Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2020

Supporting Information

Enhanced nuclear accumulation of pyrrole-imidazole polyamides by incorporation of the tri-arginine vector

Takuya Hidaka,[a] Yutaro Tsubono,[a] Kaori Hashiya,[a] Toshikazu Bando,[a] Ganesh N. Pandian,[b] Hiroshi Sugiyama[*ab]

† Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo, Kyoto 606-8502, Japan

‡ Institute for Integrated Cell-Material Science (WPI-iCeMS), Kyoto University, Sakyo, Kyoto 606-8501, Japan.

Table of contents **Page**

Experimental methods

General information of synthesis

The reagents were purchased from standard suppliers. The solid phase synthesis was performed using an automated synthesizer PSSM-8 (Shimadzu) with a computer assisted operation system. Reversed-phase high performance liquid chromatography (HPLC) was performed on a JASCO HPLC system (JASCO Engineering UV2075 UV/vis detector and a PU-2089 plus gradient pump) equipped with Chemcobond 5-ODS-H reversed-phase column (4.6 × 150 mm). H_2O (+0.1% trifluoroacetic acid) and acetonitrile were used as the mobile phase and the program was a linear gradient of 0 to 100% acetonitrile in 40 min at a flow rate of 1.0 mL/min. Absorbance at 254 nm was monitored. MALDI-TOF mass spectroscopy was performed with the microflex system (Bruker).

Synthesis of SOX2i-R3, SOX2i, Ctrl-R3, AP2i-R3 and AP2i

The Fmoc solid-phase synthesis was performed as described previously.[1,2] Each Fmoc monomer units (Fmoc-D-Arg(Pbf)-OH, Fmoc-β-alanine-OH, Fmoc-GABA-OH, Fmoc-*N*-methylpyrrole (Py)-OH and Fmoc- *N*-methylimidazole (Im)-OH) was introduced sequentially to Fmoc-D-Arg(Pbf)-Alko resin for synthesis of SOX2i-R3, Ctrl-R3 and AP2i-R3 and to PyIm-coupled clear resin or Im-coupled oxime resin for synthesis of SOX2i and AP2i, respectively. Fmoc-ImPy-OH dimer units were also used in the synthesis of SOX2i-R3 to improve the synthesis efficiency. N-terminal was capped with acetyl group by mixing with 20% acetic anhydride in *N*, *N*-dimethylformamide. Synthesized compounds were cleaved with *N*, *N*-dimethylaminopropylamine at 55 °C for 3 hours. The reaction solution was drained into ether and the resulting solid was dried *in vacuo*. Protecting group of arginine residues (Pbf group) of SOX2i-R3, Ctrl-R3 and AP2i-R3 were removed with deprotection cocktail (trifluoroacetic acid: triisopropylsilane: water (95:2.5:2.5 v/v%)) at room temperature for 30 minutes. The crude sample was purified by reversed-phase HPLC using a JASCO HPLC system (JASCO Engineering UV2075 UV/vis detector and a PU-2089 plus gradient pump) with a preparative C18 (ODS) column (COSMOSIL $5C_{18}$-MS-II, 10ID x 150 mm). H_2O (+0.1% trifluoroacetic acid) and acetonitrile were used as the mobile phase. HPLC and MALDI-TOF mass spectroscopy was performed for the characterization of the purified compounds.

Synthesis of SOX2i-R3-TAMRA and SOX2i-TAMRA

After the solid phase synthesis, the compounds were cleaved from the resin with 3, 3'-diamino-*N*-methyldipropylamine at 55 °C for 3 hours. The crude samples were purified by reversed-phase column chromatography using the CombiFlash Rf system equipped with 4.3 g C18 RediSep Rf reversed-phase flash column (Teledyne Isco, Inc). H_2O (+0.1% trifluoroacetic acid) and acetonitrile were used as the mobile phase. The coupling reaction was performed by adding 1.3 eq of 5-TAMRA-NHS ester and 3 eq of *N*,*N*-diisopropylethylamine (DIEA) and shaking at room temperature overnight. Powderization, deprotection, purification and characterization were performed as described above.

Cell culture

HeLa (JCRB9004) and 201B7 cells were provided by JCRB Cell Bank (Japan) and the RIKEN BRC (Japan) respectively. SKBR-3 cells were purchased from ATCC. All cells were maintained in a humidified CO_2 incubator at 37°C.

HeLa cells were maintained in Dulbecco's Modified Eagle Medium (ThermoFisher Scientific) supplemented with 10 % fetal bovine serum (Sigma) and 1% MEM Non-Essential Amino Acids Solution (ThermoFisher Scientific).

201B7 iPS cells were cultured on Matrigel Matrix (hESC-qualified, Corning) in mTeSR1 medium (Stemcell Technologies) supplemented with 0.5x penicillin/streptomycin (Nacalai Tesque). Cells were passaged using TrypLE Express Enzyme (no phenol red, ThermoFisher Scientific) as dissociation reagent and seeded in medium supplemented with 2.5 μM of Y-27632 (Wako). Medium change with fresh medium (without Y-27632) was performed every day from the next day of the passaging.

SKBR-3 cells were maintained in McCoy's 5A Medium (ThermoFisher Scientific) supplemented with 10 % fetal bovine serum (Sigma).

Live cell imaging

Exponentially growing HeLa cells were trypsinized and seeded on 8-well ibi-treat μ-slides (ibidi) at the density of 1.5 x 10^4 cells/ well one day prior to the treatment. DMSO solution of SOX2i-R3-TAMRA and SOX2i-TAMRA was prepared and the concentration was determined using an extinction coefficient of 91000 M^{-1} cm^{-1} at λ_{max} near 565 nm. The medium was changed with OPTI-MEM (ThermoFisher Scientific) containing 2% FBS (Sigma) and 5 μM of each compound (DMSO: 0.5%) and cells were treated for 24 hours. After the treatment, the culture medium was removed and FluoroBrite DMEM (ThermoFisher Scientific) supplemented with 10% FBS (Sigma) and Hoechst33342 (1 μg/mL) was added and incubated for 10 minutes. Cells were washed twice with FluoroBrite DMEM and imaged by the FV1200 Laser Scanning Microscope (Olympus). The same imaging conditions were adopted for all samples. Image analysis was performed using FV10-ASW (Olympus).

Flow cytometry analysis

Exponentially growing HeLa cells were trypsinized and seeded on a 12-well plate (NIPPON Genetics) at the concentration of 5 x 10^4 cells/ well one day prior to the treatment. The medium was changed with OPTI-MEM (ThermoFisher Scientific) containing 2% FBS (Sigma) and 5 μM of each compound (DMSO: 0.5%) and the treatment was performed for 24 hours. After the treatment, the cells were detached from the plate by trypsinization and subjected to D-PBS wash twice. The washed cells were resuspended in D-PBS. Samples were analyzed using a BD FACS AriaII (BD Biosciences) after removal of cell clumps using polystyrene tubes with cell strainer caps (Corning). 20000 events were recorded for each sample, and FSC (Forward Scatter)/ SSC (Side Scatter) plot was used for population gating to remove data points obtained from debris and electrical noise. After the selection, signal intensity of TAMRA of 13,082 events of the negative control

sample, 12,330 events of the SOX2i-R3-TAMRA treated sample and 13,834 events of the SOX2i-TAMRA treated sample were plotted in the histogram.

Electrophoretic mobility shift assay

DMSO solution of SOX2i-R3, SOX2i and Ctrl-R3 was prepared and the concentration was determined from the calculation formula below using the maximum absorbance in 300-310 nm measured by a Nanodrop ND-1000 (ThermoFisher Scientific):

$$c = \frac{Abs}{9900 \times a \times d}\,(M)$$

(a: total number of pyrrole and imidazole rings, d (cm): optical path length (0.1 cm for Nanodrop ND-1000), Abs = Maximum absorbance in 300-310 nm)

Each template DNA (1 μM) was mixed with 1 μM of SOX2i-R3, SOX2i or Ctrl-R3 in aqueous buffer containing 10 mM sodium chloride, 10 mM sodium cacodylate and 2.5 v/v% DMSO at pH 7.0. Before electrophoresis samples were annealed by heating to 95°C for 3 min and cooled to 25°C at speed of -0.5°C/ 5 seconds. 8 μL of each sample was mixed with 2 μL of Novex Hi-Density TBE Sample Buffer (ThermoFisher Scientific) and 1 μL of each loading mixture was loaded. Native-PAGE was performed with 20% polyacrylamide gel at 200 V for 60 min in TBE buffer. The gel was stained with SYBR Gold (ThermoFisher Scientific) and imaged with the FLA-3000 system (Fujifilm).

Quantitative reverse-transcription PCR (RT-qPCR) analysis of 201B7 cells

201B7 iPS cells were seeded on a Matrigel-coated 24-well plate (Greiner) in mTeSR1 medium supplemented with 2.5 μM of Y-27632 at the concentration of 6×10^4 cells/ well one day prior to the treatment. The medium was replaced with differentiation medium (Advanced RPMI 1640 (ThermoFisher Scientific) supplemented with 0.2% FBS and 1x L-glutamine) containing each compound and 0.1% DMSO. 48 hours later, total RNA was extracted from each well using FastGene RNA Basic Kit (NIPPON Genetics) and reverse transcription was performed from 500 ng of total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) following the manufacturer's instructions. Reaction mixture was prepared with Thunderbird SYBR qPCR mix (Toyobo) and qPCR reaction was performed and monitored on LightCycler 480 System II (Roche). The relative expression level of each gene was calculated from the average Cp value of three replicates of each samples and normalized to the mean value of negative control samples. The primer pairs used in this experiment are listed in Table S1.

Quantitative reverse-transcription PCR (RT-qPCR) analysis of SKBR-3 cells

SKBR-3 cells were seeded on a 12-well plate (Greiner) in growth medium at the concentration of 1×10^5 cells/ well one day prior to the treatment. The medium was replaced with fresh growth medium containing each compound and 0.1% DMSO and the cells were treated for 48 hours. The successive RNA extraction, reverse transcription and qPCR assay were performed as explained above.

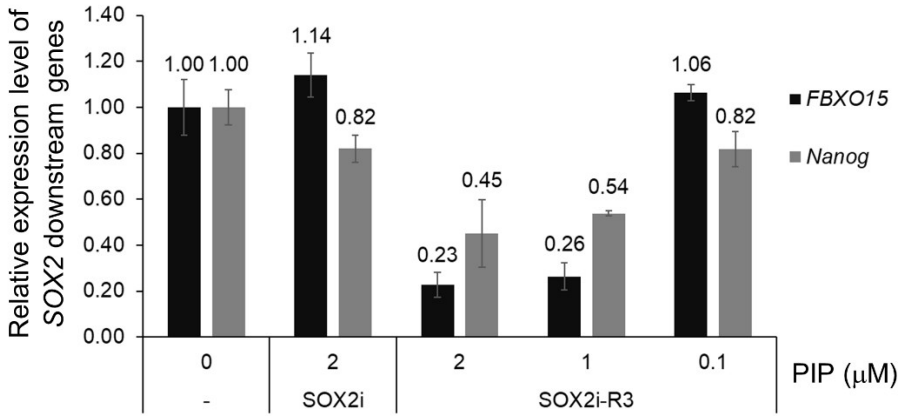


Figure S1. Relative RNA expression level of SOX2 downstream genes in 201B7 cells treated with SOX2i and SOX2i-R3 for 48 hours. Mean values calculated from three culture wells are indicated as a bar chart. Each value is normalized to the control samples. HPRT1 was used as a housekeeping gene. Error bars represent ±SD (n=3).

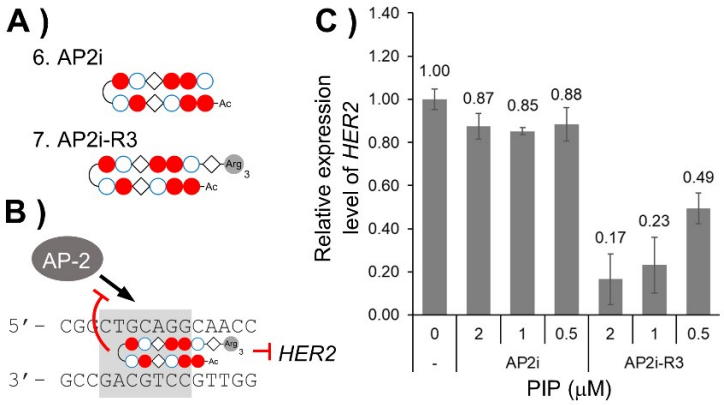
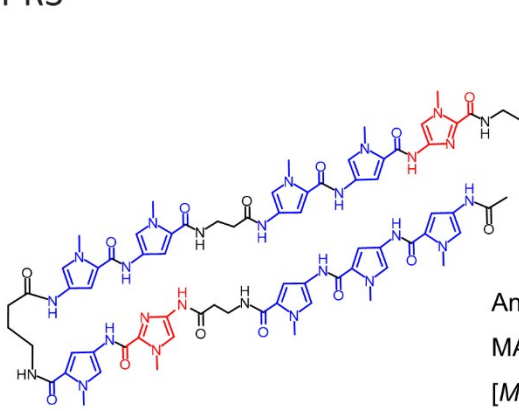


Figure S2. (A) Design of AP2i and AP2i-R3 targeting AP2 binding sequence in *HER2* promoter region (chemical structures are shown in Fig. S3). (B) Schematic illustration of *HER2* repression by AP2i-R3. (C) Relative expression level of *HER2* in SKBR-3 cells treated with AP2i and AP2i-R3 for 48 hours. Mean values calculated from three culture wells are shown and each value is normalized to the control samples. *ACTB* was used as a housekeeping gene. Error bars represent ±SD (n = 3).

Characterization data of synthesized compounds

1. SOX2i-R3



Analytical HPLC t_R = 15.6 min

MALDI-TOF MS m/z for $C_{96}H_{133}N_{40}O_{18}^+$

$[M + H]^+$ calcd 2134.07, found 2134.06

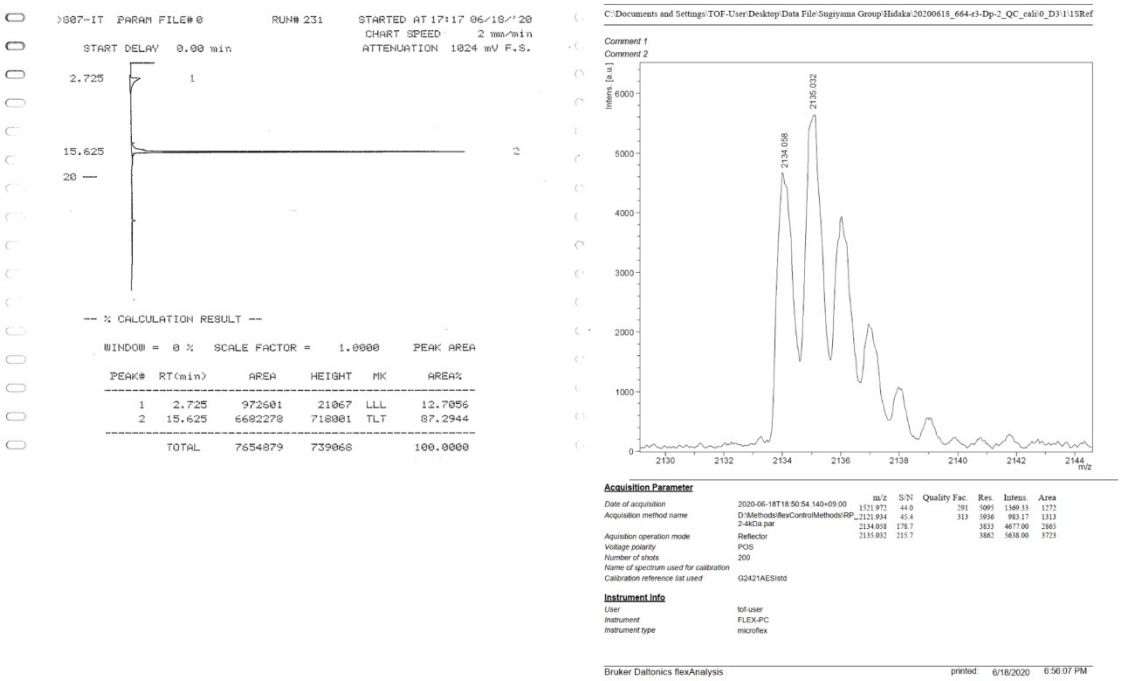
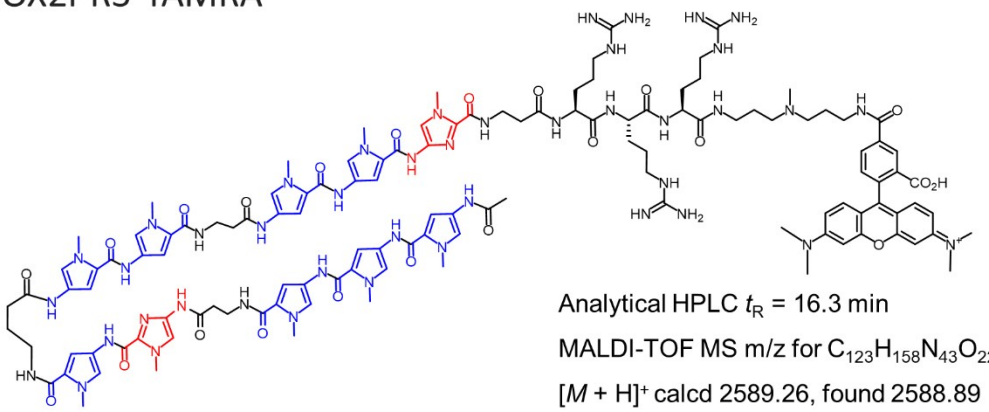


Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

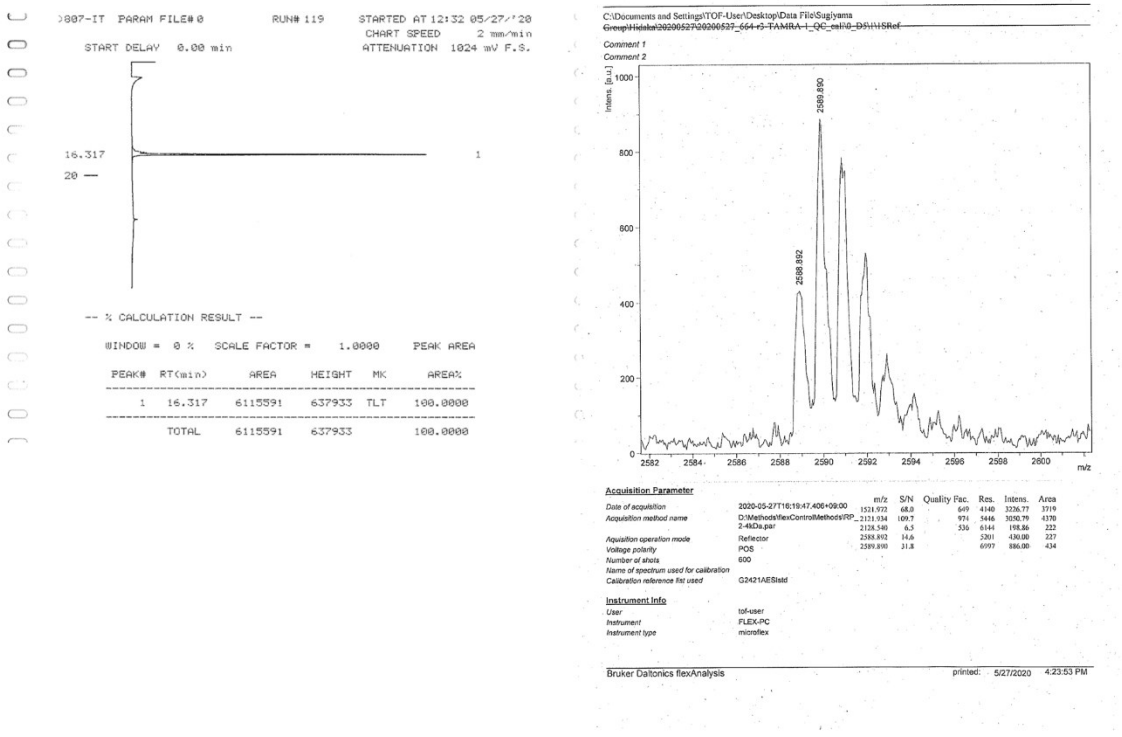
2. SOX2i-R3-TAMRA



Analytical HPLC t_R = 16.3 min

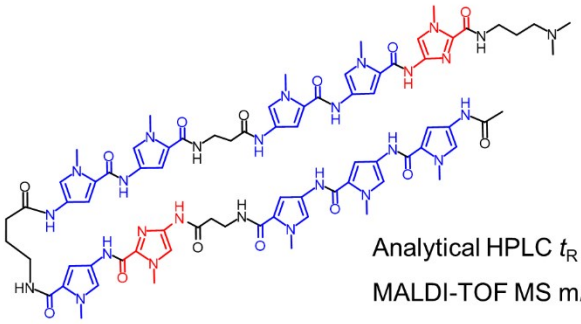
MALDI-TOF MS m/z for $C_{123}H_{158}N_{43}O_{22}^+$

$[M + H]^+$ calcd 2589.26, found 2588.89



(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

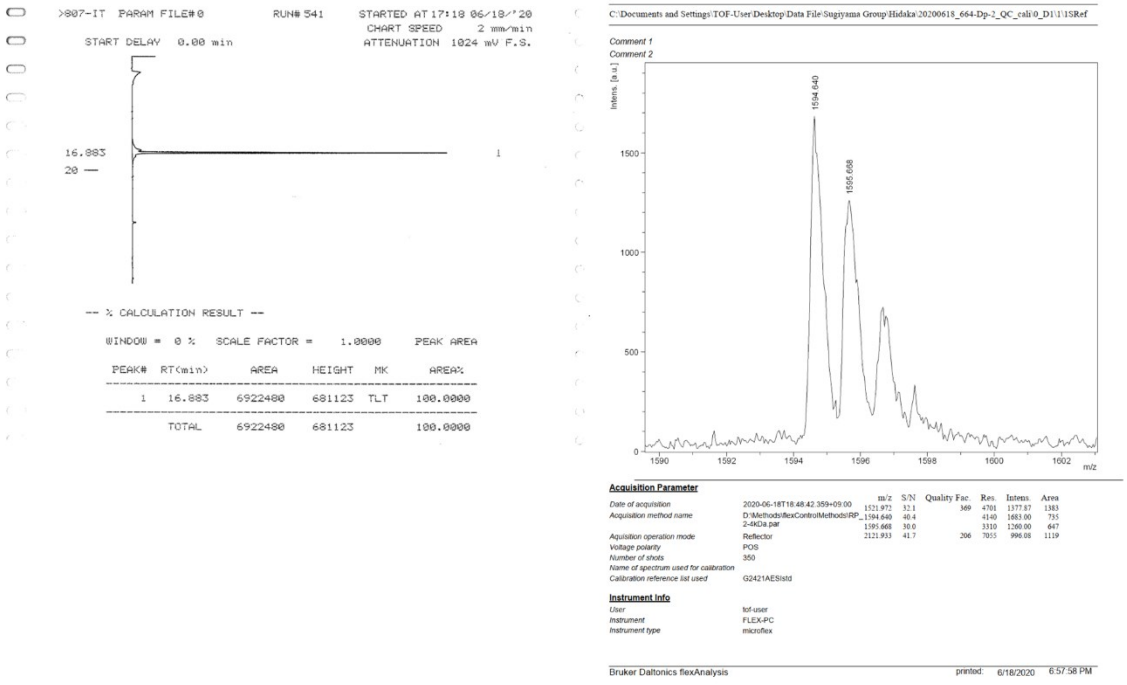
3. SOX2i



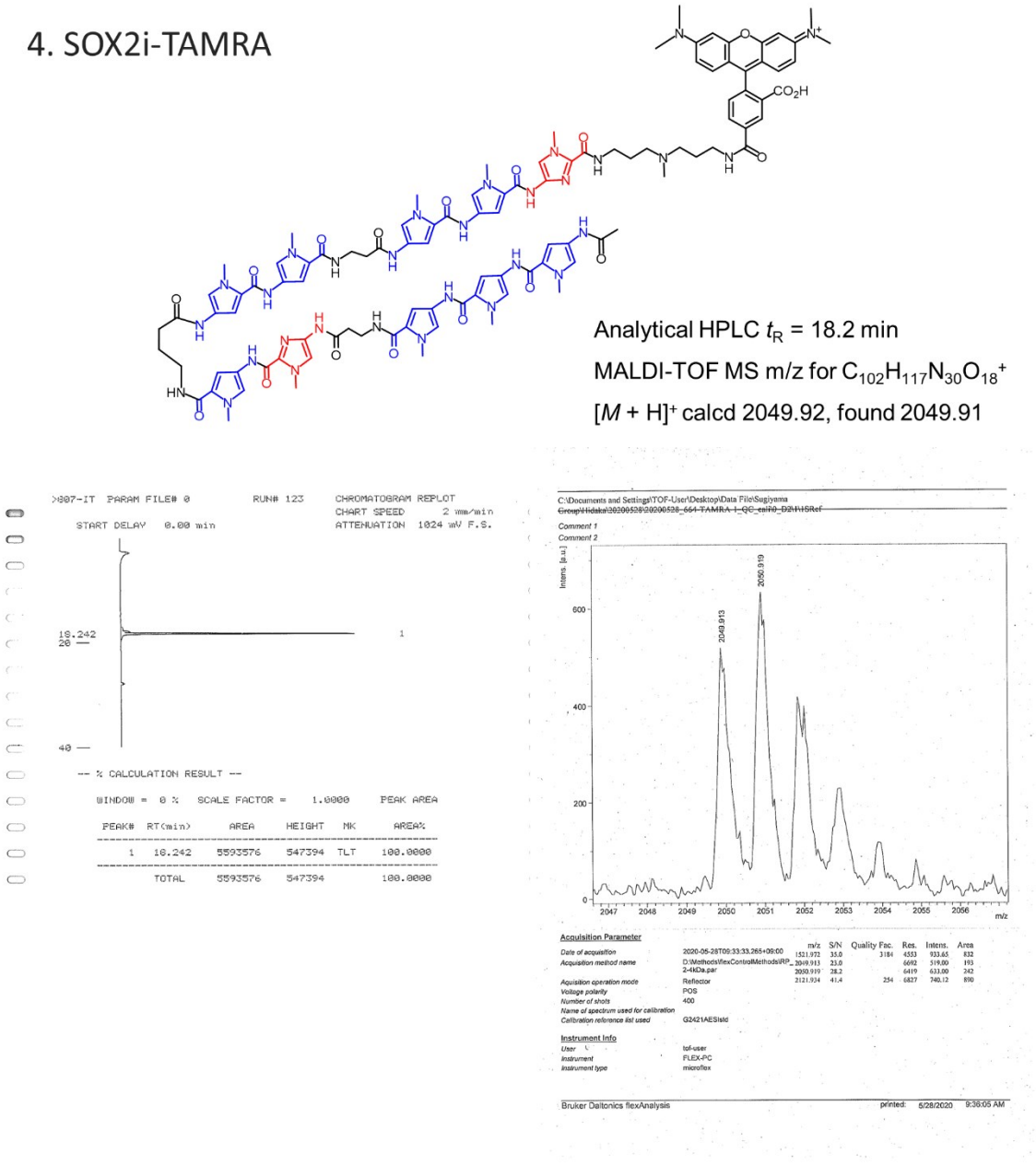
Analytical HPLC t_R = 16.9 min

MALDI-TOF MS m/z for $C_{75}H_{92}N_{27}O_{14}^+$

$[M + H]^+$ calcd 1594.73, found 1594.64

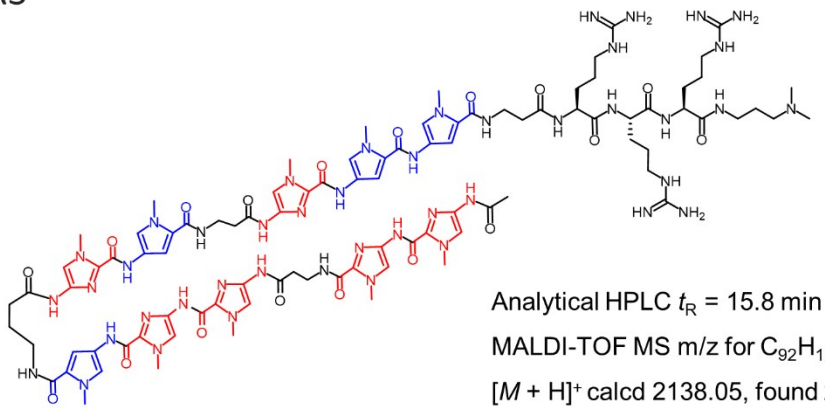


(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.



(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

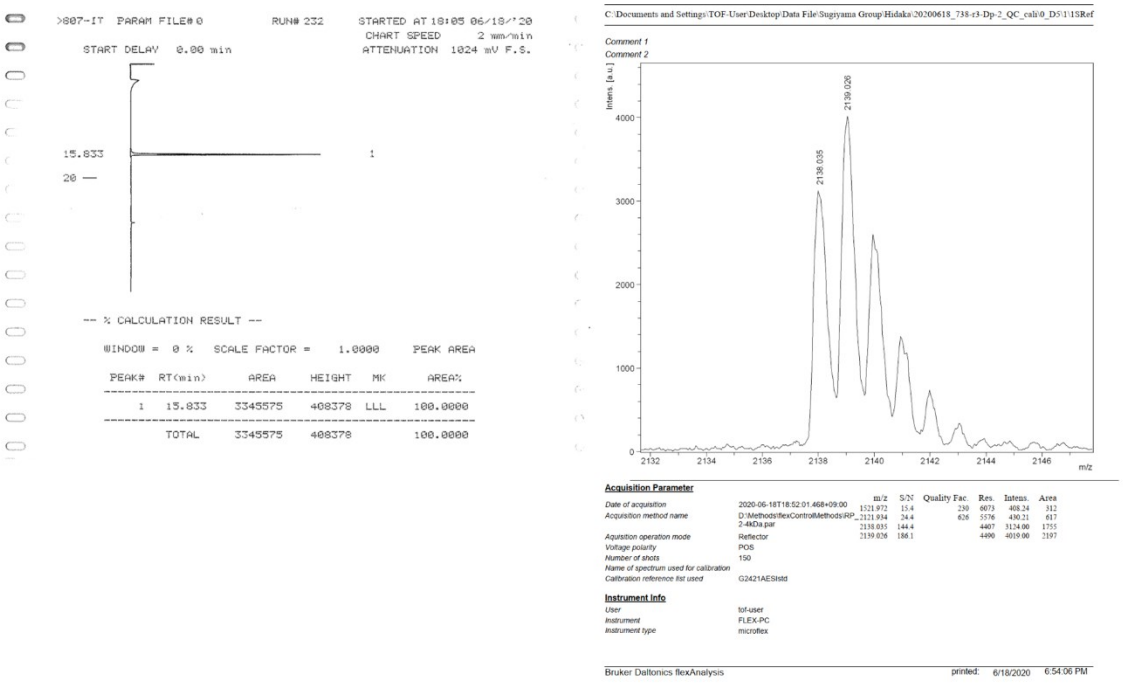
5. Ctrl-R3



Analytical HPLC t_R = 15.8 min

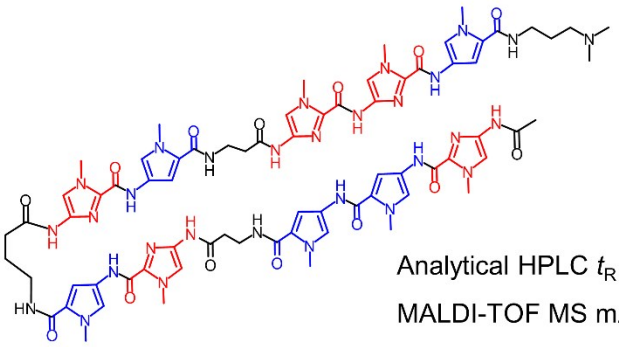
MALDI-TOF MS m/z for $C_{92}H_{129}N_{44}O_{18}^+$

$[M + H]^+$ calcd 2138.05, found 2138.04



(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

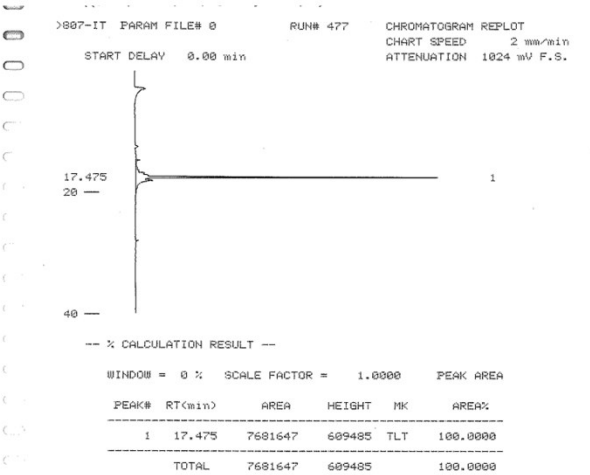
6. AP2i

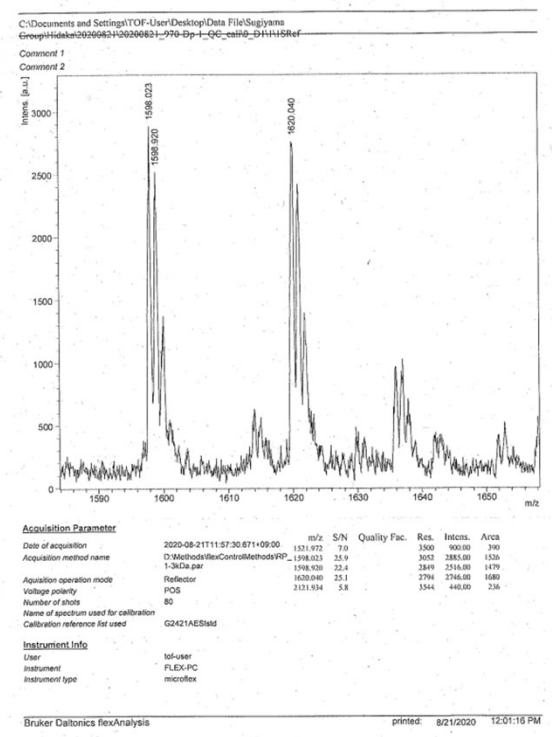


Analytical HPLC t_R = 17.5 min

MALDI-TOF MS m/z for $C_{72}H_{89}N_{30}O_{14}^+$

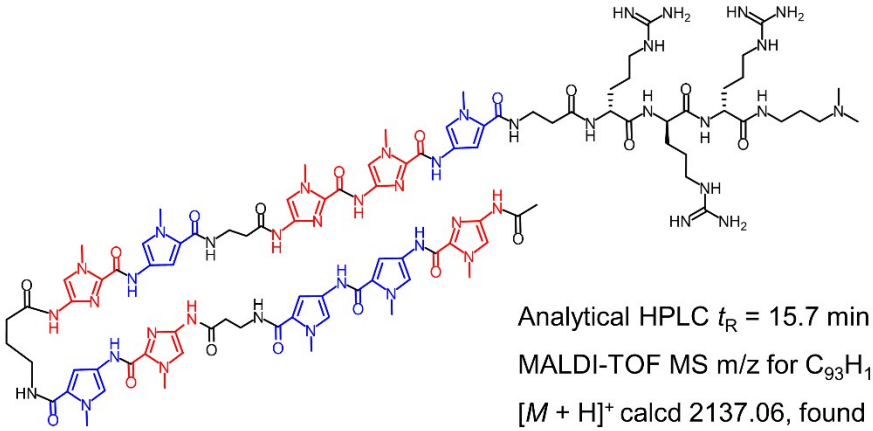
$[M + H]^+$ calcd 1597.72, found 1598.02





(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

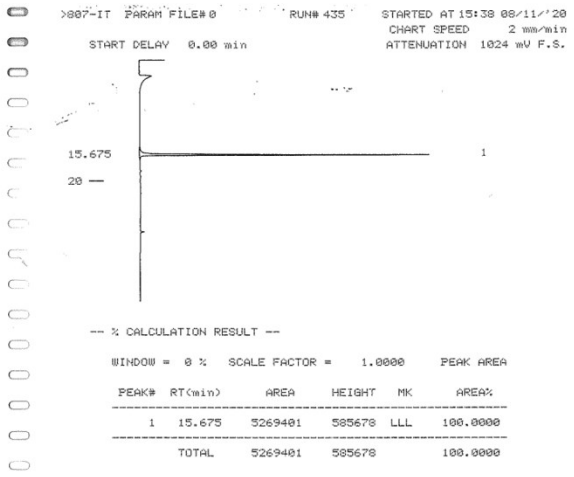
7. AP2i-R3



Analytical HPLC t_R = 15.7 min

MALDI-TOF MS m/z for $C_{93}H_{130}N_{43}O_{18}^+$

$[M + H]^+$ calcd 2137.06, found 2136.89



>807-IT PARAM FILE# 0 RUN# 435 STARTED AT 15:38 08/11/'20
CHART SPEED 2 mm/min
START DELAY 0.00 min ATTENUATION 1024 mV F.S.

15.675

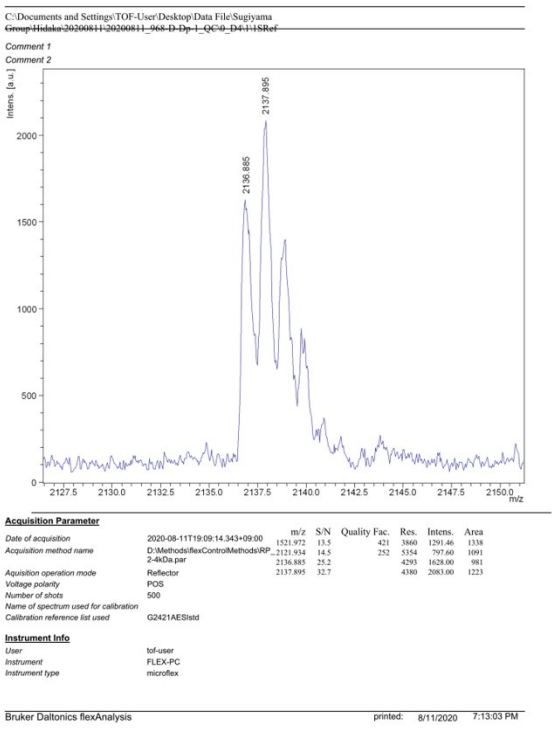
20

1

-- % CALCULATION RESULT --

WINDOW = 0 % SCALE FACTOR = 1.0000 PEAK AREA

PEAK#	RT(min)	AREA	HEIGHT	MK	AREA%
1	15.675	5269401	585678	LLL	100.0000
	TOTAL	5269401	585678		100.0000



C:\Documents and Settings\TOF-User\Desktop\Data File\Sugiyama
Group\Hidaka\20200811-20200811_968-D-Dp-1_QC-0_D4-1-1SRef

Comment 1
Comment 2

Intens. [a.u.]
2000
1500
1000
500
0
2136.885
2137.895
2127.5 2130.0 2132.5 2135.0 2137.5 2140.0 2142.5 2145.0 2147.5 2150.0
m/z

Acquisition Parameter

Date of acquisition	2020-08-11T19:09:14.343+09:00
Acquisition method name	D:\Methods\flexControl\Methods\RP_2-4kDa.par
Aquisition operation mode	Reflector
Voltage polarity	POS
Number of shots	500
Name of spectrum used for calibration	
Calibration reference list used	G2421AESIstd

m/z	S/N	Quality Fac.	Res.	Intens.	Area
1521.972	13.5	421	3860	1291.46	1338
2121.934	14.5	252	5354	797.60	1091
2136.885	25.2		4293	1628.00	981
2137.895	32.7		4380	2083.00	1223

Instrument Info

User	tof-user
Instrument	FLEX-PC
Instrument type	microflex

Bruker Daltonics flexAnalysis printed: 8/11/2020 7:13:03 PM

(Continued) Figure S3. Chemical structure, HPLC chart and mass spectra of compound 1-7.

Primers used for qPCR

Table S1. The primer list for qPCR experiments

primer	Sequence
HPRT1 forward	5’- GGCGAACCTCTCGGCTTTC
HPRT1 reverse	5’- TCATCACTAATCACGACGCCA
SOX2 forward	5’- GGGAAATGGGAGGGGTGCAAAAGAGG
SOX2 reverse	5’- TTGCGTGAGTGTGGATGGGATTGGTG
18S forward	5’- AAACGGCTACCACATCCAAG
18S reverse	5’- CCTCCAATGGATCCTCGTTA
Nanog forward	5’- AATACCTCAGCCTCCAGCAGATG
Nanog reverse	5’- TGCGTCACACCATTGCTATTCTTC
FBXO15 forward	5’- TGGCTGTGACAGACTCATTCGG
FBXO15 reverse	5’- GATAGTAGCCGAGCCTAATGTGC
HER2 forward	5’- GAGCACCCAAGTGTGCAC
HER2 reverse	5’- TTGGTTGTGAGCGATGAG
ACTB forward	5’- CAATGTGGCCGAGGACTTTG
ACTB reverse	5’- CATTCTCCTTAGAGAGAAGTGG

References

1. Y. Sawatani, G. Kashiwazaki, A. Chandran, S. Asamitsu, C. Guo, S. Sato, K. Hashiya, T. Bando, H. Sugiyama, *Bioorganic Med. Chem.* 2016, **24**, 3603.
2. J. Syed, G. N. Pandian, S. Sato, J. Taniguchi, A. Chandran, K. Hashiya, T. Bando, H. Sugiyama, *Chem. Biol.* 2014, **21**, 1370.