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EXPERIMENTAL METHODS

Synthesis

General materials and methods

The syntheses of compounds DB847 and DB1454 were reported in previous papers.[1,2] The syntheses of compounds DB2429, DB2457, DB2430, DB2432, DB2465, DB1300, DB2325, DB2501, DB2464, and DB2454 are described in Scheme 1 and below. The purity of all compounds were verified by NMR, HRMS and elemental analysis. Melting points (uncorrected) were determined on a Mel-Temp 3.0 melting point apparatus. TLC analysis was performed with silica gel 60 F254 precoated aluminum sheets; UV light was used for detection. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (^{1}H data was obtained at 400MHz and ^{13}C data obtained at 100.6 MHz) using the indicated solvents. Mass spectra was obtained from the Georgia State University Mass Spectrometry Laboratory, Atlanta, GA. Elemental analysis were performed by Atlantic Microlab Inc., Norcross, GA, and are within ±0.4 of the theoretical values. The compounds reported as salts frequently analyzed correctly for fractional moles of water and/or other solvents; in each case ^{1}H NMR spectra was consistent with the analysis. Reactants and solvents were purchase from Aldrich Chemical Co., VWR International, or Combi-Blocks, Inc.

2-[5-(Amidino)-*N*-methylbenzimidazolyl]-5-[4-amidinophenyl] thiophene trihydrochloride (DB2429)

A mixture of the 5-(4-cyanophenyl) thiophene-2-carboxaldehyde[3] (2.13 g, 0.01 mol), 3-amino-4-methylaminobenzonitrile (1.47 g, 0.01 mol) and sodium metabisulfite[4] (2.85 g , 0.015 mol) in 25 mL dry DMF was heated at reflux (under N_2) for 18 h, cooled, diluted with 75 mL water, stirred, filtered, and washed with water. The solid was collected, dried and stirred with a 1:3 mixture of ethanol/ether for 2 h, filtered, washed with ether, and dried under reduced pressure at 80 ^{o}C for 12 h to yield 2.6 g (76%) of the yellow 2-[5-(cyano)-*N*-methylbenzimidazolyl]-5-(4-cyanophenyl)thiophene, mp >300 °C dec.; ^{1}H NMR (DMSO-d_6) δ 8.43 (brs, 1H), 8.32 (brs, 1H), 8.21 (brs, 1H), 8.08 (d, 2H, J = 8 Hz), 7.94 (d, 2H, J = 8 Hz), 7.88 (d, 1H, J = 8.4 Hz), 7.69 (d, 1H, J = 8.4 Hz), 4.17 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 149.5, 141.6, 140.7, 139.6, 138.7, 133.0, 132.9, 127.8, 127.3, 127.0, 125.9, 123.6, 119.8, 118.9, 112.1, 109.9, 104.4, 32.2; The compound was used directly in the next step without further characterization. To a cold and stirred suspension

of the above di-nitrile (0.34 g, 0.001 mol) in 25 ml dry THF was added 6.0 ml, (0.006 mol) 1M $LiN(TMS)_2$[5] in THF, stirring was continued for 24 h at rt, the mixture was cooled, carefully acidified with saturated ethanolic-HCl, the precipitated solid was stirred for 2 h, the solvent removed under reduced pressure, suspended in ether, and filtered. The solid was collected, suspended in10 mL ice water, and basified with 2M NaOH, the yellow precipitate was filtered, washed with water and dried. The solid was suspended in anhydrous ethanol (25 mL), 5 ml of saturated ethanolic HCl was added and the mixture was stirred for 6 h, concentrated under reduced pressure, dry ether was added and the solid was filtered. The solid was dried under reduced pressure at 80 °C for 12 h to yield 0.32 g (64%) of the diamidine tri hydrochloride as yellow solid, mp >300 °C dec.; ^{1}H NMR (DMSO-d_6/50 °C) δ 9.45 (s, 2H), 9.35 (s, 2H), 9.26 (s, 2H), 9.16 (s, 2H), 8.27 (s, 1H), 8.03 (d, 2H, J = 8.8 Hz), 7.98 (d, 2H, J = 8.8 Hz), 7.97 (d, 1H, J = 3.6 Hz), 7.91 (d, 1H, J = 3.6 Hz), 7.91 (d, 1H, J = 8.8 Hz), 7.82 (s, 1H, J = 8.8 Hz), 4.15 (s, 3H); MS: HRMS: Calcd. 376.1391, found: 375.1387 (M^++1); Anal. calcd. for $C_{20}H_{18}N_6S\cdot 3HCl\cdot 1.0H_2O$: C, 47.99; H, 4.63; N, 16.80; Found: C, 48.00; H, 4.78; N, 16.41.

5-(4-Amidinophenyl)-4-methyl-2- [**2 (1-*N*-methyl-5-amidinobenzimidazolyl)] thiophene tri hydrochloride (DB2464)**

Reaction of 4-cyanophenylboronic acid (1.76 g, 0.12 mol), 2-bromo-3-methyl-thiophene-5-carboxaldehyde (2.05 g, 0.01 mol), 12 mL 2M Na_2CO_3 (0.24 mol) and $Pd(PPh_3)_4$ (0.27 g, 2 mol %) in 50 mL dioxane under standard Suzuki conditions[6] yielded yellow 5-(4-cyanophenyl)-4-methylthiophene-2-carboxaldehyde, 1.64 g (72%); mp 159-160 °C; ^{1}H NMR (DMSO-d_6) δ 9.91 (s, 1H), 7.99 (d, 2H, J = 8 Hz), 7.97 (s, 1H), 7.78 (d, 2H, J = 8Hz), 2.37 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 184.7, 144.8, 142.0, 141.9, 138.0, 137.2, 133.4, 129.9, 118.9, 111.7, 15.4. The compound was used directly in the next step without further characterization. A mixture of the 5-(4-cyanophenyl)-3-methyl-2-thiophene aldehyde (2.27 g, 0.01 mol), 3-amino-4-methylaminobenzonitrile 1.47 g (0.01 mol) and sodium metabisulfite (2.85 g, 0.015 mol) in 25 mL dry DMF was heated at reflux (under N_2) for 18 h, cooled, diluted with water was added and filtered. The solid was collected, stirred with a 1:3 mixture of ethanol/ ether for 20 min., filtered, washed with ether and dried under reduced pressure at 70 °C for 12 h to yield 5-(4-cyanophenyl)-3-methyl-2- [2 (1-N-methyl-5-cyano-benzimidazolyl)] thiophene as a yellow solid, 2.7 g (76%), mp >245-6 °C dec. ^{1}H NMR (DMSO-d_6) δ 8.20 (s, 1H), 7.97 (d, 2H, J = 8.4 Hz), 7.87 (d, 1H, J

= 8.4 Hz), 7.85 (s, 1H), 7.79 (d, 2H, *J* = 8.4 Hz), 7.69 (d, 1H, *J* = 8.4 Hz), 4.12 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 149.7, 141.5, 139.2, 138.4, 137.5, 135.8, 132.9, 132.3, 130.1, 128.7, 125.3, 123.0, 119.1, 117.9, 111.4, 110.2, 104.2, 31.6, 14.5; MS: HRMS: Calcd. 355.1017, found 355.1017 (M^++1). The compound was used directly in the next step without further characterization. Reaction of the above *bis*-nitrile (0.354 g, 0.001 mol) in 20 mL dry THF ,6.0 mL, (0.006 mol) 1M LiN(TMS)$_2$ in THF, following the above work-up gave the trihydrochloride as yellow solid 0.33 g (64%), mp >330 °C dec.; ^{1}H NMR (DMSO-d_6) δ 9.61 (s, 2H), 9.49 (s, 2H), 9.40 (s, 2H), 9.27 (s, 2H), 8.25 (s, 1H), 8.01 (d, 2H, *J* = 8 Hz), 7.92 (d, 1H, *J* = 8.8 Hz), 7.90 (s, 1H), 7.83-7.81 (br, d, 3H), 4.14 (s, 6H), 2.44 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 166.3, 165.6, 149.5, 141.4, 140.5, 139.6, 138.7, 136.8, 134.1, 130.5, 129.4, 129.1, 127.6, 122.9, 122.2, 119.5, 111.8, 32.9, 15.7; MS:HRMS Calcd. 389.1548, found 389.1542. Anal. calcd. for $C_{21}H_{20}N_6S\cdot 3HCl\cdot 1.25H_2O$: C, 48.59; H, 4.95; N, 16.20**;** Found: C, 48.67; H, 5.13; N, 16.34.

2-[5-(Amidino)-*N*-methylbenzimidazolyl]-5-[4-amidino phenyl] furan tri hydrochloride (DB2430)

A mixture of the 5-(4-cyanophenyl)furan-2-carboxaldehyde[7] (1.97 g, 0.01 mol), 3-amino-4-methylamino-benzonitrile (1.47 g, 0.01 mol) and sodium metabisulfite (2.85 g, 0.015 mol) in 25 mL dry DMF was heated at reflux (under N_2) for 18 h, cooled, water was added, and filtered. The solid was collected and stirred with a 1:3 mixture of ethanol/ether for 20 min., filtered, washed with ether, and dried under reduced pressure at 70 °C for 12 h to yield 5-(4-cyanophenyl)-2- [2 (1-*N*-methyl-5-cyano-benzimidazolyl)] furan as brown solid, 1.94 g (62%), mp >270 °C dec.; **^{1}**H NMR (DMSO-d_6) δ 8.19 (s, 1H), 8.01 (d, 2H, *J* = 8.8 Hz), 7.93 (d, 2H, *J* = 8.8 Hz), 7.86 (d, 1H, *J* = 8.4Hz), 7.67 (dd, 1H, *J* = 1.2 Hz, *J* = 8.4 Hz), 7.52 (brs, 2H), 4.15 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 153.8, 146.4, 145.5, 142.6, 139.6, 133.6, 133.4, 126.4, 125.1, 124.2, 120.1, 119.1, 116.7, 112.4, 111.7, 110.9, 105.2, 32.5; MS: HRMS: Calcd. 313.1089, 313.1078 (M^++1). The compound was used directly in the next step without further characterization. Reaction of the above *bis*-nitrile (0.312 g, 0.001 mol) in 20 mL dry THF, with 6.0 mL (0.006 mol) 1M Li N(TMS)$_2$ in THF, following the above work-up gave the trihydrochloride as yellow solid, 0.32 g (62%); mp >270 °C dec.; ^{1}H NMR (DMSO-d_6) δ 9.59 (s, 2H), 9.55 (s, 2H), 9.36 (s, 2H), 9.30 (s, 2H), 8.29 (s, 1H), 8.19 (d, 2H, *J* = 8.4 Hz), 8.04 (d, 1H, *J* = 8.4 Hz), 8.02 (d, 2H, *J* = 8.4Hz), 7.87 (d, 1H, *J* = 8.4Hz), 7.75 (d, 1H, *J* = 3.2 Hz), 7.64 (d, 1H, *J* = 3.2 Hz), 4.24 (s, 3H); ^{13}C NMR (DMSO-d_6) δ 165.7,

164.9, 154.4, 144.8, 143.1, 138.9, 138.7, 133.5, 129.2, 127.4, 124.5, 123.3, 122.8, 118.3, 118.1, 111.8, 111.5, 32.7; MS: HRMS: Calcd. 359.1620, found 359.1620 (M^++1); Anal. calcd. for $C_{20}H_{18}N_6O \cdot 3HCl \cdot 3.0H_2O$: C, 46.14; H, 5.23; N, 16.15; Found: C, 46.26; H, 5.01; N, 16.24.

2-(4-amidinophenyl)-6-[2-*N*-methyl-5-amidimo) benzimidazolyl] pyridine trihydrochloride (DB2465).

2-(4-Cyanophenyl)pyridine-6-carboxaldehyde[8] (2.08 g, 0.01 mol), 3-amino-4-methylaminobenzonitrile (1.47 g, 0.01 mol) and sodium metabisulfite (2.85 g, 0.015 mol) in 25 mL dry DMF was heated at reflux (under N_2) for 18 h, cooled, diluted with 75 mL water, stirred, filtered and washed with water. The solid was collected, dried and stirred with 1:3 mixture of ethanol/ether for 2 hr., filtered, washed with ether, and dried under reduced pressur at 80 °C to yield 2.5 g (72%) yellow solid, mp >260 °C dec.; ^{1}H NMR (DMSO-d_6) δ 8.38 (brd, 3H, *J* = 8.0 Hz), 8.31 (s, 1H), 8.27 (d, 1H, *J* = 8.0 Hz), 8.19 (dd, 1H, *J* = 8.0 Hz), 8.03 (d, 2H, *J* = 8.0 Hz), 7.93 (d, 1H, *J* = 8.4 Hz), 7.75 (d, 1H, *J* = 8.4 Hz), 4.37(s, 3H); ^{13}C NMR (DMSO-d_6) δ 153.5, 151.8, 149.1, 142.0, 141.2, 139.5, 138.4, 132.4, 127.1, 125.8, 124.0, 123.96, 121.6, 119.1, 118.0, 111.9, 111.6, 104.4, 32.8; MS: HRMS: Calcd. 336.1249, found 336.1241 (M^++1). The compound was used directly in the next step without further characterization. The above bis-nitrile (0.347 g, 0.001 mol) in 25 mL dry THF , 6.0 mL, (0.006 mol) 1M LiN $(TMS)_2$ in THF, following the above work-up yielded the beige hydrochloride salt 0.31 g (61%); mp >260 °C dec.; ^{1}H NMR (DMSO-d_6) δ 9.54 (s, 2H), 9.43 (s, 2H), 9.32 (s, 2H), 9.17 (s, 2H), 8.44 (d, 2H, *J* = 8.4 Hz), 7.52 (d, 1H, 8.0 Hz), 8.37 (s, 1H), 8.32 (d, 1H, *J* = 8.0 Hz), 8.23 (dd, 1H, *J* = 8.0 Hz), 8.05 (d, 2H, *J* = 8.4 Hz), 7.99 (d, 1H, *J* = 8.4 hz), 7.87 (d, 1H, *J* = 8.0 Hz), 4.42 (9s, 3H); ^{13}C NMR (DMSO-d_6) δ 166.7, 166.0, 154.7, 152.9, 149.8, 143.6, 142.0, 141.2, 139.4, 129.2, 128.9, 127.7, 124.8, 123.2, 122.5, 122.2, 120.6, 112.1, 33.7; MS: HRMS: Calcd. 370.1780, found 370.1769 (M^++1). Anal. calcd. for $C_{21}H_{19}N_7 \cdot 3HCl \cdot 1.75H_2O$: C, 49.54; H, 5.05; N, 19.27; Found: C, 49.88; H, 5.19; N, 19.13.

2, 5-Bis {2-[5-(Amidino)-*N*-methylbenzimidazolyl]}-thiophene tetrahydrochloride (DB1300).

A well stirred solution of 2, 5-thiophene dicarboxaldehyde (0.07 g, 0.0005 mol), 3-amino-4-methylaminobenzamidine hydrochloride (0.20 g, 0.001 mol), and 1,4-benzoquinone (0.108 g, 0.001 mol) in ethanol (30 mL) was heated at reflux for 12 h (under N_2). The reaction mixture was

cooled, concentrated to ca. 10 mL and stirred with 50 mL acetone, filtered, washed with dry ether and dried to yield the dihydrochloride salt. This salt was stirred in 1:1 mixture of hot ethanol/methanol (50 mL), filtered, the volume reduced to ca. 15 mL and acidified with HCl-saturated ethanol (3 mL), stirred for 2 h, concentrated under reduced pressure, dry ether was added and the solid filtered. The solid was washed with ether and dried under reduced press at 80 °C (12 h) to yield a greenish solid, 0.2 g (69%); mp >320 °C dec.; ^{1}H NMR (DMSO-d_6) δ 9.43 (s, 4H), 9.10 (s, 4H), 8.25 (s, 2H), 8.01(s, 2H), 7.91 (d, 2H, *J* = 8.4 Hz), 7.80 (d, 2H, *J* = 8.4 Hz), 4.15 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 165.9,148.7, 141.0, 140.0, 134.1, 130.2, 122.3, 121.9, 119.1, 111.3, 32.2; MS: HRMS: Calcd. 429.1609, found 429.1626 (M^++1); Anal. calcd. for $C_{22}H_{20}N_8S\cdot4HCl\cdot0.5H_2O$: C, 45.43; H, 4.33; N, 19.28; Found: C, 45.56; H, 4.51; N, 18.98.

5, 5'-Bis {[5-(Amidino)-*N*-methylbenzimidazolyl]}-2, 2'-bithiophene tetrahydrochloride (DB2325)

As above, 2, 2'-bihiophene-5, 5'dicarbaldehyde[9] 0.11 g (0.0005 mol), 3-amino-4- methylamino-benzamidine hydrochloride 0.2 g (0.001 mol), and 1,4-benzoquinone 0.108 g (0.001 mol) in ethanol (30 mL) was heated at reflux for 12 h, (under N_2); following the above workup yielded the tetrahydrochloride as a violet grey solid, 0.23 g (68%); mp >320 °C dec.; ^{1}H NMR (DMSO-d_6/75 °C) δ 9.32 (s, 4H), 9.02 (brs, 4H), 8.25 (s, 2H), 7.94 (d, 2H, *J* = 3.6 Hz), 7.91 (d, 2H, *J* = 8.8 Hz), 7.79 (d, 2H, *J* = 8.8 Hz), 7.69 (d, 2H, *J* = 3.6 Hz), 4.16 (s, 6H); MS: HRMS: Calcd. 511.1487, found 511.1416 (M^++1); Anal. calcd. for $C_{26}H_{22}N_8S_2\cdot4HCl\cdot1.0H_2O$: C, 46.42; H, 4.20; N, 16.66; Found: C,46.54; H, 4.42; N, 16.73.

5-(4-carboxamidophenyl)-2-[5-(amidino)-*N*-methylbenzimidazolyl}-thiophene dihydrochloride (DB2432)

5-(4-Carboxamidophenyl)-2-thiophene carbaldehyde[10] (0.231 g, 0.001 mol), 3-amino-4-methylaminobenzamidine hydrochloride (0.2 g, 0.001 mol) and 1,4-benzoquinone (0.108 g, 0.001 mol) in 30 mL ethanol was heated at reflux for 12 h, (under N_2), following the above workup yielded the dihydrochloride a violet grey solid, 0.3 g (64%); mp >300 °C dec.; ^{1}H NMR (DMSO-d_6/75 °C) δ 9.39 (s, 2H), 9.14 (s, 2H), 8.25 (s, 1H), 8.07 (br, 1H), 8.01-7.97 (m, 3H), 7.94 (d, 1H, *J* = 8.4 Hz), 7.88 (d, 2H, *J* = 8. 0Hz), 7.85 (d, 1H, *J* = 4.0 Hz), 7.80 (d, 1H, *J* = 8.4 Hz), 7.43(1H,), 4.16 (s, 3H); ^{13}C NMR (DMSO-d_6/ 75 °C) δ 166.9, 165.9, 149.2, 145.7, 141.3, 139.9, 134.9, 133.8,

130.9, 130.1, 128.0, 125.3, 125.0, 121.8, 121.3, 118.8, 110.6, 31.3; MS: HRMS: Calcd. 376.1232, found 376.1221 (M^++1); Anal. calcd. for $C_{20}H_{17}N_5OS \cdot 2HCl \cdot 1.0H_2O$: C, 51.60; H, 4.55; N, 15.05; Found: C, 51.75; H, 4.73; N, 14.73.

4-(5-(5-(4-Carbamimidoylphenyl)-1-methyl-1H-benzo[d]imidazol-2-yl)thiophen-2-yl)benzimidamide (DB2457)

Sodium metabisulphite (1.9 g, 10 mmol) was added to a solution of 3'-amino-4'-(methylamino)-[1, 1'-biphenyl]-4-carbonitrile[8] (1.11 g, 5 mmol) and 5-(4-cyanophenyl) thiophene-2-carboxaldehyde[3] (1.17 g, 5.5 mmol) in DMF (15 mL) and the mixture was heated at 110 ^oC for 12 h. The reaction mixture was poured into water, filtered and dried. Purification was by crystallization from DMF which yielded a yellow solid (1.2 g, 58 %). mp > 250 ^oC ; ^{1}HNMR (DMSO-d_6) δ 8.07 (br s, 1H), 7.98-7.97 (m, 4H), 7.93-7.91 (m, 6H), 7.80 (d, J = 8.0 Hz, 1H), 7.75 (d, J = 8.0 Hz, 1H), 4.11 (s, 3H); ESI-HRMS: m/z calculated for $C_{26}H_{16}N_4S$: 417.1168, found: 417.1161. The compound was used directly in the next step without further characterization. The above bis-nitrile (0.33 g, 0.8 mmol) was suspended in freshly distilled THF (5 mL), and treated with a 1M LiN $(TMS)_2$ THF solution (4 mL and 4.0 mmol) and the mixture was stirred for two days at room temperature. The reaction mixture was cooled to 0 ^oC and HCl saturated ethanol (3 mL) was carefully added. The mixture was stirred for 24 h, ether was added and the resultant solid was collected by filtration. The diamidine was purified by neutralization with 1*M* sodium hydroxide solution followed by filtration of the resultant solid, washed with water and dried. The free base was stirred with ethanolic HCl for 24 h, ether was added, and the solid which formed was filtered and dried to yield a yellow solid (0.45 g, 95 %), mp > 300 ^oC ; ^{1}HNMR (DMSO-d_6) δ 9.36 (s, 4H), 9.32 (s, 4H), 8.08 (br s, 1H), 8.01-8.00 (m, 5H), 7.97-7.95 (m, 4H), 7.88(d, J = 3.6 Hz, 1H), 7.96 (d, J = 8.0 Hz, 2H), 4.14 (s, 3H); ESI-HRMS: m/z calculated for $C_{26}H_{23}N_6S$: 451.1699, found: 451.1693 (M^++1); Anal. Calcd. for $C_{26}H_{22}N_6S \cdot 3\ HCl \cdot 2\ H_2O$: C, 52.39; H, 4.90; N, 14.10. Found: C, 52.27; H, 5.03; N, 13.81.

4-(5-(5-(4-Carbamimidoylphenyl)-1-methyl-1H-benzo[d]imidazol-2-yl)furan-2-yl)benzimidamide (DB2501)

Sodium metabisulphite (1.9 g, 10 mmol) was added to a solution of 3'-amino-4'-(methylamino)-[1, 1'-biphenyl]-4-carbonitrile[1] (1.11 g, 5 mmol) and 5-(4-cyanophenyl) furan-2-

carboxaldehyde[4] (1.08 g, 5.5 mmol) in DMF (15 mL) and the mixture was heated at 110 ^{o}C for 12 h. The reaction mixture was poured into water, filtered and dried. Purification was by crystallization from DMF which yielded the bis-nitrile as a yellow solid (1.1 g, 55 %). mp > 250 ^{o}C ; ^{1}HNMR (DMSO-d_6) δ 8.08 (d, *J* = 8.0 Hz, 2H), 7.98 (d, *J* = 8, 2H), 7.91 (br s, 1H), 7.75 (d, *J* = 8, 1H), 7.36-7.62 (m, 3H), 7.55 (br s, 1H), 7.47 (br s, 1H), 7.30 (d, *J* = 8.0 Hz, 2H), 4.17 (s, 3H). The compound was used directly in the next step without further characterization. The above bis-nitrile (0.32 g, 0.8 mmol) was suspended in freshly distilled THF (5 mL), and treated with $LiN(TMS)_2$ (4 mL 1M THF, 4.0 mmol). The mixture was stirred for two days at room temperature. The reaction mixture was cooled to 0 ^{o}C and HCl saturated ethanol (3 mL) was carefully added. The mixture was stirred for 24 h, ether was added and the resultant solid was collected by filtration. The diamidine was purified by neutralization with 1*M* sodium hydroxide solution followed by filtration of the resultant solid, washed with water and dried. Thee free base was stirred with ethanolic HCl for 24 h, ether was added, and the solid which formed was filtered and dried to yield a yellow solid (0.4 g, 85 %), mp > 300 ^{o}C ; ^{1}HNMR (DMSO-d_6) δ 9.52 (s, 4H), 9.25 (s, 4H), 8.29 (m, 2H), 8.04-8.02 (m, 3H), 7.96 (d, *J* = 8.8, 2H), 7.84 (br s, 1H), 7.72 (br s, 1H), 7.65 (d, *J* = 8.8 Hz, 2H), 7.34(m, 2H), 4.26 (s, 3H); ESI-HRMS: m/z calculated for $C_{26}H_{23}N_6O$: 435.1961, found: 435.1970 (M^{+}+1); Anal. Calcd. for $C_{26}H_{22}N_6O{\cdot}3.0HCl{\cdot}3.0H_2O$ C, 52.22; H, 5.22; N, 14.05. Found: C, 51.85; H, 4.97; N, 13.73.

4-(1-Methyl-2-(thiophen-2-yl)-1H-benzo[d]imidazol-5-yl) benzimidamide (DB2454)

Sodium metabisulphite (1.9 g, 10 mmol) was added to a solution of 3'-amino-4'-(methylamino)-[1, 1'-biphenyl]-4-carbonitrile (1.11 g, 5 mmol) and thiophene-2-carboxaldehyde (0.6 g, 5.5 mmol) in DMF (15 mL). The mixture was heated at 110 ^{o}C for 12 h. The reaction mixture was poured into water, filtered and dried. Purification was by column chromatography on silica gel using hexanes/ ethyl acetate (70/30, v/v) to yield a white solid (1g, 68 %). mp 201-203 ^{o}C ; ^{1}HNMR (DMSO-d_6) δ 8.06 (br s, 1H), 7.89 (d, *J* = 8.8 Hz, 2H), 7.93 (d, *J* = 8.8 Hz, 2H), 7.85 (m, 2H), 7.78 (d, *J* = 7.6 Hz, 1H), 7.71 (d, *J* = 7.6 Hz, 1H), 7.31 (br s, 1H), 4.08 (s, 3H); ESI-HRMS: m/z calculated for $C_{19}H_{14}N_3S$: 316.0903, found: 316.0896. The compound was used directly in the next step without further characterization. The above nitrile (0.25 g, 0.8 mmol) was suspended in freshly distilled THF (5 mL), and $LiN(TMS)_2$ (2 mL 1M THF, 2.0 mmol) was added, and the mixture was stirred for two days at room temperature. The reaction mixture was cooled to 0 ^{o}C

and HCl saturated ethanol (3 mL) was carefully added. The mixture was stirred for 24 h, diluted with ether and the resultant solid was collected by filtration. The amidine was purified by neutralization with 1M sodium hydroxide solution followed by filtration of the resultant solid, washed with water and dried. The free base was stirred with ethanolic HCl for 24 h, ether was added, and the solid which formed was filtered and dried to give a yellow solid (0.29 g, 89 %). mp > 300 ^{o}C ; ^{1}HNMR (DMSO-d_6) δ 9.49 (s, 2H), 9.26 (s, 2H), 8.11-8.09 (m, 3H), 8.05 (d, J = 8.4, 2H), 8 (d, J = 8.4, 2H), 7.98 (br s, 1H), 8.05 (d, J = 8.4, 1H), 7.42 (t, J = 4.4, 1H), 4.15 (s, 3H); ESI-HRMS: m/z calculated for $C_{19}H_{17}N_4S$: 333.1168, found: 333.1164 (M^++1); Anal. Calcd. for $C_{19}H_{16}N_4S{\cdot}2.0HCl{\cdot}0.75\ H_2O$: C, 54.48; H, 4.69; N, 13.37. Found: C, 54.44; H, 4.77; N, 13.24.

Biophysical Experimental

Materials

In the DNA thermal melting (T_m), circular dichroism (CD), fluorescence emission spectroscopy and electrospray ionization mass spectrometry (ESI-MS) experiments, the following hairpin oligomer sequences were used with the hairpin loop underlined.

AAATTT [5'-CCAAATTTGCCTCTGCAAATTTGG-3'],

AAA**G**TTT [5'-CCAAA**G**TTTGCTCTCAAACTTTGG-3'],

AAA**GC**TTT [5'-CCAAA**GC**TTTGCTCTCAAAGCTTTGG-3'],

AAT**G**AAT [5'-CCAAT**G**AATGCTCTCATTCATTGG-3'],

ATA**G**TAT [5'-CCATA**G**TATGCTCTCATACTATGG-3'],

AATT**G**AATT [5'-CCAATT**G**AATTGCTCTCAATTCAATTGG-3'],

In the electrospray ionization mass spectrometry (ESI-MS) experiment, another sequence, AAA**G**TTT [5'-GCCAAA**G**TTTGCCTCTGCAAACTTTGGC-3'], was used in order to avoid the same molecular weight as other test sequences in the ESI-MS competition experiments. In SPR experiments, 5′-biotin labeled hairpin DNA oligomers were used including AAA**I**TTT [5'-Biotin-CCAAA**I**TTTGCTCTCAAACTTTGG-3']. All DNA oligomers were obtained from Integrated

DNA Technologies, Inc. (IDT, Coralville, IA) with reverse-phased HPLC purification and mass spectrometry characterization.

The buffer used in T_m, CD and fluorescence emission spectroscopy experiments was 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4. The biosensor-surface plasmon resonance (SPR) experiments were performed in filtered, degassed 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4 with 0.05% (v/v) surfactant P20. 150 mM ammonium acetate buffer with 5% or 10% MeOH was used in ESI-MS experiments.

UV-vis Thermal Melting (T_m)

DNA thermal melting experiments were performed on a Cary 300 Bio UV-vis spectrophotometer (Varian). The concentration of each hairpin DNA sequence was 3 μM in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) using 1 cm quartz cuvettes. The mixture solutions of DNA and ligands were tested with the ratio of 2:1 [ligand/DNA]. All samples were annealed prior to each experiment. The spectrophotometer was set at 260 nm with a 0.5 °C/min increase beginning at 25 °C, which is below the DNA melting temperature and ending above it at 95 °C. The absorbance of the buffer was subtracted, and a graph of normalized absorbance *vs.* temperature was created using KaleidaGraph 4.0 software. The ΔT_m values were calculated using a combination of the derivative function and estimation from the normalized graphs.

Biosensor-Surface Plasmon Resonance (SPR)

SPR measurements were performed with a four-channel Biacore T200 optical biosensor system (GE Healthcare, Inc., Piscataway, NJ). A streptavidin-derivatized (SA) CM5 sensor chip was prepared for use by conditioning with a series of 180 s injections of 1 M NaCl in 50 mM NaOH (activation buffer) followed by extensive washing with HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% P20, pH 7.4). Biotinylated-DNA samples (AAATTT,

AAA**G**TTT, AAA**GC**TTT, AAA**I**TTT, ATA**G**TAT and AATT**G**AATT hairpins) of 25-30 nM were prepared in HBS buffer and immobilized on the flow cell surface by noncovalent capture as previously described.[11,12] Flow cell 1 was left blank as a reference, while flow cells 2−4 were immobilized separately by manual injection of biotinylated-DNA stock solutions (flow rate of 1 μL/min) until the desired amount of DNA response units (RU) was obtained (300−330 RU). Ligand solutions were prepared with degassed and filtered Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4 with 0.05% (v/v) surfactant P20) by serial dilutions from a concentrated stock solution. Typically, a series of different ligand concentrations (2 nM to 500 nM) were injected over the DNA sensor chip at a flow rate of 100 μL/min until a constant steady-state response was obtained (180 s), followed by buffer flow for ligand dissociation (600−1800 s). After each cycle, the sensor chip surface was regenerated with a 10 mM glycine solution (pH 2.5) for 30 s followed by multiple buffer injections to yield a stable baseline for the following cycles. RU_{obs} was plotted as a function of free ligand concentration (C_{free}), and the equilibrium binding constants (K_A) were determined either with a one-site binding model ($K_2 = 0$) or with a two-site model, where $r = (RU_{obs}/RU_{max})$ represents the moles of bound compound/mol of DNA hairpin duplex and K_1 and K_2 are macroscopic binding constants.

$$r = (K_1 \cdot C_{free} + 2K_1 \cdot K_2 \cdot C_{free}^2)/(1 + K_1 \cdot C_{free} + K_1 \cdot K_2 \cdot C_{free}^2) \quad (1)$$

RU_{max} can be used as a fitting parameter, and the obtained value compared to the predicted maximal response per bound ligand to independently evaluate the stoichiometry.[13] Kinetic analyses were performed by globally fitting the binding results for the entire concentration series using a standard 1:1 kinetic model with integrated mass transport-limited binding parameters as described previously.[14]

Fluorescence Emission Spectroscopy

Fluorescence spectra were recorded on a Cary Eclipse Spectrophotometer, with excitation and emission slit width fixed at [2.5, 5 nm] depending on the concentrations of ligands. The free compound solutions at different concentrations were prepared in Tris-HCl buffer (50 mM Tris−HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4), and DNA sequence (AAATTT, AAA**G**TTT, and AAA**GC**TTT) aliquots were added from a concentrated stock. Each titration spectra were collected after allowing an incubation time of 10 min. DB2429 was excited at 350 nm and DB2430 was excited at 355 nm based on molecular absorbance from UV-vis spectroscopy. Emission spectra of DB2429 and DB2430 were monitored from 380 to 620 nm wavelength. All the fluorescence titrations were performed at 25 °C.

Circular Dichroism (CD)

Circular dichroism experiments were performed on a Jasco J-810 CD spectrometer in 1 cm quartz cuvette at 25 °C. A buffer scan as a baseline was collected first in the same cuvette and subtracted from the scan of following samples. The hairpin DNA sequence AAA**G**TTT (5 µM) in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) was added to the cuvette prior to the titration experiments and then the compound (DB2429, DB847 or DB2432) was added to the DNA solution and incubated for 10 min to achieve equilibrium binding for the DNA-ligand complex formation. For each titration point, four spectra were averaged from 500 to 220 nm wavelength with scan speed 50 nm/min, with a response time of 1s. Baseline subtracted graphs were created using the KaleidaGraph 4.0 software.

Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray Ionization Mass Spectrometry (ESI-MS) analyses were performed on a Waters Q-TOF micro-spectrometer (Waters Corporate, Milford, MA) equipped with an electrospray ionization source (ESI) in negative ion mode. DNA sequences for ESI-MS experiments were

dialyzed in 150 mM ammonium acetate buffer (pH 6.7) at 4 ºC with 3x buffer exchange. Test samples were prepared in 150 mM ammonium acetate with 5% or 10% v/v methanol at pH 6.7 and introduced into the ion source through direct infusion. The instrument parameters were as follows: capillary voltage of 3000 V, sample cone voltage of 20 V, extraction cone voltage of 1.0 V, desolvation temperature of 90 °C, and source temperature of 100 °C. Nitrogen was used as nebulizing and drying gas. Samples were injected at a flow rate of 5 µL/min and run for ~10 min to reach stabilization. Scanned peaks were analyzed over *m*/*z* 300-2500 range and the final 2 min averaged. Analyses and interpretation of the deconvoluted spectra were performed using MassLynx 4.1 software.

Structural Calculations

Molecular torsional angle map calculations of the compounds were performed in the *Spartan'14* software.[15] The "constrain dihedral" command was used with selected compounds to restrain four atoms to define the middle rotation bond and two terminal bonds which formed the dihedral as the calculation targets. The calculation range was set from 0° to 180° through 20 steps. Calculations were carried out with the energy profile method at ground state with density functional B3LYP/6-31G* in vacuum. After the calculations, the relative energy (rel. E) (kJ/mol) was displayed to a spreadsheet. The torsional angle map can be created with the constraint of the torsional angle as the X axis and rel. E as the Y axis by using KaleidaGraph 4.0 software.

Molecular Docking

DB2429 and DB2457 were optimized at the B3LYP/6-31*G level of theory using *Spartan'14* software.[15] The minimized ligand was assigned Gasteiger−Huckel charges by using the Autodock vina 4.02[16] software package. The *ds*-[(5′-CCAAA**G**TTTG-3') (5'-CAAACTTTGG-3')] DNA sequence was generated from the biopolymer-build DNA double helix module from the Tripos

SYBYL-X1.2 software package.[17] The duplex DNA sequence was docked with the minimized structure of DB2429 and DB2457 using AutoDock vina 4.02.[16] The center of the macromolecule is the grid center with a grid size of 68 Å×70 Å×120 Å and a grid spacing of 0.375 Å. Docking runs were performed using the Lamarckian genetic algorithm (LGA) with no modifications of docking parameters. LGA was used because of the existence of rotatable bonds in the ligands and to evaluate the correct conjugate DNA conformation to reproduce various experimental ligand−DNA complex structures. Initially, we used a population of random individuals (population size of 150), a maximal number of 2500000 energy evaluations, and a mutation rate of 0.02 fs. Two hundred independent flexible docking runs were conducted for each ligand, and then the lowest-energy dock conformation obtained from the flexible docking was resubmitted for rigid docking to remove the internal energy of the ligand (steric clashes) and retain the hydrogen bonding interaction with *ds*-DNA bases.

Figures and Tables

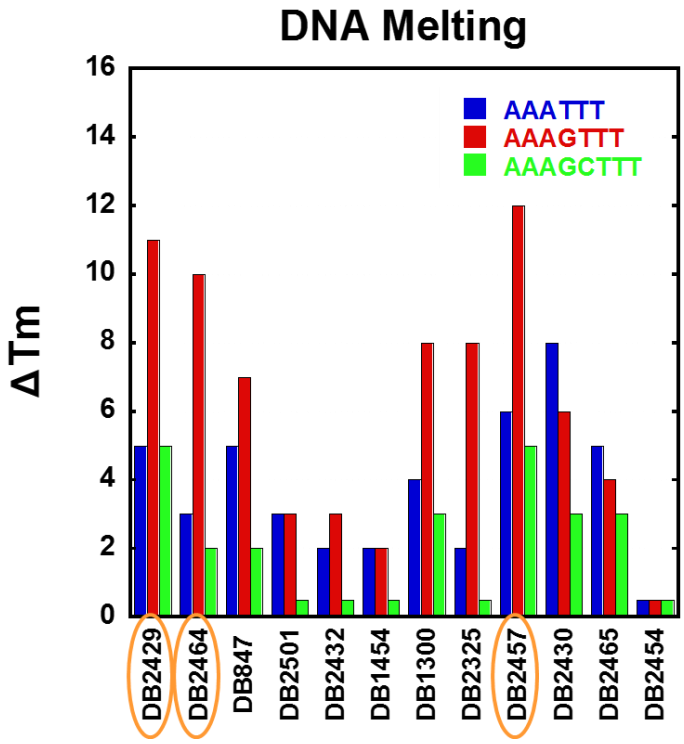


Figure S1. Comparisons of thermal melting results (ΔT_m, °C) of DB2429 and its analogues with pure AT and mixed DNA Sequences. ΔT_m = T_m (the complex) - T_m (the free DNA). 3 μM DNA sequences were studied in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) with the ratio of 2:1 [ligand/DNA]. The strong AAAGTTT binding affinity values are marked with orange circles and an average of two independent experiments with a reproducibility of ±0.5 °C.

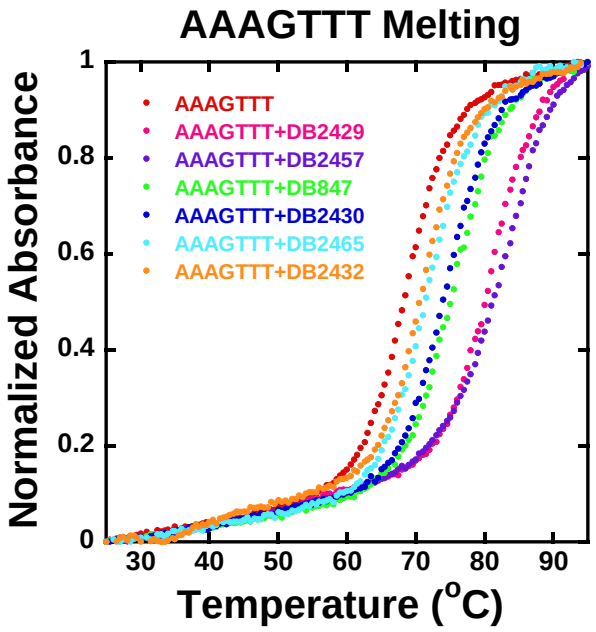


Figure S2. UV-vis thermal melting curves of DNA hairpin AAAGTTT binding with DB2429 and its analogues. 3 μM AAAGTTT sequence were studied in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) with the ratio of 2:1 [ligand/DNA].

Table S1. Thermal melting studies (ΔT_m, °C) of different AT flanking sequences of G•C bp with DB2429 and DB2457[a]

Ligands \ Sequences	AAT G AAT	ATA G TAT	AATT G AATT	AAA G TTT
DB2429	6	6	5	11
DB2457	8	6	5	12

[a] ΔT_m = T_m (the complex) - T_m (the free DNA). 3 μM DNA sequence were studied Tri-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) with the ratio of 2:1 [ligand/DNA]. An average of two independent experiments with a reproducibility of ±0.5 °C.

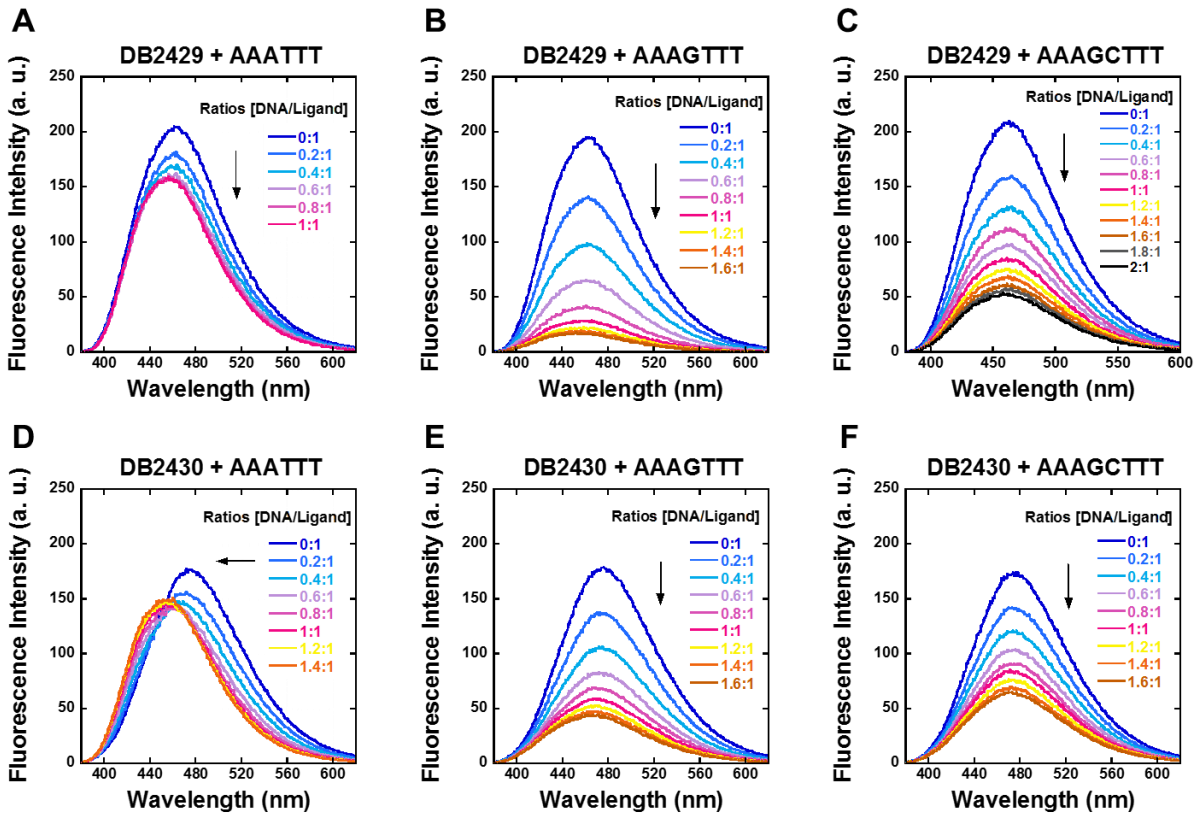


Figure S3. (A, B, C) Fluorescence emission spectra for 500 nM DB2429 titrated with sequences AAATTT, AAAGTTT and AAAGCTTT in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) at 25 °C. The excitation wavelength of DB2429 is 350 nm. (D, E, F) Fluorescence emission spectra for 500 nM DB2430 titrated with sequences AAATTT, AAAGTTT and AAAGCTTT in Tris-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4) at 25 °C. The excitation wavelength of DB2430 is 355 nm. All the data were collected in the emission range of 380~620 nm and the slit width is [2.5 nm, 5 nm]. Arrows indicate changes in fluorescence following the titration of DNA sequence.

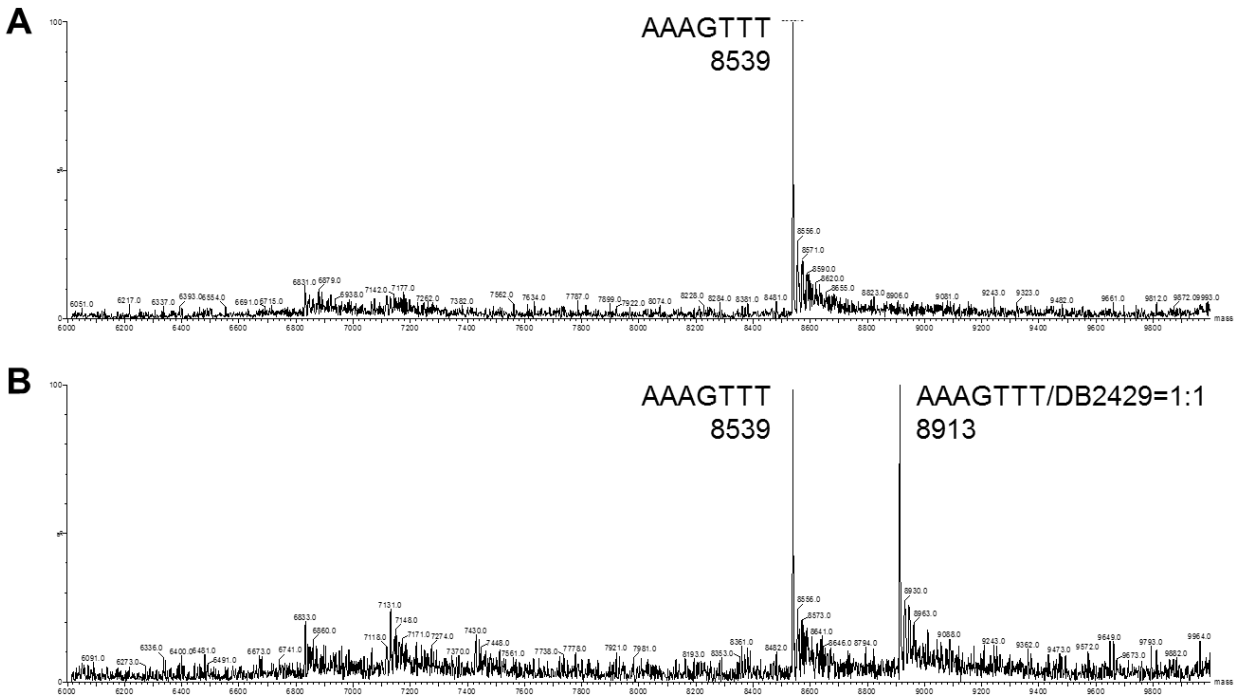
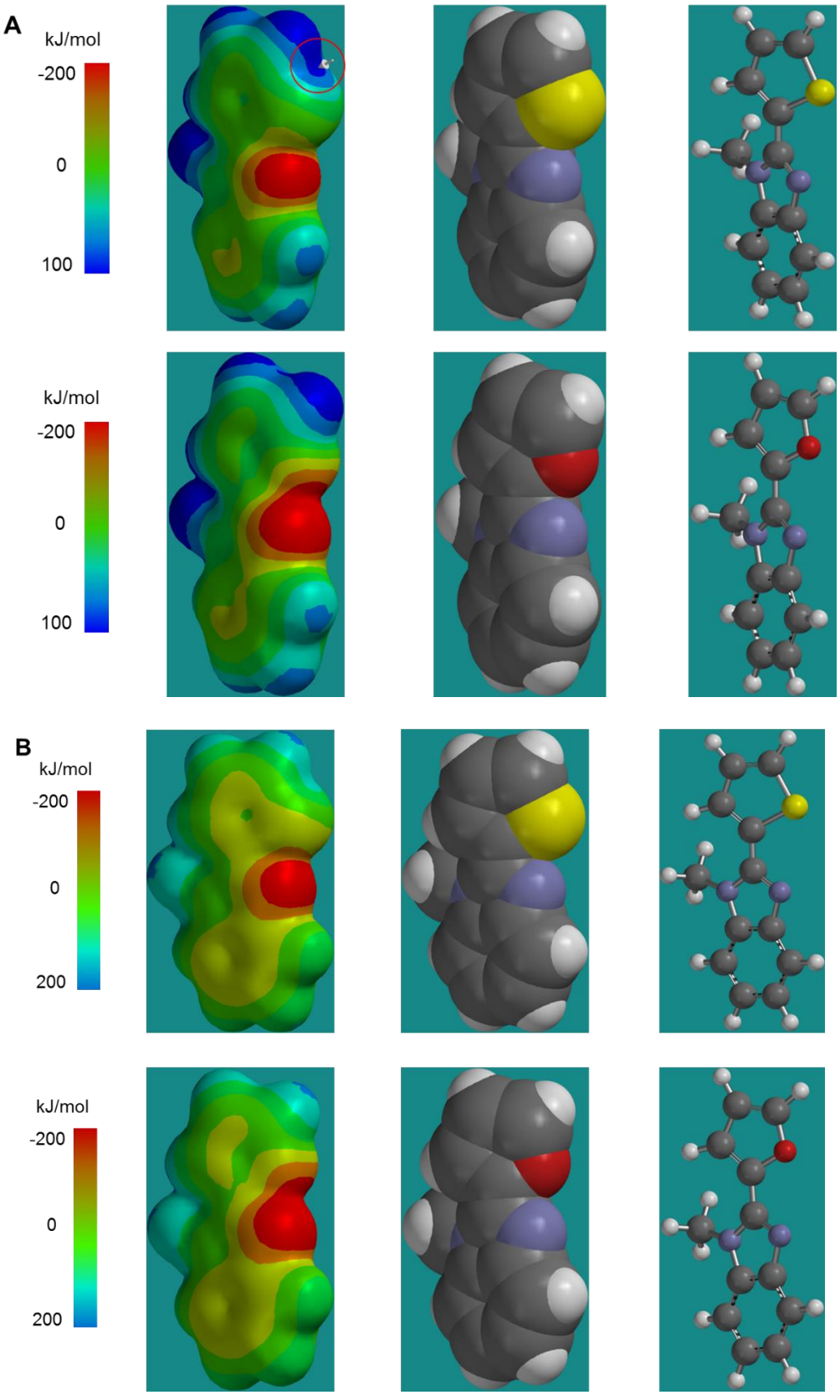


Figure S4. Comparison of ESI−MS spectra of DNA sequence AAAGTTT with DB2429 at a 4:1 ratio [ligand/DNA]. (A) AAAGTTT DNA sequence. (B) AAAGTTT with 4:1 ratio of DB2429. 10 μM DNA each was tested in ammonium acetate buffer (150 mM ammonium acetate with 10% methanol (v/v), pH 6.8). The ESI-MS results shown here were deconvoluted spectra.


A
kJ/mol
-200
0
100
kJ/mol
-200
0
100
B
kJ/mol
-200
0
200
kJ/mol
-200
0
200

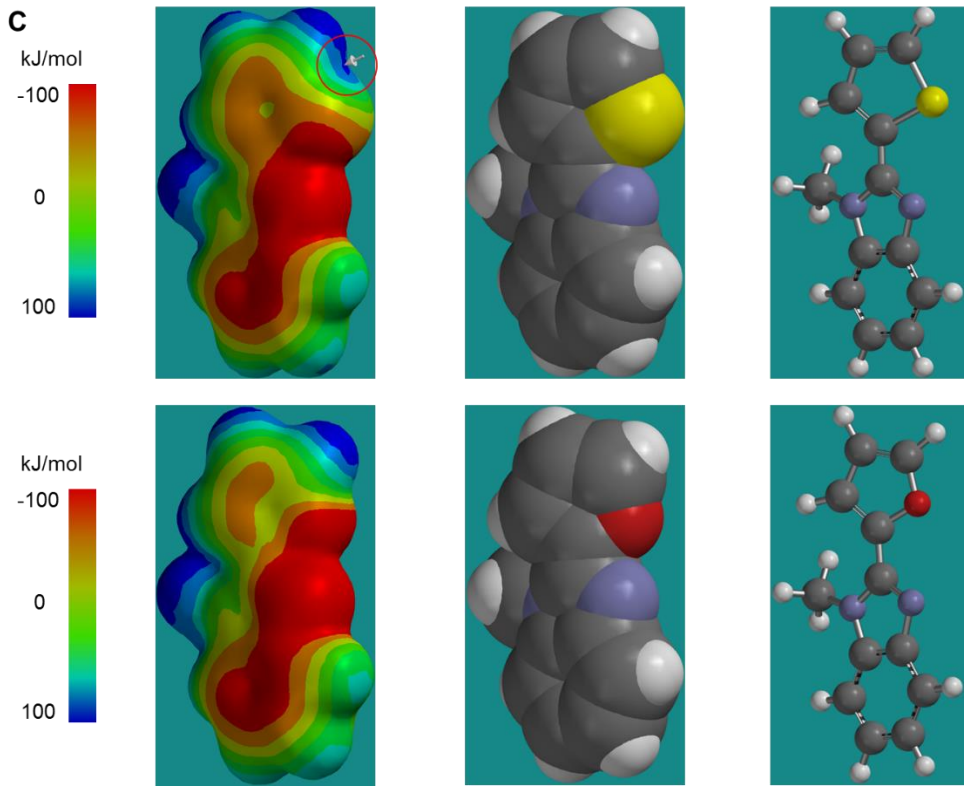


Figure S5. Molecular electrostatic potential maps (at B3LYP/6-31G* level of theory) of *N*-MeBI-thiophene module (upper) and *N*-MeBI-furan module (below). (A) The property range is set as [-200, 100] kJ/mol. From left to right is electrostatic potential maps (left), space filling model (middle), ball and spoke model (right). The σ-hole of C–S bond is shown in the red circle. (B) The property range is set as [-200, 200] kJ/mol. From left to right is electrostatic potential maps (left), space filling model (middle), ball and spoke model (right). (C) The property range is set as [-100, 100] kJ/mol. From left to right is electrostatic potential maps (left), space filling model (middle), ball and spoke model (right). The σ-hole of C–S bond is shown in the red circle.

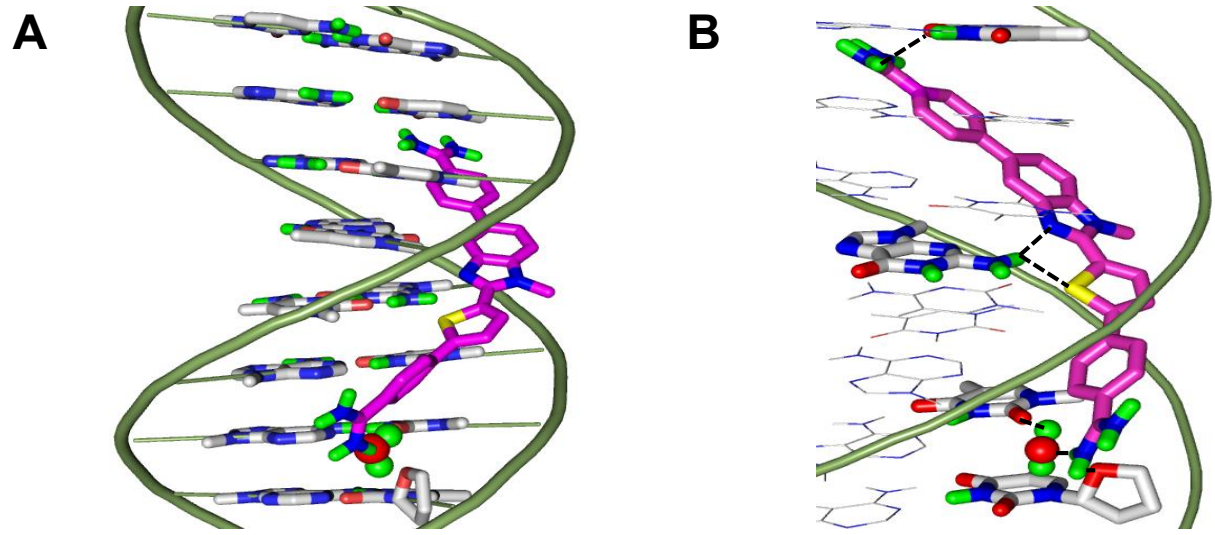


Figure S6. Minor groove views of docked conformations of (A) DB2457 with the AAA**G**TTT sequence. The compounds are shown as stick model and colored by atom type (magenta for carbon, blue for nitrogen, yellow for thiophene sulfur, and green for amidine hydrogen). The DNA backbone is represented as a tube form in light green, DNA nucleobases are represented by sticks colored by atom type (gray for carbon, blue for nitrogen, red for oxygen, green for polar hydrogen). A water molecule in the sphere is shown at the bottom amidine of each compound. (B) Important H-bond interactions between compounds and DNA nucleobases (shown with a black dashed line), the thiophene S and *N*-MeBI N form bifurcated hydrogen bonds with the exocyclic G6-NH in the minor groove, the bottom amidine group forms H-bonds with the thymine (T) carbonyl group through the water molecule, and the top amidine group forms a direct hydrogen bond with the thymine (T) carbonyl group.

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