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New Synthetic Method of 5-Formyluracil-Containing Oligonucleotides and Their Melting Behavior

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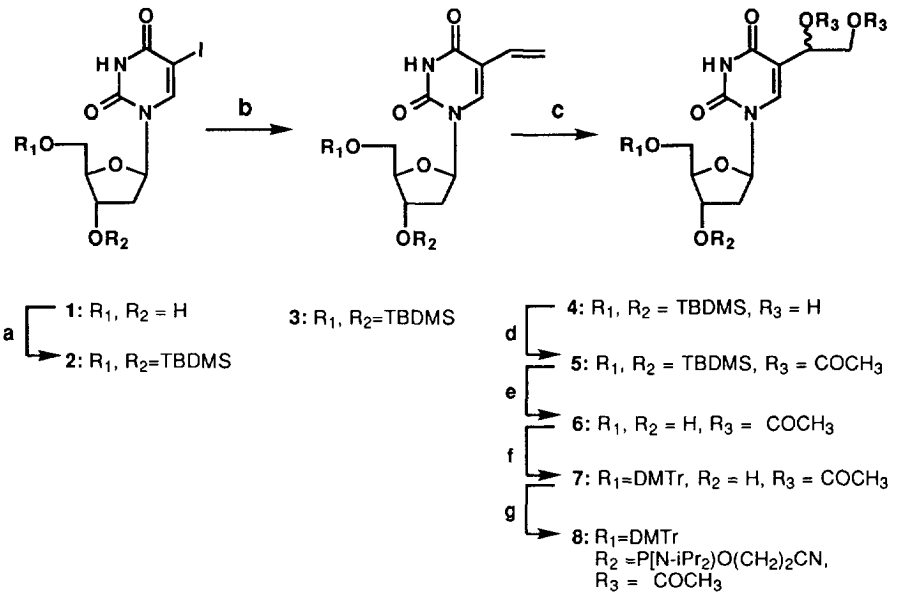
Abstract: A new method for the synthesis of 5-foU-containing oligonuleotides by phosphoramidite chemistry and subsequent post-oxidation with sodium periodate was developed. 5-(1,2-Dihydroxyethyl)uracil-containing oligomers, which are readily converted to 5-foU-containing oligomers by sodium periodate oxidation, were synthesized according to the standard β-cyanoethyl phosphoramidite chemistry. The phosphoramidite of a protected 5-(1,2-dihydroxyethyl)-2'-deoxyuridine derivative was prepared from 5-iodo-2'-deoxyuridine in 7 steps. UV melting behavior of these three oligomers demonstrated that the 5-foU-A base pair are less stable than the T-A base pair. Copyright © 1996 Published by Elsevier Science Ltd

5-Formyldeoxyuridine is a new type of oxidized thymine lesion formed in DNA by ionizing radiation[1] and by photosensitization with 2-methyl-1,4-naphthoquinone[2] or nitoro-substituted naphthalimide derivatives.[3] Recently, Bjelland *et al.* reported that *E. coli* 3-methyladenine DNA glycosylase II (AlkA) has a 5-foU DNA glycosylase activity, whereas an analogous human alkyl base DNA glycosylase (Anpg) does not show such activity.[4] More recently, 5-formyluracil (5-foU) DNA glycosylase activity was found in cell-free extracts from human,[2] mouse, and rat.[5] These results suggest a potential mutagenic effect of the 5-foU residues contained in DNA. In the previous studies the activity of 5-foU DNA glycosylase has been examined using aged tritium-labeled DNA and γ-ray radiated DNA possibly containing other types of oxidative DNA damage.[4,5] In order to facilitate the identification of DNA glycosylase activity and to understand the genetic effect of this oxidative lesion in detail, site specific incorporation of 5-foU into oligonucleotides is very important. Previously, the synthesis of 5-foU-containing oligonucleotides has been reported by Matsuda *et al.*[6] However, this method requires rather drastic conditions for the deprotection of the formyl group such as 80% CH_3COOH-overnight, which is not applicable to the synthesis of longer oligonucleotides. Here we report an entirely different method for the synthesis of 5-foU-containing oligonucleotides.

In view of the instability of the 5-foU moiety toward alkali, we employed a new method involving a combination of phosphoramidite chemistry and subsequent post-oxidation in order to obtain 5-foU-containing oligonucleotides. As precursors, 5-(1,2-dihydroxyethyl)uracil-containing oligomers which are readily oxidized to 5-foU-containing oligomers by sodium periodate were synthesized according to the standard β-cyanoethyl

phosphoramidite chemistry and subsequent deprotection. The phosphoramidite of the protected 5-(1,2-dihydroxyethyl)-2'-deoxyuridine **8** was prepared from 5-iodo-2'-deoxyuridine (**1**) in 7 steps as shown in Scheme 1. Thus, Pd-catalyzed coupling of the protected 5-iodo-2'-deoxyuridine **2** with vinyl acetate giving **3** was accomplished by the modified method reported by Walker et al.[7] Osmium oxidation of **3** followed by acetylation gave **6**.[8] Standard dimethoxytritylation and phosphitylation of **6** yielded phosphoramidite **8**.[9]

Scheme 1



(a) TBDMSiCl, imidazole, pyridine, 33 h, 99%; (b) vinyl acetate, $Pd(OAc)_2$, PPh_3, NEt_3, DMF, 70 °C, 16 h, 68%; (c)OsO_4, 4-methylmorpholine-N-oxide, acetone-H_2O-t-BuOH (4:1:1), 15 h, 44%; (d) Ac_2O, pyridine, 44 h, 96%; (e)TBAF, THF, 14 h, 75%; (f) DMTrCl, DMAP, NEt_3, pyridine, 22 h, 78%; (g) $P(N\text{-}iPr_2)_2O(CH_2)_2CN$, tetrazole, 2.5 h, quant.

After standard deprotection with conc. ammonia at 55 °C for 12 h, the oligomers were purified by reverse phase HPLC.[10] Under the deprotection conditions, the acetyl protecting group of the oligomers was completely removed. Figure 1(a) shows the HPLC profile of the purified 5-(1,2-dihydroxyethyl)uracil-containing oligomer **9** where two peaks corresponding to its diastereomers were observed. Addition of aqueous sodium periodate converted these two peaks into only one peak of 5-foU-containing hexamer **10** (Fig. (b)). Enzymatic digestion of the isolated peak provided dC, 5-foU, dG, and dA in a 2:1:2:1 ratio, confirming the structure of **10** (c). It was also found that ca. 50 equiv of sodium periodate relative to the 1,2-dihydroxyethyl moiety is necessary for the complete conversion of **9** into **10** within 1 min at 0 °C. A large excess (3000 equiv) of sodium periodate did not affect the yield of **10**. Formation of **10** was further confirmed by $NaBH_4$ reduction to 5-hydroxymethyluracil-containing oligonucleotide **11**.

Scheme 2

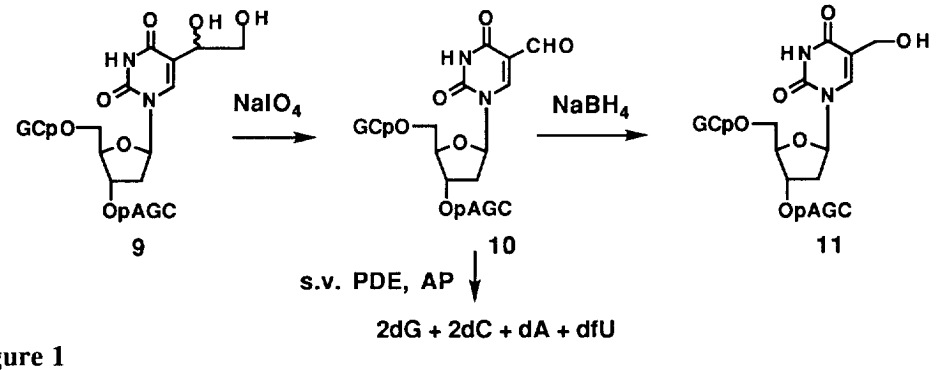


Figure 1

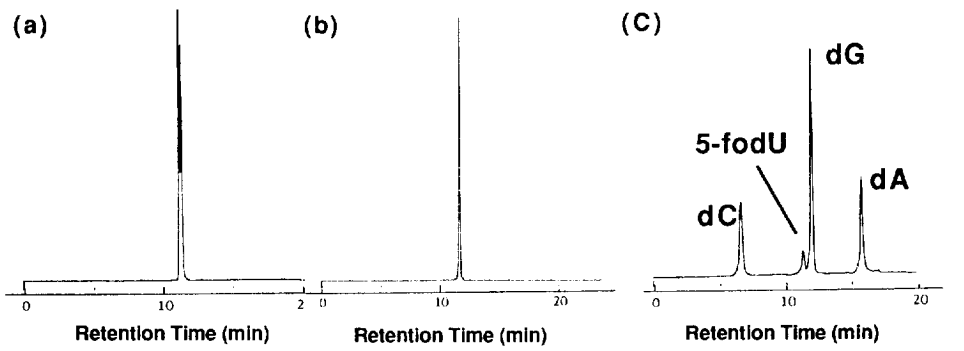


Figure 1. HPLC profiles of modified oligonucleotides; (a) 5-(1,2-dihydroxyethyl)uracil-containing hexamer **9** purified by HPLC, (b) oxidation of **9** with aq. sodium periodate, (c) enzymatic digestion of **10** with snake venom phosphodiesterase (s. v. PDE) and calf intestine alkaline phosphatase (AP). The mixture (10 μL) was analyzed by HPLC on a Cosmosil $5C_{18}$ column (4.6 x 150 mm), detected at 254 nm; elution was with 0.05 M ammonium formate, 0-10 % acetonitrile, linear gradient, 20 min, at a flow rate of 1.0 mL/min.

With respect to the mutagenecity of 5-foU, the thermal stability of a 5-foU-adenine (A) base pair has been examined by molecular modeling study.[2] In order to investigate the stability of the 5-foU-A base pair, UV melting behavior of three 5-foU-containing oligomers was compared with those of the corresponding parent thymine-containing oligomers. In contrast to the previous observation,[6a] the melting temperatures (Tm) of the three duplexes were lower (5.8-2.1 °C) than those of the parent thymine-containing oligomers (Table 1). A similar tendency was also observed under a high salt condition containing 1 M NaCl (data not shown). The small effects on Tm suggest that the incorporation of 5-foU does not significantly perturb the duplex DNA structure.

In conclusion, we have developed a convenient and efficient synthetic method of 5-foU-containing oligonuleotides. We also demonstrated that the 5-foU-A base pair is less stable than the T-A base pair. Recently, cell-free extracts of human, mouse, and rat have been shown to possess 5-foU DNA glycosylase activity.[2,5] Preliminary experiments indicated that incubation of double stranded 5-foU-containing 22 mers with cell-free extracts from Hela cells resulted in an efficient cleavage of the oligomer at the 5-foU site. Work

on isolation of 5-foU DNA glycosylase from human cell-free extract and the enzymatic incorporation experiments using 5-foU-containing oligonucleotides are in progress.

Table 1. Melting Temperatures for 5-Formyluracil-Containing Oligonucleotides[a]

Sequence	Tm[b]	ΔTm[c]
5'-d(CGCGAATTCGCG)-3'	67.5	-
5'-d(CGCGAAfUTCGCG)-3'	63.4	-4.1
5'-d(GCTAGC)-3'	25.8	-
5'-d(GCfUAGC)-3'	20.0	-5.8
5'-d(GCATGC)-3'	33.2	-
5'-d(GCAfUGC)-3'	31.0	-2.2

[a]Thermal denaturation profiles were obtained with a Jasco V-550 spectrophotometer equipped with a Peltier temperature controller. The absorbance of the sample was monitored at 260 nm from 2 °C to 82 °C at a heating rate of 1 °C per minute. [b]Tm data were at 0.1 mM base concentration of oligonucleotides in 50 mM Na cacodylate (pH 7.0). [c]Tm decrease relative to the parent thymine-containing oligomer. fU = 5-formyluracil.

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8. Low yield of **4** presumably due to oxidation of C5-C6 double bond. **6**: ^{1}H NMR (400 MHz, D_2O) δ 1.97 (s, 3 H, CH_3CO), 2.02 (s, 3 H, CH_3CO), 2.14-2.37 (m, 2 H, H2'), 3.63-3.97 (m, 2 H, H5'), 3.93-3.97 (m, 1 H, H4'), 4.23-4.38 (m, 3 H, CH_2OAc and H3'), 5.75-5.77 (m, 1 H, CHOAc), 6.15 (t, 1 H, J=6.4 Hz, H1'), 7.92 (s, 0.5 H, H6, isomer A), 7.97 (s, 0.5 H, H6, isomer B). FAB MASS: m/e 373 $(M+H)^+$.
9. **8**: ^{1}H NMR (400 MHz, D_2O) δ 0.97-1.27 (m, 12 H, $CH(CH_3)_2$), 1.47 (s, 1.5 H, CH_3COOCH), 1.50 (s, 1.5 H, CH_3COOCH), 1.93 (s, 3 H, CH_3COOCH_2), 1.97-2.17 (m, 1 H, $CH(CH_3)_2$), 2.35 (t, J=6.6 Hz, 1 H, OCH_2CH_2CN), 2.53-2.64 (m, 1 H, $CH(CH_3)_2$), 2.71 (t, J=6.1 Hz, 1 H, OCH_2CH_2CN), 3.12-3.28 (m, 1 H, H2'), 3.34-3.60 (m, 4 H, OCH_2CH_2CN,H2',H5'), 3.73 (s, 3 H, OCH_3), 3.74 (s, 3 H, OCH_3), 4.05-4.27 (m, 3 H, OCH_2CH_2CN, H4'), 4.33-4.58 (m, 1 H, H3'), 5.71 (t, J=5.1 Hz, 1 H, $CHOCH_3$), 6.15 (m, 0.5 H, H1'), 6.25 (t, J=5.8 Hz, 0.5 H, H1'), 6.75-6.83 (m, 4 H, methoxyphenyl-o), 7.17-7.40 (m, 9 H, phenyl,methoxyphenyl-m), 7,54 (s, 0.25 H, H6), 7.57 (s, 0.25 H, H6), 7.64 (s, 0.25 H, H6), 7.70 (s, 0.25 H, H6), 8.51 (s, 1 H, NH) ^{31}P NMR (121 MHz, $CDCl_3$) δ 150.33,149.99,149.71 (diastereomers). FAB MASS: m/e 875 $(M+H)^+$.
10. A variety of 5-foU-containing oligonucleotides including 22 mer for enzymatic incorporation experiment was prepared. All coupling yields, including that with **8** were higher than 97%. Generally, more than 20 OD of purified oligomer was obtained in a 1 μmol scale synthesis.

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