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The synthesis of blocked triplet-phosphoramidites and their use in mutagenesis

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ABSTRACT

A general approach for the synthesis of oligonucleotide-triplet phosphoramidites and the synthesis of four such blocks are described. A strategy was devised to minimize the number of dimer precursors needed for synthesis of a complete set of triplet-amidite blocks encoding all 20 amino acids. Whereas synthesis of 20 triplet-amidite blocks consisting of codon sequences requires 16 dimer blocks, just seven dimer blocks are required to synthesize all required antisense sequences. The antisense sequences are then converted to codons in template mediated replication. Using a mixture of four triplet-amidites and conventional automated solid-phase DNA synthesis, short (6mer) and medium length (30mer) oligonucleotide mixtures were synthesized and analyzed. The latter was replicated *in vitro* and used as a mutagenic cassette to produce four mutants of Asp 221 in the enzyme thymidylate synthase. The method establishes the direction and utility for the production and use of triplet-amidite blocks in DNA synthesis.

INTRODUCTION

Several methods have been reported for the generation of mixtures of mutants of specific residues of recombinant proteins using mixtures of mutagenic oligonucleotides (1–4). To achieve this, a mixture of synthetic oligonucleotides containing one or more degenerate codons at the target site(s) is prepared and used as a cassette or a primer in subsequent mutagenesis experiments. The minimal mixture of oligonucleotides that can be prepared on a conventional synthesizer that contains codons for all 20 natural amino acids is either NNG/C or NNA/T. These mixtures contain 32 codons. A problem encountered in this approach results from the redundancy of codons for many amino acids (i.e. 11 of 20 are redundancies in the minimal codon mixture) and the presence of one stop codon in the minimal oligonucleotide mixture. By biasing the nucleotide amidite mixture used, the content of the library can be modified to exclude one or more codons, but always at the expense of excluding other desired codons (5).

It would be desirable to have a method available by which the content of an oligonucleotide mixture could be controlled to contain only those codons that are desired. Such a method is possible using a mixed resin approach, analogous to that used in peptide synthesis (6,7); however, this requires aliquoting the resin, coupling each aliquot and recombining the resins for each base mixture and would be extremely tedious to apply practically. A method is also available using solution phase oligonucleotide synthesis, where blocked oligonucleotide triplets rather than monomers can be used in condensations (8). Using triplets as the monomeric unit, one can introduce only those codons desired and avoid the problems of amino acid redundancies and stop codons which accompany methods using mononucleotide synthetic units. To combine the utility of triplet building blocks with automated synthesis of oligonucleotides, a method is needed to prepare the appropriately blocked triplet phosphoramidite reagents.

Recently, the use of triplet phosphoramidites for preparation of mutants of a protein has been reported (9). However, the synthesis of the phosphoramidite reagents was not described and the cited commercial source for the reagents is no longer in existence. Furthermore, the yields of oligonucleotide were low and aberrant deletions were present in the final products. While this work was in progress, the synthesis of trinucleotide phosphoramidites representing codons for all 20 amino acids was achieved through only seven dinucleotide intermediates (10). The reported synthesis was in the 3′ to 5′ direction, while the synthesis method utilized here is in the 5′ to 3′ direction.

In the present work we describe the design and synthesis of phosphoramidite triplet codons and their use in the synthesis of oligonucleotide mixtures by conventional methods. One such mixture was cloned as a cassette into a synthetic gene for thymidylate synthase and the resultant plasmids sequenced to verify the authenticity of the mutants.

MATERIALS AND METHODS

General

Fast atom bombardment mass spectra (FAB-MS) were obtained on a JEOL JMS-HX110 at an ionizing voltage of 70 eV. TLC was done on Merck Kieselgel F254 precoated plates (Merck). The silica gel used for column chromatography was YMC gel 60A (70–230 mesh, YMC Co. Ltd). UV absorbance spectra were recorded with a Shimadzu UV-240 spectrophotometer. Blocked

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dinucleoside diphosphates were obtained from Yamasa Co. (Choshi, Japan).

Restriction endonucleases, T4 DNA ligase and polymerase were purchased from New England Biolabs or Life Technologies, Inc. and used as recommended by the manufacturers. dNTPs and *Escherichia coli* strain DH5α were purchased from Life Technologies, Inc. and Magic DNA Clean-up system was from Promega Corporation (Madison, WI). The Thy-*E.coli* x2913recA has been previously described (11). pSCTS9 (*Not*I stuffer) was the synthetic TS gene pSCTS9 (2) containing a non-coding insert between the *Bgl*II and *Avr*II sites that had a *Not*I site for restriction purification. Methods for plasmid purification, subcloning and bacterial transformation were as described (12). DNA sequencing was performed at the Biomolecular Resource Center, UCSF with the Dyedeoxy terminator *Taq* cycle sequencing kit (Applied Biosystems) on an Applied Biosystems 373 automated DNA sequencer.

Synthesis of triplet-amidite blocks

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-benzoyl-2′-deoxyadenylyl-(3′-5′)-N-benzoyl-2′-deoxyadenosine [5].(Structures referred to numerically in brackets are given in Figure 1.) The blocked dinucleoside diphosphate *o*-chlorophenyl ester of *N*-benzoyl-5′-*O*-di-*p*-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-[triethylammonium *N*-benzoyl-2′-deoxyadenosine 3′-(*o*-chlorophenyl phosphate)] (500 mg, 0.35 mmol) was dehydrated by co-evaporation several times with anhydrous pyridine and dissolved in anhydrous pyridine (5 ml). A mixture of N^6-benzoyl-2′-deoxyadenosine (1.2 g, 3.4 mmol) and MSNT (1-mesitylene-2-sulfonyl-3-nitro-1,2,4-triazole) (500 mg, 1.7 mmol) were added to the above solution under argon pressure. The mixture was stirred for 10 min at room temperature, then absolute ethanol (5 ml) was added and the mixture was stirred for 10 min. The solution was concentrated *in vacuo*, the residue was dissolved in $CHCl_3$ and the solution was washed with saturated aqueous $NaHCO_3$. Precipitated N^6-benzoyl-2′-deoxyadenosine (620 mg) was filtered, then the filtrate was dried with anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified on a silica gel column (3 × 11 cm) with $CHCl_3$:methanol (16:1, v/v) as eluent. Fractions were concentrated to give **5** (580 mg, 96%, as a white foam). Compounds **5–10** were analyzed by TLC on Merck Kieselgel in $CHCl_3$: methanol (10:1;v/v). R_f values are given for each compound. R_f of **5** was 0.41 for double development. Exact mass for **5**: Calculated for $C_{84}H_{74}Cl_2N_{15}O_{18}P_2$: 1714.4220. Found by FABMS: 1714.4280.

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-benzoyl-2′-deoxycytidylyl-(3′-5′)-N-isobutylyl-2′-deoxyguanosine [6]. Compound **6** (585 mg, 97.5%, as a white foam) was obtained from the reaction of the *o*-chlorophenyl ester of *N*-benzoyl-5′-*O*-di-*p*-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-[triethylammonium *N*-benzoyl-2′-deoxycytidine 3′-(*o*-chlorophenyl phosphate)] [**2**] (500 mg, 0.34 mmol) with N^2-isobutyryl-2′-deoxyguanosine (1.2 g, 3.4 mmol) and MSNT (500 mg, 1.7 mmol) in pyridine (10 ml) for 10 min at room temperature. The residue was purified on a silica gel column (2.8 × 13 cm) with $CHCl_3$:methanol (16:1, v/v) as eluent. Fractions were concentrated to give **6**. R_f of **6** on TLC was 0.33 for double development. Exact mass: Calculated for $C_{80}H_{76}Cl_2N_{13}O_{20}P_2$: 1672.4120. Found by FABMS: 1672.4210.

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-isobutylyl-2′-deoxyguanylyl-(3′-5′)-N-benzoyl-2′-deoxycytidine [7]. Compound **7** (480 mg, 78%, as a white foam) was obtained from the reaction of the *o*-chlorophenyl ester of *N*-benzoyl-5′-*O*-di-*p*-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-[triethylammonium *N*-isobutylyl-2′-deoxyguanosine 3′-(*o*-chlorophenyl phosphate)] [**3**] (500 mg, 0.36 mmol) with N^4-benzoyl-2′-deoxycytidine (1.2 g, 3.6 mmol) and MSNT (500 mg, 1.7 mmol) in pyridine (10 ml) for 10 min at room temperature. The residue was purified on a silica gel column (2.8 × 10 cm) with $CHCl_3$:methanol (16:1, v/v) as eluent. Fractions were concentrated to give **7**. R_f of **7** on TLC was 0.37. Exact mass: Calculated for $C_{80}H_{76}Cl_2N_{13}O_{20}P_2$: 1672.4120. Found by FABMS: 1672.4080.

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl- 2′-deoxyadenylyl-(3′-5′)-thymidylyl-(3′-5′)-thymidine [8]. Compound **8** (515 mg, 90%, as a white foam) was obtained from the reaction of the *o*-chlorophenyl ester of *N*-benzoyl-5′-*O*-di-*p*-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-[triethylammonium thymidine 3′-(*o*-chlorophenyl phosphate)] [**4**] (500 mg, 0.37 mmol) with thymidine (0.8 g, 3.7 mmol) and MSNT (500 mg, 1.7 mmol) in pyridine (10 ml) for 10 min at room temperature. The residue was purified on a silica gel column (2.8 × 15 cm) with $CHCl_3$:methanol (15:1, v/v) as eluent. Fractions were concentrated to give **8**. R_f of **8** on TLC was 0.44. Exact mass: Calculated for $C_{70}H_{68}Cl_2N_9O_{20}P_2$: 1488.3440. Found by FABMS: 1488.3470.

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-benzoyl-2′-deoxyadenylyl-(3′-5′)-[N-benzoyl-2′-deoxyadenosine 3′-O-(cyanoethyl)(N,N-diisopropylamino) phosphine] [9]. The blocked trinucleoside diphosphate **5** (740 mg, 0.43 mmol) was dehydrated by co-evaporation with anhydrous pyridine several times. The product was dissolved in pyridine:CH_2Cl_2 (1:4, v/v, 10 ml). *N,N*-diisopropylethylamine (150 µl, 0.86 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (144 µl, 0.65 mmol) were added and the solution was stirred at room temperature. After 40 min, the same amounts of the last two reagents were added to the reaction mixture and stirring continued for a further 20 min. $CHCl_3$ and saturated aqueous $NaHCO_3$ were added to the reaction mixture and the organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified on a silica gel column (2.4 × 7 cm) with $CHCl_3$:methanol (20:1, v/v) as eluent. Fractions were concentrated to give **9** (510 mg, 63%, as a foam). R_f of **9** on TLC was 0.29. FAB-MS: *m/z* 1914 (M^++1).

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-benzoyl-2′-deoxycytidylyl-(3′-5′)-[N-isobutylyl-2′-deoxyguanosine 3′-O-(cyanoethyl)(N,N-diisopropylamino)phosphine] [10]. Compound **6** (530 mg, 0.32 mmol) in pyridine:CH_2Cl_2 (1:4, v/v, 10 ml) was stirred with a mixture of *N,N*-diisopropylethylamine (110 µl, 0.64 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (107 µl, 0.48 mmol) for 30 min at room temperature. The same amounts of the last two reagents were added to the reaction mixture and the whole mixture was stirred for a further 30 min. After work-up as described above, the residue was chromatographed over a silica gel column (2.4 × 7 cm) with $CHCl_3$:methanol (20:1, v/v) as eluent. Fractions were concentrated to give **10** (440 mg, 75%, as

a foam). R_f of **10** on TLC was 0.37 for double development. FAB-MS: *m/z* 1872 (M^++1).

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-N-isobutylyl-2′-deoxyguanylyl-(3′-5′)-[N-benzoyl-2′-deoxycytidine 3′-O-(cyanoethyl)(N,N-diisopropylamino) phosphine] [11]. Compound **7** (530 mg, 0.28 mmol) in pyridine:CH_2Cl_2 (1:4, v/v, 10 ml) was stirred with a mixture of *N,N*-diisopropylethylamine (97 µl, 0.56 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (94 µl, 0.42 mmol) for 30 min at room temperature. The same amounts of the last two reagents were added to the reaction mixture after 20 min and the whole mixture was stirred for a further 20 min. After work-up as described above, the residue was chromatographed over a silica gel column (2.4 × 7 cm) with $CHCl_3$:methanol (20:1, v/v) as eluent. Fractions were concentrated to give **11** (420 mg, 79%, as a foam). R_f of **11** on TLC was 0.34. FAB-MS: *m/z* 1872 (M^++1).

o-Chlorophenyl ester of N-benzoyl-5′-O-di-p-methoxytrityl-2′-deoxyadenylyl-(3′-5′)-thymidylyl-(3′-5′)-[thymidine 3′-O-(cyanoethyl)(N,N-diisopropylamino)phosphine] [12]. Compound **8** (650 mg, 0.44 mmol) in pyridine:CH_2Cl_2 (1:4, v/v, 10 ml) was stirred with a mixture of *N,N*-diisopropylethylamine (153 µl, 0.88 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (147 µl, 0.66 mmol) for 30 min at room temperature. After work-up as described above, the residue was purified on a silica gel column (2.4 × 10 cm) with $CHCl_3$:methanol (20:1, v/v) as eluent. Fractions were concentrated to give **12** (500 mg, 68%, as a foam) R_f of **12** on TLC was 0.47 for double development. FAB-MS: *m/z* 1686 (M^++1).

Synthesis of oligonucleotides

Preparation of a mixed triplet-amidite solution. Compounds **9** (48 mg, 0.025 mmol), **10** (72 mg, 0.038 mmol), **11** (47 mg, 0.025 mmol) and **12** (42 mg, 0.025 mmol) were dissolved in acetonitrile:DMF (5:1, v/v, 1.2 ml) to give a mixed triplet-amidite solution **X** which was used for oligonucleotide synthesis.

Synthesis of oligonucleotides. Oligonucleotides (5′-TXAT-3′ and 5′-CACGCCTAGGAAGATXAGCAGATCTGCT-3′) were synthesized on an Applied Biosystems 381A DNA synthesizer by the cyanoethyl phosphoramidite method. Synthesis was at the 1.0 µmol scale and 170 µl of 0.094 M **X** was used in the appropriate coupling step. Coupling efficiency was monitored at each cycle by spectrophotometric measurement of released dimethoxytrityl cation at 500 nm. The coupling yield of **X** was 90% using 30 min for coupling time. A 1 M solution of tetramethylguanidine and pyridine-2-aldoxime in dioxane (1 ml) was added to the oligonucleotide linked to the resin and the mixture was kept at room temperature overnight. Concentrated NH_4OH was added, the mixture was heated at 55°C for 5 h and the released oligonucleotide was chromatographed on a C-18 silica gel column (1 × 10 cm, Waters, Marlboro, MA) with a linear gradient of (0–35%) acetonitrile in 0.1 M TEAA buffer (pH 7.0). Fractions were concentrated and the residue was treated with 80% acetic acid at room temperature for 20 min. The solution was concentrated and the residue was co-evaporated with water. The residue was dissolved in water, the solution was washed with ethyl ether and the aqueous layer was concentrated to give the deprotected oligonucleotide.

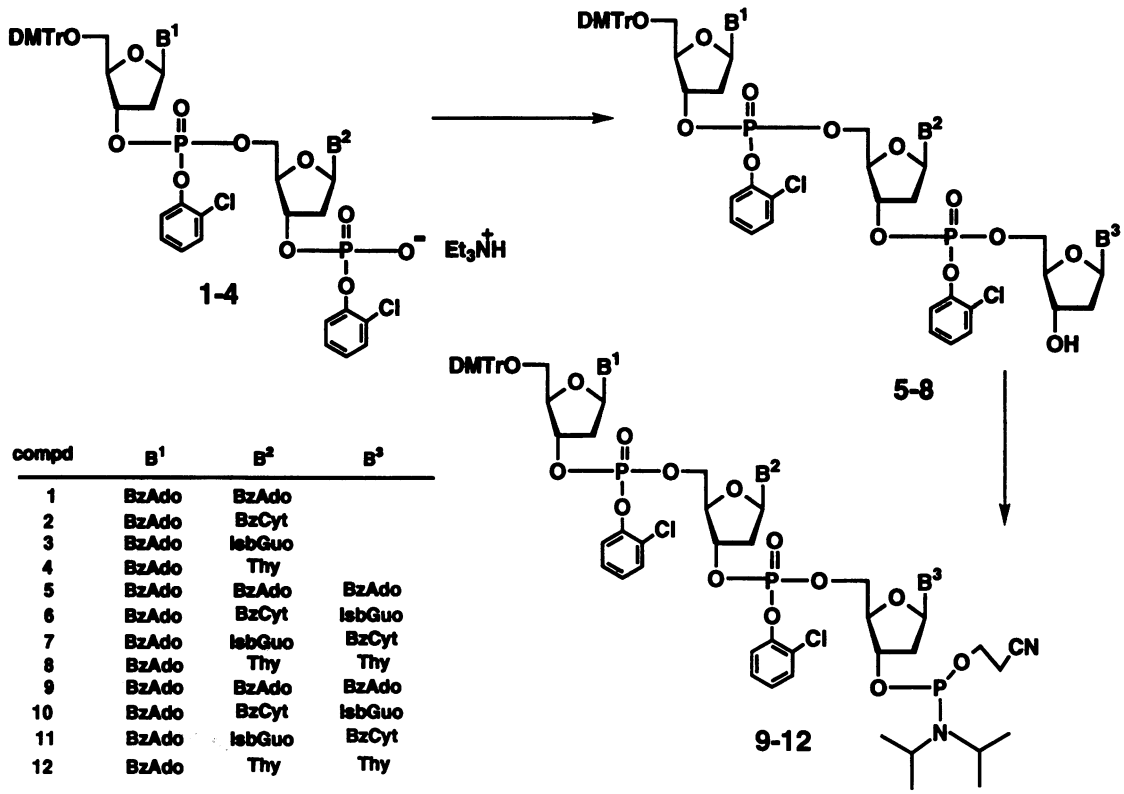
Thymidylate synthase Asp 221 mutagenesis. A solution (430 ml) containing 5 mg oligonucleotide and 100 ml T4 DNA polymerase buffer (250 mM Tris–HCl, pH 8.0, 25 mM $MgCl_2$, 25 mM dithiothreitol and 500 mg/ml bovine serum albumin) was heated at 100°C for 3 min and then allowed to cool to room temperature. Nucleoside triphosphates (2 mM) and T7 DNA polymerase (20 U) were added and the solution was incubated at 37°C for 20 min. The oligonucleotide was extracted with phenol/chloroform, precipitated with ethanol in the presence of tRNA carrier and purified by 20% PAGE. The purified oligonucleotide was ligated to the purified *Avr*II/*Bgl*II large fragment of pSCTS9 (*Not*I stuffer) (Magic DNA clean up; Promega) at a ratio of 2.5:1; the DNA was restriction purified with *Not*I and transformed into *E.coli* DH5α. Plasmid DNA was isolated and digested with *Not*I to destroy the parent vector and *Bgl*II to eliminate multiple cassettes and linearize plasmids (3). After re-ligation, the DNA was transformed into x2913 recA and 335 colonies were isolated on LB plates containing Thy and ampicillin. Colonies were randomly selected for DNA sequencing of plasmids.

RESULTS AND DISCUSSION

Oligonucleotide mixtures as codons for mutagenesis have utility in producing (i) a limited number of selected mutants in one experiment and (ii) all 20 amino acids in a random library. The production of selected mutants can be achieved to some extent by limiting the bases introduced into specific positions of codons; however, conventional methods of introducing mononucleotide mixtures by automated synthesis severely limit the codons which can be made in such mixtures. While mixtures of 32 oligonucleotides (as NNC/G or NNA/T) yield all 20 codons and are simple to incorporate in synthetic oligonucleotides, they have two shortcomings as sources of codons for obtaining peptide sequence diversity. First, each codon mixture contains a stop codon which generates an unnecessary member of the peptide mixture. Moreover, when preparing a combinatorial peptide library, the stop codon of each triplet mixture results in termination of 1/32 of the preceding peptide sequences generated and thus destruction of a like number of complete sequences; the terminations increase with the codons replaced and ultimately prevent achieving all of the theoretically possible members of a combinatorial library. Secondly, the 32 DNA sequences in the mixtures contain 11 redundant codons, which increase the size of the library that must be obtained to assure the presence of each of the unique amino acid members. In a random hexapeptide library, generated, for example, on a coat protein of a phage, the 20^6 different peptides are represented on 32^6 phage particles. It is clear that for the purpose of generating peptides or mutants, it would be desirable to introduce codons as a unit during oligonucleotide synthesis of mixtures. In this manner, one could introduce only those codons desired and avoid stop codons and codon redundancies in the mixture.

Design and synthesis of antisense triplet-amidite blocks

The triplet-amidite blocks synthesized in the present study are shown in Figure 1. The triplet-amidite blocks [**9–12**] consist of the appropriately protected nucleosides linked by the *o*-chlorophenyl phosphotriesters (13) and containing a 3′ cyanoethyl phosphoramidite moiety on the 3′ hydroxyl group (14). The



compd	B¹	B²	B³
1	BzAdo	BzAdo	
2	BzAdo	BzCyt	
3	BzAdo	IsbGuo	
4	BzAdo	Thy	
5	BzAdo	BzAdo	BzAdo
6	BzAdo	BzCyt	IsbGuo
7	BzAdo	IsbGuo	BzCyt
8	BzAdo	Thy	Thy
9	BzAdo	BzAdo	BzAdo
10	BzAdo	BzCyt	IsbGuo
11	BzAdo	IsbGuo	BzCyt
12	BzAdo	Thy	Thy

Figure 1. Structures and synthetic scheme for the triplet-amidite blocks.

o-chlorophenyl group has been widely used in the liquid phase phosphotriester method (13). The *o*-chlorophenyl phosphotriesters are sufficiently stable for the reaction, work up and purification procedures used for liquid phase oligomer synthesis and smoothly deblocked by oximate treatment (15). The cyanoethyl phosphoramidite (14) moiety has been widely used for the solid phase oligonucleotide synthesis. Consequently, advantages of the phosphotriester and the phosphoramidite methods, sufficient stability of the *o*-chlorophenyl phosphotriesters and high coupling efficiency of the phosphoramidite, are incorporated in the triplet-amidite blocks. The blocks should be useful reagents for DNA synthesis performed on a conventional automated DNA synthesizer in combination with the commercially available nucleoside cyanoethyl phosphoramidites.

We anticipated that a time-consuming part of the synthesis of the triplet blocks would be the synthesis of the dimer blocks. Therefore, we designed the base sequences of the 20 triplet-amidite blocks to minimize the number of dimer blocks needed. The approach is based on the fact that the third base of codons is the most degenerate. Thus, a set of antisense sequences can be designed using a fewer number of different bases at the first position (third base of corresponding codon) and hence require a fewer number of dimer blocks than the corresponding codons. The set of codons, antisense sequences and amino acids shown in Table 1 confirms that if one synthesizes 20 triplet-amidite blocks consisting of the codon sequences, 16 dimer blocks (underlined) must be prepared. In contrast, just seven dimer blocks (AA, AG, AT, AC, TT, CC, CA) need be prepared for the synthesis of 20 triplet-amidite blocks for the antisense sequences. We therefore synthesized the antisense oligonucleotide sequences of certain genes; these were converted into sense strands by *in vitro* replication methods. RNA oligomers consisting of sense sequences could also be prepared by *in vitro* transcription (16) of such DNA antisense oligomers. Consequently, triplet-amidite blocks consisting of antisense sequences are not only easily synthesized but are also useful for a variety of biochemical studies. While this work was in progress, a report appeared on the synthesis of triplet amidite blocks via seven amidite dimers, using the strategy described above (10).

In this study, each of four triplet-amidite blocks [**9–12**] were synthesized in three steps starting from the corresponding phosphotriester dimer blocks [**1–4**] as shown in Figure 1. The dimer blocks, synthesized as described by Broka *et al.* (13) were coupled with appropriately protected nucleosides to afford the trimer blocks [**5–8**] in good yields. Although the 3′- and 5′-hydroxyl groups of the nucleosides were not protected, the use of a large excess of nucleosides afforded selective reaction of the 5′-hydroxyl group (primary alcohol) with the activated phosphate groups at the 3′-end of the dimer blocks. The position of coupling of the nucleosides in the trimer blocks was verified by NMR (data not shown). The signals for the 3′-hydroxy groups were clearly present, whereas signals for the 5′-hydroxy groups were not detectable. The results showed that the 5′-hydroxy groups of the nucleosides were selectively coupled with the phosphodiester residues of the dimer blocks. The 3′-hydroxy group of the trimer blocks was then phosphotilated (14) to yield the triplet-amidite blocks [**9–12**] in acceptable yields.

Table 1. Dinucleoside blocks necessary to synthesize a complete set of 20 antisense sequences of the genetic code[a]

DNA sequence of codon (5′–3′)	Amino acid	Antisense sequence (5′–3′)
TTT	Phe	aaa
CTT	Leu	aag
ATT	Ile	aat
GTT	Val	aac
TCT	Ser	aga
CCT	Pro	aga
ACT	Thr	agt
GCT	Ala	agc
TAT	Tyr	ata
CAT	His	atg
AAT	Asn	att
GAT	Asp	atc
TAT	Cys	aca
CGT	Arg	acg
GGT	Gly	acc
CAA	Gln	ttg
AAA	Lys	ttt
GAA	Glu	ttc
TGG	Trp	cca
ATG	Met	cat

[a]The dimers required for synthesis are underscored. In the text, as in this table, codons in capital refer to DNA sequence of codon, while those in lower case refer to the antisense sequence.

Synthesis of oligonucleotides with triplet-amidite blocks

For DNA oligomer synthesis, the four triplet-amidite blocks [**9–12**] were dissolved in acetonitrile:DMF (5:1, v/v) and labeled solution **X**. The blocks did not completely dissolve in acetonitrile, the solvent usually used for monomer amidites. The concentrations of the blocks were 0.02 M for **9**, **11** and **12** and 0.03 M for **10**. The concentration of the ACG block [**10**] was higher than the others since we expected that **10**, containing G at the 3′-end, would be relatively less reactive. The overall coupling yield of the solution **X** was 90%, which is considerably higher than the 1–3% yields previously obtained using solid-phase synthesis with blocks containing methyl phosphotriesters (9). The yield for coupling a T-monomer to the 5′ position of **X** was >99%.

In order to determine coupling efficiencies of the triplet-amidite blocks, a short DNA oligomer mixture, 5′-TXAT-3′, was synthesized. When all four blocks are incorporated into the oligomer, a mixture of four oligomers (TACGAT, TAGCAT, TAAAAT and TATTAT) should be obtained. After deprotection

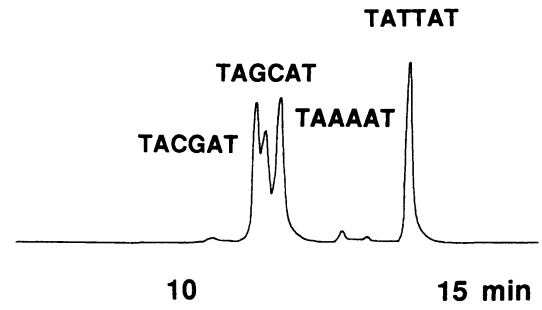


Figure 2. HPLC analysis of TXAT with a C-18 column (Inertsil ODS-2, GL Science, Japan). The oligomers were eluted with a linear gradient from 9.5 to 22.5% acetonitrile in 0.1 M triethylammonium acetate, pH 6.8, over 20 min. Flow rate was 1 ml/min.

and purification, the mixture separated into four HPLC peaks which were assigned by co-elution with authentic samples synthesized using monomer amidites (Fig. 2). The results show that all four blocks were incorporated into the oligomer to afford the desired mixture. The ATT block [**12**] showed the highest and the AAA block [**9**] showed the next highest coupling efficiency as determined by the area of the peaks. The coupling efficiency of the ACG block [**10**] was lower than that of the AAA block, even though the concentration of **10** was higher than those of other blocks. The AGC block showed the lowest coupling efficiency, which was not expected. The results suggested that the coupling efficiency of the blocks varied and depended on the sequence as well as the nucleosides at the 3′-ends.

To test the practicality of using the triplet-amidite blocks in a complete mutagenesis procedure, a 30mer oligonucleotide containing the four triplet blocks was synthesized and used to prepare four Asp 221 mutants of thymidylate synthase. The mutants were chosen (i) to test the relative reactivities of the four 3′ phosphoramidites nucleotide units towards the 5′ hydroxyl of a terminal A of a resin-bound oligonucleotide and (ii) to provide a minimal diversity of side chains which might yield structure-function information about the enzyme. The oligonucleotide mixture was designed to contain 12 self complementary nucleotides at the 5′ end which, when annealed, produced a *Bgl*II site and a sequence near the 3′ end which would produce an *Avr*II site upon production of the opposite strand. The oligonucleotide was annealed through its complementary 5′ ends, extended with DNA polymerase and excised with *Bgl*II and *Avr*II endonucleases to produce the mutagenic cassette. The oligonucleotide duplex was cloned into the large *Bgl*II/*Avr*II fragment of the synthetic thymidylate synthase gene and transformed into *E.coli* DH5α and then x2913 recA. Thirty colonies were randomly chosen and their plasmid DNA was sequenced. The results (Table 2) show that all four triplet blocks were incorporated into the oligomer to afford the desired mixture. Of the plasmids sequenced, 28 had single cassettes possessing the expected mutations, whereas two had multimers of the mutagenic cassette.

Asn(AAT-att) and Phe (TTT-aaa) codons were present in equal numbers. As observed with the model 5′-TXAT-3′ described above, the Ala (GCT-agc) was lower than expected. The Arg (CGT-acg) was higher than expected based on the model synthesis but in accord with the higher yield expected based on the

concentration of triplet-phosphoramidite used. It should be emphasized that we do not know where in the multi-step procedure encompassing synthesis through sequencing the bias arises, or whether in fact there is a bias.

Table 2. Codons obtained by DNA sequencing of 30 random clones of thymidylate synthase Asp 221 mutants

Codon (mutant)[a]	Number[b]	Fraction
AAT (Asn)	9	0.27
GCT (Ala)	3	0.1
CGT (Arg)	12	0.36
TTT (Phe)	9	0.27

[a]Prepared from the mixture of anticodon triplet blocks as described in the text.
[b]All but two clones had a single oligonucleotide insert. One clone had a dimer and one had a trimer; since the multimers must have resulted from enzymatic conversions, their codons are reported in the table.

SUMMARY

We have developed a new method for the synthesis of blocked trinucleotide 3′ phosphoramidites which is suitable for use in solid phase oligonucleotide synthesis. We also developed an approach for the synthesis of oligonucleotide duplexes containing trinucleotide codon blocks which is based on (i) the chemical synthesis of corresponding antisense sequences, (ii) chemical incorporation into a single strand oligonucleotide and (iii) enzymatic synthesis of the sense strand. The use of antisense sequences permits synthesis of all 20 triplet amidite blocks from only seven dinucleotide precursors, a considerable advantage over the 20 dinucleotides necessary for synthesis of blocks with codon sequences. Four trinucleotide antisense blocks were synthesized by the current method and used as a mixture to synthesize corresponding mixtures of the 6mer and 30mer oligonucleotides by conventional solid phase methods. The complementary strand of the 30mer was prepared enzymatically and used to simultaneously convert a single residue of thymidylate synthase to the four desired mutants. Although coupling reactions require more study to ascertain conditions optimal for equimolar incorporation of the triplet blocks, our results clearly establish the directions and feasibility of using such reagents for oligonucleotide synthesis. We believe that the ability to introduce codons as units into oligonucleotides by conventional automated synthesis will be of great utility in creating molecular diversity by mutagenesis.

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