Research Article

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

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Received 8 October 2022; Revised 6 February 2023; Accepted 18 February 2023; Published 14 March 2023

Academic Editor: Ashis Basu

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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 Tm 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-aminooethyl 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’-d(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 (Tm) 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.
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 (Py₄-) 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 polymides (Py₄- and Py₃-) using various aminomethyl linkers in terms of binding affinity for complementary DNA (Figure 2).

In addition to pyrrole polymides, which possess high affinity for A-T base pairs of dsDNA, pyrrole-imidazole polymides, 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 polymides (imidazole polyamide (Im₃-) and pyrrole-γ-imidazole...
polyamide hairpin motif (Py3-γ-Im3-) 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% CH3CN in water (0.01 M TEAA, pH 7) was used. 1H-NMR and 13C-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 = CBz) and 2′-deoxynucleoside 3′-phosphoramidites 17 (B = T, C, A, G) were purchased from Glen...
Research Corporation. 2′-Deoxy-2-fluorinosine 3′-phosphoramide 17 (B = 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-1H-Pyrrole-2-Carbonyl]Amino-1H-Pyrrole-2-Carbonyl]Amino-1H-Pyrrole-2-Carboxylate (6). Compound 2 [10–13] (2.83 g, 9.66 mmol) and 1-methyl-1H-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)carbo dimidazole 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, H2O (150 mL x 3), 5% NaHCO3 aq. (150 mL x 3), and H2O (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 0.5–5% methanol/chloroform solvent system to give 6 (2.79 g, 80% yield) as a slightly brown glass. 1H-NMR (DMSO-d6): 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 x 3), 6.06 (dd, 1H, J = 2.6, 3.9 Hz, Py-H), 3.88 (s, 3H, NCH3), 3.85 (s, 3H, NCH3), 3.84 (s, 3H, NCH3), 3.74 (s, 3H, OCH3); 13C-NMR (DMSO-d6): δ 160.8, 158.6, 158.5, 128.1, 125.8, 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: calc for C17H18N5O2 (M + H)+ 384.1672, found 384.1667; UV (CH3OH): λmax 399, 238 nm, λmin 260, and 222 nm; and ε208 1.8 × 104.

2.2. 2′-[9H-Fluoren-9-yl]Methoxy carbonylamino[Ethanaminium Chloride (8), 4-[9H-Fluoren-9-yl]Methoxy carbonylamino]Butaniminum Chloride (10), and 5-[9H-Fluoren-9-yl]Methoxy carbonylaminopentaniminum Chloride (11). Compounds 8, 10, and 11 were prepared according to the synthetic procedure of 3-[9H-Fluoren-9-yl]methoxy carbonylamino]propanaminium chloride (9) [10–13].

2.3. (9H-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 7 (0.19 g, 20% yield) as a white powder. 1H-NMR (CD3OD): 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, CHCH2 of the Fmoc group), 4.22 (t, 1H, J = 6.6 Hz, CHCH2 of the Fmoc group), 3.32–3.30 (m, 2H, NCH3), and 2.92–2.90 (t, 2H, J = 6.0 Hz, NCH3); 13C-NMR (CD3OD): 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: calc for C17H16NO2(M + H)+ 283.1548, found 283.1455.

Compound 10: (9H-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. 1H-NMR (CD3OD): 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, CHCH2 of the Fmoc group), 4.20 (t, 1H, J = 6.8 Hz, CHCH2 of the Fmoc group), 3.15 (t, 2H, J = 6.6 Hz, NCH3), 2.93 (t, 2H, J = 7.4 Hz, NCH3), and 1.66–1.55 (m, 4H, CH2 × 2); 13C-NMR (DMSO-d6): δ 156.4, 144.1, 140.9, 127.8, 127.3, 125.4, 120.3, 65.5, 46.9, 39.8, 38.6, 26.4, and 24.9; HRMS (ESI-TOF) m/z: calc for C19H23N2O2(M + H)+ 311.1760, found 311.1752.

Compound 11: (9H-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. 1H-NMR (CD3OD): 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, CHCH2 of the Fmoc group), 4.17 (t, 1H, J = 6.8 Hz, CHCH2 of the Fmoc group), 3.08 (t, 2H, J = 6.9 Hz, NCH3), 2.87 (t, 2H, J = 7.6 Hz, NCH3), 1.64–1.60 (m, 2H, CH2), 1.51–1.47 (m, 2H, CH2), and 1.38–1.34 (m, 2H, CH2); 13C-NMR (CD3OD): 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: calc for C20H25N2O2(M + H)+ 325.1916, found 325.1925.
Pyrrrole amide tetramer (481 mg, 1.74 mmol) was dissolved in methanol (8.7 mL), and then 2M 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 Py₃C-carboxylic acid (856 mg, quantitative yield), which was subsequently used without purification. ¹H-NMR (DMSO-d₆): δ 121.31 (s, 1H, COOH), 9.95 (s, 1H, and CONH), 9.91 (s, 1H, and CONH), 9.84 (s, 1H, and CONH), 7.46 (d, 1H, J = 1.9 Hz, and Py-H), 7.70-7.24 (m, 2H, and Py-H × 2), 7.07 (d, 1H, J = 1.8 Hz, and Py-H), 7.05 (d, 1H, J = 1.8 Hz, and Py-H), 6.95 (d, 1H, J = 2.2 Hz, and Py-H), 6.93-6.91 (m, 1H, and Py-H), 6.85 (d, 1H, J = 1.9 Hz, and Py-H), 6.06 (dd, 1H, J = 2.6 Hz, and J = 3.8 Hz, and Py-H), 3.88 (s, 3H, and NCH₃), 3.86 (s, 3H, and NCH₃), 3.85 (s, 3H, and NCH₃), and 3.82 (s, 3H, and NCH₃). Py₃C-carboxylic acid (492 mg, 1.0 mmol) and diisopropylethylamine (190 μL, 1.09 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 2M HCl aq. (100 mL × 3), H₂O (100 mL), 5% NaHCO₃ aq. (100 mL × 3), and H₂O (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. ¹H-NMR (DMSO-d₆): δ 9.99 (s, 1H, and CONH), 9.88 (s, 1H, and CONH), 9.82 (s, 1H, and CONH), 7.98-7.95 (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), 7.41 (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), 7.30-7.24 (m, 3H, CONH, and Py-H × 2), 7.18 (s, 1H, and Py-H), 7.05-7.04 (m, 2H, and Py-H × 2), 6.95-6.94 (m, 1H, and Py-H), 6.93-6.91 (m, 1H, and Py-H), 6.87 (s, 1H, and Py-H), 6.06 (dd, 1H, J = 2.6 Hz, J = 3.9 Hz, and Py-H), 4.29 (d, 2H, J = 6.9 Hz, and CHCH₂ of the Fmoc group), 4.21 (t, 1H, J = 6.9 Hz, and CHCH₂ of the Fmoc group), 3.89 (s, 3H, and NCH₃), 3.86 (s, 3H, and NCH₃), 3.85 (s, 3H, and NCH₃), 3.80 (s, 3H, and NCH₃), 3.18-3.13 (m, 2H, and NHCH₂), 3.01-2.96 (m, 2H, and NHCH₂), 1.52-1.39 (m, 4H, and CH₂ × 2); ¹³C-NMR (DMSO-d₆): 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, 121.11, 122.0, 120.1, 118.4, 117.7, 112.6, 106.6, 104.72, 104.69, 104.2, 65.2, 46.8, 38.1, 36.2, 35.9, 27.0, and 26.7; HRMS (ESI-TOF) m/z: calculated for C₄₃H₄₆N₉O₆ (M + H)⁺ 798.3728, found 798.3732.

2.5. (9H-Fluoren-9-yl)Methyl 5-{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]Aminobutyrylcarbamate (15). Pyrrrole amide triamide (515 mg, 1.34 mmol) was dissolved in methanol (6.7 mL), and then 2M 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 Py₃C-carboxylic acid (495 mg, quantitative yield), which was subsequently used without purification. ¹H-NMR (DMSO-d₆): δ 121.16 (s, 1H, COOH), 9.93 (s, 1H, and CONH), 9.86 (s, 1H, and CONH), 7.46 (d, 1H, J = 1.9 Hz, and Py-H), 7.26 (d, 1H, J = 1.8 Hz, and Py-H), 7.08 (d, 1H, J = 1.8 Hz, and Py-H), 6.95-6.94 (m, 1H, and Py-H), 6.92 (dd, 1H, J = 1.7, and 3.9 Hz, and Py-H), 6.85 (d, 1H, J = 1.9 Hz, and Py-H), 6.06 (dd, 1H, J = 2.5,
and 3.9 Hz Py-H), 3.92 (s, 3H, and NCH3), 3.89 (s, 3H, and NCH3), and 3.86 (s, 3H, and NCH3); 13C-NMR (DMSO-d6): 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 C18H20N5O4 (M + H)+ 370.1515, found 370.1509.

Py3-carboxylic acid (495 mg, 1.34 mmol), 10 (697 mg, 2.01 mmol), and 1-hydroxybenzotriazole (362 mg, 2.68 mmol) were dissolved in DMSO (13.4 mL) and then DCC (553 mg, 2.68 mmol) and N-ethylisopropylamine (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 x 3), H2O (100 mL), 5% NaHCO3 aq. (100 mL), and then the precipitate was removed by filtration. 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. 1H-NMR (DMSO-d6): 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), 7.78 (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 CHCH3 of the Fmoc group), 4.20 (t, 1H, J = 6.8 Hz, and CHCH3 of the Fmoc group), 3.88 (s, 3H, and NCH3), 3.85 (s, 3H, and NCH3), 3.80 (s, 3H, and NCH3), 3.19-3.14 (m, 2H, and NHCH3), 3.02-2.98 (m, 2H, and NHCH3), and 1.33-1.37 (m, 4H, and CH2-x2). 13C-NMR (DMSO-d6): 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, 121.0, 118.4, 117.7, 117.6, 112.6, 106.6, 104.1, 104.1, 65.2, 46.8, 38.1, 36.2, 36.1, 35.9, 27.0, and 26.7; HRMS (ESI-TOF) m/z: calculated for C37H40N5O8 (M + H)+ 662.3091, found 662.3054.

2.6. Ethyl 1-Methyl-4-Nitromidazole-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 nitrite (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% NaHCO3 aq. (300 mL) was added. The mixture was stirred under a slight positive pressure of hydrogen for C3H12N3O2 (M + H)+ 170.0938, found 170.0938.

2.7. 1-Methylimidazole-2-Carboxylic Acid (23). Ethyl 1-methyl-1H-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. 1H-NMR (CD3OD): δ 7.49 (s, 1H, and Im-H), 7.39 (s, 1H, and Im-H), 4.15 (s, 3H, and NCH3); 13C-NMR (CD3OD): δ 158.0, 141.9, 126.6, 119.6, and 37.1; HRMS (ESI-TOF) m/z: calculated for C3H11N2O2 (M + H)+ 127.0508, found 127.0512.

2.8. Ethyl 4-Amino-1-Methylimidazole-2-Carboxylate (24). Ethyl 4-nitro-1H-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. 1H-NMR (CDCl3): δ 6.37 (s, 1H, and Im-H), 4.38 (2H, q, J = 7.1 Hz, and OCH2CH3), 3.92 (3H, s, and NCH3), 2.96 (brs, NH2, and 2H), and 1.40 (3H, t, J = 7.1 Hz, and OCH2CH3); 17C-NMR (CDCl3): δ 159.0, 145.6, 131.6, 109.5, 61.3, 35.7, and 14.5; HRMS (ESI-TOF) m/z: calculated for C3H11N2O2 (M + H)+ 170.0930, found 170.0938.

2.9. Ethyl 4-(tert-Butyloxycarbonyl)-1-Methyl-1H-Imidazole-2-Carboxylate (25). Compound 24 (2.09 g, 12.4 mmol) was dissolved in DMSO (15 mL), and then a solution of di-tert-butylcarbdiurate (5.42 g, 24.8 mmol) in DMSO (10 mL) was added. After stirring for 19 h at room temperature, H2O (50 mL) was added to the solution. The solution was diluted with chloroform (300 mL) and washed with H2O (100 mL x 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 (3:5-1:1 v/v) solvent system to give 25 (2.28 g, 59% yield) and ethyl 1-methyl-5-nitromidazole-2-carboxylate (22) (920 mg, 24% yield).
using an ethyl acetate/hexane (2:3 v/v) solvent system to give 25 (3.34 g, quantitative yield) as a white powder. 1H-NMR (DMSO-d6): δ 9.68 (s, 1H, and CONH), 7.30 (s, 1H, and Im-H), 4.25 (q, 2H, J = 7.2 Hz, and OCH2CH3), 3.88 (s, 3H, and NCH3), 1.44 (s, 9H, and OC(CH3)3), and 1.29 (t, 3H, J = 7.2 Hz, and OCH2CH3); 13C-NMR (DMSO-d6): 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 C12H19N3O4(M + H)+ 293.1365, found 293.1366.

Im2-amine compound (1.30 mmol) and 23 (214 mg, 1.70 mmol) were dissolved in DMF (13 mL), and then EDCl (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 H2O (10 mL), 5% NaHCO3 aq. (100 mL × 2) and H2O (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. 1H-NMR (CDCl3): 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 OCH2CH3), 4.10 (s, 3H, and NCH3), 4.03 (s, 3H, and NCH3), and 1.45 (t, 3H, and J = 7.1 Hz, and OCH2CH3); 13C-NMR (CDCl3): 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 C12H19N3O4(M + H)+ 401.1686, found 401.1682; UV (CH3OH): λmax 311 nm, λmin 236 nm, ε260 1.2 × 104.

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-Carboxylic Acid (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 Im2-carboxylic acid (450 mg, quantitative yield) as a white powder, which was subsequently used without purification. 1H-NMR (CDCl3): 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), 10.45 (1H, s, and CONH), 7.54 (s, 1H, and Im-H), 4.28 (q, 2H, J = 7.1 Hz, and OCH2CH3), 3.99 (s, 3H, and NCH3), 3.94 (s, 3H, and NCH3), and 1.29 (t, 3H, J = 7.1 Hz, and OCH2CH3); 13C-NMR (DMSO-d6): δ 158.1, 154.9, 153.7, 143.0, 139.9, 137.9, 136.9, 136.0, 135.0, 134.3, 127.7, 127.0, 114.6, 113.8, 35.4, 35.2, and 35.1; HRMS (ESI-TOF) m/z: calcd for C15H17N8O4(M + H)+ 373.1373, found 373.1366.

Im3-carboxylic acid (330 mg, 0.90 mmol) and 9 (440 mg, 1.30 mmol) were dissolved in DMF (9 mL), and then N-ethylidiisopropylamine (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 H2O (50 mL × 3). The organic layer was dried over anhydrous magnesium nitrate.
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. 1H-NMR (DMSO-d6): δ 10.05 (s, 1H, and CONH), 9.56 (s, 1H, and CONH), 8.31-8.29 (m, 1H, and Ar-H ×2 of the Fmoc group), 7.69 (d, 2H, J = 7.4 Hz, and 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 CHCH2 of the Fmoc group), 4.22 (t, 1H, J = 6.9 Hz, and CHCH2 of the Fmoc group), 4.02 (s, 3H, and NCH3), 4.00 (s, 3H, and NCH3), 3.96 (s, 3H, and NCH3), 3.24-3.20 (m, 2H, and NHCCH3), 3.05-3.01 (m, 2H, and NHCCH3), and 1.66-1.59 (m, 2H, and CH2); 13C-NMR (CDCl3): 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: calcld for C35H39N10O5 (M + H)+ 651.2792, found 651.2802.

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)Butylicarbamate (30). Im3-carboxylic acid (740 mg, 2.00 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 H2O (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. 1H-NMR (DMSO-d6): 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 CHCH2 of the Fmoc group), 4.19 (t, 1H, J = 6.9 Hz, and CHCH2 of the Fmoc group), 4.01 (s, 3H, and NCH3), 4.00 (s, 3H, and NCH3), 3.95 (s, 3H, and NCH3), 3.23-3.18 (m, 2H, and NHCCH3), 3.00-2.95 (m, 2H, and NHCCH3), 1.54-1.39 (m, 4H, and CH2 × 2), and 1.30-1.24 (m, 2H, and CH2); 13C-NMR(CDCl3): 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: calcld for C35H39N10O5 (M + H)+ 679.3085, found 679.3085.

2.15. (9H-Fluoren-9-yl)Methyl 5-(1-Methyl-4-(1-Methyl-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxamido)Pentylicarbamate (31). Im3-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 H2O (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 32 (1.56 g, 82% yield) as a slightly brown glass. 1H-NMR(CDCl3): 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(CH$_3$)$_3$), 3.98 (s, 3H, and NCH$_3$), 3.93 (s, 3H, and NCH$_3$), 3.90 (s, 3H, and NCH$_3$), 3.43-3.39 (m, 2H, and NHCH$_2$-), 2.41 (t, 2H, J = 7.1 Hz, and COCH$_2$-), 1.95-1.88 (m, 2H, and -CH$_2$C$_2$H$_5$), 1.25 (t, 3H, J = 7.2 Hz, and OCH$_2$CH$_2$-); 13C-NMR(CDC$_3$): δ 173.8, 161.8, 159.5, 158.9, 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$_{2}$H$_3$N$_2$O$_5$ (M + H)$^+$ 483.2356, found 483.2354.

2.17. Ethyl 1-Methyl-4-(1-Methyl-4-(tert-Butoxycarbonylamino)-1H-Imidazole-2-Carboxamido)-1H-Imidazole-2-Carboxylate (34). 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 at 2 h at 40 °C, the reaction solution was concentrated in vacuo to give Im$_3$-amine compound, which was subsequently used without purification.

Im$_3$-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 14 h, the solution was diluted with chloroform (500 mL) and washed with H$_2$O (100 mL), 5% NaHCO$_3$ aq. (100 mL × 2) and H$_2$O (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) solvent system to give Compound 25 (54 mg, 0.104 mmol) as a slightly brown solid, which was subsequently used without purification.

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.

Im$_3$-amine compound (0.104 mmol) and Py$_3$-NH(CH$_2$)$_3$CO$_2$H (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$_2$O (20 mL), 5% NaHCO$_3$ aq. (20 mL × 2) and H$_2$O (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 35 (50 mg, 57% yield) as a slightly brown glass. 1H-NMR (CD$_3$OD): 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$_2$CH$_2$-), 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$_2$CH$_2$-); 13C-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$_9$O$_4$(M + H)$^+$ 455.2043, found 455.2039.
and 14.2; HRMS (ESI-TOF) m/z: calcd for C_{39}H_{36}N_{15}O_{8}(M + H)^+ 852.3654, found 852.3651; UV (CH$_3$OH): $\lambda_{\text{max}}$ 300 nm, $\epsilon_{260}$ 3.0 $\times$ 10$^4$.

2.19. (9H-Fluoren-9-yl)Methyl-(3-(1-Methyl-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)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$_6$): $\delta$ 10.48 (s, 1H, and CONH), 10.22 (s, 1H, and CONH), 9.93 (s, 1H, and CONH), 9.34 (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$_3$), 3.84 (s, 3H, and NCH$_3$), 3.80 (s, 3H, and NCH$_3$), 3.22-3.16 (m, 2H, and NHCH$_2$-), 2.39 (t, 2H, J = 7.2 Hz, and COCH$_2$-), and 1.82-1.79 (m, 2H, and -CH$_2$C$_{2}$H$_5$).

$^{13}$C-NMR(DMSO-d$_6$): $\delta$ 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$_{54}$H$_{58}$N$_{17}$O$_9$(M + H)$^+$ 818.3341, found 824.3347.

Py$_3$-γ-Im$_3$-carboxylic acid (338 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$_2$O (20 mL), 5% NaHCO$_3$ aq. (20 mL $\times$ 2), and H$_2$O (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$_6$): $\delta$ 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), 6.92-6.69 (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$_3$ of the Fmoc group), 4.21 (t, 1H, J = 6.8 Hz, and CHCH$_3$ of the Fmoc group), 3.97 (s, 3H, and NCH$_3$), 3.95 (s, 3H, and NCH$_3$), 3.88 (s, 3H, and NCH$_3$), 3.80 (s, 3H, and NCH$_3$), 3.22-3.20 (m, 4H, and NHCH$_2$-), 2.33-2.31 (m, 2H, and COCH$_2$-), 1.82-1.78 (m, 2H, and -CH$_2$C$_{2}$H$_5$), and 1.56-1.44 (m, 2H, and -CH$_2$C$_{2}$H$_5$); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ 170.0, 161.4, 158.7, 158.5, 158.5, 156.2, 155.6, 154.9, 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, 121.8, 115.9, 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, 35.3, 32.7, 25.3; HRMS (ESI-TOF) m/z: calcd for C$_{99}$H$_{98}$$^{13}$N$_{17}$O$_9$(M + H)$^+$ 1088.4603, found 1088.4604.
2.21. (9H-Fluoren-9-yl)Methyl (4-(1-Methyl-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)Butyl)Carbonate (37). Py$_3$y-Im$_2$carboxylic acid (41 mg, 0.048 mmol) and 10 (28 mg, 0.080 mmol) were dissolved in dichloromethane (3 mL), and then EDCI (20 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$_2$O (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. H-NMR (DMSO-$d_6$): $\delta$ 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$\times 2$), 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, CH$_2$CH$_2$ of the Fmoc group), 4.19 (t, 1H, $J = 7.4$ Hz, CH$_2$Fmoc 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$_3$), 3.27-3.20 (m, 4H, NHCH$_2$CH$_2$-), 3.00-2.99 (m, 2H, and NHCH$_2$CH$_2$-), 2.38-2.35 (m, 2H, and COCH$_2$CH$_2$-), 1.81-1.78 (m, 2H, and -CH$_2$CH$_2$H$_2$), 1.46-1.30 (m, 4H, and -CH$_2$CH$_2$CH$_2$H$_2$); HRMS (ESI-TOF): m/z calcd for C$_{56}$H$_{62}$N$_7$O$_y$(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(GCGTENPE$^\text{J}$$^\text{F}$$^\text{NPE}$$^\text{J}$$^\text{GC}$))$^{-3}$ was synthesized using a syringe-based system. CPG support-bound 2'-deoxynucleoside 16 (B = CBz, 1000 Å, purchased from Applied Biosystems Pty Ltd.) (2 μmol) was treated with 3% w/v Cl$_2$CO$_2$H 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 = CBz) in acetonitrile (0.5 mL) and 0.5 M 1H-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$_2$O/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, B = A, B = G, B = I, and I$^\text{F}$$^\text{NPE}$$^\text{J}$), as described above, the terminal DMTr protecting group of the oligonucleotide was removed by treatment with 3% w/v Cl$_2$CO$_2$H in dichloromethane (1.0 mL × 2) for 1 min, and the CPG support was washed with acetonitrile (2.0 mL × 2). Resultant CPG support-bound oligonucleotide 18 was treated with 0.1 M Fmoc-NH(CH$_2$)$_2$NH-MGB polyamide (12, 13, 14, 15, 29, 30, 31, 35, 36, or 37) in 1:5 Et$_3$N/1,4-dioxane (1.0 mL) at 60°C for 24 h and then washed with acetonitrile (2.0 mL × 2). Following this, the CPG support-bound oligomer was treated with 0.5 M DBU in pyridine (2.0 mL) at room temperature for 12 h and washed with acetonitrile (2.0 mL × 2). The generated oligomer was then cleaved from the CPG support by treatment with conc. NH$_2$OH (1.5 mL × 2) for 2 h at room temperature. The resulting solution was then heated in a sealed vial at 55°C for 6 h. Following evaporation, the residue was dissolved in H$_2$O (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(CCGAATTTGGC))$^{-3}$, $G$ = Py$_3$-NH(CH$_2$)$_n$-$G$ (n = 3 - 5).

ON 1 (n = 3) yields 48.8 A$_{260}$ units from 16 (B = CBz) (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 43.4 A$_{260}$ units from 16 (B = CBz) (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 = CBz) (2 μmol). HRMS (ESI-TOF) m/z calcd for C$_{137}$H$_{173}$N$_{50}$O$_{69}$P$_{10}$ (M + 3H)$^{3+}$ 1960.5314, found 1960.4405.

ON 2 (5'-d(CCGAACCTTGGC))$^{-3}$, $G$ = Py$_3$-NH(CH$_2$)$_n$-$G$ (n = 4).

ON 2 (n = 4) yields 22.5 A$_{260}$ units from 16 (B = CBz) (2 μmol). HRMS (ESI-TOF) m/z calcd for C$_{136}$H$_{165}$N$_{48}$O$_{68}$P$_{10}$ (M + 3H)$^{3+}$ 1265.2768, found 1265.2252.

ON 3 (5'-d(CCGAACCCCTGGC))$^{-3}$, $G$ = Im$_3$-NH(CH$_2$)$_n$-$G$ (n = 3 - 5).
ON 3 \((n = 3)\) yields 21.8 A_{260} units from 16 \((B = C^{Br})\) \((2 \mu\text{mol})\). 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 \((B = C^{Br})\) \((2 \mu\text{mol})\). 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 \((B = C^{Br})\) \((2 \mu\text{mol})\). 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 \((n = 2)\) yields 8.4 A_{260} units from 16 \((B = C^{Br})\) \((1 \mu\text{mol})\). HRMS (ESI-TOF) \(m/z\) calcd for \(C_{142}H_{158}N_{51}O_{67}P_{10}(M + H)^+\) -4165.9555, found 4165.9580. ON 4 \((n = 3)\) yields 11.0 A_{260} units from 16 \((B = C^{Br})\) \((1 \mu\text{mol})\). HRMS (ESI-TOF) \(m/z\) calcd for \(C_{145}H_{162}N_{51}O_{67}P_{10}(M + H)^+\) -4179.9712, found 4179.9858. ON 4 \((n = 4)\) yields 11.3 A_{260} units from 16 \((B = C^{Br})\) \((1 \mu\text{mol})\). HRMS (ESI-TOF) \(m/z\) calcd for \(C_{146}H_{164}N_{51}O_{67}P_{10}(M + H)^+\) -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 Na2EDTA 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 \(\mu\text{M}\) [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 Na2EDTA 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 \(\mu\text{M}\) [5].
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(CGGAATTTGCG)-3′; G = Py-NH(CH₂)₅-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).
sequence (5').

The stability of modified dsDNAs was investigated by Tm analysis (Table 1). ON 1 (n = 3) showed high binding ability for complementary DNA, forming dsDNAs and respective Tm values. Mismatch base: a: adenine; c: cytosine. n.d.: not detected. It was confirmed that ON 1 (n = 3) formed stable dsDNA with complementary DNA.

Conjugates ON 1 (n = 3, 4, and 5) were purified by reversed-phase HPLC to yield 48.8, 34.0, and 31.4 A260 units, respectively, from 16 (B = C6H5) (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 Tm and CD analyses. From the Tm values, it was found that the stability of modified dsDNA was greatly influenced by the linker length (ON 1 (n = 3, Tm = 59.5°C, ΔTm = +25.4°C), (n = 4, Tm = 60.2°C, ΔTm = +26.1°C), and (n = 5, Tm = 52.9°C, ΔTm = +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(GCCTCcAACCG)-5’), which contains a mismatch base in the recognition sequence (5’-d(AATT)-3’/3’-(TTAA)-5’) of the pyrrole amide moiety, and 2-base mismatch DNA (3’-d(GCaTTcAACCG)-5’) which does not form dsDNA with the unmodified DNA, the DNA sequence recognition ability of ON 1 (n = 4) was investigated by Tm analysis (Table 1). ON 1 (n = 4) formed dsDNA with single-base mismatch DNA and displayed stabilization of the dsDNA (Tm = 45.6°C, ΔTm = +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.

<table>
<thead>
<tr>
<th>dsDNAs</th>
<th>Complementary DNA</th>
<th>Mismatch DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3’-d(GCCTTAAACCG)-5’</td>
<td>3’-d(GCCTTcAACCG)-5’</td>
</tr>
<tr>
<td>Unmodified DNA</td>
<td>34.1</td>
<td>22.1</td>
</tr>
<tr>
<td>Modified DNA a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ON 1 (n = 3)</td>
<td>59.5</td>
<td>+25.4 b)</td>
</tr>
<tr>
<td>ON 1 (n = 4)</td>
<td>60.2</td>
<td>+26.1 b)</td>
</tr>
<tr>
<td>ON 1 (n = 5)</td>
<td>52.9</td>
<td>+18.8 b)</td>
</tr>
<tr>
<td>ON 2 (n = 4)</td>
<td>50.8</td>
<td>+16.7 b)</td>
</tr>
</tbody>
</table>

a) modified DNA: 5’-d(CGGAATTTGGC)-3’, ON 1 (G = Py3-NH(CH2)6-G); ON 2 (G = Py3-NH(CH2)6-G). b) dsDNA (4.3 μM) in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na2EDTA. c) Tm [modified dsDNA] - Tm (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].
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. 

\[ \text{ON}_2 \] 

\[ \begin{align*} \text{ON}_3 \quad (n=3) & \quad 43.7 +2.3 \\ \text{ON}_3 \quad (n=4) & \quad 46.5 +5.1 & \quad 35.4 +1.5 \quad 37.1 +1.6 \\ \text{ON}_3 \quad (n=5) & \quad 43.6 +2.2 \\ \text{ON}_4 \quad (n=2) & \quad 63.3 +21.9 \\ \text{ON}_4 \quad (n=3) & \quad 53.6 +12.2 & \quad 41.1 +7.2 \quad 55.8 +20.3 \\ \text{ON}_4 \quad (n=4) & \quad 49.8 +8.4 \end{align*} \]

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. 

\[ \text{ON}_2 \quad (n=4) \quad (5'\cdot\text{d(CGGAATTTGGC})-5') \quad \text{G=Py}_3\text{-NH(CH}_2)_n\text{-G}} \] 

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

**Scheme 5:** Synthesis of imidazole polyamide derivatives. 

Reagents and conditions: i (CH\textsubscript{3})\textsubscript{4}N\textsuperscript{+}NO\textsubscript{3}\textsuperscript{−}, TFAA, CHCl\textsubscript{3}, 0°C-rt, 21 (59%), and 22 (24%). ii 1 M NaOH aq./EtOH/pyridine, rt; Dowex 50WX8 (H\textsuperscript{+}-form), 23 (quant.); 24 (quant.). iii H\textsubscript{2}, 10% Pd/C, 1:1 AcOEt/EtOH, rt, 24 (quant.). iv (Boc)\textsubscript{2}O, DMF, rt, 25 (quant.); (v) DCI, HOBt, DIEA, DMF, rt, 27 (88%). vi AcCl, EtOH/CHCl\textsubscript{3}, rt-40°C. vii 23, EDCI, DMAP, DMF, rt, 28 (84%). viii 9, 10, or 11, DCI, HOBr, 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 \( \Delta T_m \) values.

<table>
<thead>
<tr>
<th>dsDNAs</th>
<th>Complementary DNA</th>
<th>Mismatch DNA\textsuperscript{(d)}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( 3'-\text{d(GCCTGGGACCG)}-5' )</td>
<td>( 3'-\text{d(GCCTGaGACCG)}-5' )</td>
</tr>
<tr>
<td>Unmodified DNA</td>
<td>( T_m )\textsuperscript{(b)}</td>
<td>( \Delta T_m )\textsuperscript{(c)}</td>
</tr>
<tr>
<td>ON 3 ( (n=3) )</td>
<td>41.4</td>
<td>—</td>
</tr>
<tr>
<td>ON 3 ( (n=4) )</td>
<td>43.7 +2.3</td>
<td>—</td>
</tr>
<tr>
<td>ON 3 ( (n=5) )</td>
<td>46.5 +5.1</td>
<td>—</td>
</tr>
<tr>
<td>ON 4 ( (n=2) )</td>
<td>43.6 +2.2</td>
<td>—</td>
</tr>
<tr>
<td>ON 4 ( (n=3) )</td>
<td>63.3 +21.9</td>
<td>—</td>
</tr>
<tr>
<td>ON 4 ( (n=4) )</td>
<td>53.6 +12.2</td>
<td>—</td>
</tr>
</tbody>
</table>

a) modified DNA: \( 5'-\text{d(CGACCTGGCG)}-3' \), ON 3 \( (G=\text{Im}_3\text{-NH(CH}_2)_n\text{-G}) \), ON 4 \( (G=\text{Py}_3\text{-Im}_3\text{-NH(CH}_2)_n\text{-G}) \). b) dsDNA (4.3 \( \mu \text{M} \)) in 10 mM sodium phosphate buffer (pH 7.0) containing 10 mM NaCl and 0.1 mM Na\textsubscript{2}EDTA. c) \( \Delta T_m \textsuperscript{(c)} = T_m \textsuperscript{(modified dsDNA)} - T_m \textsuperscript{(unmodified dsDNA)} \). d) mismatch base, a: adenine, t: thymine.
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\(^{12}\)) \((2 \mu\text{mol})\) (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: \(T_m = 50.8^\circ\text{C}, \Delta T_m = +16.7^\circ\text{C}\)) was lower compared with modified dsDNA (ON 1 (n = 4)/complementary DNA: \(T_m = 60.2^\circ\text{C}, \Delta T_m = +26.1^\circ\text{C}\)). 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 (\(T_m = 39.1^\circ\text{C}, \Delta T_m = +17.0^\circ\text{C}\)). 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-methylpyrrolyl (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) \(5’\text{-d(CGACCCTGGC)}:G = \text{Im}3\text{-NH(CH}_2\text{n-G)}\) and ON 4 \(G = \text{Py}3\text{-Im}3\text{-NH(CH}_2\text{n-G)}\) as model modified oligonucleotides, which form dsDNA with complementary DNA (\(3’\text{-d(GCCTGGGACCG})\)) 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-amino-butyl, 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-amino-butyl, 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-amino-butyl, 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 \((B = C^{12})\) \((2 \mu\text{mol})\).

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 \(\Delta T_m = +5.1^\circ\text{C}\) compared with ON 3 \((n = 3, T_m = 43.7^\circ\text{C}, \Delta T_m = +2.3^\circ\text{C})\) and \((n = 5, T_m = 45.6^\circ\text{C}, \Delta T_m = +2.2^\circ\text{C})\) (Table 2). Moreover, it was determined that the imidazole amide moeity of ON 3 (n = 4) was bound in the minor groove of dsDNA, since an induced CD band of the
imidazole amide moiety centered at 314 nm was observed (Figure 4(II)) [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) (ΔT_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(GCCTGAGCCG)-5′ (mismatch base, a: adenine), which have a mismatch base in the sequence recognized by the imidazole amide moiety, and 3′-d(GCCTGGAGCCG)-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, ΔT_m = +1.5°C, and T_m = 37.1°C, ΔT_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(CGGACCCTGCG)-3′: G = Pyγ-Im3-NH(CH2)n-G) with conjugated pyrrole-imidazole polyamide hairpin motifs, which recognize C-G base pairs via a pyrrole/imidazole amine 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 Å260 units, respectively, from 16 (B = Cβ-) (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, ΔT_m = +21.9°C). Furthermore, we attempted an examination of a modified oligonucleotide using the amidomethyl 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].
It was found that ON 4 \((n = 2)\) possessed higher DNA sequence recognition ability, since the mismatch dsDNA \([\text{ON } 4 (n = 2)/3^\prime -d(GCCTGGAGAgG)-5^\prime \), \(T_m = 55.8^\circ C, \Delta T_m = +20.3^\circ C\)] possessed higher stability compared with the mismatch dsDNA \([\text{ON } 4 (n = 2)/3^\prime -d(GCCTGaGACGCG)-5^\prime \), \(T_m = 41.1^\circ C, \Delta T_m = +7.2^\circ C\)].

4. Conclusions

We synthesized MGB polyamide-oligonucleotide conjugates with linked MGB polymides at the 2-exocyclic amino group of a guanine base using various aminooalkyl 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 polymides comprised pyrrole polymides (Py3- and Py4-), which possess binding affinity for A-T base pairs, and imidazole (Im3-) and pyrrole-\(\gamma\)-imidazole (Py3-\(\gamma\)-Im3-) 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. Py3- and Py4-oligonucleotide conjugates \([\text{ON } 1 (n = 4)\) and ON 2 \((n = 4)\)] containing the 4-aminoethyl linker formed stable dsDNA with complementary DNA via binding of the MGB polyamide moiety. Although Im3-oligonucleotide conjugate ON 3 \((n = 4)\) containing the 4-aminoethyl 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 Py3-\(\gamma\)-Im3-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-\(\gamma\)-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.

Acknowledgments

The authors would like to thank Dr. Yasuo Shida and Mr. Haruhiko Fukaya for the elemental analyses and mass measurements (Analytical Center, Tokyo University of Pharmacy and Life Sciences).

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)

References


