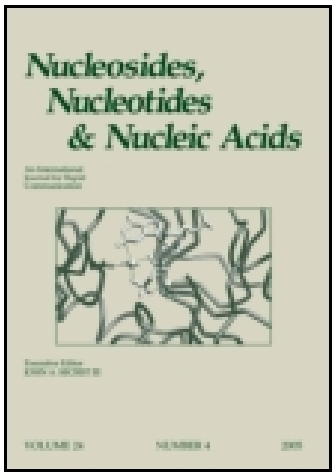

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Synthesis of a Convenient Thymidine Glycol Phosphoramidite Monomer and Its Site-specific Incorporation into DNA Fragments

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SYNTHESIS OF A CONVENIENT THYMIDINE GLYCOL PHOSPHORAMIDITE MONOMER AND ITS SITE-SPECIFIC INCORPORATION INTO DNA FRAGMENTS

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□ *An original phosphoramidite building block of the thymidine glycol lesion has been prepared taking into account the additional diol function and the high lability of this oxidatively induced nucleobase damage. Then the modified nucleoside was site-specifically inserted into DNA fragments by solid support assembling followed by a "one-step" mild final deprotection treatment.*

Keywords Oxidative DNA lesion; Thymine glycol; Oligodeoxyribonucleotide; Chemical synthesis; Protecting groups

INTRODUCTION

Various oxidative stress agents including ionizing radiation, ultraviolet-A light and several chemicals are able to oxidize the sugar and nucleobase moieties of DNA giving rise to a large set of lesions.[1–3] In that respect, 5,6-dihydroxy-5,6-dihydrothymine **1** (thymine glycol or thymine diol) (Scheme 1) has been shown to be a major °OH and one-electron mediated oxidation product of thymine. It was shown that the amount of thymine glycol increases substantially when DNA is exposed to oxidative events.[4] The lesion **1** is produced in DNA or at the nucleoside level as a mixture of two pairs of *cis* and *trans* diastereomers, which are in equilibrium two by two in solution through epimerization at C6.[5] Thymidine glycol **1**, which may block DNA polymerases during the replication process,[6] is efficiently removed by the base excision repair (BER) pathway involving DNA *N*-glyco-

This paper is dedicated to the memory of Dr. John A. Montgomery.

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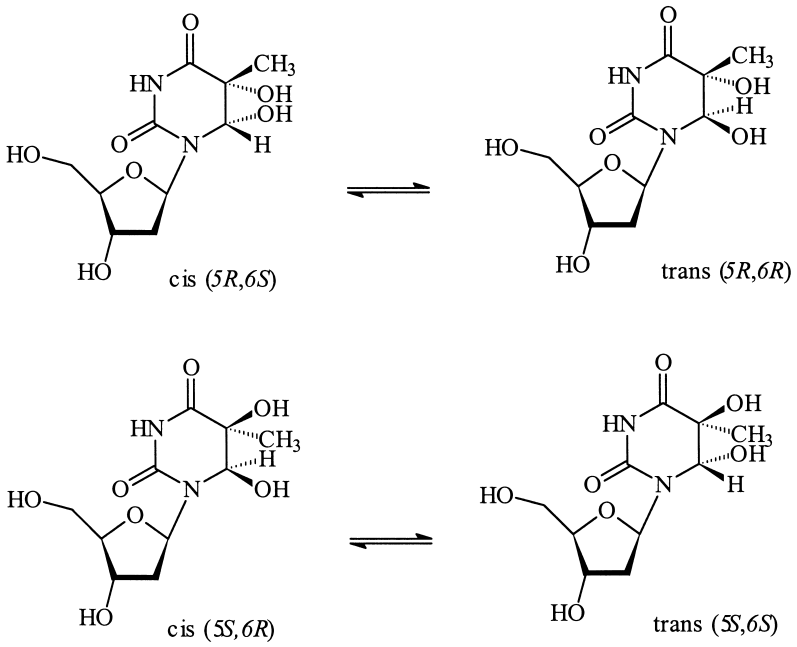


SCHEME 1 Structure of the four 5,6-dihydroxy-5,6-dihydrothymidine isomers (**1**).

sylases,[7] and also by the nucleotide excision repair (NER) system.[8] It was found that the two *cis* thymine glycols were excised in a stereoselective manner from site-specifically oxidized oligonucleotides by a wide set of DNA *N*-glycosylases including Endo III, endo VIII, yNTG1, mNTH and mNEIL1 but not yNTG2.[9] Recently, Friedberg et al. have shown that human DNA polymerase kappa bypasses and extends behond thymine glycol during translesional synthesis in vitro by incorporating correct nucleotides.[10]

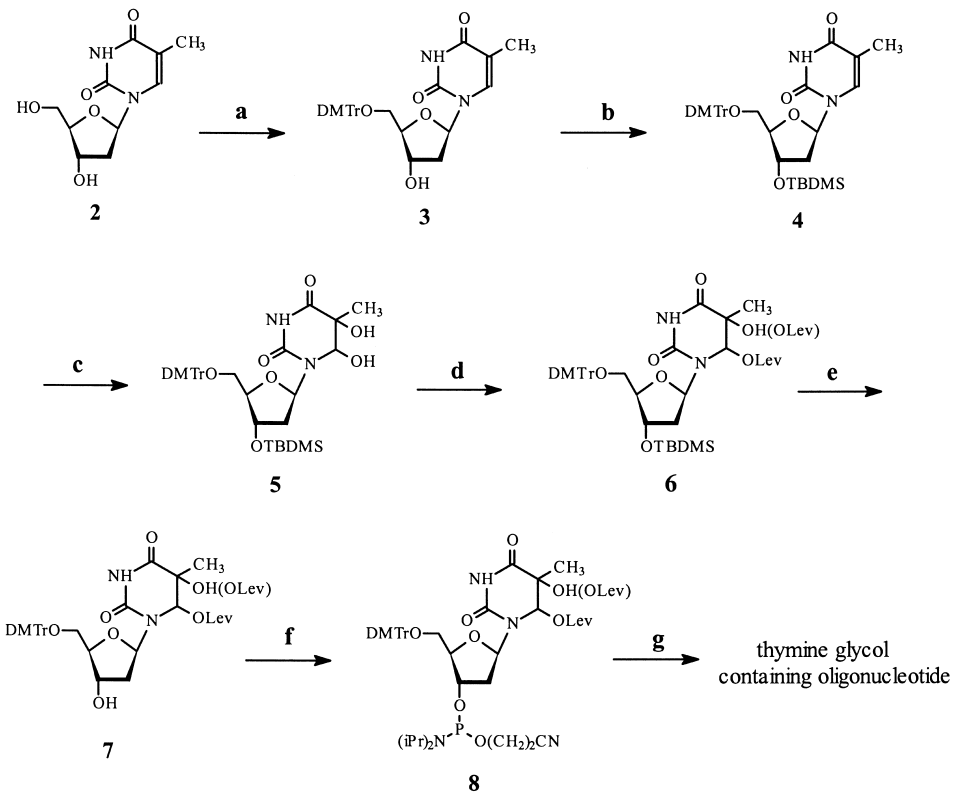
In order to obtain further biological and structural information on this major oxidative alteration of DNA, it is necessary to prepare oligodeoxynucleotides (ODNs) that contain **1** at defined sites as probes or substrates. The presence of hydroxyl groups within the thymine base together with 5,6-saturation of the ring, confer a high instability to the nucleoside particularly under alkaline conditions. The latter aspect together with the necessity to protect the additional hydroxyl functions of the base make the insertion of **1** into defined sequence oligonucleotides a challenging objective. The usual approach to prepare thymine glycol-containing ODNs consists in the post-oxidation, by either osmium tetroxide or potassium permanganate, of a thymine residue incorporated into a single strand DNA fragment.[7d,11] However the post-modification approach shows several limitations that concern the stereochemical composition, the chain length, the sequence, the yield and also the quantity of modified ODNs thus prepared. To overcome

partly some of these difficulties, Saito and coworkers recently reported a site-selective post-oxidation of a thymine residue within a DNA fragment in the presence of bipyridine-tethered complementary ODN.[12]

To our best knowledge, only one total chemical approach for incorporating **1** into DNA fragments has been yet reported by Iwai.[13] The latter synthesis, consists in the preparation of a phosphoramidite building block, that contains one or two *t*-butyldimethylsilyl (TBDMS) protective groups on the nucleobase hydroxyl sites. Then, the subsequent incorporation of the protected oxidized nucleoside into modified ODNs was achieved by solid-phase assembling. The final support cleavage and deprotection are then performed by a mild ammonia treatment at room temperature followed by an additional desilylation step by fluoride ions.

Herein we report the synthesis of a suitable phosphoramidite monomer **8** that contains the levulinyl (Lev) as the nucleobase protective group (Scheme 2). Prior to the preparation of the phosphoramidite synthon **8**, the stability of thymidine glycol **1** was checked at room temperature under several conditions used during DNA solid support synthesis: 30% aqueous ammonia, 80% acetic acid and a 0.02 M commercial oxidizing solution of iodine. Less than 10% of degradation of **1** was observed after 10 h of incubation in the acid and oxidizing solutions. However **1** was fully decomposed when left for 4 h in aqueous ammonia. The problem of instability was circumvented using a 0.05 M methanolic solution of K_2CO_3 as an alternative mild alkali deprotection system. Under the latter conditions, no detectable degradation was observed after 4 h at room temperature. Moreover, the levulinyl-protected thymine glycol derivative was quantitatively converted into the parent compound **1** upon K_2CO_3 treatment for 2 h at room temperature. The kinetic and stability studies show the compatibility of **1** with the latter alkali deprotection conditions, which may be used in combination with the "Pac-phosphoramidite" chemistry.[14] Furthermore, it was expected that the protection of the additional hydroxyl functions of **1** together with its insertion in the oligonucleotide chain might increase the stability of the base moiety.

The synthesis of the target phosphoramidite **8** (Scheme 2) started using 5′-O-DMTr-3′-O-TBDMS-thymidine (**4**), prepared by classical dimethoxytritylation (step a) and silylation (step b) of thymidine (**2**), respectively. Then, sugar-protected thymidine glycol (**5**), preferentially in its *cis*-5*R*,6*S* configuration,[15] was obtained in a high yield by oxidation with osmium tetroxyde in the presence of methylmorpholine-*N*-oxide in a t-BuOH/THF/H_2O solution.[16] Compound **5** was then converted into a mixture of O^6-monolevulinyl and O^5,O^6-dilevulinyl derivatives **6** after treatment with levulinic acid in the presence of DCC and DMAP in THF. The levulinyl group, which has been already successfully used for the chemical synthesis of modified oligonucleotides that contain nucleobase damages,[17,18] has been selected



SCHEME 2 **a)** DMTr-Cl (2 eq), pyridine, 16 h, 76%; **b)** TBDMS-Cl (2 eq), imidazole (2.5 eq), pyridine, 24 h, 82%; **c)** OsO_4 (1/19 eq), methylmorpholine-N-oxide (2 eq), t-BuOH, THF, H_2O, 45°C, 24 h, 85%; **d)** Levulinic acid (5 eq), DCC (5 eq), DMAP (0.6 eq), THF, 40°C, 24 h, 71%; **e)** TBAF (4 eq), THF, 3 h, 60%; **f)** 2-cyanoethyl-*N,N,N′,N′*-tetraisopropyldiamidite (1.1 eq), diisopropylammonium tetrazolate (0.5 eq), CH_2Cl_2, 4 h, 75%; **g)** oligonucleotide solid-phase assembling and deprotection.

for the following suitable properties: high reactivity toward hydroxyl functions, good stability during acidic and oxidizing treatments, easy removal in neutral or mild alkali conditions. Due to the weak reactivity of the tertiary hydroxyl function at the position 5 of the nucleobase, the O^6-mono-protected residue (Figure 1) was obtained in a large excess (O^6-monoLev/O^5,O^6-diLev = 9/1 from the ^{1}H-NMR and HPLC analyses). This, in agreement with previous fundings relative to the lack of reactivity of such tertiary alcohol functions,[4d,19] allows the use of the pure O^6-monosubstituted compound or in mixture with the O^5,O^6-dilevulinyl derivative in the subsequent reaction pathways. Thus, the TBDMS group was selectively removed by treating compounds **6** with 1 M tetrabutylammonium fluoride in THF, yielding the 3′-hydroxy derivatives **7**. Standard phosphitylation of **7** gave phosphoramidite building blocks **8**, which have been used for the preparation of defined-sequence ODNs that contain the thymine glycol lesion.

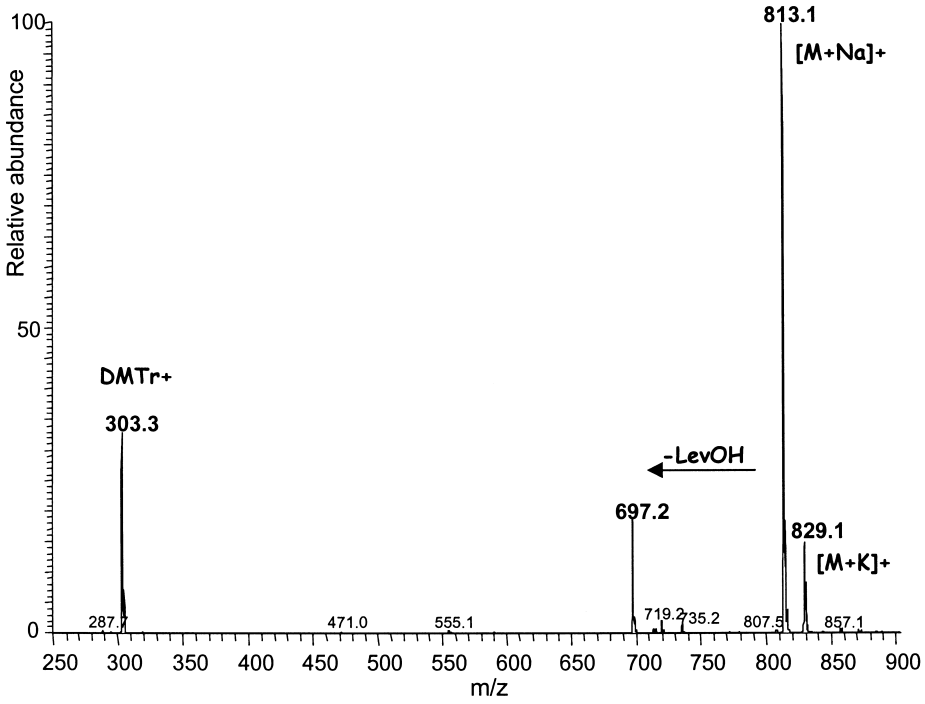


FIGURE 1 ESI mass spectrum, in the positive mode, of RP-HPLC purified O^6-monolevulinyl derivative **6** (molecular weight: 790 g·mol^{-1}).

Thus, several thymine glycol-containing oligonucleotides (5-, 14-, and 34-mers) were prepared on a DNA synthesizer with few modifications in the standard procedure: 1) the phenoxyacetyl (pac) group was used for the amino-protection of the unmodified bases (pac for dA and dG and isobutyryl for dC, respectively) in combination with anhydride phenoxyacetic as the capping reagent, 2) the oxidation step was performed with either a diluted 0.02 M iodine solution or a 10-camphorsulfonyl-oxaziridine solution,[20] 3) the final support cleavage and total deprotection of modified DNA fragments were achieved using a 0.05 M K_2CO_3 solution in methanol at room temperature for 4 h as a mild alkali treatment. After RP-HPLC purification, the purity and the homogeneity of the modified DNA oligomers were checked by analytical RP-HPLC, polyacrylamide gel electrophoresis, electrospray and MALDI-TOF mass spectrometry measurements (ESI MS and MALDI-TOF/MS) (Figure 2). The latter mass spectrometry analyses, which were in complete agreement with the calculated molecular weights,[21] confirm the presence and the integrity of the thymidine glycol residue within the synthetic oligonucleotides.

In conclusion, the synthesis reported herein provides an improved method for the preparation of oligonucleotides containing the thymidine

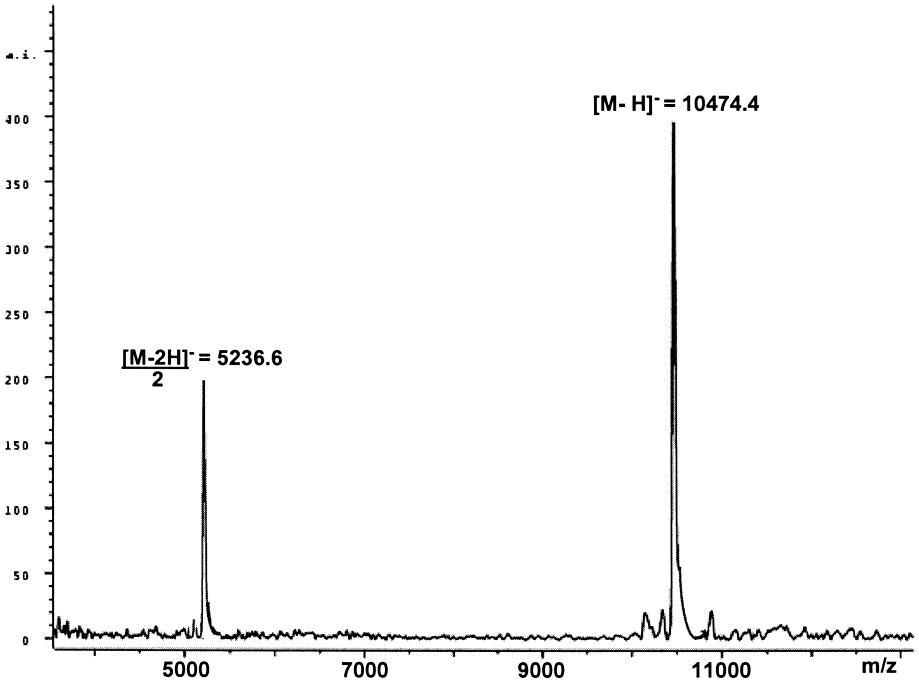


FIGURE 2 MALDI-TOF mass spectrum, in the negative mode, of the purified 34-mer thymine glycol-containing oligonucleotide (molecular weight: 10,474.8 g·mol^{-1}).

glycol damage at specific positions by a total chemical approach that involves a mild "one step" final alkali deprotection step. The latter modified DNA sequences will be used as either substrates or probes to study the biological properties of thymine glycol base lesions together with their structural features within DNA.

EXPERIMENTAL SECTION

General Procedures and Materials

The silica gel (70–200 μm) used for the low-pressure column chromatography was purchased from SDS (Peypin, France). TLC was carried out on Merck DC Kieselgel 60 F-254 plastic sheets (Darmstadt, Germany). All reagents used were of the highest available purity. Anhydrous solvents were purchased from SDS. Acetonitrile and methanol (HPLC grade) were obtained from Carlo Erba (Milan, Italy). Buffers for HPLC were prepared using water purified with a Milli-Q system (Milford, MA). The Hypersil ODS column (5 μm, 4.6 $\times$ 250 mm I.D.) was purchased from Interchim (Montlucon, France). Functionalized CPG supports and unmodified 2′-deoxyribo-

nucleoside 3′-phosphoramidites, protected with phenoxyacetyl for dAdo, isopropyl-phenoxyacetyl for dGuo and acetyl for dCyd, were from Glen Research (Sterling, Virginia).

Mass Spectrometry Measurements

All modified and unmodified oligonucleotides were characterized by electrospray ionization-mass spectrometry measurements (ESI-MS) on an LCQ ion trap model spectrophotometer (Finnigan). Typically, 0.1 AU_{260nm} of the sample (approximately 3 μg) was dissolved in a solution of acetonitrile and water (50/50, v/v) containing 1% triethylamine prior to be analyzed in the negative mode (voltage 5 kV). The modified protected nucleosides were analyzed by ESI-MS in both the negative and positive modes. For the positive mode analysis, the sample was dissolved in a solution of methanol and water (50/50, v/v) that contained 0.5% formic acid.

MALDI mass spectra were obtained with a commercially available time-of-flight mass spectrometer (Biflex, Bruker) equipped with a 337 nm nitrogen laser. Spectra were recorded in the linear and positive modes. For the matrix, a mixture of 3-hydroxypicolinic acid and picolinic acid in a 4/1 (w/w) ratio was dissolved in aqueous acetonitrile solution (50%) that contained a small amount of Dowex-50W 50X8-200 cation exchange resin (Sigma). Typically, 1 μL of the sample was added to 1 μL of the matrix and the resulting mixture was stirred. Then, the solution was then placed on the top of the target plate and allowed to dry by itself. The spectra were calibrated with 1 pmol/μL solution of myoglobin (m/z 16952), using the same conditions that were described for the analysis of oligonucleotides.

Synthetic Procedures

5′-O-dimethoxytrityl-thymidine (**3**) and 5′-O-dimethoxytrityl-3′O-*tert*-butyldimethylsilyl-thymidine (**4**) were prepared from thymidine (**2**) nucleoside by using the chloride derivatives of protecting groups and classical procedures.

5′-O-Dimethoxytrityl-3′-O-*tert*-butyldimethylsilyl-5,6-dihydroxy-5,6-dihydrothymidine (5). 5.6 g of 5′-O-dimethoxytrityl-3′O-*tert*-butyldimethylsilyl-thymidine (**4**, 8.5 mmol) are dissolved in a mixture of THF (24 mL), *tert*-butanol (20 mL), and water (3 mL). Then, 2 g of methylmorpholine-*N*-oxide (2 eq, 2 g, 17 mmol) and osmium tetraoxide (0.05 eq, 0.425 mmol) were added to the solution under stirring. The reaction was placed at 45° C for 24 h. The mixture was cooled in an ice-water bath and neutalized by addition of an aqueous 20% sodium thiosulfate solution (5 mL). The resulting solvents were evaporated to dryness. The oily residue was dissolved

in ethyl acetate (50 mL), washed with saturated $NaHCO_3$ aqueous solution (50 mL) and water (50 mL × 2). Then the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The reaction mixture was resolved by silica gel column chromatography (0–3% step gradient of methanol in AcOEt/hexane 25/75) to afford the title compound **5** in a yield of 85%, 4.98 g, 7.20 mmol), as a white powder. Rf (AcOEt/hexane/MeOH: 25/75/10) = 0.45. ESI-MS (positive mode): m/z = 715.1 $[M+Na]^+$; 303.3 ($DMTr^+$). ^1H NMR (200.13 MHz, CD_3COCD_3): δ: 7.65–7.30 (m, 9H, H DMTr); 7.01 (d, 4H ^3J(H,H) = 9 Hz, DMTr); 6.37 (pseudo-t, 1H, ^3J(H,H) = 7 Hz, 1H, H-1′); 5.17 (m, 1H, H-3′); 4.85 (s, 1H, H-6); 4.55 (m, 1H, H-4′); 3.92 (s, 6H, C$\underline{H}_3$O-DMTr); 3.39 (m, 2H, H-5′ and H-5″); 2.59 (m, 2H, H-2′ and H-2″); 1.51 (s, 3H, C$\underline{H}_3$-dT); 0.95 (s, 9H, *t*Bu-TBDMS); 0.16 (s, 3H, C$\underline{H}_3$-TBDMS); 0.10 (s, 3H, C$\underline{H}_3$-TBDMS).

5′-O-Dimethoxytrityl-3′-O-*tert*-butyldimethylsilyl-5,6-O-levulinyl-thymidine Glycol (6). 680 mg of compound **5** (0.98 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and evaporated to dryness (×2). Then, the residue was dissolved in dry THF (30 mL) under stirring and an argon atmosphere. DCC (1.02 g, 5 eq, 4.95 mmol), DMAP (78 mg, 0.6 eq, 0.6 mmol) and levulinic acid (500 μL, 5 eq, 4.88 mmol) was added and the solution was placed at 40°C for 24 h. After a TLC analysis, the mixture was cooled in an ice-water bath and the reaction stopped by addition of methanol (2 mL). After 15 min, the resulting DCU formed was eliminated by filtration. The residue was washed with saturated $NaHCO_3$ aqueous solution (50 mL) and water (50 mL × 2). Then the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. After purification by flash chromatography on a silica gel column, using a step gradient of methanol (0 to 2%) in CH_2Cl_2 as the mobile phase, the title compound **6** was obtained as a white foam in a 71% yield (550 mg, 0.70 mmol) that contains a mixture of mono- and di-substituted derivatives (O^6-monolev/O^5,O^6-dilev = 9/1). Rf (CH_2Cl_2/MeOH: 94/6) = 0.55 (mono-Lev) and 0.58 (di-Lev). ESI-MS (positive mode): m/z = 911.1 $[M+Na]^+$ for di-Lev; 813.1 $[M+Na]^+$ for di-mono-Lev ; 303.3 ($DMTr^+$). O^6-monolevulinyl derivative **6**: ^1H-NMR (200 MHz, CD_3COCD_3): δ = 7.52–7.31 (m, 9H, DMTr); 6.98 (d, 4H ^3J(H,H) = 9 Hz, DMTr); 6.24 (pseudo-t, 1H, ^3J(H,H) = 7 Hz, 1H, H-1′); 4.78 (s, 1H, H-6); 4.46 (m, 1H, H-3′); 3.93 (m, 1H, H-4′); 3.90 (s, 6H, C$\underline{H}_3$O-DMTr); 3.43 (m, 2H, H-5′ H-5″); 2.84 (m, 2H, CH_2-Lev); 2.68 (m, 4H, H-2′ H-2″, C$\underline{H}_2$-Lev); 2.24 (s, 1H, CH_3-Lev); 1.89 (s, 3H, C$\underline{H}_3$-dT); 0.93 (s, 9H, *t*Bu-TBDMS); 0.14 (s, 3H, C$\underline{H}_3$-TBDMS); 0.08 (s, 3H, C$\underline{H}_3$-TBDMS).

5′-O-Dimethoxytrityl-5,6-O-levulinyl-thymidine Glycol (7). 215 mg of compounds **6** (0.27 mmol) was dissolved in dry THF (5 mL) under stirring and an argon atmosphere. Then, 1 mL of TBAF in THF (1 M solution, 4 eq,

1.08 mmol) was added and the solution was stirred at room temperature for 3 h. After a TLC analysis, the reaction was stopped by addition of water (1 mL). After 5 min, 15 mL of dichloromethane was added and the residue was washed with saturated $NaHCO_3$ aqueous solution (20 mL) and water (20 mL × 2). Then the organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. After purification by flash chromatography on a silica gel column, using a step gradient of methanol (0 to 4%) in CH_2Cl_2 as the mobile phase, the title compound **7** was obtained as a white foam in a 60% yield (110 mg, 0.165 mmol) (O^6-monolev/O^5,O^6-dilev = 9/1). Rf (CH_2Cl_2/MeOH: 94/6) = 0.42 (mono-Lev) and 0.45 (di-Lev). ESI-MS (positive mode): *m/z* = 797.2 $[M+Na]^+$ for di-Lev; 699.2 $[M+Na]^+$ for mono-Lev ; 303.3 ($DMTr^+$).

Thymidine Glycol Phosphoramidite Synthon (8). Compound **7** (100 mg, 0.148 mmol) was co-evaporated twice with dry pyridine and subsequently dissolved in anhydrous CH_2Cl_2 (15 mL) under an argon atmosphere. Diisopropylammonium tetrazolate (12.7 mg, 0.074 mmol, 0.5 eq) and 2-cyanoethyl-*N,N,N′,N′*-tetraisopropyldiamidite (49 μl, 0.163 mmol, 1.1 eq) were added to the solution under stirring. The course of the reaction was monitored by TLC (CH_2Cl_2/MeOH/TEA 95/5/1). After 4 h at room temperature, the mixture was diluted with ethyl acetate (25 mL) and washed with saturated $NaHCO_3$ aqueous solution (30 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuum. The resulting residue was purified by flash chromatography on a silica gel column with a step gradient of methanol (0–3%) in $CHCl_3$/TEA (99/1, v/v) as the mobile phase. Then, the collected fractions corresponding to the diastereomers were dried to afford compound **8** as a white foam (yield 75%, 0.111 mmol, 108 mg). Phosphoramidite **8** (O^6-monolevulinyl derivative): Rf (CH_2Cl_2/MeOH/TEA 95/5/1) = 0.6. ^{31}P-NMR (161.9 MHz, CD_3CN) δ = 149.5–149.9 (two diastereoisomers). ESI-MS (positive mode): *m/z* = 997.1 $[M+Na]^+$, 1013.1 $[M+K]^+$.

Stability Studies of the Thymine Glycol Nucleoside (1) Under the Alkaline Conditions Used for Oligonucleotides Chemical Synthesis

Aqueous ammonia (32%; 500 μL) was added to 0.5 AU_{230nm} of compound **1** in sealed tubes. The solutions were placed at either room temperature or 55°C. Then, the reactions were stopped at increasing time intervals (0, 1, 2, 4, 8, 16, and 24 h, respectively) by freezing in liquid nitrogen and subsequent lyophilization. Samples were analyzed by HPLC using a C18 Hypersil column. The elution was achieved with a 0 to 10% linear gradient of acetonitrile in 25 mM ammonium formiate buffer over 30 min (flow rate: 1 mL/min; UV detection at 230 nm).

Stability Studies of the Thymine Glycol Nucleoside (1) Under the Acid Conditions Used for Oligonucleotides Chemical Synthesis

A similar procedure, as described above for the alkali-stability assay, was used. This involved incubation of compounds **1** in a 80% acetic acid aqueous solution for 0, 1, 2, 4, 8, 16, and 24 h, respectively, at room temperature.

Stability Studies of the Thymine Glycol Nucleoside (1) Under Oxidizing Conditions Used for Oligonucleotides Chemical Synthesis

Similarly, compound **1** was incubated in a 0.02 M oxidizing solution of iodine for 0, 1, 2, 4, 8, 16, and 24 h, respectively, at room temperature.

Solid-phase Synthesis of Oligodeoxyribonucleotides

The synthesis of thymine glycol containing oligodeoxyribonucleotides was performed at 1 μmol scale using the "Pac phosphoramidite" chemistry[14], with retention of the 5′ terminal DMTr group (trityl-on mode). The standard 1 μmol DNA cycle was used, on an Applied Biosystems Inc. 392 DNA synthesizer, with slight modifications. The duration of condensation was increased by a factor of 4 for the modified nucleoside phosphoramidite **8** (120 s instead of 30 s for normal nucleoside phosphoramidites). Under these conditions a coupling efficiency of more than 90% for the modified monomer **8** was achieved. A 0.3 M solution of phenoxyacetic anhydride in tetrahydrofuran and a 0.02 M solution of iodine in water/pyridine/tetrahydrofuran were used for the capping and the oxidation steps, respectively.

Deprotection and Purification of Modified Oligodeoxyribonucleotides

Upon completion of the synthesis, the alkali-labile protecting groups of the thymine glycol containing oligodeoxyribonucleotides were removed by treatment with concentrated aqueous ammonia (32%) at room temperature for 4 h. Solvents were removed by evaporation under vacuum. Then, the crude 5′-DMTr-oligomers were purified and deprotected on-line by reverse-phase HPLC using a polymeric support, as previously described.[22] The modified 34-mer oligonucleotide, used in biochemical studies, was further purified by preparative polyacrylamide gel electrophoresis and, then, was desalted using a NAP-25 sephadex column (Pharmacia, Uppsala, Sweden).

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