2′,4′-BNA bearing a 2-pyridine nucleobase for CG base pair recognition in the parallel motif triplex DNA†

Yoshiyuki Hari,*[a] Sachiko Matsugu,[a] Hiroyasu Inohara,[a] Yuri Hatanaka,[a] Masaaki Akabane,[a] Takeshi Imanishi[a] and Satoshi Obika*[a,b]

Received 6th April 2010, Accepted 16th June 2010
First published as an Advance Article on the web 21st July 2010
DOI: 10.1039/c004895j

We succeeded in the synthesis of triplex-forming oligonucleotides (TFOs) that contain a deoxyribonucleotide (Py) bearing a 2-pyridine nucleobase or the 2′,4′-BNA congener (Py^B). By UV melting experiments, it was found that 2-pyridine was a very promising nucleobase for the sequence-selective recognition of a CG base pair within double-stranded DNA (dsDNA) in a parallel motif triplex. Moreover, Py^B in TFOs showed stronger affinity to a CG base pair than Py with further increase in the selectivity. Using TFO including multiple Py^B units, triplex formation with dsDNA containing three CG base pairs was observed.

Introduction

Triplex-forming oligonucleotides (TFOs) that sequence-specifically bind to double-stranded DNA (dsDNA) have received considerable attention because of the possibility of their application to various technologies such as gene therapy and gene diagnosis.[1] There are two motifs for recognition of dsDNA by TFOs, namely parallel and antiparallel ones. In the former, homopyrimidine TFOs bind to the homopurine tract of dsDNA through Hoogsteen hydrogen bonds in the major groove to form T-AT and protonated C (C^+H)-GC base triplets (Fig. 1). In the latter, purine-rich TFOs form A–AT (or T–AT) and G–GC base triplets with the homopurine tract of dsDNA by making reverse-Hoogsteen hydrogen bonds. In other words, in either motif, the recognition is restricted to the homopurine tract within dsDNA. Thus, over the past few decades, the development of nucleic acids bearing artificial nucleobases that sequence-selectively and stably form base triplets with pyrimidine-purine (CG or TA) base pairs in dsDNA has been carried out.[2] However, to date, successful examples number only a few and are limited to 2-pyridone (**P**),[3,4] 5-methylpyrimidin-2-one (^{4H}**T**)[5] and a pyrrolo[2,3-*d*]pyrimidin-2-one derivative (^{A}**PP**),[6] developed by us and other groups on the basis of the fact that T has a degree of affinity to CG base pairs (Fig. 2).[7] These nucleobases can sequence-selectively bind to a CG base pair, though the affinity is not high. For practical applications, the development of nucleobases having stronger binding affinity and higher selectivity to a CG base pair, *e.g.*, T for an AT base pair, is still required.

In our exploration for a new nucleobase toward the recognition of a CG base pair, we designed 2-pyridine (Fig. 3). Since the nitrogen of pyridine is basic, pyridine as a nucleobase is expected to act as a strong hydrogen acceptor compared to pyridone- or pyrimidinone-based nucleobases shown in Fig. 2. Meanwhile, we previously discovered that a 2′,4′-BNA (or LNA) modification in TFO (Fig. 3) dramatically improved triplex-forming ability with dsDNA in terms of both sequence-selectivity and stability.[8,9] Thus, we synthesized TFOs containing a deoxyribonucleotide (Py) with 2-pyridine or the 2′,4′-BNA congener (Py^B) and evaluated the sequence-selectivity and affinity of the TFOs to dsDNA targets in the parallel motif triplex formation. The details of our results are described in this paper.[10]

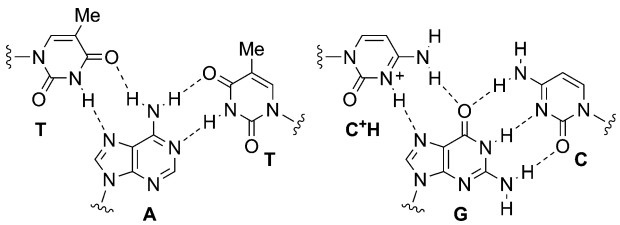


Fig. 1 T–AT and C^+H–GC base triplets.

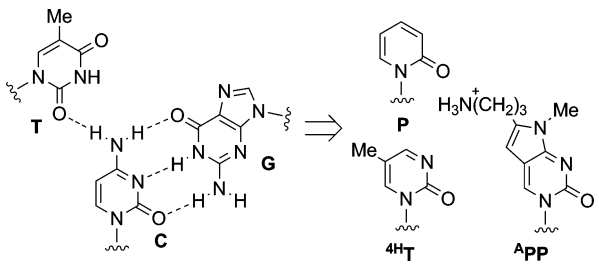


Fig. 2 T–CG base triplet and nucleobases for CG base pair recognition.

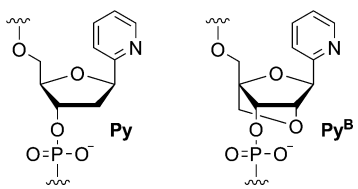


Fig. 3 Oligonucleotides designed and used in the present study.

[a]*Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan. E-mail: hari@phs.osaka-u.ac.jp, obika@phs.osaka-u.ac.jp; Fax: +81 6 6879 8201, +81 6 6879 8204; Tel: +81 6 6879 8201, +81 6 6879 8200*
[b]*PRESTO, JST, Sanbancho Building, 5-Sanbancho, Chiyodaku, Tokyo, 102-0075, Japan*

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C (or ^{31}P) spectra for new compounds. See DOI: 10.1039/c004895j

Modified TFOs

10-Py 5'-TTTTT^{m}CT**Py**T^{m}CT^{m}CT^{m}CT-3'
10-Py^{B} 5'-TTTTT^{m}CT**Py^{B}**T^{m}CT^{m}CT^{m}CT-3'
11-Py^{B} 5'-TTTTT^{m}CT**Py^{B}**C^{m}CT^{m}CT^{m}CT-3'

12-Py^{B} 5'-TTTTT^{m}C^{m}C**Py^{B}**T^{m}CT^{m}CT^{m}CT-3'
13-Py^{B} 5'-TTTTT^{m}C^{m}C**Py^{B}**C^{m}CT^{m}CT^{m}CT-3'
14-Py^{B} 5'-TTTTT**Py^{B}**T**Py^{B}**T**Py^{B}**TCTCT-3'

Natural TFOs

10-T 5'-TTTTT^{m}CT**T**T^{m}CT^{m}CT^{m}CT-3'
11-T 5'-TTTTT^{m}CT**T**C^{m}CT^{m}CT^{m}CT-3'
12-T 5'-TTTTT^{m}C^{m}C**T**T^{m}CT^{m}CT^{m}CT-3'
13-T 5'-TTTTT^{m}C^{m}C**T**C^{m}CT^{m}CT^{m}CT-3'
14-T 5'-TTTTT**T**T**T**T**T**TCTCT-3'

dsDNA targets

15 5'-GCTAAAAAGA**X**AGAGAGATCG-3'
3'-CGATTTTTCT**Y**TCTCTCTATC-5'
(**XY** = CG, GC, TA or AT)

16 5'-GCTAAAAAGA**C**GGAGAGATCG-3'
3'-CGATTTTTCT**G**CCTCTCTAGC-5'

17 5'-GCTAAAAAGG**C**AGAGAGATCG-3'
3'-CGATTTTTCC**G**TCTCTCTAGC-5'

18 5'-GCTAAAAAGG**C**GGAGAGATCG-3'
3'-CGATTTTTCC**G**CCTCTCTAGC-5'

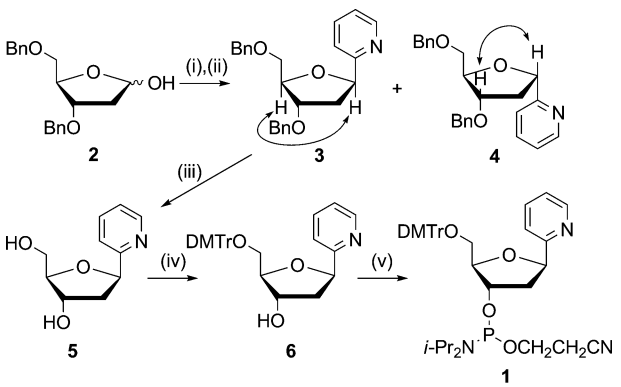
19 5'-GCTAAAAA**C**A**C**A**C**AGAGATCG-3'
3'-CGATTTTT**G**T**G**T**G**TCTCTAGC-5'

Fig. 4 TFOs and dsDNA used in the present study. ^{m}C indicates 2′-deoxy-5-methylcytidine.[13]

Results and discussion

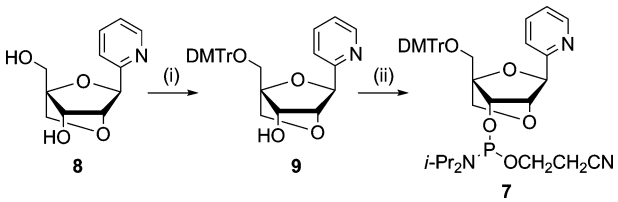
Synthesis

The phosphoramidite **1** combined with a deoxyribose and a 2-pyridine nucleobase was synthesized as shown in Scheme 1. Coupling of the dibenzylated 2′-deoxy-D-ribose **2**[11] with 2-pyridyllithium, prepared from 2-bromopyridine and *n*-BuLi, followed by the Mitsunobu reaction using 1,1′-azobis(*N*,*N*-dimethylformamide) and *n*-Bu_3P gave a 2 : 1 separable mixture of desired β-isomer **3** and the α-isomer **4** in 60% yield from **2**. The structure of each isomer was confirmed by the NOESY correlations shown in Scheme 1. Debenzylation of **3** gave the desired monomer **5** in 60% yield. Compound **5** was reacted with DMTrCl in pyridine to afford **6** in 49% yield, which was phosphitylated to give the phosphoramidite **1**, a suitable building block for the oligonucleotide synthesis, in 78% yield. The synthesis of the 2′,4′-BNA phosphoramidite **7** bearing pyridine as an unnatural nucleobase was achieved from the 2′,4′-BNA monomer **8** that we previously reported (Scheme 2).[12] Compound **9** was obtained



Scheme 1 Reagents and conditions: (i) 2-pyridyllithium, Et_2O–THF, −78 °C, 1.5 h; (ii) 1,1′-azobis(*N*,*N*-dimethylformamide), *n*-Bu_3P, CH_2Cl_2, rt, 1.5 h (60% for 2 steps, **3** : **4** = 2 : 1); (iii) 20% $Pd(OH)_2$-C, cyclohexene, EtOH, reflux, 3 h (60%); (iv) DMTrCl, pyridine, rt, 2.5 h (49%); (v) *i*-$Pr_2NP(Cl)OCH_2CH_2CN$, *i*-Pr_2NEt, CH_2Cl_2, rt, 1.5 h (78%). Two-headed arrows indicate observed NOESY correlations.

in 90% yield by treatment of **8** with DMTrCl in pyridine and phosphitylation of **9** gave the desired phosphoramidite **7** in 89% yield. The obtained phosphoramidites **1** and **7** were introduced into TFOs using a standard phosphoramidite protocol on an automated DNA synthesizer. The purity of the prepared TFOs **10–14** including Py or Py^{B}, shown in Fig. 4, was verified using



Scheme 2 Reagents and conditions: (i) DMTrCl, pyridine, rt, 3 h (90%); (ii) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, diisopropylammonium tetrazolide, CH_3CN–THF, rt, 5 h (89%).

Table 1 T_m values (°C) of triplexes between TFOs **10** and four dsDNA targets **15**.[a,b]

TFO	XY			
	CG	TA	GC	AT
10-T	25 (44)	17 (33)	20 (35)	44 (59)
10-Py	24 (41)	17 (34)	15 (35)	19 (35)
10-Py^{B}	36 (56)	22 (33)	18 (38)	21 (44)

[a] Conditions: 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl and 10 mM $MgCl_2$. The final concentration of each oligonucleotide used was 1.5 μM. [b] T_m values, shown in the parenthesis, were obtained when 5 mM spermine instead of 10 mM $MgCl_2$ was used.

reversed-phase HPLC and their compositions were determined by MALDI-TOF-MS analysis.

Evaluation

Triplex-forming ability of the TFOs **10–14** containing Py or Py^{B} was evaluated on the basis of a T_m value calculated by UV melting experiments. Initially, the UV melting experiment using TFOs **10-T**, **10-Py** and **10-Py^{B}**, and four dsDNA targets **15** (XY = CG, TA, GC and AT), was performed in 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl and 10 mM $MgCl_2$, and the results are summarized in Table 1. As described in the introduction, T has an affinity for CG base pairs. The TFO **10-T** had a T_m value of 25 °C against dsDNA **15** (XY = CG). However, the triplex between **10-T** and **15** (XY = AT) containing a stable T–AT base triplet naturally showed a higher T_m value (44 °C). By contrast, TFO **10-Py**, which bears a 2-pyridine nucleobase, showed the highest T_m value against **15** (XY = CG) among four targets **15** and the stability was as much as that of **10-T** and **15** (XY = CG). This result suggests that a 2-pyridine nucleobase effectively recognizes a CG base pair in dsDNA (Fig. 5). Next, the properties of Py^{B}, combined with 2′,4′-BNA modification and a 2-pyridine nucleobase, instead of Py, were examined (Table 1 and Fig. 6). The T_m value of **10-Py^{B}**

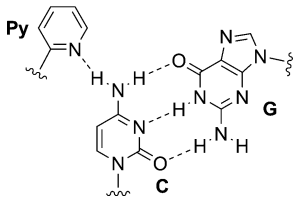


Fig. 5 Plausible recognition pattern of a CG base pair by 2-pyridine nucleobase (**Py**).

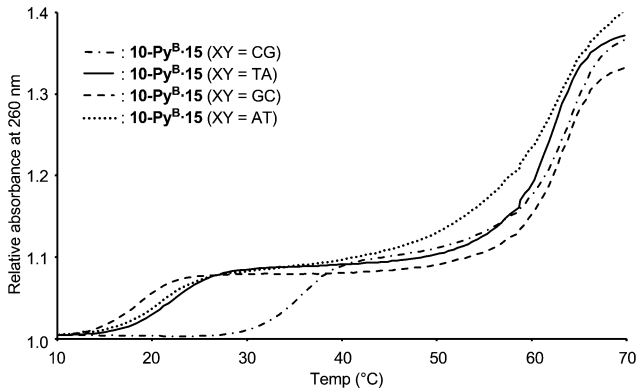


Fig. 6 UV melting profiles (260 nm) for triplexes **10-Py^B·15** (XY = CG, TA, GC and AT). The presented melting curves were normalized.

with **15** (XY = CG) was 36 °C, an increase of 12 °C compared to that of **10-Py**. On the sequence-selectivity of **10-Py^B**, the T_m value with **15** (XY = CG) was 14–18 °C higher than those with the other targets **15** (XY = TA, GC or AT), while, on the sequence-selectivity of **10-Py**, the T_m difference, ΔT_m, was only 5–9 °C. The dramatic improvement of selectivity and affinity to a CG base pair by Py^B is attributed to the synergy between recognition of a CG base pair by 2-pyridine and stabilization of the triplex by 2′,4′-BNA modification. Interestingly, under the same conditions, the ability of Py^B slightly exceeded that of 2′,4′-BNA bearing a 2-pyridone nucleobase, P^B, which we previously developed.[14,15] On the other hand, under conditions using 5 mM spermine in place of 10 mM $MgCl_2$, the T_m and ΔT_m values of the triplex between **10-Py^B** and **15** (XY = CG), 56 °C and over 12 °C respectively, were found to be almost comparable to those of natural triplex between **10-T** to **15** (XY = AT).

In view of the potential general applications, we examined the influence of neighboring sites of a Py^B-CG base triplet under neutral conditions containing 5 mM spermine (Table 2). Triplexes between **11** and **16**, **12** and **17**, and **13** and **18** have C–GC base triplets at the 3′-site, 5′-site, and both 3′- and 5′-sites, respectively. Although these triplexes were less stable than that of TFO **10** with **15** (XY = CG) because of an increase in the number of labile ^{m}C–GC base triplets at neutral pH, it was clarified, by comparison with the corresponding T–CG base triplet, that Py^B can effectively recognize a CG base pair.

Finally, to verify the practicality of Py^B further, we investigated the stability of the triplexes of TFO **14-Py^B** with dsDNA **19**, including three Py^B-CG base triplets (Table 2). In the case of the triplex between **14-T** and **19** including three T-CG base triplets, triplex formation was not observed at all. However, TFO **14-Py^B** formed the relatively stable triplex with dsDNA **19** and the T_m value was 22 °C, equal to that of TFO containing P^B in the same context.[3b]

Table 2 UV melting experiments of TFOs **10–14** and dsDNA targets **15–19**.[a]

TFO	dsDNA target	T_m value/°C
10-T	**15** (XY = CG)	44
10-Py^B	**15** (XY = CG)	56
11-T	**16**	39
11-Py^B	**16**	49
12-T	**17**	34
12-Py^B	**17**	46
13-T	**18**	15
13-Py^B	**18**	34
14-T	**19**	—[b]
14-Py^B	**19**	22

[a] Conditions: 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl and 5 mM spermine. The final concentration of each oligonucleotide used was 1.5 μM. [b] No triplex formation was observed.

Conclusions

We have synthesized TFOs that contain a deoxyribonucleotide (Py) bearing a 2-pyridine nucleobase or the 2′,4′-BNA congener (Py^B) and their triplex-forming ability was evaluated by UV melting experiments. From the results, Py^B is a promising candidate for recognition of a CG base pair in triplex formation though more detailed experiments are naturally needed. The ability of Py^B is the same or more than that of 2′,4′-BNA bearing a 2-pyridone nucleobase, P^B, which we previously developed.[3] It is interesting to note that 2-pyridine has a new type of nucleobase structure, differing from the pyridone- or pyrimidinone-based nucleobases, like P, ^{4H}T and ^{A}PP. This indicates that 2-pyridine is attractive as a nucleobase core to improve recognition ability by additional introduction of functional groups, *i.e.*, to sequence-selectively and stably recognize a CG base pair through two or more hydrogen bonds.

Experimental

General

All chemicals were purchased from chemical suppliers. For column chromatography, Fuji Silysia silica gel PSQ-100B and FL-100D were used. All melting points were measured on a Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on a JEOL ECS400 spectrometer. IR spectra were recorded on JASCO FT/IR-200 and JASCO FT/IR-4200 spectrometers. Optical rotations were recorded on a JASCO DIP-370 instrument. Mass spectra were measured on a JEOL JMS-600 or JEOL JMS-700 mass spectrometer. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer.

2-(2-Deoxy-3,5-di-*O*-benzyl-β-D-ribofuranosyl)pyridine (3) and 2-(2-deoxy-3,5-di-*O*-benzyl-α-D-ribofuranosyl)pyridine (4)

Under a nitrogen atmosphere, a solution of compound **2**[12] (321 mg, 1.02 mmol) in anhydrous THF (5 mL) was added to a solution of 2-pyridyllithium, prepared from 2-bromopyridine (0.60 mL, 6.12 mmol) and *n*-BuLi (1.53 M in hexane, 4 mL, 6.12 mmol) in

anhydrous Et_2O (19 mL), at −78 °C and the mixture was stirred at −78 °C for 1.5 h. After addition of water, the mixture was extracted with AcOEt. The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (n-hexane–AcOEt = 1 : 2) to give appropriate compounds (294 mg, R_f = *ca.* 0.2), which were dissolved in CH_2Cl_2 (8 mL) and 1,1′-azobis(*N*,*N*-dimethylformamide) (193 mg, 1.12 mmol) and *n*-Bu_3P (0.28 mL, 1.12 mmol) were added at room temperature. After being stirred at room temperature for 1.5 h, the mixture was diluted with Et_2O and filtered through a pad of Celite®. The filtrate was concentrated *in vacuo* and the residue was purified by flash silica gel column chromatography (*n*-hexane–AcOEt = 5 : 1) to give a mixture of β-isomer **3** and α-isomer **4** (230 mg, 60%, **3** : **4** = 2 : 1). Compound **3**, pale yellow oil; $[\alpha]_D^{24}$ +50.84 (*c* 1.00, $CHCl_3$); IR (KBr) *ν*: 2861, 1592, 1454, 1436, 1100, 1028 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) *δ* 2.06 (1H, ddd, *J* = 6.0, 10.1 and 13.3 Hz), 2.56 (1H, ddd, *J* = 1.8, 6.0 and 13.3 Hz), 3.60 (1H, dd, *J* = 5.0 and 10.1 Hz), 3.67 (1H, dd, *J* = 4.6 and 10.1 Hz), 4.15–4.17 (1H, m), 4.34 (1H, ddd, *J* = 2.3, 4.6 and 5.0 Hz), 4.53, 4.59 (2H, AB, *J* = 12.0 Hz), 4.58 (2H, s), 5.26 (1H, dd, *J* = 6.0 and 10.1 Hz), 7.14–7.18 (1H, m), 7.26–7.36 (10H, m), 7.51 (1H, d, *J* = 7.8 Hz), 7.64 (1H, dt, *J* = 1.8 and 7.8 Hz), 8.53 (1H, m); ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 39.05, 70.96, 71.05, 73.39, 81.00, 81.12, 84.11, 120.33, 122.31, 127.60, 127.62, 127.65, 128.34, 128.39, 136.63, 138.06, 138.15, 148.85, 161.55; MS (FAB): *m*/*z* 376 (MH^+); HRMS (FAB): 376.1912 (MH^+, calcd for $C_{24}H_{26}NO_3$: 376.1913). Compound **4**, pale yellow oil; $[\alpha]_D^{24}$ +5.89 (*c* 1.00, $CHCl_3$); IR (KBr) *ν*: 2861, 1591, 1454, 1361, 1102 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) *δ* 2.34 (1H, ddd, *J* = 4.6, 6.4 and 12.8 Hz), 2.70 (1H, ddd, *J* = 6.4, 7.3 and 12.8 Hz), 3.62 (2H, d, *J* = 4.6 Hz), 4.19–4.22 (1H, m), 4.37–4.45 (3H, m), 4.59, 4.61 (2H, AB, *J* = 11.9 Hz), 5.25 (1H, dd, *J* = 6.4 and 7.3 Hz), 7.10–7.16 (3H, m), 7.23–7.35 (8H, m), 7.59 (1H, d, *J* = 7.8 Hz), 7.67 (1H, dt, *J* = 1.8 and 7.8 Hz), 8.52 (1H, d, *J* = 4.6 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 38.69, 70.76, 71.16, 73.45, 80.52, 80.95, 83.52, 119.83, 121.86, 127.50, 127.59, 128.23, 128.24, 128.34, 136.54, 137.90, 138.09, 148.67, 162.80; MS (FAB): *m*/*z* 376 (MH^+); HRMS (FAB): 376.1912 (MH^+, calcd for $C_{24}H_{26}NO_3$: 376.1913).

2-(2-Deoxy-β-D-ribofuranosyl)pyridine (5)[16]

A solution of compound **3** (82 mg, 0.22 mmol), 20% $Pd(OH)_2$-C (85 mg) and cyclohexene (1.6 mL, 16 mmol) in EtOH (2 mL) was refluxed for 3 h. The mixture was filtered through a paper filter and the filtrate was concentrated in vacuo. The residue was purified by flash silica gel column chromatography (AcOEt–MeOH = 20 : 1 to 10 : 1) to give compound **5** (26 mg, 60%) as a colorless oil.^1H NMR (400 MHz, CD_3OD) *δ* 2.04 (1H, ddd, *J* = 5.9, 9.6 and 13.3 Hz), 2.31 (1H, ddd, *J* = 1.8, 6.4 and 13.3 Hz), 3.67 (1H, dd, *J* = 4.3 and 11.9 Hz), 3.73 (1H, dd, *J* = 3.7 and 11.9 Hz), 4.01–4.02 (1H, m), 4.35–4.36 (1H, m), 5.20 (1H, dd, *J* = 6.4 and 9.6 Hz), 7.32 (1H, dd, *J* = 3.7 and 7.3 Hz), 7.58 (1H, d, *J* = 7.8 Hz), 7.83 (1H, dd, *J* = 7.3 and 7.8 Hz), 8.47 (1H, d, *J* = 3.7 Hz).

2-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]-pyridine (6)

Under a nitrogen atmosphere, DMTrCl (200 mg, 0.59 mmol) was added to a solution of compound **5** (58 mg, 0.29 mmol) in anhydrous pyridine (1 mL) at room temperature and the mixture was stirred for 2.5 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography (*n*-hexane–AcOEt–Et_3N = 50 : 50 : 1) to give compound **6** (70 mg, 49%) as a white powder. M.p. 45–49 °C; $[\alpha]_D^{23}$ +32.59 (*c* 1.00, $CHCl_3$); IR (KBr) *ν*: 3379, 2931, 1606, 1509, 1250, 1177 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) *δ* 2.01 (1H, br s), 2.22 (1H, ddd, *J* = 6.2, 9.0 and 13.2 Hz), 2.41 (1H, ddd, *J* = 2.9, 6.4 and 13.2 Hz), 3.29 (1H, dd, *J* = 5.5 and 9.7 Hz), 3.36 (1H, dd, *J* = 4.4 and 9.7 Hz), 3.78 (6H, s), 4.10–4.14 (1H, m), 4.39–4.42 (1H, m), 5.29 (1H, dd, *J* = 6.6 and 9.0 Hz), 6.82 (4H, d, *J* = 8.6 Hz), 7.15–7.46 (10H, m), 7.53 (1H, d, *J* = 7.9 Hz), 7.64 (1H, m), 8.53 (1H, d, *J* = 4.6 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 42.11, 55.18, 64.34, 74.12, 80.56, 86.16, 86.22, 113.07, 120.17, 122.27, 126.74, 127.78, 128.14, 130.05, 135.96, 135.98, 136.69, 144.80, 148.75, 158.41, 161.78; MS (FAB): *m*/*z* 520 (MH^+); HRMS (FAB): 520.2100 (MNa^+, calcd for $C_{31}H_{31}NNaO_5$: 520.2100).

2-[3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-2-deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]pyridine (1)[14]

Under a nitrogen atmosphere, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (94 μL, 0.54 mmol) was added to a solution of compound **9** (211 mg, 0.40 mmol) and *i*-Pr_2NEt (94 μL, 0.54 mmol) in anhydrous CH_2Cl_2 (1 mL) at 0 °C and the mixture was stirred for 1.5 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash silica gel column chromatography (*n*-hexane–AcOEt = 3 : 1 to 2 : 1) to give compound **1** (46 mg, 78%) as a colorless oil.^{31}P NMR (162 MHz, $CDCl_3$) *δ* 147.93, 148.47.

2-[5-*O*-(4,4′-Dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]pyridine (9)

Under a nitrogen atmosphere, DMTrCl (197 mg, 0.58 mmol) was added to a solution of compound **8** (100 mg, 0.45 mmol) in anhydrous pyridine (2 mL) at room temperature and the mixture was stirred for 3 h. After addition of saturated aqueous $NaHCO_3$ solution, the mixture was extracted with AcOEt. The organic extracts were washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash silica gel column chromatography (*n*-hexane–AcOEt = 1 : 1) to give compound **9** (212 mg, 90%) as a white powder. M.p. 68–73 °C; $[\alpha]_D^{28}$ −5.60 (*c* 0.77, $CHCl_3$); IR (KBr) *ν*: 3334, 3007, 2944, 1605, 1508, 1251, 1035 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) *δ* 2.00 (1H, br s), 3.50, 3.53 (2H, AB, *J* = 11.5 Hz), 3.80 (6H, s), 4.01, 4.10 (2H, AB, *J* = 8.2 Hz), 4.22 (1H, d, *J* = 6.0 Hz), 4.47 (1H, s), 5.18 (1H, s), 6.85 (4H, d, *J* = 8.7 Hz), 7.18–7.52 (10H, m), 7.65 (1H, d, *J* = 8.2 Hz), 7.69–7.73 (1H, m), 8.56 (1H, d, *J* = 4.1 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) *δ* 55.21, 60.06, 72.20, 72.80, 82.58, 83.93, 86.29, 86.37, 113.18, 120.77, 122.40, 126.88, 127.89, 128.12, 130.08, 130.11, 135.69, 135.75, 136.74, 144.71, 149.07, 158.53, 158.83; MS (FAB): *m*/*z* 548 (MNa^+); HRMS (FAB): 548.2050 (MNa^+, calcd for $C_{32}H_{31}NNaO_6$: 548.2049)

2-[3-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4′-dimethoxytrityl)-2-*O*,4-*C*-methylene-β-D-ribofuranosyl]pyridine (7)

Under a nitrogen atmosphere, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (0.19 mL, 0.60 mmol) was added to a solution of compound **9** (211 mg, 0.40 mmol) and diisopropylammonium tetrazolide (97 mg, 0.48 mmol) in anhydrous MeCN–THF (3 : 1, 10 mL) at room temperature and the mixture was stirred for 5 h. The solvent was removed under reduced pressure and the residue was purified by flash silica gel column chromatography (*n*-hexane–AcOEt = 1 : 1) to give compound **7** (256 mg, 89%) as a white powder. M.p. 48–51 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83, 0.93 (6H, 2d, J = 6.4 Hz and J = 6.9 Hz), 1.07, 1.08 (6H, 2d, J = 6.9 Hz and J = 6.9 Hz), 2.31, 2.45 (2H, 2ddd, J = 1.4, 6.0 and 6.4 Hz and J = 2.7, 6.4 and 6.9 Hz), 3.37–3.72 (6H, m), 3.79, 3.80 (6H, 2 s), 3.93, 3.96 (1H, 2d, J = 7.3 Hz and J = 7.8 Hz), 4.10–4.33 (2H, m), 4.56, 4.67 (1H, 2 s), 5.23, 5.24 (1H, 2 s), 6.82–6.86 (4H, m), 7.17–7.33 (4H, m), 7.39–7.43 (4H, m), 7.51–7.54 (2H, m), 7.67–7.76 (2H, m), 8.54–8.56 (1H, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 147.71, 148.39; MS (FAB): m/z 726 (MH^+); HRMS (FAB): 726.3307 (MH^+, calcd for $C_{41}H_{49}N_3O_7P$: 726.3308).

Synthesis of TFOs 10–14

The synthesis of TFOs **10–14** was performed on a 0.2 μmol scale on an automated DNA synthesizer (Applied Biosystems Expedite™ 8909) using the common phosphoramidite protocol except for a prolonged coupling time of 5–30 min for unnatural phosphoramidites. TFOs synthesized on DMTr-ON mode were cleaved from the CPG resin by treatment with 28% aqueous NH_3 solution at room temperature for 1.5 h. Additional treatment with 28% aqueous NH_3 solution at 55 °C for 15 h underwent the removal of all protecting groups on TFOs. The obtained crude TFOs were purified with Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XTerra® MS C_{18} 2.5 μm, 10 × 50 mm). The composition of the TFOs was confirmed by MALDI-TOF-MS analysis. MALDI-TOF-MS data ([M − H]$^-$) for TFOs **10–14**: **10-Py**, found 4449.57 (calcd 4448.97); **11-Py^B**, found 4475.83 (calcd 4476.04); **12-Py^B**, found 4475.07 (calcd 4475.07); **13-Py^B**, found 4475.04 (calcd 4475.07); **14-Py^B**, found 4475.03 (calcd 4474.08); **15-Py^B**, found 4441.88 (calcd 4442.05).

UV melting experiments (T_m measurements)

UV melting experiments were carried out on a Shimadzu UV-1650PC spectrophotometer equipped with T_m analysis accessory. The UV melting profiles were recorded in 7 mM sodium phosphate buffer (pH 7.0), 140 mM KCl and 10 mM $MgCl_2$ or 10 mM spermine from 5 °C to 85 °C at a scan rate of 0.5 °C min^{-1} at 260 nm. The final concentration of each oligonucleotide used was 1.5 μM. A T_m value was designated the maximum of the first derivative calculated from the profile.

Acknowledgements

This work was supported in part by Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) and by a Grant-in-Aid for Science Research (KAKENHI) from the Japan Society for the Promotion of Science (JSPS).

Notes and references

1 For reviews: (*a*) S. Buchini and C. J. Leumann, *Curr. Opin. Chem. Biol.*, 2003, **7**, 717; (*b*) M. Duca, P. Vekhoff, K. Oussedik, L. Halby and P. B. Arimondo, *Nucleic Acids Res.*, 2008, **36**, 5123.
2 For reviews: (*a*) S. O. Doronina and J.-P. Behr, *Chem. Soc. Rev.*, 1997, **26**, 63; (*b*) D. M. Gowers and K. R. Fox, *Nucleic Acids Res.*, 1999, **27**, 1569.
3 (*a*) S. Obika, Y. Hari, M. Sekiguchi and T. Imanishi, *Angew. Chem., Int. Ed.*, 2001, **40**, 2079; (*b*) S. Obika, Y. Hari, M. Sekiguchi and T. Imanishi, *Chem.–Eur. J.*, 2002, **8**, 4796; (*c*) H. Torigoe, Y. Hari, S. Obika and T. Imanishi, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 1097.
4 We also reported a 1-isoquinolone nucleobase, a 2-pyridone analog, for CG base pair recognition: (*a*) Y. Hari, S. Obika, M. Sekiguchi and T. Imanishi, *Tetrahedron*, 2003, **59**, 5123; (*b*) H. Torigoe, Y. Hari, S. Obika and T. Imanishi, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 1571.
5 (*a*) I. Prévot-Halter and C. J. Leumann, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 2657; (*b*) S. Buchini and C. J. Leumann, *Tetrahedron Lett.*, 2003, **44**, 5065; (*c*) S. Buchini and C. J. Leumann, *Angew. Chem., Int. Ed.*, 2004, **43**, 3925; (*d*) S. Buchini and C. J. Leumann, *Eur. J. Org. Chem.*, 2006, 3152.
6 (*a*) R. T. Ranasinghe, D. A. Rusling, V. E. C. Powers, K. R. Fox and T. Brown, *Chem. Commun.*, 2005, 2555; (*b*) D. A. Rusling, V. E. C. Powers, R. T. Ranasinghe, Y. Wang, S. D. Osborne, T. Brown and K. R. Fox, *Nucleic Acids Res.*, 2005, **33**, 3025.
7 (*a*) K. Yoon, C. A. Hobbs, J. Koch, M. Sardaro, R. Kuntny and A. L. Weis, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 3840; (*b*) I. Radhakrishnan and D. J. Patel, *J. Mol. Biol.*, 1994, **241**, 600.
8 (*a*) S. Obika, Y. Hari, T. Sugimoto, M. Sekiguchi and T. Iminishi, *Tetrahedron Lett.*, 2000, **41**, 8923; (*b*) H. Torigoe, Y. Hari, S. Obika and T. Imanishi, *J. Biol. Chem.*, 2001, **276**, 2354; (*c*) S. Obika, T. Uneda, T. Sugimoto, D. Nanbu, T. Minami, T. Doi and T. Imanishi, *Bioorg. Med. Chem.*, 2001, **9**, 1001.
9 (*a*) B.-W. Sun, B. R. Babu, M. D. Sørensen, K. Zakrzewska, J. Wengel and J.-S. Sun, *Biochemistry*, 2004, **43**, 4160; (*b*) E. Brunet, P. Alberti, L. Perrouault, R. Babu, J. Wengel and C. Giovannangeli, *J. Biol. Chem.*, 2005, **280**, 20076.
10 A part of the results was reported at the 4th International Symposium on Nucleic Acids Chemistry. S. Matsugu, H. Inohara, S. Obika and T. Imanishi, *Nucleic Acids Symp. Ser.*, 2005, **49**, 159.
11 W. Wierenga and H. I. Skulnick, *Carbohydr. Res.*, 1981, **90**, 41.
12 Y. Hari, S. Obika, M. Sakaki, K. Morio, Y. Yamagata and T. Imanishi, *Tetrahedron*, 2002, **58**, 3051.
13 It is known that ^{m}C has higher binding affinity to a GC base pair than C at physiological pH. T. J. Povsic and P. B. Dervan, *J. Am. Chem. Soc.*, 1989, **111**, 3059.
14 Under the same conditions, selectivity (ΔT_m) and affinity (T_m) of P^B to a CG base pair were 10–19 °C and 33 °C, respectively[3a].
15 Although T^B showed the T_m value of 35 °C for a CG base pair under the same conditions, T^B has no selectivity to a CG base pair because the T_m value of T^B for an AT base pair was 57 °C. S. Obika, Y. Hari, T. Sugimoto, M. Sekiguchi and T. Imanishi, *Tetrahedron Lett.*, 2000, **41**, 8923.
16 Y. Kim, A. M. Leconte, Y. Hari and F. E. Romesberg, *Angew. Chem., Int. Ed.*, 2006, **45**, 7809.

