Supporting Information

Multivalent dendritic DNA aptamer molecules for the enhancement of therapeutic effect

Yusuke Kawamoto[†,*], You Wu[†], Soyoung Park[‡], Kumi Hidaka[§], Hiroshi Sugiyama[§,‖], Yuki Takahashi[†], and Yoshinobu Takakura[†,*]

[†]Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimoadachicho, Sakyo-ku, Kyoto 606-8501, Japan
[‡]Immunology Frontier Research Center, Osaka University, Yamadaoka, Suita, Osaka 565-0871, Japan
[§]Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan
[‖]Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida-ushinomiyacho, Sakyo-ku, Kyoto 606-8501, Japan

Corresponding Authors
(Y.K.) y.kawamoto@pharm.kyoto-u.ac.jp
(Y.T.) takakura@pharm.kyoto-u.ac.jp

Table of Contents

Materials and Methods

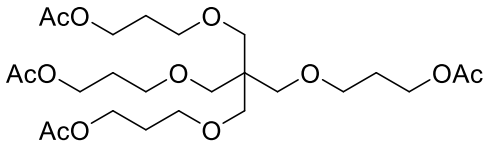
1, General Materials, Methods and Equipment

Except otherwise noted, reagents were purchased from Sigma-Aldrich, Tokyo Chemical Industry (TCI), Nacalai Tesque (Nacalai), and FUJIFILM Wako Pure Chemicals (Wako) and solvents were from Nacalai and Wako. They were used without further purification. Chloroform-*d* ($CDCl_3$) including 0.03% tetramethylsilane (TMS) was purchased from Eurisotop. Reversed phase high performance liquid chromatography (HPLC) analyses and purifications were performed using LC-20AD pump and SPD-20A detector (Shimadzu) equipped with COSMOSIL 5C18 AR-II 10 ID × 150 mm reversed phase column (Nacalai) at 40 °C. Nuclear magnetic resonance (NMR) spectra were obtained on JNM-ECZ600R (JEOL). Electrospray ionization mass spectra (ESI MS) were obtained on Thermo Exactive plus spectrometer (Thermo Fischer Scientific). Matrix assisted laser desorption/ionization coupled to time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Microflex-KSII (Bruker). When performing analytical polyacrylamide gel electrophoresis (PAGE), gels were stained with SYBR Gold (Thermo Fischer Scientific) and then visualized by use of WSE-6100 LuminoGraph I (ATTO). Concentrations of oligonucleotides used in this study were determined by measuring the absorbance at λ = 260 nm, and each DNA solution was stored in distilled water (Otsuka Pharmaceutical) at -20 °C until use.

2, Synthesis of Phosphoramidites

All reactions were monitored with Silicagel 70 F_{254} TLC plate (Wako) using iodine (I_2) vapor and UV irradiation for the detection, and column chromatography was carried out using Wakosil 60, 64–210 μm (Wako).

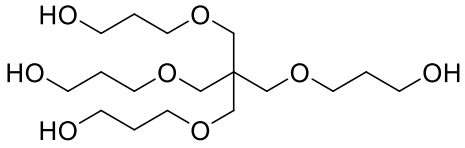
Synthesis of Compound 1



To pentaerythritol tetraallyl ether (5.00 g, 16.9 mmol, 1.0 eq.) was added dropwise 0.5 M of 9-borabicyclo[3.3.1]nonane (9-BBN) in tetrahydrofuran (THF) (135 mL, 67.5 mmol, 4.0 eq.), then the reaction was carried out at room temperature for 18 h under argon atmosphere. The reaction was opened to the air, and then water (10 mL) was added. After cooling down to 0 °C, 3 M of aqueous sodium hydroxide (NaOH)

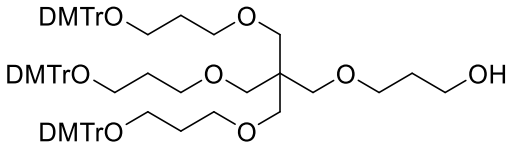
solution (67 mL, 20.2 mmol, 1.2 eq.) was added. Subsequently, 40 mL of 30% of aqueous hydrogen peroxide (H_2O_2) was carefully added at 0 °C. The resulting mixture was stirred for 1 h at room temperature, then precipitation was removed by filtration. The aqueous phase was extracted three times with THF. After concentration of organic phase *in vacuo*, the resulting residue was dissolved in pyridine (25 mL) and acetic anhydride (25 mL). The reaction mixture was stirred at room temperature for 3 h. After concentration of the mixture *in vacuo*, the resulting crude residue was purified by column chromatography with hexane/ethyl acetate (EtOAc) (4:1 to 2:3 gradient) to afford compound **1** (7.59 g, 14.1 mmol, 83%) as colorless liquid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.13 (t, *J* = 6.6 Hz, 8H), 3.44 (t, *J* = 6.1 Hz, 8H), 3.35 (s, 8H), 2.05 (s, 12H), 1.86 (quint, *J* = 6.4 Hz, 8H). ^{13}C NMR (151 MHz, $CDCl_3$) d 171.14, 69.62, 67.60, 61.83, 45.32, 28.87, 21.00, ESI-HR MS calculated for $C_{25}H_{44}NaO_{12}^+$ $[M+Na]^+$ 559.2725, found 559.2721.

Synthesis of Compound 2



Compound **1** (2.39 g, 4.45 mmol*)* was dissolved in 28% sodium methoxide methanol solution and then stirred at room temperature for 1 h. After concentration of the mixture *in vacuo*, the resulting residue was purified by column chromatography with dichloromethane (DCM)/methanol (MeOH) (12:1) to afford compound **2** (1.67 g, 4.53 mmol, quant) as colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.75 (t, *J* = 5.5 Hz, 8H), 3.59 (t, *J* = 5.5 Hz, 8H), 3.39 (s, 8H), 3.27 (brs, 4H), 1.80 (quint, *J* = 5.5 Hz, 8H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 70.90, 70.38, 61.37, 44.60, 31.63. ESI-HR MS calculated for $C_{17}H_{36}O_8^+$ $[M+H]^+$ 369.2483, found 369.2487.

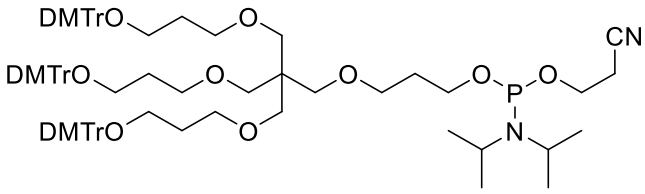
Synthesis of Compound 3



Compound **2** (306 mg, 0.830 mmol, 1.0 eq.) was dissolved in anhydrous pyridine 3.0 mL. To the solution were added 4,4′-dimethoxytrityl chloride (702 mg, 2.07 mmol, 2.5 eq.) and 4-dimethylaminopyridine (DMAP) (10 mg, 0.083 mmol, 0.10

eq.), and then the reaction was carried out at room temperature for 3 h under argon atmosphere. The resulting mixture was partitioned between EtOAc and saturated aqueous sodium bicarbonate ($NaHCO_3$) solution. The aqueous phase was extracted three times with EtOAc. Combined organic phase was washed with brine, dried over sodium sulfate (Na_2SO_4) and then evaporated to dryness. The resulting crude residue was purified by column chromatography with hexane/EtOAc (4:1 to 1:1 gradient) to afford compound **3** as a pale yellow solid (275 mg, 0.216 mmol, 26%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.41 (d, J = 7.6 Hz, 6H), 7.29 (d, J = 8.9 Hz, 12H), 7.24 (d, J = 7.7 Hz, 4H), 7.16 (t, J = 7.3 Hz, 4H), 6.79 (d, J = 8.9 Hz, 13H), 3.75 (s, 18H), 3.67 (q, J = 5.5 Hz, 2H), 3.40 (m, 8H), 3.23 (s, 2H), 3.21 (s, 6H), 3.07 (t, J = 6.5 Hz, 6H), 2.73 (t, J = 5.6 Hz, 2H), 1.79 (quint, J = 6.5 Hz, 6H), 1.70 (quint, J = 5.5 Hz, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 158.29, 145.28, 136.63, 129.99, 128.19, 127.70, 126.58, 112.96, 85.74, 71.25, 71.02, 69.99, 68.63, 62.50, 60.62, 55.16, 45.00, 31.75, 30.20. ESI-HR MS calculated for $C_{80}H_{90}NaO_{14}^+$ $[M+Na]^+$ 1297.6223, found 1297.6221.

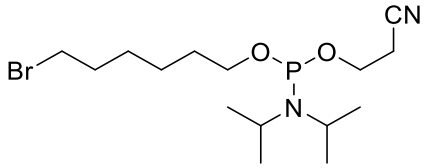
Synthesis of Compound 4



Compound **3** (301 mg, 0.236 mmol, 1.0 eq.) was dissolved in anhydrous DCM 2.0 mL. *N, N*-Diisopropylethylamine (DIPEA) (123 μL, 0.708 mmol, 3.0 eq.) was added to the solution. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (105 μL, 0.471 mmol, 2.0 eq.) was subsequently added to the reaction solution at 0 °C, and the mixture was stirred at room temperature for 1 h under argon atmosphere. The reaction mixture was diluted by saturated aqueous $NaHCO_3$ solution and the aqueous layer was extracted three times with DCM. The combined organic layer was dried over Na_2SO_4 and then evaporated under reduced pressure. The resulting residue was purified by column chromatography with hexane/EtOAc (3:1 to 2:1 gradient) containing 1% triethylamine (TEA) to afford compound **4** (307 mg, 0.208 mmol, 88%) as a pale yellow residue. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.40 (d, *J* = 7.8 Hz, 6H), 7.29 (d, *J* = 8.7 Hz, 12H), 7.25 – 7.22 (m, 4H), 7.16 (t, *J* = 7.4 Hz, 4H), 6.79 (d, *J* = 8.6 Hz, 13H), 3.78 – 3.72 (m, 21H), 3.68 – 3.53 (m, 3H), 3.39 (t, *J* = 6.5 Hz, 6H), 3.32 (t, *J* = 6.2 Hz, 2H), 3.22 (s, 6H), 3.20 (s, 2H), 3.06 (t, *J* = 6.4 Hz, 6H), 2.57 (td, *J* = 6.6, 3.2 Hz, 2H), 1.78 (quint, *J* = 6.1 Hz, 8H), 1.17 (d, *J* = 6.8 Hz, 6H), 1.14 (d, *J* = 6.9 Hz, 6H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 158.26, 145.26, 136.62, 129.96, 128.18, 127.68, 126.56, 117.65,

112.94, 85.71, 69.79, 69.66, 68.53, 67.79, 60.87, 60.75, 60.63, 58.38, 58.25, 55.15, 45.21, 42.99, 42.88, 31.44, 31.40, 30.26, 24.67, 24.61, 24.56, 20.31, 20.27. ^{31}P NMR (243 MHz, $CDCl_3$) δ 147.99. ESI-HR MS calculated for $C_{89}H_{108}N_2O_{15}P^+$ $[M+H]^+$ 1475.7482, found 1475.7478.

Synthesis of Compound 6



6-Bromo-1-hexanol (200 mg, 1.11 mmol, 1.0 eq.) was dissolved in anhydrous DCM 3.5 mL. Then DIPEA (577 μL, 3.31 mmol, 3.0 eq.) was added to the solution. Subsequently, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (493 μL, 2.21 mmol, 2.0 eq.) was added to the reaction solution at 0 °C, and the mixture was stirred at room temperature for 1 h under argon atmosphere. The reaction mixture was diluted by saturated aqueous $NaHCO_3$ solution and the aqueous layer was extracted three times with DCM. The combined organic layer was washed with saturated aqueous $NaHCO_3$ solution, dried over Na_2SO_4 and evaporated under reduced pressure. The resulting residue was purified by column chromatography with hexane/EtOAc (100:3) containing 1% triethylamine (TEA) to afford compound **6** (294 mg, 0.772 mmol, 70%) as colorless liquid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.89 – 3.77 (m, 2H), 3.70 – 3.57 (m, 4H), 3.41 (t, *J* = 6.7 Hz, 2H), 2.65 (t, *J* = 6.5 Hz, 2H), 1.87 (quint, *J* = 7.0 Hz, 2H), 1.63 (quint, *J* = 6.9 Hz, 2H), 1.48 – 1.44 (m, 2H), 1.42 – 1.38 (m, 2H), 1.20 – 1.17 (m, 12H). ^{13}C NMR (151 MHz, $CDCl_3$) δ.117.66, 63.52, 63.40, 58.40, 58.20, 43.01, 42.92, 33.88, 32.71, 31.00, 30.96, 27.84, 25.15, 24.67, 24.63, 24.60, 24.55, 20.39, 20.35. ^{31}P NMR (243 MHz, $CDCl_3$) δ 147.91. ESI-HR MS calculated for $C_{15}H_{31}BrN_2O_2P^+$ $[M+H]^+$ 381.1301, found 381.1304.

3, Solid-phase Synthesis of Azide-modified Oligonucleotides

For solid-phase synthesis of oligonucleotides, phosphoramidites and controlled pore glasses were purchased from Glen Research, and other reagents were from Wako, Sigma-Aldrich and ChemGenes. Using M-2-MX DNA/RNA synthesizer (Nihon Techno Service), solid-phase synthesis was performed at 1 μmol scale on a Glen UnySupport™ 1000 controlled pore glass (CPG) for non-labeled oligonucleotides or 3'-(6-FAM) CPG for FAM-labelled ones. Phosphoramidites of natural bases were dissolved in anhydrous acetonitrile (ACN) to prepare 100 mg/ mL (~0.15 M for thymine and ~0.12 M for guanine respectively) solution. Trebler phosphoramidite **4**

(synthesized following Scheme S1) and doubler phosphoramidite **5** (Figure S2, purchased from Glen Research) were used to incorporate branching structures. For the incorporation of azide group at the 5'-end, 6-bromohexyl phosphoramidite **6** (synthesized following Scheme S2) was used. Phosphoramidites **4**-**6** were dissolved in anhydrous ACN to prepare 0.13~0.14 M solution. Coupling of thymine and guanine was performed for 10 sec × 2. Coupling of phosphoramidites **4**-**6** was performed for 10 min × 3.

In order to replace the bromide group at the 5'-end, oligonucleotide-loaded CPG equivalent to 1 μmol bromide group was suspended with a solution of sodium azide (100 mM) and sodium iodine (100 mM) dissolved in anhydrous *N*,*N*-dimethylformamide (DMF) 1.6-1.7 mL. The suspension was shaken at 65 °C for 90 min. After removal of solution by centrifugation, CPG was washed with DMF × 2 and ACN × 2.

Cleavage from the solid support and deprotection of oligonucleotides was accomplished by treating the solid support with 1.0 mL of 50:50 of methylamine ($MeNH_2$) in 40 wt. % in water and ammonia (NH_3) in 28 wt. % in water at room temperature for 15 min and then at 65 °C for 15 min. After concentration using a speed vac, oligonucleotides were purified using reversed phase HPLC at a flow rate of 3.0 mL/min. Solvent A consisted of 50 mM triethylamine acetic acid (TEAA) pH 7.0 dissolved in water and solvent B was ACN. A gradient from 5% to 75% of solvent B over 30 min was employed for the purification of 3-Branched N_3 and 3-Branched N_3-FAM. A gradient from 20% to 40% of solvent B over 20 min was employed for the purification of 6-Branched N_3, 6-Branched N_3-FAM, 9-Branched N_3 and 9-Branched N_3-FAM. After purification with HPLC followed by lyophilization, products were characterized by ESI MS. The yields and ESI MS results are summarized in Table S2.

4, Synthesis of 3'-DBCO-modified Oligonucleotides

Solid-phase synthesis of 3'-amine-modified oligonucleotides was performed as describe above using 3'-PT-Amino-Modifier C3 CPG at 1 μmol scale. After cleavage, deprotection and concentration, 3'-amine-modified oligonucleotides were isolated by ethanol precipitation as follows: (i) 0.1 volume of 3 M sodium acetate solution and 2 volumes of ice-cold ethanol were subsequently added to the solution, and the solution was mixed before incubation at -20 °C for 1 h. (ii) The precipitate was pelleted by centrifugation at 4 °C for 30 min, and the supernatant was removed. (iii) To wash the pellet, 70% ice cold ethanol was added, and centrifugation was performed again at 4 °C for 10 min, followed by removal of the supernatant. (iv) The pellet was dried at room temperature. For the ethanol precipitation procedure, each centrifugation step was

performed at the maximum speed of the centrifugal machines (approximately 15,000 rpm). The obtained 3'-amine-modified oligonucleotides were used for the next DBCO-conjugation reaction without further purification.

DIPEA (7.50 μL, 43.1 μmol) was added to the solution of 3'-amine-modified crude oligonucleotides dissolved in distilled water 228 μL. Then 20 eq. of DBCO-NHS (BroadPharm) dissolved in DMF 228 μL was added, followed by mixing at room temperature overnight. The reaction mixture was subsequently purified using reversed phase HPLC (5% to 75% ACN in 50 mM TEAA (pH 7.0) buffer over 30 min at flow rate of 3.0 mL/min). After purification with HPLC followed by lyophilization, products (AS1411-DBCO or cAS1411-DBCO) were confirmed by ESI MS. The yields and ESI MS results are summarized in Table S2.

5, Synthesis of DNA Dendrons

General Procedure

A solution of 5'-azide-modified oligonucleotides (1.0 eq.) in water was added to the solution of 3'-DBCO-modified oligonucleotides (≥2.5 eq. per azide group) in water. The resulting solution was mixed with 3 M aqueous sodium chloride (NaCl) solution to make a final concentration of 1.5 M NaCl. The reaction mixture was shaken at 37 °C overnight. After confirming completion of the reaction by using denaturing polyacrylamide gel electrophoresis (PAGE), 2 volumes of ice-cold ethanol were added to the mixture and crude DNA was isolated by ethanol precipitation as described previously. The product was purified by denaturing PAGE run on 8% gel containing 7 M urea. The product band visualized by UV shadowing was excised, pulverized, and extracted in distilled water by shaking at 37 °C overnight. The gels were removed by filtration through empty Micro Bio-Spin columns (Bio-Rad Laboratories). The purified DNAs were isolated from the supernatant by ethanol precipitation as described previously and were characterized by denaturing PAGE run on 8% gel containing 7 M urea (Figure 1B). 3- and 6-branched DNA dendrons could also be analyzed by MALDI-TOF MS (Table S3) following our previous method.[1]

Synthesis of 3-Branched AS1411

A 56.0 μL solution of 3-Branched N_3 (60.0 nmol, 1.0 eq.) and the 359 μL solution of AS1411-DBCO (450 nmol, 7.5 eq.) were used. The obtained product was 52.8 nmol (88% yield for this step, 19% from solid support).

Synthesis of 3-Branched AS1411-FAM

A 15.8 μL solution of 3-Branched N_3-FAM (20.0 nmol, 1.0 eq.) and the 89.2 μL solution of AS1411-DBCO (150 nmol, 7.5 eq.) were used. The obtained product was 13.1 nmol (66% yield for this step, 17% from solid support).

Synthesis of 6-Branched AS1411

A 46.4 μL solution of 6-Branched N_3 (30.0 nmol, 1.0 eq.) and 258 μL solution of AS1411-DBCO (450 nmol, 15 eq.) were used. The obtained product was 26.3 nmol (88% yield for this step, 9.7% from solid support).

Synthesis of 6-Branched AS1411- FAM

A 47.6 μL solution of 6-Branched N_3-FAM (18.0 nmol, 15 eq.) and the 196 μL solution of AS1411-DBCO (270 nmol, 15 eq.) were used. The obtained product was 12.9 nmol (72% yield for this step, 8.6% from solid support).

Synthesis of 9-Branched AS1411

A 41.0 μL solution of 9-Branched N_3 (20.0 nmol, 1.0 eq.) and the 410 μL solution of AS1411-DBCO (450 nmol, 22.5 eq.) were used. The obtained product was 15.0 nmol (75% yield for this step, 7.5% from solid support).

Synthesis of 9-Branched AS1411-FAM

A 48.9 μL solution of 9-Branched N_3-FAM (20.0 nmol, 1.0 eq.) and 268 μL solution of AS1411-DBCO (450 nmol, 22.5 eq.) were used. The obtained product was 7.25 nmol (36% yield for this step, 4.3% from solid support).

Synthesis of 9-Branched cAS1411-FAM

A 17.0 μL solution of 9-Branched N_3-FAM (8.00 nmol, 1.0 eq.) and 213 μL solution of cAS1411-DBCO (200 nmol, 25 eq.) were used. The obtained product was 4.20 nmol (52% yield for this step, 6.2% from solid support).

6, CD Spectroscopy

DNAs equivalent to 27 μM of the aptamer moiety (27 μM, 9.0 μM, 6.0 μM and 3.0 μM for AS1411, 3-Branched AS1411, 6-Branched AS1411 and 9-Branched AS1411 respectively) were prepared in a 10 mM Tris-HCl (pH 7.5) and 100 mM KCl buffer. Prior to analysis, samples were annealed by heating to 95°C for 5 min, followed by gradual cooling to room temperature. The CD spectra were measured on a J-820

spectropolarimeter (JASCO) in a 0.2 cm path length quartz cell. All experiments were performed in triplicate and their average was used. The CD spectra were corrected by subtracting the blank and then smoothed. Molar ellipticity based on the amount of aptamer moiety in solution (27 μM) is shown in Figure 2A.

7, UV-Melting Analysis of G-quadruplex

DNAs equivalent to 5.0 μM of the aptamer moiety (5.0 μM, 1.7 μM, 0.83 μM and 0.56 μM for AS1411, 3-Branched AS1411, 6-Branched AS1411 and 9-Branched AS1411 respectively) were prepared in 10 mM Tris-HCl (pH 7.5) buffer containing 100 mM KCl. Prior to analysis, DNA samples were annealed by heating to 95°C for 5 min, followed by gradual cooling to room temperature. Thermal denaturation analyses of G-quadruplex were performed on a UV-1800 spectrometer (Shimadzu) having a cell path length of 1 cm equipped with a TMSPC-8 temperature controller. Denaturation profiles were recorded by measuring the absorbance at λ = 295 nm from 25 to 95 °C at a rate of 0.5 °C/min. Reported melting temperature (T_m) values shown in Figure 2B are the averages of three individual measurements, and representative melting profiles are shown in Figure S5. T_m values were calculated by average method built in Tm Analysis System (Shimadzu).

8, Fluorescence Spectroscopy

The steady-state fluorescence spectra of 0.50 μM of FAM-labelled DNAs dissolved in distilled water were measured at the room temperature on a Fluoromax-4 (Horiba) setting both the excitation and the emission slits at 5 nm. The excitation wavelength was at 488 nm.

9. Atomic Force Microscopy (AFM)

AFM imaging was carried out using Nano Live Vision (RIBM) with a USC-F12-K0.15-10 cantilever (NanoWorld). Total concentrations of DNA dendrons were adjusted to ~50 nM by addition of observation buffer (20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 1 mM EDTA). The sample solution was deposited onto a mica surface coated with 0.1% (3-aminopropyl)triethoxysilane and incubated for 5 min at room temperature. The mica surface was then washed gently with the same buffer. AFM observation was performed at room temperature.

10, Serum Stability of DNA

Solution of DNA was mixed with non-heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) to make reaction mixtures containing 100 ng/μL DNA and

50% FBS. Immediately the mixtures were distributed equally to PCR tubes (10 μL/tube) and then incubated at 37 °C to proceed the nuclease digestion. After 0, 2, 4, 8, 12, or 24 h of incubation, 20 μL/tube of 0.5 M ethylenediaminetetraacetic acid (EDTA) solution was mixed to stop the digestion and then stored at -20 °C until use. The concentration of polyacrylamide gels and the running time were changed depending on the type of DNA to see full length DNA and the entire degradation products (15% polyacrylamide gel including 7 M urea and 1 h running for AS1411, 8% polyacrylamide gel including 7 M urea and 50 min running for 3-Branched AS1411 and 6-Branched AS1411, 8% polyacrylamide gel including 7 M urea and 2 h running for 9-Branched AS1411). Gels were stained with SYBR Gold (Thermo Fischer Scientific) and then visualized by use of WSE-6100 LuminoGraph I (ATTO). The exposure time to take gel images was 0.10 sec. The representative bands images were shown in Figure S7a. Quantification of bands intensity was carried out with ImageJ. Since the fluorescence intensity of DNA bands stained by SYBR Gold varies depending on the number of SYBR Gold molecules bound to DNA, the band intensity can vary among the four types of DNA. Therefore, we utilized the ratio of band intensity of remaining full-length DNA to that at 0 h to assess the serum stability of DNA. The average ratio of three independent experiments were shown in Figure S7b.

11, Cell Culturing

HeLa cells (RIKEN Cell Bank) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Shimadzu Diagnostics) supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Nacalai). Following ATCC's guideline, MCF-10A cells (ATCC) were cultured using the Mammary Epithelial Basal Medium (MEBM) and associated additives included in MEGM™ Mammary Epithelial Cell Growth Medium BulletKit (Lonza) except GA-1000 solution. Further, MEBM was supplemented with 100 ng/mL cholera toxin (Sigma-Aldrich). Cells were cultured at 37 °C in humidified air containing 5% CO_2.

12, Cellular Uptake of DNA

Cells were seeded on 24-well plates at a density of 5.0×10^4 cells/well and cultured for 1 day prior to the treatment. The medium was replaced with Opti-MEM (Thermo Fisher Scientific) containing 100 nM FAM-labelled oligonucleotides. The cells were incubated for 4 h at 37 °C in humidified air containing 5% CO_2. Then cells were washed with phosphate buffered saline (PBS) and then harvested by trypsinization. The cells were suspended with PBS and then applied to the flow cytometry

(FACSCalibur, BD Biosciences) to evaluate fluorescence intensity of the cells by counting 10,000 events. Relative mean fluorescence intensity (MFI) values normalized to medium were shown. All experiments were performed in triplicate.

13, Endocytosis Inhibition Study

HeLa cells were seeded on 48-well plates at a density of 5.0×10^4 cells/well and cultured for 1 day prior to the treatment. For competition experiments to show the nucleolin-mediated uptake, the cells were pretreated with 20 μM AS1411 or nonactive cAS1411 for 2 h at 37 °C followed by cotreatment of 50 nM FAM-labelled oligonucleotides and the free aptamer for 4 h at 37 °C. For the endocytosis pathway study, HeLa cells were pretreated with 2.0 μM cytochalasin D (actin polymerization inhibitor), 20 μM chlorpromazine (clathrin-mediated endocytosis inhibitor) or 200 μM genistein (caveolae-mediated endocytosis inhibitor) for 2 h at 37 °C followed by cotreatment of 50 nM 9-Branched AS1411-FAM and the inhibitor as described above. Then trypsinized cells were subjected to SH800S Cell Sorter (Sony) for the evaluation of the uptake of FAM-labeled oligonucleotides. Relative MFI values normalized to the control were shown. All experiments were performed in triplicate.

14, Confocal Microscopic Observation

Cells were seeded on a 8-well chamber slide (Watson) at a density of 2.0×10^4 cells/well and cultured for one day prior to the treatment. FAM-labelled oligonucleotides (100 nM) diluted in Opti-MEM were added to each well and incubated with shielding the light for 4 h at 37 °C in humidified air containing 5% CO_2. After washing twice with PBS, the cells were fixed with 4% paraformaldehyde in PBS (Nacalai) for 20 min at room temperature and washed twice again with PBS. Then cells were treated with 1.2 μM of 4',6-diamino-2-phenylindole (DAPI) in PBS for 10 min, and then washed twice with PBS. Finally, the slide glass was mounted with Fluoro-KEEPER Antifade Reagent (Nacalai) and sealed with manicure, followed by observation with a confocal microscope FV1200 (Olympus). For lysosomal colocalization analysis, HeLa cells were co-treated with 100 nM 9-Branched AS1411-FAM and 100 nM LysoTracker Red DND-99 (Thermo Fischer Scientific) diluted in Opti-MEM for 4 h at 37 °C, and observation was performed with a confocal microscope A1R-MP (Nikon).

15, Anti-proliferative Effect

The WST-8 assay was performed to evaluate the cell viability. Cells were seeded in a 96-well plate at a density of 2.5×10^3 cells/well and incubated for 1 day

before treatment. The medium was replaced with fresh culturing medium including the aptamer and cells were incubated for 72 h at 37 °C in humidified air containing 5% CO_2. Then, 10 μL/well of Cell Count Reagent SF (Nacalai) was added to each well and the cells were incubated at 37 °C, followed by measuring the absorbance at 450 nm using Multiskan FC Microplate Photometer (Thermo Fisher Scientific) to evaluate the cell viability. All experiments were performed in triplicate.

16, Statistical Analysis

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Tukey−Kramer tests for multiple comparisons, and Welch's t-test for comparison between two groups.

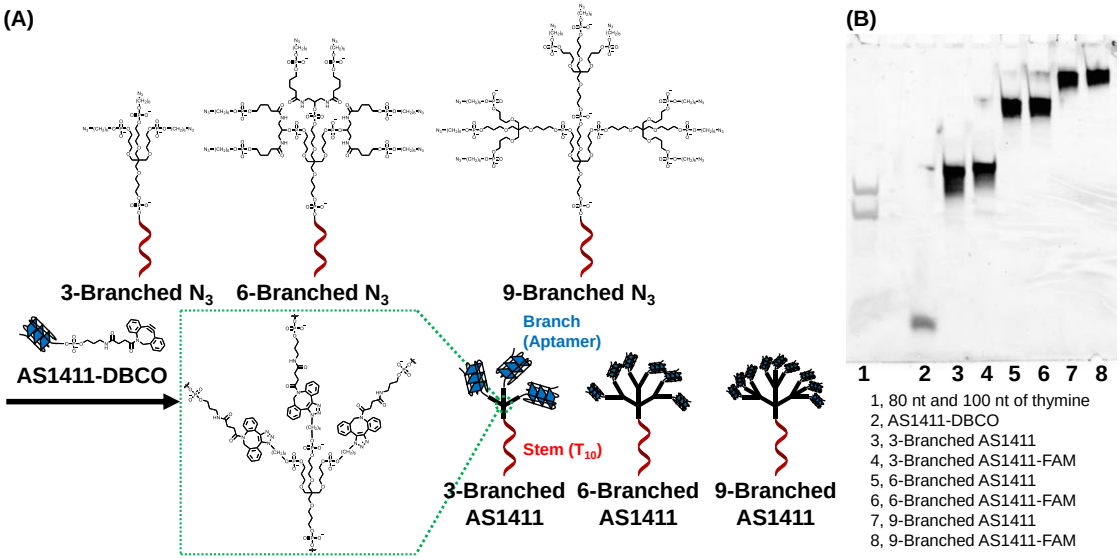
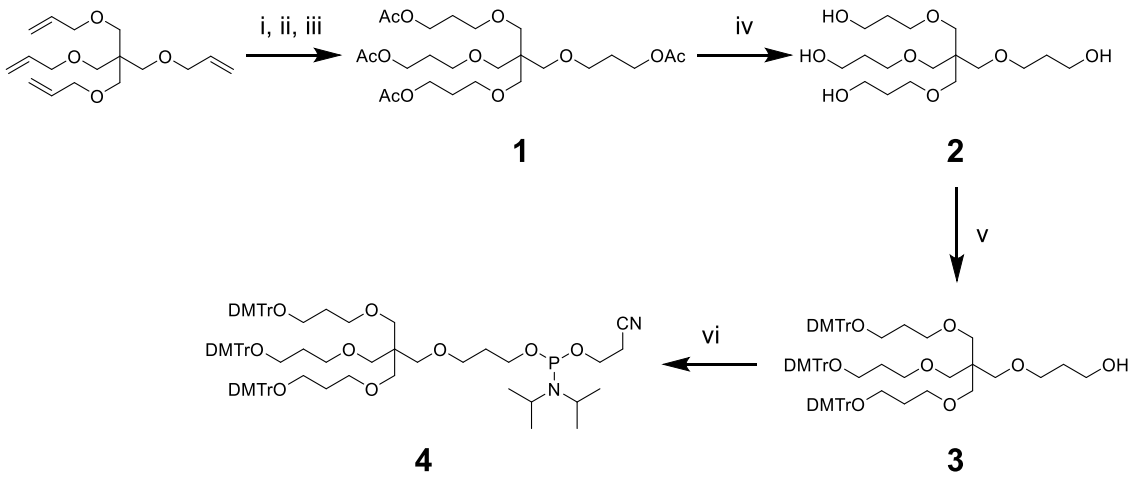


Figure S1. (A) Structures and synthesis of DNA dendrons composed of the multivalent aptamer via SPAAC. (B) Denaturing polyacrylamide gel electrophoresis analysis of synthesized DNA dendrons.



Scheme S1. Synthesis of trebler phosphoramidite **4**.[2] Reagents and conditions: (i) 9-BBN, THF, RT. (ii) NaOH, H_2O_2, 0 °C to RT. (iii) Ac_2O, Pyridine, RT. (iv) MeONa, MeOH, RT. (v) 4,4′-Dimethoxytrityl chloride, DMAP, Pyridine, RT. (vi) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, DCM, 0 °C to RT.

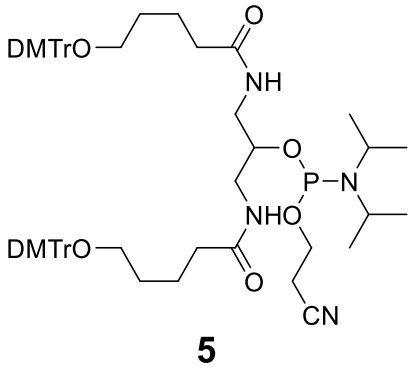
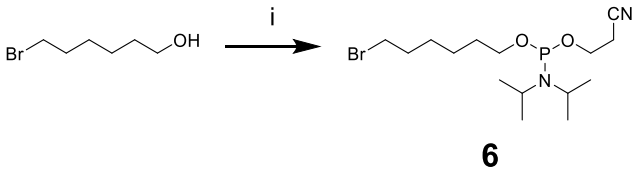


Figure S2. Structure of doubler phosphoramidite **5**.



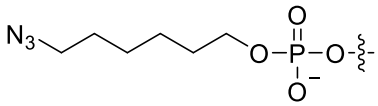
Scheme S2. Synthesis of 6-bromohexyl phosphoramidite **6**.[3] Reagents and conditions: (i) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, DCM, 0 °C to RT.

Table S1. Sequences of oligonucleotides[abc]

Name	Sequence (5' to 3')
AS1411	GGTGGTGGTGGTTGTGGTGGTGGTGG
cAS1411	CCTCCTCCTCCTTCTCCTCCTCCTCC
AS1411-DBCO	GGTGGTGGTGGTTGTGGTGGTGGTGG-DBCO
cAS1411-DBCO	CCTCCTCCTCCTTCTCCTCCTCCTCC-DBCO
AS1411-FAM	GGTGGTGGTGGTTGTGGTGGTGGTGGTTTTTTTTTT-FAM
cAS14111-FAM	CCTCCTCCTCCTTCTCCTCCTCCTCCTTTTTTTTTT-FAM
3-Branched N_3	N_3-Trebler-TTTTTTTTTT
3-Branched N_3-FAM	N_3-Trebler-TTTTTTTTTT-FAM
6-Branched N_3	N_3-Doubler-Trebler-TTTTTTTTTT
6-Branched N_3-FAM	N_3-Doubler-Trebler-TTTTTTTTTT-FAM
9-Branched N_3	N_3-Trebler-Trebler-TTTTTTTTTT
9-Branched N_3-FAM	N_3-Trebler-Trebler-TTTTTTTTTT-FAM
3-Branched AS1411	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Trebler-TTTTTTTTTT
3-Branched AS1411-FAM	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Trebler-TTTTTTTTTT-FAM
6-Branched AS1411	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Doubler-Trebler-TTTTTTTTTT
6-Branched AS1411-FAM	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Doubler-Trebler-TTTTTTTTTT-FAM
9-Branched AS1411	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Trebler-Trebler-TTTTTTTTTT
9-Branched AS1411-FAM	GGTGGTGGTGGTTGTGGTGGTGGTGG-Linker-Trebler-Trebler-TTTTTTTTTT-FAM
9-Branched cAS1411-FAM	CCTCCTCCTCCTTCTCCTCCTCCTCC-Linker-Trebler-Trebler-TTTTTTTTTT-FAM

[a]Non-modified linear AS1411 and cAS1411 were purchased from Integrated DNA Technologies. FAM-labelled linear oligonucleotides AS1411-FAM and cAS1411-FAM were purchased from Japan Bio Services.

[b]N_3 represents the following structure:



[c]Linker represents the following structure:

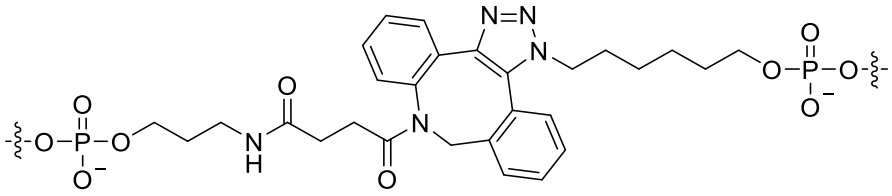


Table S2. ESI-MS values and yield from CPG of oligonucleotides

Name	Ion	MS		Yield from CPG
		Calcd.	Found	
AS1411-DBCO	$[M-11H]^{11-}$	789.217	789.217	75%
cAS1411-DBCO	$[M-9H]^{9-}$	889.256	889.256	63%
3-Branched N_3	$[M-5H]^{5-}$	803.770	803.770	21%
3-Branched N_3-FAM	$[M-8H]^{8-}$	573.117	573.117	26%
6-Branched N_3	$[M-10H]^{10-}$	631.825	631.825	11%
6-Branched N_3-FAM	$[M-9H]^{9-}$	695.059	695.059	12%
9-Branched N_3	$[M-10H]^{10-}$	653.477	653.477	10%
9-Branched N_3-FAM	$[M-10H]^{10-}$	710.388	710.388	12%

Table S3. MALDI-TOF MS values of dendritic oligonucleotides[a]

Name	Ion	MS	
		Calcd.	Found
3-Branched AS1411	$[M-2H]^{2-}$	15057.03	15235.17
3-Branched AS1411-FAM	$[M-2H]^{2-}$	15341.76	15377.72
6-Branched AS1411	$[M-2H]^{2-}$	28938.34	29239.85
6-Branched AS1411-FAM	$[M-2H]^{2-}$	29223.07	29250.77

[a]MS signal of 9-Branched dendritic DNAs could not be observed.

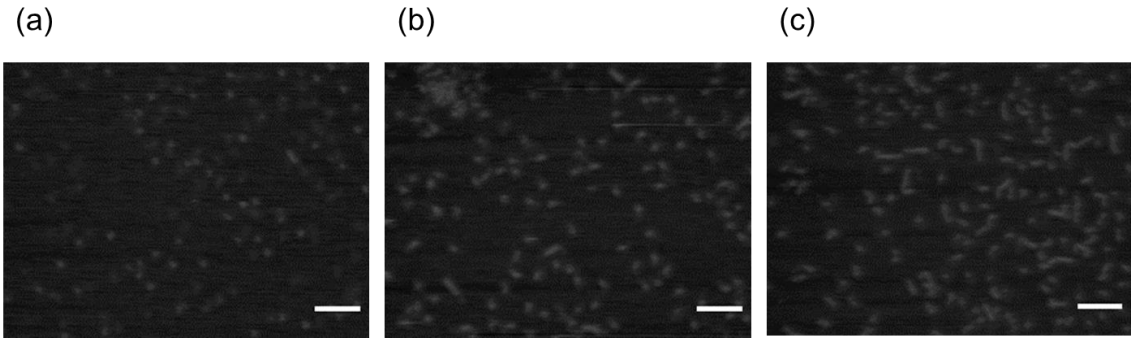


Figure S3. AFM images of (a) 3-Branched AS1411, (b) 6-Branched AS1411, and (c) 9-Branched AS1411. Scale bars: 50 nm

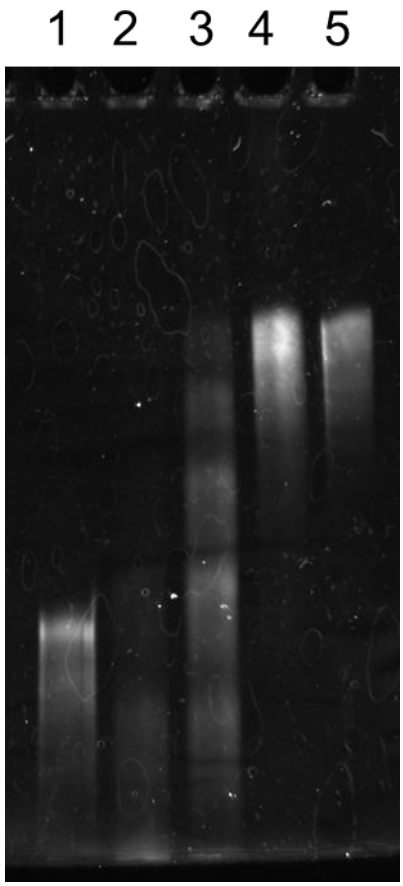


Figure S4. Denaturing polyacrylamide gel electrophoresis of the reaction mixture of 9-Banched N_3 and varied equivalency of AS1411-DBCO. (lane 1) 6-Branched AS1411, (lane 2) the mixture of 1.0 eq. of 9-Banched N_3 and 3.0 eq. of AS1411-DBCO, (lane 3) the mixture of 1.0 eq. of 9-Banched N_3 and 6.0 eq. of AS1411-DBCO, (lane 4) the mixture of 1.0 eq. of 9-Banched N_3 and 9.0 eq. of AS1411-DBCO, and (lane 5) 9-Branched AS1411.

Denaturing PAGE analysis of the reaction mixture of 9-Banched N_3 and varied equivalency of AS1411-DBCO demonstrated the bands derived from partially reacted 9-Banched N_3 (Figure S4). Because the fourth band from the top in lane 3 was close to that of 6-Branched AS1411 (lane 1), this band was supposed to be derived from 9-Branched N_3 in which six of nine azide groups reacted with AS1411-DBCO. Three bands were seen above this band and the position of the top band was consistent with that of 9-Branched AS1411 (lane 5). The position of the band in lane 4 was also consistent with that of 9-Branched AS1411 and no bands were seen above this. The result suggests one molecule of 9-Branched N_3 can react with nine molecules of AS1411-DBCO and 9-Branched AS1411 is composed of nine AS1411 branches.

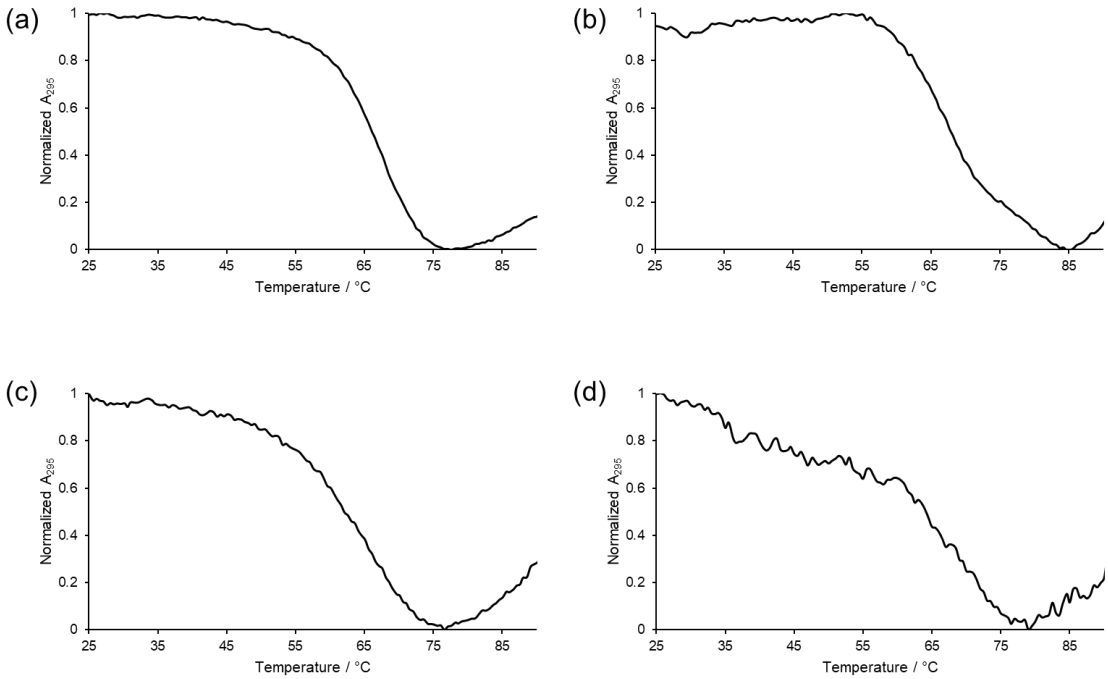


Figure S5. Thermal denaturation curves of G-quadruplex-forming aptamer (equivalent to 5.0 μM AS1411) within a dendritic molecule in 10 mM Tris-HCl (pH 7.5) buffer containing 100 mM KCl. (a) AS1411, (b) 3-Branched AS1411, (c) 6-Branched AS1411, (d) 9-Branched AS1411.

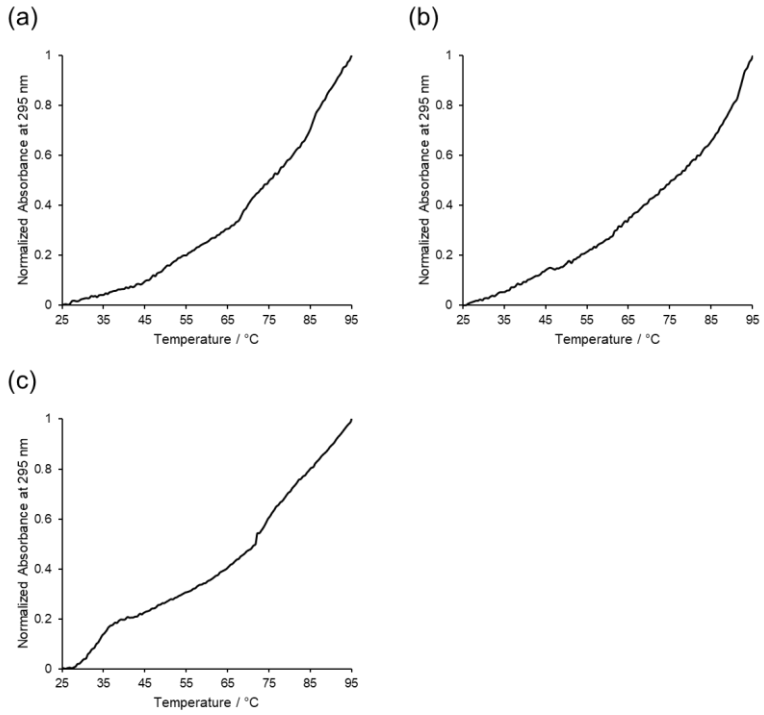


Figure S6. Temperature-dependent change of absorbance at 295 nm of 5 μM stem DNAs (a) 3-Branched N_3, (b) 6-Branched N_3 and (c) 9-Branched N_3 in 10 mM Tris-HCl (pH 7.5) buffer containing 100 mM KCl.

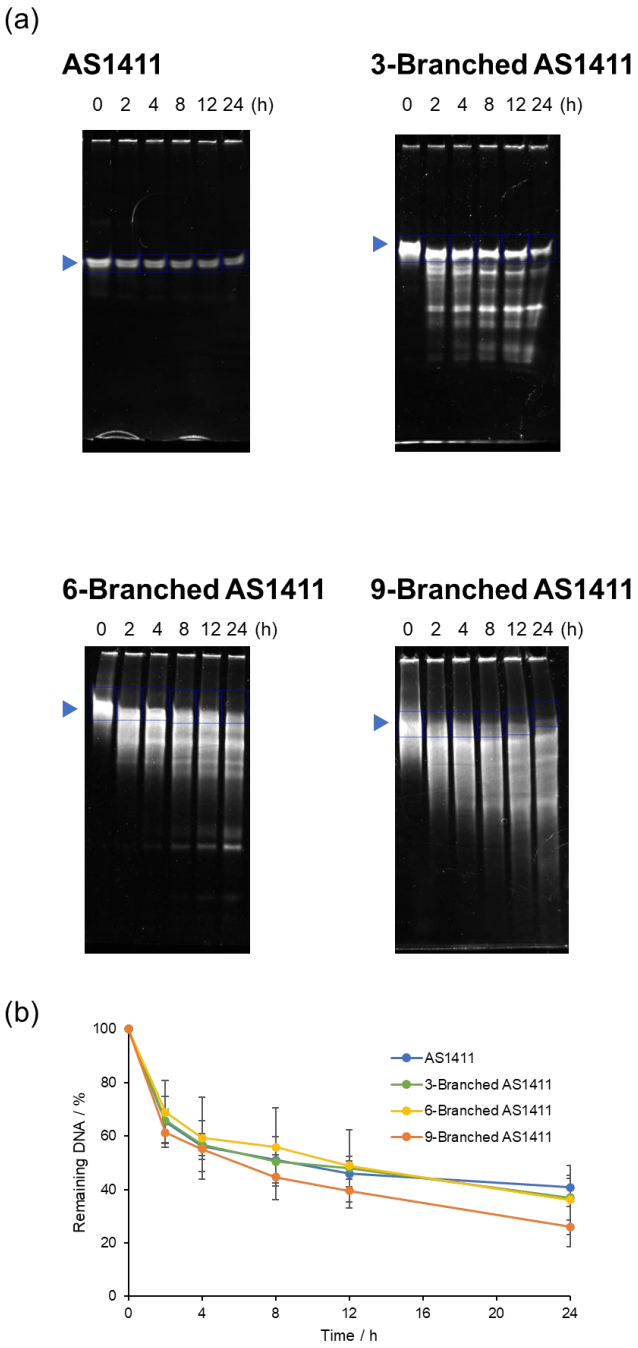


Figure S7. Serum stability of dendritic aptamers in 50% FBS solution. (a) Representative gel images of degraded aptamers. Deltas indicate the position of full-length and squares indicate the areas quantified for (b). (b) Ratio of the remaining full length DNA plotted against incubation time. The mean of the three independent experiments is shown; error bar represents their standard deviation.

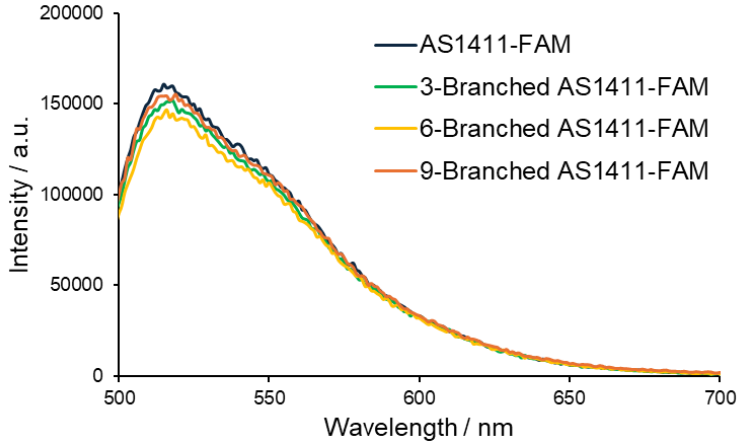


Figure S8. Fluorescence spectra of AS1411-FAM, 3-Branched AS1411-FAM, 6-Branched AS1411-FAM and 9-Branched AS1411-FAM at 0.50 μM in water.

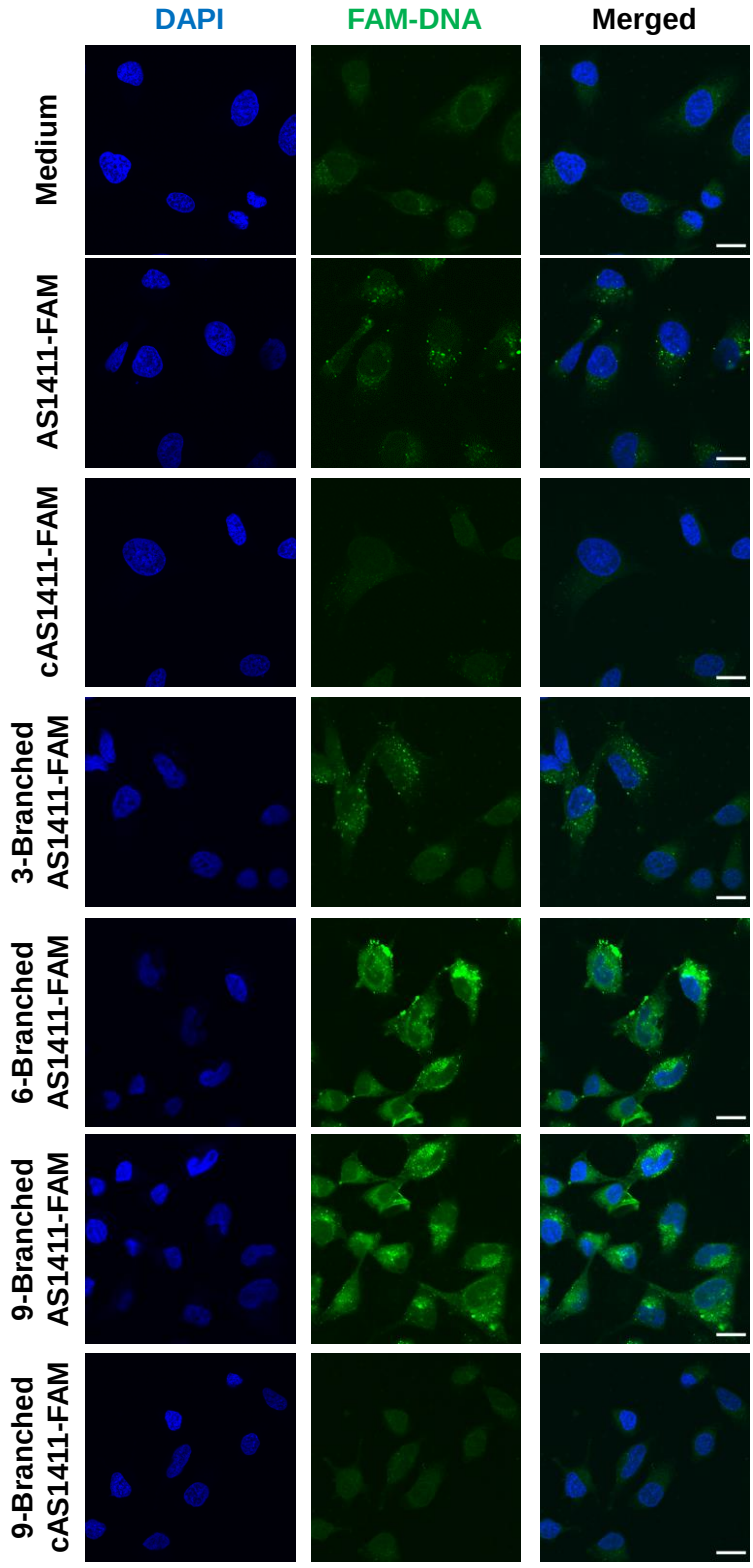


Figure S9. Confocal microscopic images of HeLa cells treated with FAM-labelled aptamers AS1411-FAM, 3-Branched AS1411-FAM, 6-Branched AS1411-FAM and 9-Branched AS1411-FAM and their negative control cAS1411-FAM and dendritic 9-Branched cAS1411-FAM. Scale bars: 20 μm.

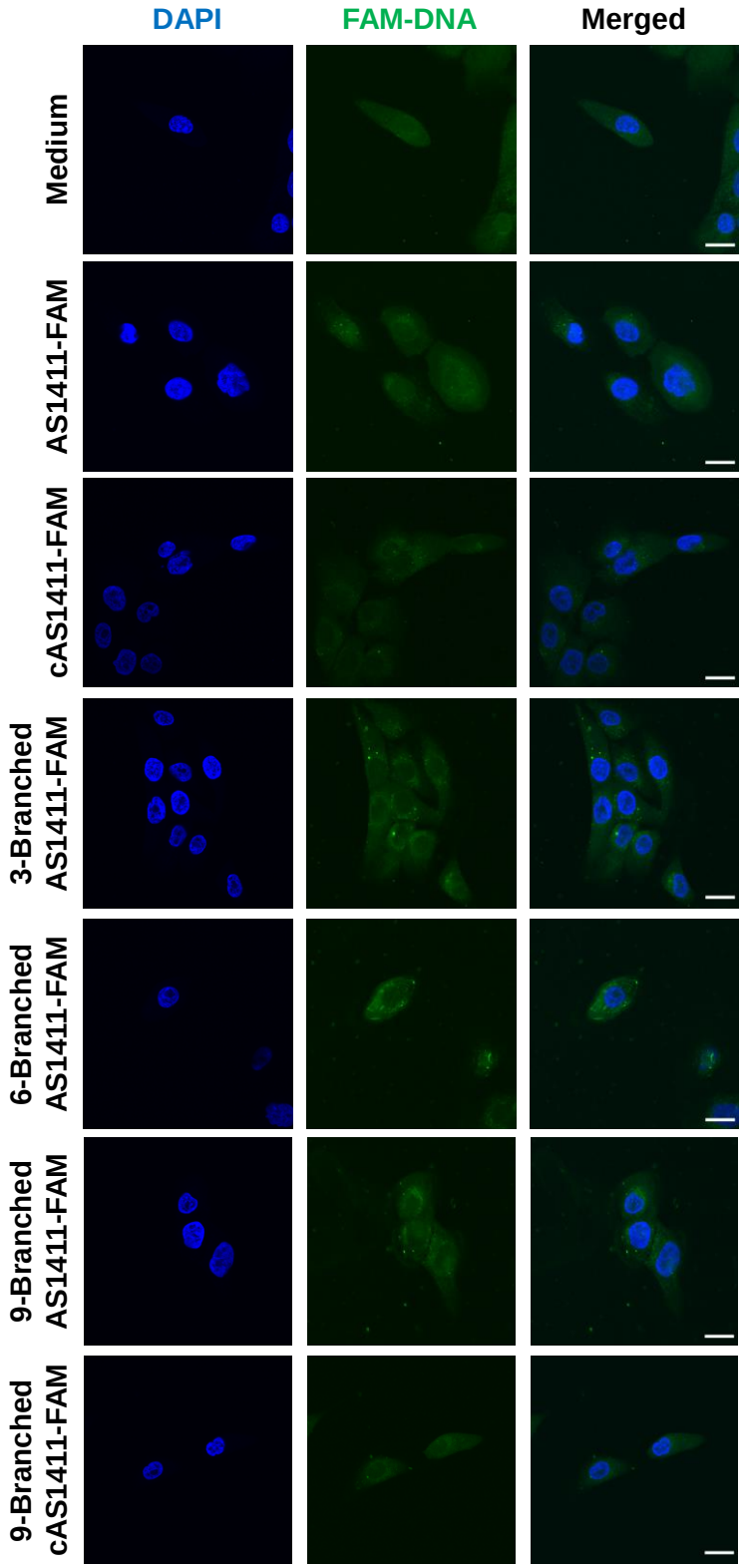


Figure S10. Confocal microscopic images of MCF-10A cells treated with FAM-labelled aptamers AS1411-FAM, 3-Branched AS1411-FAM, 6-Branched AS1411-FAM and 9-Branched AS1411-FAM and their negative control cAS1411-FAM and dendritic 9-Branched cAS1411-FAM. Scale bars: 20 μm.

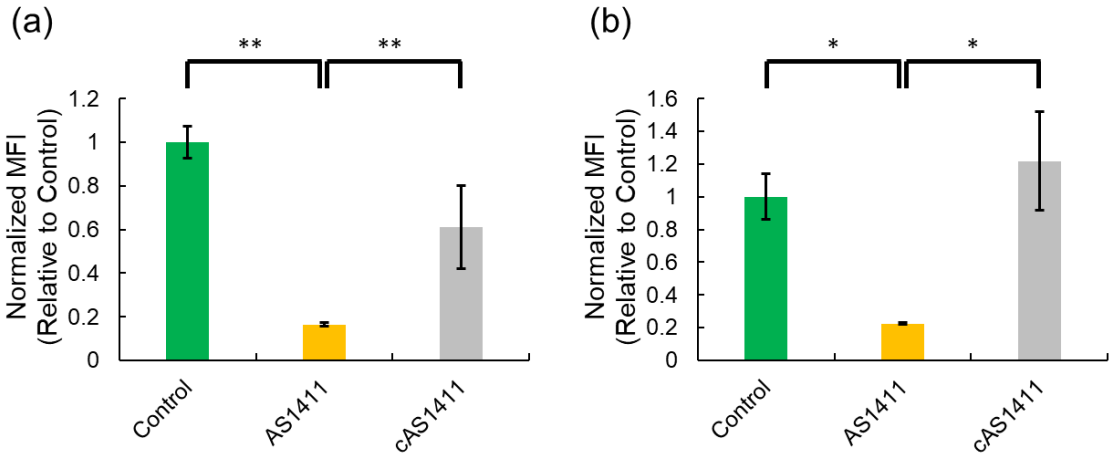


Figure S11. Flow cytometry analysis of competition assays between free aptamers and (a) AS1411-FAM or (b) 9-Branched AS1411-FAM. $^{*}P < 0.05$, $^{**}P < 0.005$.

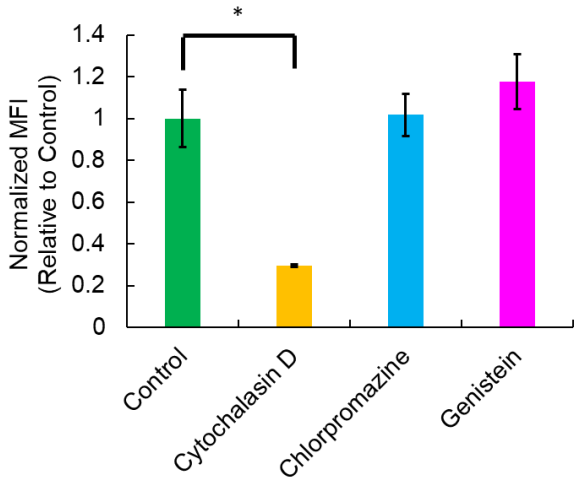


Figure S12. Endocytosis inhibition of 9-Branched AS1411-FAM with various inhibitors for its pathway study. $^{*}P < 0.05$.

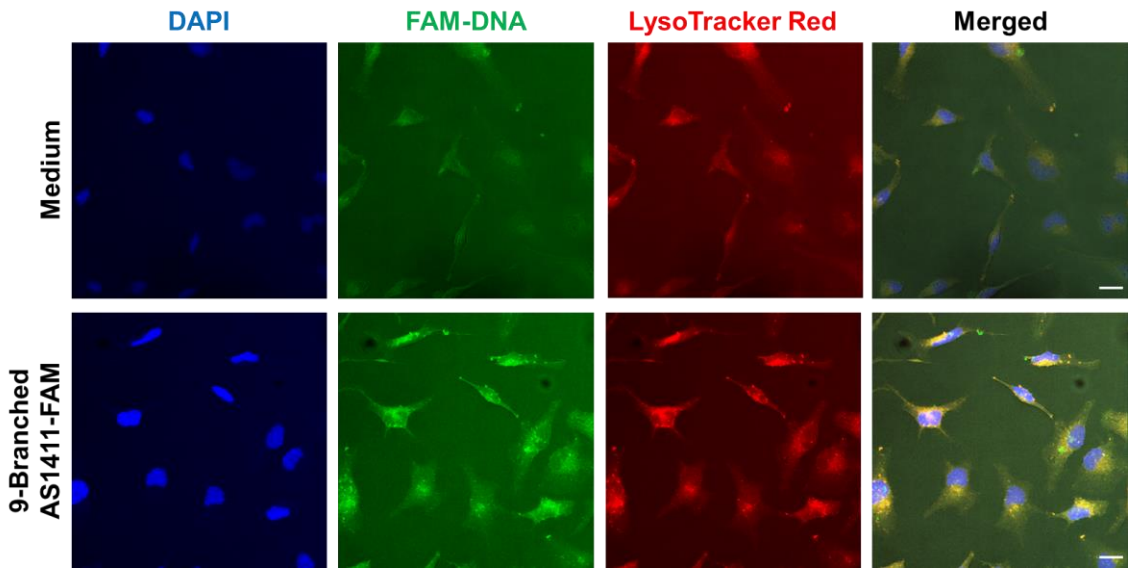


Figure S13. Confocal microscopic images of lysosomal colocalization with 9-Branched AS1411-FAM in HeLa cells. Scale bars: 20 μm

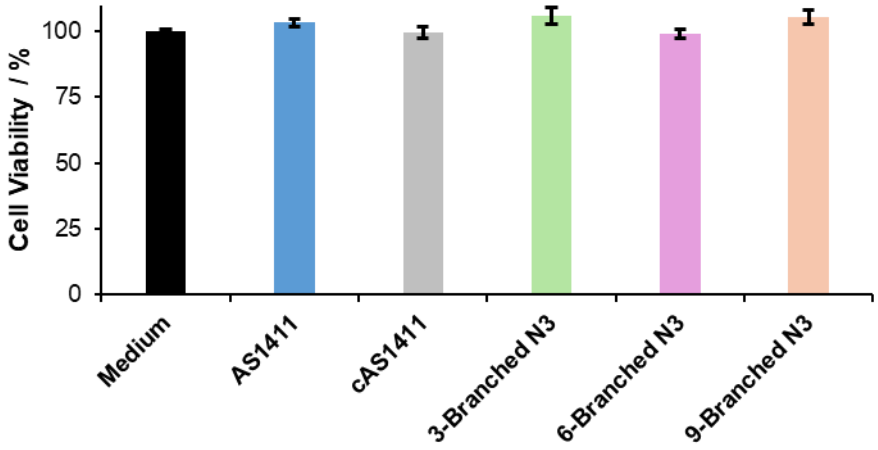


Figure S14. Cell viability of HeLa cells treated with 3 μM of AS1411, cAS1411, 3-Branched N_3, 6-Branched N_3 or 9-Branched N_3.

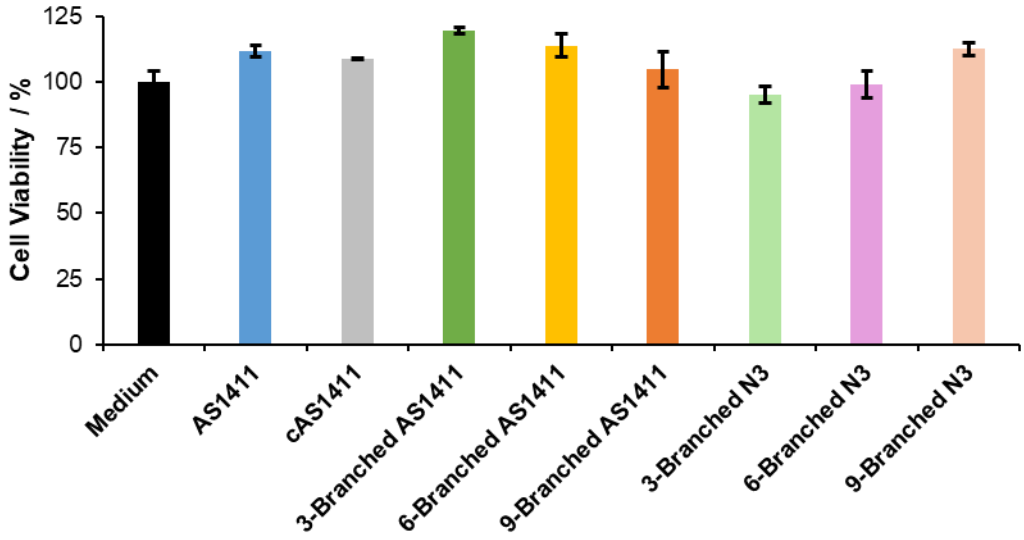


Figure S15. Cell viability of MCF-10A cells treated with antiproliferative aptamers and stem DNAs when the total dendron concentration was at 3 μM. Mean of three independent experiments is shown and error bars represent their standard deviation.

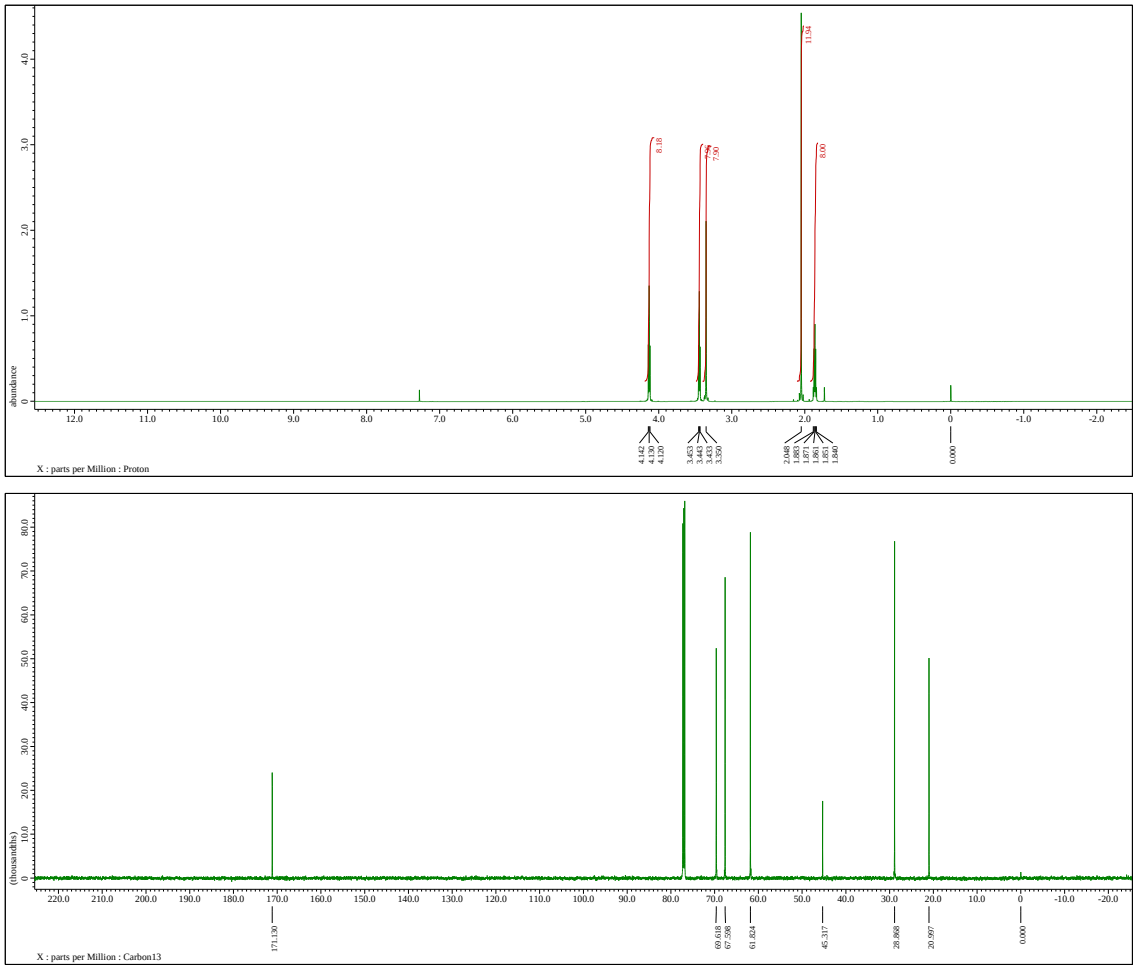


Figure S16. ^{1}H-NMR and ^{13}C-NMR of the compound **1** in $CDCl_3$ with 0.03% TMS.

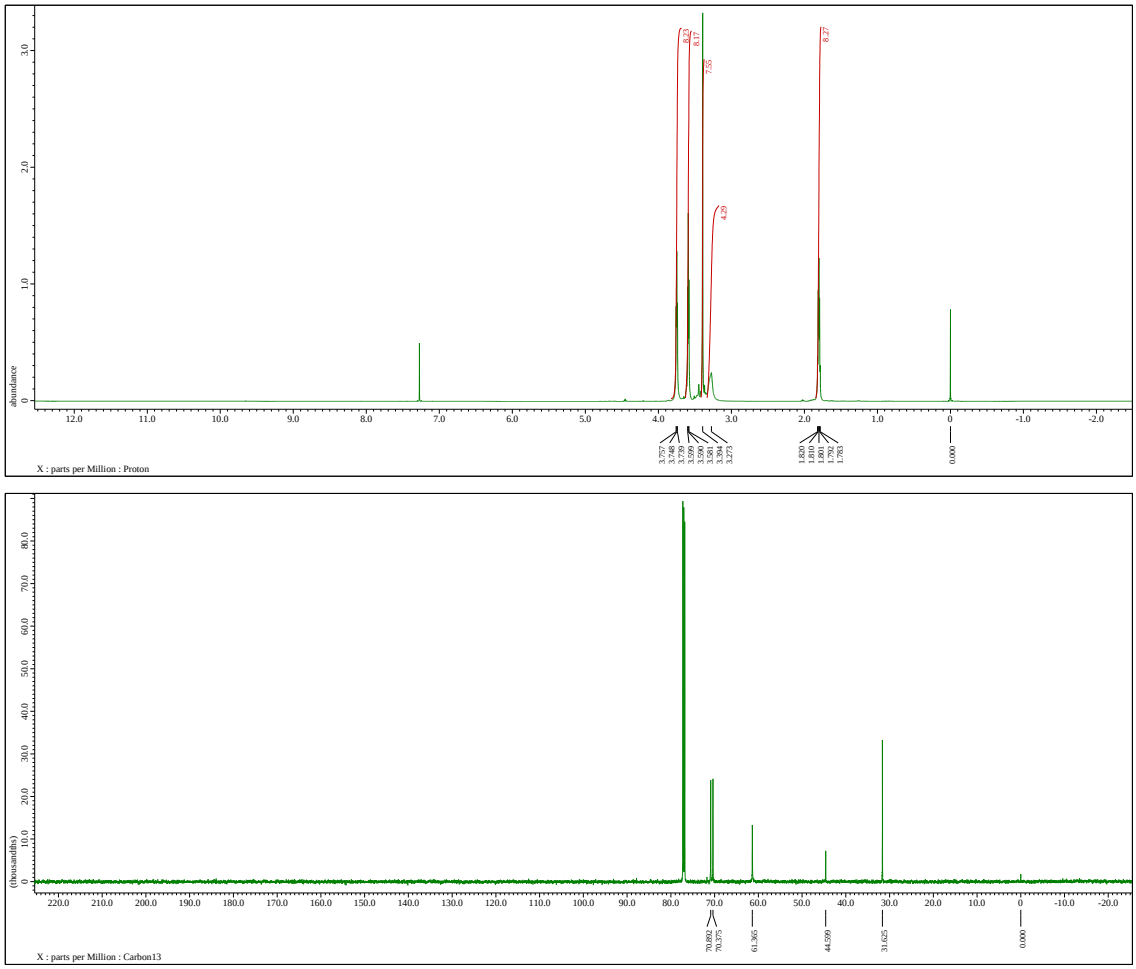


Figure S17. ^{1}H-NMR and ^{13}C-NMR of the compound **2** in $CDCl_3$ with 0.03% TMS.

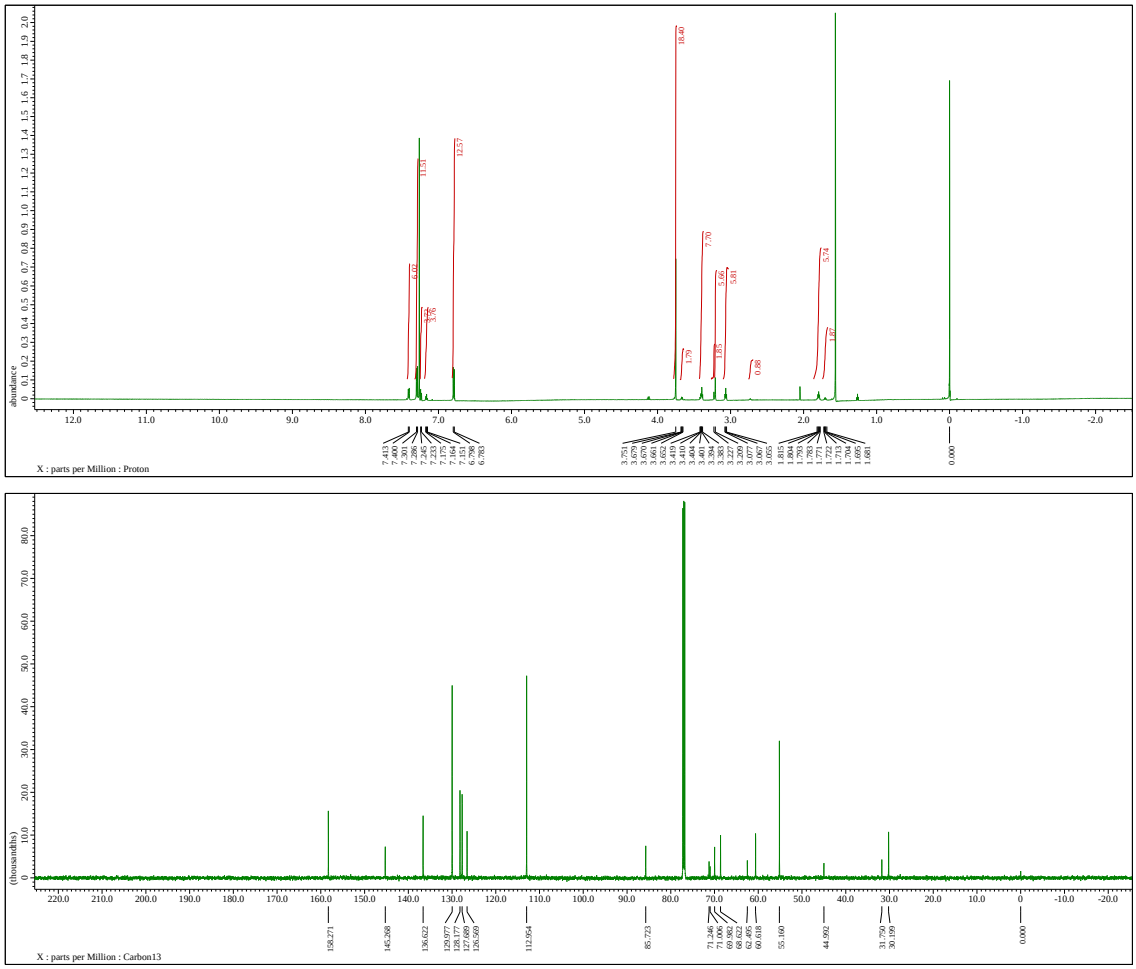


Figure S18. ^{1}H-NMR and ^{13}C-NMR of the compound **3** in $CDCl_3$ with 0.03% TMS.

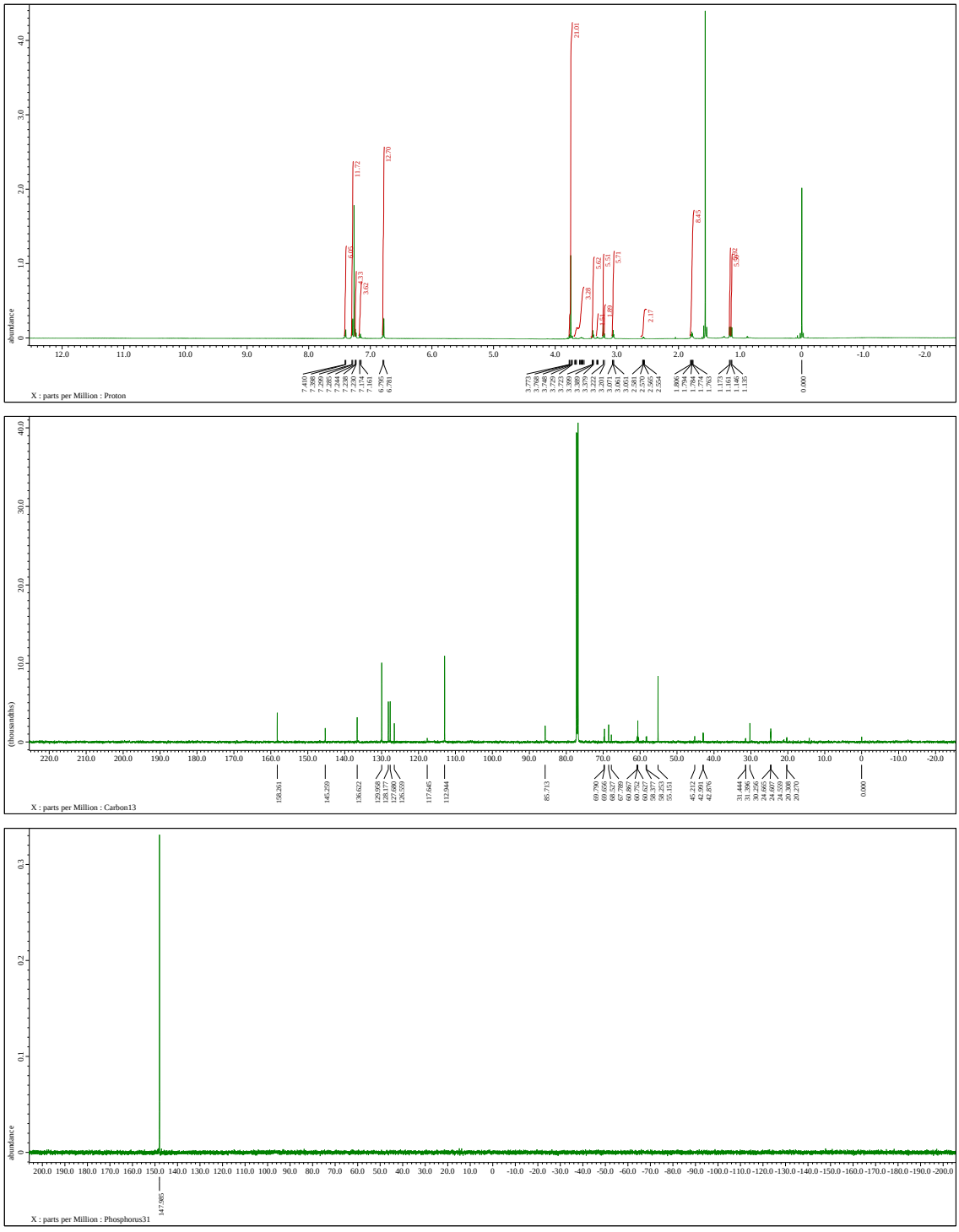


Figure S19. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR of the compound **4** in $CDCl_3$ with 0.03% TMS.

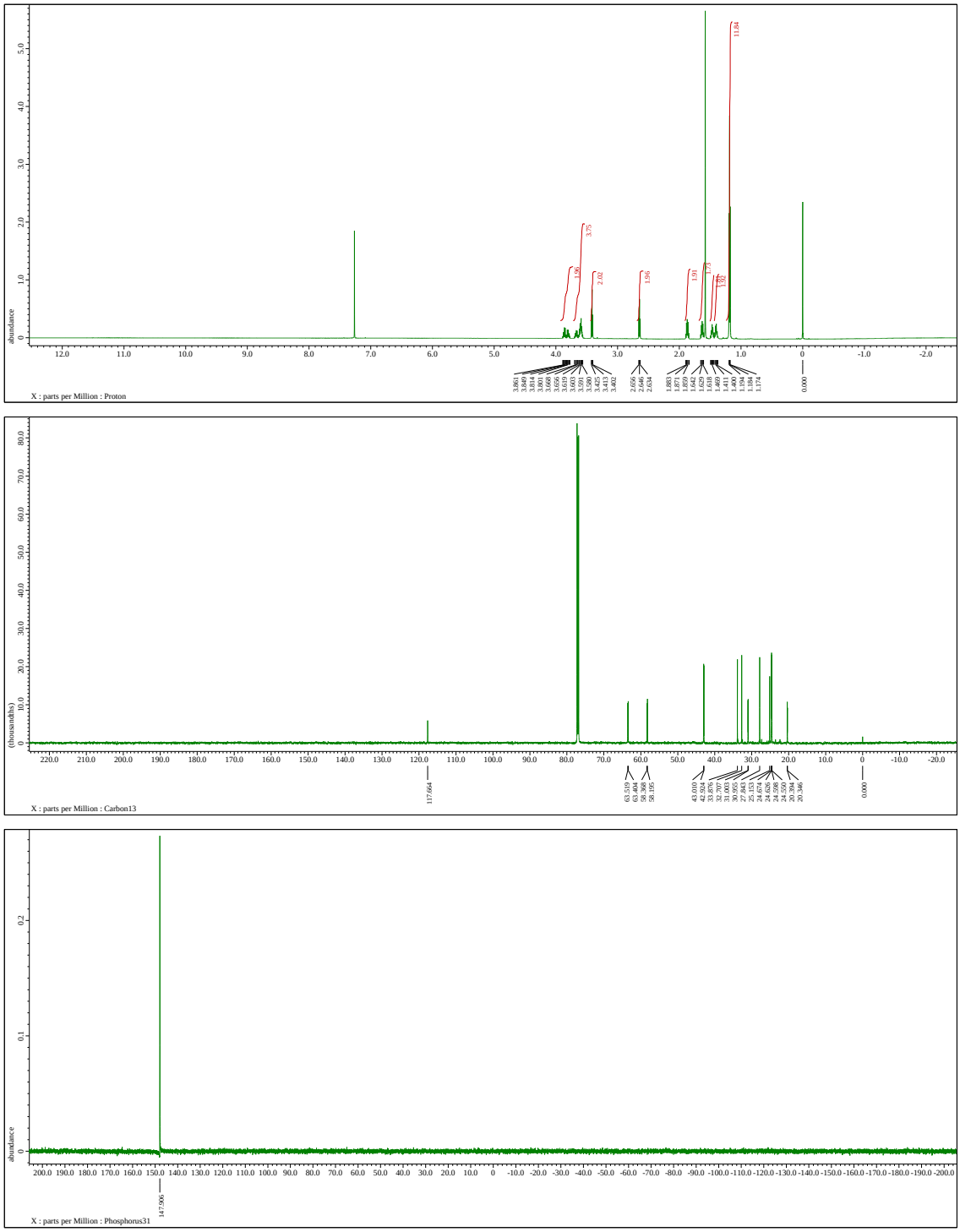


Figure S20. ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR of the compound **6** in $CDCl_3$ with 0.03% TMS.

References

1. Y. Kawamoto, W. Liu, J. H. Yum, S. Park, H. Sugiyama, Y. Takahashi and Y. Takakura, *ChemBioChem*, 2022, **23**, e202100583.
2. A. D. Shaller, W. Wan, B. M. Zhao and A. D. Q. Li, *Chem. Eur. J.*, 2014, **20**, 12165-12171.
3. J. Lietard, A. Meyer, J. J. Vasseur and F. Morvan, *Tetrahedron Lett.*, 2007, **48**, 8795-8798.