stated preparative HPLC conditions, except that the methanol concentration was 22.5%. All fractions corresponding to epimer A were combined and lyophilized to a very pale yellow powder (127 mg, 8.9%): mp >250 °C dec; IR (KBr) 3600–2600 (broad), 1705, 1615, 1600, 1570, 1510, 1385, 1320, 1230, 1220, 1170, 1120, 1020, 990, 770 cm^{-1}; λ_{max} (0.1 N aqueous HCl) 339 (ϵ 7850), 267 (13700) nm; λ_{max} (0.1 N aqueous NaOH) 343 (ϵ 9280), 274 (9990), 249.0 (16200) nm; ^{19}F NMR (470 MHz, DMSO-d_6, CF_3COOH as external standard) δ 12.47 (q, 3 F, J = 6.8 Hz) and -7.65 (q, 3 F, J = 6.8 Hz); FABMS (glycerol–0.5 M HCl matrix) m/e (relative intensity) 453 (100, MH^+); high-resolution FABMS calcd for $C_{13}H_{15}F_6N_4O_7$ m/e 453.0845 (MH^+), found 453.0844.

7-Hydroxy-8-D-ribityl-6,7-bis(trifluoromethyl)-7,8-dihydropteridine-2,4(1*H*,3*H*)-dione (14), Epimer B. All fractions corresponding to epimer B were combined and lyophilized to a very pale yellow powder (117.2 mg, 8.2%): mp >250 °C dec; IR (KBr) 3600–2600 (broad), 1620, 1510, 1390, 1325, 1240, 1230, 1170, 1115, 1020, 940, 885, 775 cm^{-1}; λ_{max} (0.1 N aqueous HCl) 341 (ϵ 7120), 338 (7120), 268 (13400) nm; λ_{max} (0.1 N aqueous NaOH) 345.0 (ϵ 8940), 274.0 (9650), 248.5 (14900) nm; ^{19}F NMR (470 MHz, DMSO-d_6, CF_3COOH as external standard) δ 12.65 (q, 3 F, J = 6.4 Hz) and -6.59 (q, 3 F, J = 6.4 Hz); FABMS (glycerol–0.5 M HCl matrix) m/e (relative intensity) 453 (74.4, MH^+), 185 (100, glycerol); high-resolution FABMS calcd for $C_{13}H_{15}F_6N_4O_7$ m/e 453.0845 (MH^+), found 453.0855.

Enzyme. Light riboflavin synthase was purified from the derepressed mutant H94 of *B. subtilis* by published procedures.[15]

Equilibrium Dialysis. Experiments were performed at 4 °C using microdialysis cells from Dianorm and Visking dialysis tubing. The buffer contained 170 mM phosphate (pH 6.8) and 10 mM sodium sulfite. The protein concentration was 2.7 mg/mL. The dialysis cells were allowed to equilibrate for 5 h at 4 °C under slow rotation. Protein was precipitated by the addition of trichloroacetic acid to a final concentration of 5%. Subsequently, the ligand concentration was determined by HPLC analysis using a column of Nucleosil RP18 (4 × 250 mm) with an eluent containing 25% methanol in 0.1% trifluoroacetic acid. The effluent was monitored photometrically (365 nm). The retention times of epimers A and B were 5 and 6 min, respectively. The flow rate was 2 mL/min.

Enzyme Assay. Riboflavin synthase activity was assayed at 37 °C according to published procedures.[32] Assay mixtures for the determination of initial rates contained 0.1 M phosphate (pH 7.4), 10 mM sodium sulfite, 10 mM EDTA, 5–45 μM 6,7-dimethyl-8-ribityllumazine, 30–200 μM enzyme inhibitor, and protein. Aliquots were removed at 1-min intervals and quenched by the addition of trichloroacetic acid to a final concentration of 5%. The concentration of riboflavin was monitored by HPLC on a column of Nucleosil RP 18, 4 × 250 mm. The eluent was 100 mM ammonium formate containing 35% methanol. For fluorensence detection, excitation was at 470 nm and emission was at 530 nm.

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Supplementary Material Available: HPLC analyses of each epimer of 14 (2 pages). Ordering information is given on any current masthead page.

New Nucleoside Phosphoramidites and Coupling Protocols for Solid-Phase RNA Synthesis

Matthew H. Lyttle,*,† Peter B. Wright,† Nanda D. Sinha,† J. D. Bain,‡ and A. Richard Chamberlin*,‡

Milligen/Biosearch, Division of Millipore, Inc., 81 Digital Drive, Novato, California 94949, and Department of Chemistry, University of California, Irvine, California 92717

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The 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diethylphosphoramidites) **3**, **5**, **7**, and **9**, modified monomers for RNA synthesis, were prepared from 2-cyanoethyl *N*,*N*-diethylchlorophosphoramidite (**1**). In conjunction with newly developed coupling protocols for automated solid-phase synthesis, they afforded synthetic oligoribonucleotides up to 74 base units in length. The performance of the new compounds was compared to the analogous 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites) **4**, **6**, **8**, and **10**. Complete removal of benzoyl groups from N^2-benzoylguanosine, which was incorporated into some of the synthetic oligoribonucleotides, was demonstrated. Purification procedures by reverse phase HPLC and PAGE methods are also presented.

Introduction

There is a continuing interest in refining techniques for the solid-phase chemical synthesis of RNA. The well-established internucleotide coupling protocols that have been developed for DNA synthesis, including methyl phosphoramidites, 2-cyanoethyl phosphoramidites, and H-phosphonates,[1] have been proven to be much less satisfactory for RNA. The necessity of protecting the 2′-hydroxyl group of RNA[2] introduces additional steric bulk that reduces the efficiency of internucleotide phosphate bond formation. This problem has led to the use of relatively reactive methyl *N*,*N*-diisopropylphosphoramidites (MAs),[3,4] which results in rapid and efficient coupling (>96% within 2 min). Unfortunately, subsequent oxidation of the resultant phosphinate generates a methyl phosphate triester intermediate that is a potent alkylating agent that has been shown to methylate thymidine and guanosine residues during DNA synthesis.[5,6] It is therefore likely

† Milligen/Biosearch.
‡ University of California.

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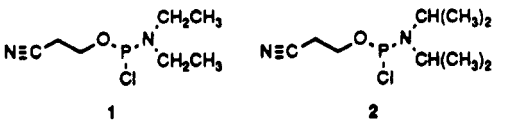
that long chains of RNA prepared with the MA procedure would contain significant amounts of methylated bases.

2-Cyanoethyl *N,N*-diisopropylphosphoramidite (CEA) coupling methods have been developed in order to avoid the generation of reactive alkylating agents during oxidation.[2,10] There are several practical considerations, however, that limit the application of the CEA method in RNA synthesis. As a corollary of their higher stability and lower tendency to generate side products, they are less reactive than their MA counterparts, and even with coupling times of 15 min lower coupling efficiencies (ca. 95%) are obtained, limiting the length of oligoribonucleotides that can be prepared by this method. There are reports of increased coupling efficiencies by activation with (dimethylamino)pyridine (DMAP) and tetrazole mixtures,[7] as well as with (*p*-nitrophenyl)tetrazole.[8] Unfortunately, DMAP gives rise to fluorescent byproducts via guanosine modification and is not to be recommended.[9] Additionally, there are problems associated with the preparation of the ribonucleoside 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidites). 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite[10] is a high boiling material that is very difficult to obtain in pure form. More seriously, the reaction of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)-3-hydroxyribonucleosides with this phosphorylating reagent is slow.[11]

On the basis of these considerations, a delicate balance in RNA synthesis must be struck between steric bulk of the 2′-hydroxyl protecting group, the reactivity of the phosphoramidite in the coupling step, and the stability of the triester oxidation product of the coupled phosphinate. Wishing to retain the desirable characteristics of the 2-cyanoethyl substituent while at the same time increasing the reactivity of the phosphoramidite, we reasoned that reducing the steric bulk of the nitrogen substituents would have the desired effect. We have therefore examined the application of 2-cyanoethyl *N,N*-di*ethyl*phosphoramidites to RNA synthesis in combination with trialkylsilyl protection of the 2′-hydroxyl group. In comparison to the corresponding diisopropyl derivatives, they are sufficiently stable, easier to prepare, and give higher coupling efficiencies in solid-phase RNA synthesis. We also have defined sequence-specific programs for automated synthesis with these derivatives, as well as conditions for complete cleavage of exocyclic amino protecting groups, in order to optimize yields for the oligoribonucleotides produced. The important question of appropriate workup and isolation techniques has also been addressed.

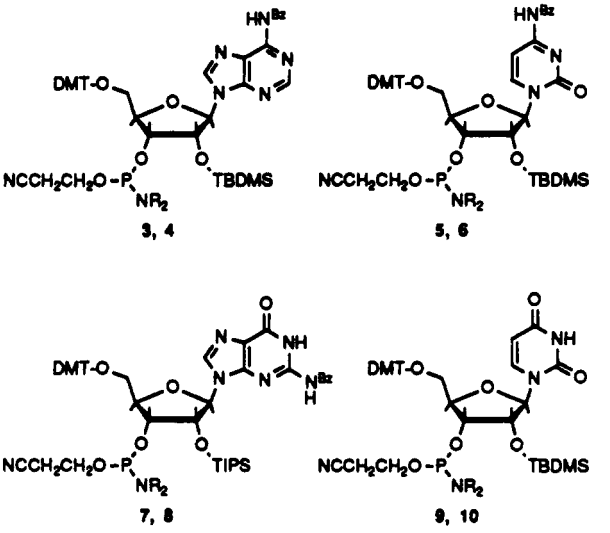
Results and Discussion

Strategy of Synthesis. The ease of synthesis and purification of 2′-protected ribonucleoside intermediates is a major factor in considering new RNA synthesis schemes, and many modes of protection have been advocated. In previous studies[12] we examined *p*-methoxybenzyl[13] and benzoyl[14] for 2′-protection and found them to be unsatisfactory. In contrast, 2′-trialkylsilyl protection proved to be advantageous,[4] and we have made the 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidites) **4**, **6**, **8**, and **10** commercially available. This same *N*-benzoyl-2′-trialkylsilyl protection was retained for the current studies.



1 **2**

Figure 1. Chlorophosphoramidite reagents for amidite synthesis.



3, 4 **5, 6** **7, 8** **9, 10**

Figure 2. RNA phosphoramidites.

We have chosen to maintain benzoyl protection for the amine substituents of the bases (Figure 1). This selection was made on practical grounds, since the intermediate N-protected nucleosides are obtained in high yields as nicely crystalline materials. A potential disadvantage of this strategy is that the prolonged treatment with ammonia required for deprotection might also remove the 2′-protecting groups and/or result in internucleotide cleavage.[1] This important issue was examined carefully, as described below.

Amidite Synthesis. The phosphorylating reagent **1** (Figure 2), prepared by modification of the published procedure for the corresponding diisopropyl derivative **2**,[15] can be obtained in high purity by simple vacuum distillation. As expected, **1** reacts with 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleosides[16] faster than does **2** (approximately 5 times more rapidly) and gives better yields of the desired ribonucleotide phosphoramidite (**3**, **5**, **7**, and **9**) with less isomerization of the trialkylsilyl protecting group.

Oligoribonucleotide Synthesis. Comparison of *N,N*-Dialkylphosphoramidites. With the new monomers **3**, **5**, **7**, and **9** in hand, a systematic study of their use in solid-phase RNA synthesis was carried out. The following issues were examined: (1) compare their performance with that of the corresponding *N,N*-diisopropyl-

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(16) Made as in ref 4, except with 4,4′-dimethoxytrityl chloride.

Table I. Short-Fragment Synthesis for Comparison of Diethyl and Diisopropyl RNA Amidites

sequence	amidite employed	crude OD	purified OD	coupling efficiency, %
ACCCUGGACCCCGCCUGCUGCGCCGGCGCUUCGC	diethyl	92	57	96.3
ACCCUGGACCCCGCCUGCUGCGCCGGCGCUUCGC	diisopropyl	89	58	93.3
UCCACGUCAUCGAGGUCAUC	diethyl	79	30	95.0
UCCACGUCAUCGAGGUCAUC	diisopropyl	69	36	93.3

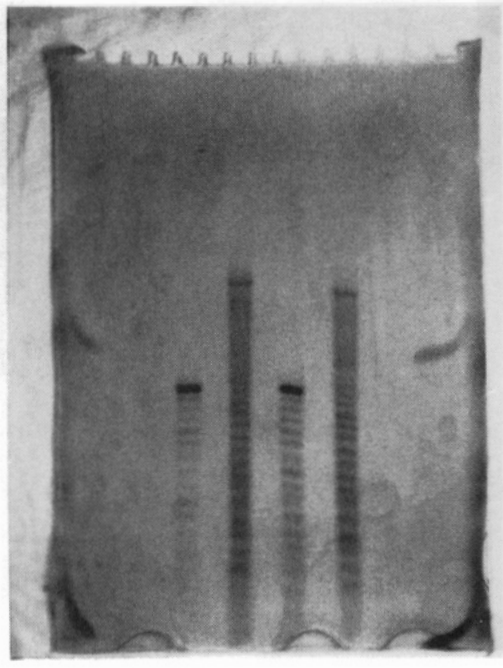

Figure 3. 20% PAGE of 20- and 35-mers made with diethyl and diisopropyl RNA amidites, after partial purification by G-25 size exclusion chromatography: from left to right, 20-mer with diethyl RNA amidites, 35-mer with diethyl RNA amidites, 20-mer with diisopropyl RNA amidites, 35-mer with diisopropyl RNA amidites.

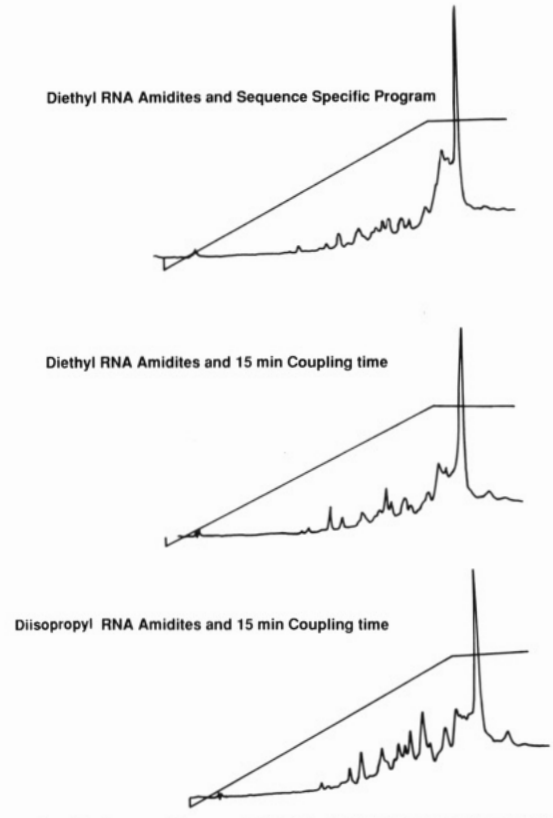

Figure 4. Anion exchange HPLC of UCCACGUCAUCGAGGUCAUC: top, diethyl RNA amidites with sequence-dependant synthesis programs; middle, diethyl RNA amidites with 15-min coupling time; bottom, diisopropyl RNA amidites with 15-min coupling time.

phosphoramidites, (2) ascertain whether sequence-specific protocols are beneficial, (3) find optimal conditions for deprotection.

For a performance comparison of **3**, **5**, **7**, and **9** with their *N*,*N*-diisopropyl counterparts **4**, **6**, **8**, and **10**, identical synthesis programs and conditions were applied. Fifteen-minute coupling times were employed to make two 35-mers and two 20-mers (Table I), and the coupling efficiencies and yields were determined.

Quantitative colorimetric assay of the effluent from DMT cleavage showed that better coupling efficiencies were obtained when *N*,*N*-diethylphosphoramidites **3**, **5**, **7**, and **9** were employed compared to the diisopropyl derivatives **4**, **6**, **8**, and **10**. In addition, PAGE analysis (Figure 3) and anion exchange HPLC of the 20-mers both showed that the 20-mer made with the *N*,*N*-diethylphosphoramidites (Figure 4, middle trace) was superior to that made with the *N*,*N*-diisopropylphosphoramidites (Figure 4, bottom trace). There was, however, little difference between the 35-mers prepared by both methods when this non-sequence-specific protocol is employed.

The synthesis of a 3′-truncated tRNA (74-mer)[17] from the monomers **3**, **5**, **7**, and **9** was attempted under similar conditions in order to extend the size limits for RNA oligomer synthesis. For this experiment, a 1000-Å CPG resin was utilized rather than the standard 500-Å solid support. A 7-min coupling time proved to be sufficient, and the resultant HPLC-purified oligoribonucleotide (Figure 6) compared well with RNA with an identical sequence obtained by run-off transcription.[22] When the analogous *N*,*N*-diisopropylphosphoramidites **4**, **6**, **8**, and **10** were employed to make the same 74-mer, the DMT effluent colors slowly declined in intensity until they were almost totally clear, indicative of a poor synthesis result. PAGE showed that no 74-mer product was obtained. The coupling efficiency was 96.4%, and only 8 OD units eluted from the G-25 column, compared with 99.2% coupling efficiency and 36 OD units for the diethyl amidites.

Table II. Coupling Times and Efficiencies for 20-mer Homopolymers

synthesis	coupling time	coupling efficiency, %
A_{20}	15:40	98.6
C_{20}	14:10	95.8
G_{20}	11:40	96.1
U_{20}	4:30	97.8

Sequence-Specific Coupling Protocols. Although the data obtained thus far indicate that the *N*,*N*-diethylphosphoramidites are superior to the corresponding diisopropyl derivatives, further improvements are realized

(17) This structure, based upon the sequence of *Escherichia coli* $tRNA^{Gly3}$, is missing the 3′-terminal cytidine and adenosine moieties of the intact tRNA. The 3′-truncated tRNA depicted in Figure 5 has been successfully employed to produce chemically misacylated tRNAs, accomplished by T4 RNA ligation with a synthetic 2′(3′)-*O*-(aminoacyl)-dinucleotide. This method has been successfully employed to incorporate non-natural amino acids site-specifically into proteins [(a) Bain, J. D.; Diala, E. S.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1989**, *111*, 8013–8014. (b) Bain, J. D.; Wacker, D. A.; Kuo, E. E.; Chamberlin, A. R. *Tetrahedron* **1991**, *47*, 2389–2400].

Table III. Different Times Employed for Each Coupling

	base coupled (to)			
	A	C	G	U
A	15:40	14:50	12:20	6:30
C	15:00	14:10	11:40	5:50
G	15:00	13:50	11:40	5:50
U	13:40	12:50	10:20	4:50

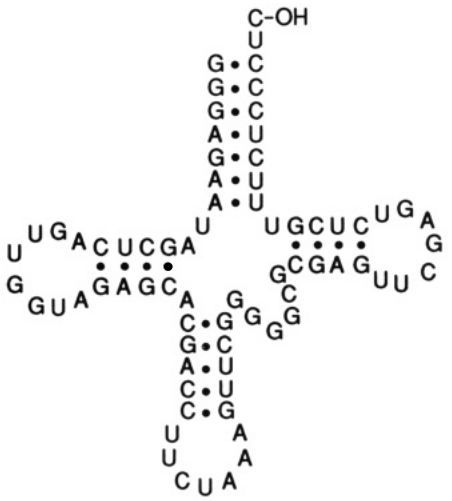

Figure 5. Sequence of 3′-truncated $tRNA^{Gly}_{CUA}$-C_{OH} (74-mer) missing the terminal adenosine and cytidine moieties.

by determining and employing the optimal coupling time for each specific protected nucleoside. New synthesis programs for testing the performance **3**, **5**, **7**, and **9** were written so that the coupling efficiency for each of the four 20-mer homopolymers would be above 95%, based on quantitative colorimetric assays of the DMT-containing effluents from the deblocking steps of synthesis cycles 8, 9, 18, and 19.[18] The coupling time and efficiency for the preparation of each homopolymer are shown in Table II.

Although the reasons for the disparity in reactivity between the individual RNA *N*,*N*-diethylphosphoramidite monomers are not clear, the differences suggested that further refinement could be made by basing the coupling time not only on the monomer added but also on the identity of the nucleotide found at the nucleophlic 5′-terminus of the growing oligomer. Thus, each of the 16 different possibilities could have a distinct coupling time. With this in mind, a series of programs was written in which the exposure time of each added base was dependent on both of these factors. These coupling times are shown in Table III.

A truly rigorous optimization of each of these time parameters would be prohibitive in terms of time spent and materials employed. Thus, the values were derived from individual coupling efficiencies for each of the 16 possible combinations, based upon quantitative colorimetric assay of the effluent from DMT cleavage of the oligonucleotides listed in Table I and Figure 5 (additionally, the parameters derived from the homopolymer couplings were employed). Reaction times were then adjusted so that coupling efficiencies were >95%. The programs were applied to the syntheses of those fragments listed in Table IV. The immediate advantage—over the use of a constant extended coupling time for all couplings—is in the saving of time and unnecessary exposure of the growing chain to the coupling reagents.

(18) The coupling efficiencies are computed by using the expression CE = exp[(ln *B*/*A*)/*n*], where *B* is the last color(s), *A* is a preceding color or colors, and *n* is the number of couplings done between the two measurements. The order of capping and oxidation was reversed in syntheses where the coupling efficiencies were measured. This modification gives more reliable values.

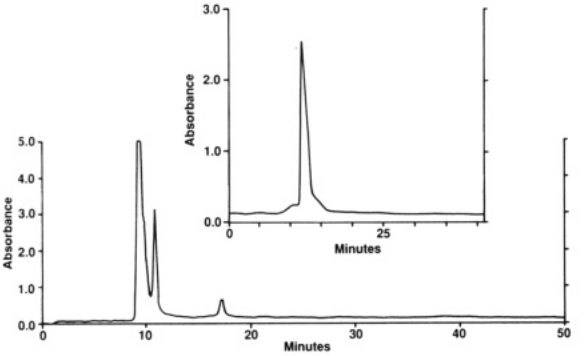

Figure 6. Reverse phase purification of 74-mer oligoribonucleotides. Insert shows re-injection of purified product, after DMT removal.

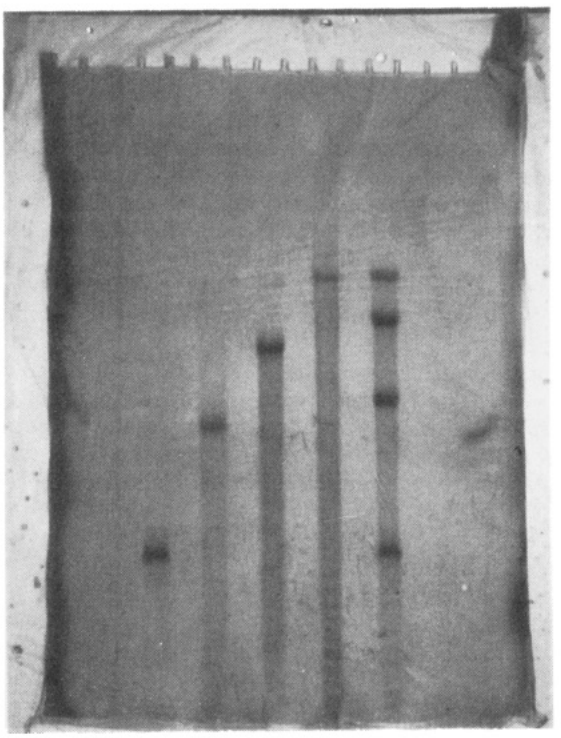

Figure 7. 15% PAGE of 20-, 35-, 50-, and 74-mer oligoribonucleotides after partial purification by G-25 size exclusion chromatography. The right lane contains a mixture of 2-, 40-, 60-, and 80-mer uridine homopolymers.

When the new sequence and base specific programs and the diethyl amidites were used to synthesize the same 20- and 35-mers, only small, faint failure bands are observed relative to the product (lanes 1 and 2, Figure 7). There is concern that the trialkylsilyl 2′-protecting groups might be removed by the ammonia treatment used to remove the exocyclic amine protecting groups, leading to strand cleavage.[1] Therefore, it was important to determine the source of the failure sequences observed. If undesired cleavage due to premature removal of the trialkylsilyl groups by ammonia were the major factor in generating these failures, then these RNA crudes should look very similar to the RNA fragments shown in Figure 3. They do not.

The failure bands in Figure 3 are probably due to low coupling efficiency, in the case of the diisopropyl RNA amidites and the use of an unoptimized synthesis program for the diethyl amidites. We have seen that long exposure of an RNA chain to reactive DNA amidites tends to di-

Table IV. RNA Sequences Made with Diethyl RNA Amidites and Sequence-Specific Programs[a]

sequence	length	coupling efficiency	yield, OD
GGGAGAAUAGCUCAGUUGGUAGAGCACGACCUUCUAAAGUUCGGGGGCGCGAGUUCGAGUCUC-GUUUCUCCCUC	74	99.2	42
GCUGACAACGCUGCUGCUGCUGCUGCUGCUACUGCUGCUGAACGCAUCCG	50	98.4	59
ACCCUGGACCCCGCCUGUCUGCGCCGGCGACUUCGC	35	98.1	69
UCCACGUCAUCGAGGUCAUC	20	96.7	50

[a] Coupling efficiency was calculated with DMT colors 9, 10, $n-2$, and $n-1$. The yield is in OD units, after the G-25 column. PAGE analysis of the four fragments is shown in Figure 5.

Table V. Deprotection of G-20 Homopolymers

sample	temp (°C)	exposure time (h)	% debenzoylation
A	rt	1	26
B	rt	24	56
C	rt	48	76
D	55	6	83
E	55	24	>99

minish product quality,[19] and the 15-min exposure to the reactive uridine *N,N*-diethylphosphoramidite 8 may be responsible here. There is, however, a fair correlation between the coupling efficiency and synthesis quality: lane 1 of Figure 7 is 96.7%, lane 2 is 98.1%, lane 1 of Figure 3 is 95.0%, and lane 2 is 96.3%. The failure bands that are present in the gel of Figure 3 are therefore generated mostly during the synthesis and not during the ammonia treatment.

This is reaffirmed by the SAX HPLC traces shown in Figure 4. The synthesis with the highest coupling efficiency shows the best product quality. Again, if undesired cleavage were the major contributor to the generation of failure sequences, then the top trace would look more like the bottom trace, as regards the relative amounts of failure to product peak area.

N^2-Benzoylguanosine Deprotection Study. After the automated synthesis of a protected RNA fragment, treatment with a 3:1 solution of ammonia and ethanol is used[4] for CPG cleavage and removal of protecting groups. It is known that removal of the N^2-benzoyl protecting group from guanosine[20] is the slow step for this process. Incomplete protecting group removal is highly undesirable; therefore, the conditions required for the complete removal of the benzoyl group from guanosine were carefully studied.

Five 1.0-μmol syntheses of G-20 were performed. The CPG from these runs—with RNA fragments attached—was pooled, mixed, and divided into five samples of 25 mg each. Each samples was exposed to 1.5 mL of a 3:1 solution of 30% NH_4OH/ethanol at either 55 °C or room temperature for various times. The samples were worked up and enzymatically hydrolyzed. Each sample—along with the deprotection conditions employed—is listed in Table V.

Three HPLC traces are displayed in Figure 8. The top trace is a mixture of guanosine and N^2-benzoylguanosine standards. Guanosine elutes first, and the more hydrophobic N^2-benzoylguanosine elutes later. The injection of sample D under the same conditions is seen in the middle trace, and below this, the trace of sample E injection is shown.

From the HPLC results it is evident that room temperature deprotection, even after 48 h, is insufficient to completely remove the benzoyl group from the N^2-benzoylguanosine. However, deprotection at 55 °C works well. After 24 h, the benzoyl groups are better than 99% removed. It can be seen from these data that 18–24 h at 55 °C with the ammonia/ethanol mixture is adequate for complete debenzoylation. Furthermore, internucleotide phosphate linkages of synthetic products from monomers 4, 6, 8, and 10 have the correct 3′-5′ disposition under these conditions, as demonstrated by enzymatic and ^1H NMR studies.[19]

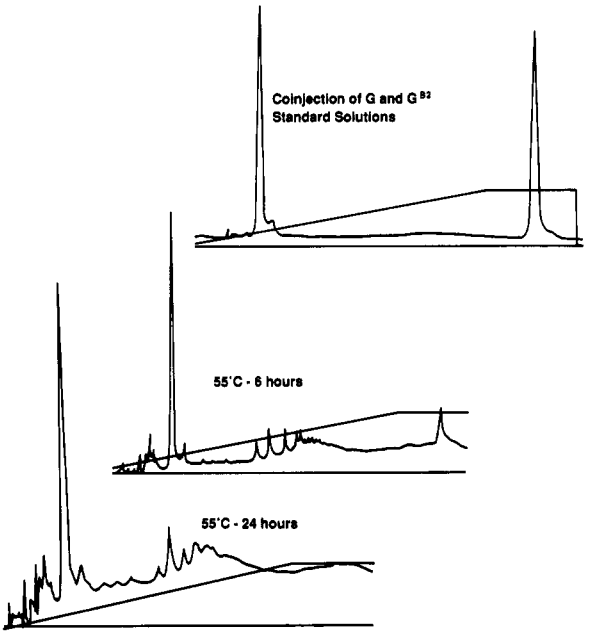

Figure 8. Reverse phase HPLC analysis of guanosine debenzoylation enzyme digests: top, control injection; middle 55 °C for 6 h; bottom, 55 °C for 24 h.

Conclusion

The new diethyl amidite compounds 3, 5, 7, and 9, in conjunction with a new sequence-dependent programming protocol, lead to a significant improvement over the analogous diisopropyl amidites 4, 6, 8, and 10 in RNA yield and purity, especially when long oligoribonucleotides are prepared. The presently described protection scheme is adequate to give good performance up to chain lengths in excess of 70 residues, even with guanosine-rich sequences, where incomplete side-chain deblocking and problems associated with prolonged ammonia treatment are exacerbated. A small amount of strand cleavage due to premature removal of trialkylsilyl groups by the ammonia treatment does occur under these conditions; however, this problem is a relatively minor one that does not affect the capability of synthesizing intermediate length RNA fragments. Indeed, our studies define rather harsh conditions for the removal of the *N*-benzoyl side-chain protecting groups, yet even so, biologically active sequences over 70 residues long have been prepared. The use of these new materials and methods should give molecular biologists and

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other nonchemists easy access to RNA fragments of the tRNA size range. The fact that all of the RNA amidites presented could be isolated in nearly analytical purity should increase confidence in these starting materials.

Experimental Section

Materials. The following biological products and reagents were used: calf intestinal alkaline phosphatase (Boehringer Mannhiem Biochemicals; 73023, Sigma), RNA T2 (*Aspergillus oryzae*, R3751). Benzoylated (diethylaminoethyl)cellulose and Poly-Prep 2-mL disposable polypropylene columns (731-1550, Bio-Rad) and Sephadex G-25 Select-D columns (5301-730608/725608, 5 Prime 3 Prime, Inc.) were purchased from the manufacturers indicated.

Solvents (Baxter Corporation) were dried prior to use when necessary. Acetonitrile was dried by heating at reflux over K_2CO_3 (5 g/L) for 2 h, distilled, and stored over 3-Å molecular sieves for at least 7 days prior to use. Diethylamine, diisopropylethylamine (DIPEA), ethyl acetate, and pyridine were dried by heating at relfux over CaH_2 (5 g/L) for 2 h and distilled. Diethyl ether and tetrahydrofuran (THF) were dried by heating at reflux over CaH_2 (5 g/L), distilled, and then redistilled from sodium metal and benzophenone. All other solvents used were of the highest commericial grade obtainable.

Special care was taken whenever handling oligoribonucleotides due to the ubiquitous nature of RNA-destroying enzymes.[4] Strictly asceptic techniques were used. All solutions were pretreated by the addition of 1% diethyl pyrocarbonate (v/v in absolute EtOH to a final concentration of 0.1%) for 24 h at room temperature and then sterilized by autoclaving at 225 °C and 18 psi for at least 30 min. Solutions for column chromatography were preserved by the addition of NaN_3 (0.001% final concentration) to preclude bacterial contamination. All glassware was treated with diethyl pyrocarbonate for at least 4 h and subsequently autoclaved; alternatively, glassware was heated to 250 °C for a minimum of 4 h. Glass pipets and minicolumns were siliconized with Sigmacote (Sigma) prior to sterilization.

The following compounds were prepared by previously reported methods: 2-cyanoethyl phosphorodichloridite and 2-cyanoethyl *N,N*-diisopropylchlorophoramidite,[10] N^6-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*O*-(2 cyanoethyl *N,N*-diisopropylphosphoramidite), N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) and 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite),[15] and N^2-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)-guanosine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite).[16]

General Methods. NMR spectra are reported in ppm from internal trimethyl phosphate. Thin-layer chromatography (TLC) was performed on 0.25-mm E. M. Reagents precoated aluminum-backed silica 60 gel plates (5534). The following chromatographic systems were employed: S_1, 0.1% pyridine in 1:1 hexanes/EtOAc, or S_2, 1:3 EtOAc/CH_2Cl_2. Flash chromatography was performed on E. M. Reagents 70–200-mesh silica gel (7734-7) as described by Still et al.[21] High-performance liquid chromatography (HPLC) was performed on a reverse phase Vydac C-4 preparative column (5 μm packing, 10 mm i.d. × 250 mm length).

Amidite Synthesis. 2-Cyanoethyl *N,N*-Diethylchlorophosphoramidite (1). 2-Cyanoethyl phosphorodichloridite (300 g, 1.74 mol) and anhydrous Et_2O (3 L) were added to a flame-dried round-bottom three-neck flask (5 L) under argon fitted with a mechanical stirrer. The stirred solution was cooled to −78 °C followed by addition of diethylamine (255 g, 3.49 mol) over a 90-min period. The resulting white slurry was stirred and allowed to slowly warm to room termperature overnight. The mixture was filtered rapidly through a large coarse sintered glass funnel and concentrated in vacuo to a pale yellow oil. Caution was observed, due to the explosive and pyrophoric nature of this compound when in contact with moisture. The oil was distilled with a s/t 24/40 "cow" fraction cutter distillation apparatus. A small forerun was observed (bp 50–95 °C, 200 μm, vacuum) followed by the main fraction, which was collected under vacuum (200 μm) at 105–112 °C to yield 229 g (63%) of 1 as a clear oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.15–4.0 (dd, J = 6.2 Hz, 2 H), 3.3–3.0 (m, 4 H), 2.8–2.7 (t, J = 6.2 Hz, 2 H), 1.3–1.1 (t, J = 7.1 Hz, 6 H); ^{31}P NMR (81 MHz, $CDCl_3$) δ 175.96; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 208 (M^+, 230, 195 (26), 193 (77), 173 (100), 138 (30), 136 (84), 120 (13); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_7H_{14}N_2OCl$ 208.0530, found 208.0533.

Representative Procedure for the Sythesis of 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(trialkylsilyl)ribonucleoside 3′-*O*-(2-Cyanoethyl *N,N*-dialkylphosphoramidites). N^6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*O*-(2-Cyanoethyl *N,N*-diethylphosphoramidite) (3). N^6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine (15 g, 19.0 mmol), anhydrous THF (200 mL), and DIPEA (10 mL) were added to a round-bottom flask (500 mL) and stirred under argon. The solution was stirred for an additional 2 h following rapid addition of 2-cyanoethyl *N,N*-diethylchlorophosphoramidite (1; 4.76 g, 22.8 mmol). The mixture was filtered to remove the white precipitate, concentrated in vacuo, and dissolved in EtOAc (200 mL), which had been purged with argon. The filtrate was washed with saturated aqueous $NaHCO_3$ (2 × 100 mL)—previously purged with argon—dried over Na_2SO_4, filtered, and concentrated in vacuo. This material was subjected to high vacuum overnight, followed by flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc) to yield 10.27 g (57%) as a white solid, R_f 0.4 (system S_1, two diastereomeric spots): ^{31}P NMR (81 MHz, $CDCl_3$) δ 150.51, 149.97; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 960 (M^+, 88), 721 (44), 600 (31), 583 (46), 303 (100), 240 (80), 136 (48); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{51}H_{62}N_7O_8PSi$ 960.4245, found 960.4212. Anal. Calcd for $C_{51}H_{62}N_7O_8PSi$: C, 63.74; H, 6.50; N, 10.20. Found: C, 63.88; H, 6.21; N, 9.93.

N^6-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine 3′-*O*-(2-Cyanoethyl *N,N*-diisopropylphosphoramidite) (4). Addition of undistilled 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (2; 25 mL, 106.0 mmol) to a solution containing N^6-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)adenosine (49 g, 62 mmol), THF (300 mL), and DIPEA (10 mL) was followed by 2 of stirring h to yield 36 g (58%) of 4 as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.44 (system S_1, two diastereomeric spots): ^{31}P NMR (162 MHz, $CDCl_3$) δ 148.65, 146.71; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 988 (M^+, 52), 749 (30), 699 (14), 668 (10), 303 (100), 240 (20), 201 (21); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{49}H_{56}N_7O_8PSi$ 988.4558, found 988.4588. Anal. Calcd for $C_{49}H_{56}N_7O_8PSi$: C, 64.37; H, 6.73; N, 9.91. Found: C, 64.22; H, 6.65; N, 9.79.

N^4-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine 3′-*O*-(2-Cyanoethyl *N,N*-diethylphosphoramidite) (5). Addition of 2-cyanoethyl *N,N*-diethylchlorophosphoramidite (1; 3.28 g, 15.7 mmol) to a solution containing N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine (10 g, 13.1 mmol), THF (200 mL), and DIPEA (5 mL) was followed by 2 h of stirring to yield 5.7 g (46%) of 5 as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.4 (system S_1, two diastereomeric spots): ^{31}P NMR (81 MHz, $CDCl_3$) δ 150.49, 149.22; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 936 (M^+, 21), 721 (127), 442 (25), 303 (100), 216 (43), 173 (69); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{50}H_{62}N_5O_9PSi$ 936.4133, found 936.4103. Anal. Calcd for $C_{50}H_{62}N_5O_9PSi$: C, 64.16; H, 6.67; N, 7.48. Found: C, 64.12; H, 5.91; N, 7.45.

N^4-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine 3′-*O*-(2-Cyanoethyl *N,N*-diisopropylphosphoramidite) (6). Addition of undistilled 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (2; 40 g, 170 mmol) to a solution containing N^4-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)cytidine (31 g, 40 mmol), THF (500 mL), and DIPEA (50 mL) was followed by 18 h of stirring to yield 17 g (44%) of 6 as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/

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EtOAc), R_f 0.45 (system S_1, two diastereomeric spots): ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.75, 146.82; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 964 (M^+, 28), 749 (54), 644 (14), 442 (8), 303 (100), 216 (16), 201 (23), 117 (9); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{52}H_{66}N_5O_9PSi$ 964.4446, found 964.4442. Anal. Calcd for $C_{52}H_{66}N_5O_9PSi$: C, 64.79; H, 6.90; N, 7.26. Found: C, 64.46; H, 6.65; N, 7.18.

N^2-Benzoyl-5′-*O*-(4,4-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine 3′-*O*-(2-Cyanoethyl *N,N*-diethylphosphoramidite) (7). Addition of 2-cyanoethyl *N,N*-diethylchlorophosphoramidite (1; 10 g, 47.3 mmol) to a solution containing N^2-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine (20 g, 23.6 mmol), THF (200 mL), and DIPEA (10 mL) was followed by 4 h of stirring to yield 12.8 g (53%) of **7** as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.5 (system S_2): ^{31}P NMR (81 MHz, $CDCl_3$) δ 152.25, 149.11; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 1018 (M^+, 22), 763 (19), 445 (25), 303 (100), 289 (29), 273 (27), 256 (54); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{51}H_{62}N_7O_8PSi$ 1018.4664, found 1018.4618. Anal. Calcd for $C_{51}H_{62}N_7O_8PSi$: C, 63.71; H, 6.73; N, 9.63. Found: C, 63.58; H, 6.55; N, 9.35.

N^2-Benzoyl-5′-*O*-(4,4-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine 3′-*O*-(2-Cyanoethyl *N,N*-diisopropylphosphoramidite) (8). Addition of undistilled 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (2; 40 g, 70 mmol) to a solution containing N^2-benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(triisopropylsilyl)guanosine (57.5 g, 68 mmol), THF (500 mL), and DIPEA (80 mL) was followed by 18 h of stirring to yield 38.6 g (54%) of **8** as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.5 (system S_2): ^{31}P NMR (162 MHz, $CDCl_3$) δ 148.90, 146.25; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 1046 (M^+, 38), 791 (16), 401 (10), 303 (100), 256 (13), 201 (12), 133 (27); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{56}H_{72}N_7O_9PSi$ 1046.4977, found 1046.5011. Anal. Calcd for $C_{56}H_{72}N_7O_9PSi$: C, 64.30; H, 6.93; N, 9.37. Found: C, 63.86; H, 6.76; N, 9.34.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(2-Cyanoethyl *N,N*-diethylphosphoramidite) (9). Rapid addition of 2-cyanoethyl *N,N*-diethylchlorophosphoramidite (1; 3.8 g, 18.2 mmol) to a solution containing 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine (10 g, 15.2 mmol), THF (150 mL), and DIPEA (5 mL) was followed by 2 h of stirring to yield 7.7 g (61%) of **9** as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.6 (system S_1): ^{31}P NMR (162 MHz, $CDCl_3$) δ 150.35, 149.47; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 833 (M^+, 13), 760 (15), 721 (16), 529 (100), 513 (12), 438 (10); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{43}H_{57}N_4O_9PSi$ 833.3711, found 833.3716. Anal. Calcd for $C_{43}H_{57}N_4O_9PSi$: C, 62.00; H, 6.90; N, 6.73. Found: C, 61.94; H, 6.69; N, 6.69.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine 3′-*O*-(2-Cyanoethyl *N,N*-diisopropylphosphoramidite) (10). Addition of undistilled 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (2; 60 g, 300 mmol) to a solution containing 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)uridine (45 g, 68 mmol), THF (500 mL), and DIPEA (80 mL) was followed by 18 h of stirring to yield 48 g (82%) of **10** as a white solid after workup and flash chromatography (silica gel, 0.1% pyridine in 1:1 hexane/EtOAc), R_f 0.6 (system S_1): ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.53, 147.25; LRMS (FAB, *p*-nitrobenzyl alcohol), *m/e* (relative intensity) 861 (12), 557 (24), 303 (100), 185 (11), 133 (32); HRMS (FAB, *p*-nitrobenzyl alcohol) calcd for $C_{56}H_{72}N_7O_9PSi$ 861.4024, found 861.4060. Anal. Calcd for $C_{56}H_{72}N_7O_9PSi$: C, 62.78; H, 7.14; N, 6.51. Found: C, 62.82; H, 6.92; N, 6.61.

The solid supports for RNA synthesis are the same as those described in the ref 4, except that the unattached ribose hydroxyl groups are acetylated. The loadings were 25 to 35 μmol per g.

Oligomer Synthesis. All of the syntheses were done with 1 μmol of immobilized nucleoside on the support. The syntheses of RNA oligomers were carried out on a MilliGen/Biosearch Model 8750. A series of 20-, 40-, 60-, and 80-mer uridine homopolymers for use as a PAGE sizing ladder was synthesized on a Milligen/Biosearch Cyclone.

The RNA amidite synthons described above frequently contained small amounts of material that was insoluble in acetonitrile. Solutions of these were filtered through Millipore 0.2-μm disposable filters (no. SLFG025NS). The concentration of amidites was 50 mg/mL in acetonitrile and the amidite consumption was 25 mg per coupling. The synthesis programs were similar to those previously published.[19] Base- and sequence-specific programming allowed different coupling times to be used for RNA synthesis with the diethyl RNA amidites. Coupling times ranged from 16 min for adenosine–adenosine couplings to 5 min for uridine–uridine.

Workup and Analysis of Products. Cleavage and Protecting Group Removal. The CPG from each test synthesis was placed in a screw cap Eppendorf tube and treated with 1.5 mL of a 3:1 solution of 30% aqueous NH_4OH/ethanol at 55 °C for 24 h. The ammonia solution was cooled to −20 °C and transferred to another Eppendorf tube (the CPG was discarded). The ammonia solution was lyophilized, leaving a solid pellet. The pellet was dissolved in 0.4 mL of 1.0 M tetrabutylammonium fluoride in THF and allowed to stand 16 h at room temperature. The reaction was quenched with 0.4 mL of sterile 1.0 M triethylammonium acetate (TEAA) (pH 7).

Sample Desalting. Bio-Rad Low Pressure Econo Columns (0.7 × 20 cm) were filled with a slurry of Sephadex G-25 and sterile aqueous 0.05 M ammonium acetate. The RNA solutions were reduced under vacuum to about 0.5 mL and loaded on to the column. Six 1.0-mL fractions were collected and assayed at 260 nm on a spectrophotometer. The RNA was usually found in fractions 3 and 4. The RNA-containing fractions are lyophilized and stored dry at −20 °C.

Gel Electrophoresis. A 15 or 20% acrylamide/urea gel solution was poured to a thickness of 2 mm and allowed to polymerize for 1 h. The gel slab was pre-electrophoresed for 1 h at 80–90 mA using a tris-borate running buffer. From 1 to 5 OD units (depending upon synthesis length and quality) of dry crude RNA sample was dissolved in 7 μL of formamide loading buffer. The samples were heated at 90 °C for 3 min and rapidly cooled by using a centrifuge. The samples were loaded into 9 × 2 mm wells and the gel run at 50–60 mA for 3–4 h. The RNA bands were visualized with use of UV shadowing.

Reverse Phase HPLC Purification. DMT-positive RNA was cleaved from the CPG, desilylated, and desalted as above. Alternatively, extraction and ethanol precipitation were used[22] to remove the TBAF and salts. Crude 5′-terminal DMT oligoribonucleotides (40–60 OD units) were dissolved in TEAA (0.1 M, pH 7) and injected onto a Vydac C-4 column equilibrated in TEAA (0.1 M, pH 7) in H_2O/CH_3CN (9:1). Gradient elution with CH_3CN (10–50% CH_3CN in 50 min) in TEAA resolved the desired peak, which eluted last, with base-line separation. Detection was at 260 nm. The purified material was collected, lyophylized, and treated with 80% HOAc for 20 min at room temperature to remove the DMT. The sample was concentrated under vacuum, redissolved in TEAA buffer, and injected onto the column. The same buffers were used, and a gradient (10–20% CH_3CN in 40 min) eluted the desired peak.

Strong Anion Exchange HPLC Analysis. 5′-Hydroxy ribonucleotides (0.5–1 OD units) obtained as fraction 4 from the G-25 desalting columns above were injected into a Beckman Ultrasil AX column equilibrated with 20% CH_3CN, 0.003 M phosphate, pH 6.8. Gradient elution with phosphate (0.003–0.32 M in 20 min) eluted the product as well as failure sequences. Detection was at 254 nm.

Enzymatic Hydrolysis of G-20 Homopolymers and HPLC Analysis. The guanosine homopolymer (10 OD units) was treated with 0.1 unit of snake venom phosphodiesterase, 40 units of calf alkaline phosphatase (both from Sigma Co.), and 0.4 mL of 0.1 M TEAA pH 10. The samples were heated at 55 °C for 2 h. The samples were cooled and spun through Millipore ultrafree-MC 10,000 MW exclusion filters to remove the digestion enzymes. The filtrate was lyophilized and the solid dissolved in 0.4 mL of water.

Reverse phase HPLC was used to analyze the samples: from 1 to 3 OD units of the material obtained was injected onto a Waters C-18 column equilibrated with 0.1 M TEAA, pH 7. Gradient elution with acetonitrile (0–15%) over 15 min and 15% for 10 min separated guanosine from N^2-benzoylguanosine. Detection was at 254 nm.

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Preparation of Chemically Misacylated Semisynthetic Nonsense Suppressor tRNAs Employed in Biosynthetic Incorporation of Non-Natural Residues into Proteins

J. D. Bain,† Dean A. Wacker,† Eric E. Kuo,† Matthew H. Lyttle,‡ and A. Richard Chamberlin*,†

Department of Chemistry, University of California, Irvine, California 92717, and Milligen/Biosearch, Division of Millipore, Inc., 81 Digital Drive, Novato, California 94949

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Both run-off transcription and direct chemical synthesis were employed to prepare semisynthetic, nonhypermodified $tRNA^{Gly}$ nonsense suppressors acylated with non-natural residues. L-3-Iodotyrosyl-$tRNA^{Gly}_{CUA}$-dCA (**3**) was prepared by four separate methods, and each of the resultant suppressors was evaluated for biological activity during in vitro translation of mRNA containing a nonsense suppression site (e.g., a UAG termination codon). Direct comparison of the individual translation experiments clearly demonstrates that all four methods yield acylated tRNA products that are biologically equivalent for the their intended purpose: site-specific incorporation of non-natural residues into proteins.

Introduction

Several groups have recently embarked upon a project whose ultimate goal is to develop general techniques for the design and expression of unique proteins containing site-specific non-natural residues.[1,2] The general strategy involves engineering into a gene a termination codon (TAG, TGA, or TAA) at the desired position of incorporation, i.e., a *nonsense mutation site*. Under normal circumstances, translation of mRNA transcribed from this modified gene would result in truncated protein product, since termination codons have no corresponding tRNAs capable of participating in the ribosome-catalyzed process. However, if the translation system is provided with a semisynthetic nonsense suppressor tRNA (i.e., a tRNA whose anticodon is complementary to one of the termination codons) charged with the desired non-natural residue, incorporation of this residue at the specified site in the protein is the result.

The key to success in this project is the development of relatively straightforward procedures for the construction of such nonsense suppressor tRNAs. Based on earlier reports of "chemical misacylation" of wild-type tRNAs,[3,4] it is known that selective acylation of tRNA can be accomplished by T4 RNA ligation of a synthetic 2′(3′)-O-acylated dinucleotide (**2**) and a 3′-truncated tRNA (**1**) lacking the 3′-terminal cytidine and adenosine moieties, as depicted in Scheme I. Building on this theme, we have developed several methods for the synthesis of 2′(3′)-O-acylated nonsense suppressor tRNAs, including the tRNA portion, and have compared the biological activities of the resultant products.

Results and Discussion

A strategy to synthesize acylated nonsense suppressor tRNAs requires that three chemical components be prepared and then joined: (1) a pCpA dinucleotide,[5] (2) an appropriately protected non-natural residue, and (3) a 3′-truncated tRNA ($tRNA\text{-}C_{OH}$), which upon ligation with the acylated dinucleotide creates the intact, chemospecifically acylated tRNA. In addition, a route that yields large quantities of the final product is desirable because the acylated nonsense suppressor tRNA would act stoichiometrically in the translation of mRNAs during the in vitro protein synthesis. In this approach, illustrated retrosynthetically in Scheme II, the initial disconnection of acylated $tRNA^{Gly}_{CUA}$-dCA (**4**) leaves the 3′-truncated tRNA $tRNA^{Gly}_{CUA}\text{-}C_{OH}$ (**1**) and the acylated dinucleotide **5**, which is further simplified to an appropriately protected dinucleotide (**6**) and an N-protected non-natural residue (**7**).

Design Criteria for the Preparation of Chemically Misacylated $tRNA^{Gly}_{CUA}$-dCA. The chemical design of the

† University of California.
‡ Milligen/Biosearch.

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(5) The following nomenclature has been employed throughout this manuscript: pCpA, 5′-*O*-phosphorylcytidylyl(3′-5′)adenosine; pdCpA (or dCA), represents the analogous deoxycytidine derivative 5′-*O*-phosphoryl-2′-deoxycytidylyl(3′-5′)adenosine; $tRNA\text{-}C_{OH}$, signifies a tRNA missing the 3′-terminal cytidine and adenosine moieties; $X\text{-}tRNA^{Y}_{Z}$, designates a tRNA normally aminoacylated with Y in vivo, containing the anticodon Z, and acylated at the 3′-terminus with the residue X (If Z is not present, the anticodon of the wild-type tRNA is employed.) $X\text{-}tRNA^{Y}_{Z}$-dCA, those tRNAs containing a deoxycytidine residue coupled to an adenosine moiety on the 3′-terminus rather than the normal all-ribose sequence.

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