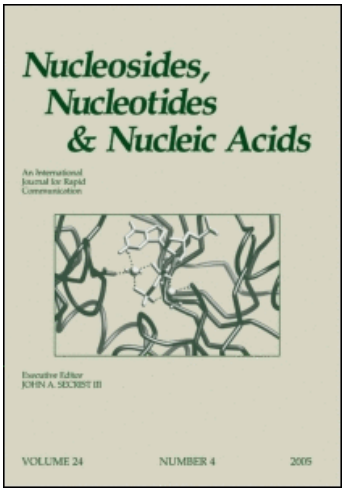

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A New Phosphoramidite Reagent for the Incorporation of Diazaphenoxazinone Nucleoside With Enhanced Base-Pairing Properties into Oligodeoxynucleotides

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A NEW PHOSPHORAMIDITE REAGENT FOR THE INCORPORATION OF DIAZAPHENOXAZINONE NUCLEOSIDE WITH ENHANCED BASE-PAIRING PROPERTIES INTO OLIGODEOXYNUCLEOTIDES

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ABSTRACT

New phosphoramidite reagent **7** suitable for incorporation of dC analogue was synthesized. ODNs containing diazaphenoxazinone residues in defined positions were prepared. The stability of duplexes formed was increased up to 3-5°C per modified base. Preliminary results of molecular biological testing were reported.

INTRODUCTION

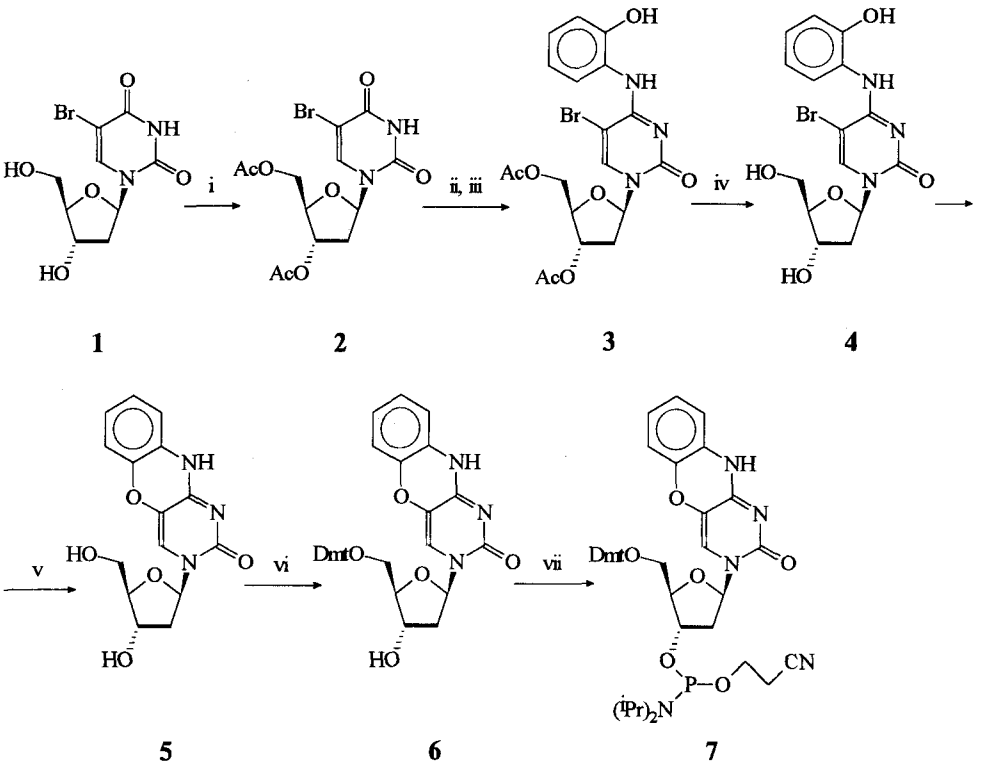
Strongly binding oligonucleotides (SBOs) containing modified heterocyclic bases have attracted considerable attention in recent few years as improved antisense agents[1-4], hybridization probes[5-7], primers for PCR amplification[8] and DNA sequencing[9]. It has been demonstrated[9] that substitution of 5-methyl deoxycytidine (m^5dC)[10,11] and 2-amino deoxyadenosine (n^2dA)[12-14] for deoxycytidine and deoxyadenosine increased the T_m of ODN:DNA duplexes by more than 1°C per modified base. ODNs containing m^5dC and n^2dA were successfully used for different purposes and appeared to be better DNA polymerase primers[6-9] and hybridization probes[5-7] than unmodified ODNs. However, the enhancement of stability of ODN:DNA duplexes proved to be inadequate in some cases[9]. Recently a new tricyclic analogue of deoxycytidine containing novel diazaphenoxazinone heterocycle provided substantially higher ΔT_ms up to 3°C per isolated modified base was described[15]. When the modifications were contiguous, ΔT_m reached 6°C[15]. Extremely high increase in

duplex stability was asigned to extended stacking interaction between adjacent phenoxazine rings. Thus, new tricyclic nucleoside may be promising candidate for different applications including antisense technology. However, H-phosphonate synthon applied by M. D. Matteucci *et al.*[15] could be adapted to common automated protocols based on standard phosphoramidite monomers only in limited extent.

In this paper we describe preparation of 5'-dimethoxytrityl 2,4-diazaphenoxazine-3-one 2'-deoxyriboside 3'-β-cyanoethyl-N,N-diisopropyl phosphoramidite **7** and its use in automated ODN synthesis. The increase in stability of duplexes formed by modified ODNs has been demonstrated to agree with earlier described data[15].

RESULTS AND DISCUSSION

Synthesis of 2,4-diazaphenoxazine-3-one synthon **7** (see **SCHEME 1**) was started from commercially available 5-bromo-2'-deoxyuridine **1**. Starting material was protected as 3',5'-diacetate **2** and then converted to N^4-2-hydroxyphenyl-5-BrdC **3** with phosphorus oxychloride and N-methylimidazole[16]. This procedure provided better results than method described earlier[15]. The product **3** was isolated with 84% yield after the column purification. Noteworthy it was necessary to use the tenfold excess of 2-aminophenol to avoid the side reactions resulted in unusual bridged *bis*-nucleoside **8** formation (see **FIG. 1**) which structure was deduced from NMR and MS data. We concluded that the reaction first led to substitution of *in situ* formed N^4-imidazolium salt[16] by 2-aminophenoxide catalysed by NEt_3 because in absence of strong tertiary base (pyridine alone) the conversion to **3** didn't take place at reasonable time. After addition of NEt_3 two new major spots were detected on TLC: one corresponding to **3** (system B, R_f 0.6) and another slowly disappearing while the reaction proceeded. We explained it as indication that intermediate O^4-2-aminophenyl-dU formed initially slowly rearranged to more thermodynamically stable final product **3**. After deacetylation of **3** with conc. ammonia-ethanol (2:1) compound **4** obtained was refluxed with CsF in ethanol to give desired tricyclic nucleoside **5**. We found that CsF gave better yield and cleaner reaction mixture than KF proposed in ref.[15], presumably due to homogeneous conditions. After 48 h of refluxing, TLC revealed complete disappearance of starting material and formation of highly fluorescent **5**. Product **5** was



i) Ac_2O, NMI, MeCN, 60°C, 1 h; ii) $POCl_3$, NMI, MeCN, -10°C, 15 min; iii) 2-aminophenol, NEt_3, Py, RT, 24 h; iv) conc.NH_4OH, EtOH, RT, 4 h; v) CsF, EtOH, reflux, 48 h; vi) Dmt-Cl, Py, RT, 6 h; vii) $NC(CH_2)_2OP[N(^iPr)_2]_2$, diisopropylammonium tetrazolide, CH_2Cl_2, RT, 4 h.

SCHEME 1

purified, protected as 5'-dimethoxytrityl derivative and converted to 2-cyanoethyl N,N-diisopropyl phosphoramidite **7** by standard methods[17,18]. Phosphoramidite **7** was applied in the automated synthesis of ODNs **9-13** (**TABLE 1**) according to the conventional protocol[17].

The binding affinity of oligonucleotides **9-13** to target DNA was determined by thermal denaturation analysis (see **TABLE 1**). UV absorption of the duplexes formed by the modified ODNs with complementary decamer **14** as a function of temperature was typical for the short duplexes (data not shown). Melting experiments demonstrated that insertion of 2,4-diazaphenoxazine-3-one 2'-deoxyriboside increased T_m by 3-5°C per modified base (depending on its position) as compared with unmodified oligomers,

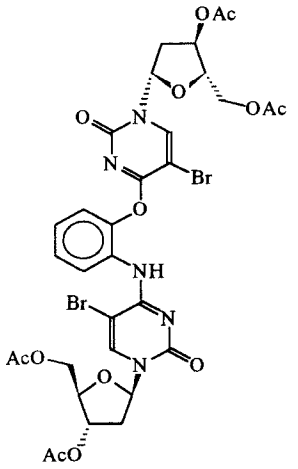


FIG. 1. The structure of the by-product **8**.

TABLE 1. T_ms of duplexes formed by modified ODNs **9-13** containing diazaphenoxazinone deoxyriboside with 5'-**d(GAGAGGGAGA)**-3' (**14**).

No.	ODN sequence	T_m, °C	ΔT_m (°C/mod.)
9	**TCTCCCTCTC**	50	-
10	**TCTCC*CTCTC**	54	4
11	**TC*TCC*CTC*TC**	60	3.3
12	**TCTC*C*C*TCTC**	65	5
13	**TCC*C*C*CTCTC**	48	-

C* indicates the position of 2,4-diazaphenoxazine-3-one 2'-deoxyriboside.

with extra-stabilization effect of contiguous tricycles especially impressive in **12**. We also observed high selectivity in base-pairing of **5** in case of ODN **13** with the order of mismatch discrimination (ΔT_m -17°C) similar to m^5dC:dA mismatch (-16.5°C)[15]. No evidence was found of possible tautomerization of **5** into 3-*H* isomer which could form Watson-Crick pair with dA[15].

In order to evaluate the utility of diazaphenoxazinone deoxyriboside as possible SBO building block several hexamers bearing novel modification in addition to the known 5-(propynyl-1)-dU[1-4] and n^2dA[5-9,12-14] were synthesized and tested in DNA sequencing in the procedure of primer walking by continuous strings[9,19]. Sequencing was successfully primed[20] by the string of 5'-**A*GGA*A*G/C*A*T*C*T*G/T*C*T*C*C*T***-3' (where **T*** designated 5-(propynyl-1)-dU, **A*** - n^2dA and **C*** - **5**). It was the first evidence that DNA polymerase could recognize such heavily base-modified ODN which contained in particular the pair of adjacent tricyclic nucleotides **5** proximal to the oligonucleotide 3'-terminus.

We demonstrated that new phosphoramidite monomer **7** can be used in standard automated DNA synthesis with coupling yields exceeding 98%. The synthesis of compound **7** is efficient and straightforward, and can be reproduced in large scale. We confirmed the previous observation[15] that ODNs containing tricyclic nucleoside **5**

possessed an increased affinity to complementary sequences and for the first time demonstrated that diazaphenoxazinone **5** containing ODNs were consistent with the common procedures of polymerase elongation and DNA sequencing. SBOs which incorporate tricyclic nucleoside **5** could be promising agents for application in further molecular biological experiments.

EXPERIMENTAL

All reagents and solvents of highest available purity were purchased from Sigma, Aldrich, Fluka and Merck. Pyridine, triethylamine, N-methylimidazole and acetonitrile were distilled from calcium hydride. Ethanol was distilled first from sodium and next from magnesium. Phosphorus oxychloride was used freshly distilled and stored under argon. 2-Aminophenol was recrystallized from boiling water and dried *in vacuo* over P_2O_5 prior to use. Cesium fluoride was dried *in vacuo* at 80°C.

TLC analysis was performed on Kieselgel 60 F254 plates (Merck). The spots were visualized under UV source, sugar-containing spots were stained upon spraying with 1M cysteine in 50% sulfuric acid with subsequent heating. The presence of 4,4'-dimethoxytrityl group was detected with trifluoroacetic acid vapours. TLC plates were eluted in the following systems: A (MeCN), B (EtOAc), C (MeCN-H_2O 9:1), D ($CHCl_3$-EtOH-NEt_3 85:5:0.5), E ($CHCl_3$-EtOH-NEt_3 90:10:0.5).

Column chromatography was performed on silica gel 60 (0.040-0.063 mm, Merck) with UV monitoring (UVICORD 83000, LKB). Analytical HPLC was performed on Separon SGX C-18 RP-column (Tessek) by gradient of acetonitrile in water. The NMR spectra were recorded on Varian VXR-400 NMR spectrometer using either DMSO-D_6 or CD_3CN as solvent and TMS as internal standard.

3',5'-Diacetyl-5-bromo-2'-deoxyuridine (2). 5-Bromo-2'-deoxyuridine **1** (1 g, 3.26 mmol), dried by co-evaporation with pyridine, was dissolved in 20 ml of acetonitrile, acetic anhydride (1.85 ml, 19.5 mmol) and N-methylimidazole (0.533 ml, 6.7 mmol) were added and the solution was kept stirring at 60°C. After complete conversion of starting material to **2** on TLC (~1 h, system A, R_F (**2**) 0.8) the mixture was cooled to 20°C and 2 ml of methanol were added. After 30 min of additional stirring the solution was evaporated to small volume and re-evaporated with 10 ml of ethanol. The residue was poured in water (100 ml) and extracted with $CHCl_3$ (3x20 ml). Organic phase was

dried by Na_2SO_4 and evaporated to dryness. Almost pure **2** (1.251 g) was isolated in 98% yield. Mass spectrum (m/z): 392 and 390 (M^+), 259, 257, 201, 176, 174, 141, 99, 81, 68, 53.

3',5'-Diacetyl-N^4-(2-hydroxyphenyl)-5-bromo-2'-deoxycytidine (3). The solution of N-methylimidazole (2.54 ml, 32 mmol) in 7.5 ml of MeCN was cooled to -10°C under argon. Then the solution of $POCl_3$ (0.887 ml, 9.6 mmol) in 7.5 ml of MeCN was added dropwise with stirring and reaction mixture was kept at -10°C for 15 min. The solution of **2** (1.251 g, 3.2 mmol), dried by repeated co-evaporations of dry MeCN, in 7 ml of acetonitrile was slowly added and then the reaction mixture was allowed to warm to 20°C. The solution of 2-aminophenol (3.488 g, 32 mmol), dried twice by co-evaporation of pyridine, in 10 ml of pyridine and 4.452 ml of triethylamine was added and the reaction mixture was stirred during 4 h at 20°C and left overnight at 5°C. After TLC revealed the completion of reaction (system B, R_F (**3**) 0.6), the mixture was diluted with 5 ml of water and evaporated to small volume. The residue was re-evaporated with 10 ml of EtOH three times, dissolved in 50 ml of $CHCl_3$, washed with aq. $NaHCO_3$ (3x25 ml), saturated aq. NaCl (25 ml), dried by Na_2SO_4 and evaporated to small volume. The residue was purified by flash chromatography on silica gel column eluted with ethanol gradient (0-10%) in $CHCl_3$. Product **3** (1.3 g) was isolated in 84% yield. ^{1}H-NMR (DMSO-D_6; δ, ppm): 2.08 (d, 6H, CH_3CO), 2.37-2.44 (m, 2H, 2'-H_{a-b}), 4.23 (m, 1H, 4'-H), 4.29 (m, 2H, 5'-H_{a-b}), 5.20 (m, 1H, 3'-H), 6.15 (t, 1H, 1'-H), 6.83-6.87, 6.92-6.94, 7.0-7.04, 8.15 (m, 4H, ArH), 8.09 (s, 1H, 6-H), 8.40 (s, 1H, NH), 10.20 (s, 1H, OH).

3',5'-Diacetyl-N^4-2-(3',5'-diacetyl-β-2'-deoxyribofuranosyl-2-oxo-5-bromopyrimidin-4-yloxy)phenyl-5-bromo-2'-deoxycytidine (8). The solution of of N-methylimidazole (0.794 ml, 10 mmol) in 5 ml of acetonitrile was cooled to -10°C under argon. Then $POCl_3$ (0.28 ml, 3 mmol) was added dropwise with stirring and resulted yellow suspension was kept at -10°C for 15 min. The solution of **2** (0.391 g, 1 mmol) in 4 ml of acetonitrile was slowly added and then the reaction mixture was allowed to warm to 20°C with stirring until TLC revealed complete disappearance of starting material and formation of product with R_F 0 (system B). The solution of 2-aminophenol (0.327 g, 3 mmol) in 3 ml of pyridine was added and the reaction mixture was left at 20°C. After 48 h TLC revealed only traces of **3** along with almost undiminished zero mobility spot. To bring the reaction to completion triethylamine (0.42 ml, 3 mmol) was added and

after 4 h two major spots were detected on TLC (system B, R_F (**3**) 0.6, R_F (**8**) 0.28). After standing overnight the mixture was evaporated to dryness, dissolved in 20 ml of $CHCl_3$, washed with aq. $NaHCO_3$ (3x15 ml), saturated aq. NaCl (10 ml), dried by Na_2SO_4 and evaporated to small volume. The residue was purified by chromatography on silica gel column eluted by increasing amounts of ethanol (0-10%) in $CHCl_3$. After evaporating of combined appropriate fractions two products were isolated: **3** (0.162 g, 33.6%) and **8** (76 mg, 17.8%). ^{1}H-NMR spectrum of **8** (DMSO-D_6; δ, ppm): 2.09 (d, 12H, CH_3CO), 2.35-2.49 (m, 4H, 2'-H_{a-b}), 4.23 (m, 2H, 4'-H), 4.28-4.32 (m, 4H, 5'-H_{a-b}), 5.16-5.23 (m, 2H, 3'-H), 6.04-6.10 (m, 2H, 1'-H), 7.34-7.42, 7.62-7.67 (m, 4H, ArH), 8.02, 8.28 (2s, 2H, 6-H), 8.65 (s, 1H, NH). MS (m/z): 376, 331, 281, 264, 238, 202, 185, 160, 140, 98, 81, 53.

N^4-2-Hydroxyphenyl-5-bromo-2'-deoxycytidine (4). Compound **3** (1.3 g) was suspended in 15 ml of ethanol, and 30 ml of 25% ammonium hydroxide was added. Mixture was stirred for 4 hours at 20°C until the solution became clear, then it was evaporated to dryness. The residue was rinsed with $CHCl_3$ and water and dissolved in EtOH. The solution was passed through a short silica gel column and evaporated. Product **4** (1 g) was isolated (TLC, system C, R_F 0.6) in almost quantitative yield. ^{1}H-NMR (DMSO-D_6; δ, ppm): 2.03-2.22 (m, 2H, 2'-H_{a-b}), 3.56-3.69 (m, 2H, 5'-H_{a-b}), 3.80-3.84 (m, 1H, 4'-H), 4.22-4.28 (m, 1H, 3'-H), 5.17-5.24 (m, 2H, 3'-OH, 5'-OH), 6.10 (t, 1H, 1'-H), 6.72-7.04, 8.16-8.20 (m, 4H, ArH), 8.45 (s, 1H, 6-H).

2,4-Diazaphenoxazine-3-one 2'-deoxyriboside (5). Compound **4** (1.120 g, 2.8 mmol), dried by repeated co-evaporations of absolute ethanol and 4.5 g (28 mmol, 10 eq) of CsF was suspended in 30 ml of absolute ethanol and refluxed for 48 h. Reaction was monitored by TLC (system C, R_F (**5**) 0.5). When the reaction was complete, the mixture was evaporated to dryness, and the residue was rinsed with water. Then the residue was dissolved in EtOH and passed through a short column with silica gel. Resulting solution was evaporated and 0.6 g (68%) of **5** was isolated. ^{1}H-NMR (DMSO-D_6; δ, ppm): 1.95-2.12 (m, 2H, 2'-H_{a-b}), 3.57 (m, 2H, 5'-H_{a-b}), 3.78 (m, 1H, 4'-H), 4.23 (t, 1H, 3'-H), 5.12 (t, 1H, 5'-OH), 5.24 (d, 1H, 3'-OH), 6.12 (t, 1H, 1'-H), 6.77-6.86 (m, 4H, ArH), 7.55 (s, 1H, 6-H). MS (m/z): 317 (M^+), 202, 201, 173, 146, 119, 118, 98, 90, 81, 76.

5'-O-4,4'-Dimethoxytrityl-2,4-diazaphenoxazine-3-one 2'-deoxyriboside (6). Compound **5** (0.6 g, 1.73 mmol), dried by repeated co-evaporations of dry pyridine, was dissolved in 15 ml of pyridine and 4,4'-dimethoxytrityl chloride (0.5 g, 1.39 mmol)

was added. The solution was stirred for 4 hours at 20°C and additional amount (0.205 g, 0.61 mmol) of 4,4'-dimethoxytrityl chloride was added. The reaction was monitored by TLC (system E, R_F (**6**) 0.61). After two hours of stirring 0.5 ml of CH_3OH was added, the solution was evaporated and dissolved in 25 ml $CHCl_3$. Organic phase was washed with 5% aq. $NaHCO_3$ (2x15 ml), saturated aq. NaCl (10 ml) and concentrated *in vacuo*. The residue was purified by chromatography on silica gel column eluted by increasing amounts (0-15%) of ethanol in $CHCl_3$-NEt_3 (200:1). Pure **6** (0.5 g) was isolated in 43% yield. ^{1}H-NMR (DMSO-D_6; δ, ppm): 2.14-2.18 (m, 2H, 2'-H_{a-b}), 3.10-3.13, 3.24-3.29 (m, 2H, 5'-H_{a-b}), 3.72 (d, 6H, CH_3O), 3.89 (m, 1H, 4'-H), 4.28 (m, 1H, 3'-H), 5.31 (d, 1H, 3'-OH), 6.12 (t, 1H, 1'-H), 6.54-6.58, 6.77-6.92, 7.20-7.43 (m, 18H, ArH and 6-H).

5'-O-4,4'-Dimethoxytrityl-2,4-diazaphenoxazine-3-one 2'-deoxyriboside 3'-β-cyanoethyl-N,N-diisopropyl phosphoramidite (7). Diisopropylammonium tetrazolide (0.16 g) was added to solution of **6** (476 mg, 0.62 mmol) in 10 ml of CH_2Cl_2. β-Cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (0.24 ml, 0.74 mmol) was added with stirring. The reaction mixture was stirred for 4 h. The reaction was monitored by TLC (system D, R_F (**7**) 0.61). Then it was diluted by 15 ml of CH_2Cl_2, washed with saturated aq. $NaHCO_3$ (2x15 ml), saturated aq. NaCl (10 ml), dried by Na_2SO_4 and evaporated. The residue was dissolved in 1 ml of CH_2Cl_2 and precipitated into 200 ml of cold (-20°C) pentane. The precipitate was collected by filtration, washed with cold pentane and dried *in vacuo*. 0.605 g (96%) of **7** was obtained. The product appeared to be sufficiently pure (~95%, as judged by ^{31}P-NMR) and was used in automated synthesis without column purification. ^{31}P-NMR (CD_3CN-MeCN 1:1; 80% aq. H_3PO_4; δ, ppm): 151.64 (unresolved pair of P-chiral diastereomers).

Oligonucleotide synthesis. ODNs **9-14** and set of hypermodified hexamers were synthesized using commercial β-cyanoethyl phosphoramidites and LCAA-CPG supports (A,C,G,T were from Sigma, 5-(propynyl-1)-dU was from Glen Research) on ASM-102U DNA synthesizer (Biosan Ltd, Russia). 5'-Dimethoxytrityl-2,6-*bis*-N,N-dimethylacetamidinopurine 2'-deoxyriboside 3'-β-cyanoethyl-N,N-diisopropyl phosphoramidite was synthesized as described[9]. After cleavage and deprotection by conc. ammonia at 20°C for 20 h, Dmt-on ODNs were purified by RP-HPLC on Separon C-18 column with subsequent removal of Dmt group by 80% aq. AcOH and

desalting. The purity of oligonucleotides was checked by analytical RP-HPLC (data not shown).

Melting experiments. T_ms were determined for duplexes formed by modified ODNs and complementary decamer 5'-**d(GAGAGGGAGA)**-3' (**14**) by tracing melting curves in TM buffer (140 mM KCl, 5 mM Na_2HPO_4, 5 mM $MgCl_2$, pH 7.0) on Perkin-Elmer 552 UV/VIS spectrophotometer at 265 nm.

Sequencing. DNA sequencing by primer walking with hypermodified hexamers was carried out essentially as described previously[9] using Sequenase version 2.0 (USB) according to 2-step Sequenase protocol with [α-^{33}P]dATP[19,21]. Double-stranded recombinant plasmid pGEM 7zf(+) used as a template was denatured by alkaline method. An equilibrium mixture contained 0.5 μg DNA, 3 μg SSB protein and 50 pmol of each hexamer in standard buffer[21].

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