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Oligonucleotides containing fluorescent 2′-deoxyisoinosine: solid-phase synthesis and duplex stability

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ABSTRACT

The fluorescent nucleoside 2′-deoxyisoinosine (2, $isol_d$) has been incorporated into oligonucleotides. For this purpose the phosphonate 3a and the phosphoramidite 3b, as well as the polymer-linked 3d, have been synthesized and oligonucleotides were prepared by P(III) solid-phase chemistry. One or two $isol_d$-residues were introduced into the oligomer $d(T_{12})$, replacing dT either in the middle or at the 3′- and 5′-ends. The $isol_d$-containing oligomers were hybridized with a modified $d(A)_{12}$ containing the conventional nucleosides (dA, dT, dG and dC) opposite to $isol_d$. The replacement of one dT by $isol_d$ in the centre of the duplex reduced the T_m value by ~15°C and a decrease of ~25°C was found when two $isol_d$ residues were incorporated. Thermodynamic data were determined from the melting curves. The destabilization was almost independent of the four naturally occurring nucleosides located opposite to $isol_d$. The $isol_d$ (2) seems to be stacked in the duplex when dT–dA base pairs are the nearest neighbours; an internal loop is formed in the case of oligomers containing two consecutive $isol_d$ residues.

INTRODUCTION

Inosine (**1a**) occurs naturally in the wobble position of the anticodon of some tRNAs, where it appears to pair with adenosine, cytidine and uridine of the codon of mRNA (1,2). 2′-Deoxyinosine (dI, **1b**) is widely used as an ambiguous nucleoside in oligonucleotide mixed probes and primers (3–5). It is also useful in nucleic acid sequencing to reduce band compression (6,7). However, it has been shown that the base pair stability of 2′-deoxyinosine with the four conventional bases decreases in the order d(I–C) > d(I–A), d(I–G) and d(I–T) (8). The base pairing was studied by X-ray analysis (9–14) and NMR spectroscopy (15–17). It has been reported that wobble base pairs, as well as Hoogsteen base pairs, are formed (8,9,12,13,15,16). Furthermore, the stability of base pairs has been shown to be sequence-dependent (3) and it has also been found that the T_m value of a dI-containing oligonucleotide is higher when the nearest neighbour is dA and not dT. In order to form non-selective base pairing, other deoxyinosine derivatives (18), as well as other ambiguous nucleosides (19), have been synthesized (Scheme 1).

2′-Deoxyisoinosine (**2**), which is isomeric to 2′-deoxyinosine, has recently been synthesized (20). The molecule shows strong fluorescence (20) similar to 2-aminopurine 2′-deoxynucleoside (21). 2′-Deoxyisoinosine (**2**) is also related to 2′-deoxyisoguanosine, but lacks the 6-amino function. Oligonucleotides containing 2′-deoxyisoguanosine have been synthesized chemically (22) and its triphosphate can be incorporated enzymatically opposite to dT residues in a template (23,24). For the $isoG_d$–dT base pair the structure shown in Scheme 2 was suggested. A similar structure can be considered for the $isol_d$–dT base pair, but a pairing mode with the keto form of the nucleoside should also be possible.

Furthermore, various base pairing patterns of $isol_d$ with the three other conventional nucleosides, including Hoogsteen pairs, can be constructed. As nothing is known about the base pair stability of isoinosine with the four conventional bases in antiparallel duplex DNA, oligonucleotides were synthesized containing $isol_d$ opposite to dT, dC, dA and dG. For this purpose the phosphonate (**3a**) and phosphoramidite (**3b**) were prepared and employed in solid-phase oligonucleotide synthesis. The $isol_d$ base pair stability is derived from duplex melting and the data on the modified oligomers will be compared with those of the parent compounds.

MATERIALS AND METHODS

General

TLC. Aluminium sheets coated with a 0.2 mm layer of silica gel 60 F_{254} (Merck, Germany). Solvent systems CH_2Cl_2/MeOH 9:1 (A), CH_2Cl_2/MeOH/Et_3N 78:20:2 (B), CH_2Cl_2/AcOEt/Et_3N 20:70:10 (C). Flash chromatography (FC) was carried out at 0.5 bar. A Uvicord S (LKB instruments, Sweden) was used for detection.

UV spectra. Hitachi-150-20 spectrometer (Hitachi, Japan).

NMR spectra. Bruker-AC-250 and AMX-500 spectrometer; δ values in p.p.m. relative to tetramethylsilane as internal standard (^1H and ^{13}C) or to external phosphoric acid (^{31}P). Elemental

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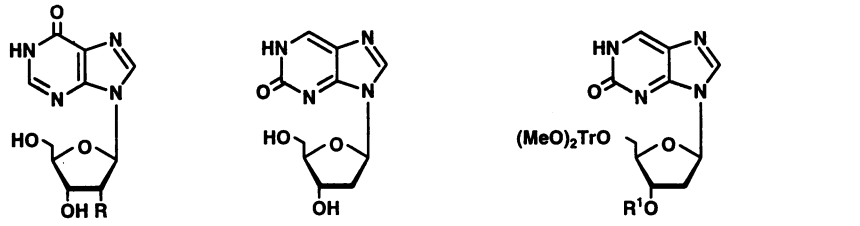
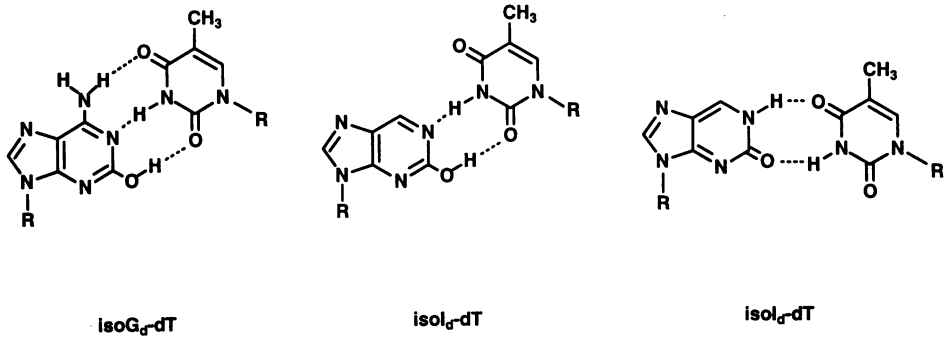


Scheme 1.



Scheme 2.

analyses were performed by Mikroanalytisches Laboratorium Beller (Göttingen, Germany). Oligonucleotide synthesis was carried out on a DNA synthesizer, model 381 A (Applied Biosystems, Weiterstadt, Germany), on a 1 μmol scale.

HPLC separation (25). Solvent gradients consisting of 0.1 M (Et_3NH)OAc (pH 7.0)/MeCN 95:5 (A) and MeCN (B) were used in the following order: gradient I, 3 min 15% B in A, 7 min 15–40% B in A, 5 min 40% B in A, 5 min 40–15% B in A, flow rate 1 ml/min; gradient II, 20 min 0–20% B in A, 5 min 20–0% B in A, flow rate 1 ml/min; gradient III, 30 min 100% A, flow rate 0.6 ml/min.

The enzymatic hydrolysis of the oligonucleotides was carried out as described (25), using alkaline phosphatase and snake venom phosphodiesterase. The mixture was analyzed on reversed-phase HPLC (RP-18, gradient III). Quantification of the material was made on the basis of the peak areas, which were divided by the extinction coefficients of the nucleoside constituents (ε_{260}: A_d 15 400, C_d 7300, G_d 11 700, T_d 8800.ε_{290}: $isoI_d$ 2100, T_d 2000).

Determination of T_m values were carried out with a Cary-1E UV/VIS spectrophotometer (Varian, Melbourne, Australia), according to Seela *et al.* (26). The temperature was increased by 60°C/h. The T_m values were determined using the software package 2hDNA (Dr Apel, Varian, Darmstadt, Germany). Thermodynamic data were calculated from one melting profile according to Kehrhan (27).

9-[2-Deoxy-5-*O*-(4,4-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-9*H*-purin-2-one (4)

Compound **2** (500 mg, 2.0 mmol) was dried by repeated co-evaporation from anhydrous pyridine, suspended in anhydrous pyridine (14 ml) and reacted with $(MeO)_2TrCl$ (860 mg, 2.5 mmol) under stirring at room temperature overnight. After addition of MeOH (10 ml) the reaction mixture was treated with 5% aqueous $NaHCO_3$ solution (100 ml). It was extracted with CH_2Cl_2 (3 × 40 ml), the combined organic layer was dried (anhydrous Na_2SO_4) and evaporated. The residue was dissolved in CH_2Cl_2 and submitted to flash chromatography (silica gel, column 12 × 3 cm, solvent system A containing traces of Et_3N). The main zone was isolated yielding a colourless powder (967 mg, 88%). TLC (silica gel, solvent system A): R_f 0.54. ^{1}H-NMR [d_6-DMSO]: 2.27, 2.70 [2*m*, H-C(2′)]; 3.16 [*m*, H-C(5′)]; 3.71 (*m*, CH_3O); 3.92 [*m*, H-C(4′)]; 4.40 [*m*, H-C(3′)]; 5.36 [*m*, OH-C(3′)]; 6.18 [*t*, *J* = 6.00 Hz, H-C(1′)]; 6.8–7.3 (*m*, aromatic H); 8.28 [*s*, H-C(8)]; 8.45 [*s*, H-C(6)]; 11.95 (*m*, NH). Analysis caclulated for $C_{31}H_{30}N_4O_6$ (554.58): C 67.13, H 5.45, N 10.10; found: C 67.02, H 5.48, N 10.12.

9-[2-Deoxy-5-*O*-(4,4-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-9*H*-purin-2-one, 3′-triethylammonium phosphonate (3a)

To a solution of PCl_3 (540 μl, 6.2 mmol) and *N*-methylmorpholine (7.0 ml) in CH_2Cl_2 (40 ml), 1,2,4-triazole (1.43 g, 20 mmol) was added. After 30 min the solution was cooled to 0°C and **4** (700 mg, 1.26 mmol), dissolved in CH_2Cl_2 (40 ml), was added slowly. After stirring for 30 min at room temperature the reaction mixture was poured into 1 M (Et_3NH)HCO_3 (70 ml), shaken and separated. The aqueous layer was extracted with CH_2Cl_2 (3 × 40 ml). The organic extracts were dried with anhydrous Na_2SO_4 and concentrated to dryness. The residue was submitted to FC (silica gel, colmun 2 × 12 cm, solvent system B), the fraction of the main zone was collected and evaporated to give a colourless foam (580 mg, 64%). TLC (silica gel, solvent system B): R_f = 0.26. ^{1}H-NMR

[d_6-DMSO]: 0.98 (*t*, *J* = 7.1 Hz, CH_3CH_2); 2.59 (*q*, *J* = 7.1, CH_3CH_2); 2.82 [*m*, H-C(2′)]; 3.11 [*m*, H-C(5′)]; 3.70 (*s*, CH_3O); 4.11 [*m*, H-C(4′)]; 4.74 [*m*, H-C(3′)]; 6.17 [*t*, H-C(1′)]; 6.61 [*d*, *J* (P, H) = 293, PH]; 6.8–7.3 (*m*, aromatic H); 8.25 [*s*, H-C(8)]; 8.45 [*s*, H-C(6)]. ^{31}P-NMR [d_6-DMSO]: 0.52 [*d*, *J*(P, H) = 587]. Analysis calculated for $C_{37}H_{46}N_5O_8P$ (719.75): C 61.74, H 6.44, N 9.73; found: C 61.80, H 6.68, N 9.61.

9-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-9*H*-purin-2-one 3′-[(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite] (3b)

To a solution of **4** (45 mg, 0.08 mmol) and (i-Pr)$_2$EtN (56 µl, 0.29 mmol) in anhydrous CH_2Cl_2 (1 ml), chloro(2-cyanoethoxy)(diisopropylamino)phosphane (115 µl, 0.51 mmol) was added at room temperature. After stirring for 30 min, the mixture was diluted with CH_2Cl_2 (10 ml) and quenched by adding 5% aqueous $NaHCO_3$ solution (5 ml). Then the aqueous layer was extracted with CH_2Cl_2 (3 × 10 ml), the combined organic layers dried (Na_2SO_4), filtered and evaporated. The residue was applied to flash chromatography (silica gel, column 10 × 2 cm, solvent system C) and a colourless foam (50 mg, 82%) obtained. TLC (silica gel, solvent system C) R_f 0.14. ^{31}P-NMR [d_6-DMSO]: 149.0, 148.3.

9-[2-Deoxy-5-*O*-(4,4-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-9*H*-purin-2-one, 3′-(3-carboxypropanoate) (3c)

To a solution of **4** (200 mg, 0.36 mmol) in anhydrous CH_2ClCH_2Cl (720 µl), 4-(dimethylammino)pyridine (43.2 mg, 0.32 mmol), succinic anhydride and triethylamine (98.4 µl, 0.72 mmol) were added. The mixture was stirred for 30 min at 50°C. Then the solution was diluted with CH_2ClCH_2Cl (10 ml), washed three times with an ice-cold aqueous solution of 10% citric acid (3 × 8 ml) followed by water (10 ml). The combined organic layers were dried (Na_2SO_4) and evaporated. The residue was precipitated from CH_2Cl_2/ether as a colourless powder (195 mg, 83%). TLC (silica gel, solvent system A): R_f = 0.32. ^{1}H-NMR [d_6-DMSO]: 1.08 (*t*, *J* = 7.0, 2H-CH_2COO); 3.00 [*m*, 2H-C(2′)]; 3.18 [*m*, 2H-C(5′)]; 3.71 (*s*, 2 × CH_3O); 4.12 [*br*, H-C(4′)]; 5.27 [*br*, H-C(3′)]; 6.20 [*t*, *J* = 6.0, H-C(1′)]; 6.8–7.4 (*m*, aromatic H); 8.28 [*s*, H-C(8)]; 8.54 [*s*, H-C(6)]. Analysis calculated for $C_{35}H_{34}N_4O_9$(654.7): C 64.21, H 5.23, N 8.56; found: C 64.21, H 5.33, N 8.46.

9-[2-Deoxy-5-*O*-(4,4-dimethoxytrityl)-β-D-erythro-pentofuranosyl]-9*H*-purin-2-one 3′-(3-*N*-fractosil carbamoyl)propanoate (3d)

A solution of **3c** (85 mg, 0.13 mmol) in 1,4-dioxane containing 5% pyridine (1 ml) was treated with 4-nitrophenol (33 mg, 0.24 mmol) and *N*,*N*-dicyclohexylcarbodiimide (50 mg, 0.25 mmol). The mixture was stirred for 2 h at room temperature and dicyclohexylurea filtered off. The filtrate was added to a suspension of amino-linked silica gel (Fractosil 200/450 µequiv. NH_2/g, 200 mg) in dry DMF (1 ml). After addition of Et_3N (200 µl) the suspension was shaken for 4 h at room temperature. Then Ac_2O (60 µl) was added and shaken for another 30 min. The Fractosil derivative was filtered, washed with DMF, EtOH and Et_2O and finally dried in vacuum. The ligand concentration was determined according to Seela *et al.* (25) and was found to be 90 µmol/g.

Solid-phase synthesis of oligonucleotides 6–19

The synthesis was carried out on a 1 µM scale using the phosphonate **3a**, as well as the solid support **3d**. The synthesis of oligomers **6–19** followed the phosphonate protocol described recently (28). The DMT-oligomers were purified by HPLC on a RP-18 column (gradient I), $(MeO)_2$Tr residues were removed by treatment with 2.5% $Cl_2CHCOOH$ in CH_2Cl_2 for 5 min at room temperature. HPLC purification was carried out as described above, but using gradient II. The oligomers were desalted on a 4 × 25 mm HPLC cartridge (RP-18, silica gel) using H_2O (10 ml) for elution of the salt, while the oligomer was eluted with MeOH/H_2O 3:2 (5 ml). The nucleotides were lyophilized on a Speed-Vac evaporator. The colourless solids were dissolved in H_2O (100 µl) and stored frozen at –18°C. The retention times and composition of oligonucleotides **6–8** are given in Table 1.

Table 1. Retention times and composition of oligonucleotides **6–8**

Oligomer	t_R (min)[a]	Composition	
		T_d	iI_d
6	12.5	5.0	1
7	12.6	10.8	1
8	12.5	4.8	1

[a]Gradient II.

RESULTS AND DISCUSSION

Monomers

2′-Deoxyisoinosine (**2**) has been synthesized previously from 2′-deoxyguanosine by a three-step route (20). It is a very polar molecule. The base, 2-hydroxypurine (29,30) can act as an electrophile, adding other molecules at the 6-position (31). The UV spectrum of 2′-deoxyisoinosine (**2**) shows a λ_{max} at 315 nm in water, which is at an even longer wavelength to that of 2′-deoxyisoguanosine (291 nm, water) and is far from other nucleosides including 2′-deoxyinosine (**1**, 248 nm). The UV maximum of **2** in dioxane shows two maxima above 250 nm (337 and 284 nm, ratio 1.5:1) and a change is observed when water is added to the dioxane solution of **2** (Fig. 1). As the UV maximum of 2-methoxy-9-β-D-ribofuranosylpurine at 282 nm (32) coincides with that of the short wavelength maximum of $isoI_d$ in dioxane (284 nm), the UV absorption can be traced back to the lactim form. Then the absorption at 337 nm (dioxane) belongs to the lactam form. When water is added to dioxane solutions of 2′-deoxyisoinosine the absorption at 284 nm disappears and the 337 nm main maximum (dioxane) is slightly shifted to 314 nm. This indicates that the lactim form is unfavourable in aqueous solution. Similar observations have been made in the case of 9-methylisoguanine (33) and also recently for 2′-deoxyisoguanosine (34). According to the hydrophobic environment of base pairs within a DNA duplex it is expected that the lactim, as well as the lactam, form of 2′-deoxyisoinosine can contribute to base pairing.

The synthesis of $isoI_d$-containing oligonucleotides was carried out on a solid support using the phosphonate **3a** (35). For this purpose a 4,4′-dimethoxytrityl residue was introduced into the 5′-position of compound **2** under standard conditions, furnishing

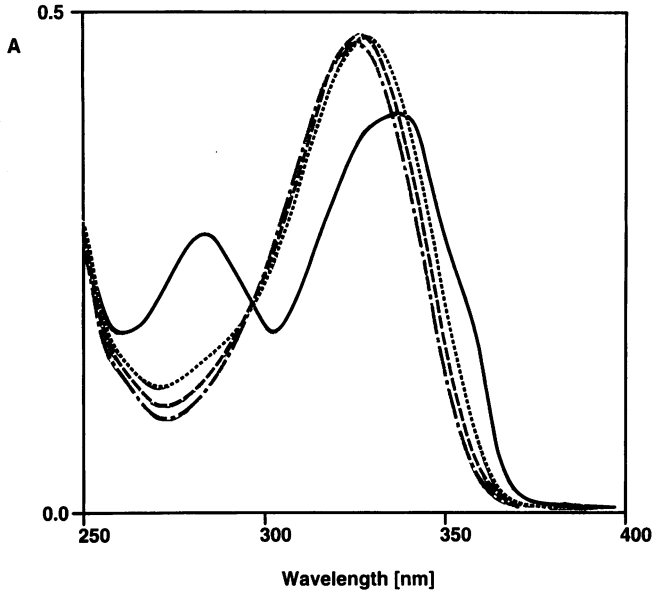
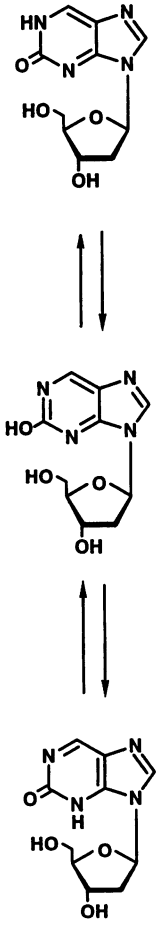
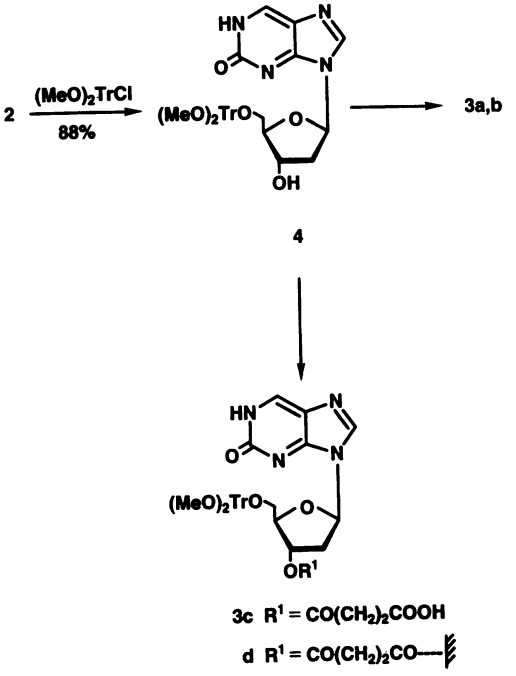


Figure 1. UV spectra of isol$_d$ (**2**) in dioxane (——); dioxane/H2O, 97:3 (·····); dioz'xane/H2O, 94:6 (-----); dioxane/H2O, 91:9 (-·-·-·).



Scheme 3.

the DMT-derivative **4** (88% yield). Tritylation was confirmed by the ~3 p.p.m. downfield shift of the C(5′) in the ^{13}C-NMR spectrum. The phosphonate **3a** was prepared from **4** with



Scheme 4.

PCl_3/*N*-methylmorpholine/1*H*-1,2,4-triazole. Compound **3a** was purified chromatographically and isolated as the triethylammonium salt (64% yield). The phosphoramidite **3b** (82% yield, diastereomeric mixture) was also prepared. Succinylation of **4** (36) gave **3c** (83% yield), which was activated to the 4-nitrophenylester and linked to amino-functionalized Fractosil (**3d**) (37). The ligand concentration was 90 µmol/g. The structure of the 2′-deoxyisoinosine derivatives was proven by ^{13}C-NMR spectroscopy. The data are summarized in Table 2. The ^{13}C-NMR chemical shifts of compound **2** are almost the same in DMSO and water, therefore a reversible water addition, as described for related purine derivatives (38), can be excluded.

Table 2. ^{13}C-NMR chemical shifts of 2′-deoxyisoinosine and derivatives in d_6-DMSO[a]

Compound	C-2	C-6	C-5	C-8	C-4	CH_3O
2	156.1	139.4	123.6	145.5	158.8	
2[b]	157.8	139.1	124.5	147.4	159.0	
4	155.9	139.5	123.6	145.7	159.0	55.1
3a	156.0	139.5	123.6	145.4	158.9	55.1
3c	155.8	139.3	123.6	145.5	159.0	55.1
Compound	C-1′	C-2′	C-3′	C-4′	C-5′	COOH
2	82.9		70.9	87.9	61.7	
2[b]	84.2	38.8	71.4	87.6	61.8	
4	82.4		70.5	85.5	64.2	
3a	82.7		72.5	85.0	63.9	
3c	82.8		74.8	83.6	65.0	173.5

[a] δ values in p.p.m. relative to Me_4Si as internal standard.
[b]Measured in water.

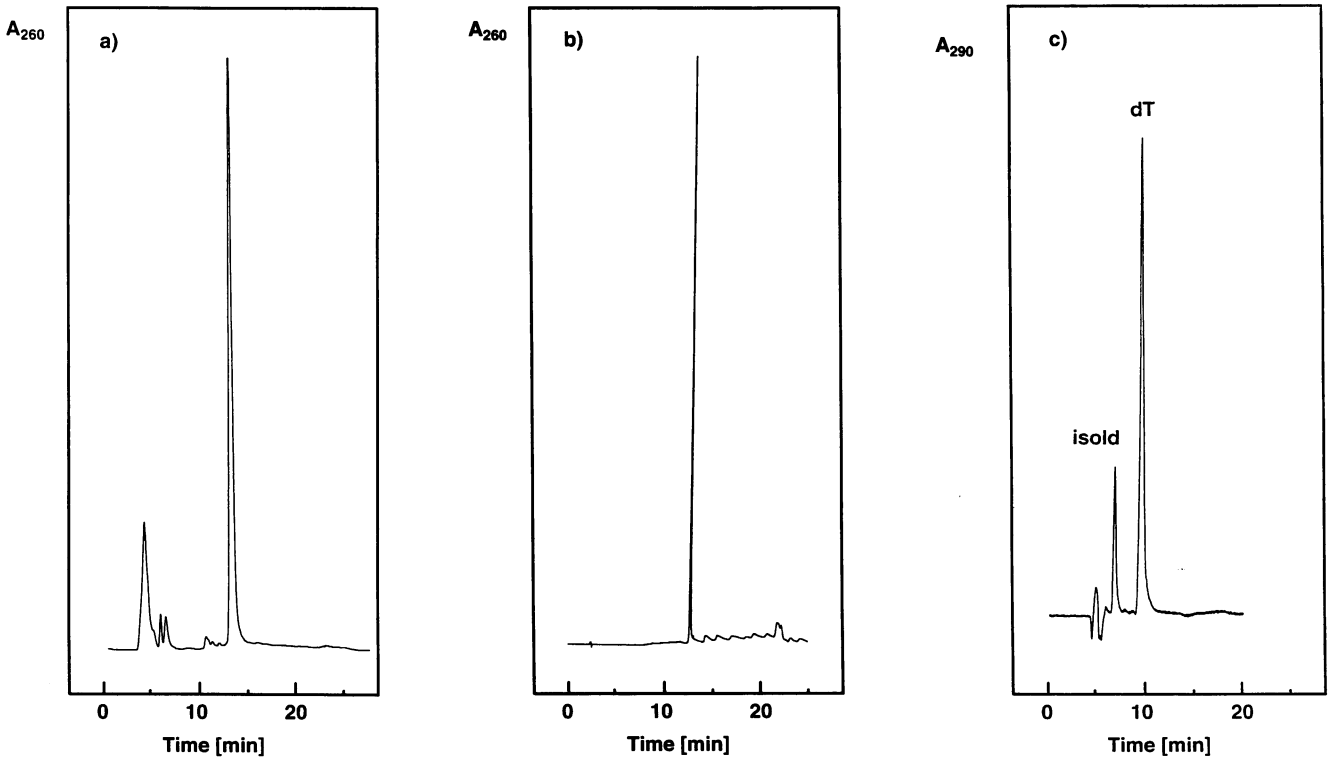


Figure 2. HPLC profiles of oligomer **6**. (**a**) Crude DMT-protected **6**, gradient I, at 260 nm; (**b**) non-protected **6**, gradient II, at 260nm; (**c**) enzymatic digest of **6**, gradient III, at 290 nm.

Oligonucleotides

Solid-phase oligonucleotide synthesis was carried out using the phosphonate **3a** together with the regular building blocks in an automated DNA synthesizer. The cycles followed the protocol published recently (39). Compared with regular phosphonates, the trityl-coupling yield went down to 85% when phosphonate **3a** was attached to a regular DNA constituent of the growing chain and to 80% when **3a** was attached to an $isoI_d$ residue. The phosphoramidite **3b** gave extremely poor coupling yields during solid-phase oligonucleotide synthesis. The 5′-DMT-protected oligomers were purified by reverse-phase HPLC, detritylated, purified again on reverse-phase chromatography and desalted. The following oligomers have been synthesized.

6 5′-d(TTTTTiIiITTTTT) **7** 5′-d(TTTTTiITTTTTT)
8 5′-d(iITTTTTTTTTTiI) **9** 5′-d(AAAAAAAAAAAA)
10 5′-d(AAAAAGGAAAAA) **11** 5′-d(AAAAATTAAAAA)
12 5′-d(AAAAACCAAAAA) **13** 5′-d(AAAAAAGAAAAA)
14 5′-d(AAAAAATAAAAA) **15** 5′-d(AAAAAACAAAAA)
16 5′-d(GAAAAAAAAAAG) **17** 5′-d(TAAAAAAAAAAT)
18 5′-d(CAAAAAAAAAAC) **19** 5′-d(TTTTTTTTTTTT)

Figure 2 shows a representative HPLC profile of the crude oligonucleotide **6** as the DMT-derivative; Figure 2 gives the profile of the deprotected purified oligomer **6**. The composition of oligonucleotides was proved by determination of the nucleoside content after hydrolysis with snake venom phosphodiesterase followed by alkaline phosphatase and RP-18 HPLC (Fig. 2c). As compound **2** shows UV maxima at 242 (2900) and 314 nm (4600) (20), which are separated from those of the other nucleosides, the $isoI_d$-content can be determined from the UV spectrum of oligonucleotide **6** (Fig. 3).

2′-Deoxyisoinosine shows strong fluorescence at ~382 nm which is shifted in MeOH to 391. This bathochromic shift is similar to that of the UV spectrum. The strong fluorescence is also observed in the case of the oligonucleotides (Table 3).

In order to investigate the stability of duplexes containing $isoI_d$ opposite to the four conventional bases the following hybrids were formed: oligomer **6** with **9**, **10**, **11** and **12**; oligomer **7** with **9**, **13**, **14** and **15**; oligomer **8** with **9**, **16**, **17** and **18**. The melting profiles were cooperative in all cases. Figure 4 shows typical melting curves obtained for duplex **7·14**. From the melting curves the T_m data were determined, which are summarized in Table 4. It can be seen that the replacement of one dT residue by $isoI_d$ in the centre of the duplex reduced the T_m value by ~15 °C and a decrease of ~25 °C was found when two residues were incorporated. The T_m decrease obtained with one $isoI_d$ residue is similar to that observed by Tinoco (3) for 2′-deoxyinosine incorporated opposite to dT, dG or dI within the centre of $d(CT_6G):d(CA_6G)$. In this case duplex melting was also strongly reduced (10 °C). Nevertheless, 2′-deoxyinosine shows base pairing selectivity and pairs much more strongly with dC than with the other nucleosides. The duplex destabilization of $isoI_d$ is almost independent of the four conventional bases located opposite to it. This would satisfy the requirement for $isoI_d$ as a universal base. As expected, the T_m decrease was small when the modified base was located at the 3′- and 5′-ends of the oligonucleotide (Table 6), which is the result of a weaker base pairing and the end of a duplex (breathing of the terminal bases).

Table 3. Fluorescence data of compound **2** and corresponding oligonucleotides[a]

Compound	Excitation (nm)	Emission (nm)
2 (H_2O)	320	382
2 (MeOH)	325	391
6 (H_2O)	318	382
7[b]	320	382
8 (H_2O)	318	383
7·9[b]	318	382

[a]Measured at 25°C.
[b]Measured in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM sodium cacodylate (pH 7.0).

Table 4. T_m values and thermodynamic data for 5′-d(XTTTTTTTTTTX)·5′-d(YAAAAAAAAAAY)[a]

X·Y	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol/K)	ΔG (25°C) (kcal/mol)	h (%)
(19·9) T·A[b]	44	–89	–281	–5.3	
(7·9) iI·A	30	–84	–276	–1.8	18
(7·13) iI·G	28	–84	–280	–0.6	20
(7·14) iI·T	31	–85	–282	–1.0	21
(7·15) iI·C	28	–88	–294	–0.4	21

[a]Measured at 260 nm in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM sodium cacodylate (pH 7.0) at 10 μM single-strand concentration.
[b]At 7.5 μM single-strand concentration.

Table 5. T_m values and thermodynamic data for 5′-d(TTTTTXXTTTTT)·5′-d(AAAAAYYAAAAA)[a]

XX·YY	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol/K)	ΔG (25°C) (kcal/mol)	h (%)
(19·9) TT·AA[b]	44	–89	–281	–5.3	
(6·9) iIiI·AA	21	–31	–107	0.9	21
(6·10) iIiI·GG	20	–59	–203	0.8	20
(6·11) iIiI·TT	19	–55	–190	1.6	24
(6·12) iIiI·CC	15	–53	–184	1.8	17

[a,b]See Table 4.

Table 6. T_m values and thermodynamic data for 5′-d(XTTTTTTTTTTX)·5′-d(YAAAAAAAAAAY)[a]

XX·YY	T_m (°C)	ΔH (kcal/mol)	ΔS (cal/mol/K)	ΔG (25°C) (kcal/mol)	h (%)
(19·9) AA·TT[b]	44	–89	–281	–5.3	
(8·9) iIiI·AA	41	–67	–213	–3.5	24
(8·16) iIiI·GG	40	–74	–239	–2.8	25
(8·17) iIiI·TT	39	–67	–215	–2.9	24
(8·18) iIiI·CC	39	–82	–264	–3.3	23

[a,b]See Table 4.

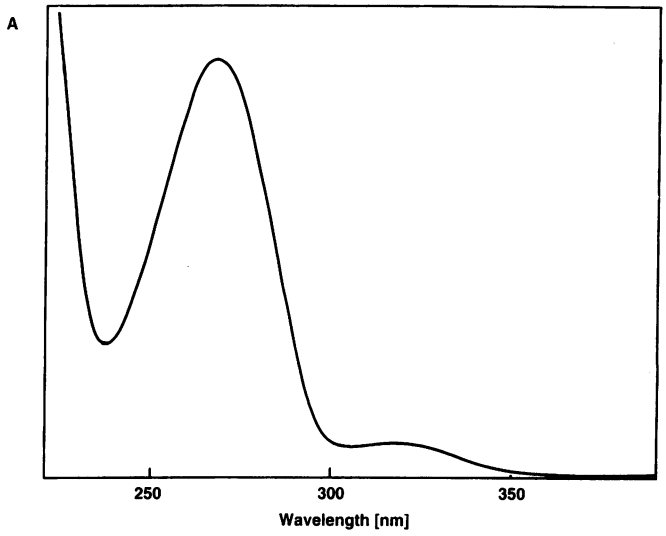


Figure 3. UV spectrum of oligomer **6** in H_2O.

The melting curve thermodynamic data for helix–coil transitions of all duplexes were calculated from the absorbance curves as reported earlier (40,41) and are given in Table 4–6. The concentration-dependent T_m values were also determined and $1/T_m$ was plotted against log c. As can be seen from the concentration dependence of the T_m value, duplex melting was confirmed (Fig. 4b). The thermodynamic data determined from this graph were in good agreement with those obtained from a single melting profile [**7·9**: ΔH = –83 kcal/mol, ΔS = –262 cal/mol/K (from Fig. 4b); ΔH = –84 kcal/mol, ΔS = –276 cal/mol/K (from Table 4)]

Despite the fact that the T_m value was decreased by ~15°C in the case of oligomers containing one $isoI_d$ residue in place of dT (internal position) the enthalpy was only slightly affected. This was not expected, but was again similar to the situation found for oligonucleotides containing one dI residue opposite to dA, dT or dG within a stretch of dT (3). The enthalpy data imply that in the case of the incorporation of one $isoI_d$ residue opposite to dA, dT, dG or dC a stacked duplex is formed. In this case a dT–dA base pair is located on both sides of $isoI_d$. It remains an open question whether hydrogen bonding occurs. At least from model building, non-Watson–Crick base pairs can be realized, but none of them seem to be very strong. Only NMR experiments or X-ray analysis data can give a more detailed picture.

The situation changes when two $isoI_d$ residues are located in the centre of a duplex (duplexes **6·9**, **6·10**, **6·11** and **6·12**). According to Table 5 a strong loss of reaction enthalpy is observed in this case, which is partly compensated for by a favourable entropy term. It is anticipated that a loop is formed containing at least two $isoI_d$ residues. Accordingly, nearest neighbour interaction of two consecutive $isoI_d$ residues is very unfavourable for duplex formation. The incorporation of one residue seems to be accommodated by the duplex structure. It seems likely that the polar molecular structure of $isoI_d$ is accepted between two regular residues, but when two of those polar residue become nearest neighbours, they loop out. $IsoI_d$ may bind more water than the conventional nucleic acid residues. This water coordination can be considered to occur on the single-stranded molecule. During duplex formation more water will be released than in the case of

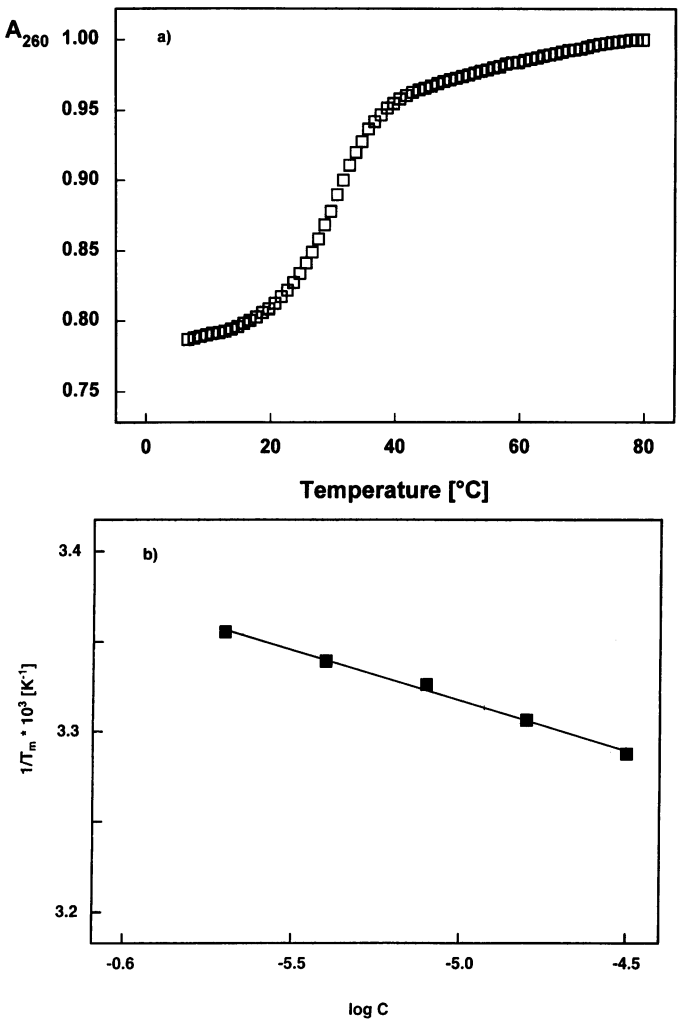


Figure 4. (**a**) Normalized melting profiles of duplex **7·14**. The curves were measured at 260 nm in 1 M NaCl, 100 mM $MgCl_2$ and 60 mM sodium cacodylate (pH 7.0) at a 10 μM single-strand concentration. (**b**) Plot of $1/T_m$ versus log *c* of duplex **7·9** [same buffer as in (a)].

the conventional nucleosides, which results in a favourable entropy term.

As discussed above, base pairing modes of $isoI_d$ with the four conventional bases are possible, but the experiments described do not favour hydrogen bonded structures within an antiparallel duplex. Nevertheless, base pairing of $isoI_d$ with dC may occur in alternating $d(isoI–C)_n$ having a parallel chain orientation. This is likely, as the related $d(isoG–C)_3$ forms a stable duplex in 1 M NaCl with a T_m of 32°C (42,43). Studies on this subject, as well as on oligomers containing more than two 2′-deoxyisoinosine residues as nearest neighbours, are in progress. Improvements of the $isoI_d$–building block structure (oxygen protection) may be necessary to increase coupling yields, which is still a problem when consecutive $isoI_d$ residues have to be incorporated into an oligonucleotide.

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