Spermine Moiety Attached to the C-5 Position of Deoxyuridine Enhances the Duplex Stability of the Phosphorothioate DNA/Complementary DNA and Shows the Susceptibility of the Substrate to RNase H

Tomohisa MORIGUCHI, Hideaki SAKAI, Hideo SUZUKI, and Kazuo SHINOZUKA*

Graduate School of Engineering, Gunma University; 1–5–1 Tenjincho, Kiryu, Gunma 376–8515, Japan.
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Novel phosphorothioate-modified oligodeoxynucleotides (S-ODNs) containing a deoxyuridine derivative bearing a spermine moiety at the C-5 position were synthesized. The study of the thermal stability and the thermodynamic stability showed that the modified S-ODNs have been able to form the stable duplexes with the complementary DNA. It was also found that the duplex composed of the modified S-ODN and its complementary RNA strand is the substrate for *Escherichia coli* RNase H, and the cleavage of the RNA strand by the enzyme was almost similar as in the case of the unmodified one.

Key words nucleic acid; antisense DNA; phosphorothioate DNA; spermine; duplex stability

Phosphorothioate-modified oligodeoxynucleotides (S-ODNs) have attracted considerable attention because of their potential as antisense inhibitors of gene expression. The effectiveness of an antisense oligonucleotide in inhibiting gene expression depends on many factors, of which three that have been typically evaluated are: (i) the nuclease resistance of the antisense oligonucleotides, (ii) the thermal stability of the antisense oligonucleotides-RNA hybrid formed with the antisense oligonucleotides, and (iii) the ability of RNase H to degrade the RNA of the hybrid.[1—5] One major problem in the use of S-ODNs as antisense molecules is their low thermal stability to the complementary RNAs. In order to overcome this problem, several types of modification such as introduction of a cationic residue,[6—15] enhancement of the stacking interaction[2,16—20] and fixation of the sugar conformation[21—25] were explored. Especially, cation-conjugation of oligonucleotide makes amphiphilic molecules to reduce the net negative charge on oligonucleotides, and, therefore, it would bring about improvement of the cell permeability. As the result, it is expected that cation-conjugation enables oligonucleotides to be a better antisense therapeutic agent.

Previously, we have reported the synthesis and physicochemical as well as biological properties of novel S-ODNs containing the branched polyamine-tethering nucleoside analogs.[26,27] It was disclosed that the S-ODNs possess greatly enhanced selective hybridization ability to the complementary RNA and the susceptibility of the DNA/RNA heteroduplex to RNase H hydrolysis. Moreover, such S-ODN exhibited potent antisense activity targeted to the *rev* gene of HIV-1, even with a short oligonucleotide length (15-mer), similar to the case of the unmodified longer (27-mer) S-ODN.[28] These results prompted us to investigate further polyamine-conjugation of S-ODN. In this paper, we would like to report the synthesis of a novel phosphorothioate DNA containing a spermine-tethering deoxyuridine derivative in the place of thymidine. Spermine is a biogenic polyamine,[30] and is present at millimolar total concentration in the nuclei of eukaryotic cells where it stabilizes the double-stranded structure of DNA against denaturation.[30—33] Several properties of the spermine-conjugated S-ODNs, such as thermodynamic properties and susceptibility to the substrate for the RNase H-mediated RNA cleavage are also reported.

Results and Discussion

Oligodeoxynucleotides containing a C-5 spermine-tethering deoxyuridine derivative were synthesized on a DNA synthesizer with the modified procedure previously reported.[35] The introduction of the spermine moiety was performed by the post-synthetic modification method by the treatment of the support-bound oligonucleotides with excess amounts of spermine. Two types of modified S-ODNs (ODN-1, 2) were prepared by the automated DNA synthesizer (Fig. 1). The average coupling yield of 5-(methoxycarbonylmethyl)-2′-deoxyuridine phosphoramidite derivative was 98% using elongated coupling time of 360 s. Tetraethylthiuramdisulfide (TETD) was used as a sulfurization reagent and the sulfurization time was 900 s. The fully protected S-ODNs linked to the solid support were treated with 2.5 M spermine/EtOH at 55 °C for 48 h in order to introduce the spermine moiety into the C-5 position of the deoxyuridine residue and release from the CPG support, as well as deprotection of the base and phosphate protecting groups. The phosphoramidite derivative of the *N*-acetylated 2′-deoxycytidine derivative was used instead of the standard *N*-benzoyl derivative to prevent the possible transamination at the C-4 position of the cytosine base during the treatment.[35] Consequently, no C-4-sperminated deoxycytidine derivative was observed in the experiments. Additionally, the complete removal of the isobutyryl group of the guanine moiety was confirmed in an independent experiment by the treatment of *N*-2-isobutyryl-2′-deoxyguanosine under the same conditions. The HPLC analysis showed that the deprotected product gave a single peak and gave the same retention time on 2′-deoxyguanosine. It should be

ODN-N: 5′-TCG TCG CTG TCT CCG-3′
ODN-1: 5′-TCG TCG C**U**G TCT CCG-3′
ODN-2: 5′-TCG **U**CG CTG TC**U** CCG-3′

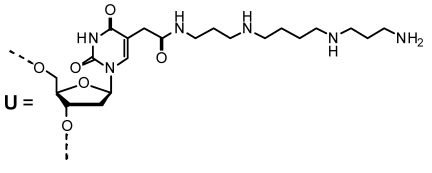

Fig. 1. Sequences of S-ODNs Synthesized
U = 5-(sperminocarbonylmethyl)-2′-deoxyuridine.

* To whom correspondence should be addressed. e-mail: sinozuka@chem-bio.gunma-u.ac.jp
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noted that although the spermine treatment of the support-bound S-ODNs was effective for the release and deprotection of the oligomers, the released oligomers were quite insoluble into the spermine/EtOH solution. Therefore, the treatment of the residue with an appropriate buffer to recover the oligomers was required (see Experimental). The S-ODNs having a 5′-DMTr group were purified by reversed-phase HPLC after removal of the excess amounts of spermine by gel filtration. The isolated compound was treated with 20% acetic acid by the usual procedure to remove the DMTr group followed by desalting to give the desirable oligomers with satisfactory yields. The structure of the purified S-ODNs was analyzed by the ESI-mass spectroscopy, since the synthesized S-ODNs were not able to be analyzed by the nucleoside composition analysis because of the nuclease resistance of S-ODN. The observed ion peaks were identified with the predicted values by calculation (Table 1). Furthermore, we tried to synthesize the S-ODN bearing three spermine-tethered deoxyuridine derivative, however, the unexpected aggregation of S-ODN occurred after the introduction of the spermine moiety and no further purification carried out.

Stable duplex formation of the antisense agent with the target mRNA is considered as a major important factor in antisense therapy. Thus, we examined the thermal stability of the duplexes on phosphorothioate DNA and the complementary oligodeoxynucleotide. For the application to the antisense therapy, the duplex forming properties of polyamine-modified phosphorothioate DNA must be investigated by using RNA as a complementary strand, however, it seems that the several properties of the modified duplex were almost equal to that of complementary DNA instead of RNA. The UV melting profiles of all duplexes exhibited helix-to-coil transitions whose shapes were similar to that of the unmodified duplex. The T_m values were determined from the maximum values of the first deviation of the UV melting profiles and are shown in Table 2.

The T_m values increased with the duplex concentration, and the relationship between the T_m values and the duplex concentration is given by the van't Hoff equation which may be expressed in logarithmic form.[36] The T_m values of the duplexes containing C-5 substituted deoxyuridine derivatives were higher than that of the unmodified duplex at lower concentration ($<0.3\,\mu$M); to the contrary, at higher concentrations ($>0.6\,\mu$M), the unmodified heteroduplex was more stable than the modified one. The introduction of the spermine moiety at the C-5 position of the deoxyuridine derivative was found to stabilize the S-ODN/DNA heteroduplex under the physiological conditions, *i.e.* nanomolar concentrations. It seems that the stabilizing effects are due to the neutralization of polyanionic charges on the internucleotide phosphate groups, which is a disadvantageous factors for the formation of double-stranded oligonucleotides, by the introduction of the polycation of the polyamine moiety. The reason for the inversion phenomenon of the T_m values between modified duplexes and unmodified ones is impossible to elucidate only by comparison of the T_m values among the different duplex concentrations.

The magnitude of the stabilization effects was greater than in the case in which the introduction of spermine was carried out at the C-4 position of a deoxycytidine derivative,[10] but was lower than at the C-2 position of the deoxyinosine derivative.[6] This could be attributed to the fact that spermine introduced at the C-2 position of the purine base exists in the minor groove of double helical DNA and forms additional hydrogen bonds between the protonated amine groups and N^3 of purine bases or O-2 of pyrimidine bases, in addition to the possible neutralization of negative charges on the backbone.[40] On the other hand, the spermine moiety attached at the C-5 position of the deoxyuridine derivative exists in the major groove of the heteroduplex, and, therefore, additional stabilization effects, such as hydrogen bonding described above, may not exist. No marked influence of the existence of the spermine moiety on the overall structure of the heteroduplexes was observed by the CD measurement (data not shown). The result indicates that no structural perturbation was induced by the existence of the polyamine molecule. This is contrary to the fact that the externally added spermine alters the DNA duplex conformation from B-type to A-type[37]; the spermine introduced to the C-5 position of the deoxyuridine does not exhibit such effect.

Next, in order to investigate the role of the spermine moiety on the duplex stability, we determined the thermodynamic parameters for the formation of three types of heteroduplexes. The thermodynamic parameters ($\Delta H°$, $\Delta S°$, and $\Delta G°$) of the duplexes were determined by calculations based on the slope of a $1/T_m$ *versus* $\ln(C_T/4)$ plot, where C_T is the total concentration of single strands (Fig. 2). All plots showed to be linear over the entire 100-fold range in concen-

Table 1. Isolated Yields and ESI-Mass Spectra of S-ODNs

	Isolated yield (%)	Observed	Calculated
S-ODN-1	37.6	4964.08	4964.21
S-ODN-2	31.1	5192.33	5192.14

Table 2. T_m Values for the Duplexes of S-ODNs/cDNA

Concentration	S-ODN-N (°C)	S-ODN-1 (°C)	S-ODN-2 (°C)
10 μM	61.3	58.3	56.2
5 μM	59.3	57.5	56.0
2.5 μM	57.5	56.3	54.2
1.3 μM	56.1	55.4	53.5
0.63 μM	54.9	54.1	52.9
0.31 μM	52.6	53.2	52.3
0.16 μM	51.4	52.6	51.6

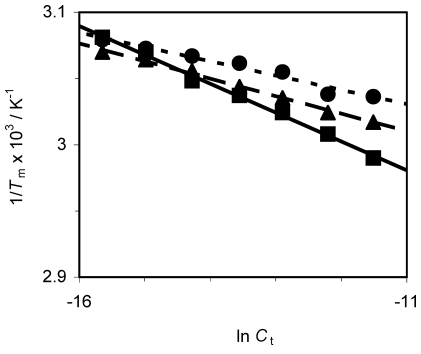

Fig. 2. The van't Hoff Plots of S-ODNs/cDNA Duplexes

Square plot and solid line show S-ODN-N/cDNA duplex. Triangle plot and broken line show S-ODN-1/cDNA duplex. Circle plot and dotted line show S-ODN-2/cDNA duplex.

Table 3. Thermodynamic Parameters for the Duplexes of S-ODNs/cDNA

ODNs	$\Delta H°$ (kcal/mol)	$\Delta\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol·K)	$\Delta\Delta S°$ (cal/mol·K)	$\Delta G°_{298}$ (kcal/mol)	$\Delta\Delta G°_{298}$ (kcal/mol)
S-ODN-N	−92.0		−249.5		−17.6	
S-ODN-1	−148.2	−56.2	−421.3	−172.2	−22.5	−4.9
S-ODN-2	−183.4	−91.4	−531.3	−281.8	−25.0	−7.4

trations, and interestingly, all lines showed intersect between the observed ranges, since no such intersection is observed generally within the usual oligonucleotide concentrations. This observation seems to be characteristic for effect by the introduction of spermine moiety into S-ODNs.

$\Delta G°$ values were gradually decreased with the increase in the number of the spermine moieties as those are shown in Table 3. Thus, the spermine moiety indeed acts as the stabilizer of the double helixes, and the stabilization effects are enhanced additively according to the number of spermine moieties. $\Delta H°$ values behaved similarly to that of $\Delta G°$, indicating the spermine moiety stabilizes the duplex mainly through neutralization of the phosphate anions by the protonated polyamine species. And the favorable effects in terms of $\Delta H°$ values of the spermine are consistent with the increasing number of the cations of protonated spermine moieties. In contrast, the spermine moiety destabilizes the duplex in terms of entropy, since $\Delta S°$ decreased with the increase of the number of spermine moieties. This could be attributed to the fact that the introduction of the spermine polycations causes DNA to assume a rigid coiled structure because of the electrostatic interaction between polyamines and phosphates leading to the constrained duplex structure.

One disadvantage of the antisense therapy using phosphorothioate oligonucleotides is the low accuracy in recognition of the target RNA sequence. Accuracy in the recognition of the target RNA sequences is one of the most necessary properties required of the antisense agents. In general, the most indistinguishable mismatch base pair is the G–T base pair, which enables the formation of a relatively stable Wobble-type base pair. Accurate G–T mismatch base pair recognition could contribute to preventing the non-target specific inhibition of the gene expression. We have reported previously a case of the phosphorothioate oligodeoxynucleotides containing a C-5 trisamine modified deoxyuridine derivative.[28)]

We compared the difference in the T_m values between fully matched DNA/S-ODN duplexes and the duplexes containing the single G–T mismatch sequence (M1-DNA and M2-DNA) (Table 4). It should be noted that the mismatched guanine base was placed at the opposite position of the deoxyuridine derivative attaching the spermine moiety at the C-5 position. As is clear from Table 4, all duplexes showed the apparent mismatch penalty by the introduction of the G–T(**U**) base pairs compared to the canonical A–T(**U**) base pairs. However, no apparent enhancement of the mismatch penalty was observed by the introduction of the spermine moiety under these conditions.

Comparison of the thermodynamic parameters between the unmodified duplexes and the spermine-modified duplexes (Table 5), however, gave very interesting results. In the case of the unmodified DNA/S-ODN duplexes, little destabilization was observed on both $\Delta H°$ and $\Delta S°$ by the introduction

Table 4. T_m Values of the Mismatched Duplexes of S-ODNs/cDNA[a)]

	cDNA	M1-DNA[b)] (ΔT_m) (°C)	M2-DNA[c)] (ΔT_m) (°C)
S-ODN-N	57.5	53.1 (−4.4)	52.2 (−5.3)
S-ODN-1	56.3	52.6 (−3.7)	—
S-ODN-2	54.2	—	47.3 (−6.9)

a) Each oligonucleotide concentration is 2.5 μM. 2.5 μM each oligonucleotides, 10 mM sodium phosphate buffer. *b*) M1-DNA=d(CGG AGA CGG CGA CGA). *c*) M2-DNA=d(CGG AGA CAG CGG CGA).

Table 5. Thermodynamic Parameters for the Mismatched Duplexes of S-ODNs/cDNA

Entry	Duplexes	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol·K)	$\Delta G°_{298}$ (kcal/mol)
1	S-ODN-N/cDNA	−92.0	−249.5	−17.6
2	S-ODN-N/M1-DNA[a)]	−90.5	−249.0	−16.3
3	S-ODN-1/cDNA	−148.2	−421.3	−22.5
4	S-ODN-1/M1-DNA[a)]	−79.3	−215.2	−15.1

a) M1-DNA=d(CGGAGACGGCGACGA).

of the G–T mismatch base pair at the central position of the DNA/S-ODN duplex. (entry 1 and 2) However, a much more drastic destabilization in terms of $\Delta H°$ was observed by the introduction of the G–**U** mismatch base pair for the spermine-modified duplexes (entry 3 and 4). In the case of entry 4, $\Delta H°$ was reduced to almost half as compared to that of the fully matched duplex (entry 3). Although $\Delta S°$ showed the completely opposite behavior, overall effects of both $\Delta H°$ and $\Delta S°$ become unfavorable (increase in $\Delta G°$) for the duplex stability. Thus, the introduction of the spermine bearing the deoxyuridine derivative to S-ODN is effective for the discrimination of the mismatched DNA/S-ODN heteroduplexes. It should be noted that we could not observe any drastic change in the CD spectra of the heteroduplexes (data not shown). Therefore, we can not present a conclusive specific behavior of the thermodynamic parameters at this moment.

It has been postulated that the activity of antisense ODNs is due, at least in part, to the cleavage of the target RNA strand of a DNA–RNA duplex by RNase H.[38)] Therefore, we next examined the susceptibility of the duplexes of the S-ODNs and their complementary RNA strands by the method according to the procedure reported by Stein *et al.* as shown in Fig. 3.[39)] The gradual increase of the observed absorption in Fig. 3 indicates the proceeding of the cleavage of the complementary RNA strand by the RNase H activity.

RNase H activity of duplexes of S-ODNs with complementary RNA 15 mer is almost similar among all duplexes. No negative effects were observed by the introduction of the spermine moiety attached to the C-5 position of a deoxyuridine derivative. It is predicted that the positively charged

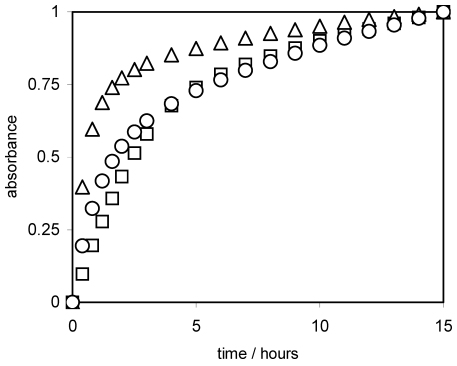

Fig. 3. Relative Absorbance of Time Course of the Treatment of S-ODNs/cRNA Duplexes with RNase H

Square plot shows S-ODN-N/cRNA duplex. Triangle plot shows S-ODN-1/cRNA duplex. Circle plot shows S-ODN-2/cRNA duplex.

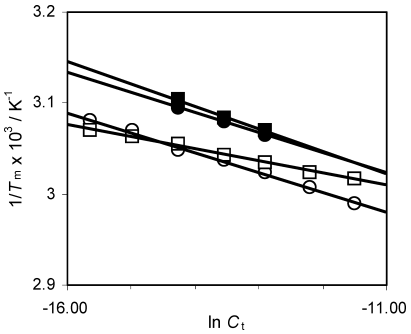

Fig. 4. The van't Hoff Plots of S-ODNs/Matched and Mismatched Duplexes

Circle plot shows S-ODN-N/cDNA duplex. Filled circle plats shows S-ODN-N/M1-DNA duplex. Square plot shows S-ODN-1/cDNA duplex. Filled square plats shows S-ODN-1/M1-DNA duplex.

polyamine moiety may affect the binding of the heteroduplex with RNase H, because of the electrostatic repulsion of the positive charge with the basic region of the nucleic acid binding site of the enzyme.[40] However, no distinct difference in the susceptibility to RNase H was observed between the unmodified S-ODN and the modified S-ODNs bearing the spermine moieties. This could be attributed to the fact that the minor groove of the DNA/RNA duplex is a major determinant of the binding of the RNase H.[41,42] In Addition, no information about the cleavage site of RNA strand by RNase H was given in these experiments.

Experimental

General Procedures HPLC analysis was carried out using a Wakosil 5C18 column (Wako Pure Chemicals, 4×250 mm) on a JASCO880-SC system controller with a JASCO 880-PU pump unit and JASCO 875-UV UV detector. Oligonucleotide synthesis was carried out using a Applied Biosystems DNA synthesizer 392A. ESI-TOF mass spectra were obtained on a Perkin Elmer Sciex API-100.

Synthesis of S-ODNs S-ODNs were synthesized on a DNA/RNA synthesizer by the modified phosphoramidite method. The coupling time of the 5-methoxycarbonylmethyl-2′-deoxyuridine phosphoramidite was extended to 360 s. Tetraethylthiuramdisulfide (TETD) was used as a sulfurization reagent, and the reaction time with TETD treatment was 900 s. The introduction of spermine, the deprotection and the cleavage from CPG support were carried out by the treatment with 2.5 M spermine/EtOH at 55 °C for 48 h. After removal of the excess amounts of spermine by gel filtration on a Sephadex G-25 column, the S-ODNs having the 5′-DMTr group were purified by reversed-phase HPLC on a column using 50 mM TEAA (pH 7.0) with a gradient of 15—40% acetonitrile in 25 min. The isolated compound was treated with 20% acetic acid by the usual procedure to remove the DMTr group followed by desalting on a Sephadex G-25 column. The eluents were collected and lyophilized to give the pure S-ODNs. The determination of their structure was performed by the observation with ESI-TOF mass spectra.

T_m Measurements UV absorbance was measured with a Hitachi UV-3000 spectrophotometer equipped with a Hitachi Temperature Controller SPR-10. The solution temperature in a cuvette was measured directly with a temperature data controller AM-7002 (Anritsu Meter Co., Ltd.). Absorbance and temperature data were recorded on NEC personal computer PC-9821. The rate of heating or cooling was 0.5 °C/min. T_m values were obtained in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl and 1 mM EDTA.

Thermodynamic Parameters The thermodynamic parameters of all duplexes were determined by plotting the reciprocal of the melting temperature, T_m^{-1}, *versus* $\log(C_t/4)$, where C_t is the total concentration of strands.[43]

$$T_m^{-1} = (2.303R/\Delta H)/\log(C_t/4) + \Delta S/\Delta H$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

RNase H Hydrolysis RNase H, RNase inhibitor and RNA were obtained from BRL, Wako Chemicals Co., and GENSET Co., respectively. RNA and S-ODN were pre-incubated at room temperature for 12 h in a buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 50 mM NaCl, and 5% sucrose, after heating at 90 °C for 5 min. To this mixture were further added RNase H (10 units), 2 mM DTT, and RNase inhibitor (200 units). The absorption at 260 nm was continuously monitored at 25 °C for 1000 min.

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Reference

1) Inoue H., Hayase Y., Iwai S., Ohtsuka E., *FEBS Lett.*, **215**, 327—330 (1987).
2) Wagner R. W., Matteucci M. D., Lewis J. G., Gutierrez A. J., Moulds C., Froehler B. C., *Science*, **260**, 1510—1513 (1993).
3) Mercola D., Cohen J. S., *Cancer Gene Ther.*, **2**, 47—59 (1995).
4) Eckstein F., *Antisense Nucleic Acid Drug Dev.*, **10**, 117—121 (2000).
5) Barnes T., Turner D., *J. Am. Chem. Soc.*, **123**, 4107—4118 (2001).
6) Potier P., Abdennaji A., Behr J.-P., *Chem. Eur. J.*, **6**, 4188—4194 (2000).
7) Haginoya N., Ono A., Nomura Y., Ueno Y., Matsuda A., *Bioconjugate Chem.*, **8**, 271—280 (1997).
8) Ueno Y., Kumagai I., Haginoya N., Matsuda A., *Nucleic Acids Res.*, **25**, 3777—3782 (1997).
9) Ueno Y., Mikawa M., Matsuda A., *Bioconjugate Chem.*, **9**, 33—39 (1998).
10) Prakash T. P., Barawkar D. A., Kumar V., Ganesh K. N., *Bioorg. Med. Chem. Lett.*, **4**, 1733—1738 (1994).
11) Godzina P., Adrych-Rozek K., Markiewicz W. T., *Nucleosides Nucleotides*, **18**, 2397—2414 (1999).
12) Heydtek L. E., Zhou H.-Q., Dande P., Gold B., *J. Am. Chem. Soc.*, **120**, 12165—12166 (1998).
13) Lin K.-Y., Matteucci M. D., *J. Am. Chem. Soc.*, **120**, 8531—8532 (1998).
14) Sund C., Puri N., Chattopadhyaya J., *Tetrahedron*, **52**, 12275—12290 (1996).
15) Sund C., Puri N., Chattopadhyaya J., *Nucleosides Nucleotides*, **16**, 755—760 (1997).
16) Gutierrez A. J., Terhorst T. J., Matteucci M. D., Froehler B. C., *J. Am. Chem. Soc.*, **116**, 5540—5544 (1994).
17) Buhr C. A., Wagner W. R., Grant D., Froehler B. C., *Nucleic Acids Res.*, **24**, 2974—2980 (1996).
18) Seela F., Ramzaeva N., Zulauf M., *Nucleosides Nucleotides*, **16**, 963—966 (1997).
19) Balow G., Mohan V., Lesnik E. A., Johnston J. F., Monia B. P., Acevedo O. L., *Nucleic Acids Res.*, **26**, 3350—3357 (1998).
20) Lin K. Y., Jones R. J., Matteucci M. D., *J. Am. Chem. Soc.*, **117**, 3873—3874 (1995).
21) Koshkin A. A., Nielsen P., Meldgaard M., Rajwanshi V. K., Singh S. K., Wngel J., *J. Am. Chem. Soc.*, **120**, 13252—13253 (1998).

22) Obika S., Morio K., Nanbu D., Imanishi T., *Chem. Commun.*, **1997**, 1643—1644 (1997).
23) Tarköy M., Bolli M., Leumann C., *Helv. Chim. Acta*, **77**, 716—744 (1994).
24) Steffens R., Leumann C., *Helv. Chim. Acta*, **80**, 2426—2439 (1997).
25) Renneberg D., Leumann C. J., *J. Am. Chem. Soc.*, **124**, 5993—6002 (2002).
26) Sawai H., Makamura A., Sekiguchi S., Yumoto K., Endo M., Ozaki H., *J. Chem. Soc. Chem. Commun.*, **1994**, 1997—1998 (1994).
27) Ozaki H., Nakamura A., Arai M., Endo M., Sawai H., *Bull. Chem. Soc. Jpn.*, **68**, 1981—1987 (1995).
28) Matsukura M., Okamoto T., Miike T., Sawai H., Shinozuka K., *Biochem. Biophys. Res. Commun.*, **293**, 1341—1347 (2002).
29) Tabor C. W., Tabor H., *Annu. Rev. Biochem.*, **53**, 749—790 (1984).
30) Tabor H., *Biochemistry*, **1**, 496—501 (1962).
31) Basu H. S., Schwietert H. C., Feuerstein B. G., Marton L. J., *Biochem. J.*, **269**, 329—334 (1990).
32) Esposito D., Delvecchio P., Bareno G., *J. Am. Chem. Soc.*, **119**, 2606—2613 (1997).
33) Schmid N., Behr J. P., *Biochemistry*, **30**, 4357—4361 (1991).
34) Shinozuka K., Umeda A., Aoki T., Sawai H., *Nucleosides Nucleotides*, **17**, 291 (1998).
35) Miller P. S., Reddy M. P., Murakami A., Blake K. R., Lin S. B., Agris C. H., *Biochemistry*, **25**, 5092 (1986).
36) Aboul-ela F., Koh D., Tinoco I., Jr., Martin F. H., *Nucleic Acids Res.*, **13**, 4811—4825 (1985).
37) Robinson H., Wang A. H.-J., *Nucleic Acids Res.*, **26**, 676—682 (1996).
38) Mirabelli C. K., Bennett C. F., Anderson K., Crooke S. T., *Anti-Cancer Drug Design*, **6**, 647—661 (1991).
39) Stein C. A., Subasinghe S., Shinozuka K., Cohen J. S., *Nucleic Acids Res.*, **16**, 3209—3221 (1988).
40) Zamaratski E., Pradeepkumar P. I., Chattopadhyaya J., *J. Biochem. Biophys. Methods*, **48**, 189—208 (2001).
41) Fedoroff O.-Y., Salazar M., Reid B. R., *J. Mol. Biol.*, **233**, 509—523 (1993).
42) Salazar M., Fedoroff O.-Y., Miller J. M., Ribeiro N. S., Reid B. R., *Biochesmitry*, **32**, 4207—4215 (1993).
43) Borer P. N., Dengler B., Tinoco I., Jr., Uhlenbeck O. C., *J. Mol. Biol.*, **241**, 246—262 (1974).