Russian Chemical Bulletin, International Edition, Vol. 53, No. 2, pp. 463—470, February, 2004

Detection of point mutations using pyrene-labeled DNA probes

N. N. Dioubankova,[a] A. D. Malakhov,[a] D. A. Stetsenko,[b] and V. A. Korshun[a]★

[a] M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences,
16/10 ul. Miklukho-Maklaya, 117997 Moscow, Russian Federation
Fax: +7 (095) 330 6738. E-mail: korshun@mail.ibch.ru
[b] MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
Fax: +44 (1223) 402070. E-mail: ds@mrc-lmb.cam.ac.uk

Pyrene-labeled oligodeoxyribonucleotide probes were shown to be suitable for the detection of point mutations. Reagents based on homochiral 2,4-dihydroxybutyramides were used to introduce pyrene residues at the 3´- and 5´- ends of oligonucleotide pairs. The oligonucleotide pair forms a tandem complex with a complementary target, giving rise to an excimer signal (λ_{max} 470—490 nm) in the fluorescence spectra when the pyrene residues come into close proximity. The maximum ratio of the intensity of the excimer signal to the monomer signal (λ_{max} 380—400 nm) is attained when (*S*)-*N*-(1-pyrenylmethyl)-3,3-dimethyl-2,4-dihydroxybutyramide is used to introduce the pyrene residue. The excimer fluorescence completely disappears with an increase in the distance between the pyrene residues (upon the introduction of an additional nucleotide in the target) or in the presence of a mismatch near the contact site of the probes.

Key words: modified oligonucleotides, pyrene fluorophore, detection of point mutations, excimer fluorescence.

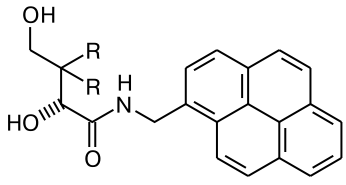
In recent years, highly efficient methods for detection of DNA sequences, urgent for medical diagnostics, have been developed on the basis of fluorescent dyes. The commanding lead of fluorophores among the labels for nucleic acids is mainly determined by the possibility of automatic detection of fluorescence. The use of a pair of interacting dyes (donor—acceptor[1—3] or fluorophore—quencher[4—6]) allows one to detect hybridization (and, hence, the presence of sequences complementary to the probes) in solution in a real-time mode.

A pair spectrally sensitive to hybridization can be composed of identical fluorophores able to form excimers (excited dimers). The long-wavelength excimer fluorescence will be observed only in the case of close proximity (direct contact) of the dyes. A tetracyclic aromatic hydrocarbon, pyrene, tends to form excimers.[7—9] The pyrene excimer is widely used to study the conformations and to detect the interactions of biomolecules.[7—10] Numerous examples of hybridization-sensitive fluorescence of excimers of pyrene derivatives attached to nucleic acids are known.[11—29]

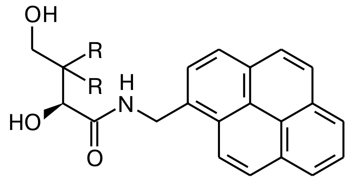
Since excimer formation is only possible where two pyrene residues approach each other to a distance of 2—5 Å, it appeared pertinent to use a pair of pyrene-labeled probes that form a tandem complex on the target. The probes contain pyrene residues at the 3´- and 5´-ends attached in such a way that upon hybridization, they approach each other, giving rise to excimer fluorescence.[27—29] If the target has an additional nucleotide or a mismatch near the contact site of the probes, the pyrene residues are unable to approach each other to the required distance and no long-wavelength fluorescence of the excimer can be observed. A sequence of two pyrene-labeled probes can be selected for any detectable mutation. The mode of pyrene attachment to the oligonucleotides is also important. For example, it was shown that the efficiency of excimer formation depends on the length of the linker between pyrene and the oligonucleotide.[29]

Recently, we developed a method of synthesis of homochiral pseudo-nucleosides based on (*R*)-2,4-dihydroxybutyramides,[30] in particular, pyrene derivatives **1** and **2** and described their incorporation into oligodeoxyribonucletides. These compounds are readily available and optically pure; the "natural" distance between the hydroxy groups in the nucleosides is obeyed in their structures; and the *R*-configuration of the chiral carbon atom mimics the configuration of C(3´) in 2´-deoxyribonucleosides. These pseudo-nucleosides can be easily attached to both the 5´- and the 3´-ends of oligonucleotides. It was of interest to verify the applicability of such pyrene reagents for detecting the hybridization of the tandem complex on the basis of excimer fluorescence.

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 2, pp. 443—449, February, 2004.



1, **2**



3, **4**

R = H (**1**, **3**), Me (**2**, **4**)

Results and Discussion

(*S*)-2,4-Dihydroxybutyramides **3** and **4** were prepared and converted into reagents for the synthesis of modified oligodeoxyribonucleotides, namely, phosphoramidites and solid supports were obtained by procedures similar to those reported previously.[30] The synthesis of the reagents will be described in detail elsewhere.* Commercially available (*S*)-(−)-2-hydroxy-γ-butyrolactone and (*S*)-(+)-2-hydroxy-3,3-dimethyl-γ-butyrolactone were used as chiral precursors. The presence of two additional methyl groups in compounds **2** and **4** hinders free rotation of both the acyclic pseudosugar fragment and the pyrene residue and also increases the solubility of pseudo-nucleosides and their derivatives in organic solvents.

In this study, the automated solid-phase synthesis of 3´- or 5´-pyrene-labeled oligonucleotides was carried out using reagents based on diols **1**—**4**. The pairs of probes were hybridized to a complementary DNA target and the fluorescence spectra of the resulting complexes were recorded. With appropriate arrangement of the pyrene residues, the spectra exhibited an emission band of the pyrene excimer with a broad maximum at 480 nm.

A 20-mer DNA fragment (**M1**) corresponding to the TAR region of the HIV RNA able to bind the Tat-protein was used as the model.[31,32] We also synthesized a number of nonmodified DNA targets containing one (**M2**), two (**M3**), and three (**M4**) additional nucleotides between positions 10 and 11 in the sequence of the **M1** target, a single nucleoside deletion in position 11 of the **M1** fragment (**M5**), and a mutation in position 8 (**M6**) (Table 1). Four pairs (**A** and **B**) of 10-mer oligodeoxyribonucleotide probes complementary to sequences 1—10 (**A**) and 11—20 (**B**) of the **M1** target having pyrene pseudonucleosides **1**—**4** attached to the 5´- and 3´-ends, respectively, were used as probes. Oligonucleotides were purified by electrophoresis in a denaturing polyacrylamide gel (PAAG) and by reversed-phase HPLC (pyrene-containing conjugates) and characterized by MALDI-TOF mass spectrometry (see Table 1).

Pyrene-labeled oligonucleotide conjugates are easily isolated in a pure state (typical mass spectra and HPLC profiles are presented in Figs. 1 and 2). The introduction of two additional methyl groups into the butyramide backbone results in an increase in the HPLC retention time of the conjugates by approximately 5 min (see Table 1).

Pyrene-containing oligonucleotides exhibit characteristic fluorescence spectra (λ_{max} 381 and 398 nm), the pyrene fluorescence being quenched more considerably by the neighboring thymidine (probes **B**) than by the neighboring deoxycytidine (probes **A**); this effect (Fig. 3) is in line with previous reports on quenching of pyrene fluorescence by nucleic bases.[33]

In the hybridization of two 3´-,5´-labeled probes with the **M1** target, the pyrene fluorophores are close to each

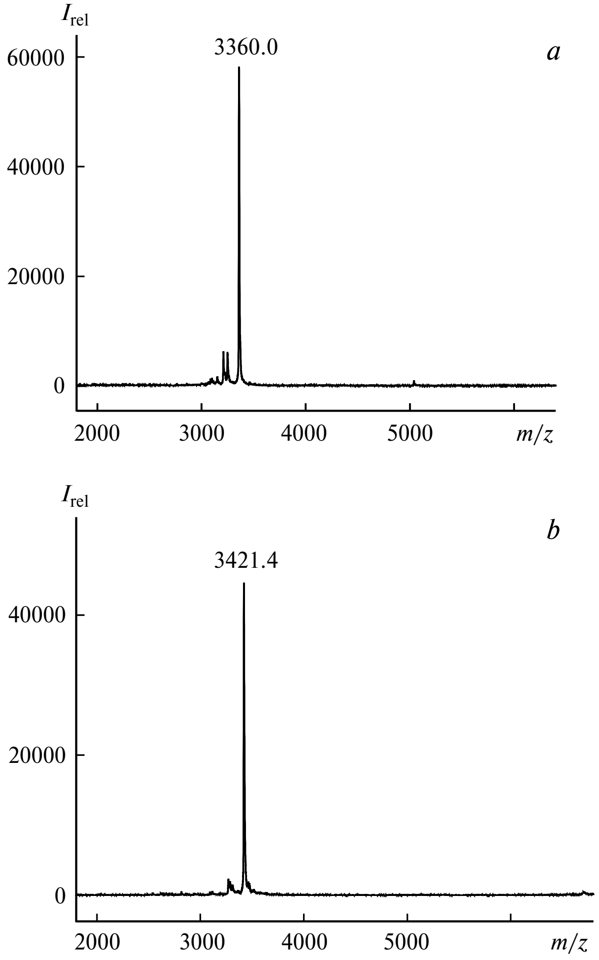


Fig. 1. Mass spectra of **B1** (*a*) and **A3** (*b*) conjugates.

---

* The material is being prepared for publication.

Table 1. Primary structures and properties of oligonucleotides

Oligo-nucleotide	5´→3 sequence[a]	M Found/Calculated	τ/min[b]
A1	**1**CAAATCTGGT	3419.8/3423.4	21.4
B1	CTCCCAGGCT**1**	3360.0/3360.3	24.8
A2	**2**CAAATCTGGT	3453.9/3451.4	26.7
B2	CTCCCAGGCT**2**	3384.5/3388.4	29.1
A3	**3**CAAATCTGGT	3421.4/3423.4	21.8
B3	CTCCCAGGCT**3**	3360.9/3360.3	24.6
A4	**4**CAAATCTGGT	3448.2/3451.4	25.6
B4	CTCCCAGGCT**4**	3383.4/3388.4	29.2
	10 20		
M1	ACCAGATTTGAGCCTGGGAG	6173.2/6183.0	—[c]
M2	ACCAGATTTGAAGCCTGGGAG	6491.0/6496.2	—
M3	ACCAGATTTGAGAGCCTGGGAG	6820.9/6825.4	—
M4	ACCAGATTTGAGAAGCCTGGGAG	7135.4/7138.6	—
M5	ACCAGATTTGGCCTGGGAG	5867.0/5869.8	—
M6	ACCAGAT***C***TGAGCCTGGGAG	6166.9/6168.0	—

[a] The nucleotides inserts in the targets with respect to the **M1** oligonucleotide are underlined; the mismatch nucleotide of the **M6** target with respect to probe **A** is italicized.

[b] For conditions, see Experimental; τ is the retention time. [c] Not determined.

other; hence, an excimer fluorescence signal appears in the fluorescence spectrum at 480 nm (Fig. 4). The proportion of the excimer fluorescence varies depending on the butyramide linker. In the case of *S*-isomers, the signal of the excimer fluorescence is more intense than for the *R*-isomers, and the additional methyl groups in the butyramide backbone increase the proportion of the excimer signal (Table 2). Apparently, the *S*-configuration of the chiral center ensures coplanarity of the pyrene chromophores and the maximum overlap of the π-electron shells, while the additional methyl groups restrict the mobility and thus fix the fluorophores in this position. Thus, the **A4—B4** pair of probes gives the highest excimer signal when hybridized to the **M1** target. The reduction of the target length by one nucleotide (Fig. 5), which formally brings the fluorophores closer to each other, leads to a decrease in the excimer signal (hybridization with the **M5** target). The pattern of dependence of the excimer signal intensity on the type of backbone is retained. A longer target (**M2**) proved to be the most favorable for the interaction of fluorophores. Further increase in the distance entails a decrease in the excimer

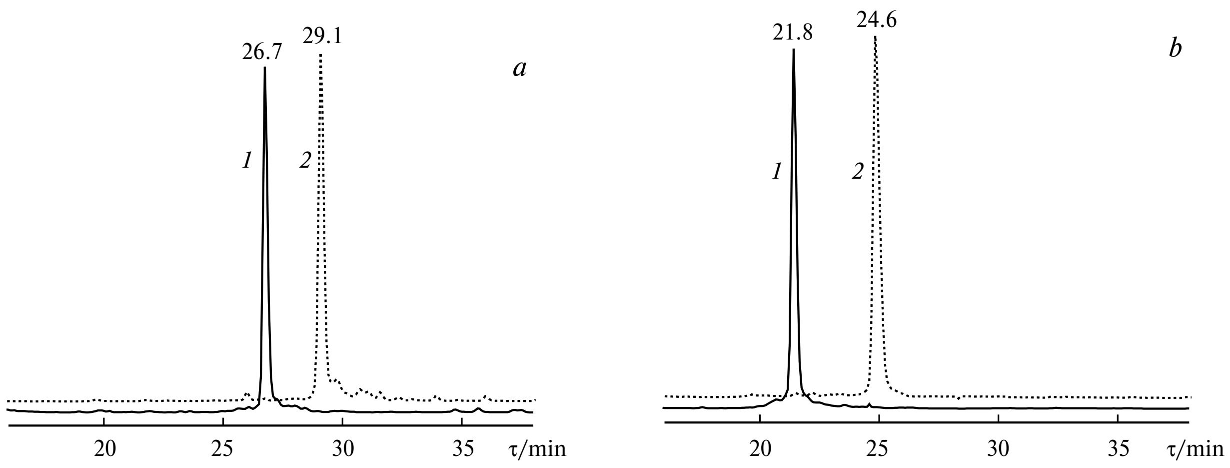


Fig. 2. Analytical HPLC of pyrene conjugates: (*a*) **A2** (*1*), **B2** (*2*); (*b*) **A3** (*1*), **B3** (*2*). The conditions are given in the Experimental, τ is the retention time.

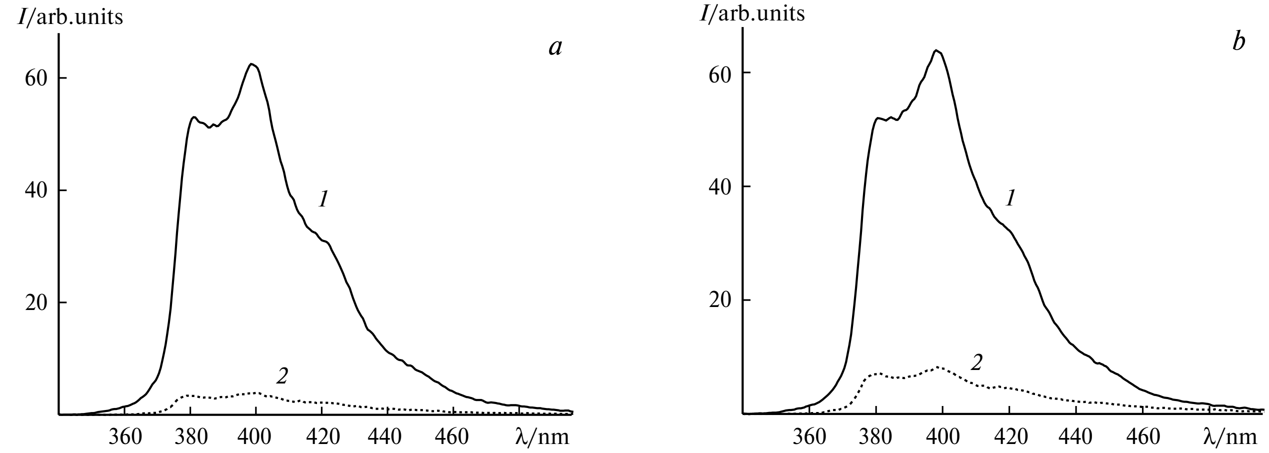


Fig. 3. Fluorescence spectra of modified oligonucleotides: (*a*) **A1** (*1*), **B1** (*2*); (*b*) **A2** (*1*), **B2** (*2*) in the hybridization buffer; λ_{excit} 330 nm; oligonucleotide concentration, $3 \cdot 10^{-6}$ mol L^{-1}.

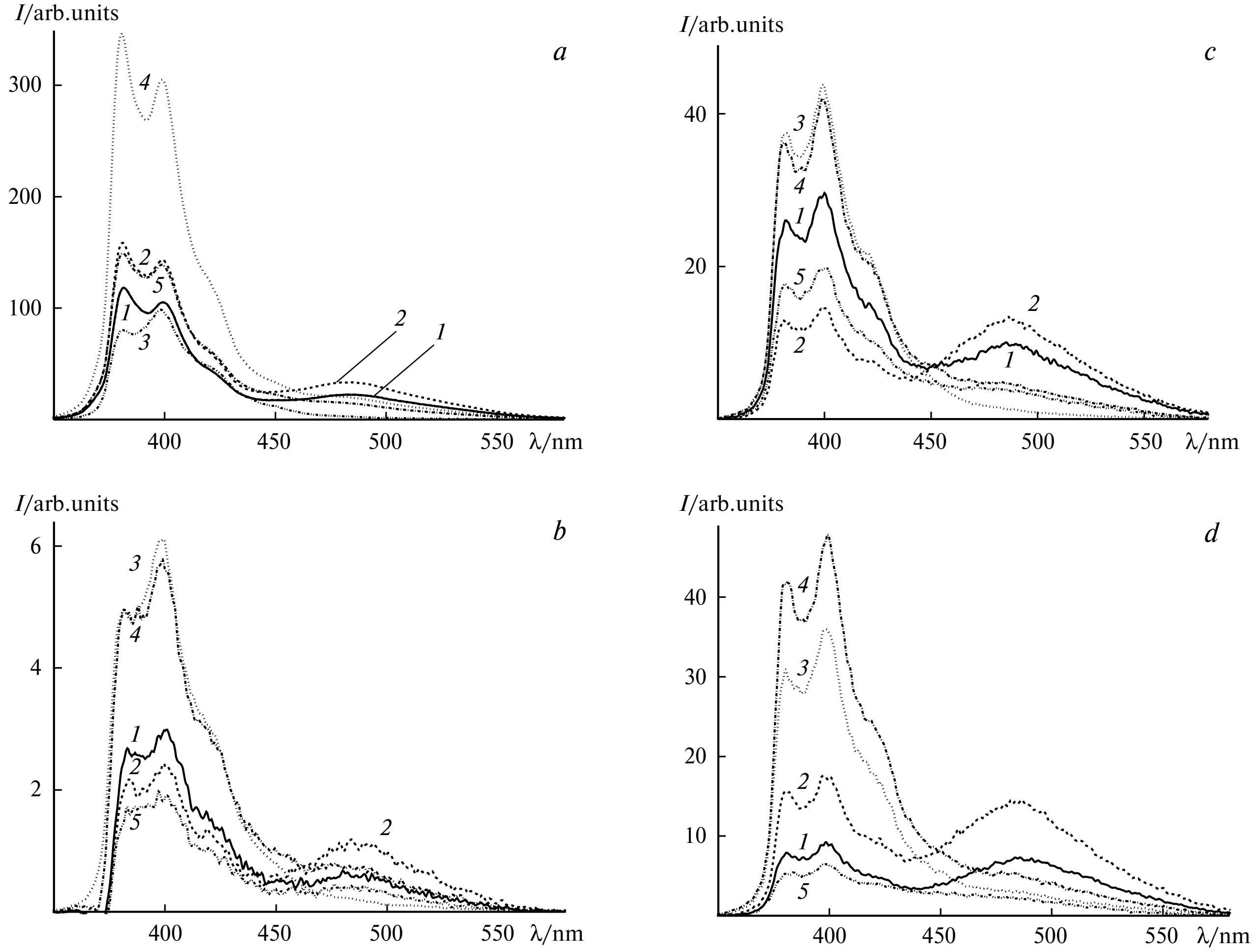


Fig. 4. Fluorescence spectra of the tandem complexes of fluorescence probes on complementary targets of different lengths: **A1** and **B1** (*a*), **A2** and **B2** (*b*), **A3** and **B3** (*c*), **A4** and **B4** (*d*) with **M1** (*1*), **M2** (*2*), **M3** (*3*), **M4** (*4*), **M5** (*5*). Conditions: hybridization buffer; λ_{excit} 330 nm; oligonucleotide concentration, $3 \cdot 10^{-6}$ mol L^{-1}.

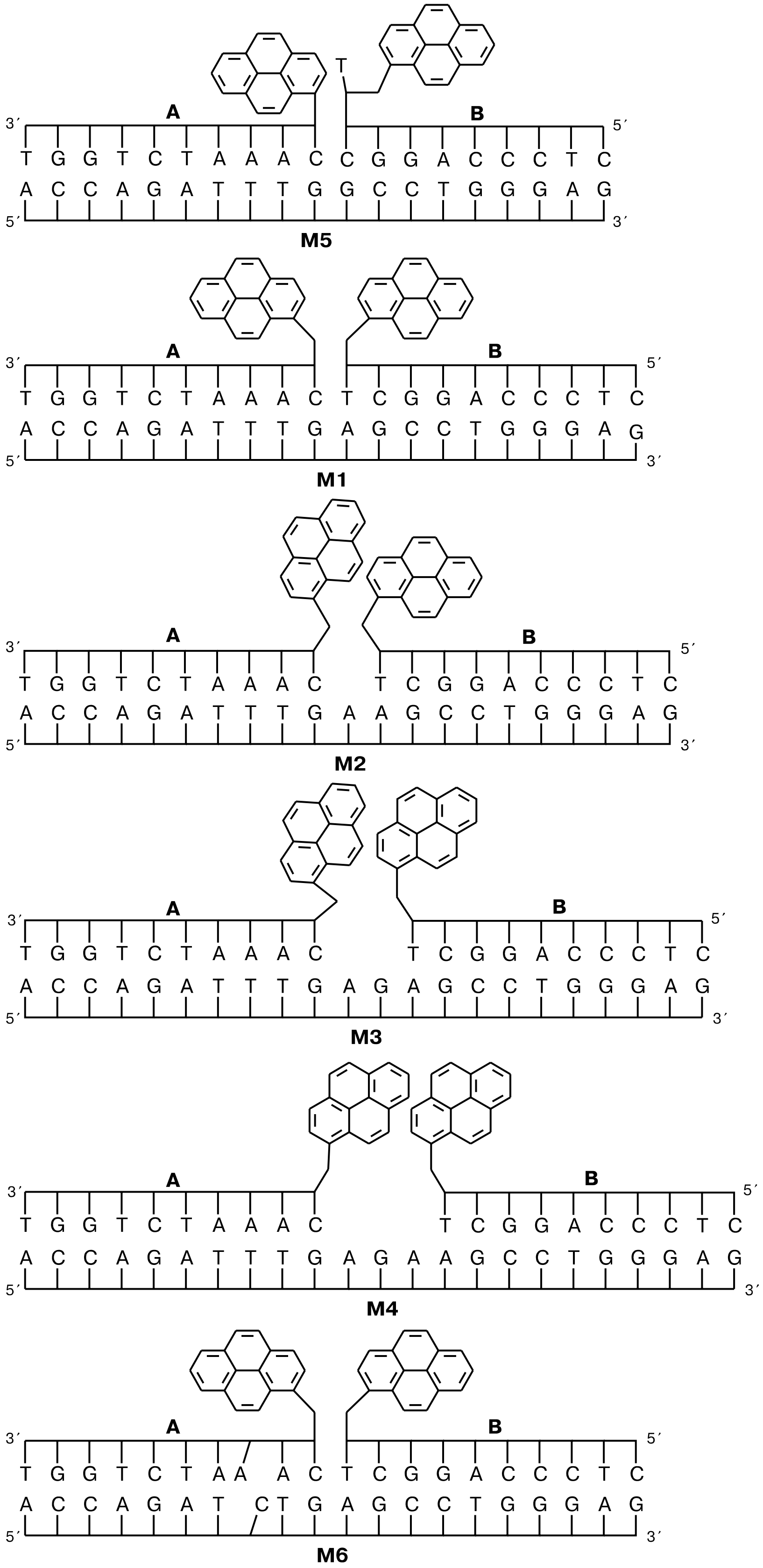


Fig. 5. Scheme of the arrangement of pairs of the oligonucleotide probes on complementary targets of different lengths.

Table 2. Ratio of the intensities of the excimer-to-monomer fluorescence in the **A**•**B**•**M** tandem complexes

Match target	Monomer used for the incorporation of pyrene			
	1	**2**	**3**	**4**
M5	0.10	0.23	0.20	0.33
M1	0.19	0.24	0.34	0.79
M2	0.21	0.54	0.92	0.81
M3	0.03	0.04	0.03	0.09
M4	0.06	0.15	0.11	0.11

signal (**M3**), and in the case of the **M4** target, only the monomer signal remains in the spectrum (see Fig. 4 and Table 2).

The hybridization of the probe pairs with the targets containing at least one mismatch results in disappearance of the long-wavelength excimer signal (Fig. 6). This can be used for detecting the mutant DNA in solutions. Oligonucleotides **A4—B4** proved to be the probe pair of choice for this purpose; the pyrene residues were introduced using (*S*)-2,4-dihydroxy-3,3-dimethylbutyramide derivative **4**.

Thus, we developed a new system for the detection of point mutations in solution based on changes in pyrene fluorescence. The system is based on the principle proposed previously.[27—29] Using pyrene-containing dihydroxybutyramide *seco*-pseudonucleosides, one can observe excimer fluorescence upon tandem hybridization of two probes on a complementary target. The method for the introduction of the pyrene fluorophore into the terminal positions of oligonucleotides used in this study is less expensive and less laborious than the procedures used previously.[27—29] The modes of optimal orientation of dyes on the target providing the highest excimer-to-monomer signal intensity ratio are described.

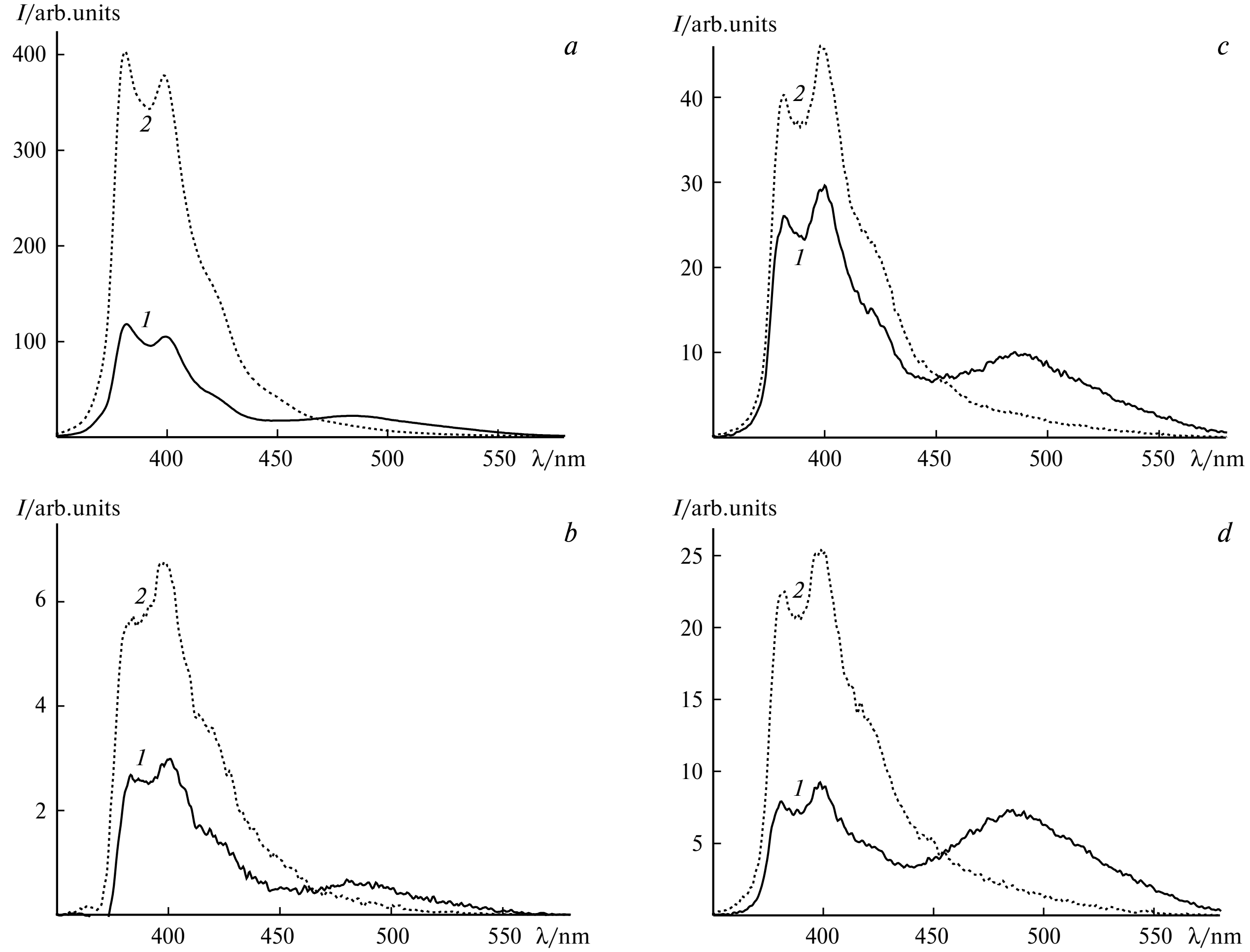


Fig. 6. Fluorescence spectra of the duplexes of the **A1** and **B1** (*a*), **A2** and **B2** (*b*), **A3** and **B3** (*c*), **A4** and **B4** (*d*) probe pairs on complementary (**M1**, curve *1*) and noncomplementary (**M6**, curve *2*) targets. Conditions: hybridization buffer; λ_{excit} 330 nm; oligonucleotide concentration, $3 \cdot 10^{-6}$ mol L^{-1}.

Experimental

The syntheses of compounds **1** and **2** and reagents based on them were reported previously.[30] The enantiomeric diols **3** and **4** and the reagents derived from them were prepared from (*S*)-(–)-2-hydroxy-γ-butyrolactone and (*S*)-(+)-2-hydroxy-3,3-dimethyl-γ-butyrolactone in a similar way.

Oligonucleotides A1—A4, **B1—B4**, and **M1—M6**. The solid-phase oligonucleotide synthesis was carried out on a 1 μmol scale using a standard phosphoramidite protocol according to manufacturer´s guidelines on an automated ABI 380 DNA/RNA Synthesizer using standard reagents (Cruachem). Modified phosphoramidites were dissolved in anhydrous acetonitrile; the coupling step lasted for 10 min. After the synthesis, the support with the oligonucleotides containing protective groups was treated with 25% aqueous ammonia for 6 h at 55 °C for deprotection. The resulting solution was concentrated, the residue was dissolved in 0.4 mL of a 1 *M* aqueous solution of $LiClO_4$, and the mixture was filtered through a centrifuge-type filter (Spin-X, COSTAR) and reprecipitated with a fivefold volume of acetone. The oligonucleotides were isolated by electrophoresis in a denaturing 20% PAAG with 7 *M* urea; the bands containing the target product were visualized at 260 nm and cut out, the products were eluted with a 0.5 *M* solution of $LiClO_4$ (3×1.5 mL), the eluates were concentrated, and the residues were desalinated on NAP-10 columns (Amersham Biosciences).

The purity of the oligonucleotides was checked by reversed-phase HPLC using a Phenomenex RP-C18 column (3.90×300 mm) and buffer A (MeCN, 5 % v/v) in a 0.1 *M* aqueous solution of triethylammonium acetate; buffer B: MeCN, flow rate 1 mL min^{-1}; B→A gradient: 0→5%, 5 min; 5→15%, 10 min; 15→40, 30 min; 40→80%, 10 min; and 80→0%, 10 min. The products were detected at 254 nm. The MALDI-TOF mass spectra of each oligonucleotide were recorded on a Voyager-DE BioSpectrometry Workstation instrument (PerSeptive Biosystems) using a 1 : 1 (v/v) mixture of solutions of 2,6-dihydroxyacetophenone (40 mg in 1 mL of methanol) and diammonium citrate (80 mg in 1 mL of 50% aqueous acetonitrile) as the ionization matrix. A fresh mixture was prepared prior to each measurement.

UV spectra were measured on a Perkin-Elmer Lambda 40 UV/VIS spectrometer. The concentration of oligonucleotides was determined on the basis of absorption of their aqueous solutions at 260 nm taking the pyrene extinction coefficient at this wavelength to be 22300 L mol^{-1} cm^{-1}.[34]

To prepare **duplexes**, oligonucleotides (3 nmol of each) were mixed in 0.5 mL of the hybridization buffer: 100 *mM* NaCl, 10 *mM* Na phosphate, 0.1 *mM* EDTA, pH 7.0. The mixture was kept for 5 min at 96 °C and cooled to room temperature for 2.5—3 h.

The fluorescence spectra were recorded on a Perkin-Elmer LS 50B luminescence spectrometer using an excitation wavelength of 330 nm, 200 μL cells, and an excitation and recording slit of 5 nm. The concentrations of oligonucleotides in the hybridization buffer were $3 \cdot 10^{-6}$ mol L^{-1}.

This work was financially supported by the Russian Foundation for Basic Research (Project No. 03-03-32196). The authors are grateful to M. J. Gait (Laboratory of Molecular Biology), V. A. Efimov, K. R. Birikh, and I. A. Prokhorenko (Laboratory of the Chemistry of Nucleic Acids, M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry of the RAS) for useful discussion. N. N. Dyubankova wishes to thank the Laboratory of Molecular Biology of the Medical Research Council (Cambridge, UK) for the support of her visit to Cambridge in 2002. A. D. Malakhov is grateful to the AstraZeneca company for supporting his research work at the Laboratory of Molecular Biology (Cambridge, UK) in 2002—2003.

References

1. P. R. Selvin, *Nature Struct. Biol.,* 2000, **7**, 730.
2. D. M. J. Lilley, and T. J. Wilson, *Curr. Opin. Chem. Biol.,* 2000, **4**, 507.
3. M. Lorenz, A. Hillisch, and S. Diekmann, *Rev. Mol. Biotechnol.*, 2002, **82**, 197.
4. S. Tyagi and F. R. Kramer, *Nature Biotechnol.*, 1996, **14**, 303.
5. S. Gelmini, C. Orlando, R. Sestini, G. Vona, P. Pinzani, L. Ruocco, and M. Pazzagli, *Clin. Chem.*, 1997, **43**, 752.
6. D. Whitcombe, J. Theaker, S. P. Guy, T. Brown, and S. Little, *Nature Biotechnol.*, 1999, **17**, 804.
7. F. M. Winnik, *Chem. Rev.*, 1993, **93**, 587.
8. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Kluwer Academic, Plenum, New York, 1999, 698 p.
9. R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, Molecular Probes, Eugene, OR, 2002, 966 p.
10. S. S. Lehrer, *Meth. Enzymol.*, 1997, **287**, 286.
11. H. Fritzsche, A. Akhebat, E. Taillandier, K. Rippe, and T. M. Jovin, *Nucleic Acids Res.*, 1993, **21**, 5085.
12. G. Tong, J. M. Lawlor, G. W. Tregear, and J. Haralambidis, *J. Am. Chem. Soc.*, 1995, **117**, 12151.
13. F. D. Lewis, Y. Zhang, and R. L. Letsinger, *J. Am. Chem. Soc.*, 1997, **119**, 5451.
14. K. V. Balakin, V. A. Korshun, I. A. Prokhorenko, G. V. Maleev, I. A. Kudelina, S. V. Gontarev, and Yu. A. Berlin, *Bioorg. Khim.*, 1997, **23**, 33 [*Russ. J. Bioorgan. Chem.*, 1997, **23**, 28 (Engl. Transl.)].
15. K. V. Balakin, V. A. Korshun, I. I. Mikhalev, G. V. Maleev, A. D. Malakhov, I. A. Prokhorenko, and Y. A. Berlin, *Biosensors Bioelectronics*, 1998, **13**, 771.
16. S. Mohammadi, A. Slama-Schwok, G. Léger, D. El Manouni, A. Shchyolkina, Y. Leroux, and E. Taillander, *Biochemistry*, 1997, **36**, 14836.
17. A. Slama-Schwok, K. Zakrewska, G. Léger, Y. Leroux, M. Takahashi, E. Käs, and P. Debey, *Biophys. J.*, 2000, **78**, 2543.
18. E. Kostenko, M. Dobrikov, D. Pyshnyi, V. Petyuk, N. Komarova, V. Vlassov, and M. Zenkova, *Nucleic Acids Res.*, 2001, **29**, 3611.
19. A. Mahara, R. Iwase, T. Sakamoto, K. Yamana, T. Yamaoka, and A. Murakami, *Angew. Chem. Int.*, *Ed. Engl.*, 2002, **41**, 3648.
20. J. Michel, K. Bathany, J.-M. Schmitter, J.-P. Monti, and S. Moreau, *Tetrahedron*, 2002, **58**, 7975.

21. V. A. Korshun, D. A. Stetsenko, and M. J. Gait, *J. Chem. Soc.*, *Perkin Trans. 1*, 2002, 1092.
22. U. B. Christensen and E. B. Pedersen, *Nucleic Acids Res.*, 2002, **30**, 4918.
23. K. Yamana, T. Iwai, Y. Ohtani, S. Sato, M. Nakamura, and H. Nakano, *Bioconjugate Chem.*, 2002, **13**, 1266.
24. U. B. Christensen and E. B. Pedersen, *Helv. Chim. Acta.*, 2003, **86**, 2090.
25. K. Yamana, Y. Ohtani, H. Nakano, and I. Saito, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 3429.
26. N. N. Dioubankova, A. D. Malakhov, D. A. Stetsenko, M. J. Gait, P. E. Volynsky, R. G. Efremov, and V. A. Korshun, *ChemBioChem*, 2003, **4**, 841.
27. K. Ebata, M. Masuko, H. Ohtani, and M. Kashiwasake-Jibu, *Photochem. Photobiol.*, 1995, **62**, 836.
28. P. L. Paris, J. M. Langenhan, and E. T. Kool, *Nucleic Acids Res.*, 1998, **26**, 3789.
29. M. Masuko, H. Ohtani, K. Ebata, and A. Shimadzu, *Nucleic Acids Res.*, 1998, **26**, 5409.
30. N. N. Dioubankova, A. D. Malakhov, D. A. Stetsenko, V. A. Korshun, and M. J. Gait, *Org. Lett.*, 2002, **4**, 4607.
31. A. Arzumanov, F. Godde, S. Moreau, J.-J. Toulmé, A. Weeds, and M. J. Gait, *Helv. Chim. Acta*, 2000, **83**, 1424.
32. A. Arzumanov, A. P. Walsh, V. K. Rajwanshi, R. Kumar, J. Wengel, and M. J. Gait, *Biochemistry*, 2001, **40**, 14645.
33. M. Manoharan, K. L. Tivel, M. Zhao, K. Nafisi, and T. L. Netzel, *J. Phys. Chem.*, 1995, **99**, 17461.
34. R. A. Friedel and M. Orchin, *Ultraviolet Spectra of Aromatic Compounds*, Wiley, New York, 1951.

Received October 14, 2003;
in revised form December 15, 2003