^3,N^3-tetramethyl-

Table I. Electrophoretic Mobilities of Products and Standard Specimens (Electrophoresis with 0.05 M Sodium Hydrogen Citrate, pH 3.5)

compd	electrophoretic mobility[a]	compd	electrophoretic mobility[a]
Cp	−1.00	G-G-A, **19a**	+0.32
Gp	+3.45	G-C-A, **19b**	−0.77
A	−9.26	G-G-A-Phe, **20a**	−1.97
Phe	−4.29	G-C-A-Phe, **20b**	−4.18

[a] Mobility of Cp = 1.00; (−) = migration toward cathode; (+) = migration toward anode.

guanidine (0.33 M) and 2-nitrobenzaldoxime (0.38 M) in dry acetonitrile (1 mL). After 3 h at room temperature, the reaction product was precipitated with an excess of dry ether, centrifuged, washed twice with dry ether, and dried in vacuo over P_2O_5. The residue was treated with cold 80% formic acid (2.0 mL) at 0 °C for 30 min. The product was precipitated with an excess of dry ether, isolated by centrifugation, dissolved in cold 80% acetic acid (0.2 mL), and reprecipitated with excess ether. The solid product was dissolved in ammonium acetate buffer (5 mM, pH 4.5; 1 mL) and purified on a C_{18} SPE Baker column (3 mL) using a step gradient of acetonitrile (0, 5, 10, 20, 50, and 100%) in 5 mM ammonium acetate (pH 4.5). Product **20** was eluted with 20% acetonitrile; the eluate was evaporated in vacuo and coevaporated with 80% acetic acid at low temperature to remove the last traces of salt. The yields of chromatographically and electrophoretically (see Table I) uniform products **20** were determined spectrophotometrically and were in the 20–30% range. The products were also characterized by UV spectroscopy in 0.01 N HCl (**20a**: λ_{max} = 256 nm; λ_{min} = 237 nm; $A_{250/260}$ = 0.94; $A_{280/260}$ = 0.53; $A_{290/260}$ = 0.35; **20b**: λ_{max} = 258 nm; λ_{min} = 245 nm; $A_{250/260}$ = 0.86; $A_{280/260}$ = 0.73; $A_{290/260}$ = 0.45), mild alkaline hydrolysis to phenylalanine and parent oligonucleotides and enzymatic digestion with appropriate nucleases. Product **20a** was quantitatively digested with RNAse T_1 (ratio of Gp/A = 1.94) and with snake venom phosphodiesterase (ratio of G + pG/A + pA = 1.99). Product **20b** was quantitatively digested with RNAse T_1 and RNAse A (ratio of Gp/Cp/A = 1.01/0.98/1.00) and with snake venom phosphodiesterase (ratio of G/C + pC/A + pA = 1.02/1.06/1.00). Product **20b** was also identical in several electrophoretic systems with a previously prepared G-C-A-Phe.[1a]

A Highly Efficient and Large-Scale Synthesis of (2*S*,3*S*)-[2,3-$^2H_2$]- and (2*S*,3*R*)-[3-^{2}H]Aspartic Acids via an Immobilized Aspartase-Containing Microbial Cell System

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Received October 11, 1988

This paper describes the preparation and utilization of an immobilized aspartase-containing *Escherichia coli* system for the large-scale synthesis of (2*S*,3*S*)-[2,3-$^2H_2$]- and (2*S*,3*R*)-[3-^{2}H]aspartic acids from the appropriately labeled fumaric acids in >95% isolated yield, with >97% deuterium incorporation at the C-3 center and optically pure at both the C-2 and C-3 centers.

The synthesis of both L-aspartic acids (2*S*) stereospecifically labeled at the C-3 position with deuterium[1-3] and their utilization for the preparation of other stereospecifically labeled biologically important compounds, such as serine, glutamic acid, and amino ethanol, are well documented.[4-8] The method for the synthesis of the L-aspartic acids stereospecifically labeled at the C-3 position is not only unduly laborious and limited to the production of small quantities but requires the use of the expensive enzyme aspartase [EC 4.3.1.1][9] and the use of highly noxious and poisonous hydrogen sulfide gas in the workup of the enzyme reaction. In view of our interest in utilizing stereospecifically labeled L-aspartic acid as a synthon for the preparation of stereospecifically labeled L-homoserines and L-homoserine lactones,[10] L-methionines,[11] L-discadenines,[12] and *S*-adenosyl-L-methionines,[13] all of biological importance, we have developed a more efficient, less laborious and more cost effective method to prepare large quantities of aspartic acids stereospecifically labeled with deuterium at the C-3 position. In this paper we report the application of an immobilized aspartase-containing microbial cell system designed for that purpose.

Results and Discussion

The enzyme aspartase [EC 4.3.1.1] catalyzes the reversible addition of NH_2 to the *si* face and H, from the solvent, to the *re* face of fumarate in an overall anti fashion to yield L-aspartic acid.[1-3] If the reaction is performed in

(1) Englard, S. *J. Biol. Chem.* **1958**, *233*, 1003–1009.
(2) Krasna, A. I. *J. Biol. Chem.* **1958**, *233*, 1010–1013.
(3) Englard, S.; Hanson, K. R.; In *Methods in Enzymology*; Colowick, S. P., Kaplan, N. O., Eds.; Vol. XIII, Lowensteine, J. M., Ed.; Academic Press, Inc.: New York, 1969; pp 578–580.
(4) Field, S. J.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1979**, 1163–1165.
(5) Gani, D.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1982**, 867–869.
(6) Field, S. J.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2387–2392.
(7) Gani, D.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2393–2398.
(8) Gani, D.; Young, D. W. *J. Chem. Soc., Chem. Commun.* **1983**, 576–578.
(9) Aspartase is available from the Sigma Chemical Co. at a cost of ≈$167.00/100 units: one unit of enzyme will convert 1.0 μmol of L-aspartic acid to fumarate per minute at pH = 8.5 at 30 °C.
(10) Ramalingam, K.; Woodard, R. W. *J. Org. Chem.* **1988**, *53*, 1900–1903.
(11) Ramalingam, K.; Woodard, R. W. Manuscript in preparation.
(12) Son, J.-K.; Ramalingam, K.; Woodard, R. W. *Synthesis* **1988**, 240–242.
(13) Lee, K.-M.; Ramalingam, K.; Woodard, R. W. Unpublished results.

0022-3263/89/1954-3195$01.50/0