Peptide-nucleic acids (PNAs) with pyrimido[4,5-*d*]pyrimidine-2,4,5,7-(1*H*,3*H*,6*H*,8*H*)-tetraone (PPT) as a universal base: their synthesis and binding affinity for oligodeoxyribonucleotides†

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Peptide-nucleic acids (PNAs) including pyrimido[4,5-*d*]pyrimidine-2,4,5,7-(1*H*,3*H*,6*H*,8*H*)-tetraone (PPT) as a nucleobase were synthesized, and their binding affinity for the complementary oligodeoxyribonucleotides was investigated. We found that PNAs with one or two PPT(s) and natural nucleobases (*i.e.*, adenine, cytosine, guanine, or thymine) have excellent binding affinity for oligodeoxyribonucleotides with complementary bases at the positions facing the natural nucleobases, and with adenine, cytosine, guanine, and thymine at the positions opposite PPT in PNAs. The binding affinity of the PPT-containing PNA is higher than or comparable to that of a PNA consisting of all complementary natural nucleobases, *viz*. a PNA with a suitable natural nucleobase in place of PPT in the PPT-containing PNA. Consequently, it was concluded that PPT serves as a useful universal base that can recognize all natural nucleobases.

Introduction

In the field of nucleic acid research, much effort has been expended to synthesize a universal base that is able to recognize natural nucleobases, *i.e.*, adenine, cytosine, guanine, and thymine (or uracil),[1] in order to form stable pairs with these bases. Such a universal base would be useful for various investigations dealing with nucleic acids that cannot be easily carried out using only the four kinds of natural nucleobases. Such investigations include discrimination of single-nucleotide polymorphisms (SNPs) in DNA hybridization by means of artificial mismatches,[2,3] design of oligonucleotide primers or hybridization probes, where the identity of one or more bases in the target sequence is unknown,[4–8] and sequence-specific recognition of duplex DNA, thereby blocking gene expression.[9,10] Various candidates have been proposed as potential universal bases. These include hypoxanthine (inosine),[4] 3-nitropyrrole,[5] 5-nitroindole,[11] isocarbostyril derivatives,[12] (3,4-dihydropyrimido)oxazine derivatives,[13] 9-deazaguanine,[14] 8-aza-7-deazapurine derivatives,[15] pyridazine derivatives,[16] 4-*N*-(*N*-quinon-5-yl)carbamoylcytosine,[17] and 2-oxoimidazole-4-carboxamide.[18]

However, these bases are not useful in all situations. With the exception of 3-nitropyrrole and 5-nitroindole, all of the above bases form a stable pair with only some of the natural nucleobases. For example, inosine, which was the first universal base and thus has been most frequently used, discriminates cytosine and forms a stable base with it, but does not do so with the other natural nucleobases.[19,20] In contrast, 3-nitropyrrole and 5-nitroindole have quite good universality, recognizing and forming base pairs with all four natural nucleobases, but the stability of the resulting base pairs is not always high. The reason for this low stability is considered to be because 3-nitropyrrole and 5-nitroindole are hydrophobic bases and thus form base pairs through π–π stacking interactions (space or intercalative interactions). However, such interactions are not always strong enough to allow satisfactory base-recognizing ability. Therefore, in order to create a base pair that possesses high binding affinity to natural nucleobases, it is necessary to design a base that forms pairs with all the natural nucleobases through not only the π–π stacking interactions but also the hydrogen-bond interactions that are observed in natural base pairs.

As such a base, we recently designed pyrimido[4,5-*d*]pyrimidine-2,4,5,7-(1*H*,3*H*,6*H*,8*H*)-tetraone (PPT).[21] As we have reported,[21] this artificial base indeed serves as a perfect universal base to form a stable 1:1 complex with all the natural nucleosides. Initially, we planned to prepare oligonucleotides containing a ribonucleoside with PPT (PPT-nucleoside) (**1**). However, trials for preparation of the targeted nucleotide resulted in failure, because the PPT-nucleoside is too labile in the presence of acids to use as a building unit for the synthesis of oligonucleotides. For example, the PPT-nucleoside underwent cleavage of the glycoside bond by treatment with dichloroacetic acid in dichloromethane, which is generally used for removal of the 5′-*O*-*p*,*p*′-dimethoxytrityl protecting group in oligonucleotide synthesis. In addition, conversion of the PPT-nucleoside to the phosphoramidite could not be achieved, because the PPT-nucleoside is poorly soluble in various organic solvents, such as acetonitrile. Given the problems encountered with the stability and solubility of the PPT-nucleoside chemistry, it was decided to investigate the applicability of our system in peptide/nucleic acids (PNAs) containing the PPT group as the nucleobase.

As is well known, PNAs – oligonucleotide analogs in which the phosphate backbone of the oligonucleotide is replaced

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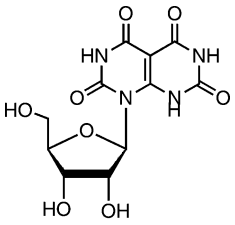
by an uncharged polyamide backbone consisting of an *N*-(2-aminoethyl)glycine unit[22] – form duplexes with complementary DNA. PNAs have numerous advantages over DNA for forming a duplex with DNA, because the polyamide backbone of the PNA is neutral. The advantages include (i) the higher thermodynamic stability of the PNA–DNA duplex than a DNA–DNA duplex, (ii) the higher sensitivity of PNA for recognizing mismatched bases, and (iii) the relative independence of PNA from the salt concentration of a solution.[23–25] Therefore, PNAs are important as biotechnological, diagnostic, and medicinal agents,[23–25] and are frequently employed in various biological investigations, such as studies investigating antisense chemotherapy.[23–25] Accordingly, the PPT-containing PNAs are substances of great interest. In this study, we prepared a PPT-containing PNA and investigated its binding affinity for deoxyribonucleotides with complementary natural nucleobases.



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Results and discussion

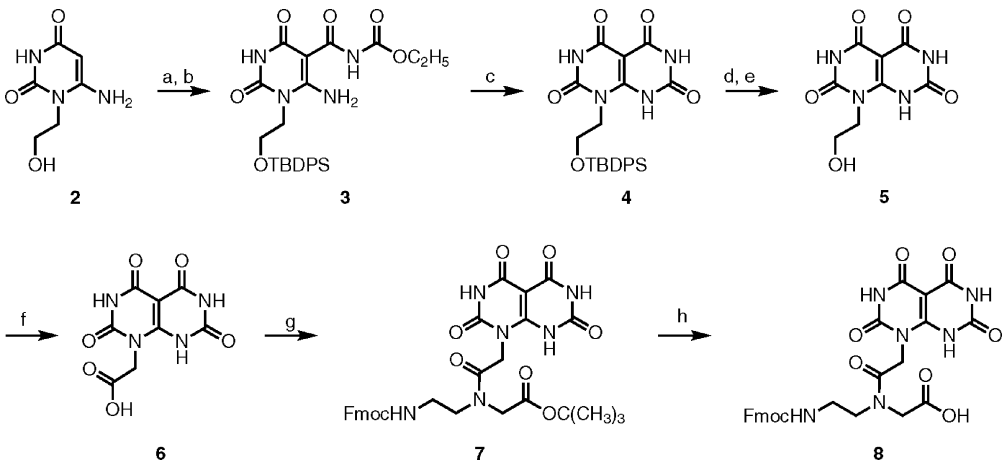
Preparation and stability of the PPT-containing amino acid

We first prepared an amino acid with PPT (PPT-amino acid), to be used as a building block for the PPT-containing PNA, and checked its stability of the PPT-amino acid under acidic and basic conditions.

The synthesis of the PPT-amino acid **8** was carried out according to the procedure shown in Scheme 1. Treatment of 1-hydroxyethyl-6-aminouracil (**2**)[26] with *tert*-butyldiphenylsilyl chloride (TBDPSCl) in the presence of imidazole in DMF (60 °C, 1.5 h) and then with ethyl isocyanatoformate ($O{=}C{=}NCOOC_2H_5$)[27] in DMF (25 °C, 24 h) gave **3** in 92% overall yield. Subsequently, **3** was reacted with sodium ethoxide in refluxing ethyl alcohol for 17 h to provide **4** as a mono-sodium salt[28] in 92% yield. Subsequently, the *O*-TBDPS protecting group of **4** was removed using triethylamine trihydrofluoride [$(C_2H_5)_3N{\cdot}3HF$] (25 °C, 24 h) to give **5** in 82% yield. The alcohol **5** was converted to the carboxylic acid **6** in 52% yield by oxidation using a mixture of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), NaBr, and NaClO (25 °C, 1 h). The product **6** was condensed with *tert*-butyl *N*-[2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl]glycinate[29] [$FmocNH(CH_2)_2NHCH_2COOC(CH_3)_3$] using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride as a promoter in DMF (25 °C, 24 h) to provide **7** in 74% yield. Finally, the *O*-*tert*-butyl protecting group of **7** was removed by treatment with a 1:2 (v/v) mixture of dichloromethane and trifluoroacetic acid (TFA) (25 °C, 24 h) to give **8** in 87% yield. The PPT-substituted amino acid skeleton – the parent skeleton of **7** and **8** – was not decomposed at all by this acid treatment. Removal of the *N*-Fmoc protecting group of **8** was also achieved without decomposition of the parent skeleton by using a 2:2:96 (v/v/v) mixture of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), piperidine and DMF (25 °C, 15 min). These results indicated that the PPT-substituted amino acid is stable under the usual acidic and basic conditions required for the synthesis of PPT-containing PNAs.

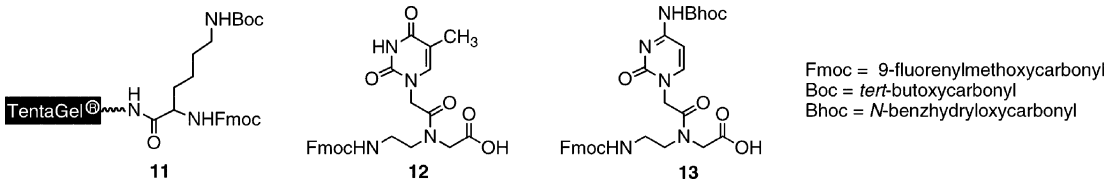
Synthesis of PPT-containing PNA oligomers

We next prepared two PPT-containing PNAs, H_2N-Lys-CCT(PPT)TCC-Gly-H (**9**) and H_2N-Lys-CCTTTCC(PPT)(PPT)-Gly-H (**10**), on a solid phase, starting from commercially available TentaGel® S-RAM with lysine **11** (0.20 mmol/g) and using the amino acids **8**, **12**,[29] and **13**[30] as building units according to a typical Fmoc solid-phase peptide synthesis. The resulting crude product was purified by HPLC on a COSMOSIL $5C_{18}$-AR-300 column to give **9** and **10** in 40% and 41% yields, respectively. The HPLC profile of **9** is shown in Fig. 1. In a similar manner, H_2N-Lys-CCTTTCC-Gly-H (**14**), which is required for the control experiment, was separately synthesized.



2 **3** **4** **5** **6** **7** **8**

Scheme 1 *Reagents and conditions*: (a) TBDPSCl, imidazole, DMF, 60 °C, 1.5 h; (b) $OCNCH_2COOC_2H_5$, DMF, 25 °C, 24 h; (c) C_2H_5ONa, C_2H_5OH, reflux, 17 h; (d) $(C_2H_5)_3N{\cdot}3HF$, 25 °C, 24 h; (e) (f) TEMPO, NaBr, NaClO, KOH, H_2O, 25 °C, 2 h; (g) $FmocNH(CH_2)_2NHCH_2COOC(CH_3)_3$, EDC·HCl, DMF, 25 °C, 24 h; (h) TFA, CH_2Cl_2, 25 °C, 24 h.



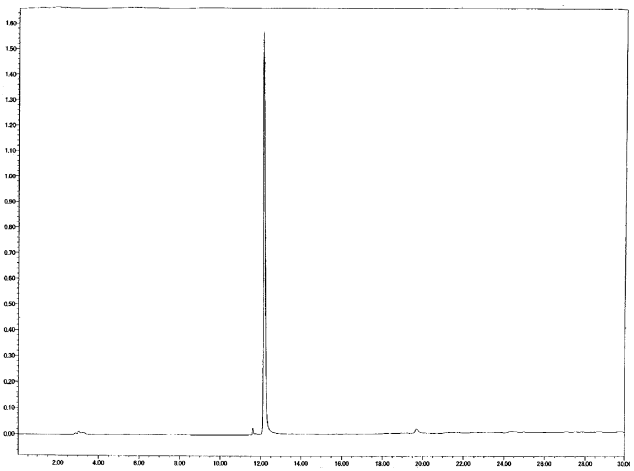


Fig. 1 HPLC profile of the compound **9**. *Conditions*: COSMOSIL 5C18-MS column (4.6 × 250 mm); eluent A, 0.1% TFA/water; eluent B, 0.1% TFA/acetonitrile; gradient, linear 0–100% B over 35 min; detection 260 nm; flow rate, 1 mL/min; 55 °C.

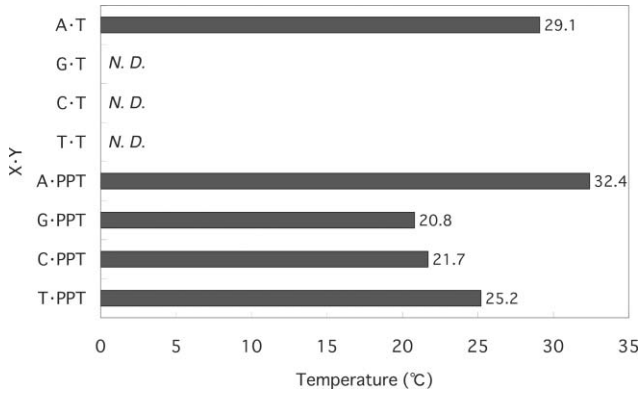


Fig. 2 T_m values of a 1:1 molar mixture of 5′-GGA**X**AGG-3′ (**X** = A, G, C, or T) (**15**, **16**, **17** or **18**) and H_2N-Lys-CCT**Y**TCC-Gly-H (**Y** = T or PPT) (**14** or **9**). N.D. = not detected.

Binding affinity of the PPT-containing PNA for a complementary DNA

First, we investigated the hybridizing ability of the PNA **9**, which contains one PPT, for complementary DNAs **15**, **16**, **17**, and **18** (Table 1) as follows. We prepared a duplex of the DNA and **9**, by mixing 2.0 μmol of **15**, **16**, **17** or **18** and **9** in 10 mM phosphate buffer (pH 7) (1.0 mL), and estimated the stability of each of the resulting duplexes by thermal denaturation experiments using absorbance spectroscopy. In this experiment, all absorbance–temperature curves were measured at 260 nm, and melting temperatures (T_m) were determined on the basis of the resulting melting curves by parallel melting data analysis software (JASCO, version 1.01.01). In order to confirm the universality of PPT as a nucleobase, we also carried out similar experiments using **14** as a PNA, where PPT in **9** is replaced by T. The T_m of a 1:1 molar mixture of the DNA (**15**, **16**, **17** or **18**) and **14** was measured and compared with that of a 1:1 molar mixture of the DNA and **9**. The results obtained by these experiments are shown in Fig. 2.

Table 1

DNA	5′-GGA**X**AGG-3′
15	**X** = A
16	**X** = G
17	**X** = C
18	**X** = T
PNA	H_2N-Lys-CCT**Y**TCC-Gly-H
9	(**Y** = PPT)
14	(**Y** = T)

Fig. 2 indicates that **9** and **14** have quite different binding affinities compared to **15**, **16**, **17** and **18**. Thus, as predicted, **14** makes a stable duplex only with **15**, and does not form a stable duplex with **16**, **17** or **18**. In contrast, **9** forms stable duplexes with **15**, **16**, **17** and **18**, though the T_m value (*i.e.* the binding ability), differs slightly according to the particular nucleobase. ΔG values of the duplex estimated[31,32] on the basis of the observed T_m values are −35.9 kJ/mol for the duplex of **9** and **15**, −34.2 kJ/mol for the duplex of **9** and **16**, −35.3 kJ/mol for the duplex of **9** and **17**, and −35.0 kJ/mol for the duplex of **9** and **18**. These ΔG values are all similar, indicating that, as expected, PPT serves as a universal base that recognizes all natural nucleobases, regardless of whether they are purine or pyrimidine bases.

Subsequently, the ability of the PNA **10**, which includes two PPTs, to hybridize the complementary DNA, 5′-GGAAAGG**Y**^{1}**Y**^{2}-3′ (type I) (**Y**^{1}, **Y**^{2} = A, G, C or T) or 3′-GGAAAGG**Y**^{1}**Y**^{2}-5′ (type II) (**Y**^{1}, **Y**^{2} = A, G, C or T), was examined in a manner similar to that described above. Thus, we produced duplexes of **10** and complementary DNAs of type I or type II and measured the T_m values of the resulting duplexes (Fig. 3). We found that the PNA **10** forms duplexes with both DNAs, and that these duplexes have similar T_m values. Thus, the difference between the maximum and minimum T_m values ($T_{m,max}$ and $T_{m,min}$) is only 5.8 °C. This is smaller than the difference between $T_{m,max}$ and $T_{m,min}$ in the duplexes between PNA **9** (which contains one PPT) and the complementary DNA **15**. This shows that, when two PPTs are incorporated in PNAs, the resulting PNAs form duplexes that have closer stabilities than those with one PPT, regardless of whether the nucleobase facing the PPT unit is purine or pyrimidine. Consequently, we consider that an increase in the number of PPTs enhances the binding affinity for all natural nucleobases, and thus allows the

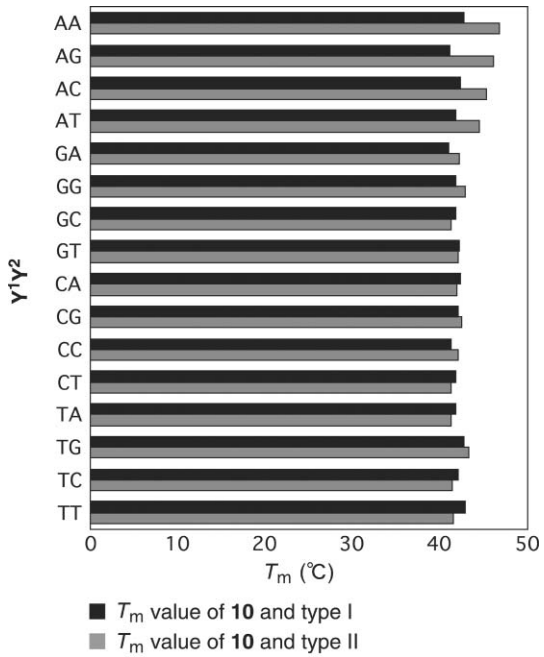


Fig. 3 T_m values of a 1:1 molar mixture of H_2N-Lys-CCTTT-CC(PPT)(PPT)-Gly-H (**10**) and 5′-GGAAAGG**Y**^{1}**Y**^{2}3′ (type I) or 3′-GGAAAGG**Y**^{1}**Y**^{2}5′ (type II) (**Y**^{1}, **Y**^{2} = A, G, C or T).

PNA to form more stable duplexes with the complementary DNA.

Conclusions

We designed pyrimido[4,5-*d*]pyrimidine-2,4,5,7-(1*H*,3*H*,6*H*,8*H*)-tetraone (PPT) as a new universal base and investigated its universality using PPT-containing PNAs. The results revealed that PPT serves as an excellent universal base to recognize all four natural nucleobases, A, G, C, and T. It is important to determine how PPT recognizes the these different kinds of natural nucleobases – in other words, to clarify the configuration in which PPT forms base pairs with the natural nucleobases. At present, we have no direct observations that could answer this query. However, based on the finding that the T_m values of the duplex of the PPT-containing PNA and the complementary DNA are comparable to those of duplexes of fully complementary PNAs and DNAs, such as **9** and **15–18**, we think that PPT forms the base pairs by not only by π–π stacking interaction, but also by hydrogen-bond formation through dynamic transformations such as conformational change and keto–enol tautomerization. Here, we think that the conformational change allows PPT to take a pyrimidine base form when forming hydrogen bonds with a purine base, *i.e.*, A or G, but a purine base form when forming hydrogen bonds with a pyrimidine base, *i.e.*, C or T. At the same time, the keto–enol tautomerization allows PPT to have structures suitable for forming base pairs with natural nucleobases *via* hydrogen-bonding interaction. In conclusion, we have developed a PNA with PPT as a universal base and demonstrated that this PPT-containing PNA has the ability to form a duplex with a complementary DNA, in which all of the four natural nucleobases face the PPT. Thus, these PPT-containing PNAs are expected to be useful in various investigations dealing with nucleic acids.

Experimental

General procedures, materials, and solvents

UV spectra were measured in DMSO on a JASCO V-550 spectrometer. IR spectra were obtained in a KBr tablet on a JASCO FT/IR-5300 spectrometer. NMR spectra were taken in DMSO-d_6 on a JEOL JNM-α400 or ECA-500 instrument. The ^{1}H- and ^{13}C-NMR chemical shifts are described as δ values in ppm relative to ^{1}H and ^{13}C of $(CH_3)_4Si$. ESI-TOF mass spectra were obtained on Applied Biosystems Mariner spectrometers. HPLC analysis was carried out using a COSMOSIL $5C_{18}$-MS column (ODS-5 mm, 4.6 × 250 mm; Nacalai Tesque, Kyoto, Japan) on a Waters 2695 Separations Module chromatograph with a Waters 2996 Photodiode Array detector. Column chromatography was performed using Nacalai Tesque silica gel 60 (neutrality, 75 mm). Unless otherwise noted, synthetic reactions were carried out at ambient temperature. The reactions requiring anhydrous conditions were carried out under an argon atmosphere in flasks dried by heating at 400 °C under 133–400 Pa, or by washing with a 5% solution of dichlorodimethylsilane in dichloromethane followed by anhydrous dichloromethane, and then heating at 100 °C. DMF and dichloromethane were distilled from calcium hydride. Other organic reagents were used as commercially supplied without any purification. Solid and amorphous organic substances were used after drying over P_2O_5 at 50–60 °C for 8–12 h under 133–400 Pa. The Fmoc protected C-monomer **13** was purchased from Applied Biosystems (Foster City, USA). DNA oligomers were purchased from Gene Design Inc, Japan.

Ethyl {6-Amino-1-[2-(*tert*-butyldiphenylsilanyloxy)ethyl]-2,4-dioxo-1,2,3,4-tetrahydro-pyrimidine-5-carbonyl}carbamate (3). To a suspension of 1-hydroxyethyl-6-aminouracil (**2**) (1.0 g, 5.8 mmol) in anhydrous DMF (6.0 mL) were added imidazole (0.88 g, 13 mmol) and *tert*-butyldiphenylchlorosilane (1.8 mg, 6.4 mmol). The resulting mixture was stirred at 60 °C for 1.5 h and then was added dropwise to water (1.0 L). The resulting precipitate was collected by filtration and dried under reduced pressure to give 1-(2-*tert*-butyldiphenylsilanyloxy)ethyl-6-aminouracil (2.2 g, 92% yield) as a colorless powder: mp 250 °C; ^{1}H NMR (400 MHz) 0.96 (s, 9H), 3.76 (br, 2H), 4.04 (br, 2H), 4.60 (br, 1H), 6.70 (br, 2H), 7.41 (m, 6H), 7.58 (m, 4H), 10.32 (br, 1H); ^{13}C NMR (100 MHz) 19.1, 26.9, 42.1, 61.6, 76.2, 128.0, 128.3, 130.3, 130.1, 134.9, 135.5, 151.9, 158.8, 162.9. This product (14 g, 33 mmol) was suspended in anhydrous DMF (40 mL) and to the resulting suspension was added ethyl isocyanatoformate (3.9 g, 34 mmol) dropwise over 30 min at 25 °C. The mixture was stirred at 25 °C for 24 h and concentrated *in vacuo*. The resulting solid material was washed with ethyl acetate, collected by filtration, and dried under reduced pressure to give **3** (7.0 g, 40% yield) as a colorless powder: mp 275 °C; 1H NMR (400 MHz) 0.94 (s, 9H), 1.22 (t, J = 7.2 Hz, 3H), 3.81 (d, J = 4.8 Hz, 2H), 4.12 (q, J = 4.8 Hz, 2H), 4.18 (t, J = 4.8 Hz, 2H), 7.42 (m, 6H), 7.53 (M, 4H), 8.36 (br, 1H), 10.85 (br, 1H), 11.35 (br, 1H), 12.39 (br, 1H); 13C NMR (100 MHz) 14.7, 19.1, 26.9, 43.1, 60.6, 61.1, 81.1, 128.3, 130.4, 132.9, 135.4, 148.8, 150.8, 159.9, 164.5, 166.2; IR 3389, 3111,

1735, 1626, 1508, 1426, 1288, 1203, 1113, 1024 cm^{-1}; UV λ_{max} 272 nm (ε 30,200).

1-[2-(*tert*-Butyldiphenylsilanyloxy)ethyl]-1*H*,8*H*-pyrimido[4,5-*d*]pyrimidine-2,4,5,7-tetraone (4). A solution of sodium ethoxide in ethanol prepared using sodium (60 mg, 2.6 mmol) and dry ethanol (20 mL) was added to **3** (0.60 g, 1.1 mmol). The mixture was refluxed for 17 h and then cooled to 25 °C. The resulting precipitate was collected by filtration. The collected solid was washed with a 1.0 M aqueous HCl solution until the pH of the filtrate became 7 and then dried under reduced pressure to give **4** (0.50 g, 92% yield) as a colorless powder: mp 300 °C; ^{1}H NMR (400 MHz) 0.94 (s, 9H), 3.83 (t, J = 4.8 Hz, 2H), 4.23 (t, J = 4.8 Hz, 2H), 7.38 (m, 6H), 7.57 (m, 4H), 9.79 (br, 1H), 10.53 (br, 1H); ^{13}C NMR (100 MHz) 19.2, 27.0, 42.3, 61.1, 86.0, 128.3, 130.1, 133.6, 135.4, 151.6, 157.9, 161.6, 162.6, 164.8; MS (ESI$^+$) calcd for $C_{24}H_{25}N_4O_5Si$ (M + H$^+$) 479.21, found 479.17; IR 3424, 1693, 1542, 1507, 1427, 1263, 1228, 1112, 1058 cm^{-1}; UV λ_{max} 279 nm (ε 20,000).

1-(2-Hydroxyethyl)-1*H*,8*H*-pyrimido[4,5-*d*]pyrimidine-2,4,5,7-tetraone (5). Compound **4** (1.7 g, 3.6 mmol) was mixed with triethylamine trishydrofluoride (4.9 g, 31 mmol) in a polypropylene flask and the mixture was stirred at 25 °C for 24 h. The resulting mixture was poured into a 2 M aqueous KOH solution in a glass flask, and then to this mixture was added a 2 M HCl solution to adjust the pH of the mixture to 7. The precipitated solid was collected by filtration and dried under reduced pressure to give **5** (0.70 g, 82% yield) as a colorless powder: mp 295 °C; ^{1}H NMR (400 MHz) 3.49 (br, 2H), 4.02 (br, 2H), 4.88 (br, 1H), 9.44 (br, 1H), 10.20 (br, 1H); ^{13}C NMR (100 MHz) 43.0, 58.8, 86.1, 151.5, 157.9, 161.3, 162.7, 164.5; MS (ESI$^+$) calcd for $C_8H_8N_4O_5$ (M + H$^+$) 241.05, found 241.06;; IR 2961, 2812, 1746, 1636, 1536, 1418, 1263, 1066 cm^{-1}; UV λ_{max} 278 nm (ε 32,200).

(2,4,5,7-Tetraoxo-3,4,5,6,7,8-hexahydro-2*H*-pyrimido[4,5-*d*]-pyrimidin-1-yl)acetic acid (6). Compound **5** (1.0 g, 4.2 mmol) was suspended in water (70 mL) containing 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (0.70 g, 4.2 mmol) and sodium bromide (0.92 g, 8.4 mmol). The suspension was adjusted to pH 10 by adding a 0.4 M aqueous NaOH solution. To this suspension was added an 11% aqueous sodium hypochlorite solution (5.4 mL), and the pH of the resulting mixture was adjusted to 11 by adding a 0.4 M aqueous NaOH solution. The resulting mixture was stirred at 25 °C for 1.5 h and then ethanol (400 mL) was added to the mixture. The precipitated solid was collected by filtration. The solid was dissolved in water (30 mL). To this solution was added a 2 M HCl solution. The precipitated solid was collected by filtration and dried under reduced pressure to give **6** (0.53 g, 50% yield) as a colorless powder: mp 296 °C; ^{1}H NMR (400 MHz) 4.71 (s, 2H), 11.23 (br, 1H), 11.44 (br, 1H), 13.22 (br, 1H); ^{13}C NMR (100 MHz) 44.6, 87.5, 149.6, 150.3, 155.4, 158.5, 159.7, 169.1; MS (ESI$^+$) calcd for $C_8H_6N_4O_6$ (M + H$^+$) 255.04, found 255.03; IR 3385, 3218, 3071, 2931, 2857, 1697, 1653, 1599, 1490, 1428, 1395, 1285, 1181, 1112, 1007 cm^{-1}; UV λ_{max} 275 nm (ε 30,800).

***tert*-Butyl *N*-[2-(*N*-9-Fluorenylmethoxycarbonyl)amino ethyl]-*N*-[(2,4,5,7-tetraoxo-3,4,5,6,7,8-hexahydro-2*H*-pyrimido[4,5-*d*]-pyrimidin-1-yl)acetyl]glycinate (7).** To a solution of *tert*-butyl *N*-[2-(*N*-9-fluorenylmethoxycarbonyl)aminoethyl]glycinate (1.1 g, 2.8 mmol) in anhydrous DMF (10 mL) was added **6** (0.72 g, 2.8 mmol), and the mixture was stirred to dissolve **6**. To the resulting solution was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.1 g, 5.6 mmol). The mixture was stirred at 25 °C for 24 h and then concentrated under reduced pressure. Water (30 mL) was added to the resulting viscous oil. The mixture was stirred at 25 °C for several minutes to produce a white solid. The solid was collected by filtration and dried under reduced pressure. This crude product was subjected to flash chromatography and eluted with a 19:80:1 (v/v/v) mixture of methanol and dichloromethane and acetic acid to give **7** (1.3 g, 74%) as a colorless powder: mp 297 °C; ^{1}H NMR (400 MHz) 1.37–1.45 (m, 9H), 2.75 (br, 1H), 3.00–3.42 (m, 4H), 3.89–3.92 (br, 1H), 4.14–4.33 (m, 4H), 4.73–4.94 (m, 2H), 7.30–7.41 (m, 5H), 7.66–7.68 (m, 2H), 7.86–7.91 (m, 2H), 9.87 (br, 1H), 10.6 (br, 1H); ^{13}C NMR (100 MHz) 28.1, 42.3, 47.2, 47.5, 49.5, 65.4, 66.0, 81.4, 82.4, 83.6, 86.5, 120.6, 125.5, 125.6, 127.5, 128.1, 141.2, 144.3, 144.4, 150.5, 156.6, 156.8, 166.3, 167.4, 168.6, 168.9; MS (ESI$^+$) calcd for $C_{31}H_{32}N_6O_9$ (M + H$^+$) 633.29, found 633.23; IR 3386, 3216, 3071, 2975, 2822, 1719, 1547, 1508, 1449, 1255, 1204, 1146, 1039 cm^{-1}; UV λ_{max} 268 nm (ε 36,000).

***N*-[2-(*N*-9-Fluorenylmethoxycarbonyl)aminoethyl]-*N*-[(2,4,5,7-tetraoxo-3,4,5,6,7,8-hexahydro-2*H*-pyrimido[4,5-*d*]pyrimidin-1-yl)acetyl]glycine (8).** To a suspension of **7** (1.9 g, 3.0 mmol) in anhydrous dichloromethane (10 mL) was added trifluoroacetic acid (20 mL). This mixture was stirred at 25 °C for 24 h. The resulting solution was concentrated to give a residual oil, to which was added ether with stirring to afford a precipitate. This was collected by filtration, dried under reduced pressure, and recrystallized from a 4:1 (v/v) mixture of methanol and ether to provide **8** (1.5 g, 87%) as a colorless powder: mp 295 °C; ^{1}H NMR (400 MHz) 2.75 (br, 1H), 3.10–3.64 (m, 4H), 3.99 (br, 1H), 4.23–4.37 (m, 4H), 4.79–4.97 (m, 2H), 7.28–7.46 (m, 5H), 7.65–7.69 (m, 2H), 7.87–7.91 (m, 2H), 10.68 (br, 1H), 11.16 (br, 1H), 12.66 (br, 1H); ^{13}C NMR (100 MHz) 47.2, 47.4, 48.3, 54.4, 65.4, 66.0, 87.0, 87.1, 120.6, 120.7, 125.4, 125.5, 125.6, 127.5, 128.1, 128.2, 141.2, 144.2, 144.3, 150.1, 156.6, 156.8, 159.2, 167.0, 169.9, 170.9, 171.2 ppm; MS (ESI$^+$) calcd for $C_{27}H_{24}N_6O_9$ (M + H$^+$) 577.19, found 577.17; IR 3420, 3252, 3068, 2979, 2818, 1698, 1550, 1504, 1451, 1369, 1240, 1154, 1124, 1036 cm^{-1}; UV λ_{max} 269 nm (ε 24,400).

Solid-phase synthesis of PNA oligomers

2-(*N*-9-Fluorenylmethoxycarbonyl)amino-6-(*tert*-butoxycarbonylamino)hexanoic acid-loaded Tentagel S-RAM resin (37 mg, 10 μmol of the amino group) was used as a solid support for the solid-phase synthesis. A Teflon syringe with a frit at the bottom of a 5 mL polypropylene cartridge was used as reactor.

The chain elongation of PNA was carried out as follows: (1) the resin in the cartridge was washed with anhydrous DMF (0.5 mL) at 25 °C; (2) the resin was mixed with 0.5 mL of a 2:2:96 (v/v/v) mixture of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) and piperidine and anhydrous DMF at 25 °C for 7 min; (3) steps (1) and (2) were repeated; (4) the resin was washed with anhydrous DMF (0.5 mL) at 25 °C; (5) step (4) was repeated three times; (6) the resin was washed with 0.5 mL of a 1:1 (v/v) mixture of anhydrous *N*-methylpyrrolidone and anhydrous DMF at 25 °C; (7) step (6) was repeated twice; (8) the resin was treated

with a homogeneous mixture of an Fmoc-protected monomer [**8** (17 mg, 30 μmol) or **12** (15 mg, 30 μmol) or **13** (21 mg, 30 μmol)], *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (10 mg, 27 μmol), a 0.4 M solution of *N*,*N*′-diisopropylethylamine in anhydrous DMF (83 μL), a 0.6 M solution of 2,6-lutidine in anhydrous DMF (55 μL), and a 1:1 (v/v) mixture of anhydrous *N*-methylpyrrolidone and anhydrous DMF (0.2 mL) at 25 °C for 1.5–2.0 h; (9) the resin was collected by filtration and washed with anhydrous DMF (0.5 mL) at 25 °C; (10) the resin was treated with a 5:6:89 (v/v/v) mixture of acetic anhydride, 2,6-lutidine, and anhydrous DMF (0.5 mL) at 25 °C for 5 min; (11) steps (9) and (10) were repeated. Steps (1) to (11) were then repeated until a chain of the desired length was obtained.

The resin in the cartridge was washed with anhydrous DMF (0.5 mL) at 25 °C, and then treated with 0.5 mL of a 2:2:96 (v/v) mixture of DBU, piperidine, and anhydrous DMF at 25 °C for 7 min. These two procedures were repeated twice. The resin was washed with anhydrous DMF (0.5 mL) at 25 °C three times and with 0.5 mL of a 1:1 (v/v) mixture of anhydrous *N*-methylpyrrolidone and anhydrous DMF at 25 °C twice. The resin was treated with a mixture of (*N*-9-fluorenylmethoxycarbonylamino)acetic acid (12 mg, 40 μmol), *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (14 mg, 36 μmol), a 0.4 M solution of *N*,*N*′-diisopropylethylamine in anhydrous DMF (0.11 mL), a 0.6 M solution of 2,6-lutidine in anhydrous DMF (73 μL), and a 1:1 (v/v) mixture of anhydrous *N*-methylpyrrolidone and anhydrous DMF (0.2 mL) at 25 °C for 2.0 h. The resulting resin was separated by filtration, washed successively with anhydrous DMF (0.5 mL), anhydrous methanol (0.5 mL), and anhydrous dichloromethane (0.5 mL), and then dried under reduced pressure. The resin was mixed with a 1:9 (v/v) mixture of *m*-cresol and trifluoroacetic acid (1.0 mL) at 25 °C for 2 h. The resin was removed by filtration and the filtrate was concentrated. To the resulting oil was added ether (10 mL). The precipitated solid was collected by filtration. The solid product was dissolved in a 93:7 (v/v) mixture of buffer A (a 0.1% trifluoroacetic acid/water solution) and buffer B (a 0.1% trifluoroacetic acid/acetonitrile solution) and subjected to HPLC purification using a C-18 reversed phase column. Elution of products was carried out by the linear gradient elution method starting from a 93:7 (v/v) mixture of buffer A and buffer B to a 1:1 (v/v) mixture of buffer A and buffer B as eluent (flow rate 10 ml/min), where a UV detector (254, 260, and 266 nm) was used for monitoring the elution course. The fractions containing the desired product were combined and lyophilized. The resulting material was treated with a 20% piperidine/water solution at 0 °C for 45 min. The solution was again dried by lyophilization. The residue was dissolved in a 0.1% trifluoroacetic acid/water solution (8.0 mL). The resulting solution was subjected to HPLC purification on a C-18 reversed phase column, eluted with 100% of buffer A to a 1:1 (v/v) mixture of buffer A and buffer B (linear gradient over 35 min). The combined fractions containing the target product were concentrated by lyophilization. This sequence of procedures gave **9** (8.3 mg) in a 40% isolated yield [MS (ESI$^+$) calcd for $C_{82}H_{110}N_{38}O_{28}$ (M + 2H$^+$) 1038.4, found 1038.5] and **10** (11 mg) in a 41% isolated yield [MS (ESI$^+$) calcd for $C_{105}H_{136}N_{48}O_{38}$ (M + 3 H$^+$) 893.35, found 893.37].

Measurement of T_m of a PNA–DNA duplex

A solution of a PNA oligomer (2.0 μmol) and a DNA oligomer (2.0 μmol) in a 10 mM phosphate buffer solution of pH 7 (1.0 mL) was prepared. The resulting solution was placed in a quartz cuvette of 1 cm path length and heated at 95 °C for 5 min. The solution was then cooled to 5 °C at a rate of 1 °C/min, and kept at this temperature. The resulting solution was subjected to UV measurement at 260 nm from 5 °C to 60 °C or 80 °C using a JASCO V-550 spectrometer equipped with a JASCO ETC-505T Peltier temperature controller by increasing the temperature at a rate of 1 °C/min. The obtained UV absorbance was plotted to prepare the melting curve of the duplex of the PNA oligomer and the DNA oligomer. The T_m value was estimated on the basis of the resulting melting curve using parallel melting data analysis software (JASCO, version 1.01.01).

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