Supporting Information

Metalloprotein-Catalyzed Click Reaction for In Situ Generation of a Potent Inhibitor

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Contents

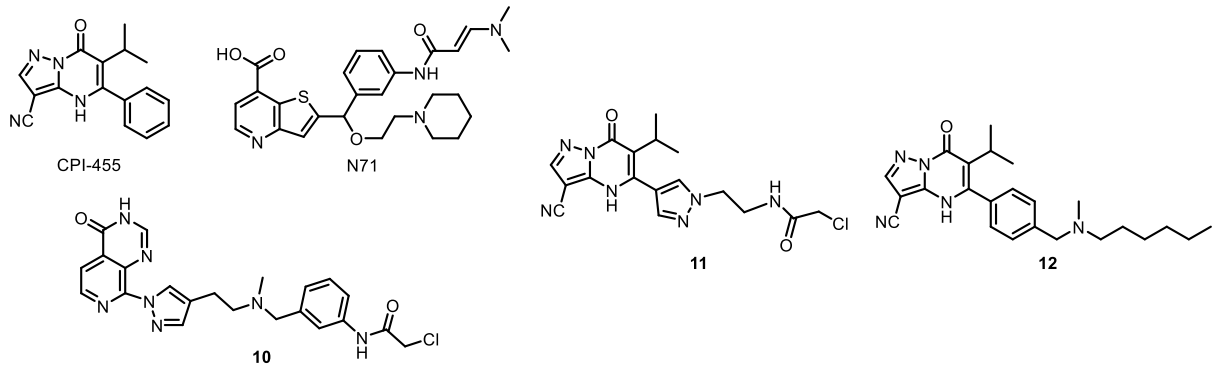


Figure S1. Structures of representative reported KDM5 inhibitors.

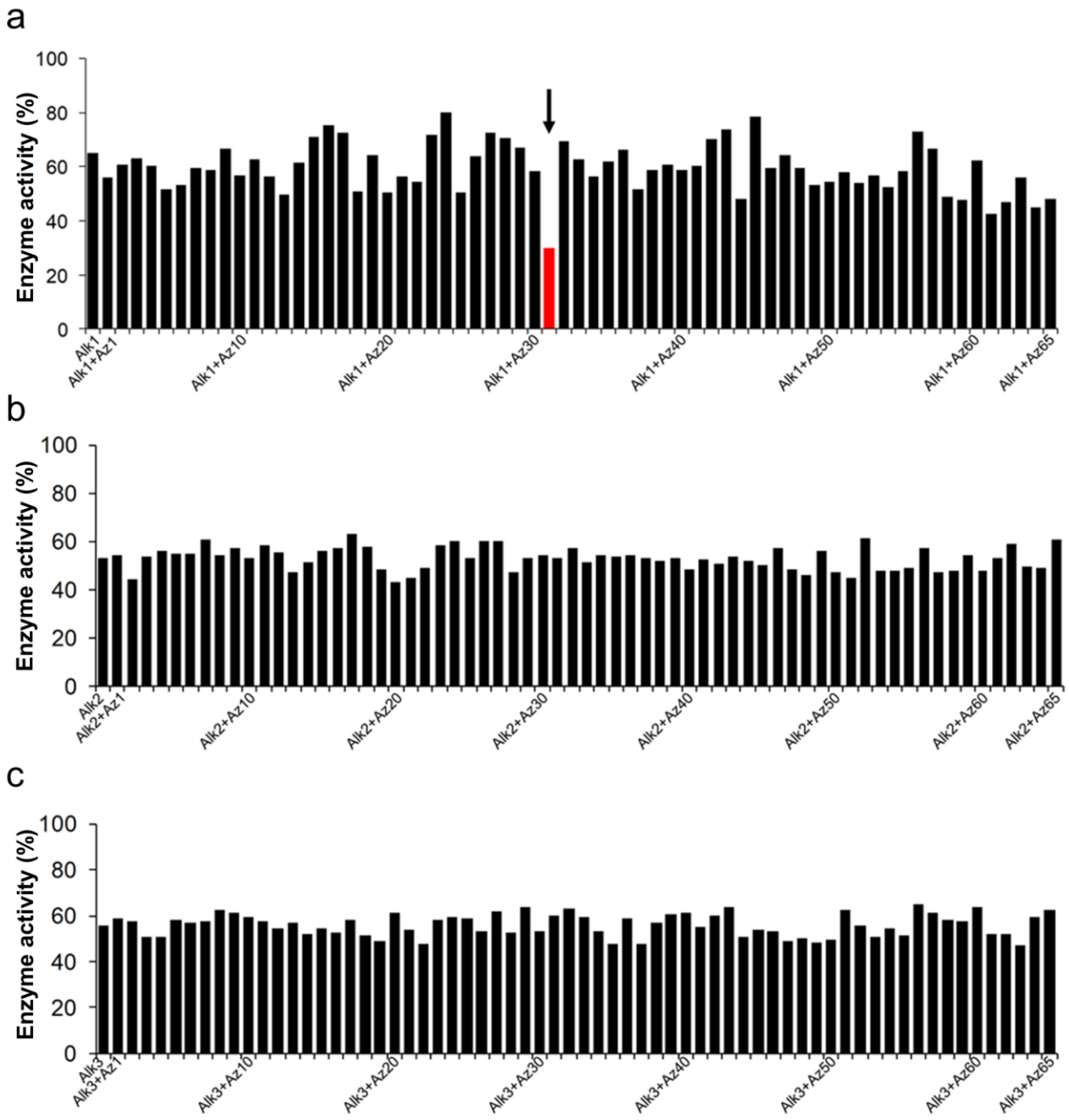


Figure S2. In situ click chemistry screenings for hit alkyne/azide combinations by KDM5C assay using AlphaLISA screening systems. The enzyme activities were evaluated in the presence of **Alk1** (150 μM) (a), **Alk2** (400 μM) (b), or **Alk3** (200 μM) (c) without azide or with **Az1**–**65** (125 μM). The hit was the combination of **Alk1** and **Az31** (arrow).

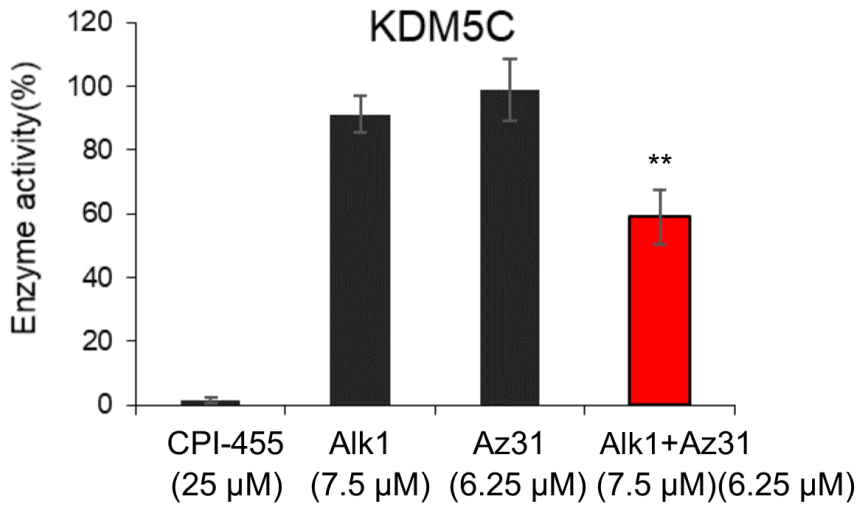


Figure S3. KDM5C activities after in situ click chemistry. The %KDM5C activities were evaluated by AlphaLISA screening assay. Values were calculated from three independent determinations. Bars represent means ± SD from three independent experiments: $^{**}P < 0.01$. ANOVA and Bonferroni-type multiple *t* test results indicated a significant difference between **Alk1** or **Az31** alone and the combination of **Alk1** with **Az31**.

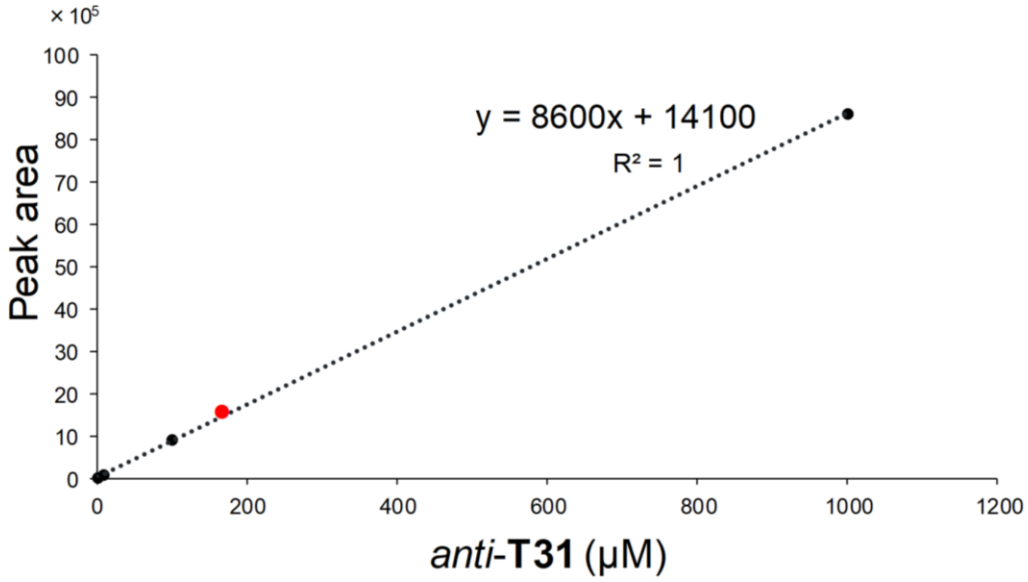


Figure S4. Standard curve of concentration of *anti*-T31 (1000 μM, 100 μM, 10 μM, 1 μM) versus peak area in HPLC analysis, and determination of the concentration of the in situ product. The red circle indicates the in situ product. From these results, the product yield was calculated to be 27%.

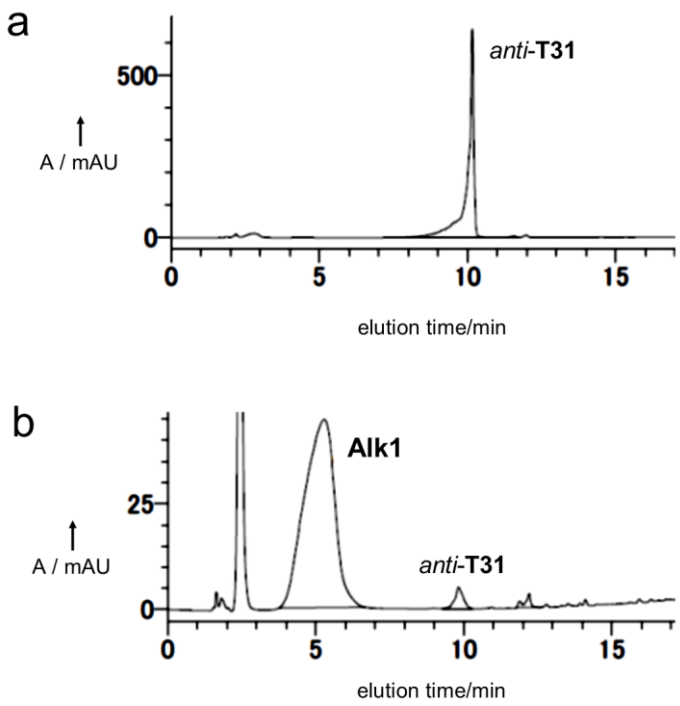


Figure S5. HPLC analysis for in situ click products in KDM5C with an excess of $MnCl_2$. (a) Authentic sample of *anti*-**T31** (10.16 min). (b) Analysis of in situ reaction products in the presence of KDM5C (150 nM) with an excess of $MnCl_2$. The peak corresponding to *anti*-**T31** was observed (9.82 min). The peak corresponding to **AlK1** was also observed (5.29 min).

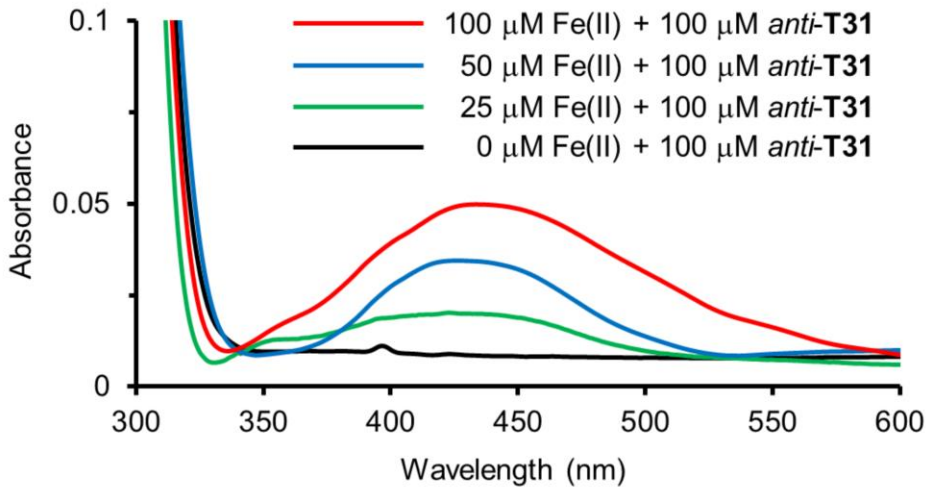


Figure S6. UV-vis absorption spectra of *anti*-**T-31** (100 μM) in the presence or absence of Fe(II) (0, 25, 50, or 100 μM).

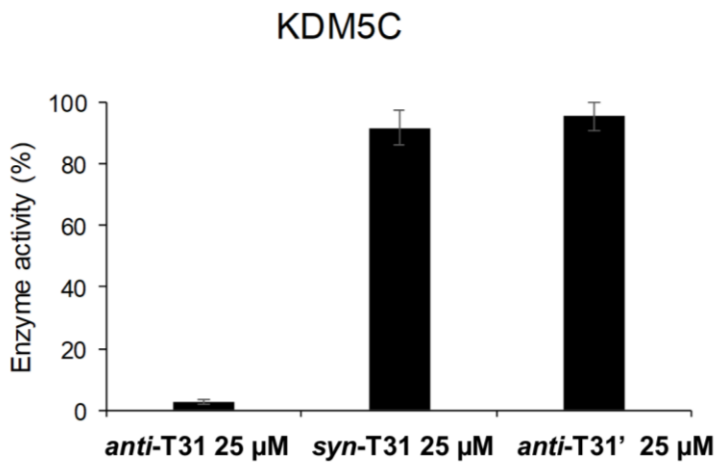


Figure S7. Evaluation of KDM5C-inhibitory activities of *anti*-**T31**, *syn*-**T31**, and *anti*-**T31'** (25 μM).

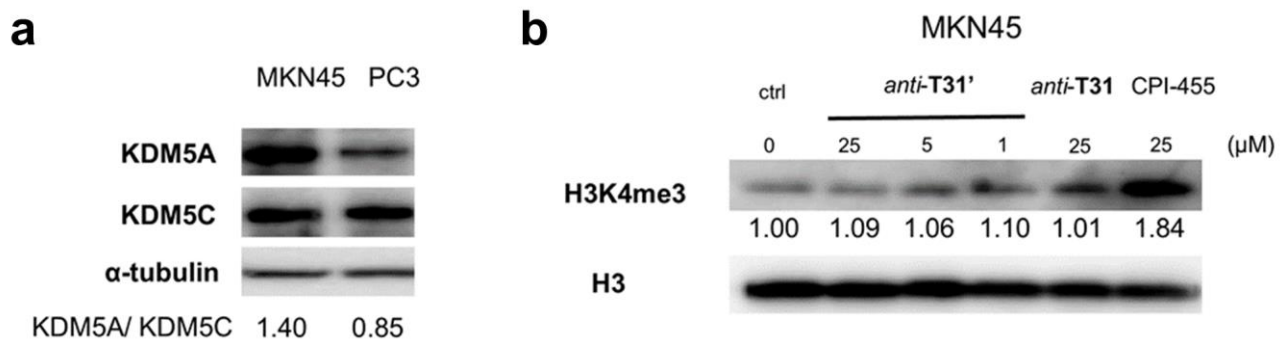
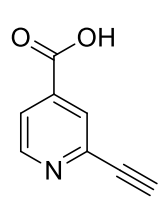
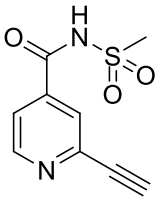


Figure S8. Western blot analysis of KDM5A, KDM5C, and H3K4me3. (a) Western blot detection of KDM5A, KDM5C and α-tubulin in MKN45 and PC3 cells. Values of the KDM5A/KDM5C ratio determined by optical density measurement of the blots are shown. (b) Western blots showing H3K4me3 and H3 levels in MKN45 cells treated with *anti*-**T31'**, *anti*-**T31** and CPI-455. The methylation levels were analyzed after 48 h incubation with the inhibitors. Values of the H3K4me3/H3 ratio determined by optical density measurement of the blots are shown.

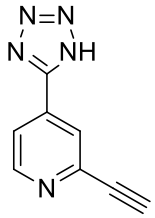
Table S1. *In vitro* KDM5C-inhibitory activities of Alk1–3.



Alk1



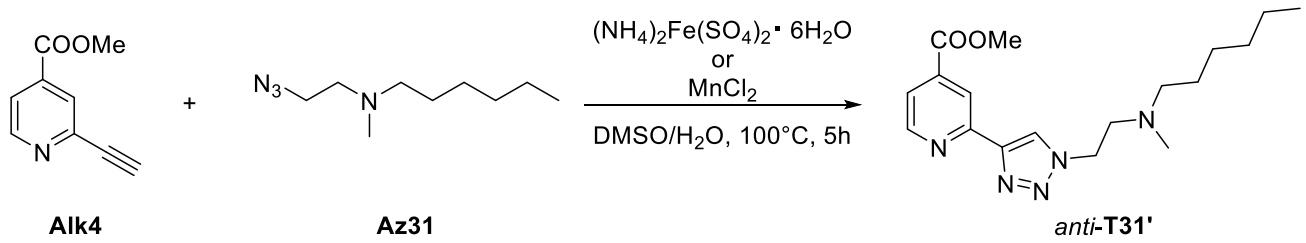
Alk2



Alk3

Compound	IC_{50} (μM)
Alk1	161±4.00
Alk2	386±13.0
Alk3	198±0.167

Table S2. Azide-alkyne cycloaddition in the presence of $(NH_4)_2Fe(SO_4)_2$ or $MnCl_2$.[a]



Alk4 **Az31** *anti*-**T31'**

entry	$(NH_4)_2Fe(SO_4)_2$ (mM)	$MnCl_2$ (mM)	Yield (%) [a]
1	-	-	15
2	2	-	13
3	10	-	36
4	15	-	44
5	20	-	52
6	40	-	65
7	-	20	57

[a] The % yields were determined by HPLC analysis.

Table S3. Alpha screen assay components.

Reagents	Supplier	Code
HEPES	Nacalai	17514-44
Tween20	Nacalai	01864-94
BSA	Nacalai	28351-85
α-Ketoglutaric acid potassium salt (2-OG)	Sigma	K2000
Ammonium iron (II) sulfate hexahydrate (Fe(II))	Sigma	215406
(+) Sodium L-ascorbate	Sigma	11140
KDM2A (FBXL11)	BPS	50156
KDM2B (FBXL10)	BPS	50120
KDM3A (JMJD1A)	BPS	50130
KDM4A (JMJD2A)	BPS	50123
KDM4B (JMJD2B)	BPS	50104
KDM4C (JMJD2C)	BPS	50105
KDM5A (JARID1A)	BPS	50110
KDM5B (JARID1B)	BPS	50121
KDM5C (JARID1C)	BPS	50112
KDM6B (JMJD3)	BPS	50115
KDM7B (PHF8)	BPS	50131
AlphaLISA anti-H3K4me2-1 acceptor beads	PerkinElmer	AL116C
AlphaLISA anti-H3K36me2 acceptor beads	PerkinElmer	AL123C
AlphaLISA anti-H3K27me2-1 acceptor beads	PerkinElmer	AL121C
AlphaLISA anti-H3K9/27 acceptor beads	PerkinElmer	AL138C
Alphascreen Protein A Acceptor beads	PerkinElmer	6760137
Alpha streptavidin donor beads	PerkinElmer	676002
AlphaLISA 5X Epigenetics Buffer 1 Kit	PerkinElmer	AL008
H3(1-21)K4(Me3)-GGK(Biotin)	Anaspec, Inc.	AS64192
H3(1-21)-K9(Me2)-GGK(Biotin)	Anaspec, Inc.	AS64359
H3(1-21)K9(Me1)-GGK(Biotin)	Anaspec, Inc.	AS64358
H3(21-44)-K36(Me3)-GK(Biotin)	Anaspec, Inc.	AS64441
H3(21-44)-K27(Me3)-GK(Biotin)	Anaspec, Inc.	AS64367
H3(21-44)-K36(Me2)-GK(Biotin)	Anaspec, Inc.	AS64442
Anti-H3K9Me1	Abcam	Ab8896
Anti-H3K36Me1	Abcam	Ab9048

Table S4. Conditions for Alpha screen assays.

Enzyme	[E]/nM	Peptide substrates	Peptide/μM	Acceptor beads	Antibody
KDM2A	15.0	H3(21-44)-K36(Me2)-GK(Biotin)	0.10	Protein A	Anti-H3K36Me1 (0.4 μg/mL)
KDM2B	20.0	H3(21-44)-K36(Me2)-GK(Biotin)	0.10	Protein A	Anti-H3K36Me1 (0.4 μg/mL)
KDM3A	1.50	H3(1-21)K9(Me1)-GGK(Biotin)	0.10	anti-H3K9/27	—
KDM4A	10.0	H3(21-44)-K36(Me3)-GK(Biotin)	0.10	anti-H3K36me2	—
KDM4B	10.0	H3(21-44)-K36(Me3)-GK(Biotin)	0.10	anti-H3K36me2	—
KDM4C	10.0	H3(21-44)-K36(Me3)-GK(Biotin)	0.10	anti-H3K36me2	—
KDM5A	120	H3(1-21)K4(Me3)-GGK(Biotin)	0.10	anti-H3K4me2-1	—
KDM5B	280	H3(1-21)K4(Me3)-GGK(Biotin)	1.0	anti-H3K4me2-1	—
KDM5C	280	H3(1-21)K4(Me3)-GGK(Biotin)	1.0	anti-H3K4me2-1	—
KDM6B	4.00	H3(21-44)-K27(Me3)-GK(Biotin)	0.10	anti-H3K27me2-1	—
KDM7B	20.0	H3(1-21)K9(Me2)-GGK(Biotin)	0.10	Protein A	Anti-H3K9Me1 (0.4 μg/mL)

Table S5. Reagents used for western blotting analysis.

Reagents	Supplier	Code	Dilution for probing [a]
Primary antibodies			
rabbit polyclonal H3K4me3 antibody	Abcam	ab8580	1:5000 dilution
rabbit polyclonal H3K4me2 antibody	CST	9725	1:5000 dilution
rabbit polyclonal H3K4me1 antibody	Abcam	ab8895	1:5000 dilution
rabbit polyclonal H3K4me0 antibody	BPS	25258	1:5000 dilution
rabbit polyclonal H3K9me3 antibody	Abcam	ab8898	1:1000 dilution
rabbit monoclonal H3K27me3 antibody	Abcam	ab192985	1:1000 dilution
rabbit polyclonal H3K36me2 antibody	Abcam	ab9049	1:1000 dilution
rabbit polyclonal histone H3 antibody	Abcam	ab1791	1:200000 dilution
rabbit monoclonal KDM5A antibody	CST	3876	1:1000 dilution
rabbit monoclonal KDM5C antibody	CST	5361	1:1000 dilution
mouse monoclonal α-tubulin antibody	sigma	T9026	1:1000 dilution
Secondary antibodies			
ECL rabbit IgG, HRP-linked whole antibody	GE Healthcare Life Science	NA934	1:2500 dilution
ECL mouse IgG, HRP-linked whole antibody	GE Healthcare Life Science	NA931	1:5000 dilution
HRP Substrate			
Immobilon™ Western Chemiluminescent HRP Substrate	Millipore	P90718	

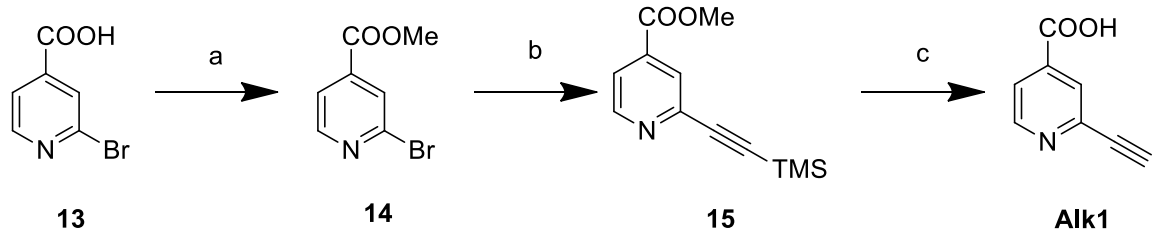
Chemical synthesis

General methods.

Melting points were determined using a Yanagimoto micro melting point apparatus or a Büchi 545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance spectra (^{1}H NMR: 300 MHz) and carbon nuclear magnetic resonance spectra (^{13}C NMR: 75 MHz) were recorded on a Bruker AVANCE300 AV spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to the internal standard, tetramethylsilane. Electrospray ionization (ESI) mass spectrometry measurements were performed on a Bruker HCT plus mass spectrometer. Reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Kishida Kagaku, Kanto Kagaku, Wako Pure Chemical Industries or Nacalai Tesque, and used without purification. Flash column chromatography was performed using silica gel (particle size 200–440 mesh) supplied by Toyotakako Silica Gel (#AP300D). HPLC was performed with a Shimadzu instrument equipped with a COSMOSIL packed column (5C18-AR-II, 4.6 ID × 150 mm, Nacalai) for analysis and the eluents were water containing 0.1% TFA (A) and MeCN containing 0.1% TFA (B). The conditions for analytical HPLC were as follows; flow rate 1.0 mL/min, detection wavelength 254 nm, gradient A/B 0 to 20 min (90/10 to 10/90), 20 to 30 min (10/90), 30 to 40 min (10/90 to 90/10).

Synthesis of 2-ethynylisoniconinic acid (Alk1)

Scheme S1. Synthesis of Alk1.[a]



[a] Reagents and conditions: (a) EDCI·HCl, CH_2Cl_2, MeOH, rt, 87%; (b) trimethylsilylacetylene, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, MeCN, rt, 70%; (c) NaOH, H_2O, MeOH, rt, 93%.

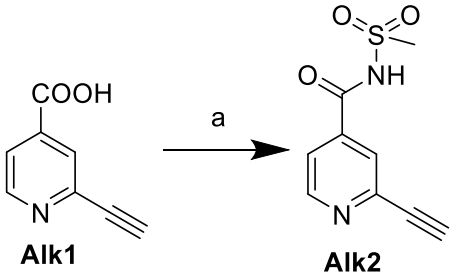
*Step 1. Preparation of methyl 2-bromoisonicotinate (**14**).*

To a solution of 2-bromoisonicotinic acid **13** (1.46 g, 7.23 mmol) in CH_2Cl_2 (10.0 mL) and MeOH (15.0 mL) was added EDCI·HCl (1.36 g, 7.20 mmol) at 0 °C. The mixture was stirred for 20 h at room temperature, then concentrated in vacuo, and the residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/10) to give 1.35 g (87%) of **14** as colorless crystals: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.52 (1H, dd, *J* = 4.2, 0.6 Hz), 8.02 (1H, t, *J* = 6.6 Hz), 7.80 (1H, dd, *J* = 3.9, 0.6 Hz), 3.97 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 151.0, 144.3, 141.2, 129.2, 123.3, 54.40; MS (ESI) m/z 215.7, 217.7 (MH^+).

*Step 2: Preparation of 4-trimethylsilylethynylpyridine-2-carboxylic acid methyl ester (**15**).*
To a solution of **14** (216 mg, 1.00 mmol), $PdCl_2(PPh_3)_2$ (36.0 mg, 51.2 μmol) and CuI (19.0 mg, 100 μmol) in Et_3N (200 μL) and MeCN (2.00 mL) was added (trimethylsilyl)acetylene (165 μL, 1.17 mmol) under argon. The mixture was stirred for 3 h at room temperature, then concentrated in vacuo, and the residue was diluted with Et_2O. Insoluble materials were removed by filtration. The filtrate was concentrated in vacuo and the residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/10) to give 102 mg (70%) of **15** as a yellow oil: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.68 (1H, dd, *J* = 4.2, 0.6 Hz), 7.97 (1H, s), 7.73 (1H, dd, *J* = 4.2, 0.6 Hz), 3.93 (3H, s), 0.26 (9H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 151.0, 144.3, 138.2, 127.1, 122.6, 103.5, 96.86, 53.35, 0.18; MS (ESI) m/z 233.8 (MH^+).

*Step 3: Preparation of 2-ethynylisonicotinic acid (**Alk1**).*
To a solution of **15** (50.0 mg, 215 μmol) in MeOH (2.00 mL) was added 2 N aqueous NaOH (236 μL, 472 μmol) at 0 °C. The mixture was stirred for 12 h at room temperature, then acidified to pH 4.0 with saturated aqueous citric acid solution. The precipitate was collected by filtration, washed with water, and dried in vacuo. The crude solid was recrystallized from MeOH/AcOEt = 2/1 to give 29.4 mg (93%) of **Alk1** as a pale white solid: mp 203–205 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ; ppm) 8.75 (1H, dd, *J* = 5.1, 0.9 Hz), 7.87–7.88 (1H, m), 7.81 (1H, dd, *J* = 4.8, 1.5 Hz), 4.45 (1H, s); ^{13}C NMR (DMSO-d_6, 75 MHz, δ; ppm) 165.0, 151.0, 143.4, 137.9, 126.1, 122.8, 82.64, 81.05; HRMS (EI) calcd. for $C_8H_5NO_2$ 147.0320, found 147.0310.;Anal. calcd. for $C_8H_5NO_2$: C, 65.31; H, 3.43; N, 9.52. found: C, 64.91; H, 3.60; N, 9.28.

Synthesis of 4-[*N*-(methylsulfonyl)]-2-ethynylpyridinecarboxamide (Alk2).
Scheme S2. Synthesis of Alk2.[a]



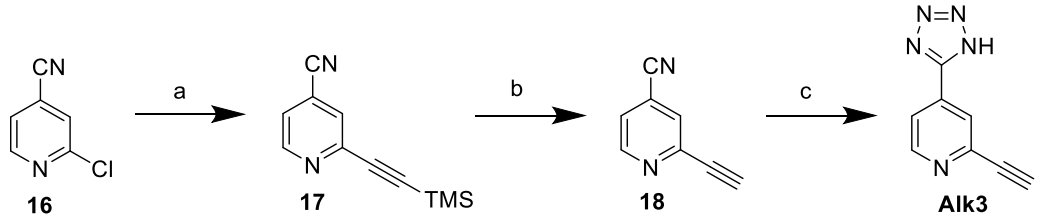
[a] Reagents and conditions: (a) methanesulfonamide, DMAP, EDCI·HCl, CH_2Cl_2, rt, 26%.

To a solution of **Alk1** (147 mg, 1.00 mmol), EDCI·HCl (388 mg, 2.00 mmol), and DMAP (244 mg, 2.00 mmol) in CH_2Cl_2 (15.0 mL) was added methyl sulfonamide (189 mg, 2.00 mmol). The mixture was stirred for 24 h at room temperature, then the reaction was quenched with water. The aqueous layer was acidified with 1 N aqueous HCl solution and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried over Na_2SO_4, filtered, and concentrated in vacuo. The

residue was purified by silica gel flash column chromatography (CH_2Cl_2/methanol = 10/1) to give 56 mg (26%) of **Alk2** as a dark brown solid: mp 141–142 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ; ppm) 8.76 (1H, dd, *J* = 4.5, 0.9 Hz), 8.00 (1H, q, *J* = 0.9 Hz), 7.82 (1H, dd, *J* = 3.6, 1.5 Hz), 4.50 (1H, s), 3.38 (3H, s); ^{13}C NMR (DMSO-d_6, 75 MHz, δ; ppm) 164.6, 151.0, 142.4, 140.0, 125.4, 121.9, 82.41, 81.39, 41.16; MS (ESI) m/z 225.1 (MH^+); HRMS (EI) calcd. for $C_9H_8N_2O_3$ 224.0256, found 224.0256; HPLC t_R = 7.58 min, purity 96.5%.

Synthesis of 4-(2H-tetrazol-5-yl)-pyridine (Alk3).

Scheme S3. Synthesis of Alk3.[a]



[a] Reagents and conditions: (a) trimethylsilylacetylene, $Pd(Ph_3)_2Cl_2$, CuI, Et_3N, MeCN, 60 °C, 98%; (b) TBAF, THF, CH_2Cl_2, 0 °C, 66%; (c) NaN_3, NH_4Cl, DMF, 80 °C, 12%.

*Step 1: Preparation of 4-cyano-2-[2-(trimethylsilyl)ethynyl]pyridine (**17**).*

Compound **17** was prepared from 2-chloro-4-cyanopyridine **16** by using a similar procedure to that described for the preparation of **Alk1** (Step 2), except for the following changes: reaction temperature, 60 °C, silica gel flash column chromatography (AcOEt/*n*-hexane = 1/10): Yield, 1.96 g (98%); a pale yellow solid; mp 46.0–48.0 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.75 (1H, dd, *J* = 4.2, 0.9 Hz), 7.67 (1H, q, *J* = 0.3 Hz), 7.44 (1H, dd, *J* = 3.6, 1.5 Hz), 0.30 (9H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 151.0, 144.6, 128.7, 124.2, 121.0, 115.9, 101.8, 98.78, 0.36; MS (ESI) m/z 201.1 (MH^+).

*Step 2: Preparation of 4-cyano-2-ethynylpyridine (**18**).*

To a solution of **17** (1.96 g, 9.79 mmol) in CH_2Cl_2 (25.0 mL) was added 1 M TBAF in THF (14.0 mL, 14.0 mmol) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C, then the reaction was quenched with water. The organic layer was separated, washed with water, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/3) to give 823 mg of **18** (66%) as a pale yellow solid: mp 104–106 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.76 (1H, dd, *J* = 4.2, 0.9 Hz), 7.68 (1H, q, *J* = 0.3 Hz), 7.49 (1H, dd, *J* = 3.6, 1.5 Hz), 3.32 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 151.1, 143.8, 128.8, 124.7, 121.1, 115.7, 81.10, 80.05; MS (ESI) m/z 129.2 (MH^+).

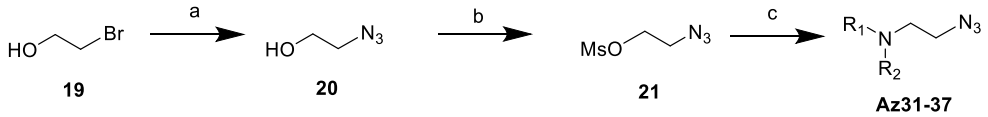
*Step 3: Preparation of 4-(2H-tetrazol-5-yl)pyridine (**Alk3**).*

To a solution of **18** (396 mg, 3.00 mmol) in anhydrous DMF (20.0 mL) was added NaN_3 (195 mg, 3.00 mmol) and NH_4Cl (162 mg, 3.00 mmol) under nitrogen. The mixture was stirred for 12 h at

80 °C under nitrogen, then cooled to room temperature, diluted with AcOEt, and washed with 4 N aqueous HCl solution twice. The organic layer was separated, washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 2/1) to give 62.0 mg (12%) of **Alk3** as a brown solid: mp 210–212 °C; ^{1}H NMR (DMSO-d_6, 300 MHz, δ; ppm) 8.79 (1H, dd, J = 4.2, 0.9 Hz), 8.10 (1H, q, J = 0.6 Hz), 8.00 (1H, dd, J = 3.3, 1.8 Hz), 4.51 (1H, s); ^{13}C NMR (DMSO-d_6, 75 MHz, δ; ppm) 154.7, 151.5, 142.7, 132.9, 124.1, 120.7, 82.35, 81.41; MS (ESI) m/z 172.1 (MH^+); HRMS (EI) calcd. for $C_8H_5N_5$ 171.0537, found 171.0545; HPLC t_R = 7.45 min, purity 96.0%.

Synthesis of azides Az31–37.

Scheme S4. Synthesis of Az31-37.[a]



[a] Reagents and conditions: (a) NaN_3, H_2O, reflux; (b) MsCl, Et_3N, CH_2Cl_2,rt, 34%; (c) amine, K_2CO_3, MeCN, reflux, 16-53% .

*Step 1: Preparation of 2-azidoethanol (**20**).*

To a solution of 2-bromoethanol **19** (581 µL, 8.1 mmol) in water (5.00 mL) was added NaN_3 (647 mg, 10.0 mmol). The mixture was stirred for 12 h at 100 °C, then cooled to room temperature, and saturated with solid NaCl. The resulting mixture was extracted with Et_2O. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to give compound **20** as a crude product. This was used in the next reaction without further purification.

*Step 2: Preparation of 1-mesyl-2-azidoethanol (**21**).*

To a solution of crude **20** in dry CH_2Cl_2 (10.0 mL) were successively added Et_3N (1.74 mL, 12.5 mmol) and methanesulfonyl chloride (967 µL, 12.5 mmol) at 0 °C. The mixture was stirred for 12 h at room temperature, filtered, and concentrated in vacuo. The residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 2/3) to give 560 mg of **21** (34%) as colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 4.26 (2H, t, J = 5.7 Hz), 3.52 (2H, t, J = 4.5 Hz), 3.01 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 67.88, 49.55, 37.17.

*Step 3: Preparation of 2-azido-1-ethylhexylmethylamine (**Az31**).*

To a suspension of **21** (189 mg, 1.15 mmol) and K_2CO_3 (482 mg, 3.50 mmol) in MeCN (5 mL) was added *N*-hexylmethylamine (151 µL, 1.00 mmol). The mixture was stirred for 12 h at 90 °C, then filtered, and the filtrate was concentrated in vacuo. The residue was purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/3) to give 50.0 mg of **Az31** (27%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 3.32 (2H, t, J = 6.0 Hz), 2.58 (2H, t, J = 6.0

Hz), 2.37 (2H, t, *J* = 7.8 Hz), 2.26 (3H, s), 1.42–1.49 (2H, m), 1.26–1.32 (6H, m), 0.89 (3H, t *J* = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 58.14, 56.52, 49.16, 42.34, 31.91, 27.36, 27.16, 22.74, 14.14; MS (ESI) m/z 185.1 (MH^+); HRMS (EI) calcd. for $C_9H_{20}N_4$ 185.1764, found 185.1761; HPLC t_R = 10.2 min, purity 97.6%.

Azides **Az32**–**37** were prepared from the corresponding amines using the same procedure described for **Az31**.

*2-Azido-1-benzylmethylethylamine (**Az32**).*

Yield 53%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 7.28–7.40 (5H, m), 3.61 (2H, s), 3.36 (2H, t, *J* = 6.0 Hz), 2.69 (2H, t, *J* = 6.0 Hz), 2.31 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 138.7, 128.9, 128.3, 127.2, 62.57, 56.26, 48.90, 42.15; MS (ESI) m/z 191.2 (MH^+). HPLC t_R = 7.87 min, purity 96.2%.

*2-Azido-1-diethylbutylamine (**Az33**).*

Yield 24%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 3.28 (2H, t, *J* = 6.3 Hz), 2.66 (2H, t, *J* = 6.3 Hz), 2.55 (2H, q, *J* = 6.9 Hz), 2.46 (2H, t, *J* = 7.8 Hz), 1.31–1.44 (4H, m), 1.04 (3H, t, *J* = 0.9 Hz), 0.93 (3H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 53.60, 52.85, 49.51, 47.81, 29.37, 20.55, 14.02, 11.80; MS (ESI) m/z 171.3 (MH^+). HPLC t_R = 22.9 min, purity 95.8%.

*2-Azido-1-ethylpentylmethylamine (**Az34**).*

Yield 37%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 3.32 (2H, t, *J* = 6.3 Hz), 2.57 (2H, t, *J* = 6.0 Hz), 2.36 (2H, t, *J* = 6.0 Hz), 2.26 (3H, s), 1.42–1.52 (2H, m), 1.28–1.32 (4H, m), 0.90 (3H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 57.95, 56.38, 49.00, 42.18, 29.52, 26.92, 22.57, 14.00; MS (ESI) m/z 171.3 (MH^+). HPLC t_R = 22.7 min, purity 98.5%.

*2-Azido-1-ethylpropylbutylamine (**Az35**).*

Yield 36%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 3.25 (2H, t, *J* = 6.0 Hz), 2.65 (2H, t, *J* = 6.3 Hz), 2.39–2.47 (4H, m), 1.30–1.50 (6H, m), 0.89 (6H, q, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 56.49, 54.20, 53.56, 49.54, 29.38, 20.52, 20.38, 14.01, 11.76; MS (ESI) m/z 185.3 (MH^+). HPLC t_R = 10.2 min, purity 97.6%.

*2-Azido-1-ethyldibutylamine (**Az36**).*

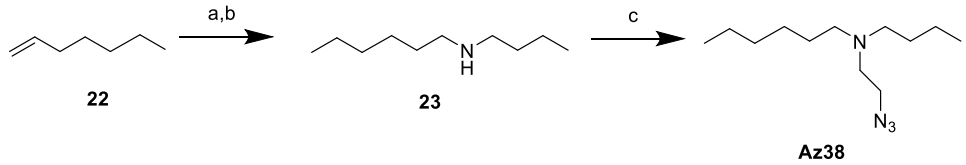
Yield 48%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 3.25 (2H, t, *J* = 6.3 Hz), 2.63 (2H, t, *J* = 6.3 Hz), 2.44 (4H, t, *J* = 7.2 Hz), 1.30–1.43 (8H, m), 0.92 (6H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 54.19, 53.52, 49.53, 29.39, 20.51, 13.99; MS (ESI) m/z 199.3 (MH^+). HPLC t_R = 9.15 min, purity 99.9%.

*2-Azido-1-ethylbutylbenzylamine (**Az37**).*

Yield 16%; a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 7.25–7.39 (5H, m), 3.65 (2H, s), 3.26 (2H, t, *J* = 6.0 Hz), 2.71 (2H, t, *J* = 6.3 Hz), 2.51 (2H, t, *J* = 7.2 Hz), 1.47–1.57 (2H, m), 1.29–1.41 (2H, m), 0.92 (3H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 139.5, 128.7, 128.2, 127.0, 59.09, 54.13, 53.31, 49.43, 29.29, 20.44, 14.01; MS (ESI) m/z 233.3 (MH^+). HPLC t_R = 11.1 min, purity 96.3%.

Synthesis of 2-Azido-1-ethylbutylhexylamine (Az38).

Scheme S5. Synthesis of Az38.[a]



[a] Reagents and conditions: (a) $BH_3{\cdot}SMe_2$, rt ; (b) sodium hypochlorite, *N*-butylamine, THF, 0 °C , 41%; (c) **21**, K_2CO_3, MeCN, reflux, 46%.

*Step 1: Preparation of N-butyl-1-hexanamine (**23**).*

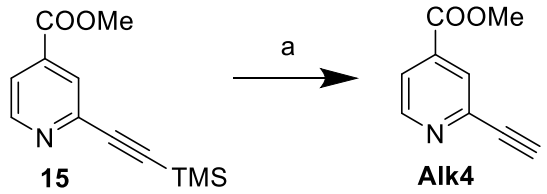
A solution of $BH_3{\cdot}SMe_2$ (1.00 mL, 10.0 mmol) was added dropwise to 1-hexene (5.00 mL, 40.0 mmol) at 0 °C under nitrogen. The mixture was warmed to room temperature and stirred for 12 h to obtain a solution of trialkylborane. A solution of sodium hypochlorite (18.4 mL, 10.0 mmol) was added dropwise to a solution of *N*-butylamine (1.00 mL, 10.0 mmol) in THF (10.0 mL) at 0 °C under nitrogen. This mixture was added quickly to the solution of trialkylborane at 0 °C. The reaction mixture was warmed to room temperature and stirred for 3 h, then acidified to pH 1.0 with 10% aqueous HCl solution, and washed with Et_2O. The free amine was obtained by adding 6 N aqueous NaOH solution to the aqueous layer (pH >13) and extracted with Et_2O. The organic layer was dried over Na_2SO_4 and concentrated to give 650 mg of **23** (41%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 2.56 (2H, t, *J* = 7.2 Hz), 2.56 (1H, t, *J* = 7.5 Hz), 1.26–1.47 (12H, m), 0.83–0.99 (6H, m); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 50.16, 49.81, 32.32, 31.79, 30.15, 27.09, 22.60, 20.52, 14.00; MS (ESI) m/z 158.3 (MH^+).

*Step 2: Preparation of 2-azido-1-ethylbutylhexylamine(**Az38**).*

Compound **Az38** was prepared from compound **23** by using a similar procedure to that described for the preparation of **Az31** (Step 3): Yield, 104 mg (46%); a colorless oil; ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm): 3.26 (2H, t, *J* = 6.0 Hz), 2.64 (2H, t, *J* = 6.3 Hz), 2.44 (2H, t, *J* = 7.2 Hz), 2.44 (2H, t, *J* = 7.2 Hz), 1.40–1.45 (4H, m), 1.27–1.32 (8H, m), 0.87–0.94 (6H, m); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm): 54.67, 54.35, 53.64, 49.68, 31.96, 29.50, 27.29, 27.24, 22.80, 20.70, 14.19; MS (ESI) m/z 227.3 (MH^+); HPLC t_R = 13.3 min, purity 98.3%

Synthesis of 2-ethynylisonicotinate (Alk4).

Scheme S6. Synthesis of Alk4.[a]

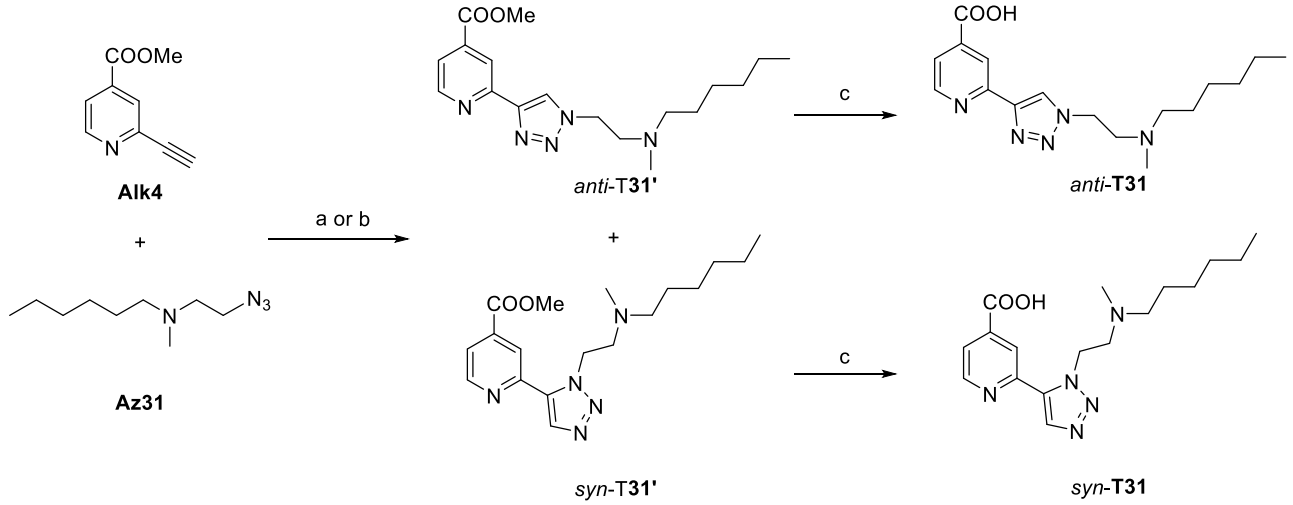


[a] Reagents and conditions: (a) TBAF, THF, 0 °C, 96%

Compound **Alk4** was prepared from 2-chloro-4-cyanopyridine **16** by using a similar procedure to that described for the preparation of **Alk1** (Step 2), except for the following changes: reaction temperature, 0 °C, silica gel flash column chromatography (AcOEt/*n*-hexane = 1/2): Yield 248 mg (96%); a colorless solid; mp 64.5–65.5 °C; ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.75 (1H, d, *J* = 5.1 Hz), 8.03 (1H, q, *J* = 0.6 Hz), 7.81 (1H, dd, *J* = 4.2, 0.9 Hz), 3.97 (3H, s), 3.23 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 165.0, 151.0, 143.4, 137.9, 126.8, 122.6, 82.21, 78.37, 53.00 ; MS (ESI) m/z 162.2 (MH^+); HPLC t_R = 11.7 min, purity 99.4%.

Synthesis of 2-(1-(2-(hexyl(methyl)amino)ethyl)-1*H*-1,2,3-triazol-4-yl)isonicotinic acid (*anti*-T31) and 2-(1-(2-(hexyl(methyl)amino)ethyl)-1*H*-1,2,3-triazol-5-yl)isonicotinic acid (*syn*-T31)

Scheme 7. Synthesis of *anti*-T31 and *syn*-T31.[a]



[a] Reagents and conditions: (a) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, TBTA, *tert*-BuOH, rt, 47% (*anti*-**T31'**); (b) toluene, 100 °C, 26% (*anti*-**T31'**), 29% (*syn*-**T31'**); (c) NaOH, H_2O, MeOH, rt, 21% (*anti*-**T31**), 29% (*syn*-**T31'**).

Synthesis of *anti*-T31

*Step 1: Preparation of methyl 2-(1-(2-(hexyl(methyl)amino)ethyl)-1H-1,2,3-triazol-4-yl)isonicotinate (anti-**T31'**)*

To a suspension of **Alk4** (16.2 mg, 100 μmol) and **Az31** (18.3 mg, 100 μmol) in *tert*-BuOH (2.50 mL) and H_2O (2.50 mL) were added $CuSO_4 \cdot 5H_2O$ (25.0 mg, 100 μmol), sodium ascorbate (20.0 mg, 100 μmol). The mixture was stirred for 12 h at room temperature, then concentrated in vacuo. The residue was poured into water and extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by silica gel flash column chromatography (AcOEt/*n*–hexane = 1/3 to CH_2Cl_2/MeOH = 5/1) to give 16.2 mg of *anti*-**T31'** (47%) as an orange oil; ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.71–8.74 (2H, m), 8.30 (1H, s), 7.78 (1H, dd, *J* = 3.6, 0.6 Hz), 4.52 (2H, t, *J* = 6.3 Hz), 3.99 (3H, s), 2.88 (2H, t, *J* = 6.0 Hz), 2.40 (2H, t, *J* = 7.5 Hz), 2.31 (3H, s), 1.22–1.42 (8H, m), 0.83 (3H, t, *J* = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 165.6, 151.7, 150.2, 147.5, 138.4, 123.3, 121.7, 119.5, 57.98, 57.03, 52.70, 48.57, 42.09, 31.75, 27.21, 27.02, 22.63, 14.02; MS (ESI) m/z 346.4 (MH^+); HRMS(EI) calcd. for $C_{18}H_{27}N_5O_2$ 345.2165, found 346.2237 (MH^+); HPLC t_R = 12.13 min, purity 95.6%.

*Step 2: Preparation of 2-(1-(2-(hexyl(methyl)amino)ethyl)-1H-1,2,3-triazol-4-yl)isonicotinic acid hydrochloride (anti-**T31**)*

To a solution of *anti*-**T31'** (16.2 mg, 47.0 μmol) in MeOH (3.00 mL) was added 2.00 mL of 2 N aqueous NaOH solution. The reaction mixture was stirred for 12 h at room temperature, then acidified to pH 1.0 by adding 1 N HCl. The precipitate was collected by filtration and the filtrate was concentrated in vacuo to give 10.0 mg of *anti*-**T31** (HCl salt) as a colorless solid (25%): ^{1}H NMR (CD_3OD, 300 MHz, δ; ppm) 8.79 (1H, d, *J* = 4.5 Hz), 8.64 (1H, s), 8.61 (1H, s), 7.89 (1H, dd, *J* = 3.6, 1.5 Hz), 4.99 (2H, t, *J* = 6.0 Hz), 3.92 (2H, s), 3.30 (2H, s), 3.02 (3H, s), 1.73 (2H, quin., *J* = 7.8 Hz), 1.38 (6H, m), 0.93 (3H, t, *J* = 7.2 Hz); ^{13}C NMR (CD_3OD, 75 MHz, δ; ppm) 166.0, 150.5, 150.2, 147.6, 139.8, 124.0, 122.4, 119.4, 56.67, 54.24, 44.53, 39.71, 30.92, 25.73, 23.56, 22.03, 12.79; MS (ESI) m/z 332.1(MH^+-HCl); HRMS (EI) calcd. for $C_{17}H_{25}N_5O_2$ 331.2008, found 331.2041; HPLC t_R = 10.10 min, purity 99.6%.

Synthesis of *syn*-T31.

*Step 1: Preparation of methyl 2-(1-(2-(hexyl(methyl)amino)ethyl)-1H-1,2,3-triazol-5-yl)isonicotinate (syn-**T31'**)*

A solution of **Alk4** (123 mg, 0.760 mmol) and **Az31** (210 mg, 1.14 mmol) in 5.00 mL of toluene was stirred at 100 °C for 30 h. The reaction mixture was concentrated in vacuo and the residue was purified by silica gel flash column chromatography (AcOEt/*n*–hexane = 1/1 to AcOEt only) to give 76.0 mg of *syn*-**T31'** (29%) as a yellow oil and 68.0 mg of *anti*-**T31'** (26%) as an orange oil.

syn-**T31'**: ^{1}H NMR ($CDCl_3$, 300 MHz, δ; ppm) 8.79 (1H, dd, *J* = 4.2, 0.9 Hz), 8.12 (1H, t, *J* = 0.9 Hz), 8.02 (1H, s), 7.80 (1H, dd, *J* = 3.6, 1.5 Hz), 4.97 (2H, t, *J* = 7.2 Hz), 3.96 (3H, s), 2.83 (2H, t, *J* = 7.2 Hz), 2.32 (2H, t, *J* = 7.2 Hz), 2.22 (3H, s), 1.15–1.29 (8H, m), 0.82 (3H, t, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz, δ; ppm) 164.9, 150.5, 148.4, 138.7, 135.3, 133.8, 122.2, 122.0, 57.75, 56.84, 53.02, 47.80, 42.34, 31.76, 27.14, 26.98, 22.64, 14.07; MS (ESI) m/z 346.4 (MH^+); HPLC t_R = 12.65 min, purity 95.6%.

*Step 2: Preparation of 2-(1-(2-(hexyl(methyl)amino)ethyl)-1H-1,2,3-triazol-5-yl)isonicotinic acid (syn-**T31**)*

To a solution of *syn*-**T31'** (50.0 mg, 145 µmol) in MeOH (3.00 mL) was added 2.00 mL of 2 N NaOH aq.. The reaction mixture was stirred for 12 h at room temperature, then acidified to pH 4.0 by adding citric acid, and extracted with AcOEt. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered and concentrated in vacuo to give 13.9 mg of *syn*-**T31** as a yellow oil (29%).

syn-**T31**: ^{1}H NMR (CD_3OD, 300 MHz, δ; ppm) 8.92 (1H, dd, *J* = 4.5, 0.6 Hz), 8.43 (1H, s), 8.40 (1H, t, *J* = 0.3 Hz), 7.97 (1H, dd, *J* = 3.6, 1.5 Hz), 5.36 (2H, t, *J* = 6.0 Hz), 3.92 (2H, s), 3.29 (2H, t, *J* = 8.4 Hz), 3.05 (3H, s), 1.75 (2H, quin., *J* = 8.1 Hz), 1.37 (6H, m), 0.93 (3H, t, *J* = 7.2 Hz); ^{13}C NMR (CD_3OD, 75 MHz, δ; ppm) 167.0, 151.8, 148.5, 141.8, 137.5, 135.2, 124.4, 123.7, 58.00, 56.08, 46.15, 41.34, 32.32, 27.14, 25.03, 23.41, 14.21; MS (ESI) m/z 332.1(MH^+); HRMS(ESI) calcd. for $C_{17}H_{25}N_5O_2$ 331.2008, found 332.2079(MH^+), HPLC t_R= 10.98 min, purity 99.5%.

ICP-AES analysis

Cu content was determined by ICP-AES analysis. Test solutions were prepared by dissolving **Alk1**, **Az31** or KDM5C in 10% DMSO aqueous solution. Solutions for the standard curve were prepared by diluting copper standard solution (1000 mg/L, Merck) with 10% DMSO aqueous solution. Analytical conditions for ICP-AES: instrument ICPS-8100 Shimadzu, RF power 1.2 kW, plasma gas flow 1.2 L/min (Ar), auxiliary gas flow 14.0 mL/min (Ar), carrier gas flow 0.7 L/min (Ar), wavelength 324.754 nm (Cu).

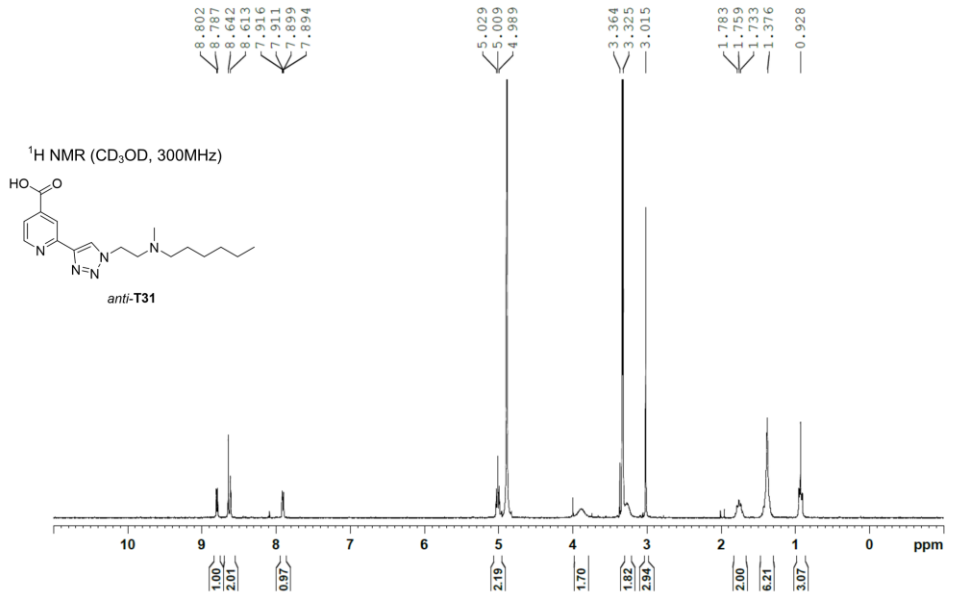
Free metal ion-catalyzed azide-alkyne cycloaddition by HPLC analysis

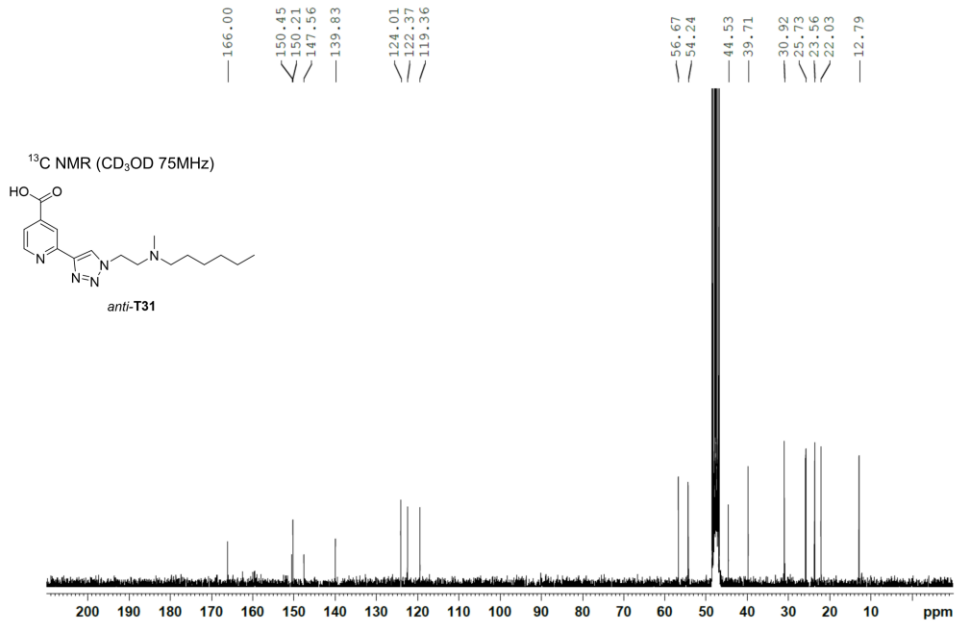
A solution of **Alk4** (20 mM) and **Az1** (40 mM) in DMSO (200 µL) was reacted in the presence/absence of $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (40, 20, 15, 10, or 2 mM) or $MnCl_2$ (20 mM) in H_2O (100 µL) at 100ºC for 5 h. The reaction mixture was diluted with CH_3CN/H_2O (200 µL) and filtered through a 0.45 µm filter. Then, the resulting mixture was analyzed by HPLC. HPLC was performed with Shimazu instrument equipped with a COSMOSIL packed column (5C18-AR-II, 4.6 ID×150 mm, nacalai) for analysis; the eluents were used water contained 0.1% TFA (A) and MeCN contained 0.1% TFA (B). The conditions for analytical HPLC were as follows; flow rate 1.0 mL/min, measuring wavelength 254 nm, gradient A/B 0 to 20 min (90/10 to 10/90), 20 to 30 min (10/90), 30 to 40 min (10/90 to 90/10) for Fe(II)-catalyzed reaction or gradient A/B 0 to 5 min (90/10), 5 to 20 min (90/10 to 10/90), 20 to 30 min (10/90), 30 to 40 min (10/90 to 90/10) for Mn(II)-catalyzed reaction.

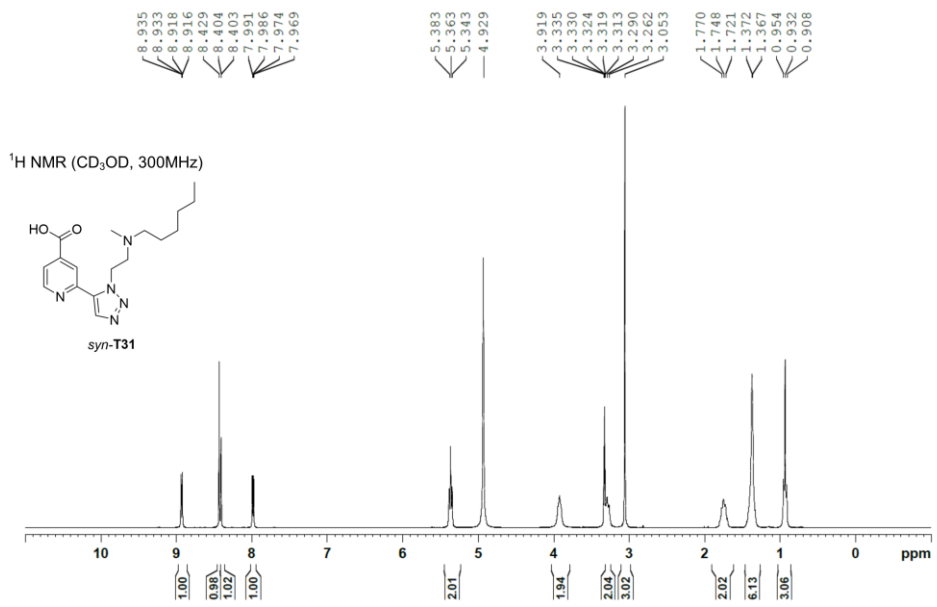
UV-vis spectral analysis

UV-Vis spectra were recorded on a JASCO V-770 spectrophotometer at room temperature. Test samples were prepared by dissolving **T31** (final concentration: 100 µM) and $(NH_4)_2Fe(SO_4)_2$ (final concentration: 0, 25, 50, or 100 µM) with 800 µL of $AcCN/H_2O$ (1:1) Absorbances of the samples were normalized by corresponding blank solutions containing $(NH_4)_2Fe(SO_4)_2$.

NMR spectrum of *anti*-T31, *syn*-T31, and *anti*-T31'



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3.330
3.324
3.319
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1H NMR (CD3OD, 300MHz)
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O
N
N
N
N
syn-T31
10
9
8
7
6
5
4
3
2
1
0
ppm
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1.94
2.04
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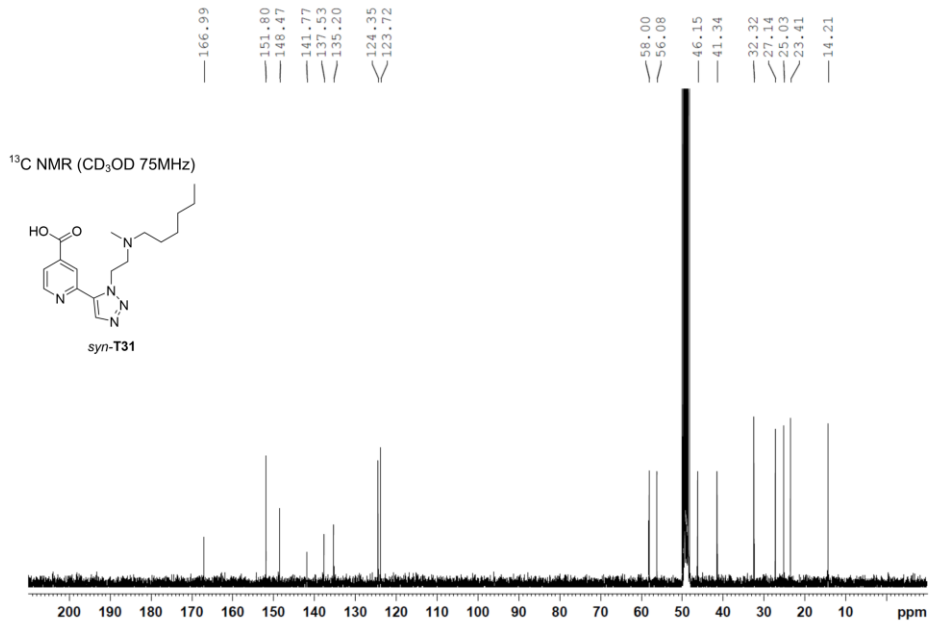
3.06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27.14
25.03
23.41
14.21
13C NMR (CD3OD 75MHz)
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O
N
N
N
N
syn-T31
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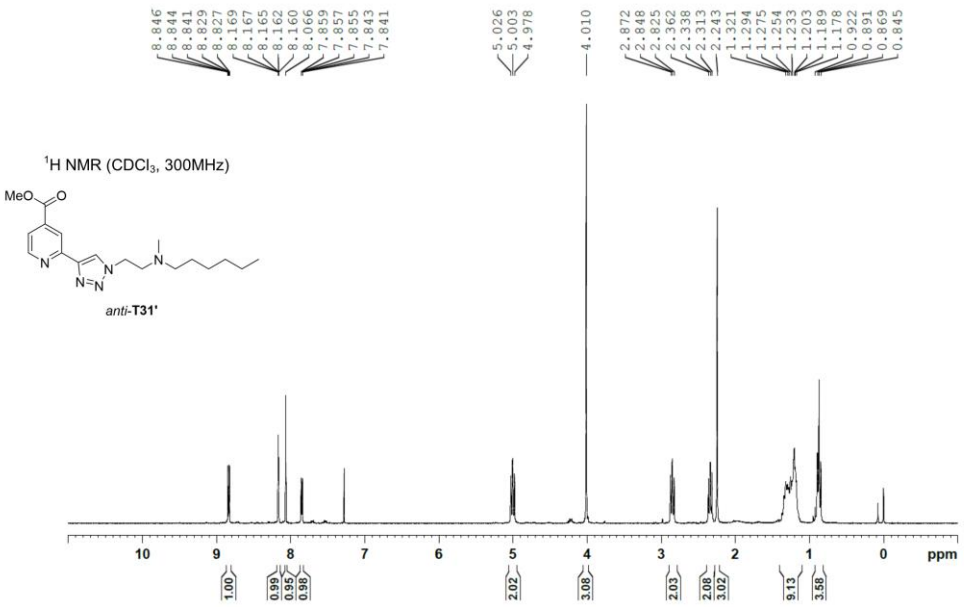
ppm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1.233
1.203
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0.845
1H NMR (CDCl3, 300MHz)
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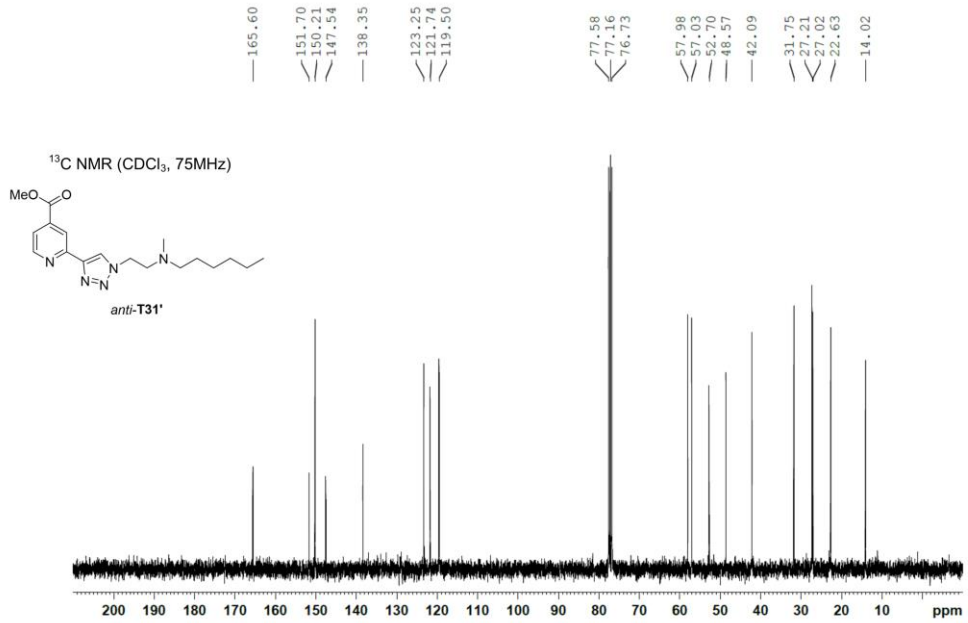
O
N
N
N
N
anti-T31'
10
9
8
7
6
5
4
3
2
1
0
ppm
1.00
0.99
0.95
0.98
2.02
3.08
2.03
2.08
3.02
9.13
3.58


165.60
151.70
150.21
147.54
138.35
123.25
121.74
119.50
77.58
77.16
76.73
57.98
57.03
52.70
48.57
42.09
31.75
27.21
27.02
22.63
14.02
13C NMR (CDCl3, 75MHz)
MeO
O
N
N
N
N
anti-T31'
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
ppm