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Hybridization triggered cross-linking of deoxyoligonucleotides

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ABSTRACT

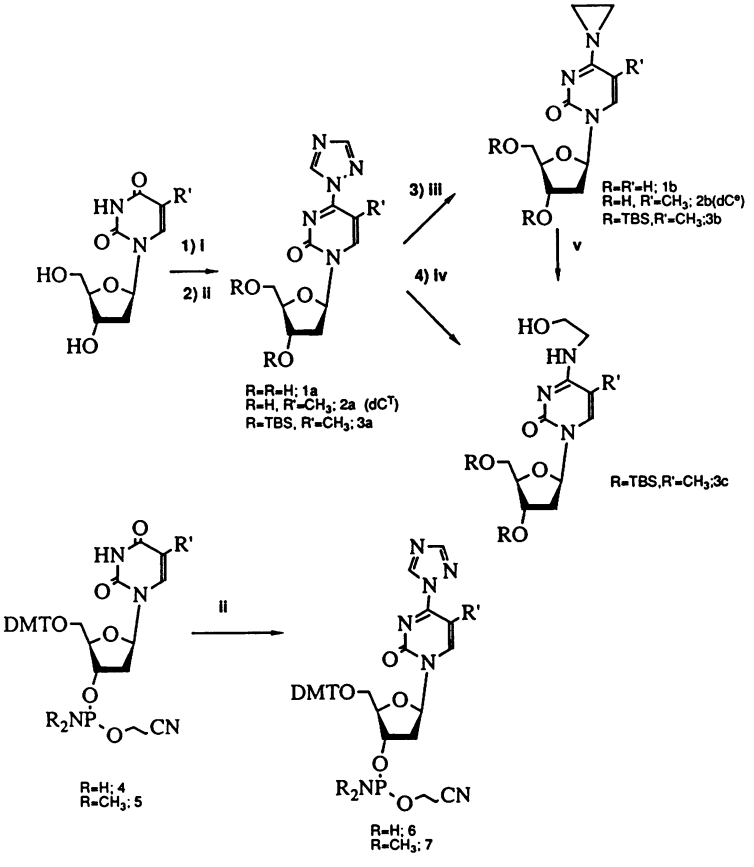
This paper reports details of the synthesis of oligodeoxynucleotides containing the modified base 5-methyl-N^4,N^4-ethanocytosine (C^e). The 9-fluorenylmethoxycarbonyl group is used as a protecting group for the exocyclic amines of dA and dC. This group can be removed rapidly under very mild conditions. Oligomers containing the C^e base form a cross-link when hybridized to their complementary deoxyoligonucleotides. Some of the scope and limitations of these cross-link forming oligonucleotides are reported.

INTRODUCTION

The high specificity of nucleic acid-nucleic acid recognition has prompted significant interest in the design of modified polynucleotides that bind irreversibly to their complementary sequence via a covalent crosslink.[1,2] One major potential application of such modified polynucleotides would be as inhibitors of specific mRNA translation.[3] The reagents which have been used previously to form specific crosslinks with a complementary sequence have suffered from at least two major difficulties: lack of specificity in the reaction with the desired sequence (due to the high reactivity of the alkylating moiety used) or the requirement of a chemical reagent (e.g. $NaBH_4$) to activate the alkylating moiety after hybridization. We have developed a novel procedure that avoids these problems by the synthesis of oligodeoxynucleotides containing the modified base 5-methyl-N^4,N^4-ethanocytosine (C^e)[4]. This base appears to react by virtue of its orientation and proximity to a base on the opposite strand of a double-stranded oligodeoxynucleotide. A preliminary communication of the results with a model system of this concept has appeared.[4] We report here details of the successful expansion of the concept of hybridization triggered alkylation to a system containing four naturally occurring DNA bases.

RESULTS AND DISCUSSION

In order to synthesize oligodeoxynucleotides containing the C^e group we used an amidite containing the 5-methyl cytosine triazole (C^T) base (2a=dC^T) (Scheme 1). This procedure is based on the work of Huynh-Dinh



i) Trimethylsilyldimethylamine or t-butyldimethylsilyl chloride, ii) $POCl_3$/triazole
iii) ethylenimine, iv) 2-aminoethanol, v) H_3O^+

Scheme 1: The Synthesis of Model Monomers;

et al.[5] These workers used an analogous procedure to prepare oligomers containing the 5-methyl cytosine triazole base. These oligomers were worked up with a mixture of oximate and conc. ammonia, to give oligonucleotide probes containing a mixture of thymidine and 5-methyl cytidine. We found that the 2'-deoxyuridine (4) or thymidine (5) amidites could be quantitatively converted to the corresponding C^T amidites 6 or 7 by slight

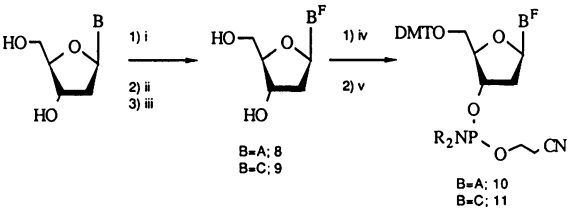
modification of the general procedure of Reese et al.[6] The C^T base is stable to the conditions of the phosphoramidite synthesis of oligomers. When monomers or oligomers containing the C^T group are treated with ethylenimine (aziridene) the C^T group is converted to a C^e group in essentially quantitative yield.[4] This enables one to introduce the acid sensitive C^e group into a synthetic oligodeoxynucleotide after all of the detritylation steps are over (see Scheme 3).

To assess the stability of the C^e group, a number of monomeric C^e derivatives were prepared. The unprotected C^T derivatives could be conveniently prepared by a variant of the transient protection procedure by using N,N-dimethylaminotrimethylsilane to give the salt-free 3',5'-bistrimethylsilylnucleosides.[10] The 3',5'-silyl nucleosides are then treated with Reese's reagent[6] (phosphoryl tristriazolide) in acetonitrile and subjected to aqueous work up. The 3',5'-silyl C^T derivatives are treated with dilute acetic acid giving the unprotected C^T nucleosides (which are isolated by crystallization) in fair yield (see experimental). Triazolides 1a or 2a were converted to the 2-hydroxyethyl-5-methyl-cytidine derivatives 1c or 2c by treatment with 2-aminoethanol. Products 1c and 2c were identical to the products derived from dilute aqueous acid treatment of the corresponding C^e derivatives 2b and 3b, as expected. The 2'-deoxyuridine derivatives 1a, 1b, and 6 were also prepared (Scheme 1) and showed reactivity indistinguishable from the corresponding thymidine derivatives.

The N^4,N^4-ethanocytosine group is relatively unreactive, however, it is not stable to the conditions (i.e. 5 hours at 55°C with conc. NH_4OH) required to deprotect the base exocyclic amine groups, when using the conventional protecting groups. We therefore required a protecting group that would be removed more rapidly and in high yield, under mild conditions. The 4-nitrophenethyloxycarbonyl group is a tempting choice, however, this group is only completely removed after ca. 18 hours by 0.5M 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) in acetonitrile.[7] Compounds 2b and 3b made it possible to assess the stability of the C^e moiety in both organic and aqueous solvents (see Scheme 1). These C^e derivatives were not completely stable to 1M DBU for the extended period of time required to remove the 4-nitrophenethyl group.

We found the 9-fluorenylmethoxycarbonyl (fmoc) group to be more suitable.[8] The fmoc group is removed by a mild base promoted β-elimination reaction. This protecting group has proven useful in peptide

chemistry[9a] and has been used to protect the exocyclic amine functions of several mononucleosides.[9b] Compounds 8 and 9 were prepared using the well-established transient trimethylsilyl (TMS) protection procedure[9b,10] (Scheme 2). Protected nucleosides 8 and 9 were converted to the

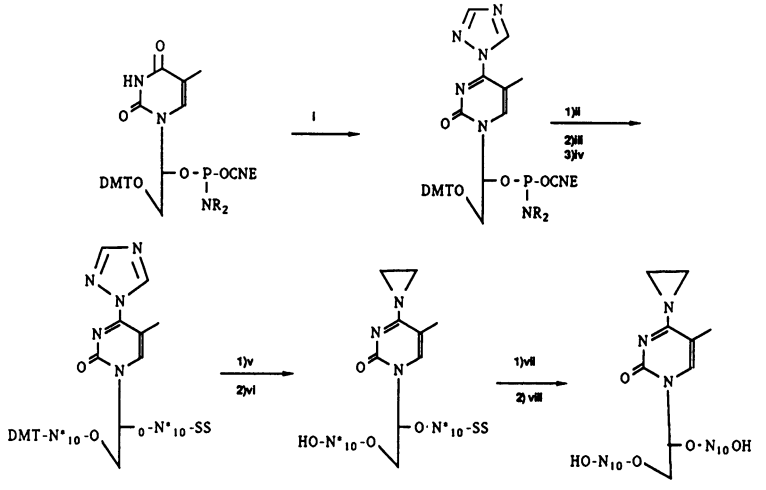


TMS = Trimethylsilyl , F = 9-Fluorenylmethoxycarbonyl , DMT = Dimethoxytrityl,
R = Isopropyl, R' = H or Me
B = Adenine or Cytosine
i = TMSCl/pyridine, ii = FCl , iii = conc. ammonia, iv = DMTCl, v = Diisopropylamino-cyanoethylphosphoramidic chloride, vi = phosphorous oxychloride/ triazole in acetonitrile

Scheme 2: The Synthesis of Fmoc Protected Amidites

corresponding 5'-dimethoxytrityl (DMT) derivatives in the usual manner.[11] The fmoc group is rapidly removed (<5 min at 22°C) from compounds 8 and 9 by 1M DBU in acetonitrile. The carbamates 8 and 9 are slowly converted to the unprotected parent nucleosides dA and dC by triethylamine[9b] in acetonitrile but are stable (no observable reaction by thin-layer chromatography (TLC) over a two hour period) to neat pyridine or 1M diisopropylethyl amine (DIPEA) in acetonitrile. This latter observation is significant since a base like DIPEA is required for conversion of 8 and 9 to their corresponding cyanoethylamidites 10 and 11.[12] Unfortunately, the corresponding 2'-deoxyguanosine derivatives did not give the desired carbamates in useful yield under any conditions investigated. For the purposes of our study, however, it was not necessary to use a guanine-containing oligomer since 2'-deoxyinosine can be substituted for a 2'-deoxyguanosine.[13] Also, the guanine bases can be located in the target oligomers (15-22), which are synthesized using conventional exocyclic amine protecting groups.

The fmoc protected nucleosides are sensitive to the acylation (capping) catalysts[14] (e.g. 4-(dimethylamino)pyridine (DMAP)) normally employed when amidites are used to prepare oligodeoxynucleotides. We found that 5 percent trimethylsilyl chloride in pyridine could be substituted for the usual capping reagent with good results. We prepared oligomers 12 and 13 (figure



DMT stands for 4,4'-dimethoxytrityl, CNE for 2-cyanoethyl, R for isopropyl, N_n stands for an oligodeoxynucleic acid, the subscript n being the number of residues in the oligomer, likewise the * denotes the CNE protecting groups on the oligomer. SS stands for the solid support (silica gel). N stands for deoxyadenosine, deoxycytidine, thymidine , or deoxyinosine. i) Triazole, $POCl_3$ in CH_3CN/ triethylamine. ii) $HO-N_{10}$*-SS/4-nitrophenyltetrazole. iii) I_2/H_2O/lutidine. iv) ten couplings with N amidite (addition of N^*_{10}). v)2.5% dichloroacetic acid in CH_2Cl_2 (detritylation). vi) ethylenimine in H_2O . vii) 1M DBU in CH_3CN. viii) conc. ammonia.

Scheme 3: SYNTHESIS OF THE OLIGOMERS CONTAINING 4,4-ETHANOCYTOSINE;

3) using the commercially available thymidine and deoxyinosine cyanoethyl-N,N-diisopropyl amidites, the triazolide amidite 9[4] and the two fmoc protected amidites 5 and 6. After addition of the required number of residues, the polymer support was treated sequentially with aqueous aziridine, 1M DBU in CH_3CN (ten min.) and then finally conc. NH_4OH for 30 min. to remove the oligomers from the support. When the crude oligomers 12 or 13 were run on a 20 percent denaturing gel they were seen (by u.v. shadowing) to be a single predominant product with the correct mobility.

When the oligomer 13 precursor (on support) containing the central cytidine triazole was treated with conc. ammonia, the oligomer 14, containing a central 5-methylcytosine, was obtained. In order to determine the integrity of oligomers synthesized using fmoc protection, oligomer 14 was tailed with 2'-deoxyadenosine triphosphate using terminal deoxynucleotidyl transferase; the tailed oligomer was hybridized with $(dT)_{18}$ and primer extended with T_4 DNA polymerase and the resulting

blunt-ended double stranded hybrid was ligated into SmaI cut M-13 DNA and sequenced using the dideoxy method.[15] The resulting clones had the correct sequence with the deoxyinosine read as a deoxyguanosine and the 2'-deoxy-5-methylcytidine being read as a 2'-deoxycytidine, as expected.[13]

Target oligomers 15-22 (figure 3) were prepared using conventional exocyclic amine protecting groups and diisopropyl methoxyamidites (see Scheme 3).[14] The syntheses were performed on a Biosearch Sam One DNA synthesizer (see experimental section for synthetic cycle). Oligomer 12 corresponds to the -40 to -30 region of the TPA cDNA insert of the pt-PAtrp12 plasmid.[16] This particular sequence was chosen since cell lines containing this plasmid are available for eventual in vitro mRNA inhibition studies.

Purified oligomers 12 and 13 were allowed to hybridize with the 5'-^{32}P-labeled target oligomers (15-22) to give sixteen separate reactions, containing four distinct contexts (see fig. 1). The time course of the reactions were followed by gel electrophoresis (20 percent denaturing gel) over a period of several days at room temperature. The half-life of the reactions are similar to that observed with the A-T model system (i.e. ~30 hours at 24°C).[4] The crosslink forming reaction shows a dramatic sensitivity to context. The selectivity for cytosine[4] is only partially conserved in the four different contexts; in one case the selectivity is A~C>>T~G (lanes 9-12, fig. 1). All four bases show the ability to form crosslinks to a significant extent in certain contexts. This latter result is in marked contrast to the system containing only A and T, where only X=C and to a much lesser extent X=T showed any significant tendency to form a crosslink.[4]

In several cases (e.g. lanes 1, 9, 10 and 11; fig. 1) two product bands are clearly evident. At least two possible explanations for these results can be proposed: that the bands represent different isomeric linkages or that they represent a crosslink to a neighbor of the "X" base. In the case of the system containing only A and T, the major crosslinked product was shown by Maxam-Gilbert sequencing to be between the C^e base and the C on the opposite strand.[4] In the present case, Maxam-Gilbert sequencing did not give useful information, presumably because of the chemical liability of the modified bases produced. We therefore, cannot determine the exact nature of the crosslink bond from our data.

In order to determine the effects of mismatches around the C^e on the crosslinking reaction we prepared oligomers 23-27 (figure 2). These

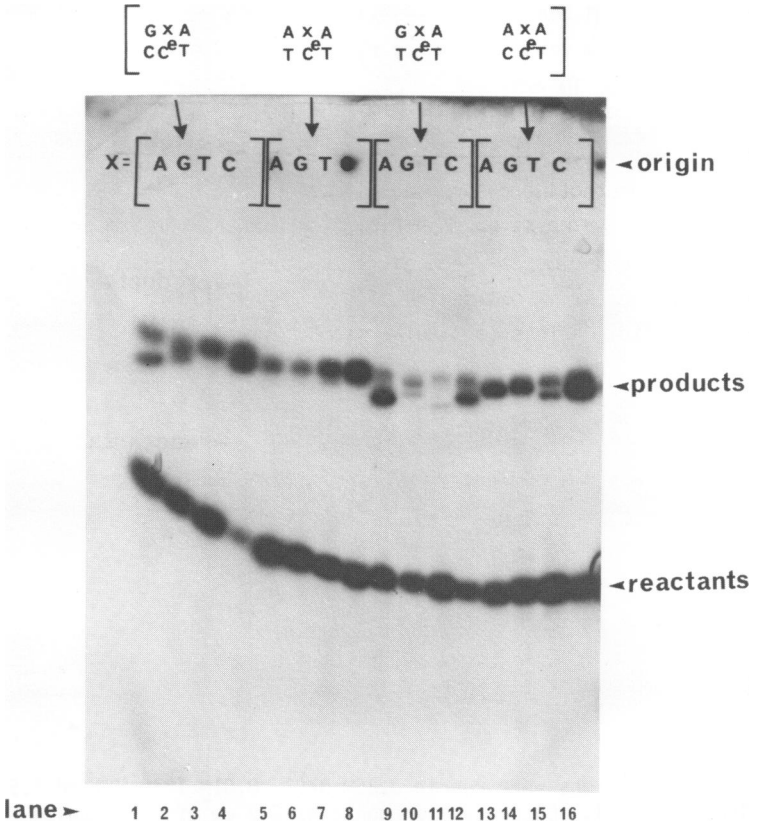


Figure 1. All reactions were run in 0.25M NaCl, 0.01M Tris HCl pH 7.5, in a total volume of 10 µl. Each reaction contains 0.2 µg of ^{32}P-labeled target oligomer (15-22) and 5 µg of the crude C^e containing oligomer (12 or 13). After 119 hours at 22°C, 1 µl of each reaction is diluted with 9 µl of formamide and run on a 20 percent denaturing gel and exposed to give the autoradiogram.

Lane	1:	Reaction of 12 with 15	Lane	5:	Reaction of 13 with 19
	2:	12 with 16		6:	13 with 20
	3:	12 with 17		7:	13 with 21
	4:	12 with 18		8:	13 with 22
	9:	13 with 15		13:	12 with 19
	10:	13 with 16		14:	12 with 20
	11:	13 with 17		15:	12 with 21
	12:	13 with 18		16:	12 with 22

oligomers were allowed to hybridize and react with 12 at 37°C (figure 2). Inspection of figure 2 shows an inverse relationship between the number of mismatches and the amount of crosslinked product produced. The effect is

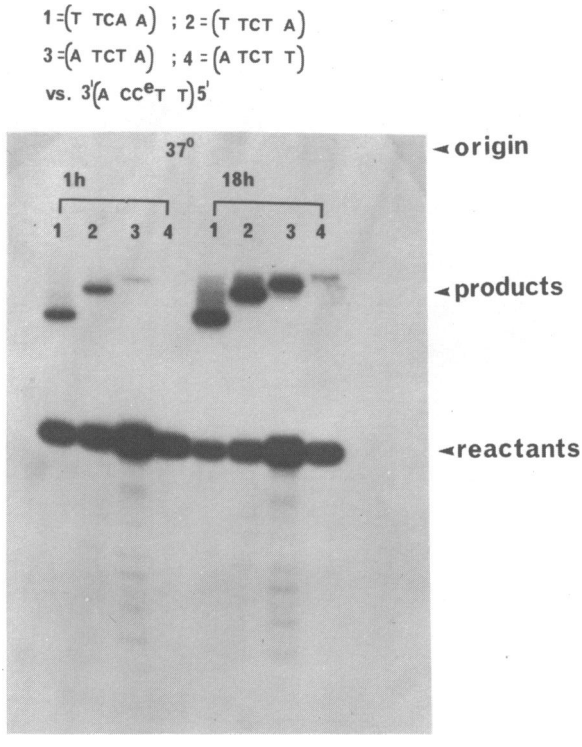


Figure 2. All reactions were run in 0.25M NaCl, 0.01M Tris HCl pH 7.5, in a total volume of 10 µl. Each reaction contains 0.2 µg of ^{32}P-labeled target oligomers (23-26) and 5 µg of crude 12. After each indicated time interval (1 hour or 18 hours) 1 µl of a reaction is diluted with 9 µl of formamide and stored at -20°C. The aliquots are then run on a 20 percent denaturing gel and exposed to give the autoradiogram.

Lane 1: Reaction of 12 with 23 [d(ATCATGGATTCAATGAAGAGA)]
Lane 2: Reaction of 12 with 24 [d(ATCATGGATTCTATGAAGAGA)]
Lane 3: Reaction of 12 with 25 [d(ATCATGGAATCTATGAAGAGA)]
Lane 4: Reaction of 12 with 26 [d(ATCATGGAATCTTTGAAGAGA)]

not as dramatic as might be expected, suggesting that there is still significant interactions between the C^e base and the central C on the opposite strand even when surrounded with mismatches. When this experiment is repeated at 22°C a significant amount of crosslinked product is observed even in the case of oligomer 26 (data not shown). This implies that the duplex 12x26 is mostly melted out at 37°C but hybridized at 22°C. It is also apparent that there is a relationship between the mobility of the crosslinked product and the number of mismatches it contains. It is

Sequences of C^e containing oligomers:

3'd(TAI TAC CTA YZT TAC TTC TCT)5'
Y=C, Z=C^e:12
Y=T, Z=C^e:13
Y=T, Z=5-methyl C:14

Sequences of the target oligomers:

5'd(ATC ATG GAT GXA ATG AAG AGA)3'
X=A:15
X=G:16
X=T:17
X=C:18

5'd(ATC ATG GAT AXA ATG AAG AGA)3'
X=A:19
X=G:20
X=T:21
X=C:22

Figure 3

possible that the crosslinked oligomers are at least partially hybridized even under the strongly denaturing conditions of the gel. One would expect the oligomers that are most correctly matched (e.g. 12x23) to be the most compact (and therefore the most mobile) and vice versa. This observation could explain why the products are resolved by the gel in the case of the presumably isomeric crosslinked oligomers (giving the double bands in lanes 1, 10, 11, 12 and 19, figure 2).

CONCLUSIONS

We have determined some of the scope and limitations of a new class of synthetic deoxyoligonucleotides which react with their corresponding complementary oligomers to form a crosslinked product. We have used the cyanoethyl group[12] for phosphate protection and used the fmoc group for the protection of the exocyclic amine moiety of adenine and cytosine[9b] in oligodeoxynucleotide synthesis. Both groups are rapidly removed by base treatment (1M DBU) of the oligomer on support. We do not recommend the use of fmoc protection for routine oligonucleotide synthesis, because of the lability of the fmoc protected monomers, the low yields in which they are obtained, and the requirement for the use of 2'-deoxyinosine instead of 2'-deoxyguanosine. The fmoc protected deoxynucleoside derivatives 10 and 11, however, may have applications in the synthesis of other sensitive DNA analogs.

Unfortunately, the rate of crosslink formation is much too slow ($t_{1/2}$=30h at room temperature) to use this system as an *in vitro* mRNA inhibitor. Computer modeling on an Evans-Sutherland/PS-300 Frodo system

suggests that a C^e-C base pair does not have the optimum interstrand reach.[17] Rather than concentrate on the N^4,N^4-ethanocytidine as an electrophile, further work should be directed toward the design of a group that would produce a much more significant rate enhancement of the crosslink forming reaction. We are currently pursuing such a course.

MATERIALS AND METHODS

Dichloroacetic acid (DCA), triazole, $POCl_3$, FmocCl, DMTCl, 2-cyanoethanol, acetic anhydride, lutidine, iodine, tetrahydrofuran (THF), 4-dimethylaminopyridine (DMAP), and phosphorous trichloride were purchased from Aldrich Chemical Co. The 5'DMT dT and 5'DMT dI N,N-diisopropyl-β-cyanoethyl amidites were purchased from American Bionuclear. Acetonitrile and pyridine (J.T. Baker Co.) were dried with activated 3Å molecular sieves (Aldrich) overnight before use. N,N-diisopropylamino-2-cyanoethyl-phosphonamidic chloride was prepared according to the published procedure.[12] ^{1}H-NMR and ^{31}P-NMR spectra were obtained using a 80 mHz IBM instrument. ^{1}H-NMR spectra are recorded as ppm (δ) from TMS internal standard, except when D_2O is the solvent an external standard (TMS = 0.0 ppm) is used. ^{31}P spectra are reported as ppm (δ) with an external standard (H_3PO_4 = 0.0 ppm). Elemental analyses were determined at the University of California (Berkeley) Chemical Analytical Services.

Synthetic Cycle for Oligodeoxynucleotides

1) Detritrylation 2.5 min. (2.5 percent dichloroacetic acid/ dichloromethane).
2) Wash 1.0 min. (acetonitrile)
3) Couple 3.75 min. (0.05 M p-nitrophenyltetrazole/0.05M DMTdN amidite in dry acetonitrile) (recycle).
4) Rinse 0.75 min. (0.1M DMAP in THF/lutidine/H_2O; 90:5:5).
5) Oxidation 0.5 min. (0.05 M I_2 in THF/lutidine/H_2O; 95:4:1).
6) Wash 1.0 min. (acetonitrile).
7) Cap 0.75 min. (0.05M DMAP, acetonitrile/acetic anhydride/lutidine; 2:1:1), or 5 percent TMSCl/pyridine when fmoc protection is used.
8) Wash 2.0 min. (acetonitrile).
9) Repeat.

Phosphoryl tristriazolide reagent.

A suspension of 6.4 g of 1,2,4-triazole in 150 ml of dry acetonitrile was cooled in an ice bath and 2.0 ml of $POCl_3$ was slowly added, with good stirring. Triethylamine (15 ml) was slowly added and this mixture was

stirred for 30 min., then 5 mMol of the appropriate thymidine or uridine derivative in ~5 ml acetonitrile is added.

4-(1,2,4-Triazol-1-yl)-2-pyrimidon-1-yl-β-D-2'-deoxyribofuranoside (1a).

A stirred suspension of 2.28 g (10 mMol) of 2'-deoxyuridine in 100 ml of dry acetonitrile was treated with 10 ml (6.24 mMol) of N,N-dimethylaminotrimethylsilane (Petrarch Systems Inc.) under argon. After 30 min. at 22°C the resulting solution was concentrated under high vacuum, and redissolved in 10 ml acetonitrile. This solution was added to the phosphoryl tristriazolide reagent (twofold scale), prepared as above. After stirring for 2 h at 22°C, this mixture was poured into 250 ml of 5 percent sodium bicarbonate and extracted (2x150 ml) with dichloromethane. The combined organic washes were concentrated and dissolved in 100 ml methanol. This solution was treated with 100 ml of 80 percent acetic acid/water (to remove the TMS groups) and stirred for 2h at 22°C. This solution was concentrated to dryness then dissolved in ethanol (~100 ml) and conc. to a small volume. The crystalline product is isolated by filtration and dried under high vacuum overnight. Yield 1.5g (59 percent). Calc. for $C_{11}H_{13}N_5O_4$-1/4 H_2O (m.w. 279.23); C: 46.56, H: 4.79, N: 24.68. Found, C: 46.50, H: 4.71, N: 24.73. ^{1}HNMR (D_2O) δ6.04 (t, 1' H), 16.98 (d, C-5H), 8.07 (s, triazole H), 8.45 (d, C-6H), 9.12 (s, triazole H). No melting was observed on heating to 200°C, only a gradual darkening.

4-(1,2,4-Triazol-1-yl)-5-methyl-2-pyrimidon-1-yl-β-D-2'-deoxyribofuranoside (2a [dC^T])

Using the same procedure as for 1a, 1.6g (55 percent). Calc. for $C_{12}H_{15}N_5O_4$ (m.w. 293.26); C: 46.56, H: 4.79, N: 24.68. Found; 46.50, 4.71, 24.73. ^{1}HNMR δ 6.14 (t, H), 8.18 (s, triazole H), 8.44 (s, C-6H), 9.12 (s, triazole H). No melting was observed on heating to 200°C; a softening at 166-169°C was noted, followed by a gradual darkening.

3',5'-Bis-(t-butyldimethylsilyl)-5-methyl-N^4,N^4-ethano-2'-deoxycytidine (3b) (Compounds 1b and 2b were made by a similar procedure.)

A solution of ~30 percent aqueous aziridine was added to 20 mg of 3',5'(TBS) C^T (2a) in 1 ml dioxane. After 5 minutes the solution was evaporated to dryness, resulting in a near quantitative yield. ^{1}HNMR ($CDCl_3$) δ0.94 (s, 2t-BuSi), δ1.06 (s, C-5:CH_3), δ1.38 (s; N^4,N^4:CH_2-CH_2), δ6.30 (t, 1' H), δ7.76 (s,C-4H). (Peaks corresponding to triazole are also present.)

3',5'-Bis-(t-butyldimethylsilyl)-N^4-(2-hydroxyethyl)-5-methyl-2'-deoxycytidine (3c).

A suspension of 256 mg (0.5 mMol) of 3',5'-$(TBS)_2$ dT^{18} in 2 ml acetonitrile was added to the phosphoryl tristriazolide reagent (0.1 fold scale), prepared as above. This mixture was stirred at 22°C for 2 h. The mixture (containing 3a) was then treated with 200 µl of water, followed by 1 ml of 2-aminoethanol. The resulting solution was allowed to stir for 1 h at 22°C, then poured into 40 ml of 5 percent sodium bicarbonate solution. This mixture was extracted (2x20 ml) with dichloromethane, the combined organic layers were dried (K_2CO_3) and concentrated under reduced pressure. The residue was dissolved in ~20 ml pet. ether and cooled to ~5°C. The resulting crystalline product was isolated by filtration and dried under high vacuum overnight. Yield 217 mg (85 percent based on 3',5'-$(TBS)_2$ dT). ^{1}HNMR ($CDCl_3$) δ 0.91 (d, 2 t-BuSi), 1.94 (s, C-5 CH_3), 6.35 (t, 1'H), 7.53 (s, C-6H).

4-(1,2,4-Triazol-1-yl)-5-methyl-2-pyrimidon-1-yl-β-D-2'-deoxyribofuranoside-3'-O-N,N-diisopropyl-β-cyanoethylphosphoramidites (6 and 7).

A solution of 100 mg of 4 or 5 in 0.5 ml of acetonitrile was added to the phosphoryl tristriazolide reagent (0.05 scale), prepared as above. The reaction could be followed by thin layer chromatography (5 percent methanol/dichloromethane), since the product (7) appears as a bright blue spot under long wave u.v. light (on silica gel 60 plates with 254 nm indicator). After thirty minutes at 22°C, the mixture was poured into 5 percent sodium bicarbonate solution and extracted 2x50 ml. The combined dichloromethane layers were dried ($MgSO_4$) and concentrated. The residue was dissolved in 500 µl of ethyl acetate and added dropwise to cooled (-40°C) well stirred pet. ether, to precipitate the product 6 or 7 in near quantitative yield. ^{31}P NMR (CD_3CN) δ 148.45.

N^6-(9-Fluorenylmethoxycarbonyl)-5'-(4,4'-dimethoxytrityl)-2'-deoxyadenosine (8)

Same procedure as for the synthesis of 9 (10 mMole scale) gave 2.0 g of 8 (26 percent based on dA). ^{1}H NMR ($CDCl_3$) δ 2.34 (s, $-CH_2O$), 3.76 (s, $2CH_3O$), 6.44 (t, 1' H), 6.75-7.9 (m, Ar), 8.08 and 8.68 (2s, adenine H's).

N^4-(9-Fluorenylmethoxycarbonyl)-5'-(4,4'-dimethoxytrityl)-2'-deoxycytidine (9)

A suspension of 2'-deoxycytidine (9.09 g, 40 mMol) in 50 ml dry pyridine, was concentrated to dryness under high vacuum. This was repeated

two more times. The resulting anhydrous dC was suspended in 200 ml dry pyridine and 20 ml TMSCl was added under argon. This mixture was allowed to stir for thirty minutes, then 10.4 g of solid fmocCl (40 mMol) was added. This mixture was stirred for 1h at 22°C, then 10 ml of methanol was added and the mixture was concentrated under high vacuum. The residue was dissolved in 250 ml of methanol and treated with 50 ml of conc. aqueous ammonia. This was stirred for 15 minutes at 22°C, then concentrated to 40 ml and extracted with 100 ml of ethyl acetate. The ethyl acetate phase was extracted (3x50 ml) with water and then treated with 150 ml of ethyl ether to precipitate nearly pure N^4-(9-fluorenylmethoxycarbonyl)-2'-deoxycytidine (fmoc dC). Yield after drying 3.2g (20 percent). This material was used directly in the next step. 2g (4.5 mMol) of this material was concentrated (3x25 ml) from pyridine, then dissolved in 25 ml dry pyridine. This solution was treated with 1.8g of 4,4'-dimethoxytrityl chloride for 2h at 22°C. After this time 10 ml of methanol was added and the solution concentrated under high vacuum. The residue was dissolved in 100 ml dichloromethane and extracted with 100 ml water. The organic phase was dried ($MgSO_4$), concentrated and subjected to flash chromatography (1 to 5 percent methanol/dichloromethane) to give 2.4 g of 9 (72 percent, based on fmoc dC). For storage of these sensitive compounds, we recommend that the products are lyophilized from benzene and stored as powders at -20°C. ^1H ($CDCl_3$) δ 1.57 (s, $-CH_2O$), 3.77 (s, $2CH_3O$), 6.25 (t, 1' H), 6.75-8.25 (m, Ar H's).

N^6-(9-Fluorenylmethoxycarbonyl)-5'-(4,4'dimethoxytrityl)-2'-deoxyadenosine-3'-O-N,N-diisopropyl-β-cyanoethyl phosphoramidite (10)

A solution of 255 mg (0.33 mMol) of 8 in dry acetonitrile (10 ml) was concentrated under reduced pressure, and this process was repeated two more times. The residue was dissolved in 10 ml dry dichloromethane (containing 300 µl diisopropylethylamine (DIPEA) and treated with 150 µl (~0.7 mMol) 2-cyanoethyl-N,N-diisopropylphosphoramidic chloride. This solution was stirred for 2h at 22°C, diluted with 100 ml brine and then poured into 50 ml of 70 percent brine/water containing 200 µl of DIPEA. This mixture was separated and the ethyl acetate phase dried ($MgSO_4$) and concentrated under reduced pressure. This was dissolved in 2 ml of ethyl acetate and added to 20 ml of -40°C pet. ether with good stirring, to precipitate 10. Yield after drying 285 mg (88 percent). ^{31}P NMR ($CDCl_3$) δ 149.2 and 149.4. ^{31}P NMR also showed small amounts of cyanoethyl hydrogen phosphonate, which did not interfere with subsequent reactions.

N^4-(9-Fluorenylmethoxycarbonyl)-5'-(4,4'dimethoxytrityl)-2'-deoxycytidine-3'-O-(N,N-diisopropyl-β-cyanoethyl phosphoramidite) (11)

Same procedure as for 10. ^{31}P NMR ($CDCl_3$) δ 149.0 and 149.6.

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