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Engineering Tethered DNA Molecules by the Convertible Nucleoside Approach

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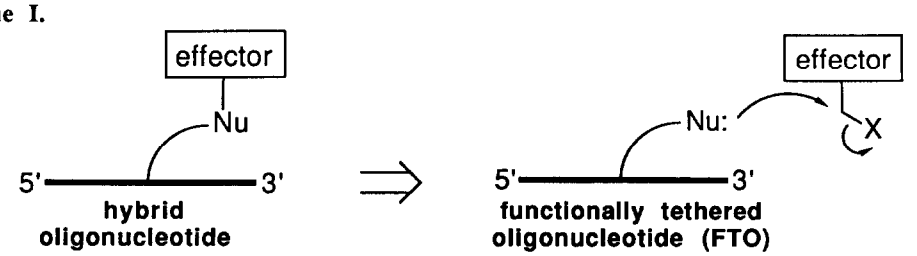
Non-natural functional groups, tethered to DNA, provide a chemical handle for the site-specific attachment of reporter and effector elements. Herein we report a general strategy for the synthesis of oligodeoxynucleotides bearing tethered functionality (functionally tethered oligonucleotides, FTOs). In this approach, the convertible nucleoside 4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine (TMP-dU) is introduced site-specifically into DNA during automated synthesis. Upon treatment with aqueous amines, the TMP-dU moiety undergoes nucleophilic substitution to yield an N^4-alkyl-dC nucleoside — the DNA product is a dC-tethered FTO. Since the tether structure is solely determined by choice of amine used in the deprotection/conversion reaction, this *convertible nucleoside approach* permits a wide variety of FTOs to be synthesized from a single precursor.

Oligodeoxynucleotides[1] bind nucleic acid targets with high affinity and exquisite sequence discrimination, but are essentially devoid of functionality that will report binding or execute chemical modification of the target. The development of efficient chemistry for oligonucleotide synthesis[2] has overcome this limitation by providing an avenue for equipping DNA molecules with non-natural effector elements. Hybrid oligonucleotides bearing attached effectors that range from small hydrocarbons to entire proteins have been designed for use *inter alia* as enzymatic[3] and nonenzymatic[4] restriction endonucleases, detection probes,[5] photoaffinity reagents,[6] and artificial repressors.[7] The most common strategy for generating hybrid oligonucleotides is presented retrosynthetically in Scheme I. An oligonucleotide that has been site-specifically modified to bear a tethered nucleophile (functionally tethered oligonucleotide, FTO) is reacted with a ligand-electrophile, usually an aliphatic amine[8] or thiol.[3,9] Owing to the critical intermediacy of FTOs in the construction of hybrid oligonucleotides, much attention has been directed at developing methods for their synthesis.

Scheme I.



Traditional routes to FTOs: the dedicated monomer strategy. Most current methods for FTO synthesis follow the strategy presented in Figure 1a.[10] A modified monomer is synthesized so as to contain a tethered functional group in protected form, in addition to the functionality required for coupling during automated DNA synthesis. This modified monomer, supplied to the automated synthesizer along with conventional reagents, is incorporated at the desired position during the oligonucleotide synthesis. The resin-bound, fully protected FTO thus obtained is then deprotected (in one or two steps) to yield the final product FTO. We have termed this a *dedicated monomer strategy*, because in it the tether is attached at the level of the monomer, and each monomer can produce only a single tether structure in DNA. Although FTO syntheses using dedicated monomers suffice in many cases, introduction of the tether at such an early stage in the synthesis has several limitations: (i) even a minor change in the structure of the tether, for example homologation by one methylene unit, requires multistep synthesis of a new monomer, followed by synthesis and purification of the new FTO; and perhaps more significantly, (ii) the use of highly functionalized tethers, peptides for example, is virtually prohibited by the need to devise and execute complex protection-deprotection schemes.

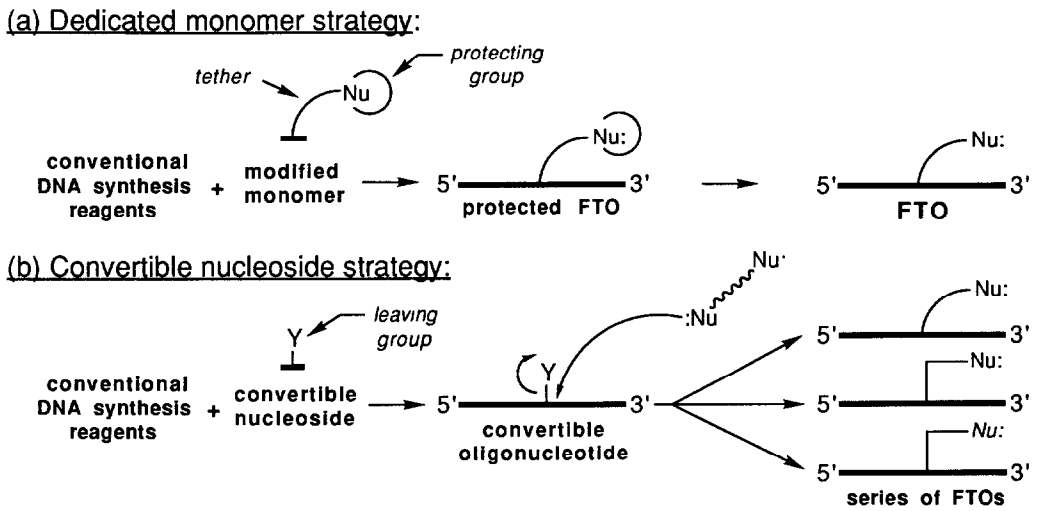


Figure 1. Synthetic strategies for FTO synthesis Y denotes a leaving group that can be displaced by the functionalized tether, resulting in attachment of the tether to DNA. Nu: and *Nu* denote different nucleophiles, for example an amine and a thiol

We have been interested particularly in systems of the type shown in Figure 2, in which a highly functionalized ligand, for example a peptide, antitumor drug, or carbohydrate, is tethered to DNA in order to increase the local concentration of ligand and thereby drive what may be an inherently unfavorable bimolecular association. In designing such systems, molecular modeling may be used to suggest tethers that will provide the most favorable ligand-DNA interaction; nevertheless, the synthetic route should be sufficiently flexible to permit the experimental evaluation of several tether permutations. Considering the dedicated monomer strategy

to be unwieldy for the purposes of our studies, we have developed an alternative approach, the *convertible nucleoside strategy*, in which the functionalized tether is introduced at the final stage of DNA synthesis.

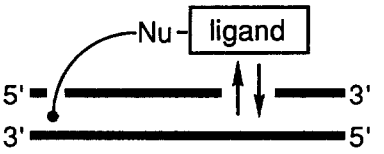


Figure 2. Use of the chelate effect to drive ligand-DNA interactions.

THE *CONVERTIBLE NUCLEOSIDE STRATEGY*: A PROCEDURE FOR INSTALLING THE FUNCTIONALIZED TETHER AT THE *END* OF THE DNA SYNTHESIS. The convertible nucleoside strategy is summarized in Figure 1b. It also employs a modified monomer, in addition to conventional synthesis reagents, but in this case the modified monomer is a nucleoside derivative containing a leaving group, Y, which can be displaced by nucleophiles. Use of this convertible nucleoside in automated synthesis yields a convertible oligonucleotide, which can be reacted with *bifunctional* nucleophiles to yield FTOs — the *bifunctional* nucleophile supplies the tether, one end becoming attached to the DNA and the other available in solution.[11] Since the convertible nucleoside approach involves installing the tether at the final stage of DNA synthesis, it should permit the synthesis of a series of sequence-related FTOs, which differ only in the tether, from one precursor. Furthermore, a single modified DNA building block—the convertible nucleoside — could serve to replace many of the dedicated monomers synthesized to date.

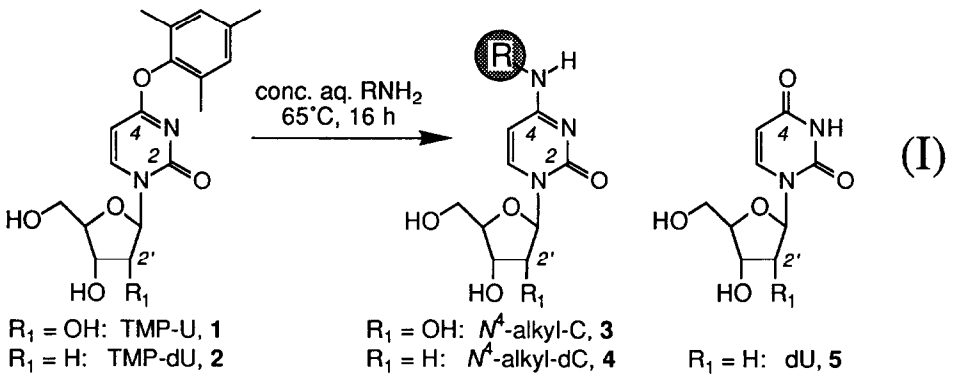


Table I.

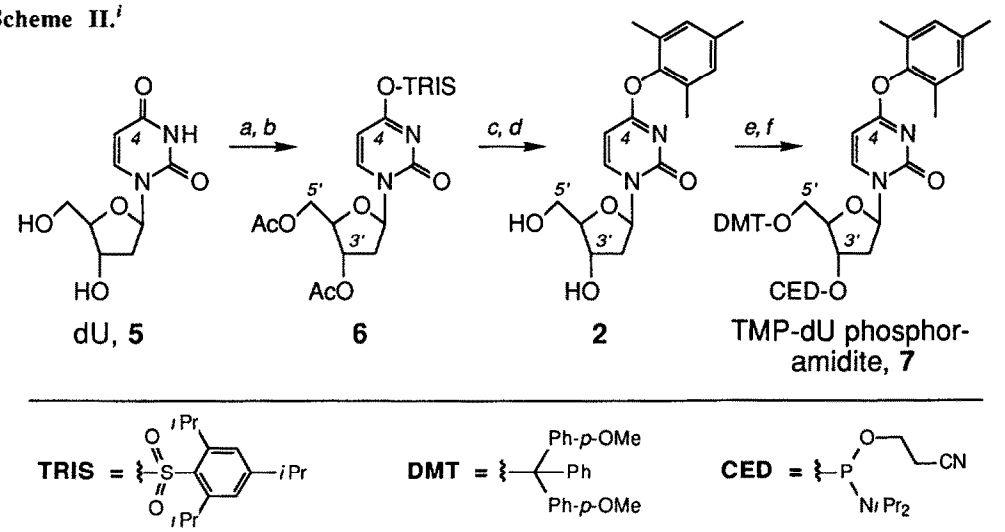
H_2NR	tether (4, R)	conversion (%)
H_2NH	H	98[a]
H_2NCH_3	H_3C	100
$H_2N(CH_2)_4NH_2$	H_2N	96
$H_2NCH_2CO_2H$	HO, O	91

[a] In this case only, the formation of 10% dU (5) was observed.

The following criteria guided our selection of convertible nucleoside: (i) it must be stable to the conditions of automated DNA synthesis; (ii) it must undergo reasonably rapid (< 24 h) and clean conversion to a tethered nucleoside under conditions tolerated by DNA; (iii) it must permit the tether to be attached in a sterically undemanding region of DNA, so that the tether will not significantly perturb DNA structure; and (iv) it should not involve modification of the 5'- or 3'-positions, since attachment of the tether at these positions would not permit access to internal sites in DNA and would preclude certain desirable enzymatic transformations (such as end-labeling and polymerization) at the tethered end.

The choice of a suitable nucleoside derivative was facilitated by earlier work,[14] in which it was demonstrated that aryl ethers of uridine could serve as latent cytidine residues by undergoing clean substitution upon treatment with ammonia [eq. I, **1** → **3** (R = H)]. We were interested in examining whether the corresponding 2'-deoxyuridine (dU) aryl ethers — as in structure **2** — would undergo analogous displacement by *alkylamines* to afford *tethered nucleosides* [eq. I, **2** → **4** (R = alkyl)]. Presumably such a conversion could be carried out in synthetic oligonucleotides to afford FTOs. Since ordinary oligonucleotides can be deprotected by treatment with alkylamines,[15] the tether incorporation and oligonucleotide deprotection reactions could in principle be accomplished in one step from a resin-bound convertible oligonucleotide.

Scheme II.[i]



[i] *a* Ac_2O, pyr, DMAP; *b*. Trisyl-Cl, NEt_3, DMAP, CH_2Cl_2; *c* NMe_3, NEt_3, CH_2Cl_2, 2,4,6-trimethylphenol; *d* NH_3/MeOH; *e*· DMT-Cl, pyr; *f*· CED-Cl, *i* Pr_2NEt, THF *i* Pr = isopropyl

Nucleoside model studies. Before proceeding with studies involving the TMP-dU moiety **2** in DNA, we first carried out nucleoside model studies in which the reaction of **2** with alkylamines was studied; in addition, we examined the stability of **2** to the reaction conditions of automated DNA synthesis. Nucleoside **2** was synthesized in high yield from the known imide sulfonate **6**,[16] using the trimethylamine-mediated

displacement reaction of Gaffney and Jones[17] (Scheme II). Nucleoside **2** was then reacted with several amines under conditions that resemble those used in standard oligonucleotide deprotection,[18] thus affording the substitution products, N^4-alkyl-dC nucleosides **4** (Table I). Although the reaction times in these model aminolyses were brief (2 h) compared to the 10-16 h period used in standard ammonia deprotection, the extent of conversion was high in all cases, which suggested that this reaction would proceed to completion during the extended reaction involving oligonucleotides. Competing hydrolysis of the TMP ether, producing 2'-deoxyuridine (**5**, eq. I), was observed only in the case of ammonia; in all other cases, the conversion of **2** to **4** proceeded cleanly within the detection limit of the HPLC analysis (~1%).

In a separate series of experiments, the convertible nucleoside **2** was exposed to the most vigorous reagent mixtures used in automated DNA synthesis: $CHCl_2CO_2H/CH_3CN$ (detritylation cycle), I_2/pyridine/H_2O/CH_3CN (oxidation), and the reactions were monitored by TLC and ^1H NMR. The convertible nucleoside remained unchanged even after periods of exposure that are significantly longer than those experienced during the longest DNA synthesis.

DEPROTECTION OF OLIGONUCLEOTIDES USING AQUEOUS AMINES. As mentioned above, similarity in the conditions for oligonucleotide deprotection and aminolysis of **2** raised the possibility that these reactions could be carried out in a one-step deprotection/conversion process to yield FTOs directly from a resin-bound convertible nucleoside. However, literature reports[19] suggested a possible problem with this approach: reaction of a protected dC residue (N^4-benzoyl-dC, **8**, Scheme III) with the amine could result in the formation of N^4-alkyl-dC (*conversion*) rather than the desired product, dC (*deprotection*). As an experimental test, we

Scheme III

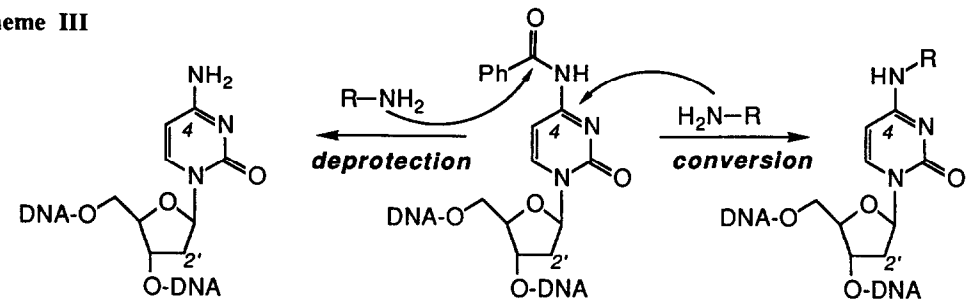


Table II

entry	rel. % dC	R	rel. % N^4-alkyl-dC
a	91	$-CH_3$	9
b	96	$-CH_2CH_2NH_2$	4
c	91	$-CH_2CH_2CH_2CH_2NH_2$	9
d	95	$-CH_2CH_2OH$	5
e	89	$-CH_2CH_2SSCH_2CH_2NH_2$	11
f	80	$-CH_2CO_2H$	20

synthesized the decamer *5'-d(N9G)-3'*[20,21] and reacted the fully protected, resin-bound oligonucleotide with a series of concentrated aqueous amines (Table II) under conditions that approximate those used in the TMP nucleoside conversion experiments. Following removal of the amine solution, the aminolysis products were enzymatically digested to component nucleosides and analyzed by HPLC (nucleoside composition analysis). In all cases, the digests showed the presence of a small but significant amount (4-20% relative to dC, Table II) of the N^4-alkyl-dC nucleoside **4**, in addition to the four naturally occurring nucleosides.[22] No other modified nucleosides were observed in the digests, which indicated that protected dA (N^6-benzoyl-dA) and dG (N^2-isobutyryl-dG) underwent only deprotection and not conversion by amines. Nonetheless, the degree of N^4-alkyl-dC production in these reactions was high enough to render direct deprotection/conversion impractical for many FTO synthesis applications. It is, however, noteworthy that the treatment of resin-bound, protected oligonucleotides with bifunctional amines may offer a simple way to generate DNA bearing a low level of statistically distributed N^4-alkyl-dC residues;[23] we expect that such a procedure could be of considerable value in, for example, footprinting protein-DNA interactions or heterobifunctional crosslinking of protein-DNA complexes.

Since the undesired conversion reaction of N^4-benzoyl-dC is dependent upon the presence of the amide substituent,[22] this problem could be circumvented by removal of the dC protecting groups before treatment of a convertible oligonucleotide with amines. However, the ammonolysis half-lives of the nucleosides N^4-benzoyl-dC (~2 h, r.t.)[24] and TMP-dU (~50 h, r.t.)[25] differ only by a factor of 25, and this window of reactivity is probably insufficient to allow cleavage of the dC protecting groups while leaving TMP-dU completely unaffected. Fortunately, DNA synthesis reagents having substantially reduced half-lives for base-deprotection have been described[24,26] and are commercially available: a dC nucleoside having the alternative N^4-isobutyryl protecting group has an ammonolysis half-life of ~30 min.[24] We thus elected in our FTO synthesis to use such labile protecting groups on all of the DNA bases, so that these could be removed by mild treatment with ammonia prior to conversion of the TMP-dU moiety.[27]

FTO SYNTHESIS USING A dC-CONVERTIBLE NUCLEOSIDE.[29] The convertible nucleoside **2** was elaborated to the corresponding "phosphoramidite" **7** (Scheme II), which is the monomer required for the incorporation of **2** into DNA.[30] Phosphoramidite **7** was then employed in the automated synthesis of a decanucleotide, ***d(GCAAG2TTGC)***.[21] The stepwise coupling yield of the TMP-dU phosphoramidite **7**, as quantified by the released DMT cation, was indistinguishable from the average yield (~96% at the 10 μmol scale) observed for the commercial phosphoramidites[24] used in the rest of the synthesis; thus, the TMP-dU phosphoramidite **7** can be used in automated DNA synthesis without modification of the coupling protocol.

The structure of the resin-bound convertible oligonucleotide ***d(GCAAG2TTGC)*** is illustrated in Scheme IV. In addition to the convertible nucleoside moiety **2**, this fully protected decamer bears a 3'-ester linkage to the resin (solid support), 2-cyanoethyl ester substituents on all the internucleotide phosphate linkages, and amide protecting groups on the exocyclic amines of dG, dC, and dA.[24] Mild treatment of ***d(GCAAG2TTGC)*** with ammonium hydroxide resulted in cleavage of all the non-natural appendages except the TMP-dU moiety, as determined by nucleoside composition analysis. The resulting convertible oligonucleotide, **d(GCAAG2TTGC)**,[21] was reacted separately with a series of amines to yield a family of FTOs having the sequence **d(GCAAG4TTGC)** (Scheme IV).

Scheme IV.

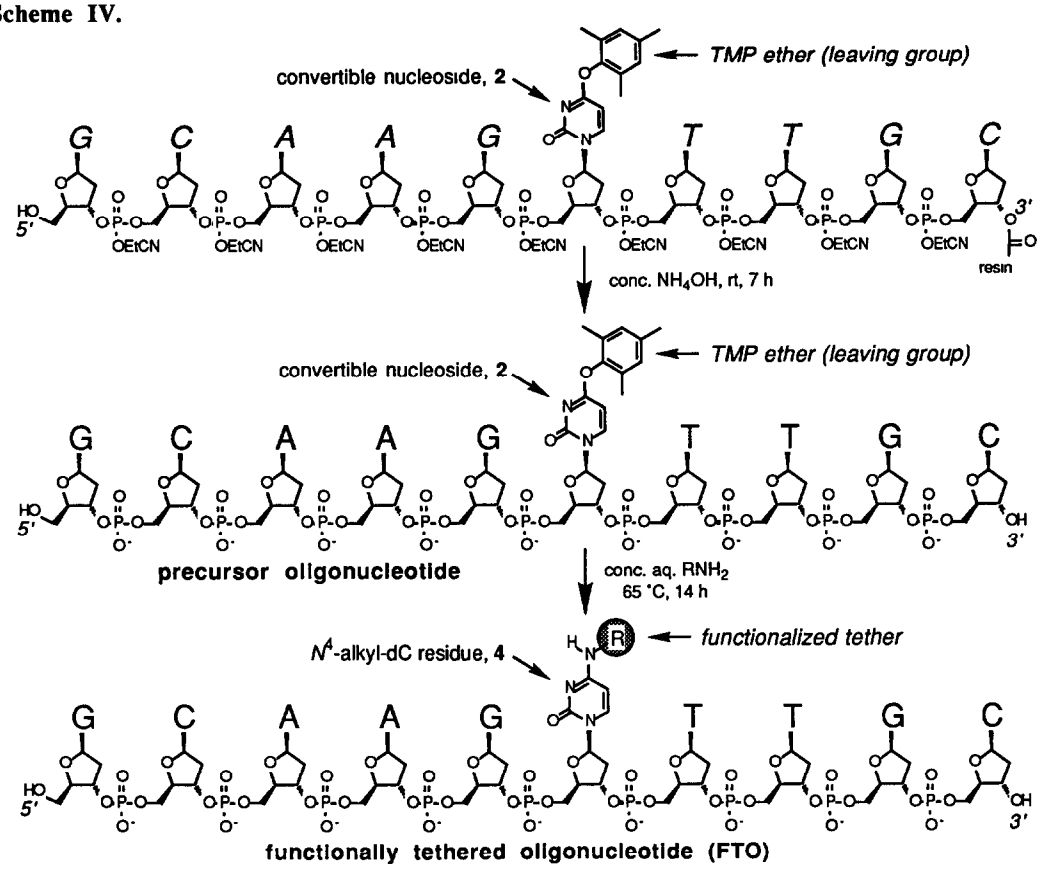


Table III[a]

entry	amine	tether	% conversion	T_m (°C)
a	H_2NH	H (none)	95	51.3
b	H_2N CH$_3$	CH$_3$	100	45.5
c	H_2N NH_2	NH_2	100	44.4
d	H_2N NH_2	NH_2	100	45.3
e	H_2N OH	OH	100	41.0
f	H_2N CO_2H	CO_2H	89	36.7
g	$(H_2N$ S$)_2$	S S NH_2	100	40.9

[a] All reactions were carried out for 14 h at 65°C, but the concentration of amine varied: entry *a*, 14 *M*; *b*, 22 *M*; *c*, 1 *M*; *d-g*, 5 *M*.

The extent of conversion, as determined by anion-exchange FPLC chromatography, was quantitative in all cases except those involving relatively non-nucleophilic amines (Table III, entries a and f); nonetheless, in those cases, the unreacted precursor oligonucleotide could readily be separated from the FTO by FPLC [We are currently evaluating more reactive phenyl ether derivatives for use with such non-nucleophilic amines.] Nucleoside composition analysis of the FTOs revealed the presence of the predicted amount of an N^4-alkyl-dC nucleoside in each of the FTOs. 2'-Deoxyuridine (5) was not present in any of the FTO digest mixtures, indicating that hydrolysis of the TMP-dU was not rate-competitive, even with ammonia. Thus, the convertible nucleoside enjoys an enhanced selectivity for amines when present in DNA.

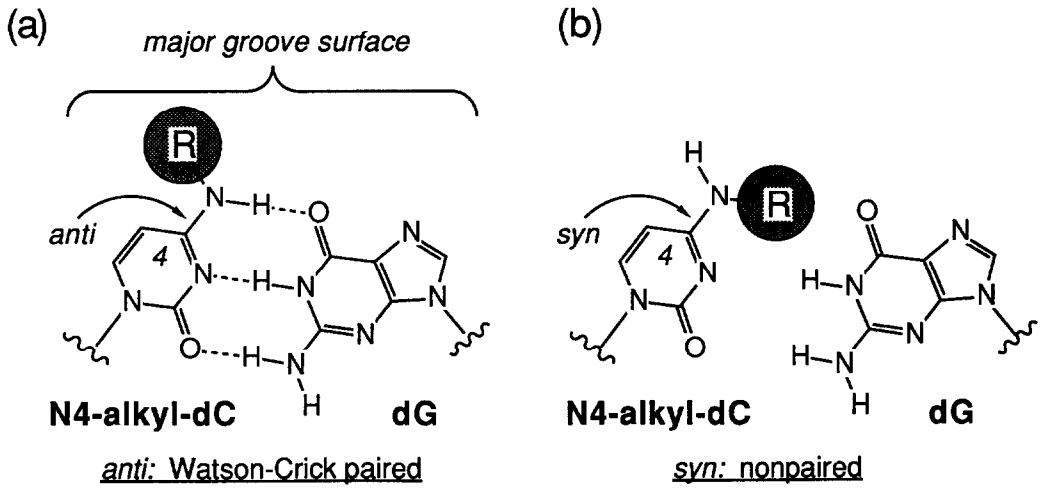


Figure 3. The *anti* rotamer of N^4-alkyl-dC (a) is thermodynamically disfavoured in the free nucleoside, but favoured in DNA because it can Watson-Crick basepair with dG, unlike the *syn* rotamer (b) which disrupts Watson-Crick basepairing.

THE STRUCTURAL EFFECTS OF N^4-ALKYL-dC. Assuming that the tether adopts the non-Watson-Crick paired position on dC residues in DNA, it protrudes directly out into the central space of the major groove (Figure 3), thus occupying one of the least sterically demanding attachment sites available in B-DNA. N^4-Methyl-dC residues are naturally present at high levels (up to 5% relative to dC) in the DNA of some bacteria, so it is reasonable to expect that that this modification is not deleterious to DNA structure. Indeed, an X-ray crystallographic structure of duplex DNA containing the related *N*-alkyl base N^6-methyladenine is almost superimposable on the X-ray derived structure of the non-methylated molecule.[31] While N-alkylated bases do not appear to cause *steric* perturbation of duplex DNA structure, N4-alkyl-dC does weakly destabilize duplex structure as the result of an *electronic* factor: the need to Watson-Crick pair via the thermodynamically disfavored *anti* rotamer about the C4-N^4 bond (Figure 3).[32] The FTOs synthesized in this study are self-complementary and should therefore spontaneously assemble into a duplex DNA molecule in which the tethers are diagonally located on adjacent basepairs:

5'-d(GCAAG4TTGC)-3'

3'-d(CGTT4GAACG)-5'

Formation of duplex DNA in the FTOs was confirmed by their exhibition of discrete, cooperative melting transitions (T_m's, Table III), as well as their exhibition of circular dischroism spectra characteristic of B-form DNA (not shown). The destabilizing effects of N^4-alkyl-dC in the FTOs are surprisingly weak (Table III), especially considering that the tethers are attached at neighboring positions in these molecules. The single case in which duplex stability is strongly perturbed, **d(GCAAG4fTTGC)** (entry f), is likely to arise from charge repulsion between the neighboring carboxylate groups. It is noteworthy that in most common FTO applications a single tether is required per duplex molecule, in which cases the degree of duplex destabilization would presumably be smaller.

CONCLUSIONS. In summary, we report a general strategy for the introduction of tethered functionality into DNA. A single phosphoramidite monomer, supplied to the automated synthesizer, can be used for the site-specific introduction of a wide variety of functional groups into DNA: in this study, amine, disulfide, thiol,[33] alcohol, and carboxyl groups were introduced into DNA from a single precursor. Since the tether is appended to a DNA base, the 5'- and 3'-ends of the molecule are available for enzymatic manipulations. The central location of the tether attachment site in the major groove of B-DNA, as well as the low degree of duplex perturbation, suggests that dC-tethered FTOs will be useful in studies involving DNA-interactive ligands, such as peptides, carbohydrates, and antitumor drugs. Applications of this methodology can be readily envisioned in the investigation of protein-DNA interactions, the creation of novel conformationally locked DNA molecules, and gene-targeted drug delivery.

ACKNOWLEDGMENTS. This research was supported by a DuPont Young Faculty Fellowship and a Camille and Henry Dreyfus Distinguished New Faculty Award, as well as generous gifts from Hoffmann-La Roche, Inc. and ICI Pharmaceuticals Group. We thank members of the Verdine Group for their valuable input, and Dr. Andrew Tyler for MS measurements. The Hewlett-Packard 1090M photodiode array HPLC system was the generous gift of the manufacturer.

EXPERIMENTAL SECTION.

General Experimental.

All solvents were dried prior to use. Methylene chloride, pyridine, and triethylamine were distilled from calcium hydride. Tetrahydrofuran was distilled from benzophenone ketyl and methanol was distilled from magnesium. All reactions were carried out under a nitrogen atmosphere. Flash chromatography was performed using Merck silica gel 60 (230-400 mesh). ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 300 and ^{31}P NMR spectra were recorded on a Bruker WM-300 instrument. UV spectra were measured on the HP 1090M photodiode array spectrophotometer. Mass spectra were measured on a JEOL JMS-AX505H instrument at the Harvard mass spectrometry facility. Analytical and Preparative HPLC were performed on a Hewlett Packard HP1090 instrument. Analytical FPLC (Fast Protein Liquid Chromatography) was performed on a Pharmacia FPLC system.

Synthesis of TMP-dU Phosphoramidite (7).

3', 5'-Diacetyl-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine. 254 mg (0.44 mmol) of 3',5'-diacetyl-4-*O*-(2,4,6-triisopropylbenzenesulfonyl)-2'-deoxyuridine[16] was added to a stirred solution of 700 mg 2,4,6-trimethylphenol (5.14 mmol) in 700 μL CH_2Cl_2. The solution was cooled in an ice bath and anhydrous trimethylamine was bubbled through for 10 min, after which was added 200 μL triethylamine. The solution was allowed to warm to room temperature and, after 2 h, the excess amine and solvent were removed *in vacuo*. Purification of the crude product by flash chromatography (5% EtOAc/toluene followed by EtOAc) yielded 161 mg (0.37 mmol, 85%) 3',5'-diacetyl-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.89 ppm [d (7.4 Hz), 1H, H_6], 6.83 [s, 2, aryl H], 6.25 [dd (5.6, 7.9), 1, $H_{1'}$], 6.10 [d (7.4), 1, H_5], 5.19 [m, 1, $H_{3'}$], 4.32 [m, 3, $H_{4'}$ & $H_{5'}$], 2.76 [ddd (2.0, 5.6, 14.4), 1, $H_{2'a}$], 2.24 [s, 3, p-Ph-CH_3], 2.08 [s, 6, o-Ph-CH_3], 2.07 [m, 1, $H_{2'b}$], 2.06 [s, 6, -$COCH_3$].

^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.81 ppm, 170.12, 169.97, 155.07, 146.78, 142.76, 135.17, 129.45, 129.19, 94.33, 86.99, 82.64, 74.00, 63.57, 38.63, 20.65, 20.54 ($\underline{CH_3}CO$, aryl CH_3), 16.15.

HRMS: found: 453.1656, calc: 453.1638 ($M + Na^+$)

4-*O*-(2,4,6-Trimethylphenyl)-2'-deoxyuridine (2). 164 mg (0.38 mmol) 3',5'-diacetyl-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine was dissolved in 2 mL methanolic ammonia (half saturated) and stirred for 2 h at rt. Following removal of the solvent *in vacuo*, the residue was dissolved in EtOAc and extracted with water (twice back extracted with EtOAc). The combined organic layers were dried over magnesium sulfate, filtered, and concentrated to yield 125 mg (0.36 mmol, 95%) 4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine (2).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.30 [d (7.4 Hz), 1H, H_6], 6.82 [s, 2, aryl H], 6.10 [d (5.8), 1, $H_{1'}$], 6.02 [d (7.4), 1, H_5], 4.41 [m, 1, $H_{3'}$], 4.13 [bs, 1, -OH], 3.93 [m, 1, $H_{4'}$], 3.74 [bs, 3, $H_{5'}$ & -OH], 2.42 [m, 1, $H_{2'a}$], 2.22 [s, 3, *p*-Ph-CH_3], 2.19 [m, 1, $H_{2'b}$], 2.03 [s, 6, o-CH_3].

^{13}C NMR ($CDCl_3$, 75 MHz): δ 171.28, 156.01, 149.42, 146.90, 145.74, 137.25, 135.54, 129.74, 129.41, 87.77, 70.13, 61.40, 41.07, 20.69, 16.26.

HRMS: found: 369.1441, calc: 369.1426 ($M + Na^+$)

5'-*O*-(4,4'-Dimethoxytrityl)-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine. A solution of 80 mg (0.23 mmol) 4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine (2) and 80 mg (0.24 mmol) 4,4'-dimethoxytrityl chloride in 200 μL pyridine was stirred for 15 h at rt. The solvent was removed *in vacuo*, and the residue was subjected to flash chromatography (80% EtOAc/19% Hexanes/1% Et_3N) to yield 140 mg (0.22 mmol, 93%) 5'-*O*-(4,4'-dimethoxytrityl)-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine.

^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.21 [d (7.4 Hz), 1H, H_6], 7.39- 6.81 [m, 15, aryl-H], 6.21 [d (5.8), 1, $H_{1'}$], 5.75 [d (7.4), 1, H_5], 4.51 [m, 1, $H_{3'}$], 4.06 [m, 1, $H_{4'}$], 3.77 [s, 6, -Ph-OCH_3], 3.48 [dd (3.1, 10.7), 1, $H_{5'a}$], 3.39 [dd (3.4, 10.7), 1, $H_{5'b}$], 2.60 [m, 1, $H_{2'a}$], 2.23 [m, 4, $H_{2'b}$ & p-Ph-CH_3], 2.04 [s, 6, o-Ph-CH_3].

^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.93, 158.80, 155.53, 147.04, 144.47, 144.15, 135.46, 130.13, 129.74, 129.36, 128.21, 127.97, 127.08, 113.35, 93.81, 87.02, 86.85, 86.07, 70.85, 62.73, 55.25, 41.94, 16.35.

HRMS: found: 671.2739, calc: 671.2733 ($M + Na^+$)

3'-*O*-(2-Cyanoethyl-N,N'-diisopropylphosphoramido)-5'-*O*-(4,4'-dimethoxytrityl)-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine (TMP-dU phosphoramidite, 7). 160 μL (0.92 mmol) of diisopropylethylamine was added to a solution of 140 mg (0.22 mmol) 5'-*O*-(4,4'-dimethoxytrityl)-4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine in 1.0 mL dry THF. Following the addition of 60 μL (0.27 mmol) 2-cyanoethyl-N,N'-diisopropylchlorophosphoramidite (Aldrich), the solution was stirred for 30 min at rt. The solvent was removed *in vacuo* and the residue purified by flash chromatography (gradient from 50% EtOAc/ 49% hexanes/ 1% Et_3N to 99% EtOAc/ 1% Et_3N). The oily residue thus obtained was dissolved in a minimal amount of EtOAc and triturated with hexanes to yield 110 mg (0.13 mmol, 60%) 7 as a gummy solid (a 50/50 mixture of diastereomers at phosphorus).

^1H NMR ($CDCl_3$, 300 MHz): δ 8.26, 8.18 [d, 2, H_{6i}, H_{6ii}], 7.41-6.80 [m, 30, ArH], 6.25 [m, 2,$H_{1'i}$,$H_{1'ii}$], 5.70 [m, 2, $H_{5'i}$,$H_{5'ii}$], 4.65 [m, 2, $H_{3'i}$,$H_{3'ii}$], 4.15 [bs, 2, $H_{4'i}$,$H_{4'ii}$], 3.78,3.77 [s, 12, -OCH_3], 2.58,2.42 [t, 4, $NCCH_2\underline{CH_2}$-], 2.24 [s,6, p-CH_3], 2.06 [s,12, o-CH_3], 1.18-1.03 [m, 24, -$CH(\underline{CH_3})_2$].

^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.74, 158.64, 155.36, 146.89, 144.24, 143.98, 135.28, 135.22, 135.02, 130.08, 130.03, 129.60, 129.19, 128.13, 127.78, 126.96, 117.32, 117.17, 113.16, 93.72, 93.64, 86.74, 85.44, 85.28, 72.64, 72.41, 71.89, 71.66, 62.34, 62.06, 58.34, 58.26, 58.08, 58.02, 55.09, 43.30, 43.18, 43.12, 40.90, 40.66, 24.52, 24.44, 24.32, 20.59, 20.28, 20.19, 20.10, 20.02, 16.20.

^{31}P NMR ($CDCl_3$, 122 MHz): δ 147.3 ppm, 146.8 (85% H_3PO_4 in H_2O as external standard)

HRMS: found: 849.4030, calc: 849.3992 ($M + H^+$)

Nucleoside Model Studies.

Approximately 2 absorbance units of 4-*O*-(2,4,6-trimethylphenyl)-2'-deoxyuridine or N^4-benzoyl-2'-deoxycytidine were treated with aqueous amine (ammonia, 14 *M*; methylamine, 13 *M*; 1,4 diaminobutane, 7 *M*; glycine, 5 *M*) in a screw capped Eppendorf tube at 65°C for two hours. Aliquots (10 μL) were removed, quenched in 2 *M* KH_2PO_4 buffer (pH 5.5), and analyzed by reverse phase HPLC (see below). Nucleoside standards, **4**, were prepared by treatment of 4-*O*-(2,4,6- trimethylphenyl)-2'-deoxyuridine with aqueous amine at 65°C for four hours. Products were purified by semi-prep reverse phase HPLC and characterized by their UV and ^1H NMR spectra.

Synthesis and Aminolysis of *5'-d(N9G)*.

The oligonucleotide was synthesized on a 10 μmol scale on an Applied Biosystems 381A instrument programmed such that an equimolar delivery of dC, dG, dA, and T amidites to the dG resin occurred for each of nine couplings. The resin was washed with acetonitrile and dried under a stream of argon. Aliquots of the resin representing 100 nmol of theoretical yield were subdivided into screw cap Eppendorf tubes and deprotected with 200 μL of amine as described in the text (5 *M* in amine except for ammonium hyroxide and methylamine for which 1 mL of 14 and 13 *M* solutions were used respectively). After 14 h at 65°C ammonia and methylamine were removed by lyophilization and the other reactions were neutralized with glacial acetic acid. Supernatants were removed, diluted with distilled water, and desalted by centrifugal filtration (Centricon 3, Amicon). This procedure also served to purify the oligonucleotide from benzamide and isobutyramide. Retentates were lyophilized and redissolved in double distilled water.

Synthesis and Aminolysis of 5'-d(GCAAG2TTGC).

The oligonucleotide was synthesized on a 10 μmol scale on the Applied Biosystems 381A Instrument utilizing the optional port for TMP-dU phosphoramidite **7** (0.1 *M* solution in dry CH_3CN). The crude 5'-tritylated oligonucleotide product was treated with conc. ammonium hydroxide for 7 h at rt, then purified on a semi-prep Hamilton PRP-1 column (Solvent A: 0.1 *M* triethylammonium acetate, pH 7.5; Solvent B: acetonitrile; gradient: 20% B to 30% B in 10 min, 30% B for 15 min). Fractions were pooled, lyophilized, and detritylated (3% aqueous acetic acid, 10 min). Approximately 15 nmol (A_{260}) of the precursor oligonucleotide was treated with aqueous amine (concentrations given in Table III) in a screw-capped Eppendorf tube with a total reaction volume of 100 μL (1 mL in the cases of ammonia and methylamine) in a 65°C oven for 14 h. In the cases of ammonia and methylamine, the amine was removed by direct lyophilization; in all other cases, the reaction mixtures were diluted to 1 mL with distilled water and neutralized by the addition of glacial acetic acid. The solutions were desalted by repeated centrifugal filtration (Centricon 3, Millipore). Retentates were lyophilized, resuspended in 50 μL of distilled water, and stored at -20°C.

Nucleoside Composition Analysis.

Oligonucleotide samples (15 nmol) were digested with nuclease P1 (Pharmacia): 10 m*M* $ZnCl_2$, 60 m*M* NaOAc pH 4.0, 55°C, 2 h, snake venom phosphodiesterase (Pharmacia): 10 m*M* $MgCl_2$, 70 m*M* NaCl, 200 m*M* Tris-HCl pH 8.0, rt, 2 h, and alkaline phosphatase (Boehringer Mannheim): 0.1 m*M* $ZnCl_2$, 40 m*M* NaCl, 10 m*M* β-mercaptoethanol, 100 m*M* Tris-HCl, pH 8.0, 37°C, 2 h. Samples were passed through a Millex 22 μ filter, then analyzed by reverse phase HPLC (Beckman Ultrasphere ODS, 4.6 x 250 mm) employing a photodiode array detector (Hewlett Packard LC 1090; solvent A: 0.02 *M* KH_2PO_4, pH 5.6; solvent B: 60:40 MeOH/H_2O; 1.5 mL/min.; elution program: isocratic A for one min, 0-25% B in 10 min, 25-100% B in 5 min, isocratic B for 10 min. Nucleosides were identified by comparison with authentic standards. Blank runs in which no oligonucleotide was added to the digest served as a control for peaks that were introduced along with the protein preparations.

FPLC Analysis.

Samples were analyzed on the FPLC system operating at 254 nm, using a Pharmacia Mono Q 5/5 column under denaturing conditions (solvent A: 0.01 *M* NaOH; Solvent B: 0.01 *M* NaOH, 1.5 *M* NaCl; 0.5 mL/min; isocratic A for 8 min, 0-100% B in 40 min).

REFERENCES AND FOOTNOTES.

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20. N is a mixture of A, C, and G, and T. This degenerate oligonucleotide mixture was chosen to have a broad range of sequence contexts represented in one experiment.
21. Italicized DNA sequences denote the presence of protecting groups and linkage to the solid support; nonitalicized sequences are deprotected and cleaved from the resin. All oligonucleotides shown herein are written in the conventional 5' (left) to 3' (right) orientation.
22. In nucleoside model studies, up to 40% of the conversion product was obtained upon treatment of N^4-benzoyl-dC with aqueous amines, and thus, the reaction is more selective for deprotection over conversion in DNA. Treatment of dC under the same conditions yielded only recovered starting material, indicating that the conversion reaction takes place only with protected dC.
23. Assuming that the ratio of conversion to deprotection is independent of sequence, and taking entry b of Table II as an example, deprotection of an oligonucleotide with ethylenediamine would yield a product in which each of the dC positions actually has 96 % dC and 4 % N^4-(2-aminoethyl)-dC. The yield of oligonucleotide bearing no modified dC residues is 0.96^n, where n is equal to the number of dC residues in the oligonucleotide.
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25. Nucleoside **2** was reacted with conc. NH_4OH at 25°C, and aliquots were removed at regular time intervals. The aliquots were neutralized with HOAc and analyzed by HPLC using the conditions described in the Experimental Section under Nucleoside Composition Analysis. The data points were fit to a pseudo first order rate equation, from which the $T_{1/2}$ was calculated.
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