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Research Article

Synthesis and Evaluation of MGB Polyamide-Oligonucleotide Conjugates as Gene Expression Control Compounds

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MGB polyamide-oligonucleotide conjugates **ON 1**-**4** with linked MGB polyamides at the 2-exocyclic amino group of a guanine base using aminoalkyl linkers were synthesized and evaluated in terms of binding affinity for complementary DNA containing the MGB polyamide binding sequence using T_m and CD analyses. The MGB polyamides comprised pyrrole polyamides (Py_4- and Py_3-), which possess binding affinity for A-T base pairs, and imidazole (Im_3-) and pyrrole-γ-imidazole (Py_3-γ-Im_3-) polyamide hairpin motifs, which possess binding affinity for C-G base pairs. It was found that the stability of modified dsDNA was greatly influenced by the linker length. Py_4- and Py_3-oligonucleotide conjugates (**ON 1** ($n = 4$) and **ON 2** ($n = 4$)) containing the 4-aminobutyl linker formed stable dsDNA with complementary DNA. Although Im_3-oligonucleotide conjugate **ON 3** ($n = 4$) containing the 4-aminobutyl linker formed stable dsDNA with complementary DNA, stabilization of dsDNA by the imidazole amide moiety of **ON 3** ($n = 4$) was lower compared with the pyrrole amide moiety of **ON 2** ($n = 4$). The Py_3-γ-Im_3-oligonucleotide conjugate **ON 4** ($n = 2$), which possesses binding affinity for C-G base pairs via a pyrrole/imidazole combination and contains a 2-aminoethyl linker, showed high binding ability for complementary DNA. Furthermore, the DNA sequence recognition of MGB polyamide-oligonucleotide conjugates was investigated using single-base mismatch DNAs, which possess a mismatch base in the MGB polyamide binding sequence. The Py_3-γ-Im_3-oligonucleotide conjugate **ON 4** ($n = 2$) showed high sequence recognition ability for complementary DNA.

1. Introduction

Numerous nucleic acid analogues have been synthesized and characterized as potential gene therapy agents [1, 2]. We previously designed and synthesized nucleoside (**Hybrid 1**) linked to pyrrole polyamide minor groove binder (MGB) comprising modified distamycin A, which possesses a high affinity for the 5′-d(AATTT)-3′/3′-(TTAAA)-5′ sequence of double-stranded DNA (dsDNA) [3–8], as a lead compound for the development of potential gene therapy agents [9–12]. When the MGB polyamide-nucleoside hybrid interacts with dsDNA, it is expected that complex formation would involve high affinity and sequence selectivity. If the hybrid is incorporated into DNA during DNA biosynthesis, it is expected that DNA replication and transcription would be obstructed through minor groove binding of the hybrid polyamide moiety. The dsDNA binding ability of **Hybrid 1** was investigated via melting temperature (T_m) and circular dichroism (CD) analyses (Figure 1) [5, 6]. It was shown that **Hybrid 1** possessed greater binding specificity compared with distamycin A [12]. Then, in an effort to examine the development of potential antisense drugs, we synthesized oligonucleotide **ON 1** ($n = 3$) conjugated to **Hybrid 2** *in lieu of* **Hybrid 1** containing the formyl group which is unstable under the basic conditions of deprotection during oligonucleotide solid-phase synthesis, and subsequently examined the binding ability of **ON 1** ($n = 3$) to complementary DNA (Figure 2) [13]. Dervan et al. [14], Zamecnik et al.

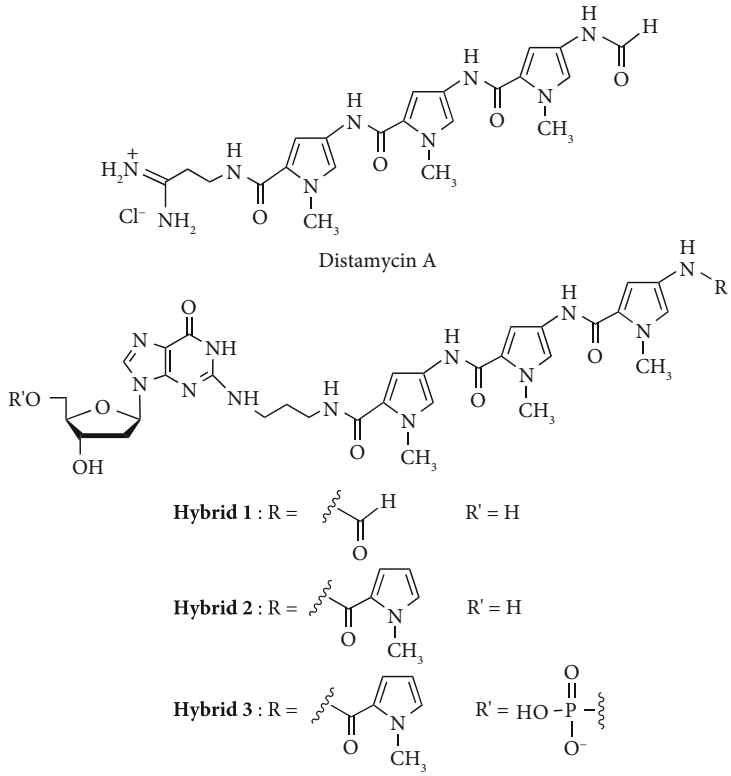


Figure 1: Structures of distamycin A, hybrids **1**, **2**, and **3**.

[15], Novopashina et al. and Boutorine et al. [16–21] have reported the synthesis and evaluation of oligonucleotides conjugated with one or two MGB polyamides to either the 5′- or 3′-ends. Sequence-specific stabilization of DNA duplexes and DNA triplexes by MGB polyamides conjugated to one DNA strand was shown. It was expected that oligonucleotides conjugated with the MGB polyamide to the 2-exocyclic amino group of a guanine base, which is positioned above the floor of the minor groove of the DNA duplex, would possess high DNA binding ability. **ON 1** ($n = 3$), which includes a modified guanosine (**G**) in the 5′ direction in the oligonucleotide chain given the preferred orientation of the polyamide in the minor groove of dsDNA (*C*-(pyrrole polyamide)-*N*/3′-(TTAAA)-5′ of the complementary target DNA) [22–24], was synthesized and evaluated as a model oligonucleotide [13]. From the T_m and CD analyses, it was found that **ON 1** ($n = 3$) formed stable dsDNA with complementary DNA via action of the pyrrole amide moiety. From this result, it is expected that MGB polyamide-oligonucleotide conjugates could be effective gene expression control compounds and that MGB polyamide-2′-deoxyguanosine hybrid might be of potential use as a sequence-specific gene therapy agent based on potential obstruction of DNA replication and transcription. The inhibition of mouse mammary carcinoma FM3A cell growth by pyrrole polyamide compounds (Hybrids and distamycin A) has been evaluated (Figure 1) [25]. It was found that hybrids induce dose-dependent inhibition of cell growth. In particular, **Hybrid 3** bearing a 5′-phosphate group, which is a suitable substrate for biosynthesis, exhibited the highest inhibition.

The binding ability of pyrrole polyamide-oligonucleotide conjugates to target DNA, and the inhibition of cell growth by pyrrole polyamide-2′-deoxyguanosine hybrids should be greatly influenced by the chain length of the pyrrole polyamide moiety and the length of the linker between the pyrrole polyamide moiety and the guanine base. Although we previously reported the synthesis of MGB polyamide-oligonucleotide conjugate **ON 1** ($n = 3$), with linked pyrrole amide tetramer (Py_4-) at the 2-exocyclic amino group of a guanine base using 3-aminopropyl linker and evaluated the stability of modified dsDNA as described above [13], an examination of the length of the chain or linker connecting the pyrrole polyamide moiety to the guanine base and the DNA sequence recognition ability had not been investigated. In an effort to improve the activity of pyrrole polyamide-oligonucleotide conjugates, we performed the synthesis and evaluation of **ON 1** and **ON 2** with linked pyrrole polyamides (Py_4- and Py_3-) using various aminoalkyl linkers in terms of binding affinity for complementary DNA (Figure 2).

In addition to pyrrole polyamides, which possess high affinity for A-T base pairs of dsDNA, pyrrole-imidazole polyamides, which possess high affinity for C-G base pairs of dsDNA, were reported by Dervan et al. [14, 22–24, 26–31]. Furthermore, the synthesis and evaluation of MGB polyamide-oligonucleotide conjugates which possess binding affinity for C-G base pairs are an important aspect of the study. We performed the synthesis and evaluation of **ON 3** and **ON 4** with conjugated MGB polyamides (imidazole polyamide (Im_3-) and pyrrole-γ-imidazole

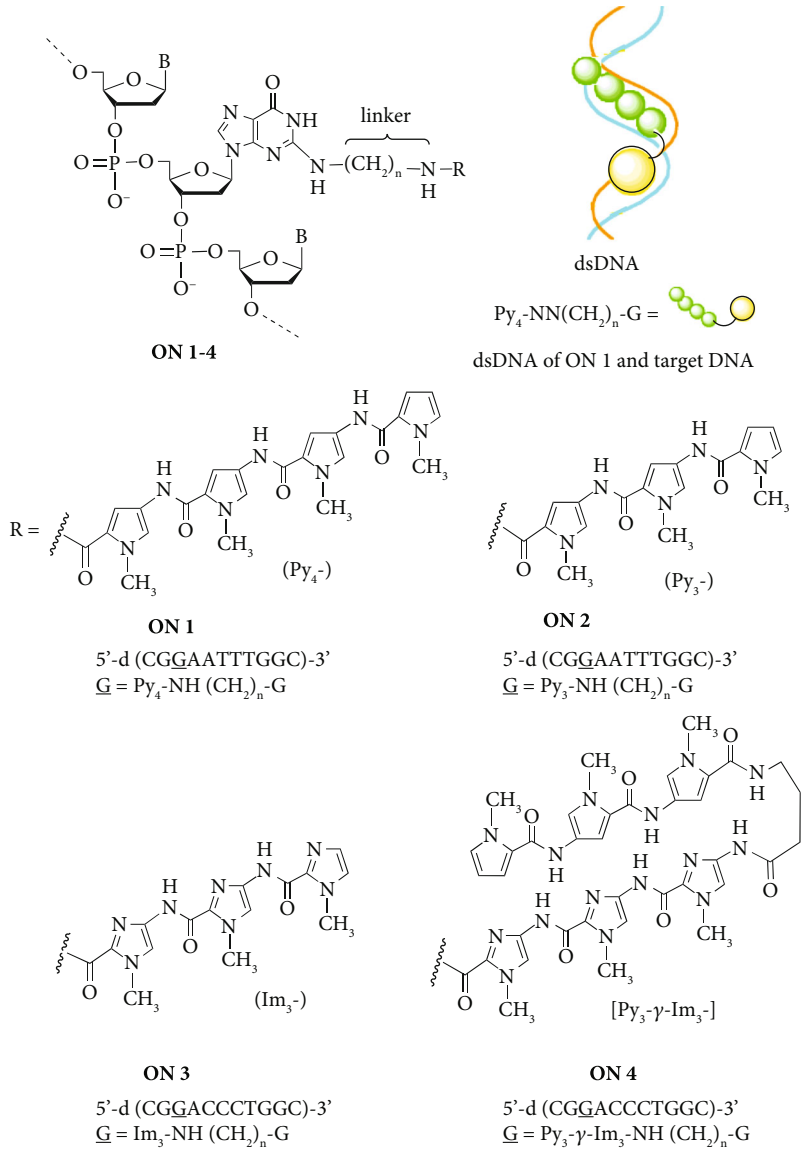


Figure 2: MGB polyamide-oligonucleotide conjugates.

polyamide hairpin motif (Py_3-γ-Im_3-)) at the 2-exocyclic amino group of a guanine base using various aminoalkyl linkers (Figure 2).

Herein, we report on the synthesis and evaluation of MGB polyamide-oligonucleotide conjugates **ON 1**-**4** using various aminoalkyl linkers.

2. Materials and Methods

Column chromatography was performed on silica gel (Kanto Chemical Silica gel N60, spherical, natural, 40-50 μm). Precoated silica gel plates with a fluorescent indicator (Merck 60 F254) were used for analytical TLC. HPLC was performed on a Waters liquid chromatograph (600E system) equipped with a UV-VIS detector (2487 Dual), data module (741 type), and fraction collector. A μBondasphare C18 5 μm 100A (3.9 mm ID × 150 mm L) column with gradients of 5-50% CH_3CN in water (0.01 M TEAA, pH 7) was used. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using Bruker DRX 400 and a Bruker Biospin AVANCE III HD 400 instruments. Mass spectra were recorded on a Micromass Q-Tof Ultima API and a Micromass LCT spectrometer with a time-of-flight analyser. Elemental analyses were performed using an Elemental Vavio EL apparatus. Circular Dichroism (CD) spectra were recorded on a JASCO J-720 spectropolarimeter. UV melting curves were measured using a Shimadzu TMSPC-8/UV-1600 apparatus. UV spectra were recorded using a Shimadzu UV-1200 apparatus. DNA oligonucleotides were purchased from Hokkaido System Science Co., Ltd. Compounds **2**, **3**, **4**, **5**, **7**, **9**, and **12** were prepared as previously described [9–13]. The CPG support-bound 2′-deoxynucleoside **16** (B = C^{Bz}) and 2′-deoxynucleoside 3′-phosphoramidites **17** (B = T, C^{Bz}, A^{Bz}, and G^{iBu}) were purchased from Glen

Research Corporation. 2′-Deoxy-2-fluoroinosine 3′-phosphoramidite **17** (B = $I^{F,NPE}$) was prepared from 2′-deoxyguanosine as previously described [32–35]. Ethyl 1-methylimidazole-2-carboxylate (**20**) was prepared according to the procedure described by Baird and Dervan. [30].

2.1. Methyl 1-Methyl-4-[1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl]Amino-1H-Pyrrole -2-Carboxylate (6). Compound **2** [10–13] (2.83 g, 9.06 mmol) and 1-methyl-1*H*-pyrrole-2-carboxylic acid (**5**) [13] (1.26 g, 9.97 mmol) were dissolved in dichloromethane (45 mL), and then 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (2.61 g, 13.6 mmol) and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) (1.66 g, 13.6 mmol) were added to the solution. After stirring at room temperature for 14 h, the solution was diluted with chloroform (300 mL) and washed with 2 M HCl aq. (150 mL x 3), H_2O (150 mL x3), 5% $NaHCO_3$ aq. (150 mL × 3), and H_2O (150 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **6** (2.79 g, 80% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 9.92 (s, 1H, CONH), 9.82 (s, 1H, CONH), 7.46 (d, 1H, J = 1.9 Hz, Py-H), 7.23 (d, 1H, J = 1.8 Hz, Py-H), 7.05 (d, 1H, J = 1.9 Hz, Py-H), 6.94-6.90 (m, 3H, Py − H × 3), 6.06 (dd, 1H, J = 2.6, 3.9 Hz, Py-H), 3.88 (s, 3H, NCH_3), 3.85 (s, 3H, NCH_3), 3.84 (s, 3H, NCH_3), 3.74 (s, 3H, OCH_3); ^{13}C-NMR (DMSO-d_6): δ 160.8, 158.6, 158.5, 128.1, 125.5, 123.0, 122.5, 122.2, 120.7, 118.5, 112.6, 108.4, 106.6, 104.8, 50.9, 36.2, and 36.1, 36.0; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{19}H_{22}N_5O_4(M+H)^+$ 384.1672, found 384.1667; UV (CH_3OH): λ_{max} 299, 238 nm, λ_{min} 260, and 222 nm; and ε_{260} 1.8×10^4.

2.2. 2-[(9H-Fluoren-9-yl)Methoxycarbonylamino]Ethanaminium Chloride (8), 4-[(9H-Fluoren-9-yl)Methoxycarbonylamino]Butanaminium Chloride (10), and 5-[(9H-Fluoren-9-yl)Methoxycarbonylamino]Pentanaminium Chloride (11). Compounds **8**, **10,** and **11** were prepared according to the synthetic procedure of 3-[(9*H*-fluoren-9-yl)methoxycarbonylamino]propanaminium chloride (**9**) [10–13].

Compound 8: (9*H*-Fluoren-9-yl)methyl phenyl carbonate (**7**) (0.95 g, 3.0 mmol) was suspended in methanol (12.5 mL), and then ethylenediamine (0.20 mL, 3.0 mmol) was added to the solution. After stirring for 4 h at room temperature, pyridinium hydrochloride (0.75 g, 6.5 mmol) was added, and the solution stirred for 10 min. The solution was concentrated *in vacuo*, and the residue was subjected to silica gel column chromatography using a methanol/chloroform (1 : 4 *v*/*v*) solvent system to give **8** (0.19 g, 20% yield) as a white powder. ^{1}H-NMR (CD_3OD): δ 7.80 (d, 2H, J = 7.5 Hz, Ar-H of the Fmoc group), 7.65 (d, 2H, J = 7.5 Hz, Ar-H of the Fmoc group), 7.40 (t, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 7.31 (t, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 4.42 (d, 2H, J = 6.6 Hz, $CHCH_2$ of the Fmoc group), 4.22 (t, 1H, J = 6.6 Hz, $CHCH_2$ of the Fmoc group), 3.32-3.30 (m, 2H, NCH_2), and 2.92-2.90 (t, 2H, J = 6.0 Hz, NCH_2); ^{13}C-NMR (CD_3OD): δ 157.9, 143.8, 141.2, 127.4, 126.8, 124.8, 119.6, 66.7, 47.1, 39.8, and 38.3; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{17}H_{19}N_2O_2(M+H)^+$ 283.1447, found 283.1455

Compound 10: (9*H*-Fluoren-9-yl)methyl phenyl carbonate (**7**) (3.16 g, 9.99 mmol) was suspended in methanol (42 mL), and then butane-1,4-diamine (1.0 mL, 9.95 mmol) was added to the solution. After stirring for 4 h at room temperature, pyridinium hydrochloride (2.51 g, 21.7 mmol) was added, and the solution stirred for 10 min. The solution was concentrated *in vacuo*, and the residue was subjected to silica gel column chromatography using a methanol/chloroform (1 : 4 *v*/*v*) solvent system to give **10** (2.11 g, 61% yield) as a white powder. ^{1}H-NMR (CD_3OD): δ 7.79 (d, 2H, J = 7.5 Hz, Ar-H of the Fmoc group), 7.64 (d, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 7.39 (t, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 7.31 (t, 2H, J = 7.3 Hz, Ar-H of the Fmoc group), 4.37 (d, 2H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 4.20 (t, 1H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 3.15 (t, 2H, J = 6.6 Hz, NCH_2), 2.93 (t, 2H, J = 7.4 Hz, NCH_2), and 1.66-1.55 (m, 4H, CH × 2); ^{13}C-NMR (DMSO-d_6): δ 156.4, 144.1, 140.9, 127.8, 127.3, 125.4, 120.3, 65.5, 46.9, 39.8, 38.6, 26.46, and 24.39; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{19}H_{23}N_2O_2(M+H)^+$ 311.1760, found 311.1752

Compound 11: (9*H*-Fluoren-9-yl)methyl phenyl carbonate (**7**) (3.16 g, 9.99 mmol) was suspended in methanol (42 mL), and then pentane-1,5-diamine (1.17 mL, 10.0 mmol) was added to the solution. After stirring for 4 h at room temperature, pyridinium hydrochloride (2.51 g, 21.7 mmol) was added and the solution stirred for 10 min. The solution was concentrated *in vacuo*, and the residue was subjected to silica gel column chromatography using a methanol/chloroform (1 : 4 *v*/*v*) solvent system to give **11** (1.64 g, 45% yield) as a white powder. ^{1}H-NMR (CD_3OD): δ 7.76 (d, 2H, J = 7.5 Hz, Ar-H of the Fmoc group), 7.60 (d, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 7.36 (t, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 7.26 (t, 2H, J = 7.4 Hz, Ar-H of the Fmoc group), 4.33 (d, 2H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 4.17 (t, 1H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 3.08 (t, 2H, J = 6.9 Hz, NCH_2), 2.87 (t, 2H, J = 7.6 Hz, NCH_2), 1.64-1.60 (m, 2H, CH_2), 1.51-1.47 (m, 2H, CH_2), and 1.38-1.34 (m, 2H, CH_2); ^{13}C-NMR (CD_3OD): δ 158.9, 145.3, 142.6, 128.7, 128.1, 126.1, 120.1, 67.5, 48.4, 41.2, 40.6, 30.3, 28.2, and 24.5; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{20}H_{25}N_2O_2(M+H)^+$ 325.1916, found 325.1925

2.3. (9H-Fluoren-9-yl)Methyl 4-{1-Methyl-4-[1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl]Amino-1H-Pyrrole-2-Carbonyl}Aminobutylcarbamate (13). Compounds **13**, **14,** and **15** were prepared according to the synthetic procedure of **12** [10–13].

Pyrrole amide tetramer **4** was prepared as previously described [13]. ^{1}H-NMR (DMSO-d_6): δ 9.96 (s, 1H, and CONH), 9.95 (s, 1H, and CONH), 9.85 (s, 1H, and CONH), 7.48 (d, 1H, J = 1.8 Hz, and Py-H), 7.25-7.24 (m, 2H, and Py-H × 2), 7.08 (d, 1H, J = 1.8 Hz, and Py-H), 7.06 (d, ^{1}H, J = 1.8 Hz, and Py-H), 6.95-6.91, (m, 3H, and Py-H × 3), 6.06 (dd, ^{1}H, J = 2.6 Hz, J = 3.8 Hz, and Py-H), 3.88 (s, 3H, and NCH_3), 3.86 (s, 3H,and NCH_3), 3.85 (s, 3H, and

NC$\underline{H}_3$), 3.84 (s, 3H, and NC$\underline{H}_3$), and 3.74 (s, 3H, and OC$\underline{H}_3$); ^{13}C-NMR (DMSO-d_6): δ 161.0, 158.8, 158.7, 128.3, 125.6, 123.2, 122.9, 122.7, 122.5, 122.3, 121.0, 118.8, 118.7, 112.9, 108.6, 106.9, 105.0, 104.9, 51.2, 36.44, 36.38, 36.32, and 36.28; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{25}H_{28}N_7O_5(M+H)^+$ 506.2152, found 506.2151; UV (CH_3OH): λ_{max} 306, 238 nm; λ_{min} 262, 223 nm; and ε_{260} 2.6×10^4.

Pyrrole amide tetramer **4** (881 mg, 1.74 mmol) was dissolved in methanol (8.7 mL), and then 2 M NaOH aq. (8.7 mL) was added to the solution. After stirring at 60°C for 3 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration and the solution evaporated to give Py_4-carboxylic acid (856 mg, quantitative yield), which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ12.12(s, 1H, COO$\underline{H}$), 9.95 (s, 1H, and CON$\underline{H}$), 9.91 (s, 1H, and CON$\underline{H}$), 9.84 (s, 1H, and CON$\underline{H}$), 7.43 (d, 1H, J = 1.9 Hz, and Py-$\underline{H}$), 7.25-7.24 (m, 2H, and Py-$\underline{H}$ × 2), 7.07 (d, 1H, J = 1.8 Hz, and Py-$\underline{H}$), 7.05 (d, 1H, J = 1.8 Hz, and Py-$\underline{H}$), 6.95 (d, 1H, J = 2.2 Hz, and Py-$\underline{H}$), 6.93-6.91 (m, 1H, and Py-$\underline{H}$), 6.85 (d, 1H, J = 1.9 Hz, and Py-$\underline{H}$), 6.06 (dd, 1H, J = 2.6 Hz, J =3.8 Hz, and Py-$\underline{H}$), 3.88 (s, 3H, and NC$\underline{H}_3$), 3.86 (s, 3H, and NC$\underline{H}_3$), 3.85 (s, 3H, and NC$\underline{H}_3$), and 3.82 (s, 3H, and NC$\underline{H}_3$); ^{13}C-NMR (DMSO-d_6): δ 162.0, 158.6, 158.51, 158.47, 128.5, 125.5, 122.77, 122.71, 122.6, 122.3, 122.1, 120.3, 119.5, 118.5, 112.7, 108.4, 106.7, 104.77, 104.72, 36.24, 36.14, 36.12, and 36.08; HRMS (ESI-TOF) *m*/*z*: calculated for $C_{24}H_{26}N_7O_5(M+H)^+$ 492.1995, found 492.1993.

Py_4-carboxylic acid (492 mg, 1.00 mmol), **10** (520 mg, 1.50 mmol), and 1-hydroxybenzotriazole (HOBt) (270 mg, 2.00 mmol) were dissolved in DMF (5 mL), and then *N*,*N*′-dicyclohexylcarbodiimide (DCC) (310 mg, 1.50 mmol) and *N*-ethyldiisopropylamine (*N*,*N*-diisopropylethylamine: DIEA) (240 μL, 1.38 mmol) were added to the solution. After stirring for 16 h, the precipitate was removed by filtration. The filtrate was diluted with chloroform (200 mL) and washed with 2 M HCl aq. (100 mL × 3), H_2O (100 mL), 5% $NaHCO_3$ aq. (100 mL × 3), and H_2O (75 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **13** (555 mg, 71% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 9.93 (s, 1H, CON$\underline{H}$), 9.88 (s, 1H, and CON$\underline{H}$), 9.82 (s, 1H, and CON$\underline{H}$), 8.00-7.97 (m, 1H, and CON$\underline{H}$), 7.88 (d, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.69 (d, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.40 (t, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.32 (t, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.30-7.23 (m, 3H, CONHCH$_2$, and Py-$\underline{H}$ × 2), 7.18 (d, 1H, J = 1.8 Hz, and Py-$\underline{H}$), 7.06-7.04 (m, 2H, and Py-$\underline{H}$ x 2), 6.95-6.95 (m, 1H, and Py-$\underline{H}$), 6.93-6.92 (m, 1H, and Py-$\underline{H}$), 6.88-6.87 (m, 1H, and Py-$\underline{H}$), 6.06 (dd, 1H, J = 2.5 Hz, J=3.9 Hz, and Py-$\underline{H}$), 4.29 (d, 2H, J = 6.9 Hz, and CHC$\underline{H}_2$ of the Fmoc group), 4.22-4.19 (t, 1H, J = 6.9 Hz, and C$\underline{H}$CH$_2$ of the Fmoc group), 3.89 (s, 3H, and NC$\underline{H}_3$), 3.86 (s, 3H, and NC$\underline{H}_3$), 3.85 (s, 3H, and NC$\underline{H}_3$), 3.80 (s, 3H, and NC$\underline{H}_3$), 3.19-3.14 (m, 2H, and NHC$\underline{H}_2$), 3.03-2.98 (m, 2H, and NHC$\underline{H}_2$), and 1.52-1.39 (m, 4H, and C$\underline{H}_2$ × 2); ^{13}C-NMR (DMSO-d_6): δ 161.2, 158.6, 158.50, 158.46, 156.1, 143.9, 140.7, 128.1, 127.5, 127.0, 125.5, 125.1, 123.0, 122.8, 122.2, 122.11, 122.09, 120.1, 118.4, 117.7, 112.6, 106.6, 104.72, 104,69, 104.2, 65.2, 46.8, 38.1, 36.2, 36.1, 35.9, 27.0, and 26.7; HRMS (ESI-TOF) *m*/*z*: calculated for $C_{43}H_{46}N_9O_6(M+H)^+$ 784.3571, found 784.3578.

2.4. (9H-Fluoren-9-yl)Methyl 5-{1-Methyl-4-[1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl-Amino-1H-Pyrrole-2-Carbonyl]Aminopentylcarbamate (14). Py_4-carboxylic acid (390 mg, 0.79 mmol), **11** (430 mg, 1.19 mmol), and 1-hydroxybenzotriazole (161 g, 1.19 mmol) were dissolved in DMF (4 mL), and then DCC (246 mg, 1.19 mmol) and *N*-ethyldiisopropylamine (190 μL, 1.09 mmol) were added. After stirring for 18 h, the precipitate was removed by filtration. The filtrate was diluted with chloroform (200 mL) and washed with 2 M HCl aq. (100 mL × 3), H_2O (100 mL), 5% $NaHCO_3$ aq. (100 mL × 3), and H_2O (75 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **14** (530 mg, 84% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 9.93 (s, 1H, and CON$\underline{H}$), 9.88 (s, 1H, and CON$\underline{H}$), 9.82 (s, 1H, and CON$\underline{H}$), 7.98-7.95 (m, 1H, and CON$\underline{H}$), 7.88 (d, 2H, J = 7.5 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.68 (d, 2H, J = 7.5 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.41 (t, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.32 (t, 2H, J = 7.4 Hz, and Ar-$\underline{H}$ of the Fmoc group), 7.30-7.24 (m, 3H, CON$\underline{H}$, and Py-$\underline{H}$×2), 7.18 (s, 1H, and Py-$\underline{H}$), 7.05-7.04 (m, 2H, and Py-$\underline{H}$ × 2), 6.95-6.94 (m, 1H, and Py-$\underline{H}$), 6.93-6.91 (m, 1H, and Py-$\underline{H}$), 6.87 (s, 1H, and Py-$\underline{H}$), 6.06 (dd, 1H, J = 2.6 Hz, J = 3.9 Hz, and Py-$\underline{H}$), 4.29 (d, 2H, J = 6.9 Hz, and CHC$\underline{H}_2$ of the Fmoc group), 4.21 (t, 1H, J = 6.9 Hz, and C$\underline{H}$CH$_2$ of the Fmoc group), 3.89 (s, 3H, and NC$\underline{H}_3$), 3.86 (s, 3H, and NC$\underline{H}_3$), 3.85 (s, 3H, and NC$\underline{H}_3$), 3.80 (s, 3H, and NC$\underline{H}_3$), 3.18-3.13 (m, 2H, and NHC$\underline{H}_2$), 3.01-2.96 (m, 2H, and NHC$\underline{H}_2$), 1.52-1.39 (m, 4H, and C$\underline{H}_2$ × 2), and 1.31-1.24 (m, 2H, and C$\underline{H}_2$); ^{13}C-NMR (DMSO-d_6): δ 161.2, 158.6, 158.50, 158.46, 156.1, 143.9, 140.7, 128.9, 128.1, 127.6, 127.3, 127.0, 125.5, 125.1, 123.1, 122.8, 122.2, 122.1, 121.4, 120.1, 118.4, 117.7, 112.6, 106.6, 104.72, 104.68, 104.1, 65.1, 46.8, 38.3, 36.2, 36.1, 35.9, 29.11, 29.05, and 23.7; HRMS (ESI-TOF) *m*/*z*: calculated for $C_{44}H_{48}N_9O_6(M+H)^+$ 798.3728, found 798.3733.

2.5. (9H-Fluoren-9-yl)Methyl 4-{1-Methyl-4-[1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carbonyl)Amino-1H-Pyrrole-2-Carbonyl]Amino-1H-Pyrrole-2-Carbonyl}Aminobutylcarbamate (15). Pyrrole amide trimer **6** (515 mg, 1.34 mmol) was dissolved in methanol (6.7 mL), and then 2 M NaOH aq. (6.7 mL) was added to the solution. After stirring for 3 h at 60°C, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration and the solution evaporated to give the Py_3-carboxylic acid (495 mg, quantitative yield), which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ12.16 (s, 1H, COO$\underline{H}$), 9.93 (s, 1H, and CON$\underline{H}$), 9.86 (s, 1H, and CON$\underline{H}$), 7.46 (d, 1H, J = 1.9 Hz, and Py-$\underline{H}$), 7.26 (d, 1H, J = 1.8 Hz, and Py-$\underline{H}$), 7.08 (d, 1H, J = 1.8 Hz, and Py-$\underline{H}$), 6.95-6.94 (m, 1H, and Py-$\underline{H}$), 6.92 (dd, 1H, J = 1.7, and 3.9 Hz Py-$\underline{H}$), 6.85 (d, 1H, J = 1.9 Hz, and Py-$\underline{H}$), 6.06 (dd, 1H, J = 2.5,

and 3.9 Hz Py-H), 3.92 (s, 3H, and NCH_3), 3.89 (s, 3H, and NCH_3), and 3.86 (s, 3H, and NCH_3); ^{13}C-NMR (DMSO-d_6): δ 162.4, 159.1, 158.9, 128.6, 125.9, 123.2, 123.1, 122.6, 120.7, 120.0, 119.0, 113.1, 108.9, 107.1, 105.2, 36.7, 36.6, and 36.5; HRMS (ESI-TOF) *m*/*z*: calculated for $C_{18}H_{20}N_5O_4(M+H)^+$ 370.1515, found 370.1509.

Py_3-carboxylic acid (495 mg, 1.34 mmol), **10** (697 mg, 2.01 mmol), and 1-hydroxybenzotriazole (362 mg, 2.68 mmol) were dissolved in DMF (13.4 mL), and then DCC (553 mg, 2.68 mmol) and *N*-ethyldiisopropylamine (300 μL, 1.74 mmol) were added. After stirring for 16 h, the precipitate was removed by filtration. The filtrate was diluted with chloroform (200 mL) and washed with 2 M HCl aq. (100 mL × 3), H_2O (100 mL), 5% $NaHCO_3$ aq. (100 mL × 3), and H_2O (100 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **15** (538 mg, 61% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 9.87 (s, 1H, and CONH), 9.81 (s, 1H, and CONH), 7.99-7.97 (m, 1H, and CONH), 7.88 (d, 2H, $J = 7.4$ Hz, and Ar-H of the Fmoc group), 7.68 (d, 2H, $J = 7.4$ Hz, and Ar-H of the Fmoc group), 7.40 (t, 2H, $J = 7.4$ Hz, and Ar-H of the Fmoc group), 7.32 (t, 2H, $J = 7.4$ Hz, and Ar-H of the Fmoc group), 7.30-7.28 (m, 1H, and CONH), 7.23 (d, 1H, $J = 1.8$ Hz, and Py-H), 7.17 (d, 1H, $J = 1.8$ Hz, and Py-H), 7.03 (d, 1H, $J = 1.8$ Hz, and Py-H), 6.95-6.94 (m, 1H, and Py-H), 6.92-6.91 (m, 1H, and Py-H), 6.87 (d, 1H, $J = 1.6$ Hz, and Py-H), 6.06 (dd, 1H, $J = 2.5$, 3.8 Hz, and Py-H), 4.29 (d, 2H, $J = 6.8$ Hz, and $CHCH_2$ of the Fmoc group), 4.20 (t, 1H, $J = 6.8$ Hz, and $CHCH_2$ of the Fmoc group), 3.88 (s, 3H, and NCH_3), 3.85 (s, 3H, and NCH_3), 3.80 (s, 3H, and NCH_3), 3.19-3.14 (m, 2H, and $NHCH_2$), 3.02-2.98 (m, 2H, and $NHCH_2$), and 1.53-1.37 (m, 4H, and $CH_2 \times 2$); ^{13}C-NMR (DMSO-d_6): δ 161.2, 158.6, 158.4, 156.1, 143.9, 140.7, 128.1, 127.6, 127.0, 125.5, 125.1, 123.0, 122.8, 122.09, 122.06, 120.1, 118.4, 117.7, 112.6, 106.6, 104.6, 104.1, 65.2, 46.8, 38.1, 36.2, 36.1, 35.9, 27.0, and 26.7; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{37}H_{40}N_7O_5(M+H)^+$ 662.3091, found 662.3054.

2.6. Ethyl 1-Methyl-4-Nitroimidazole-2-Carboxylate (21). Ethyl 1-methylimidazole-2-carboxylate (**20**) [30] (2.97 g, 19.3 mmol) was dissolved in chloroform (19 mL), and then tetramethylammonium nitrate (5.26 g, 38.6 mmol) and trifluoroacetic anhydride (10.7 mL, 77.2 mmol) were added to the solution at 0°C. After stirring for 2.5 h at room temperature, 5% $NaHCO_3$ aq. (300 mL) was added. Products were extracted with chloroform (900 mL) from the resulting solution. The organic layer was washed with 5% $NaHCO_3$ aq. (200 mL × 2) and H_2O (100 mL), dried over anhydrous magnesium sulfate, and evaporated to dryness. The residue was subjected to silica gel column chromatography using an ethyl acetate/hexane (3 : 5~1 : 1 *v*/*v*) solvent system to give **21** (2.28 g, 59% yield) and ethyl 1-methyl-5-nitroimidazole-2-carboxylate (**22**) (920 mg, 24% yield).

Compound **20**: Rf 0.20 (ethyl acetate/hexane (2 : 1 *v*/*v*) solvent system)

Compound **21** (white powder): Rf 0.42 (ethyl acetate/hexane (2 : 1 *v*/*v*) solvent system), ^{1}H-NMR (DMSO-d_6): δ 8.63 (s, 1H, and Im-H), 4.35 (q, 2H, $J = 7.1$ Hz, and CH_2CH_3), 3.99 (s, 3H, and NCH_3), and 1.33 (t, 3H, $J = 7.1$ Hz, and CH_2CH_3); ^{13}C-NMR (DMSO-d_6): δ 157.7, 144.9, 134.8, 126.8, 61.7, 36.7, and 13.9; HRMS (ESI-TOF) *m*/*z*: calculated for $C_7H_{10}N_3O_4(M+H)^+$ 200.0671, found 200.0664. Anal. Calcd for $C_7H_9N_3O_4$: C, 42.21; H, 4.55; N, 21.10, found. C, 42.23; H, 4.59; and N, 21.09

Compound **22** (white powder): Rf 0.49 (ethyl acetate/hexane (2 : 1 *v*/*v*) solvent system), ^{1}H-NMR (DMSO-d_6): δ 8.15 (s, 1H, and Im-H), 4.38 (q, 2H, $J = 7.1$ Hz, and OCH_2CH_3), 4.19 (s, 3H, and NCH_3), and 1.33 (t, 3H, $J = 7.1$ Hz, and OCH_2CH_3); ^{13}C-NMR (DMSO-d_6): δ 158.0, 140.7, 138.9, 131.4, 62.1, 35.0, and 13.9; HRMS (ESI-TOF) *m*/*z*: calcd for $C_7H_{10}N_3O_4(M+H)^+$ 200.0671, found 200.0669. Anal. Calcd for $C_7H_9N_3O_4$: C, 42.21; H, 4.55; N, 21.10, found. C, 42.13; H, 4.50; and N, 21.01

2.7. 1-Methylimidazole-2-Carboxylic Acid (23). Ethyl 1-methyl-1*H*-imidazole-2-carboxylate (**20**) (3.08 g, 20.0 mmol) was dissolved in ethanol (50 mL)/pyridine (50 mL), and then 2 M NaOH aq. (100 mL) was added to the solution. After stirring at room temperature for 1 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration, and the solution evaporated to give **23** (2.52 g, quantitative yield) as a white powder, which was subsequently used without purification. ^{1}H-NMR (CD_3OD): δ 7.49 (s, 1H, and Im-H), 7.39 (s, 1H, and Im-H), and 4.15 (s, 3H, and NCH_3); ^{13}C-NMR (CD_3OD): δ 158.0, 141.9, 126.6, 119.6, and 37.1; HRMS (ESI-TOF) *m*/*z*: calcd for $C_5H_7N_2O_2(M+H)^+$ 127.0508, found 127.0512.

2.8. Ethyl 4-Amino-1-Methylimidazole-2-Carboxylate (24). Ethyl 4-nitro-1*H*-imidazole-2-carboxylate (**21**) (2.54 g, 12.7 mmol) was dissolved in ethanol (64 mL)/ethyl acetate (64 mL), and then 10% Pd/C (0.49 g) was added. The mixture was stirred under a slight positive pressure of hydrogen at room temperature for 3 h. Pd/C was removed by filtration through celite and washed with ethyl acetate (50 mL). The filtrate was evaporated to dryness to give **24** (2.14 g, quantitative yield) as a white powder, which was subsequently used without purification. ^{1}H-NMR ($CDCl_3$): δ 6.37 (s, 1H, and Im-H), 4.38 (2H, q, $J = 7.1$ Hz, and OCH_2CH_3), 3.92 (3H, s, and NCH_3), 2.96 (brs, NH_2, and 2H), and 1.40 (3H, t, $J = 7.1$ Hz, and OCH_2CH_3); ^{13}C-NMR ($CDCl_3$): δ 159.0, 145.6, 131.6, 109.5, 61.3, 35.7, and 14.5; HRMS (ESI-TOF) *m*/*z*: calcd for $C_7H_{12}N_3O_2(M+H)^+$ 170.0930, found 170.0938.

2.9. Ethyl 4-(tert-Butoxycarbonylamino)-1-Methyl-1H-Imidazole-2-Carboxylate (25). Compound **24** (2.09 g, 12.4 mmol) was dissolved in DMF (15 mL), and then a solution of di-*tert*-butyldicarbonate (5.42 g, 24.8 mmol) in DMF (10 mL) was added. After stirring for 19 h at room temperature, H_2O (50 mL) was added to the solution. The solution was diluted with chloroform (300 mL) and washed with H_2O (100 mL × 3). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to silica gel column chromatography

using an ethyl acetate/hexane (2 : 3 *v*/*v*) solvent system to give **25** (3.34 g, quantitative yield) as a white powder. ^{1}H-NMR (DMSO-d_6): δ 9.68 (s, 1H, and CONH), 7.30 (s, 1H, and Im-H), 4.25 (q, 2H, J = 7.2 Hz, and OCH_2CH_3), 3.88 (s, 3H, and NCH_3), 1.44 (s, 9H, and $OC(CH_3)_3$), and 1.28 (t, 3H, J = 7.2 Hz, and OCH_2CH_3); ^{13}C-NMR (DMSO-d_6): δ 158.4, 152.7, 138.1, 130.9, 113.8, 78.9, 60.42, 35.4, 28.0, and 14.1; HRMS (ESI-TOF) *m*/*z*; calcd for $C_{12}H_{20}N_3O_4(M+H)^+$ 270.1454, found 270.1439; Anal. Calcd for $C_{12}H_{19}N_3O_4$: C, 53.52; H, and 7.11; N, 15.60, found. C, 53.22; H, 7.05; and N, 15.55.

2.10. 4-(tert-Butoxycarbonylamino)-1-Methyl-1H-Imidazole-2-Carboxylic Acid (26). Compound **25** (2.63 g, 9.80 mmol) was dissolved in ethanol (24.5 mL)/pyridine (24.5 mL), and then 2 M NaOH aq. (49 mL) was added to the solution. After stirring at room temperature for 1 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration, and the solution evaporated to give **26** (2.36 g, quantitative yield) as a white powder, which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ 9.52 (s, 1H, and CONH), 7.16 (s, 1H, and Im-H), 3.88 (s, 3H, and NCH_3), and 1.44 (s, 9H, and $OC(CH_3)_3$); ^{13}C-NMR (DMSO-d_6): δ 160.4, 152.5, 137.1, 133.7, 112.2, 79.0, 35.3, and 28.1; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{10}H_{15}N_3O_4Na$ $(M+Na)^+$-264.0960, found 264.0950.

2.11. Ethyl 4-(4-(tert-Butoxycarbonylamino)-1-Methyl-1H-Imidazole-2-Carboxamido)-1-Methyl-1H-Imidazole-2-Carboxylate (27). Compounds **24** (660 mg, 3.90 mmol) and **26** (1.13 g, 4.70 mmol) were dissolved in DMF (39 mL), and then *N*-ethyldiisopropylamine (1.36 mL, 7.80 mmol), 1-hydroxybenzotriazole (1.05 g, 7.80 mmol), and *N*,*N*′-diisopropylcarbodiimide (DCI) (3.60 mL, 23.3 mmol) were added to the solution. After stirring for 18 h, the reaction solution was diluted with chloroform (200 mL) and washed with 5% $NaHCO_3$ aq. (100 mL × 2) and H_2O (100 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using an ethyl acetate/hexane (2 : 1 *v*/*v*) solvent system to give **27** (1.34 g, 88% yield) as a slightly brown glass. ^{1}H-NMR ($CDCl_3$): δ 9.45 (s, 1H, and CONH), 7.54 (s, 1H, and Im-H), 7.25 (s, 1H, and Im-H), 6.71 (s, 1H, and CONH), 4.43 (q, 2H, J = 7.2 Hz, and OCH_2CH_3), 4.025 (s, 3H, and NCH_3), 4.016 (s, 3H, and NCH_3), 1.52 (s, 9H, and $OC(CH_3)_3$), 1.44 (t, 3H, J = 7.2 Hz, and OCH_2CH_3); ^{13}C-NMR ($CDCl_3$): δ 159.0, 156.2, 152.6, 137.2, 136.8, 133.2, 132.0, 114.9, 112.7, 81.1, 61.7, 36.2, 35.7, 28.5, and 14.6; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{17}H_{25}N_6O_5(M+H)^+$-393.1886, found 393.1902.

2.12. Ethyl 1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxylate (28). Compound **27** (510 mg, 1.30 mmol) was dissolved in ethanol (19.5 mL)/chloroform (6.5 mL), and then acetyl chloride (2.80 mL, 39.0 mmol) was added to the solution at room temperature. After stirring for 2 h at 40°C, the reaction solution was concentrated *in vacuo* to give Im_2-amine compound, which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ 10.45 (1H, s, and CONH), 7.71 (s, 1H, and Im-H), 7.47 (s, and 1H, Im-H), 4.28 (q, 2H, J = 7.1 Hz, and OCH_2CH_3), 3.99 (s, 3H, and NCH_3), 3.94 (s, 3H, and NCH_3), and 1.29 (t, 3H, J = 7.1 Hz, and OCH_2CH_3); ^{13}C-NMR (DMSO-d_6): δ 158.1, 154.9, 135.7, 135.0, 131.4, 129.9, 117.6, 115.9, 60.8, 48.6, 35.75, 35.66, and 14.1; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{12}H_{17}N_6O_3(M+H)^+$ 293.1362, found 293.1365.

Im_2-amine compound (1.30 mmol) and **23** (214 mg, 1.70 mmol) were dissolved in DMF (13 mL), and then EDCI (1.02 g, 5.30 mmol) and DMAP (490 mg, 4.00 mmol) were added to the solution. After stirring at room temperature for 18 h, the solution was diluted with chloroform (200 mL) and washed with H_2O (10 mL), 5% $NaHCO_3$ aq. (100 mL × 2) and H_2O (100 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using an ethyl acetate/hexane (2 : 1 *v*/*v*) solvent system to give **28** (440 mg, 84% yield) as a slightly brown glass. ^{1}H-NMR ($CDCl_3$): δ 9.48 (s, 1H, and CONH), 9.40 (s, 1H, and CONH), 7.56 (s, 1H, and Im-H), 7.50 (s, 1H, and Im-H), 7.11 (s, 1H, and Im-H), 7.02 (s, 1H, and Im-H), 4.44 (q, 2H, J = 7.1 Hz, and OCH_2CH_3), 4.10 (s, 3H, and NCH_3), 4.08 (s, 3H, and NCH_3), 4.03 (s, 3H, and NCH_3), and 1.45 (t, 3H, and J = 7.1 Hz, and OCH_2CH_3); ^{13}C-NMR ($CDCl_3$): δ 158.1, 156.5, 156.2, 138.5, 136.8, 136.0, 133.6, 132.1, 128.4, 126.4, 115.1, 114.6, 61.8, 36.2, 35.90, 35.85, and 14.6; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{17}H_{21}N_8O_4(M+H)^+$ 401.1686, found 401.1682; UV (CH_3OH): λ_{max} 311 nm, λ_{min} 236 nm, ε_{260} 1.2 × 10^4.

2.13. (9H-Fluoren-9-yl)Methyl 3-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Propylcarbamate (29). Compound **28** (500 mg, 1.20 mmol) was dissolved in ethanol (6 mL)/pyridine (6 mL) and then 2 M NaOH aq. (12 mL) was added to the solution. After stirring at room temperature for 3 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration, and the solution evaporated to give Im_3-carboxylic acid (450 mg, quantitative yield) as a white powder, which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ 10.09 (s, 1H, and CONH), 9.90 (s, 1H, and CONH), 7.61 (s, 1H, and Im-H), 7.55 (s, 1H, and Im-H), 7.44 (s, 1H, and Im-H), 7.07 (s, 1H, and Im-H), 4.01 (s, 3H, and NCH_3), 4.00 (s, 3H, and NCH_3), and 3.94 (s, 3H, and NCH_3); ^{13}C-NMR (DMSO-d_6): δ 160.2, 155.9, 155.4, 137.8, 135.3, 135.0, 134.3, 133.4, 127.7, 127.0, 114.6, 113.8, 35.4, 35.2, and 35.1; HRMS (ESI-TOF) *m*/*z*: calcd for $C_{15}H_{17}N_8O_4(M+H)^+$ 373.1373, found 373.1366.

Im_3-carboxylic acid (330 mg, 0.90 mmol) and **9** (440 mg, 1.30 mmol) were dissolved in DMF (9 mL), and then *N*-ethyldiisopropylamine (210 μL, 1.20 mmol), 1-hydroxybenzotriazole (360 mg, 2.70 mmol), and *N*,*N*′-diisopropylcarbodiimide (800 μL, 5.40 mmol) were added to the solution. After stirring for 19 h, the solution was diluted with chloroform (100 mL) and washed with H_2O (50 mL × 3). The organic layer was dried over anhydrous magnesium

sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **29** (290 mg, 50% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 10.05 (s, 1H, and CONH), 9.56 (s, 1H, and CONH), 8.31-8.29 (m, and 1H, CONH), 7.88 (d, 2H, J = 7.4 Hz, and Ar − H × 2 of the Fmoc group), 7.69 (d, 2H, J = 7.4 Hz, Ar − H × 2 of the Fmoc group), 7.64 (s, 1H, and Im-H), 7.51 (s, 1H, and Im-H), 7.45 (s, 1H, and Im-H), 7.42 (t, 2H, J = 7.4 Hz, and Ar-H × 2 of the Fmoc group), 7.34 (t, 2H, J = 7.4 Hz, and Ar − H × 2 of the Fmoc group), 7.30-7.27 (m, 1H, and CONH), 7.08 (s, 1H, and Im-H), 4.32 (d, 2H, J = 6.9 Hz, and CHCH$_2$ of the Fmoc group), 4.22 (t, 1H, J = 6.9 Hz, and CHCH$_2$ of the Fmoc group), 4.02 (s, 3H, and NCH$_3$), 4.00 (s, 3H, and NCH$_3$), 3.96 (s, 3H, and NCH$_3$), 3.24-3.20 (m, 2H, and NHCH$_2$), 3.05-3.01 (m, 2H, and NHCH$_2$), and 1.66-1.59 (m, 2H, and CH$_2$); ^{13}C-NMR ($CDCl_3$): δ 159.7, 156.9, 156.5, 156.0, 144.2, 141.4, 138.5, 135.9, 135.5, 134.4, 133.8, 128.3, 127.7, 127.1, 126.3, 125.2, 120.1, 114.6, 113.9, 66.7, 47.5, 38.0, 36.0, 35.81, 35.78, 35.76, and 30.2; HRMS (ESI-TOF) m/z: calcd for $C_{33}H_{35}N_{10}O_5(M+H)^+$ 651.2792, found 651.2820.

2.14. (9H-Fluoren-9-yl)Methyl 4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Butylcarbamate (30). Im$_3$-carboxylic acid (740 mg, 2.00 mmol) and **10** (1.04 g, 3.00 mmol) were dissolved in DMF (20 mL), and then *N*-ethyldiisopropylamine (400 μL, 2.60 mmol), 1-hydroxybenzotriazole (810 mg, 6.00 mmol), and *N*,*N*′-diisopropylcarbodiimide (1.80 mL, 12.0 mmol) were added to the solution. After stirring for 19 h, the solution was diluted with chloroform (200 mL) and washed with H_2O (100 *mL* × 3). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **30** (430 mg, 33% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 9.68 (s, 1H, and CONH), 9.56 (s, 1H, and CONH), 8.28-8.26 (m, 1H, and CONH), 7.88 (d, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.68 (d, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.64 (s, 1H, and Im-H), 7.51 (s, 1H, and Im-H), 7.45 (s, 1H, and Im-H), 7.39 (t, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.33 (t, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.29-7.297 (m, 1H, and CONH), 7.08 (s, 1H, and Im-H), 4.29 (d, 2H, J = 6.8 Hz, and CHCH$_2$ of the Fmoc group), 4.20 (t, 1H, J = 6.8 Hz, and CHCH$_2$ of the Fmoc group), 4.02 (s, 3H, and NCH$_3$), 4.00 (s, 3H, and NCH$_3$), 3.95 (s, 3H, and NCH$_3$), 3.24-3.19 (m, 2H, and NHCH$_2$), 3.02-2.98 (m, 2H, and NHCH$_2$), and 1.49-1.41 (m, 4H, and CH$_2$ × 2); ^{13}C-NMR ($CDCl_3$): δ 159.3, 156.7, 156.5, 156.0, 144.2, 141.5, 138.5, 135.9, 135.4, 134.6, 133.7, 128.4, 127.8, 127.2, 126.3, 125.2, 120.1, 114.5, 113.8, 66.7, 47.5, 40.9, 38.8, 35.86, 35.83, 35.80, 27.5, and 27.2; HRMS (ESI-TOF) m/z: calcd for $C_{34}H_{37}N_{10}O_5(M+H)^+$ 665.2948, found 665.2973.

2.15. (9H-Fluoren-9-yl)Methyl 5-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Pentylcarbamate (31). Im$_3$-carboxylic acid (450 mg, 1.20 mmol) and **11** (650 mg, 1.80 mmol) were dissolved in DMF (12 mL), and then *N*-ethyldiisopropylamine (300 μL, 1.60 mmol), 1-hydroxybenzotriazole (490 mg, 3.60 mmol) and *N*,*N*′-diisopropylcarbodiimide (1.10 mL, 7.20 mmol) were added to the solution. After stirring for 19 h, the solution was diluted with chloroform (100 mL) and washed with H_2O (50 mL × 3). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **31** (580 mg, 71% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 10.03 (s, 1H, and CONH), 9.56 (s, 1H, and CONH), 8.26-8.24 (m, 1H, and CONH), 7.87 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.67 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.64 (s, 1H, and Im-H), 7.50 (s, 1H, and Im-H), 7.45 (s, 1H, and Im-H), 7.39 (t, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.33 (t, 2H, J = 7.5 Hz, and Ar-H of the Fmoc group), 7.26-7.24 (m, 1H, and CONH), 7.08 (s, 1H, and Im-H), 4.29 (d, 2H, J = 6.9 Hz, and CHCH$_2$ of the Fmoc group), 4.19 (t, 1H, J = 6.9 Hz, and CHCH$_2$ of the Fmoc group), 4.01 (s, 3H, and NCH$_3$), 4.00 (s, 3H, and NCH$_3$), 3.95 (s, 3H, and NCH$_3$), 3.23-3.18 (m, 2H, and NHCH$_2$), 3.00-2.95 (m, 2H, and NHCH$_2$), 1.54-1.39 (m, 4H, and CH$_2$ × 2), and 1.30-1.24 (m, 2H, and CH$_2$); ^{13}C-NMR($CDCl_3$): δ 159.2, 156.6, 156.3, 155.9, 144.1, 141.3, 138.4, 135.8, 135.3, 134.6, 133.7, 128.2, 127.6, 127.0, 126.1, 125.1, 120.0, 114.4, 113.7, 66.5, 47.4, 40.9, 38.8, 35.69, 35.66, 35.64, 29.6, 29.4, and 24.1; HRMS (ESI-TOF) m/z: calcd for $C_{35}H_{39}N_{10}O_5(M+H)^+$ 679.3105, found 679.3085.

2.16. Ethyl 4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)Butanoate (32). Pyrrole amide trimer **6** (1.51 g, 3.93 mmol) was dissolved in ethanol (17 mL)/pyridine (17 mL), and then 2 M NaOH aq. (34 mL) was added to the solution. After stirring at room temperature for 3 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration, and the solution evaporated to give Py$_3$-carboxylic acid (1.45 g, quantitative yield), which was subsequently used without purification.

Py$_3$-carboxylic acid (1.45 g, 3.93 mmol) and ethyl 4-aminobutanoate (0.774 g, 5.90 mmol) were dissolved in dichloromethane (20 mL), and then EDCI (1.50 g, 7.86 mmol) and DMAP (960 mg, 7.86 mmol) were added to the solution. After stirring at room temperature for 10 h, the solution was diluted with chloroform (300 mL) and washed with H_2O (80 mL), 5% $NaHCO_3$ aq. (80 mL × 2), and H_2O (80 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using an ethyl acetate/hexane (2 : 1~8 : 1 *v*/*v*) solvent system to give **32** (1.56 g, 82% yield) as a slightly brown glass. ^{1}H-NMR($CDCl_3$): δ 7.60 (s, 1H, and CONH), 7.50 (s, 1H, and CONH), 7.15 (d, 1H, J = 1.8

Hz, and Py-H), 7.12 (d, 1H, J = 1.8 Hz, and Py-H), 6.78-6.77 (m, 1H, and Py-H), 6.71 (d, 1H, J = 1.8 Hz, Py-H), 6.68-6.66 (m, 1H, and Py-H), 6.52 (d, 1H, J = 1.9 Hz, and Py-H), 6.24-6,22 (m, 1H, and CONH), 6.13 (dd, 1H, J = 2.6 Hz, J =4.0 Hz, and Py-H), 4.15 (q, 2H, J = 7.2 Hz, and OCH_2CH_3), 3.98 (s, 3H, and NCH_3), 3.93 (s, 3H, and NCH_3), 3.90 (s, 3H, and NCH_3), 3.44-3.39 (m, 2H, and $NHCH_2CH_2$-), 2.41 (t, 2H, J = 7.1 Hz, and $COCH_2CH_2$-), 1.95-1.88 (m, 2H, and -$CH_2CH_2CH_2$-), and 1.25 (t, 3H, J = 7.2 Hz, and OCH_2CH_3); ^{13}C-NMR($CDCl_3$): δ 173.8, 161.8, 159.5, 1589.0, 128.5, 125.4, 123.4, 123.2, 121.5, 121.2, 119.3, 118.9, 112.0, 107.4, 103.8, 103.2, 60.7, 38.9, 36.8, 36.65, 36.57, 31.9, 24.7, and 14.2; HRMS (ESI-TOF) m/z: calcd for $C_{24}H_{31}N_6O_5$ $(M+H)^+$ 483.2356, found 483.2354.

2.17. Ethyl 1-Methyl-4-(1-Methyl-4-(1-Methyl-4-(tert-Butoxycarbonylamino)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxylate (33). Compound **27** (2.22 g, 5.65 mmol) was dissolved in ethanol (19.5 mL)/chloroform (6.5 mL), and then acetyl chloride (2.80 mL, 39.0 mmol) was added to the solution at room temperature. After stirring for 2 h at 40°C, the reaction solution was concentrated *in vacuo* to give Im_2-amine compound, which was subsequently used without purification.

Im_2-amine compound (5.65 mmol) and **26** (2.27 g, 8.48 mmol) were dissolved in dichloromethane (56.5 mL), and then EDCI (3.25 g, 17.0 mmol) and DMAP (2.07 g, 17.0 mmol) were added to the solution. After stirring at room temperature for 19 h, the solution was diluted with chloroform (500 mL) and washed with H_2O (100 mL), 5% $NaHCO_3$ aq. (100 mL × 2) and H_2O (100 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using an ethyl acetate/hexane (2 : 1 v/v) solvent system to give **33** (2.05 g, 70% yield) as a slightly brown glass. ^{1}H-NMR ($CDCl_3$): δ 9.53 (s, 1H, and CONH), 9.17 (s, 1H, and CONH), 7.57 (s, 1H, and Im-H), 7.49 (s, 1H, and Im-H), 7.19 (s, 1H, and Im-H), 6.92 (s, 1H, and CONH), 4.44 (q, 2H, J = 7.1 Hz, and OCH_2CH_3), 4.07 (s, 3H, and NCH_3), 4.05 (s, 3H, and NCH_3), 4.03 (s, 3H, and NCH_3), 1.53 (s, 9H, and $OC(CH_3)_3$), 1.45 (t, 3H, J = 7.1 Hz, and OCH_2CH_3); ^{13}C-NMR ($CDCl_3$): δ 158.8, 155.9, 155.8, 152.5, 137.0, 136.6, 135.7, 133.3, 133.0, 131.8, 114.8, 114.3, 112.7, 80.8, 61.5, 36.0, 35.7, 35.6, 28.3, and 14.3; HRMS (ESI-TOF) m/z: calcd for $C_{22}H_{30}N_9O_6(M+H)^+$ 516.2319, found 516.2318.

2.18. Ethyl 1-Methyl-4-(1-Methyl-4-(1-Methyl-4-(4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido) Butanamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxylate (34). Compound **32** (1.44 g, 2.99 mmol) was dissolved in ethanol (7.5 mL)/pyridine (7.5 mL), and then 2 M NaOH aq. (15 mL) was added to the solution. After stirring at room temperature for 3 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration and the solution evaporated to give Py_3-$NH(CH_2)_3CO_2H$ (1.36 g, quantitative yield), which was subsequently used without purification. ^{1}H-NMR (DMSO-d_6): δ 9.89 (s, 1H, and CONH), 9.83 (s, 1H, and CONH), 8.06-8.03 (m, 1H, and CONH), 7.23 (d, 1H, J = 1.8 Hz, and Py-H), 7.18 (d, 1H, J = 1.8 Hz, and Py-H), 7.02 (d, 1H, J = 1.9 Hz, and Py-H), 6.95-6.93 (m, 1H, and Py-H), 6.92-6.91 (m, 2H, Py − H × 2), 6.87 (d, 1H, J = 1.9 Hz, and Py-H), 6.06 (dd, 1H, J = 2.6 Hz, J = 3.9 Hz, and Py-H), 3.88 (s, 3H, and NCH_3), 3.85 (s, 3H, and NCH_3), 3.79 (s, 3H, and NCH_3), 3.19-3.16 (m, 2H, and $NHCH_2CH_2$-), 2.24 (t, 2H, J = 7.4 Hz, and $COCH_2CH_2$-), and 1.72-1.69 (m, 2H, and -$CH_2CH_2CH_2$-); ^{13}C-NMR(DMSO-d_6): δ 174.5, 161.4, 158.6, 158.5, 128.2, 125.5, 123.0, 122.8, 122.15, 122.12, 118.5, 117.8, 112.7, 106.7, 104.7, 104.2, 37.9, 36.3, 36.1, 36.0, 31.3, and 24.8; HRMS (ESI-TOF) m/z: calcd for $C_{22}H_{27}N_6O_5(M+H)^+$ 455.2043, found 455.2039.

Compound **33** (54 mg, 0.104 mmol) was dissolved in ethanol (1.56 mL)/chloroform (0.52 mL), and then acetyl chloride (220 μL, 3.14 mmol) was added to the solution at room temperature. After stirring for 3 h at 40°C, the reaction solution was concentrated *in vacuo* to give Im_3-amine compound, which was subsequently used without purification. ^{1}H-NMR (CD_3OD): δ 7.81 (s, 1H, and Im-H), 7.65 (s, 1H, and Im-H), 7.35 (s, 1H, and Im-H), 4.49 (q, 2H, J = 7.1 Hz, and OCH_2CH_3), 4.14 (s, 3H, and NCH_3), 4.11 (s, 3H, and NCH_3), 4.10 (s, 3H, and NCH_3), and 1.45 (t, 3H, J = 7.1 Hz, and OCH_2CH_3); ^{13}C-NMR (DMSO-d_6): δ 158.2, 155.6, 154.7, 135.9, 135.2, 134.8, 133.5, 131.5, 131.4, 115.9, 115.5, 115.1, 60.8, 35.7, 35.7, 35.2, and 14.1; HRMS (ESI-TOF) m/z: calcd for $C_{17}H_{21}N_9O_4(M+H)^+$ 416.1795, found 416.1788.

Im_3-amine compound (0.104 mmol) and Py_3-$NH(CH_2)_3CO_2H$ (71 mg, 0.156 mmol) were dissolved in dichloromethane (2.1 mL), and then EDCI (60 mg, 0.312 mmol) and DMAP (38 mg, 0.312 mmol) were added to the solution. After stirring at room temperature for 14 h, the solution was diluted with chloroform (50 mL) and washed with H_2O (20 mL), 5% $NaHCO_3$ aq. (20 mL × 2) and H_2O (20 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 2% methanol/chloroform solvent system to give **34** (50 mg, 57% yield) as a slightly brown glass. ^{1}H-NMR($CDCl_3$): δ 9.51 (s, 1H, and CONH), 9.24 (s, 1H, and CONH), 8.87 (s, 1H, and CONH), 7.93 (s, 1H, and CONH), 7.58-7.54 (m, 2H, Im-H, and CONH), 7.46 (s, 1H, and Im-H), 7.44 (s, 1H, and Im-H), 7.20 (d, 1H, J = 1.5 Hz, and Py-H), 7.18 (d, 1H, J = 1.5 Hz, and Py-H), 6.75-6.74 (m, 2H, and Py − H × 2), 6.60-6.59 (m, 1H, and Py-H), 6.52-6.44 (m, 2H, Py-H, and CONH), 6.10-6.09 (m, 1H, and Py-H), 4.39 (q, 2H, J = 7.1 Hz, and OCH_2CH_3), 4.02 (s, 3H, and NCH_3), 4.01 (s, 3H, and NCH_3), 4.00 (s, 3H, and NCH_3), 3.97 (s, 3H, and NCH_3), 3.93 (s, 3H, and NCH_3), 3.92 (s, 3H, and NCH_3), 3.52-3.48 (m, 2H, and $NHCH_2CH_2$-), 2.52 (t, 2H, J = 6.7 Hz, and $COCH_2CH_2$-), 2.07-2.04 (m, 2H, -$CH_2CH_2CH_2$-), and 1.40 (t, 3H, J = 7.1 Hz, and OCH_2CH_3); ^{13}C-NMR($CDCl_3$): δ 171.2, 162.6, 159.8, 159.3, 158.7, 155.9, 155.8, 136.5, 136.4, 135.8, 135.7, 133.3, 133.1, 131.9, 128.4, 125.5, 123.09, 123.05, 121.9, 121.7, 119.4, 119.0, 115.0, 114.6, 112.5, 107.3, 104.2, 103.9, 61.6, 38.9, 36.8, 36.6, 36.5, 36.1, 35.63, 35.56, 33.8, 25.5,

and 14.2; HRMS (ESI-TOF) *m/z*: calcd for $C_{39}H_{46}$-$N_{15}O_8(M+H)^+$ 852.3654, found 852.3651; UV (CH_3OH): λ_{max} 300 nm, λ_{min} 257 nm, ε_{260} 3.0×10^4.

2.19. (9H-Fluoren-9-yl)Methyl-(3-(1-Methyl-4-(1-Methyl-4-(1-Methyl-4-(4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)Butanamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Ethyl)Carbamate (35). Compound **34** (350 mg, 0.41 mmol) was dissolved in ethanol (2 mL)/pyridine (2 mL), and then 2 M NaOH aq. (2 mL) was added to the solution. After stirring at room temperature for 6 h, Dowex 50WX8 (H^+-form) was added. Dowex 50WX8 was removed by filtration, and the solution evaporated to give Py_3-γ-Im_3-carboxylic acid (338 mg, quantitative yield) as a white powder, which was subsequently used without purification. ^{1}H-NMR(DMSO-d_6): δ 10.48 (s, 1H, and CONH), 10.22 (s, 1H, and CONH), 9.93 (s, 1H, and CONH), 9.89 (s, 1H, and CONH), 9.47 (s, 1H, and CONH), 8.09-8.06 (m, 1H, and CONH), 7.62 (s, 1H, and Im-H), 7.60 (s, 1H, and Im-H), 7.54 (s, 1H, and Im-H), 7.24 (d, 1H, J = 1.5 Hz, and Py-H), 7.19 (d, 1H, J = 1.5 Hz, and Py-H), 7.06 (d, 1H, J = 1.7 Hz, and Py-H), 6.97-6.95 (m, 1H, and Py-H), 6.94-6.93 (m, 1H, and Py-H), 6.91 (d, 1H, J = 1.7 Hz, and Py-H), 6.05-6.04 (m, 1H, and Py-H), 4.01 (s, 3H, and NCH_3), 3.98 (s, 3H, and NCH_3), 3.94 (s, 3H, and NCH_3), 3.88 (s, 3H, and NCH_3), 3.84 (s, 3H, and NCH_3), 3.80 (s, 3H, and NCH_3), 3.22-3.16 (m, 2H, and $NHCH_2CH_2$-), 2.39 (t, 2H, J = 7.2 Hz, and $COCH_2CH_2$-), and 1.82-1.79 (m, 2H, and -$CH_2CH_2CH_2$-); ^{13}C-NMR(DMSO-d_6): δ 169.6, 160.8, 158.1, 158.0, 155.0, 154.9, 149.1, 136.0, 135.1, 134.5, 133.0, 132.9, 132.1, 127.6, 125.0, 123.4, 122.5, 122.3, 121.7, 121.6, 117.9, 117.3, 114.3, 114.0, 112.3, 106.1, 104.3, 103.8, 37.6, 35.8, 35.6, 35.5, 35.1, 34.8, 34.6, 32.4, and 25.0; HRMS (ESI-TOF) *m/z*: calcd for $C_{37}H_{42}N_{15}O_8(M+H)^+$ 824.3341, found 824.3337.

Py_3-γ-Im_3-carboxylic acid (330 mg, 0.40 mmol) and **8** (190 mg, 0.60 mmol) were dissolved in dichloromethane (6 mL), and then EDCI (230 mg, 1.20 mmol) and DMAP (70 mg, 0.60 mmol) were added to the solution. After stirring at room temperature for 17 h, the solution was diluted with chloroform (50 mL) and washed with H_2O (20 mL), 5% $NaHCO_3$ aq. (20 mL × 2), and H_2O (20 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **35** (240 mg, 55% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 10.39 (s, 1H, and CONH), 9.89 (s, 1H, and CONH), 9.81 (s, 1H, and CONH), 9.64 (s, 1H, and CONH), 9.60 (s, 1H, and CONH), 8.26-8.24 (m, 1H, and CONH), 8.03-8.01 (m, 1H, and CONH), 7.87 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.66 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.64 (s, 1H, and Im-H), 7.53 (s, 1H, and Im-H), 7.50 (s, 1H, and Im-H), 7.41-3.9 (m, 1H, and CONH), 7.39 (t, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.29 (t, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.22 (d, 1H, J = 1.7 Hz, and Py-H), 7.18 (d, 1H, J = 1.6 Hz, and Py-H), 7.04 (d, 1H, J = 1.6 Hz, and Py-H), 6.94 (s, 1H, and Py-H), 6.92-6.91 (m, 1H, and Py-H), 6.89 (d, 1H, J = 1.9 Hz, and Py-H), 6.05 (dd, J = 2.6 Hz, J = 3.8 Hz, 1H, and Py-H), 4.28 (d, 2H, J = 7.0 Hz, and $CHCH_2$ of the Fmoc group), 4.20 (t, 1H, J=7.0 Hz, and $CHCH_2$ of the Fmoc group), 4.01 (s, 3H, and NCH_3), 3.97 (s, 3H, and NCH_3), 3.93 (s, 3H, and NCH_3), 3.88 (s, 3H, and NCH_3), 3.85 (s, 3H, and NCH_3), 3.80 (s, 3H, and NCH_3), 3.30-3.15 (m, 6H, and $NHCH_2CH_2$- × 3), 2.36 (t, 2H, J = 6.5 Hz, and $COCH_2CH_2$-), and 1.82-1.78 (m, 2H, and -$CH_2CH_2CH_2$-); ^{13}C-NMR ($CDCl_3$): δ 171.1, 162.3, 159.7, 159.6, 159.1, 157.2, 155.7, 155.6, 143.7, 141.1, 136.4, 135.5, 135.3, 134.1, 133.5, 133.1, 128.4, 127.6, 126.9, 125.4, 124.9, 123.1, 122.8, 121.8, 121.5, 119.9, 119.5, 119.1, 115.0, 114.4, 113.9, 112.5, 107.3, 104.0, 103.9, 66.8, 47.0, 39.2, 38.92, 38.85, 38.79, 36.8, 36.6, 36.4, 35.5, 33.7, 29.7, and 25.3; HRMS (ESI-TOF) *m/z*: calcd for $C_{54}H_{58}N_{17}O_9(M+H)^+$-1088.4603, found 1088.4604.

2.20. (9H-Fluoren-9-yl)Methyl (3-(1-Methyl-4-(1-Methyl-4-(1-Methyl-4-(4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)Butanamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Propyl)Carbamate (36). Py_3-γ-Im_3-carboxylic acid (338 mg, 0.41 mmol) and **9** (205 mg, 0.62 mmol) were dissolved in dichloromethane (10 mL), and then EDCI (236 mg, 1.23 mmol) and DMAP (90 mg, 0.74 mmol) were added to the solution. After stirring at room temperature for 17 h, the solution was diluted with chloroform (50 mL) and washed with H_2O (20 mL), 5% $NaHCO_3$ aq. (20 mL × 2), and H_2O (20 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **36** (240 mg, 53% yield) as a slightly brown glass. ^{1}H-NMR((DMSO-d_6): δ 10.40 (s, 1H, and CONH), 9.88 (s, 1H, and CONH), 9.81 (s, 1H, and CONH), 9.63-9.58 (m, 2H, and CONH × 2), 8.26-8.24 (m, 1H, and CONH), 8.03-8.01 (m, 1H, and CONH), 7.87 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.68 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.64 (s, 1H, and Im-H), 7.54 (s, 1H, and Im-H), 7.51 (s, 1H, and Im-H), 7.40 (t, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.32 (t, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.30-7.27 (1H, m, and CONH), 7.22 (d, 1H, J = 1.7 Hz, and Py-H), 7.17 (d, 1H, J = 1.7 Hz, and Py-H), 7.04 (d, 1H, J = 1.7 Hz, and Py-H), 6.95-6.94 (m, 1H, and Py-H), 6.92-6.90 (m, 1H, and Py-H), 6.89 (d, 1H, J = 1.7 Hz, and Py-H), 6.05 (dd, J = 2.5 Hz, J = 3.9 Hz, 1H, and Py-H), 4.31 (d, 2H, J = 6.8 Hz, and $CHCH_2$ of the Fmoc group), 4.21(t, 1H, J = 6.8 Hz, and $CHCH_2$ of the Fmoc group), 4.01 (s, 3H, and NCH_3), 3.97 (s, 3H, and NCH_3), 3.95 (s, 3H, and NCH_3), 3.88 (s, 3H, and NCH_3), 3.84 (s, 3H, and NCH_3), 3.80 (s, 3H, and NCH_3), 3.22-3.20 (m, 4H, and $NHCH_2CH_2$- × 2), 3.03-3.01 (m, 2H, and $NHCH_2CH_2$-), 2.39-2.35 (m, 2H, and $COCH_2CH_2$-), 1.82-1.78 (m, 2H, and -$CH_2CH_2CH_2$-), and 1.64-1.62 (m, 2H, and -$CH_2CH_2CH_2$-); ^{13}C-NMR (DMSO-d_6): δ 170.0, 161.4, 158.7, 158.5, 158.5, 156.2, 155.6, 155.4, 143.9, 140.8, 136.5, 135.2, 134.6, 134.4, 133.3, 132.8, 128.1, 127.6, 127.1, 125.5, 125.1, 123.0, 122.8,

122.2, 122.1, 120.1, 118.4, 117.8, 114.8, 114.6, 114.0, 112.7, 106.7, 104.7, 104.3, 65.3, 46.8, 38.2, 37.9, 36.2, 36.1, 36.01, 35.97, 35.3, 35.1, 35.0, 32.9, 29.6, and 25.5; HRMS (ESI-TOF) *m/z*: calcd for $C_{55}H_{60}N_{17}O_9$ $(M+H)^+$ 1102.4760, found 1102.4775.

2.21. (9H-Fluoren-9-yl)Methyl (4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-4-(4-(1-Methyl-4-(1-Methyl-4-(1-Methyl-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)-1H-Pyrrole-2-Carboxamido)Butanamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Butyl)Carbamate (37). Py_3-γ-Im_3-carboxylic acid (41 mg, 0.048 mmol) and **10** (28 mg, 0.080 mmol) were dissolved in dichloromethane (3 mL), and then EDCI (28 mg, 0.14 mmol) and DMAP (12 mg, 0.096 mmol) were added to the solution. After stirring at room temperature for 14 h, the solution was diluted with chloroform (50 mL) and washed with H_2O (20 mL), 5% $NaHCO_3$ aq. (20 mL × 2) and H_2O (20 mL). The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness. The residue was subjected to chromatographic separation on a column of silica gel using a 0-5% methanol/chloroform solvent system to give **37** (14 mg, 27% yield) as a slightly brown glass. ^{1}H-NMR (DMSO-d_6): δ 10.40 (s, 1H, and CONH), 9.88 (s, 1H, and CONH), 9.81 (s, 1H, and CONH), 9.63-9.58 (m, 2H, and CONH × 2), 8.25-8.23 (m, 1H, and CONH), 8.04-8.02 (m, 1H, and CONH), 7.87 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.68 (d, 2H, J = 7.4 Hz, and Ar-H of the Fmoc group), 7.65 (s, 1H, and Im-H), 7.54 (s, 1H, and Im-H), 7.52 (s, 1H, and Im-H), 7.40 (t, 2H, J = 7.1 Hz, and Ar-H of the Fmoc group), 7.32 (t, 2H, J = 7.1 Hz, and Ar-H of the Fmoc group), 7.29-7.27 (1H, m, and CONH), 7.23 (d, 1H, J = 1.8 Hz, and Py-H), 7.18 (d, 1H, J = 1.8 Hz, and Py-H), 7.04 (d, 1H, J = 1.8 Hz, and Py-H), 6.95-6.94 (m, 1H, and Py-H), 6.93-6.91 (m, 1H, and Py-H), 6.90 (d, 1H, J = 1.8 Hz, and Py-H), 6.05 (dd, 1H, J = 2.6 Hz, J = 3.9 Hz, and Py-H), 4.29 (d, 2H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 4.19 (t, 1H, J = 6.8 Hz, $CHCH_2$ of the Fmoc group), 4.01 (s, 3H, and NCH_3), 3.97 (s, 3H, and NCH_3), 3.95 (s, 3H, and NCH_3), 3.88 (s, 3H, and NCH_3), 3.85 (s, 3H, and NCH_3), 3.80 (s, 3H, and NCH_3), 3.22-3.20 (m, 4H, $NHCH_2CH_2$- × 2), 3.00-2.99 (m, 2H, and $NHCH_2CH_2$-), 2.38-2.35 (m, 2H, and $COCH_2CH_2$-), 1.81-1.78 (m, 2H, and -$CH_2CH_2CH_2$-), 1.46-1.30 (m, 4H, and -$CH_2CH_2CH_2$- × 2); ^{13}C-NMR (DMSO-d_6): δ 170.0, 161.3, 158.6, 158.5, 158.4, 156.1, 155.6, 155.4, 143.9, 140.7, 136.5, 135.2, 134.6, 134.5, 133.3, 132.8, 128.1, 127.6, 127.0, 125.5, 125.1, 123.0, 122.8, 122.2, 122.1, 120.1, 118.4, 117.8, 114.8, 114.6, 114.0, 112.7, 106.7, 104.7, 104.2, 65.2, 46.8, 38.2, 38.1, 36.2, 36.1, 36.0, 35.3, 35.1, 35.0, 32.9, 26.9, 26.6, 25.5, and 24.0; HRMS (ESI-TOF) *m/z*: calcd for $C_{56}H_{62}N_{17}O_9$ $(M+H)^+$ 1116.1926, found 1116.4918.

2.22. Synthesis of MGB Polyamide-Oligonucleotide Conjugates ON 1-4. Conjugates **ON 1**-**4** were synthesized by the postsynthetic modification method as previously described for the synthesis of **ON 1** (n = 3) [13].

CPG support-bound oligonucleotide **18** (11-mer: 5′-d(CGI^{F,NPE}AATTTGGC)-3′ or 5′-d(CGI^{F,NPE}ACCCTGGC)-3′) was synthesized using a syringe-based system. CPG support-bound 2′-deoxynucleoside **16** (B = C^{Bz}, 1000 Å, purchased from Applied Biosystems Pty Ltd.) (2 μmol) was treated with 3% w/v Cl_3CCO_2H in dichloromethane (1.0 mL × 2) for 1 min, followed by washing with acetonitrile (2.0 mL × 2). A 0.1 M solution of 2′-deoxynucleoside 3′-phosphoramidite **17** (B = G^{iBu}) in acetonitrile (0.5 mL) and 0.5 M 1*H*-tetrazole in acetonitrile (0.5 mL) were then delivered to the column. Following 10 min, coupling agents were ejected from the column, and the CPG support was washed with acetonitrile (2.0 mL × 2) to give the phosphite dimer. Following this, 1 : 1 : 8 acetic anhydride/2,6-lutidine/THF (1.0 mL) and 16% 1-methylimidazole/THF (1.0 mL) were delivered to the column, coupling agents were ejected from the column, and the CPG support was washed with acetonitrile (2.0 mL × 2). The resultant CPG support-bound phosphite dimer was treated with 0.02 M I_2 in 1 : 2 : 7 H_2O/pyridine/THF (1.0 mL) for 1 min and washed with acetonitrile (2.0 mL × 2) to give the CPG support-bound phosphorotriester dimer. Following chain elongation using 2′-deoxynucleoside 3′-phosphoramidites **17** (B = T, C^{Bz}, A^{Bz}, G^{iBu}, and $I^{F,NPE}$), as described above, the terminal DMTr protecting group of the oligonucleotide was removed by treatment with 3% w/v Cl_3CCO_2H in dichloromethane (1.0 mL × 2) for 1 min, and the CPG support was washed with acetonitrile (2.0 mL × 2). Resultant CPG support-bound oligonucleotide **18** was treated with 0.1 M Fmoc-NH$(CH_2)_n$NH-MGB polyamide (**12**, **13**, **14**, **15**, **29**, **30**, **31**, **35**, **36**, or **37**) in 1 : 5 Et_3N/1,4-dioxane (1.0 mL) at 60°C for 24 h and then washed with acetonitrile (2.0 mL × 2). Following this, the CPG support-bound oligomer was treated with 0.5 M DBU in pyridine (2.0 mL) at room temperature for 12 h and washed with acetonitrile (2.0 mL × 2). The generated oligomer was then cleaved from the CPG support by treatment with conc. NH_4OH (1.5 mL × 2) for 2 h at room temperature. The resulting solution was then heated in a sealed vial at 55°C for 6 h. Following evaporation, the residue was dissolved in H_2O (5.0 mL) and washed with ethyl acetate (5.0 mL × 3), and the aqueous layer was evaporated. The MGB polyamide-oligonucleotide conjugates **ON 1-4** were then purified by reversed-phase HPLC.

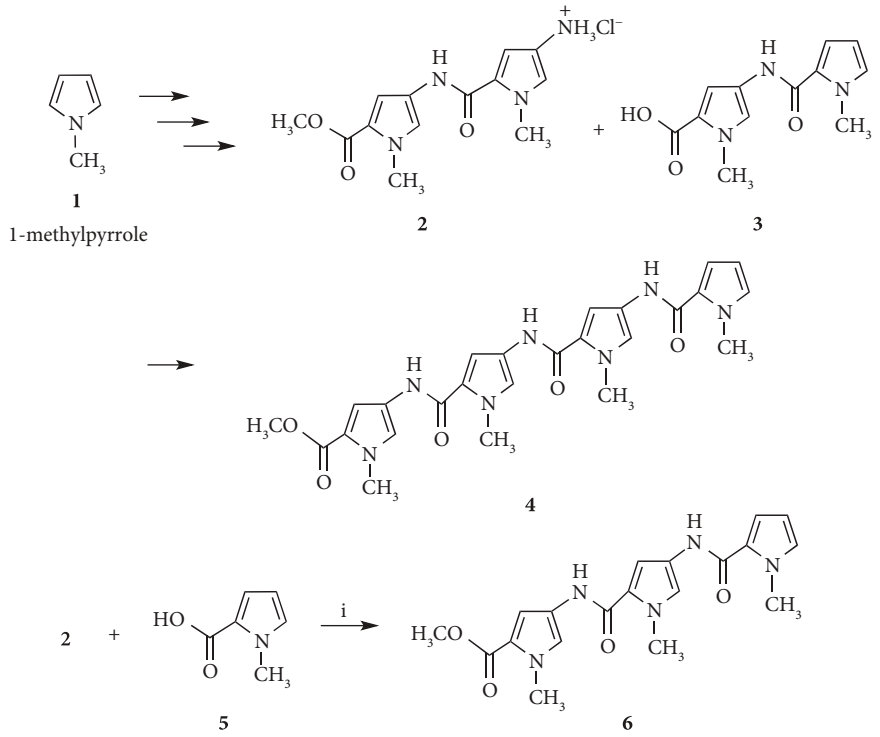
ON 1 (5′-d(CGGAATTTGGC)-3′, G = Py_4-NH$(CH_2)_n$-G (n = 3 − 5)).

ON 1 (n = 3) yields 48.8 A_{260} units from **16** (B = C^{Bz}) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{135}H_{168}N_{50}O_{69}P_{10}(M+2H)^{2+}$ 1951.4276, found 1951.4158. **ON 1** (n = 4): yields 34.0 A_{260} units from **16** (B = C^{Bz}) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{136}H_{170}N_{50}O_{69}P_{10}(M+2H)^{2+}$ -1958.4353, found 1958.4075. **ON 1** (n = 5): yields 31.4 A_{260} units from **16** (B = C^{Bz}) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{137}H_{173}N_{50}O_{69}P_{10}(M+3H)^{3+}$ 1310.6314, found 1310.5460.

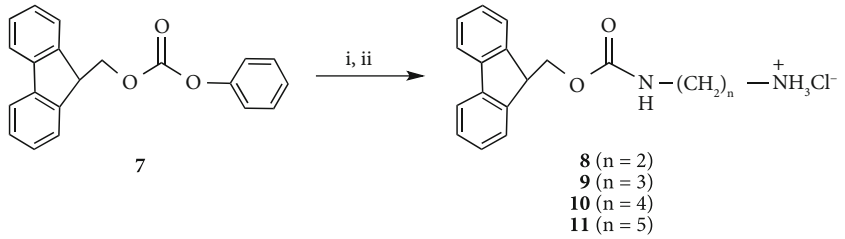
ON 2 (5′-d(CGGAATTTGGC)-3′, G = Py_3-NH$(CH_2)_n$-G (n = 4).

ON 2 (n = 4) yields 22.5 A_{260} units from **16** (B = C^{Bz}) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{130}H_{165}N_{48}O_{68}P_{10}$ $(M+3H)^{3+}$ 1265.2768, found 1265.2252.

ON 3 (5′-d(CGGACCCTGGC)-3′, G = Im_3-NH$(CH_2)_n$-G (n = 3 − 5)).



Scheme 1: Synthesis of pyrrole polyamides. Reagents and conditions: i EDCI, DMAP, CH_2Cl_2, rt, **6** (80%). Compounds **2**, **3**, **4**, and **5** were prepared as previously described [10–13].



Scheme 2: Synthesis of aminoalkyl linker reagents. Reagents and conditions: i $H_2N(CH_2)_nNH_2$, MeOH, rt; (ii) pyridinium hydrochloride, **8** (20%); **10** (61%); **11** (45%). Compound **9** was prepared as previously described [10–13].

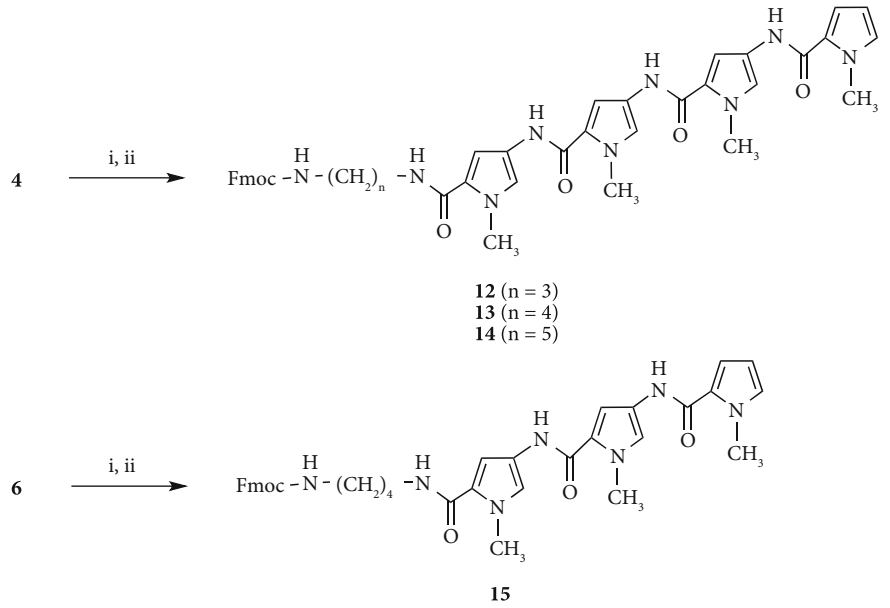
ON 3 (n=3) yields 21.8 A_{260} units from **16** ($B = C^{Bz}$) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{123}H_{156}N_{51}O_{67}P_{10}$ $(M+H)^+$ 3728.7744, found 3728.7866. **ON 3** ($n = 4$) yields 20.7 A_{260} units from **16** ($B = C^{Bz}$) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{124}H_{158}N_{51}O_{67}P_{10}$ $(M+H)^+$ 3742.7900, found 3742.7827. **ON 3** ($n = 5$) yields 28.7 A_{260} units from **16** ($B = C^{Bz}$) (2 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{125}H_{160}N_{51}O_{67}P_{10}$ $(M+H)^+$ 3756.8057, found 3756.8152.

ON 4 (5′-d(CGGACCCTGGC)-3′: G = Py_3-γ-Im_3-$NH(CH_2)_n$-G ($n = 2-4$)).

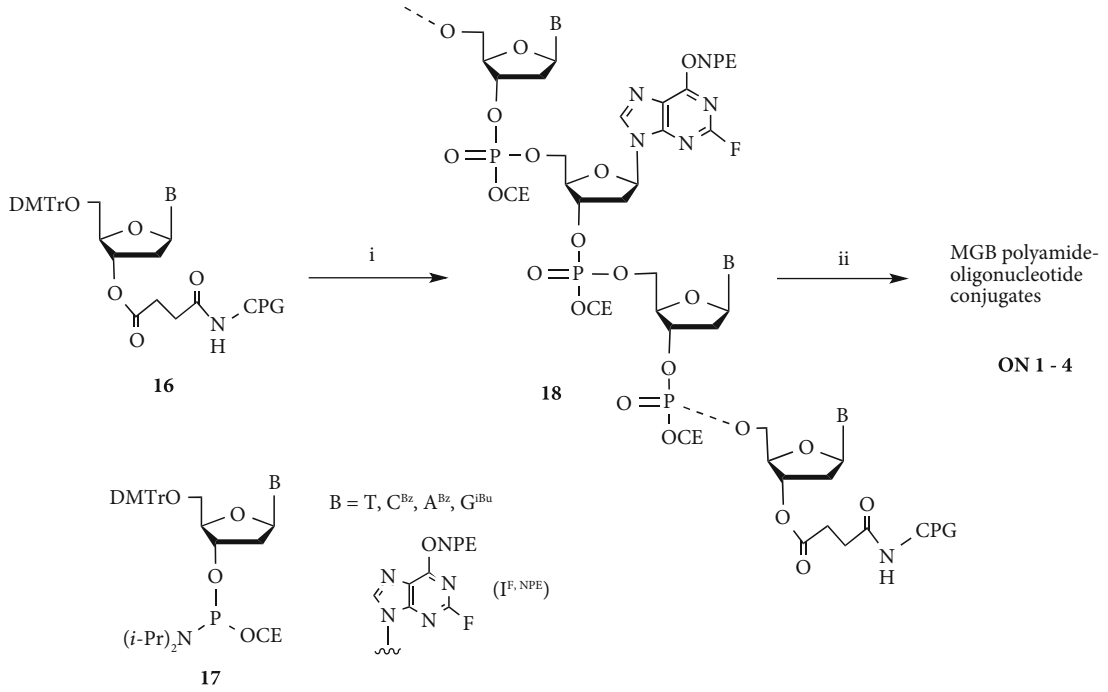
ON 4 ($n = 2$) yields 8.4 A_{260} units from **16** ($B = C^{Bz}$) (1 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{144}H_{179}N_{58}O_{71}P_{10}$ $(M+H)^+$ 4165.9555, found 4165.9580. **ON 4** ($n = 3$) yields 11.0 A_{260} units from **16** ($B = C^{Bz}$) (1 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{145}H_{181}N_{58}O_{71}P_{10}$ $(M+H)^+$ 4179.9712, found 4179.9858. **ON 4** ($n = 4$) yields 11.3 A_{260} units from **16** ($B = C^{Bz}$) (1 μmol). HRMS (ESI-TOF) *m/z* calcd for $C_{146}H_{183}N_{58}O_{71}P_{10}$ $(M+H)^+$ 4193.9868, found 4194.0024.

2.23. Melting Temperature Experiments. Absorbance versus temperature profiles of duplexes in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na_2EDTA were measured using a TMSPC-8/UV1600 (Shimadzu Co., Ltd.) instrument equipped with a thermoelectrically controlled cell holder at 260 nm and a heating rate of 1.0°C/min. The concentration of each duplex was 4.3 μM [5, 36]. From these melting curves, T_m values were obtained using a TMSPC-8 system with T_m analysis software.

2.24. Circular Dichroism (CD) Spectropolarimetry. CD spectra of duplexes in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na_2EDTA were measured using a JASCO J-720 spectropolarimeter equipped with a thermoelectrically controlled cell holder (at 20°C) and a cuvette with a path length of 10 mm. The concentration of each duplex was 5.8 μM [5].



Scheme 3: Synthesis of pyrrole polyamides bearing aminoalkyl linker. Reagents and conditions: i 1 M NaOH aq./MeOH, 60°C; Dowex 50WX8 (H^+-form); ii **9**, **10**, or **11**, DCC, HOBt, DIEA, DMF, and rt; **13** (71%); **14** (84%); **15** (61%). Compound **12** was prepared as previously described [10–13].



Scheme 4: Synthesis of MGB polyamide-oligonucleotide conjugates. Reagents and conditions: i Oligonucleotide assembly on CPG support by the phosphoramidite method, 3% Cl_3CCO_2H, CH_2Cl_2; 0.05 M phosphoramidite **17**, 0.25 M 1*H*-tetrazole, CH_3CN, or CH_2Cl_2; Ac_2O, 2,6-lutidine, 1-methylimidazole, THF; and 0.02 M I_2, H_2O/pyridine/THF. (ii) 3% Cl_3CCO_2H, CH_2Cl_2; 0.1 M FmocNH-$(CH_2)_n$NH-MGB polyamide **12**, **13**, **14**, **15**, **29**, **30**, **31**, **35**, **36**, or **37**, 1 : 5 Et_3N/1,4-dioxane, 60°C, 24 h; 0.5 M DBU, pyridine, rt, 12 h. Conc. NH_4OH, rt., 2 h–55°C, and 6 h.

3. Results and Discussion

In an effort to examine the effect of linker length or the distance between the guanine base and pyrrole polyamide on the stability of the modified dsDNA (**ON 1**/complementary DNA), we synthesized **ON 1** ($n = 3$, 4, and 5) (5′-d(CG**G**AATTTGGC)-3′: **G** = Py_4-NH$(CH_2)_n$-G) using 3-aminopropyl [13], 4-aminobutyl, and 5-aminopentyl linkers.

Pyrrole amide tetramer **4** and the linker reagent **9** were prepared as previously described (Schemes 1 and 2)

Table 1: T_m values of modified dsDNAs and respective ΔT_m values.

dsDNAs	Complementary DNA 3′-d(GCCTTAAACCG)-5′		Mismatch DNA [d] 3′-d(GCCTT**c**AACCG)-5′		3′-d(GC**a**TT**c**AACCG)-5′	
	T_m(°C)[b]	ΔT_m(°C)[c]	T_m(°C)[b]	ΔT_m(°C)[c]	T_m(°C)[b]	ΔT_m(°C)[c]
Unmodified DNA	34.1	—	22.1	—	n.d.[e]	—
Modified DNA [a]						
ON 1 ($n = 3$)	59.5	+25.4[f]				
ON 1 ($n = 4$)	60.2	+26.1	45.6	+23.5	n.d.[e]	—
ON 1 ($n = 5$)	52.9	+18.8				
ON 2 ($n = 4$)	50.8	+16.7	39.1	+17.0	n.d.[e]	—

[a]modified DNA: 5′-d(CG**G**AATTTGGC)-3′, **ON 1** (**G** = Py_4-$NH(CH_2)_n$-G); **ON 2** (**G** = Py_3-$NH(CH_2)_n$-G). [b]dsDNA (4.3 μM) in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na_2EDTA. [c]ΔT_m (°C) = T_m [modified dsDNA]-T_m (unmodified dsDNA). [d]mismatch base: **a**: adenine; **c**: cytosine. [e] n.d.: not detected. [f]it was confirmed that **ON 1** ($n = 3$) formed stable dsDNA with complementary DNA [13].

[10–13]. Linker reagents **8**, **10,** and **11** were prepared according to the synthetic procedure of **9**. Pyrrole amide tetramers **12**, **13,** and **14** bearing 3-aminopropyl, 4-aminobutyl, and 5-aminopentyl linkers were synthesized via hydrolysis of the ester moiety of **4** and coupling with linker reagents **9**, **10,** and **11**, respectively, as shown in Scheme 3. **ON 1** ($n = 3$, 4, and 5) were synthesized by a postsynthetic modification method using 2′-deoxy-2-fluoroinosine 3′-phosphoramidite **17** ($B = I^{F,NPE}$) [32–35] and pyrrole amide tetramers **12**, **13**, and **14** as previously described (Scheme 4) [13]. 2′-Deoxy-2-fluoroinosine ($I^{F,NPE}$) was incorporated into CPG support-bound oligonucleotide **18** (5′-d($CGI^{F,NPE}$AATTTGGC)-3′) using a standard procedure [37]. Resultant CPG support-bound oligonucleotide **18** was treated with 0.1 M **12**, **13**, or **14** in 1 : 5 Et_3N/1,4-dioxane at 60°C for 24 h. The generated CPG support-bound oligonucleotide was then treated with 0.5 M DBU in pyridine to remove the NPE and CE protecting groups, and then treated with concentrated NH_4OH to cleave the oligomer from the CPG support and remove the Bz and iBu protecting groups. Conjugates **ON 1** ($n = 3$, 4, and 5) were purified by reversed-phase HPLC to yield 48.8, 34.0, and 31.4 A_{260} units, respectively, from **16** ($B = C^{Bz}$) (2 μmol).

Conjugates **ON 1** ($n = 3$, 4, and 5) were converted to modified dsDNAs (**ON 1**/complementary DNA) by annealing with complementary DNA. The stability of modified dsDNAs was investigated by T_m and CD analyses. From the T_m values, it was found that the stability of modified dsDNA was greatly influenced by the linker length (**ON 1** ($n = 3$, $T_m = 59.5$°C, $\Delta T_m = +25.4$°C), ($n = 4$, $T_m = 60.2$°C, $\Delta T_m = +26.1$°C), and ($n = 5$, $T_m = 52.9$°C, $\Delta T_m = +18.8$°C)) (Table 1). **ON 1** ($n = 4$) showed high binding ability for complementary DNA, similar to **ON 1** ($n = 3$) previously reported [13]. In the CD spectrum for dsDNA [**ON 1** ($n = 4$)/complementary DNA], a strong additional CD band centered at 331 nm resulting from an induced Cotton effect of the bound pyrrole amide moiety was observed (Figure 3(I)) [5, 6, 13].

Using single-mismatch DNA (3′-d(GCCTT**c**AACCG)-5′), which contains a mismatch base in the recognition sequence (5′-d(AATTT)-3′/3′-(TT**A**AA)-5′) of the pyrrole amide moiety, and 2-base mismatch DNA (3′-d(GC**a**TT-**c**AACCG)-5′) which does not form dsDNA with the unmodified DNA, the DNA sequence recognition ability of **ON 1** ($n = 4$) was investigated by T_m analysis (Table 1). **ON 1** ($n = 4$) formed dsDNA with single-base mismatch DNA and displayed stabilization of the dsDNA ($T_m = 45.6 \circ$ C, $\Delta T_m = +23.5$°C,) by the pyrrole amide moiety. On the other hand, **ON 1** ($n = 4$) did not form dsDNA with 2-base mismatch DNA and the pyrrole amide moiety did not show any activity.

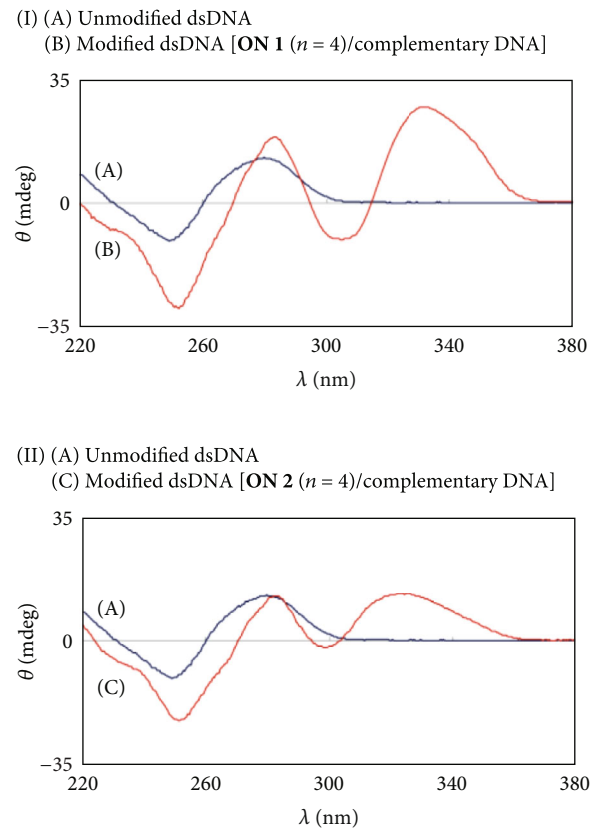
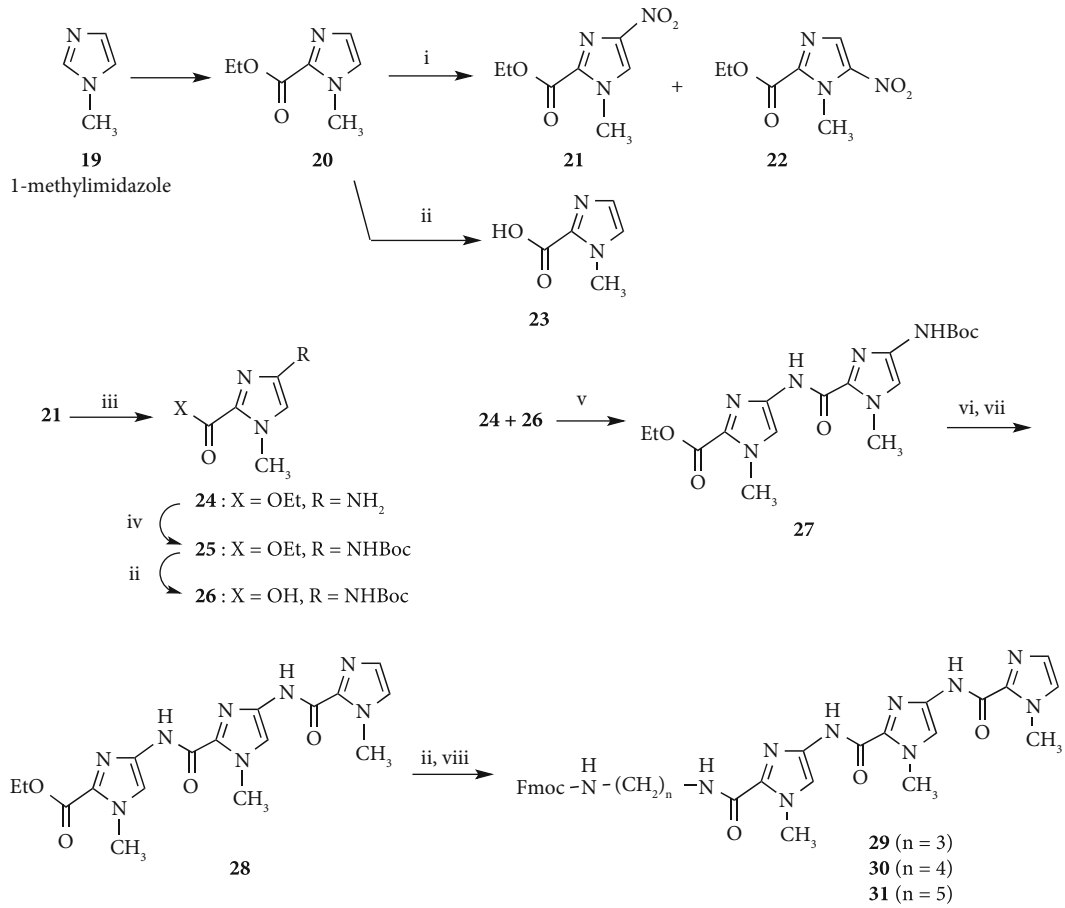


Figure 3: CD spectra of unmodified and modified dsDNAs. modified DNA: 5′-d(CG**G**AATTTGGC)-3′, **ON 1** (**G** = Py_4-$NH(CH_2)_n$-G) and **ON 2** (**G** = Py_3-$NH(CH_2)_n$-G).



Scheme 5: Synthesis of imidazole polyamide derivatives. Reagents and conditions: i $(CH_3)_4N^+ NO_3^-$, TFAA, $CHCl_3$, 0°C-rt, **21** (59%), and **22** (24%). ii 1 M NaOH aq./EtOH/pyridine, rt; Dowex 50WX8 (H^+-form), **23** (quant.); **26** (quant.). iii H_2, 10% Pd/C, 1 : 1 AcOEt/EtOH, rt, **24** (quant.). iv $(Boc)_2O$, DMF, rt, **25** (quant.); (v) DCI, HOBt, DIEA, DMF, rt, **27** (88%). vi AcCl, EtOH/$CHCl_3$, rt-40°C. vii **23**, EDCI, DMAP, DMF, rt, **28** (84%). viii **9**, **10,** or **11**, DCI, HOBt, DIEA, DMF, rt, **29** (50%); **30** (33%); **31** (71%). Compound **20** was prepared according to the procedure described by Baird and Dervan [30].

Table 2: T_m values of modified dsDNAs and respective ΔT_m values.

dsDNAs	Complementary DNA 3′-d(GCCTGGGACCG)-5′		Mismatch DNA[d)] 3′-d(GCCTG**a**GACCG)-5′		3′-d(GCCTGGGAC**t**G)-5′	
	T_m(°C) [b)]	ΔT_m(°C)[c)]	T_m(°C)[b)]	ΔT_m(°C)[c)]	T_m(°C)[b)]	ΔT_m(°C)[c)]
Unmodified DNA	41.4	—	33.9	—	35.5	—
Modified DNA[a)]						
ON 3 ($n = 3$)	43.7	+2.3				
ON 3 ($n = 4$)	46.5	+5.1	35.4	+1.5	37.1	+1.6
ON 3 ($n = 5$)	43.6	+2.2				
ON 4 ($n = 2$)	63.3	+21.9				
ON 4 ($n = 3$)	53.6	+12.2	41.1	+7.2	55.8	+20.3
ON 4 ($n = 4$)	49.8	+8.4				

a) modified DNA: 5′-d(CG**G**ACCCTGGC)-3′, **ON 3** (**G** = Im_3-$NH(CH_2)_n$-G); **ON 4** (**G** = Py_3-γ-Im_3-$NH(CH_2)_n$-G). b) dsDNA (4.3 μM) in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na_2EDTA. c) ΔT_m (°C) = T_m (modified dsDNA)-T_m (unmodified dsDNA). d) mismatch base, **a**: adenine, **t**: thymine.

We surmised that shortening the pyrrole amide chain of the modified DNA would be effective in reducing activity and increasing recognition of the target DNA sequence. **ON 2** ($n = 4$) (5′-d(CG**G**AATTTGGC)-5′: **G** = Py_3-$NH(CH_2)_n$-G) was synthesized by a postsynthetic modification method using pyrrole amide trimer **15**, which was prepared via coupling of pyrrole amide dimer **2** and pyrrole-2-carboxylic acid **5**, hydrolysis of ester product **6**, and

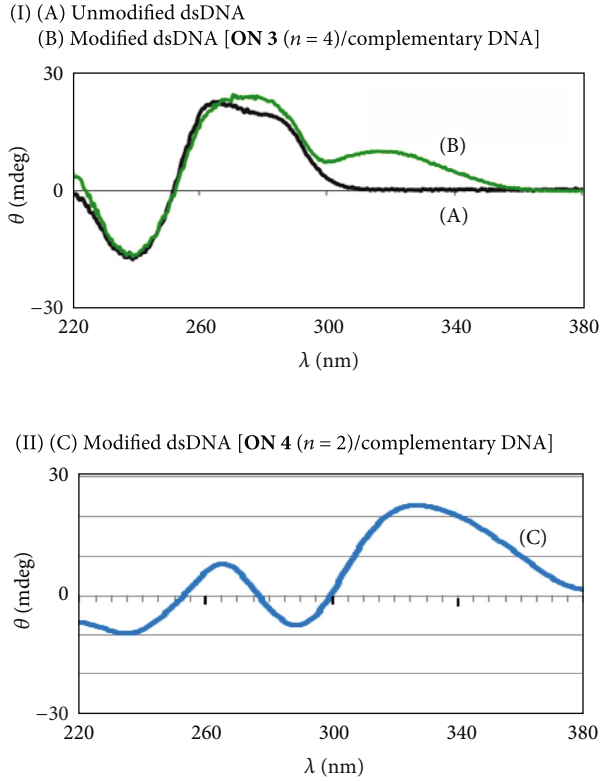


FIGURE 4: CD spectra of unmodified and modified dsDNAs. modified DNA: 5′-d(CG**G**ACCCTGGC)-3′, **ON 3** (**G** = Im_3-$NH(CH_2)_n$-G) and **ON 4** (**G** = Py_3-γ-Im_3-$NH(CH_2)_n$-G).

condensation with linker reagent **10** (Schemes 1 and 3). **ON 2** ($n = 4$) was purified by reversed-phase HPLC and yielded 22.5 A_{260} units from **16** ($B = C^{Bz}$) (2 μmol) (Scheme 4).

The stability of the modified dsDNA of **ON 2** ($n = 4$) and complementary DNA was investigated (Table 1 and Figure 3(II)). The stability of the modified dsDNA (**ON 2** ($n = 4$)/complementary DNA: $T_m = 50.8°C$, $\Delta T_m = +16.7°C$) was lower compared with modified dsDNA (**ON 1** ($n = 4$)/complementary DNA: $T_m = 60.2°C$, $\Delta T_m = +26.1°C$). The DNA sequence recognition ability of **ON 2** ($n = 4$) was investigated using single- and 2-base mismatch DNAs (Table 1). **ON 2** ($n = 4$) formed dsDNA with single-base mismatch DNA and displayed stabilization of the dsDNA ($T_m = 39.1°C$, $\Delta T_m = +17.0°C$). On the other hand, **ON 2** ($n = 4$) did not form dsDNA with 2-base mismatch DNA. The DNA sequence recognition ability of pyrrole polyamide-oligonucleotide conjugates was not improved. However, from the result of 2-base mismatch DNA, it was thought that conjugates (modified DNAs **ON 1** ($n = 4$) and **ON 2** ($n = 4$)) did not act on single-base mismatch DNA under conditions where dsDNA (unmodified DNA/single-base mismatch DNA) did not form (e.g., processing temperature > T_m (unmodified DNA/single-base mismatch DNA)).

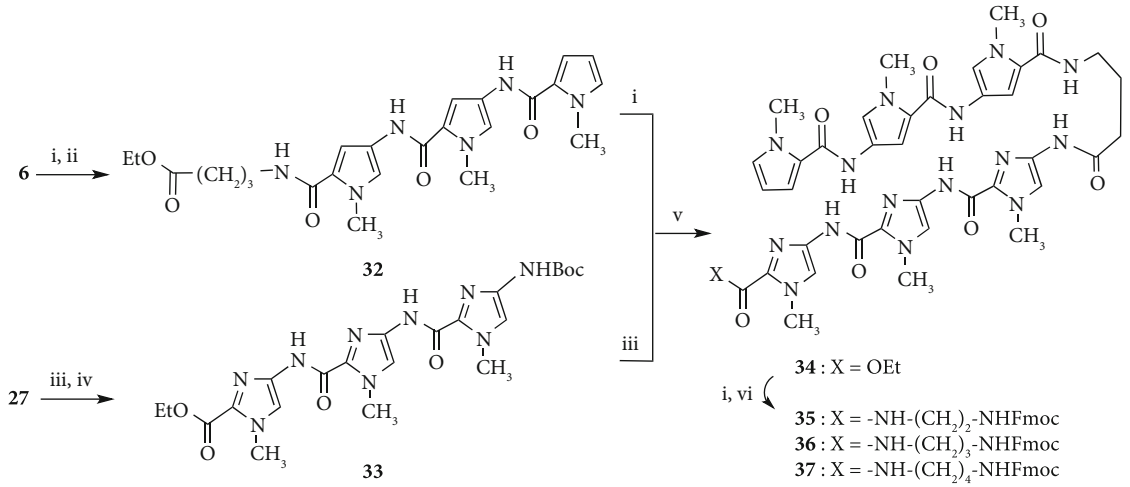
Polyamides containing 1-methylpyrrole (Py) and 1-methylimidazole (Im) can be combined in antiparallel side-by-side dimeric complexes with the minor groove of dsDNA [22–24]. An imidazole ring on one ligand complemented by a pyrrole ring on a second ligand (Im/Py combination) recognizes G-C base pairs, while a Py/Im combination targets C-G base pairs. A Py/Py combination is partially degenerate and binds either A-T or T-A base pairs. Based on the results of **ON 1** and **ON 2** described above, it was expected that imidazole polyamide-oligonucleotide conjugates should possess high binding ability for DNA that includes a guanine (G) base. Next, we synthesized and evaluated imidazole polyamide-oligonucleotide conjugates **ON 3** ($n = 3$, 4, and 5) (5′-d(CG**G**ACCCTGGC)-3′: **G** = Im_3-$NH(CH_2)_n$-G) as model modified oligonucleotides, which form dsDNA with complementary DNA (3′-d(GCCT**GGG**ACCG)-5′) that includes the imidazole polyamide binding sequence (Figure 2).

Conjugates **ON 3** ($n = 3$, 4, and 5) were synthesized by a postsynthetic modification method using imidazole amide trimers (**29**, **30** and **31**) as described above (Scheme 4). Imidazole amide trimers (**29**, **30**, and **31**) bearing 3-aminopropyl, 4-aminobutyl, and 5-aminopentyl linkers, respectively, were synthesized as shown in Scheme 5.

Baird and Dervan have reported the nitration of ethyl 1-methylimidazole-2-carboxylate (**20**) by treatment with concentrated sulfuric acid/90% nitric acid [30]. The reaction mixture was refluxed for 50 min and then quenched by pouring on ice. Ethyl 1-methyl-4-nitroimidazole-2-carboxylate (**21**) was extracted with dichloromethane and recrystallized from 21 : 1 CCl_4/ethanol in 22% yield. We attempted an improvement of the nitration method of **20** using tetramethylammonium nitrate/trifluoroacetic anhydride as a nitrating agent [38]. The reaction was performed at room temperature for 2.5 h. Following the extraction process, the reaction mixture was subjected to silica gel column chromatography using an ethyl acetate/hexane solvent system. Compound **21** and ethyl 1-methyl-5-nitroimidazole-2-carboxylate (**22**) were readily isolated in 59% and 24% yields, respectively.

Compounds **24** and **26** were prepared from **21** according to the procedure described by Baird and Dervan [30]. Imidazole amide trimers (**29**, **30**, and **31**) bearing 3-aminopropyl, 4-aminobutyl, and 5-aminopentyl linkers, respectively, were synthesized via coupling of **24** and **26** to give imidazole amide dimer **27**, deprotection of the Boc group of **27**, coupling with **23** to give imidazole amide trimer **28**, hydrolysis of the ester moiety of **28**, and condensation with 3-aminopropyl, 4-aminobutyl, and 5-aminopentyl linker reagents (**9**, **10** and **11**), respectively. Conjugates **ON 3** ($n = 3$, 4, and 5) were synthesized using imidazole amide trimers (**29**, **30,** and **31**) to yield 21.8, 20.7, and 28.7 A_{260} units, respectively, from **16** ($B = C^{Bz}$) (2 μmol).

Conjugates **ON 3** ($n = 3$, 4, and 5) were converted into modified dsDNAs by annealing with complementary DNA.The stability of modified dsDNAs was investigated by T_m and CD analyses as described above. **ON 3** ($n = 4$) formed more stable dsDNA with complementary DNA ($T_m = 46.5°C$, $\Delta T_m = +5.1°C$) compared with **ON 3** (($n = 3$, $T_m = 43.7°C$, $\Delta T_m = +2.3°C$) and ($n = 5$, $T_m = 45.6°C$, $\Delta T_m = +2.2°C$)) (Table 2). Moreover, it was determined that the imidazole amide moiety of **ON 3** ($n = 4$) was bound in the minor groove of dsDNA, since an induced CD band of the



Scheme 6: Synthesis of pyrrole-γ-imidazole polyamide derivatives. Reagents and conditions: i 1 M NaOH aq./EtOH/pyridine, rt; Dowex 50 WX8 (H^+-form). ii $H_2N(CH_2)_3CO_2Et$, EDCI, DMAP, CH_2Cl_2, rt, **32** (82%). iii AcCl, CH_3OH, rt–40°C. iv **26**, EDCI, DMAP, CH_2Cl_2, rt, **33** (70%). v EDCI, DMAP, CH_2Cl_2, rt, **34** (57%). vi **8**, **9** or **10**, EDCI, DMAP, CH_2Cl_2, rt, **35** (55%); **36** (53%); **37** (27%).

imidazole amide moiety centered at 314 nm was observed (Figure 4(I)) [5, 6, 13]. Although **ON 3** ($n = 4$) formed stable dsDNA with complementary DNA, stabilization of dsDNA by the imidazole amide moiety of **ON 3** ($n = 4$) was lower compared with the pyrrole amide moiety of **ON 2** ($n = 4$) ($\Delta T_m = +16.7$°C, Table 1)]. The DNA sequence recognition ability of **ON 3** ($n = 4$) was investigated using two single-base mismatch DNAs (3′-d(GCCTG**a**GACCG)-5′ (mismatch base, **a**: adenine), which have a mismatch base in the sequence recognized by the imidazole amide moiety, and 3′-d(GCCTGGGAC**t**G)-5′ (mismatch base, **t**: thymine)) (Table 2). **ON 3** ($n = 4$) formed modified dsDNA with two single-base mismatch DNAs and showed the same stabilization ($T_m = 35.4$°C, $\Delta T_m = +1.5$°C, and $T_m = 37.1$°C, $\Delta T_m = +1.6$°C) given the low DNA sequence recognition ability of the imidazole amide moiety.

The MGB polyamide hairpin motifs that link the side-by-side MGB polyamides using the γ-aminobutyric acid (GABA) linker to favor the heterodimeric binding site have been reported by Dervan et al. [29–31]. A code for the binding of MGB polyamide hairpin motifs has been proposed wherein Py/Im, Im/Py, Hp (3-hydroxy-1-methylpyrrole)/Py and Py/Hp combinations recognize C-G, G-C, T-A, and A-T base pairs, respectively [39–43]. MGB polyamide hairpin motifs can recognize many different sequences of dsDNA and bind in the minor groove of dsDNA according to a set of pairing rules. Novopashina et al. and Boutorine et al. have reported that oligonucleotides conjugated with MGB polyamide hairpin motifs to either the 5′- or 3′-end formed stable dsDNA with target DNA by sequence-specific dsDNA stabilization of MGB polyamide hairpin motifs [16–21].

As a further study examining stabilization and recognition abilities, modified DNAs **ON 4** ($n = 2$, 3, and 4) (5′-d(CG**G**ACCCTGGC)-3′: **G** = Py$_3$-γ-Im$_3$-NH$(CH_2)_n$-G) with conjugated pyrrole-imidazole polyamide hairpin motifs, which recognize C-G base pairs via a pyrrole/imidazole combination, at the 2-exocyclic amino group of a guanine base were synthesized and evaluated (Figure 2). Pyrrole-γ-imidazole polyamide derivatives (**35**, **36,** and **37**) were synthesized as shown in Scheme 6. Pyrrole trimer **32** was synthesized via hydrolysis of the ester moiety of pyrrole trimer **6** and coupling with ethyl 4-aminobutanoate. Pyrrole trimer **32** was converted into the carboxylic acid compound. Imidazole trimer **33** was synthesized via removal of the Boc group of imidazole dimer **27** and coupling of imidazole monomer **26**. The Boc group of imidazole trimer **33** was removed and then coupled with the carboxylic acid compound to give pyrrole-imidazole amide **34**. Following hydrolysis of the ester moiety of **34**, 2-aminoethyl, 3-aminopropyl, and 4-aminobutyl linker reagents (**8**, **9,** and **10**) were coupled to give pyrrole-γ-imidazole amide derivatives (**35**, **36,** and **37**), respectively. Using pyrrole-γ-imidazole amide derivatives (**35**, **36,** and **37**), conjugates **ON 4** ($n = 2$, 3, and 4) were synthesized by a postsynthetic modification method to yield 8.4, 11.0, and 11.3 A_{260} units, respectively, from **16** ($B = C^{Bz}$) (1 μmol) (Scheme 4).

The DNA binding ability of conjugates **ON 4** ($n = 2$, 3, and 4) were investigated by T_m analysis (Table 2). It was found that modified dsDNAs comprising **ON 4** ($n = 2$, 3, and 4)/complementary DNA possessed higher stability compared with modified dsDNA comprising **ON 3** ($n = 4$)/complementary DNA ($T_m = 46.5$°C), and that **ON 4** ($n = 2$) formed the most stable dsDNA with complementary DNA ($T_m = 63.3$ °C, $\Delta T_m = +21.9$°C). Furthermore, we attempted an examination of a modified oligonucleotide using the aminomethyl linker, although the modified oligonucleotide was not synthesized by the same synthetic procedure. From the CD spectra, it was determined that the pyrrole-γ-imadazole amide moiety of **ON 4** ($n = 2$) was bound in the minor groove of dsDNA (Figure 4(II)) [5, 6, 13].

The DNA sequence recognition ability of **ON 4** (n =2) was investigated using two single-base mismatch DNAs [3′-d(GCCTG**a**GACCG)-5′ and 3′-d(GCCTGGGAC**t**G)-5′]

(Table 2). It was found that **ON 4** ($n = 2$) possessed higher DNA sequence recognition ability, since the mismatch dsDNA [**ON 4** ($n = 2$)/3′-d(GCCTGGGAC**t**G)-5′, T_m = 55.8°C, ΔT_m = +20.3°C] possessed higher stability compared with the mismatch dsDNA (**ON 4** ($n = 2$)/3′-d(GCCTG**a**-GACCG)-5′, T_m = 41.1°C. ΔT_m = +7.2°C].

4. Conclusions

We synthesized MGB polyamide-oligonucleotide conjugates with linked MGB polyamides at the 2-exocyclic amino group of a guanine base using various aminoalkyl linkers by a post-synthetic modification method and evaluated the binding affinity for complementary DNA that included the MGB polyamide binding sequence by T_m and CD analyses. The MGB polyamides comprised pyrrole polyamides (Py_4- and Py_3-), which possess binding affinity for A-T base pairs, and imidazole (Im_3-) and pyrrole-γ-imidazole (Py_3-γ-Im_3-) polyamide hairpin motifs, which possess binding affinity for C-G base pairs. It was found that the stability of the modified dsDNA was greatly influenced by the linker length. Py_4- and Py_3-oligonucleotide conjugates (**ON 1** ($n = 4$) and **ON 2** ($n = 4$)] containing the 4-aminobutyl linker formed stable dsDNA with complementary DNA via binding of the MGB polyamide moiety. Although Im_3-oligonucleotide conjugate **ON 3** ($n = 4$) containing the 4-aminobutyl linker formed stable dsDNA with complementary DNA, stabilization of dsDNA by the imidazole amide moiety of **ON 3** ($n = 4$) was lower compared with the pyrrole amide moiety of **ON 2** ($n = 4$). The Py_3-γ-Im_3-oligonucleotide conjugates **ON 4** ($n = 2$), which possesses binding affinity for C-G base pairs via a pyrrole/imidazole combination, and contains a 2-aminoethyl linker, showed high binding ability for complementary DNA.

Furthermore, using single-base mismatch DNA, which possess a mismatch base in the pyrrole polyamide binding sequence, and 2-base mismatch DNA, which does not form dsDNA with unmodified DNA, the DNA sequence recognition of conjugates **ON 1** ($n = 4$) and **ON 2** ($n = 4$) was investigated by T_m analysis. **ON 1** ($n = 4$) formed dsDNA with single-base mismatch DNA and resulted in stabilization of the dsDNA. In the case of 2-base mismatch DNA, **ON 1** ($n = 4$) did not form dsDNA and the pyrrole amide moiety displayed no activity. Examination of **ON 2** ($n = 4$), containing a pyrrole amide moiety with short chain length, showed the same results as **ON 1** ($n = 4$). However, from the result of 2-base mismatch DNA, it was thought that modified DNA conjugates did not act on single-base mismatch DNA under conditions where dsDNA (unmodified DNA/single-base mismatch DNA) does not form. On the other hand, the DNA sequence recognition of conjugates **ON 3** ($n = 4$) and **ON 4** ($n = 2$) was investigated by T_m analysis using two single-base mismatch DNAs *in lieu* of complementary DNA. Stabilization of the duplex was observed in dsDNAs comprising **ON 3** ($n = 4$) and single-base mismatch DNA, which possess a mismatch base in the imidazole polyamide binding sequence. **ON 4** ($n = 2$) showed high sequence recognition ability for DNA that included the binding sequence of the pyrrole-γ-imidazole polyamide hairpin motif. A binding code has been proposed for MGB polyamide hairpin motifs whereby Py/Im, Im/Py, Hp/Py, and Py/Hp combinations recognize C-G, G-C, T-A, and A-T base pairs, respectively [39–43]. MGB polyamide hairpin motif-oligonucleotide conjugates may be utilized to act on dsDNA of various sequences.

It is expected that these results could lead to the development of effective gene expression control compounds and novel anticancer and/or antiviral nucleoside drugs.

Data Availability

Supporting data for the results of this report are available in the provided supplementary materials.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplementary Materials

Figure S1: mass, ^{1}H-NMR, and ^{13}C-NMR spectra of the synthesized compounds. Figure S2: UV spectra of MGB amide compounds **4**, **6**, **28,** and **34**. Figure S3: HPLC charts of MGB polyamide-oligonucleotide conjugates. Figure S4: mass spectra of MGB polyamide-oligonucleotide conjugates. Figure S5: UV melting curves of modified dsDNAs. Figure S6: CD spectra of modified dsDNAs. *(Supplementary Materials)*

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