Nucleic Acids Research, Vol. 20, No. 10 2421–2426

Chemical synthesis of 2′-deoxyoligonucleotides containing 5-fluoro-2′-deoxycytidine

Sabine Schmidt, Claus-Dietmar Pein+, Hans-Joachim Fritz[1,*] and Dieter Cech
Institut für Bioorganische Chemie, Humboldt-Universität zu Berlin, Invalidenstraße 42, O-1040 Berlin and [1]Institut für Molekulare Genetik, Georg-August-Universität Göttingen, Grisebachstraße 8, W-3400 Göttingen, Germany

Received April 8, 1992; Accepted April 22, 1992

ABSTRACT

2′-Deoxyoligonucleotides with 5-fluorocytosine residues incorporated at specific positions of the nucleotide sequence are tools of great potential in the study of the catalytic mechanism by which DNA cytosine methyltransferases methylate the 5-position of DNA cytosine residues in specific sequence contexts. Chemical synthesis of such oligonucleotides is described. Two alternative approaches have been developed, one of which proceeds *via* a fully protected phosphoramidite of 5-fluoro-4-methylmercapto-2′-deoxyuridine 2, the other *via* a fully protected phosphoramidite of 5-fluoro-2′-deoxycytidine 3. Either building block can be used in automated oligonucleotide synthesis applying standard elongation cycles and deprotection procedures exclusively. The methylmercapto function of 2 is replaced by an amino group in the final ammonia treatment used for cleavage from support and base deprotection.

INTRODUCTION

DNA cytosine methyltransferases catalyze the postreplicative methylation of DNA cytosine residues to 5-methylcytosine (1). This reaction is blocked *in vivo* by nucleosides such as 5-aza-2′-deoxycytidine, 5-fluoro-2′-deoxycytidine or their ribo-analogues (2–4). In 1983, Santi *et al.* (5) interpreted the inhibitory effect of these nucleosides by suggesting that the catalytic mechanism of DNA cytosine methyltransferases, like that of thymidylate synthase, may comprise a covalent enzyme/substrate adduct, in which a cysteine SH-function of the catalyst has undergone Michael addition to the 5,6-double bond of the nucleobase to be methylated, thus activating position C-5 as a nucleophilic centre. If this reaction occured with an inhibitor analogue of 2′-deoxycytidine incorporated into DNA, the reaction would irreversibly be blocked at the stage of the covalent adduct as, for example, depicted in Fig. 1.

In principle, this model immediately provided a rationale for the design of mechanism-based inhibitors of DNA cytosine methyltransferases. While *in vitro* crosslinking of enzyme to DNA containing 5-fluoro-2′-deoxycytidine (incorporated by DNA polymerase reaction) could be demonstrated (6 - 8), no sequence-specific inhibitors, *i.e.* chemically synthesized oligonucleotides with 5-fluorocytosine residues at specific positions, have been described to date, despite their great demand and potential as enzymological tools and despite the fact that conventional phosphoramidite methodology has permitted synthesis of oligonucleotides containing a wide variety of other modified monomers, as amply demonstrated by two recent review articles covering that field (9, 10). Previous efforts to incorporate 5-fluoro-2′-deoxycytidine into synthetic oligonucleotides were frustrated by the lability of the modified nucleotide, especially in its base-protected form; for an explicit discussion of earlier failure see Osterman *et al.* (8).

Our interest in the catalytic mechanism of methyl transfer to DNA cytosine residues prompted us to reinvestigate this problem with the results described below. We have developed two alternative approaches to the chemical synthesis of oligonucleotides containing 5-fluoro-2′-deoxycytosine residues at specific postions and have paid particular attention to the problem of suitably protecting or masking the exocyclic function at position 4 of the cytosine ring and to making the final base deprotection step compatible with the pronounced chemical sensitivity of 5-fluoro-2′-deoxycytidine. Furthermore, we strived for establishing an overall procedure that could be implemented on an automated DNA synthesizer.

MATERIALS AND METHODS

5-Fluoro-2′-deoxyuridine, 2,4-bis-(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Lawesson's reagent) and 4-chlorophenylphosphodichloridate were purchased from Sigma. Chloro-(2-cyanoethoxy)-diisopropylamino-phosphine was obtained from Aldrich, tetrazole and concentrated ammonia (32%) from Merck Darmstadt. Phosphodiesterase from *Crotalus durissus* (0.003u/μl) and calf intestine alkaline phosphatase (22u/μl) were purchased from Boehringer Mannheim. All

* To whom correspondence should be addressed

+ Present address: Pharmacia Biosystems GmbH, Munzinger Straße 9, W-7800 Freiburg, Germany

reagents for oligonucleotide synthesis were from Pharmacia. Solvents and reagents were dried as follows: 1,4-dioxan was refluxed with sodium/benzophenone until occurence of a deep purple colour, then distilled; ethanol was refluxed with sodium/phthalic acid diethylester and distilled; triethylamine and N,N-diisopropylethylamine were refluxed with calcium hydride and distilled; pyridine was refluxed and distilled first with ninhydrine, then with P_4O_{10}.

For thin layer chromatography (t.l.c.), glass silica gel plates (2×6cm) containing a 254nm fluorescent indicator (Merck Darmstadt) were used throughout. Column chromatography was performed using silica gel 60 (particle size 0.063−0.2mm) from Merck Darmstadt. Column eluates were monitored by UV absorption at 254nm and analyzed by t.l.c. ^{1}H-NMR spectra were recorded with a Bruker AM 300 spectrometer. ^{19}F-NMR spectroscopy was carried out with a Varian VXR-500S spectrometer. Mass spectrometry was performed using a Hewlett-Packard-5995A GC/MS couple. UV spectra of modified nucleosides were measured in H_2O (pH 7) using a Shimadzu 160 A UV/VIS spectrometer. h.p.l.c. was performed using an LC6A gradient system (Shimadzu). Buffers were as follows. **A**: 50% 0.1M TEAA/ 50% CH_3CN, pH 7; **B**: 98% 0.1M TEAA/ 2% CH_3CN, pH 7; **C**: 0.04M KH_2PO_4, pH 4.2; **D**: 0.04M Tris×HCl, 0.04M $MgCl_2$, pH 8.9 (TEAA: triethylammonium acetate).

5-Fluoro-4-thio-2'-deoxyuridine (6)

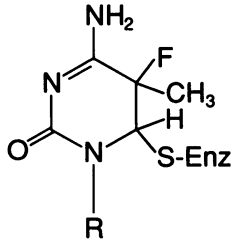
0.45g (1mmol) 3',5'-O-dibenzoyl-5-fluoro-2'-deoxyuridine (4), prepared according to (11), was dried by coevaporation with 3×10ml anhydrous pyridine and suspended in 10ml of dry dioxan/pyridine 4:1 (v/v). Lawesson's reagent (0.37g, 1.2mmol) was added and the mixture refluxed for 2h, at which point t.l.c. ($CHCl_3$/CH_3OH 95:5 v/v) showed complete conversion of the starting material to a faster moving product. The mixture was cooled on ice and 2ml H_2O were added. After standing for one hour, the mixture was evaporated to dryness, dissolved in $CHCl_3$ and extracted first with 5% $NaHCO_3$, then with saturated aqueous NaCl. The organic layer was dried over Na_2SO_4 and evaporated under vacuum. The product was purified by column chromatography using as an eluent $CHCl_3$ with a gradient of 0−5% CH_3OH. The benzoyl groups were removed by treatment with concentrated aqueous ammonia at 50°C overnight. The deprotected product was again purified by column chromatography using as an eluent $CHCl_3$ with a gradient of 0−20% CH_3OH. Yield was 0.23g (90%); the product had the following spectroscopic properties. ^{1}H-NMR (DMSO-d_6): δ[ppm] 12.8 (1H, s, N3H), 8.33 (1H, d, H6), 6.11 (1H, t, H1'), 4.3 (1H, m, H3'), 3.87 (1H, q, H4'), 3.8−3.6 (2H, m, H5',H5''), 5.3−3.4 (2H, m, OH), 2.6−2.2 (2H, m, H2',H2''); MS: m/e 262.1 (M^+, 0.7%), 145.95 (base + H^+, 100%), 117.1 (deoxyribose$^+$, 14.3%); UV absorption: λ_{max}= 336.4nm, λ_{min}= 266.6nm. m.p. 151°C.

5-Fluoro-4-methylmercapto-2'-deoxyuridine (7)

To a solution of 0.26g (1mmol) 5-fluoro-4-thio-2'-deoxyuridine (6) in 25ml 50% aqueous ethanol were added 0.31g (2.2mmol) methyliodide and 1ml of 1N sodium hydroxide. The reaction was followed by t.l.c. and UV spectroscopy. Formation of the methylmercaptoderivate is indicated by appearance of two absorbance maxima at 318 and 274nm. After completion of the reaction, the solution was adjusted with acetic acid to pH 6 and evaporated under vacuum. The product was purified by column chromatography using as an eluent $CHCl_3$ with a gradient of 0−15% CH_3OH. Evaporation of the product-containing fractions left behind a white foam. Yield was 0.23g (84%). The product had the following spectroscopic properties. ^{1}H-NMR (DMSO-d_6): δ[ppm] 8.5 (1H, d, H6), 6.07 (1H, t, H1'), 5.3 (2H, m, OH), 4.31−4.25 (1H, m, H3'), 3.9 (1H, q, H4'), 3.8−3.6 (2H, m, H5',H5''), 2.3−2.15 (2H, m, H2',H2''); MS: m/e 276 (M^+, 1.17%), 160 (base + H^+, 100%), 117 (deoxyribose$^+$, 14.02%); UV absorption: λ_{max1}= 318 nm, λ_{max2}= 274 nm, λ_{min1}= 293 nm, λ_{min2}= 245 nm. m.p. 141°C.

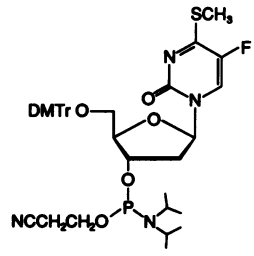
5-Fluoro-2'-deoxycytidine

5-Fluoro-2'-deoxycytidine was prepared according to (12): 0.35g (1.25mmol) p-chlorophenylphosphodichloridate was added with cooling on ice to a solution of 0.33g (1mmol) 3',5'-O-diacetyl-5-fluoro-2'-deoxyuridine (13) and 0.31g (5mmol) 1H-tetrazole in anhydrous pyridine (10ml). The solution was stirred at room temperature for 5h and the reaction was stopped with methanol. The mixture was evaporated to dryness *in vacuo*. The residue was partitioned between 2% aqueous $NaHCO_3$ and $CHCl_3$. The organic layer was washed with water, dried over Na_2SO_4 and evaporated *in vacuo* to give the 4-tetrazolo-derivate of 3',5'-O-diacetyl-5-fluoro-2'-deoxyuridine. The crude product was taken up in 20% aqueous ammonia (1.5ml) and dioxan (8ml) and stirred at room temperature for 1h. The mixture was extracted with $CHCl_3$ (8ml). The organic layer was washed with water and the solvent evaporated *in vacuo*. After removing the O-acetyl

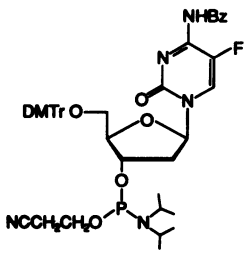


1

Figure 1: Presumed covalent adduct of DNA cytosine methyltransferase to DNA 5-fluorocytosine residue. 'R' denotes the DNA backbone to which the modified nucleobase is attached, 'Enz' the DNA cytosine methyltransfease enzyme.



2



3

Figure 2: Phosphoramidite building blocks.

groups with concentrated aqueous ammonia at 50°C for 12h, the residue was purified by column chromatography on silica gel using as an eluent $CHCl_3$ with a gradient of 0–15% CH_3OH to give 5-fluoro-2'-deoxycytidine. Yield was 0.14g (56%). The product had the following spectroscopic properties. ^{1}H-NMR (DMSO-d_6): δ[ppm] 8.09 (1H, d, H6), 7.6 (2H, s, NH_2), 6.09 (1H, t, H1'), 5.22–5.09 (2H, m, OH), 4.22–4.19 (1H, m, H3'), 3.76 (1H, q, H4'), 3.64–3.5 (2H, m, H5',H5''), 2.14–1.91 (2H, m, H2',H2''); MS: m/e 245.05 (M^+, 6.88%), 130 (base + $2H^+$, 100%), 117.05 (deoxyribose$^+$, 34.02%); UV absorption: λ_{max}= 281.4 nm, λ_{min}= 258.6 nm. m.p. 203°C [195–196.5°C (14)].

N^4-benzoyl-5-fluoro-2'-deoxycytidine

0.25g (1mmol) 5-Fluoro-2'-deoxycytidine were evaporated twice from dry pyridine and dissolved in absolute ethanol (10ml). 2.74g (12mmol) Benzoic anhydride were added and the mixture was stirred at room temperature for 15h. After completion of the reaction (controlled by t.l.c.), the solvent was removed *in vacuo*. The residue was washed with diethylether (2×5ml). Yield of pure N^4-benzoyl-5-fluoro-2'-deoxycytidine was 0.28g (80%). The product had the following spectroscopic properties. ^{1}H-NMR (DMSO-d_6): δ[ppm] 8.6 (1H, s, NH), 8.5 (1H, d, H6), 8.0–7.5 (5H, m, benzoyl), 6.1 (1H, t, H1'), 4.46–4.2 (1H, m, H3'), 3.96–3.83 (1H, q, H4'), 3.69–3.56 (2H, m, H5',H5''), 2.3–2.1 (2H, m, H2',H2''); MS m/e 247 (M-benzoyl+$2H^+$, 2.21%), 129 (base-benzoyl+$2H^+$, 16.14%), 128 (base-benzoyl+H^+, 42.19%), 117 (deoxyribose-benzoyl$^+$, 2.21%), 105 (benzoyl$^+$, 100%).

Phosphoramidite building blocks

The 5'-O-dimethoxytritylated phosphoramidites **2** and **3** of the modified nucleosides were prepared using chloro-(2-cyanoethoxy)-diisopropylamino-phosphine according to standard procedures (15, 16).

Oligonucleotide synthesis

Oligonucleotides were synthesized by the phosphoramidite method (17) with 2-cyanoethyl as the phosphate protecting group (16). An automated DNA synthesizer (Gene Assembler Plus, Pharmacia) was used for chain assembly at a 0.2μmol scale. With the methylmercapto building block **2**, benzoyl was used as the base protecting group on adenine and cytosine residues, isobutyryl on guanine. With building block **3**, phenoxyacetyl was used as the base protecting group on adenine and guanine, isobutyryl on cytosine. Modified phosphoramidite building blocks were dissolved in dry acetonitrile at a concentration of 0.1M and filtered trough 0.45μm teflon filters prior to use. Synthesis was performed 'trityl on'; deblocking was performed using 32% aqueous ammonia at 50°C overnight for oligonucleotides synthesized with building block **2** and at room temperature overnight for oligonucleotides synthesized with building block **3**.

Oligonucleotide purification

Dimethoxytrityl-containing oligonucleotides were purified by h.p.l.c. using an RP 18 column (Macherey and Nagel 250×13mm, 3ml/min) with buffer **A** and a linear gradient of 10% to 50% buffer **B** over 40min. Oligonucleotides containing a dimethoxytrityl group eluted after ca. 38min. Product-containing fractions were evaporated to dryness *in vacuo*, redissolved in 80% acetic acid (200μl) and allowed to react at room temperature for 40min. Acetic acid was removed by evaporation *in vacuo*, followed by coevaporation with water (2×100μl). The resulting product was dissolved in 200μl water and extracted with ethylacetate (2×100μl). The aqueous layer was evaporated to dryness and the oligomers stored at −20°C. Yield was about 7 O.D.$_{254}$ units per oligonucleotide.

Nucleoside composition analysis

0.2 O.D.$_{254}$ units of each oligonucleotide analyzed was dissolved in 6μl of buffer **D**. Snake venom phosphodiesterase

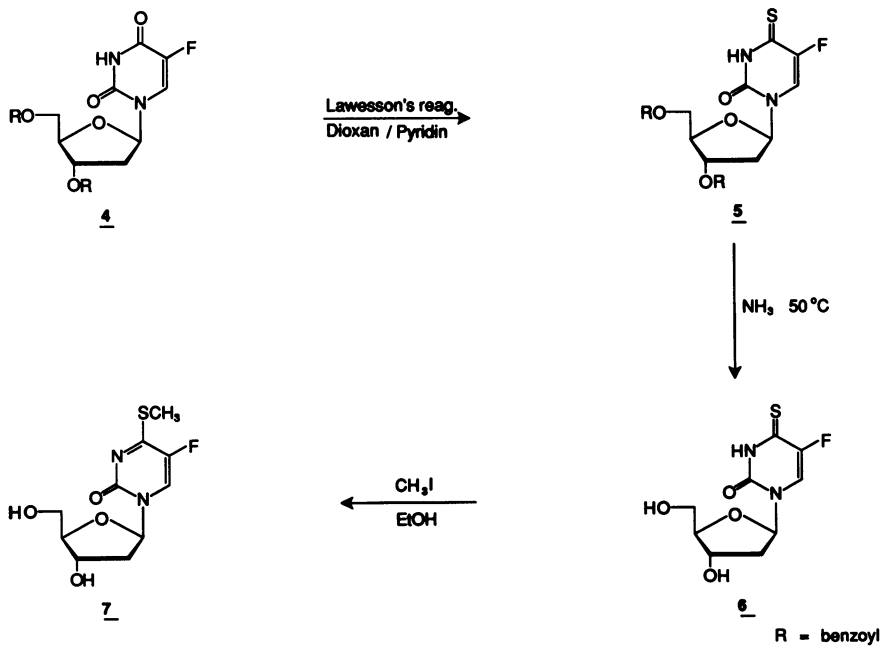


Figure 3: Synthesis of 5-fluoro-4-methylmercapto-2'-deoxyuridine.

(3μl) and alkaline phosphatase (1μl) were added and the mixture incubated at 37°C for 3h. The reaction mixtures were analyzed by h.p.l.c. using an RP 18 column (125×8mm, 1ml/min) with buffer C and a gradient of acetonitrile according to the following equation.

$$P_{AN}(t) = P_{AN1} + (P_{AN2} - P_{AN1}) \frac{e^{2t/T}-1}{e^2-1}$$

T = 10min, P_{AN1} = 0% CH_3CN, P_{AN2} = 13% CH_3CN

RESULTS AND DISCUSSION

In order to make the putative covalent intermediate illustrated in Figure 1 amenable to structural investigations, we have chemically synthesized the following natural and modified oligonucleotides.

I 5'-CGATTCCTGGGATAC-3'
II 3'-GCTAAGGACCCTATG-5'
III 5'-CGATTCFTGGGATAC-3'
IV 3'-GCTAAGGAFCCTATG-5'
V 5'-CGATTFCTGGGATAC-3'
VI 3'-GCTAAGGACFCTATG-5'

F = 2'-deoxy-5-fluorocytidine

Oligonucleotide duplex I/II contains the substrate sequence (18) of Dcm DNA cytosine methyltransferase of *E. coli* K-12 ($CC^A/_TGG$). Duplex III/IV is derived from I/II by replacing the inner cytosine residues of the Dcm cognate sequence by 5-fluorocytosine. Likewise, duplex V/VI is derived from I/II by the corresponding replacement of the outer cytosine residues. The entire set of three duplexes can thus be used to investigate possible formation of adducts such as **1** (Figure 1), including tests of sequence specificity of the crosslinking reaction.

We set out to explore two alternative routes to the synthesis of oligonucleotides III–VI. On the one hand, the direct approach *via* a phosphoramidite building block **3**, derived from N^4-benzoyl-5-fluoro-2'-deoxycytidine was reinvestigated despite earlier failure with this approach, which was attributed to extraordinary chemical sensitivity of the protected nucleotide residue under conditions of the synthesis (8). For the development of a possible backup scheme, on the other hand, we reasoned that a 4-methylmercapto-function may provide chemical stability during chain assembly while easily being replaced by an amino group during the ammonia treatment used for deprotection and cleavage of the oligonucleotide from the polymer support.

Parallel investigation of both schemes required synthesis of the two phosphoramidite building blocks **2** and **3** illustrated in Figure 2.

Figure 3 illustrates the sequence of reactions, by which 4-methylmercapto-derivative **7**, the direct synthetic precursor of building block **2**, was prepared starting from 3',5'-O-dibenzoyl-5-fluoro-2'-deoxyuridine **4**. Conversion of **4** to 3',5'-O-dibenzoyl-5-fluoro-4-thio-2'-deoxyuridine **5** by Lawesson's reagent (19) proved superior to the method of Fox *et al.* (20). O-deacylated 5-fluoro-4-thio-2'-deoxyuridine **6** can smoothly be methylated to **7**. Overall yield was very satisfactory.

Phosphoramidite building block **2** (Figure 2), prepared from **7** by standard procedures (15, 16), was used for the synthesis of oligonucleotides III and IV without any change in the synthesis protocol of the DNA synthesizer. Presence of the 5-fluorocytosine residue in the synthetic DNA was demonstrated by nucleoside composition analysis (see below).

The key intermediate in the direct approach is 5-fluoro-2'-deoxycytidine, which can be synthesized from 5-fluoro-2'-deoxyuridine without difficulties following procedures developed by Sung *et al.* (21) and in this laboratory (12). To explore use of this compound as a starting material in oligonucleotide synthesis, we turned our attention to two problems which may have precluded success with similar attempts in the past (8): first the preparation of a fully protected phosphoramidite building block **3** and, second, conditions of the chain elongation cycle and oligonucleotide deprotection.

The major problem in the development of a suitable phosphoramidite building block was protection of the exocyclic amino function of the 5-fluorocytosine ring.

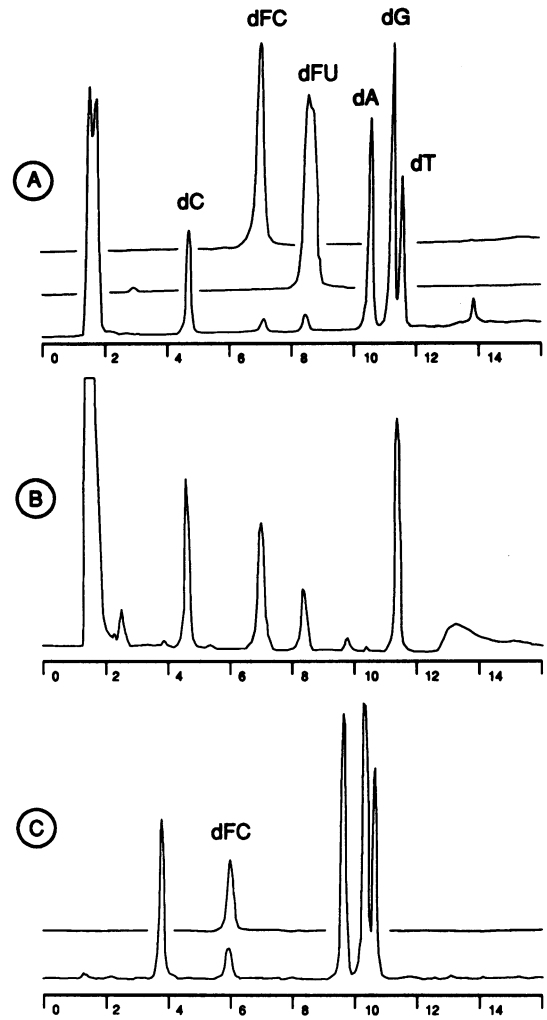


Figure 4: Nucleoside composition analysis by reverse phase h.p.l.c. Panel A: Hydrolysate of oligonucleotide III, synthesized by the indirect approach using methylmercapto-substituted phosphoramidite building block **2**. Marker nucleosides: dFC: 5-fluoro-2'-deoxycytidine. dFU: 5-fluoro-2'-deoxyuridine. Panel B: Hydrolysate of dTFFCT, synthesized by the same method. Panel C: Hydrolysate of oligonucleotide III, synthesized by the direct approach using phosphoramidite building block **3**. (A longer period of time had elapsed between h.p.l.c. analyses B and c, during which time the column changed its properties due to heavy use—hence the somewhat faster elution of all compounds in chromatogram C).

Isobutyryl protection proved to be too labile—this group was quantitatively removed already during the mild ammonia treatment used in the transient protection scheme for removal of trimethylsilyl groups (22). The observed lability may well be due to an electron withdrawing effect exerted by the fluorine atom bound to C-5. Introduction of an exocyclic benzoyl group with benzoyl chloride as the acylating agent also failed. On the other hand, benzoylation with benzoic anhydride in absolute ethanol turned out to be perfectly satisfactory and the product proved to be stable when stored dry.

Using monomeric model compounds, we next determined conditions of ammonia treatment that are strong enough to quantitatively remove the exocyclic benzoyl group and yet mild enough to leave the rather labile 5-fluorocytosine ring undamaged. Treatment of 5-fluoro-2'-deocycytidine with concentrated ammonia at 50°C overnight resulted in partial degradation of the nucleoside. Chromatography revealed the appearance of a major product which by means of comparison with authentic samples was found to be non-identical with 5-fluoro-2'-deoxyuridine, 2'-deoxycytidine or 2'-deoxyuridine. In contrast, treatment with concentrated ammonia at room temperature overnight left 5-fluoro-2'-deoxycytidine completely undamaged but lead to quantitative removal of the exocyclic benzoyl group when applied to N^4-benzoyl-5-fluoro-2'-deoxycytidine. Similar behaviour was observed with the 5-fluorocytosine residue being part of an oligonucleotide (data not shown). Apparently, the electron withdrawing effect discussed above is also responsible for the increased lability of a benzoyl group bound to the exocyclic amino function of the 5-fluorocytosine ring.

The constraints for the base deprotection reaction thus defined must also be met by the other base protecting groups used in oligonucleotide synthesis. This precludes use of the benzoyl group with 2'-deoxyadenosine and 2'-deoxycytidine and isobutyryl with 2'-deoxyguanosine. The constraints are met, however, if phenoxyacetyl ('PAC') groups are used with 2'-deoxyadenosine and 2'-deoxyguanosine and isobutyryl with 2'-deoxycytidine.

Dimethoxytritylation of N^4-benzoyl-5-fluoro-2'-deoxycytidine was carried out by standard procedures (15). Treatment with 1% trichloroacetic acid, as used in the oligonucleotide synthesis cycle, removed the dimethoxytrityl group but left the N-protected nucleoside intact. We did not observe the prohibitive lability of the nucleoside to acid described by Osterman *et al.* (8). Synthesis of phosphoramidite building block **3** was carried out in analogy to procedures described in the literature (16). Applying the conditions sketched above, automated synthesis of oligonucleotides III to VI was achieved with yields as usual for completely unmodified oligonucleotides.

Synthetic oligonucleotides were subjected to nucleoside composition analysis. To this end, samples were digested with both snake venom phosphodiesterase and alkaline phosphatase and the resulting nucleosides were separated and identified by h.p.l.c. (see Figure 4).

5-Fluoro-2'-deoxycytidine could be identified in products of both the direct and the indirect approach, which confirms successful incorporation of this modified nucleoside into the oligonucleotides analyzed. Analysis of oligonucleotides synthesized *via* methylmercapto building block **2**, however, revealed presence of both 5-fluoro-2'-deoxycytidine and 5-fluoro-2'-deoxyuridine in roughly equimolar amounts (Figure 4, panel A). The latter nucleoside was completely absent from hydrolysates of oligonucleotides synthesized using protected phosphoramidite building block **3** derived from 5-fluoro-2'-deoxy-cytidine (Figure 4, panel C).

Next, we addressed the question whether the side-reaction leading to formation of 5-fluorouracil residues took place during chain elongation or during final deprotection. In the first case, one should observe an effect of the number of synthesis cycles a protected 5-fluoro-2'-deoxycytidine is exposed to after its own chain incorporation. Therefore, the pentamer TFFCT was synthesized and subjected to nucleoside composition analysis. Its h.p.l.c. pattern (Figure 4, panel B) reveals a much smaller proportion of 5-fluoro-2'-deoxyuridine than observed with the pentadecamer (Figure 4, panel A). With this strong indication at hand of the side-reaction occuring during chain elongation, we set out to identify the individual step responsible for it. In separate reactions, nucleoside **7** (Figure 3) was treated with all the different reagents employed in the elongation cycle. Conversion of **7** to 5-fluoro-2'-deoxyuridine occured under conditions of the oxidation reaction. Apparently, treatment with iodine/collidine/water leads to partial oxidation of the methylmercapto function rendering it susceptible to nucleophilic displacement by hydroxide ion. Thus, the side-reaction described seems unescapable in the normal phosphoramidite approach. In addition, the base deprotection conditions employed here must be expected to lead to partial decomposition of deprotected 5-fluorocytosine residues (compare above). Because of the superior product quality, we clearly prefer the direct approach to the synthesis of oligonucleotides, despite the somewhat more involved synthesis of building block **3** as compared to **2** (Figure 2).

The oligonucleotides containing 5-fluorocytosine residues presented here were used successfully for the demonstration of sequence-specific, covalent crosslinking of Dcm DNA cytosine methyltransferase of *E. coli* K-12 to its correspondingly modified cognate DNA sequence (compare Figure 1; Th. Hanck, S. Schmidt and H.-J. Fritz, manuscript in preparation). A similar study of the M.HaeIII methyltransferase was published recently (23), without, however, an explicit description of the organic chemistry used for oligonucleotide synthesis.

ACKNOWLEDGEMENTS

We thank R. Machinek for measuring ^{19}F NMR spectra and D. Muster for h.p.l.c. analyses. This work was supported by Deutsche Forschungsgemeinschaft through Forschungsschwerpunkt Gentechnologie and by Volkswagen-Stiftung.

REFERENCES

1. Adams, R.L.P. (1990) *Biochem. J.* **265**, 309–320.
2. Friedman, S. (1980) *Molecular Pharmacology* **19**, 314–320.
3. Jones, P.A. and Taylor, S.M. (1980) *Cell* **20**, 85–93.
4. Creusot, F., Acs, G. and Christman, J.K. (1982) *J. Biol. Chem.* **257**, 2041–2048.
5. Santi, D.V., Garrett, C.E. and Barr, P.J. (1983) *Cell* **33**, 9–10.
6. Santi, D.V., Norment, A. and Garrett, C.E. (1984) *Proc. Nat. Acad. Sci. USA* **81**, 6993–6997.
7. Friedman, S. (1986) *Nucleic Acids Res.* **14**, 4543–4556.
8. Osterman, D.G., De Pillis, G.D., Wu, J.C., Matsuda, A. and Santi, D.V. (1988) *Biochemistry* **27**, 5204–5210.
9. Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* **90**, 544–584.
10. Goodchild, J. (1991) *Bioconjugate Chemistry* **1**, 165–187.
11. Holy, A. and Soucek, M. (1971) *Tetrahedron Letters*, 185–188.
12. Krug, A., Schmidt, S. Lekschas, J., Lemke, K. and Cech, D. (1989) *J. prakt. Chem.* **331**, 835–842.

13. Fromageot, H.P.M., Griffin, B.E., Reese, C.B. and Sulston, J.E. (1967) *Tetrahedron* **23**, 2315–2331.
14. Wempen, I., Duschinsky, R., Kaplan, L. and Fox, J.J. (1961) *J. Am. Chem. Soc.* **83**, 4755–4766.
15. Mc Bride, L.J. and Caruthers, M.H. (1983) *Tetrahedron Letters* **24**, 245–248.
16. Sinha, N.D., Biernat, J., Mc Manus, J. and Köster, H. (1984) *Nucleic Acids Res.* **12**, 4539–3557.
17. Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Letters* **22**, 1859–1862.
18. Schlagman, S., Hattman, S., May, M.S. and Berger, L. (1976) *J. Bacteriol.* **126**, 990–996.
19. Connolly, B.A. and Newman, P.C. (1989) *Nucleic Acids Res.* **17**, 4957–4974.
20. Fox, J.J., Van Praag, D., Wempen, I., Doerr, I.L., Cheong, L., Knoll, J.E., Eidinoff, M.L., Bendich, A. and Brown, G.B. (1959) *J. Am. Chem. Soc.* **81**, 178–187.
21. Sung, L.W. (1981) *Nucleic Acids Res.* **9**, 6139–6151.
22. Ti, G.S.,Gaffney, B.L. and Jones, R.A. (1982) *J. Am. Chem. Soc.* **104**, 1316–1319.
23. Chen, L., MacMillan, A. M., Chang, W., Ezaz-Nikpay, K., Lane, W.S., and Verdine, G.L. (1991) *Biochemistry* **30**, 11018–11025