Electronic Supplementary Material (ESI) for ChemComm.

Development of DACN-NHS ester and DACN-maleimide, and their application for the synthesis of artificial hybrid biomolecules

Yuuya Kawasaki,[a] Tomoya Hayashibara,[b] Yuki Seto,[b] Yutaro Taniguchi,[b] Kazunobu Igawa[a,b] and Katsuhiko Tomooka*[,a,b]

[a]*Institute for Materials Chemistry and Engineering, and IRCCS, Kyushu University, 6-1 Kasuga-koen, Kasuga, Fukuoka 816-8580, Japan*

[b]*Department of Molecular and Material Sciences, Kyushu University, 6-1 Kasuga-koen, Kasuga, Fukuoka 816-8580, Japan*

E-mail: ktomooka@cm.kyushu-u.ac.jp

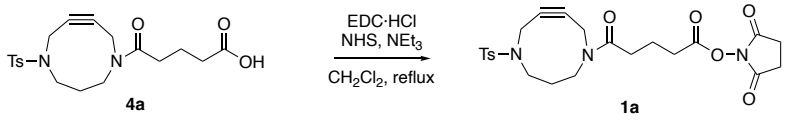
Table of Contents:

1. General Method

All reactions were carried out in heat-gun-dried glassware under an argon atmosphere unless otherwise noted. Dry CH_2Cl_2, CH_3CN, DMF, THF were purchased from Kanto Chemical Co., Inc. and used without purification. ^1H NMR spectra were recorded on Varian Mercury spectrometer (300 MHz) or JEOL JNM-ECA600 (600 MHz) and ^{13}C NMR spectra were recorded on Varian Mercury spectrometer (75 MHz), JEOL JNM-ECZ400S (100 MHz) or JEOL JNM-ECA600 (150 MHz) at ambient temperature using $CDCl_3$, DMSO-d_6 as a solvent. Chemical shifts (δ) in parts per million were referenced to the solvent residual peak as an internal standard: $CHCl_3$ for ^1H NMR (δ 7.26) and $CDCl_3$ for ^{13}C NMR (δ 77.1), DMSO-d_5 for ^1H NMR (δ 2.50) and DMSO-d_6 for ^{13}C NMR (δ 39.5), acetonitrile-d_2 for ^1H NMR (δ 1.94) and acetonitrile-d_3 for ^{13}C NMR (δ 118.3). The peak multiplicities were given as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Infrared spectra (IR) were recorded on a Fourier transfer infrared spectrophotometer (Perkin Elmer SpectrumOne) as crystals with a diffuse reflector or attenuated total reflection (JASCO, FT/IR-4600). X-ray crystallographic data were collected using a Rigaku Saturn724 diffractometer using multi-layer mirror monochromated Mo-Kα radiation (λ = 0.71075 Å) to a maximum 2θ value of 55.0º. Melting points (mp) were measured on a Yanaco Micro Melting Point Apparatus. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60 F_{254} (Merck 5715) plates and developed plates were visualized by UV (254 nm) and by heating on a hot plate after staining with a 4% solution of phosphomolybdic acid in ethanol or a 2.5% solution of *p*-anisaldehyde in ethanol. Column chromatography was performed using Fuji Silysia silica gel FL100D (neutral). PTLC was performed using Merck Silica gel 60 PF_{254} for preparative thin layer chromatography. HRMS analyses were recorded on a JEOL JMS-700 at the Analytical Center in IMCE, Kyushu University.

2. Detailed Reaction Procedures and Analytical Data

Synthesis of *N*Ts-DACN-NHS-ester (1a)



Synthesis and analytical data of *N*Ts,*N'*Gul-DACN (**4a**) were reported.[1]

To a solution of **4a** (200 mg, 0.510 mmol) in CH_2Cl_2 (4 mL) ware added *N*-hydroxysuccinimide (106 mg, 0.921 mmol), EDC·HCl (178 mg, 0.929 mmol), and NEt_3 (254 μL, 1.82 mmol) at 0 ºC. After the mixture had been stirred under reflux for 3 h, the reaction was quenched with 1M aq. HCl and extracted three times with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel chromatography ($CHCl_3$) to afford 175 mg (70%) of **1a** as colorless crystals.

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 65:35 mixture of rotational isomers): δ 7.66 (d, *J* = 8.0 Hz, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 4.22 (t, *J* = 2.5 Hz, 1.3H), 4.04 (t, *J* = 2.5 Hz, 0.7H), 3.86 (t, *J* = 2.8 Hz, 2H), 3.57 (t, *J* = 5.5 Hz, 2H), 3.27 (t, *J* = 5.8 Hz, 1.3H), 3.17 (t, *J* = 5.8 Hz, 0.7H), 2.85 (s, 2.6H), 2.82 (s, 1.4H), 2.68-2.77 (m, 2H), 2.43-2.50 (m, 5H), 2.04-2.14 (m, 4H).

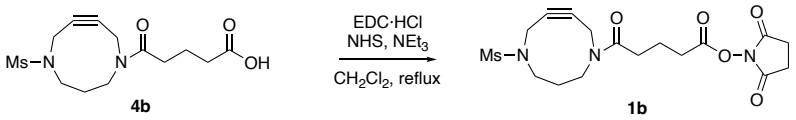
^{13}C NMR (150 MHz, $CDCl_3$): δ 172.22, 171.08, 169.18, 168.52, 143.99, 143.86, 134.64, 134.54, 130.00, 129.96, 127.42, 127.36, 89.25, 88.54, 88.33, 86.67, 45.48, 45.44, 43.85, 41.05, 40.74, 39.50, 36.63, 32.30, 31.81, 30.26, 30.11, 25.67, 21.63, 20.39, 20.11. (2 carbonyl and 5 aliphatic carbons were overlapping)

IR (reflection): 2946, 1784, 1739, 1644, 1428, 1337, 1160, 816, 754 cm^{-1}.

mp: 64.6-65.3 °C.

HRMS (EI, positive): Exact mass calc. for $C_{23}H_{27}N_3O_7S$ $[M]^+$, requires *m/z*: 489.1570, found *m/z*: 489.1570.

Synthesis of *N*Ms-DACN-NHS-ester (1b)



Synthesis and analytical data of *N*Ms,*N'*Gul-DACN (**4b**) were reported.[1]

To a solution of **4b** (3.29 g, 10.4 mmol) in CH_2Cl_2 (70 mL) ware added *N*-hydroxysuccinimide (2.15 g, 18.7 mmol), EDC·HCl (3.58 g, 18.7 mmol), and NEt_3 (5.21 mL, 37.4 mmol) at 0 ºC. After the mixture had been stirred under reflux for 3 h, the reaction was quenched with 1M aq. HCl and extracted three times with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by recrystallization from AcOEt-Et_2O to afford 3.45 g (80%) of **1b** as colorless crystals.

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 60:40 mixture of rotational isomers): δ 4.23, (t, *J* = 3.0 Hz, 1.2H), 4.06 (t, *J* = 3.0 Hz, 0.8H), 3.94 (t, *J* = 3.0 Hz, 1.2H), 3.91 (t, *J* = 3.0 Hz, 0.8H), 3.57 (t, *J* = 6.0 Hz, 2H), 3.39 (t, *J* = 6.0 Hz, 1.2H), 3.28 (t, *J* = 6.0 Hz, 0.8H), 2.83 (s, 1.8H), 2.82 (s, 1.2H), 2.81 (s, 2.4H), 2.80 (s, 1.6H), 2.69 (t, *J* = 3.0 Hz), 2.48-2.43 (m, 2H), 2.11-2.02 (m, 4H).

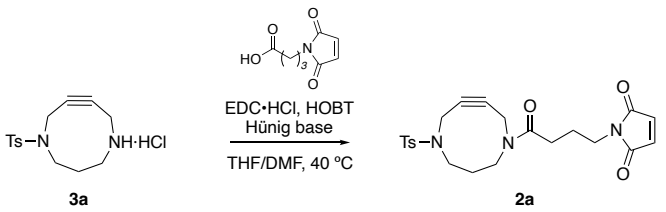
^{13}C NMR (100 MHz, $CDCl_3$): δ 172.07, 171.20, 169.32, 168.55, 89.42, 88.91, 88.04, 86.47, 45.80, 45.57, 44.14, 43.83, 40.47, 40.14, 39.47, 36.89, 36.57, 36.40, 32.24, 31.81, 31.73, 30.21, 30.16, 25.67, 20.33, 20.07. (2 aliphatic carbons were overlapping)

IR (ATR): 3472, 2934, 2361, 1815, 1738, 1620, 1320, 1146, 905, 727 cm^{-1}.

mp: 110.7-111.1 °C.

HRMS (EI, positive): Exact mass calc. for $C_{17}H_{23}N_3O_7S$ $[M]^+$, requires *m/z*: 413.1257, found *m/z*: 413.1254.

Synthesis of *N*Ts-DACN-maleimide (2a)



Synthesis and analytical data of *N*Ts,*N'*H-DACN·HCl (**3a**) were reported.[1]

To a solution of **3a** (139 mg, 0.443 mmol) in THF (2.5 mL) / DMF (0.5 mL) ware added 4-maleimidobutyric acid (89.4 mg, 0.489 mmol), EDC·HCl (127 mg, 0.661 mmol), HOBT (66.2 mg, 0.490 mmol) and Hünig base (303 μL, 1.74 mmol) at 0 ºC. After the mixture had been stirred at 40 °C for 12 h, the reaction was quenched with sat. aq. NH_4Cl and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel chromatography ($CHCl_3$) to afford 101 mg (53%) of **2a** as colorless crystals.

^{1}H NMR (300 MHz, DMSO-d_6, compound exists as a 50:50 mixture of rotational isomers): δ 7.73 (d, *J* = 8.5 Hz, 2H), 7.47 (d, *J* = 8.5 Hz, 2H), 7.03 (s, 1H), 7.01 (s, 1H), 4.15 (s, 1H), 4.12 (s, 1H), 3.95 (s, 1H), 3.91 (s, 1H), 3.48-3.39 (m, 4H), 3.21 (t, *J* = 5.5 Hz, 1H), 3.15 (t, *J* = 5.8 Hz, 1H), 2.44 (s, 3H), 2.32 (m, 2H), 1.99-1.91 (m, 2H), 1.81-1.71 (m, 2H).

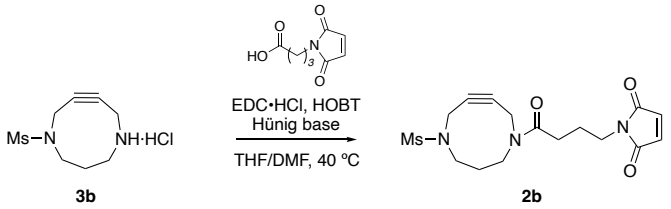
^{13}C NMR (75 MHz, DMSO-d_6): δ 171.51, 171.15, 171.01, 143.57, 134.81, 134.64, 134.58, 134.45, 129.99, 127.15, 127.04, 89.65, 89.35, 87.72, 87.04, 45.67, 45.08, 43.66, 42.96, 40.64, 36.76, 35.68, 31.49, 29.99, 29.93, 29.84, 29.70, 23.58, 23.31, 20.99. (1 carbonyl, 2 aromatic and 1 aliphatic carbons were overlapping)

IR (reflection): 3387, 2933, 1704, 1638, 1448, 1412, 1354, 914 cm^{-1}.

mp: 119.8-120.5 °C.

HRMS (EI, positive): Exact mass calc. for $C_{22}H_{25}N_3O_5S$ $[M]^+$, requires *m/z*: 443.1515, found *m/z*: 443.1516.

Synthesis of *N*Ms-DACN-maleimide (2b)



Synthesis and analytical data of *N*Ms,*N'*H-DACN·HCl (**3b**) were reported.[1]

To a solution of **3b** (4.20 g, 17.6 mmol) in THF (100 mL) / DMF (20 mL) ware added 4-maleimidobutyric acid (3.55 g, 19.4 mmol), EDC·HCl (5.06 g, 26.4 mmol), HOBT (2.63 g, 19.4 mmol) and Hünig base (12.0 mL, 68.9 mmol) at 0 ºC. After the mixture had been stirred at 40 ºC for 7 h, the reaction was quenched with sat. aq. NH_4Cl and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel chromatography ($CHCl_3$) to afford 4.58 g (71%) of **2b** as colorless crystals.

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 60:40 mixture of rotational isomers): δ 6.69 (s, 1.2H), 6.69 (s, 0.8H), 4.25 (t, *J* = 3.0 Hz, 1.2 H, 1.2 Hz), 4.02 (t, *J* = 3.0 Hz, 0.8 H), 3.98-3.95 (m , 2H), 3.61-3.55 (m, 4H), 3.43 (t, *J* = 6.0 Hz, 1.2H), 3.36 (t, *J* = 6.0 Hz, 0.8H), 2.86 (s, 1.8H), 2.85 (s, 1.2H), 2.32 (t, *J* = 6.0 Hz, 2H), 2.04-1.90 (m, 4H).

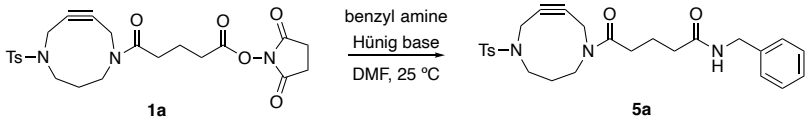
^{13}C NMR (75 MHz, $CDCl_3$): δ 172.07, 171.11, 170.96, 170.93, 134.20, 89.60, 88.87, 88.19, 86.35, 45.66, 45.48, 44.01, 43.80, 40.46, 40.24, 39.45, 37.40, 37.37, 36.80, 36.62, 36.46, 32.21, 30.94, 30.92, 30.12, 24.10, 23.85. (1 olefine carbon was overlapping)

IR (ATR): 3451, 3087, 2933, 1698, 1635, 1411, 1318, 1144, 837 cm^{-1}.

mp: decomposed at 151.0 °C.

HRMS (EI, positive): Exact mass calc. for $C_{16}H_{21}N_3O_5S$ $[M]^+$, requires *m/z*: 367.1202, found *m/z*: 367.1201.

Amidation of 1a with Benzyl Amine



To a solution of **1a** (20.0 mg, 0.0409 mmol) in DMF (2 mL) added benzyl amine (4.47 μL, 0.0409 mmol) and Hünig base (14.1 μL, 0.0818 mmol) at 25 ºC. After the mixture had been stirred at that temperature for 6 h, the reaction was quenched with H_2O and extracted three times with AcOEt. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 18.7 mg (95%) of **5a** as colorless crystals.

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 80:20 mixture of rotational isomers): δ 7.66 (d, *J* = 8.2 Hz, 2H), 7.35-7.26 (m, 7H), 6.34-5.89 (br, 1H), 4.44-4.41 (m, 2H), 4.19 (t, *J* = 2.3 Hz, 1.2H), 4.02 (t, *J* = 2.3 Hz, 0.8H), 3.87-3.84 (m, 2H), 3.55 (t, *J* = 5.6 Hz, 2H), 3.24 (t, *J* = 5.6 Hz, 1.2H), 3.15 (t, *J* = 5.6 Hz, 0.8H), 2.44-2.28 (m, 7H), 2.12-1.93 (m, 4H).

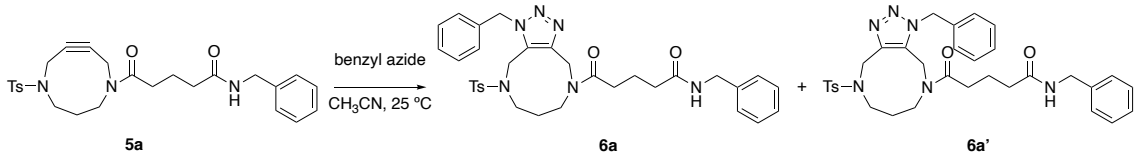
^{13}C NMR (100 MHz, $CDCl_3$): δ 173.01, 172.38, 172.05, 144.00, 143.89, 138.38, 138.34, 134.48, 134.34, 129.99, 129.94, 128.71, 127.80, 127.51, 127.36, 127.28, 89.13, 88.45, 88.29, 86.65, 45.45, 45.40, 43.86, 43.79, 43.54, 41.03, 40.70, 39.67, 36.50, 35.43, 35.26, 32.90, 32.53, 32.34, 30.08, 21.60, 21.34, 21.01. (1 carbonyl, 5 aromatic and 2 aliphatic carbons were overlapping)

IR (reflection): 3312, 2924, 1631, 1542, 1416, 1331, 1154, 916 cm^{-1}.

mp: 69.2-70.2 °C.

HRMS (EI, positive): Exact mass calc. for $C_{26}H_{31}N_3O_4S$ $[M]^+$, requires *m/z*: 481.2035, found *m/z*: 481.2035.

CFAAC of 5a with Benzyl Azide



To a solution of **5**a (63.0 mg, 0.131 mmol) in CH_3CN (6 mL) was added benzyl azide (1.63 μL, 0.131 mmol) at 25 ºC. After the mixture had been stirred at that temperature for 48 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 39.6 mg (49%, 1st eluent) of **6a'** and 38.9 mg (48%, 2nd eluent) of **6a** as colorless crystals.

6a

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 80:20 mixture of rotational isomers): δ 7.54 (d, *J* = 8.2 Hz, 0.4H), 7.12-7.41 (m, 13.6H), 6.26 (s, 1H), 5.68 (s, 0.4H), 5.50 (s, 1.6H), 4.75 (s, 0.4H), (s, 1.6H), 4.42-4.38 (m, 2H), 4.07 (s, 0.4H), 4.03 (s, 1.6H), 3.55 (t, *J* = 4.7 Hz, 1.6H), 3.24-3.18 (m, 0.4H), 3.08-2.97 (m, 0.4H), 2.93 (t, *J* = 5.9 Hz, 1.6H), 2.56 (t, *J* = 7.0 Hz, 1.6H), 2.44 (s, 3H), 2.31-2.19 (m, 2.4H), 1.81-1.94 (m, 4H).

^{13}C NMR (75 MHz, $CDCl_3$, major isomer): δ 173.62, 172.56, 144.44, 143.53, 138.55, 134.08, 133.57, 130.51, 130.06, 129.25, 128.66, 127.88, 127.38, 127.22, 52.48, 48.19, 48.05, 46.82, 43.51, 42.41, 35.86, 32.93, 27.95, 21.65, 21.31. (2 aromatic carbons were overlapping)

IR (reflection): 3301, 2931, 1955, 1639, 1539, 1496, 1340, 1160 cm^{-1}.

mp: 64.0-64.5 °C.

HRMS (EI, positive): Exact mass calc. for $C_{33}H_{38}N_6O_4S$ $[M]^+$, requires *m/z*: 614.2675, found *m/z*: 614.2676.

6a'

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 94:6 mixture of rotational isomers): δ 7.69 (d, *J* = 8.0 Hz, 2H), 7.37-7.12 (m, 12H), 6.44 (t, *J* = 5.6 Hz, 0.6H), 6.13 (d, *J* = 5.3 Hz, 0.94H), 5.90 (s, 1.88H), 5.51 (s, 0.12H), 4.57 (s, 1.88H), 4.40-4.30 (m, 4.12H), 3.46-3.21 (m, 2H), 2.98-2.87 (m, 2H), 2.45 (s, 3H), 2.33-2.24 (m, 3.88H), 1.99-1.89 (m, 4.12H).

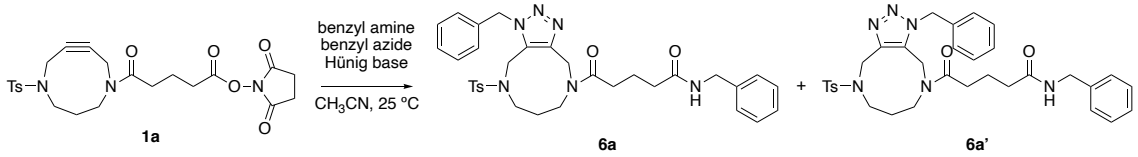
^{13}C NMR (75 MHz, $CDCl_3$, major isomer): δ 173.10, 172.34, 144.43, 142.30, 138.35, 135.79, 132.63, 130.92, 130.06, 128.93, 128.74, 128.19, 127.88, 127.76, 127.54, 127.18, 52.24, 47.67, 47.35, 43.58, 41.26, 35.36, 32.42, 30.01, 21.66, 21.04. (1 aliphatic carbon was overlapping)

IR (reflection): 3304, 2928, 1956, 1640, 1542, 1340, 1159 cm^{-1}.

mp: 66.6-67.3 °C.

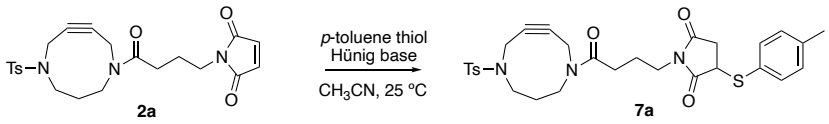
HRMS (EI, positive): Exact mass calc. for $C_{33}H_{38}N_6O_4S$ $[M]^+$, requires *m/z*: 614.2675, found *m/z*: 614.2676.

Amidation and CFAAC of 1a in Simultaneous Mixing



To a solution of **1a** (30.0 mg, 0.0613 mmol) in CH_3CN (3 mL) ware added benzyl azide (7.63 μL, 0.0613 mmol), benzyl amine (6.70 μL, 0.0613 mmol) and Hünig base (10.6 μL, 0.0623 mmol) at 25 ºC. After the mixture had been stirred at that temperature for 48 h, the reaction was quenched with H_2O and extracted three times with AcOEt. The combined organic phase was dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 17.9 mg (47%, 1st eluent) of **6a**' and 17.7 mg (47%, 2nd eluent) of **6a** as colorless crystals.

Thia Michael Reaction of 2a with *p*-Toluene Thiol



To a solution of **2a** (30.0 mg, 0.0676 mmol) in CH_3CN (3 mL) was added *p*-toluene thiol (8.40 mg, 0.0676 mmol) and Hünig base (9.20 μL, 0.0541 mmol) at 25 ºC in the shade. After the mixture had been stirred at that temperature for 12 h, the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$) to afford 38.0 mg (99%) of **7a** as colorless crystals.

^{1}H NMR (300 MHz, $CDCl_3$, compound exists as a 62.5:37.5 mixture of rotational isomers): δ 7.65 (d, J = 8.2 Hz, 2H), 7.40-7.31 (m, 4H), 7.12 (d, J = 8.2 Hz, 2H), 4.27-4.10 (m, 1.25H), 3.96-3.76 (m, 3.75H), 3.56-3.43 (m, 4H), 3.26-3.03 (m, 3H), 2.66 (ddd, J = 18.8, 3.8, 3.8 Hz, 1H), 2.43 (s, 1.88H), 2.42 (s, 0.625H), 2.32 (s, 3H), 2.23-1.73 (m, 6H).

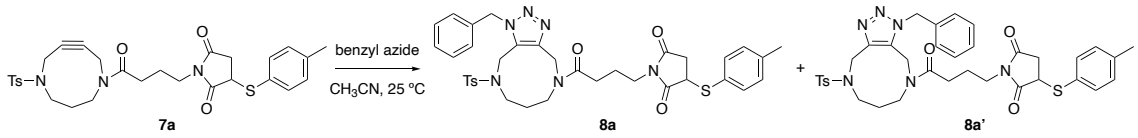
^{13}C NMR (75 MHz, $CDCl_3$): δ 175.85, 175.81, 174.81, 172.10, 170.98, 143.98, 143.85, 139.86, 139.79, 134.95, 134.78, 134.55, 130.23, 130.00, 129.96, 127.39, 127.35, 126.69, 126.52, 89.42, 88.49, 88.46, 86.67, 45.28, 44.21, 44.18, 43.72, 43.64, 41.02, 40.79, 39.38, 38.68, 38.59, 36.65, 36.15, 36.07, 32.15, 31.08, 30.96, 30.12, 29.75, 23.02, 22.84, 21.62, 21.30. (1 carbonyl, 2 aromatic and 2 aliphatic carbons were overlapping)

IR (reflection): 2924, 1704, 1645, 1335, 1159, 814, 712, 668 cm^{-1}.

mp: 55.6-57.0 °C.

HRMS (EI, positive): Exact mass calc. for $C_{29}H_{33}N_3O_5S_2$ $[M]^+$, requires *m/z*: 567.1862, found *m/z*: 567.1858.

CFAAC of 7a with Benzyl Azide



To a solution of **1ab** (43.6 mg, 0.0768 mmol) was added benzyl azide (9.55 μL, 0.0768 mmol) in CH_3CN (5 mL) at 25 ºC. After the mixture had been stirred at that temperature for 48 h, the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 40:1) to afford 24.8 mg (46%, 1st eluent) of **8a'** and 22.3 mg (42%, 2nd eluent) of **8a** as colorless crystals.

8a

^{1}H NMR (300 MHz, $CDCl_3$, trace amount of rotational isomer was observed): δ 7.41-7.09 (m, 13H), 5.52 (d, *J* = 16.0 Hz, 1H), 5.43 (d, *J* = 16.0 Hz, 1H), 4.68 (d, *J* = 16.4 Hz, 1H), 4.62 (d, *J* = 16.4 Hz, 1H), 4.12-4.07 (m, 1H), 4.04 (d, *J* = 16.4 Hz, 1H), 3.98 (d, *J* = 16.4 Hz, 1H), 3.70 (ddd, *J* = 9.5, 4.7, 4.7 Hz, 1H), 3.57-3.46 (m, 1H), 3.43-3.36 (m, 2H), 3.26-3.17 (m, 1H), 3.05-2.89 (ddd, m, 2H), 2.58 (dd, *J* = 18.5, 3.8 Hz, 1H), 2.44 (s, 3H), 2.31 (s, 3H), 1.90-1.67 (m, 4H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 176.09, 175.11, 172.82, 144.34, 143.35, 139.56, 134.63, 133.89, 133.20, 130.88, 130.10, 129.98, 129.23, 129.12, 127.45, 127.25, 126.71, 52.53, 48.75, 47.85, 46.99, 44.14, 42.80, 38.23, 36.02, 30.60, 27.95, 22.63, 21.61, 21.21.

IR (reflection): 3463, 2928, 1776, 1643, 1597, 1493, 1439, 1234, 1159, 1121 cm^{-1}.

mp: 65.1-66.0°C.

HRMS (EI, positive): Exact mass calc. for $C_{36}H_{40}N_6O_5S_s$ $[M]^+$, requires *m/z*: 700.2502, found *m/z*: 700.2500.

8a'

^{1}H NMR (300 MHz, $CDCl_3$, trace amount of rotational isomer was observed): δ 7.73 (d, *J* = 8.2 Hz, 2H), 7.49-7.10 (m, 11H), 5.97 (s, 2H), 4.51 (s, 2H), 4.42 (s, 2H), 3.65 (dd, *J* = 9.4, 4.1 Hz, 1H), 3.52 (ddd, *J* = 5.4, 5.4, 5.4 Hz, 2H), 3.44-3.40 (m, 2H), 3.09-2.97 (m, 2H), 2.82 (dd, *J* = 18.8, 9.4 Hz, 1H), 2.52 (dd, *J* = 18.8, 4.1 Hz, 1H), 2.47 (s, 3H), 2.30 (s, 3H), 2.21 (m, 2H), 1.97-1.95 (m, 2H), 1.88-1.82 (m, 2H).

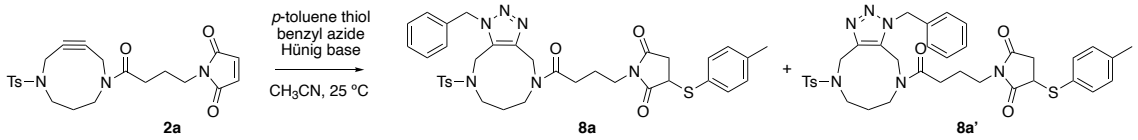
^{13}C NMR (75 MHz, $CDCl_3$): δ 175.96, 174.94, 172.40, 144.47, 142.26, 139.69, 135.82, 134.76, 132.51, 131.33, 130.19, 130.11, 129.04, 128.23, 127.91, 127.07, 126.75, 52.36, 48.19, 47.78, 46.83, 43.98, 42.35, 38.77, 36.01, 31.31, 29.42, 22.55, 21.71, 21.30.

IR (reflection): 3464, 2925, 1775, 1642, 1597, 1493, 1340, 1159, 1090 cm^{-1}.

mp: 65.4-66.2 °C.

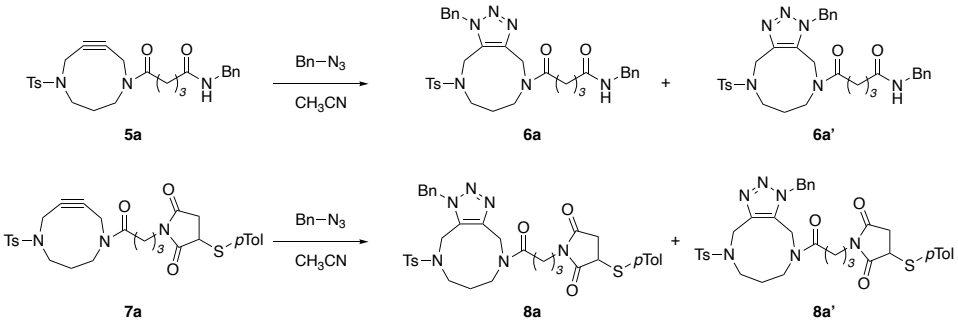
HRMS (EI, positive): Exact mass calc. for $C_{36}H_{40}N_6O_5S_s$ $[M]^+$, requires *m/z*: 700.2502, found *m/z*: 700.2501.

Thia Michael Reaction and CFAAC of 2a in Simultaneous Mixing

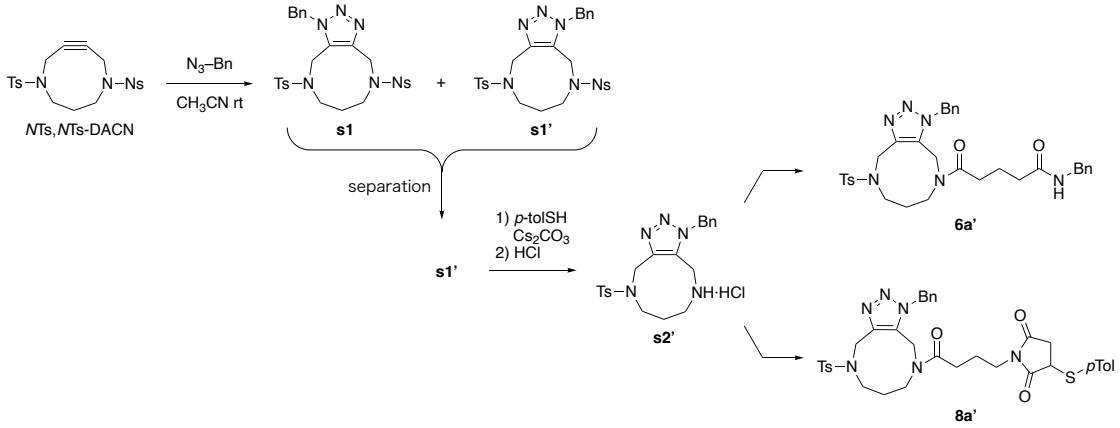


To a solution of **2a** (30.0 mg, 0.0676 mmol) in CH_3CN (3 mL) was added benzyl azide (8.41 μL, 0.0676 mmol), *p*-toluene thiol (8.40 mg, 0.0676 mmol) and Hünig base (9.19 μL, 0.0540 mmol) at 25 ºC in the shade. After the mixture had been stirred at that temperature for 48 h, the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 40:1) to afford 22.0 mg (46%, 1st eluent) of **8a'** and 22.3 mg (47%, 2nd eluent) of **8a** as colorless crystals.

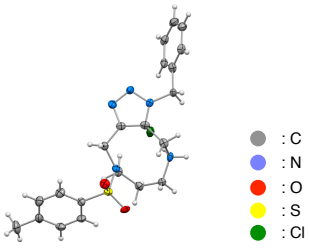
Structure Determination of Regioisomers of Huisgen Reaction



The structure of Huisgen reaction product **6a**, **6a'**, **8a** and **8a'** were determined by the comparison with authentic samples prepared as follows. Huisgen reaction products of *N*Ts,*N*Ns-DACN with benzyl azide (**s1** and **s1'**) were separated by silicagel column chromatography, and the Ns group of **s1'** was removed by treatment with *p*-tolSH and Cs_2CO_3. Thus obtained secondary amine derived from **s1'** was converted to HCl salt **s2'**, and the structure of **s2'** was determined by X-ray crystal analysis. Authentic samples of **6a'** and **8a'** were prepared from **s2'**.



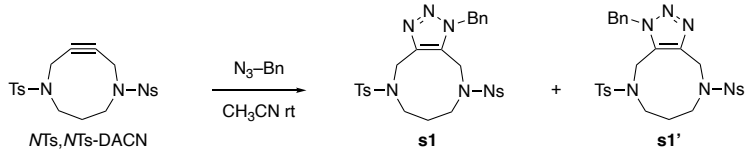
X-ray crystal structure analysis of **s2'**



ORTEP Drawing of **s2'** (50% probability ellipsoids), selected crystal data: crystal system; monoclinic, $P2_1/n$ (No. 14), a = 11.982(8) Å, b = 5.795(4) Å, c = 30.82(2) Å, α = 90°, β = 90°, γ = 90.514(15)°, V = 2140(2)Å3, T= 123K, Z= 4, Z'= 1, μ (Mo Kα)= 0.305, 17635 reflections measured, 3656 unique (R_{int} = 0.1966) which were used in all calculations. The final wR_2 was 0.2652 (all data) and R_1 was 0.1095 (I≥2 σ(I)).

CCDC Number: 2260285

Huisgen Reaction of *N*Ts,*N'*Ns-DACN with Benzyl Azide



*N*Ts,*N*Ts-DACN **s1** **s1'**

To a solution of *N*Ts,*N'*Ns-DACN (156 mg, 0.336 mmol) was added benzyl azide (42.0 μL, 0.336 mmol) in CH_3CN (3 mL) at ambient temperature. After the mixture had been stirred at that temperature for 8 h, the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography ($CHCl_3$) to afford 100 mg of first eluate **s1** (50%) and 88.6 mg of second eluate **s1'** (44%) as colorless crystals.

s1

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.93-7.89 (m, 1H), 7.78-7.61 (m, 6H), 7.33-7.22 (m, 6H), 5.72 (s, 2H), 4.76 (s, 2H), 4.47 (s, 2H), 3.41-3.37 (m, 2H), 3.00 (t, *J* = 5.7 Hz, 2H), 2.43 (s, 3H), 1.92-1.85 (br, 2H).

^{13}C NMR (100 MHz, $CDCl_3$): δ 148.49, 144.41, 141.79, 134.77, 134.34, 134.01, 132.00, 131.38, 131.13, 130.78, 130.16, 129.11, 128.56, 127.31, 127.15, 124.39, 52.21, 48.90, 47.86, 46.37, 42.57, 30.05, 21.59.

IR (reflection): 2927, 1545, 1451, 1343, 1163, 899, 817, 750, 678, 548 cm^{-1}.

mp: 94.8-95.5 °C.

HRMS (FAB, matrix; 3-nitrobenzyl alcohol, positive): Exact mass calc. for $C_{27}H_{29}N_6O_6S_2$ $[M+H]^+$, requires *m/z*: 597.1590, found *m/z*: 597.1589.

s1'

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.00-7.99 (m, 1H), 7.75-7.71 (m, 2H), 7.55-7.52 (m, 1H), 7.55-7.52 (m, 2H), 7.36-7.21 (m, 7H), 5.73 (s, 2H), 4.68 (s, 2H), 4.29 (s, 2H), 3.30 (t, *J* = 5.7 Hz, 2H), 3.24 (t, *J* = 5.7 Hz, 2H), 2.43 (s, 3H), 1.85 (tt, *J* = 5.7, 5.7 Hz, 2H).

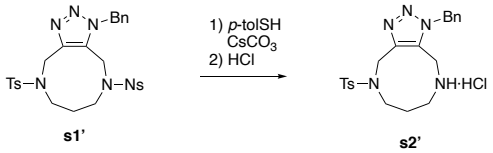
^{13}C NMR (100 MHz, $CDCl_3$): δ 148.23, 144.34, 143.04, 134.81, 134.26, 133.29, 132.06, 131.73, 130.75, 130.04, 129.46, 129.00, 128.44, 127.52, 127.50, 124.52, 52.04, 48.64, 47.70, 47.61, 41.79, 29.73, 21.58.

IR (reflection): 2930, 1542, 1456, 1342, 1162, 902, 815, 741, 668, 548 cm^{-1}.

mp: 63.9-64.2 °C.

HRMS (FAB, matrix; 3-nitrobenzyl alcohol, positive): Exact mass calc. for $C_{27}H_{29}N_6O_6S_2$ $[M+H]^+$, requires *m/z*: 597.1590, found *m/z*: 597.1590.

Remove of Ns Group of s1'



s1' **s2'**

To a solution of **s1'** (1.24 g, 2.07 mmol) in CH_3CN (20 mL) were added *p*-toluenethiol (334 mg, 2.69 mmol) and Cs_2CO_3 (878 mg, 2.69 mmol) at ambient temperature. After the mixture had been stirred at that temperature for 10 h, the reaction was quenched with 1 M aq. HCl and washed five times with Et_2O. The aqueous phase was neutralized with 1 M aq. NaOH and extracted three times with CH_2Cl_2. To the combined organic phase was added 35-37% aq. HCl (3 mL). After the mixture had been stirred at that temperature for 10 min, the solvent was removed under reduced pressure and the residual H_2O was azeotroped with CH_3CN. The resulting colorless precipitate was washed with AcOEt to afford 576 mg (62%) of **s2'** as colorless crystals.

^{1}H NMR (600 MHz, DMSO-d_6): δ 10.5-9.98 (br, 2H), 7.79 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.42-732 (m, 3H), 7.26 (m, 2H), 5.93 (s, 2H), 4.56 (s, 2H), 4.41 (s, 2H), 3.31 (t, J = 6.0 Hzz, 2H), 2.98-2.82 (br, 2H), 2.09-1.97 (m 2H).

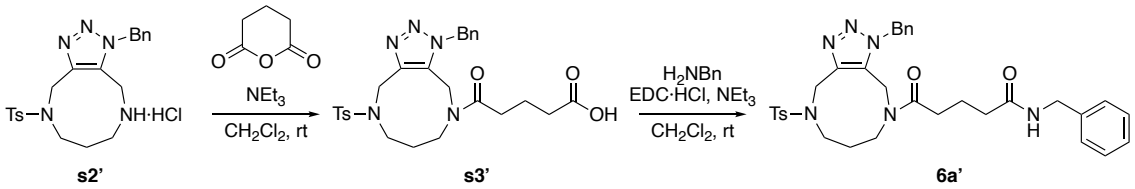
^{13}C NMR (150 MHz, DMSO-d_6): δ 145.30, 143.83, 135.33, 134.63, 130.06, 128.83, 128.16, 127.64, 127.16, 125.10, 51.76, 49.56, 47.01, 41.03, 35.03, 25.55, 21.00.

IR (reflection): 2704, 1457, 1345, 1163, 953, 816, 761, 714, 657, 551 cm^{-1}.

mp: 214.4-215.1 °C.

HRMS (EI, positive): Exact mass calc. for $C_{21}H_{25}N_5O_2S$ $[M–HCl]^+$, requires *m/z*: 411.1729, found *m/z*: 411.1727.

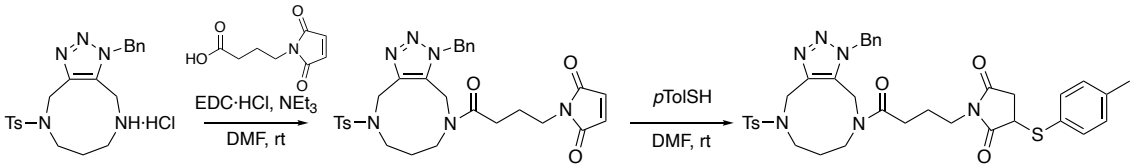
Synthesis of 6a' from s2'



s2' **s3'** **6a'**

To a solution of **s2'** (10.0 mg, 0.0226 mmol) in CH_2Cl_2 (2 mL) were added glutaric anhydride (2.60 mg, 0.0228 mmol) and NEt_3 (15.7 μL, 0.113 mmol) at ambient temperature. After the mixture had been stirred at that temperature for 20 h, benyl amine (3.70 μL, 0.339 mmol) and EDC·HCl (7.00 mg, 0.0366 mmol) were added and stirred at that temperature for 10 h. The reaction was quenched with H_2O and extracted three times with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 4.8 mg (33%) of **6a'** as colorless crystals.

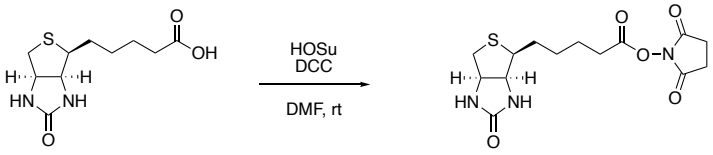
Synthesis of 8a' from s2'



s2' **s5'** **8a'**

To a solution of **s2'** (10.0 mg, 0.0226 mmol) in DMF (3 mL) were added 4-maleimidobutyric acid (6.20 mg, 0.0339 mmol), EDC·HCl (6.50 mg, 0.0339 mmol) and NEt_3 (15.7 μL, 0.113 mmol) at ambient temperature. After the mixture had been stirred at that temperature for 15 h, *p*-toluenethiol (8.40 mg, 0.0678 mmol) was added and stirred at that temperature for 3h. The reaction was quenched with H_2O and extracted three times with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 10:1) to afford 7.1 mg (43%) of **8a'** as colorless crystals.

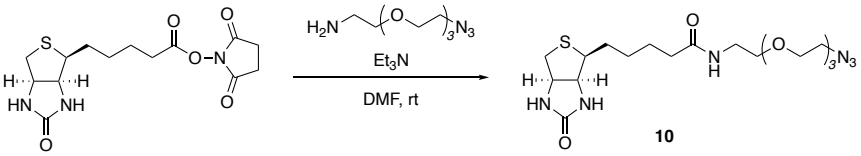
Synthesis of Biotin NHS-ester



To a solution of biotin (200 mg, 0.820 mmol) in DMF (6 mL) were added *N*-hydroxysuccinimide (94.4 mg, 0.820 mmol) and DCC (220 mg, 1.07 mmol) at ambient temperature. After the mixture had been stirred at ambient temperature for 12 h, the reaction mixture was concentrated under reduced pressure. The residue was recrystallized from $CHCl_3$ (5 mL) / *i*PrOH (20 mL) to afford 186 mg (67%) of biotin NHS ester as white crystal.

The spectroscopic data were in good agreement with the reported values.[2]

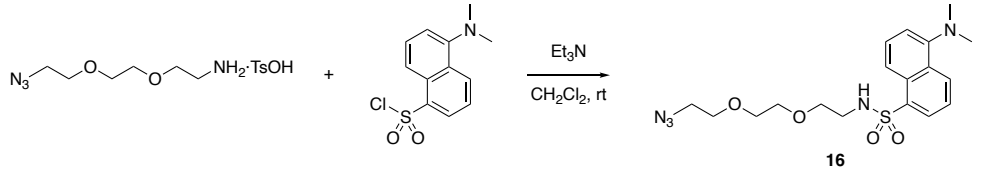
Synthesis of Biotin-PEG$_3$-N$_3$ 10



To a solution of NH_3-PEG_3-N_3 (7.0 μL, 35 μmol) in DMF (1 mL) were added Et_3N (8.2 μL, 59 μmol) and biotin NHS ester (9.8 mg, 29 μmol) at ambient temperature. After the mixture had been stirred at ambient temperature for 3 h, the reaction mixture was concentrated under reduced pressure. The residue was purified by silica-gel chromatography (acetone) to afford 10.2 mg (79%) of **10** as white crystal.

The spectroscopic data were in good agreement with the reported values.[3]

Synthesis of 16



To a solution of azido-PEG_2-amine·TsOH (87.0 mg, 0.251 mmol) in CH_2Cl_2 (2.5 mL) ware added dansyl chloride (56.4 mg, 0.209 mmol) and Et_3N (117 μL, 0.841 mmol) at 0 ºC. After the mixture had been stirred at ambient temperature for 2 h, the reaction was quenched with sat. aq. NH_4Cl and extracted with CH_2Cl_2. The combined organic phases were washed with brine, dried over Na_2SO_4, filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by silica-gel chromatography (hexane/AcOEt = 1:1) to afford 84.7 mg (99%) of **16** as green syrup.

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.56 (d, *J* = 8.7 Hz, 1H), 8.32 (d, *J* = 8.9 Hz, 1H), 8.25 (dd, *J* = 7.3, 1.3 Hz, 1H), 7.63-7.45 (m, 2H), 7.21 (d, *J* = 8.1 Hz, 1H), 5.32 (t, *J* = 6.0 Hz,1H), 3.61 (m, 2H), 3.52-3.44 (m, 2H), 3.43-3.31 (m, 6H), 3.16-3.04 (m, 2H), 2.90 (s, 6H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 135.21, 130.53, 130.01, 129.85, 129.64, 128.47, 125.07, 123.47, 119.27, 115.45, 70.54, 70.45, 70.24, 69.47, 50.78, 45.66, 43.26, 29.89.

IR (neat): 3301, 2870, 2106, 1574, 1455, 1324, 1201, 1144, 943, 792 cm^{-1}.

HRMS (FAB, matrix: 3-nitrobenzyl alcohol, positive): Exact mass calc. for $C_{18}H_{25}N_5O_4S$ $[M+H]^+$, requires *m/z*: 407.1627, found *m/z*: 407.1626.

3. Kinetic Study on CFAAC

CFAAC of **5a** and benzyl azide, **7a** and BnN_3 were performed at 1:1 molar ratio in CH_3CN (5 mM) with 4-bromo anisole as an internal standard for reversed phase high performance liquid chromatography (HPLC) analysis. The reaction temperature was controlled to 25 °C in incubator. The process of the CFAAC was monitored by reversed phase HPLC analysis attached UV-detector (λ = 254 nm). Second order rate constants for the reactions were determined by plotting the $1/[BnN_3]$ (vertical axis) versus reaction time (horizontal axis) by linear regression. All reactions were repeated in three times and the provided second order rate constants were averaged with standard error.

Reversed phase HPLC analysis were performed on a JASCO MD-2018 detectors equipped with a JASCO PU-2089 and a Shodex column oven AO-30 using Kanto Chemical Mightysil RP-18GP (4.6 mm × 250 mm, 5 μm).

Analytical conditions: 20% ammonium formate aq. / CH_3CN = 55 : 45, flow rate: 0.5 mL/min, detection: UV (220 nm), temp: 40 °C.

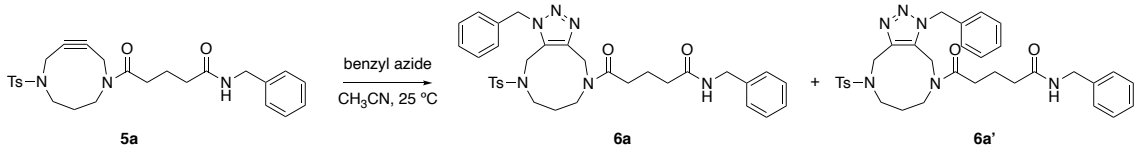
Retention time in CFAAC of **5a** and benzyl azide.

5a 12.8 min, **6a** or **6a'** 14.2 or 14.8 min, benzyl azide 18.5 min, 4-bromo anisole 23.9 min.

Retention time in CFAAC of **7a** and benzyl azide.

7a 28.0 min, **8a** or **8a'** 33.2 or 34.1 min, benzyl azide 18.0 min, 4-bromo anisole 23.0 min.

CFAAC of 5a with Benzyl Azide



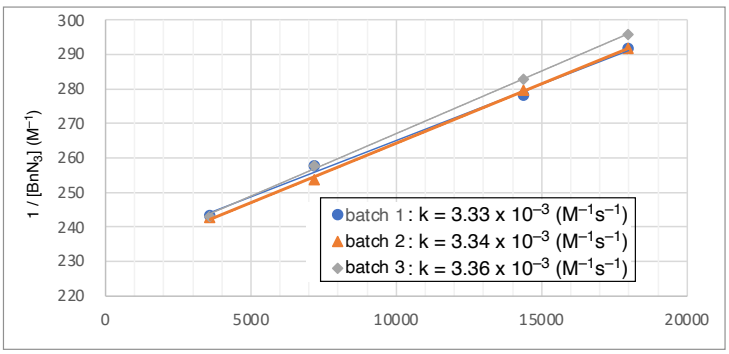
5a **6a** **6a'**

batch 1	
time (s)	1/[BnN_3] (M^{-1})
3600	243.2
7200	257.6
14400	278.3
18000	291.6
	R^2 = 0.996
	y = 0.0033x + 232.42
	k = 3.33 x 10^{-3} $M^{-1}S^{-1}$

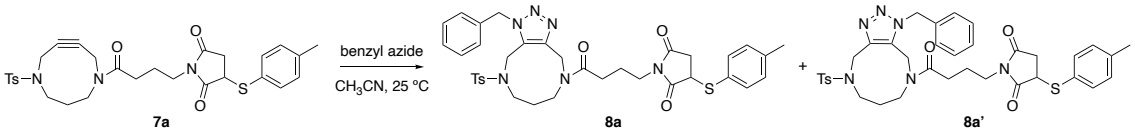
batch 2	
time (s)	1/[BnN_3] (M^{-1})
3600	242.8
7200	253.5
14400	279.7
18000	291.7
	R^2 = 0.999
	y = 0.0034x + 229.69
	k = 3.34 x 10^{-3} $M^{-1}S^{-1}$

batch 3	
time (s)	1/[BnN_3] (M^{-1})
3600	243.1
7200	257.7
14400	282.7
18000	295.9
	R^2 = 0.992
	y = 0.0036x + 230.69
	k = 3.36 x 10^{-3} $M^{-1}S^{-1}$

average of k_{5a} = 3.35±0.10 x 10^{-3} $M^{-1}S^{-1}$



CFAAC of 7a with Benzyl Azide

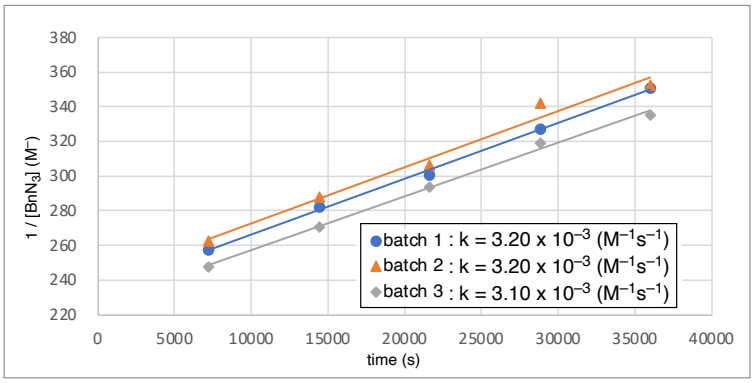


batch 1	
time (s)	1/[BnN3] (M^{-1})
7200	257.4
14400	281.7
21600	300.3
28800	327.0
36000	350.8
	R^2 = 0.998
	y = 0.0032x + 233.8
	k = 3.20 x 10^{-3} $M^{-1}S^{-1}$

batch 2	
time (s)	1/[BnN3] (M^{-1})
7200	262.3
14400	287.9
21600	306.3
28800	341.7
36000	352.1
	R^2 = 0.981
	y = 0.0032x + 240.05
	k = 3.20 x 10^{-3} $M^{-1}S^{-1}$

batch 3	
time (s)	1/[BnN3] (M^{-1})
7200	247.6
14400	270.5
21600	293.7
28800	318.7
36000	335.1
	R^2 = 0.996
	y = 0.0031x + 226.15
	k = 3.10x 10^{-3} $M^{-1}S^{-1}$

average of k_{7a} = 3.17±0.06 x 10^{-3} $M^{-1}S^{-1}$



4. LC-MS Analysis of Connection of Biomolecules

LC-MS analysis were performed on an Advion expression CMS-L equipped with a JASCO MD-4010 detectors, a JASCO PU-4180, and a JASCO column oven LCO-035 using Kanto Chemical Mightysil RP-18GP (4.6 mm × 250 mm, 5 μm).

Preparative reversed phase HPLC were performed on a JASCO MD-2018 detectors equipped with a JASCO PU-2089, and a Shodex column oven AO-30 using Kanto Chemical Mightysil RP-18GP (250 mm × 10 mm, 5 μm).

MS conditions: ESI-positive, capillary temperature: 200 °C, capillary voltage: 160 V, source voltage offset: 30 V, source voltage span: 5 V, gas temperature: 250 °C, ESI voltage: 3500 V.

Weights to two decimal places were measured using Sartorius MC5.

Connection of 9 with 10 using 1b

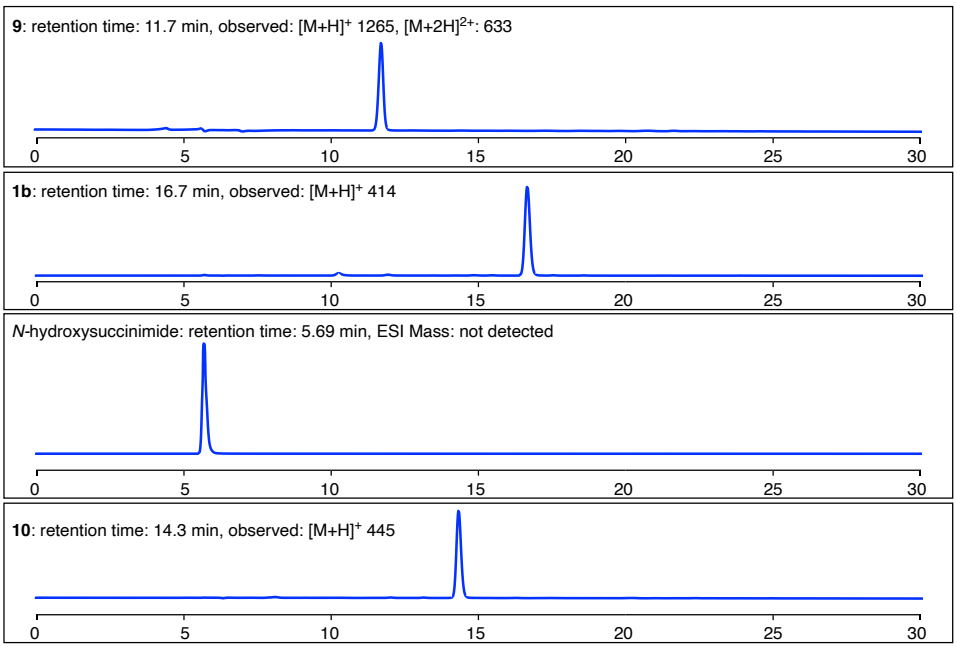
To avoid oxidation peptide the solvent was used after degassing by Ar bubbling.

To a solution of physalaemin (**9**) (2.29 mg, 1.81 μmol) in phosphate buffer (pH = 7.4, 500 μL) was added *N*Ms-DACN NHS ester **1b** (4.96 x 10^{-2} M in CH_3CN, 54.7 μL, 2.72 μmol) at 25 °C and stirred at that temperature for 2 h. After confirmation of consumption of all of **9** and generation of **10** was monitored by LC-MS analysis, **10** (4.52 x 10^{-2} M in CH_3CN, 100 μL, 4.53 μmol) was added to the mixture and stirred at that temperature for 24 h. Consumption of all of **11** and generation of **12** was confirmed by LC-MS analysis. Purification of the reaction mixture by reverse-phase HPLC and freezing-dry afforded 2.22 mg (61%) of **12** as a white amorphous.

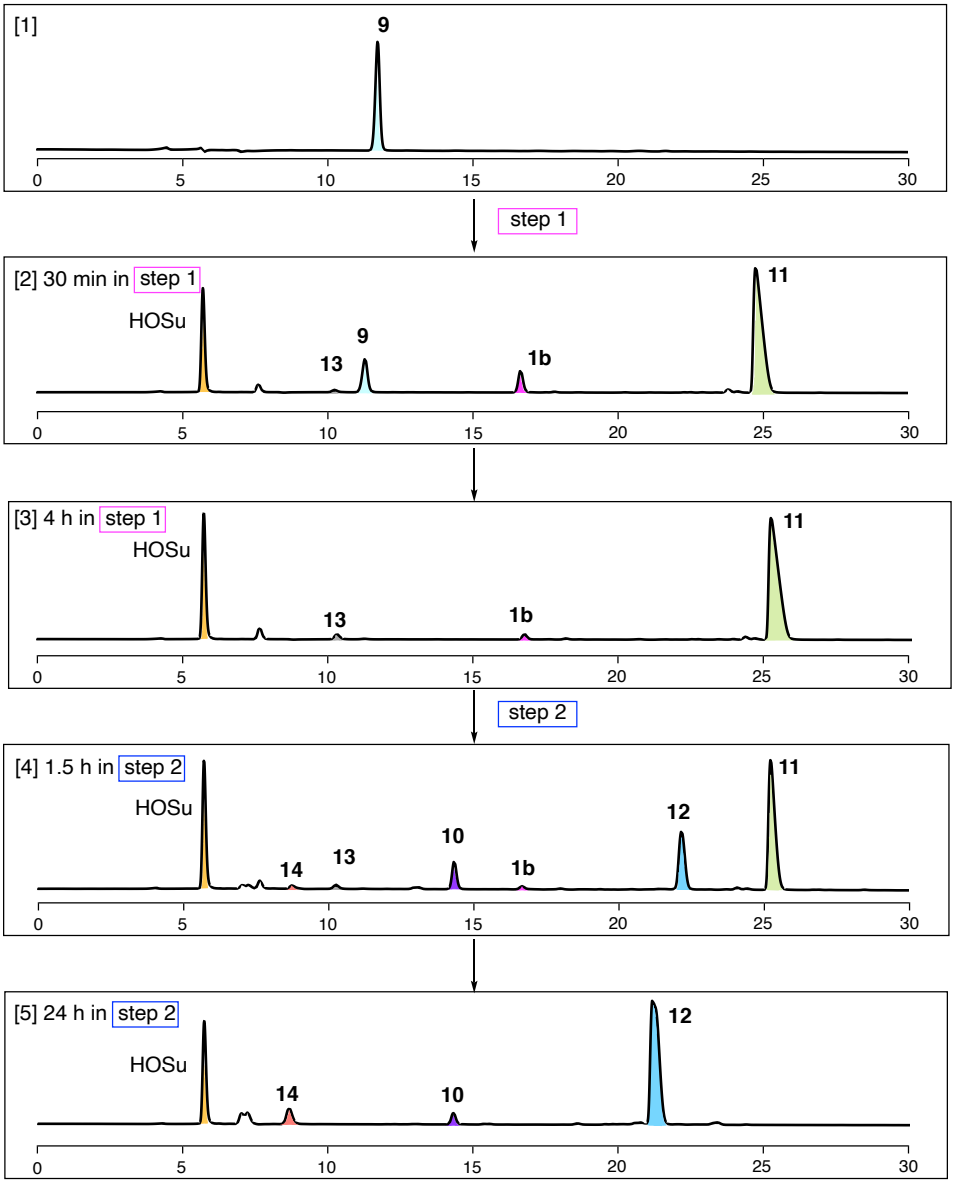
Analytical HPLC conditions: eluent: 1.0 mM formic acid aq./CH_3CN = 75:25 to 25:75 over 60 min., flow rate: 0.5 mL/min, detection: UV (220 nm) temp: 40 °C.

Preparative HPLC conditions: eluent: 5 mM formic acid aq./CH_3CN = 7:3, flow rate: 1.0 mL/min, detection: UV (220 nm) temp: 40 °C.

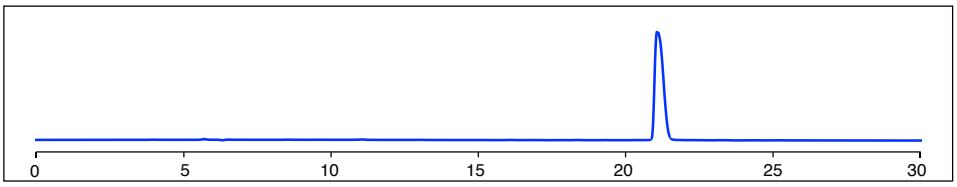
HPLC chart of 9, 1b, *N*-hydroxysuccinimide and 11



HPLC Analysis of Reaction Progress



HPLC Chart of 12 after Purification by Preparative Reverse-Phase HPLC



Retention Time and Observed Mass

9: retention time; 11.7 min, *m/z* 1265 $[M+H]^+$, 633 $[M+2H]^{2+}$.

HOSu: retention time; 5.73 min, not detected.

13: retention time; 10.3 min, *m/z* 317 $[M+H]^+$.

1b: retention time; 16.7 min, *m/z* 414 $[M+H]^+$.

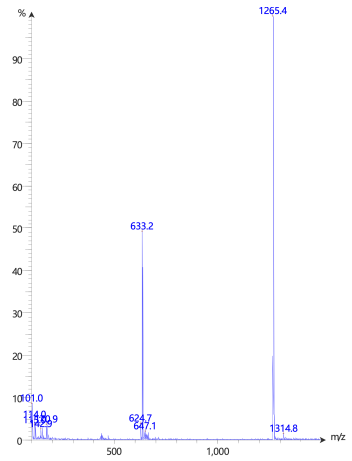
11: retention time; 25.2 min, *m/z* 1564 $[M+H]^+$, 782 $[M+2H]^{2+}$.

14: retention time; 8.66 min, *m/z* 761 $[M+H]^+$.

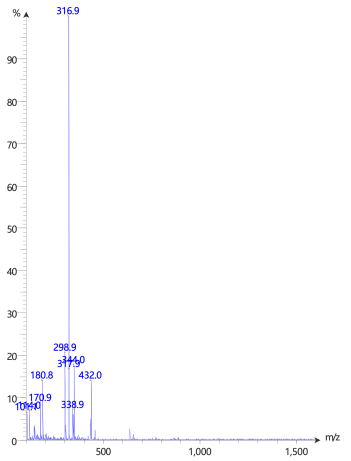
10: retention time; 14.3 min, *m/z* 445 $[M+H]^+$.

12: retention time; 21.2 min, *m/z* 1005 $[M+2H]^{2+}$.

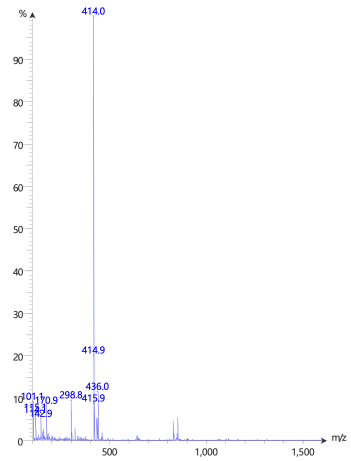
ESI-Mass Analysis



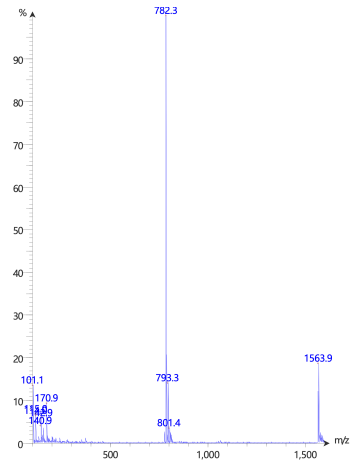
ESI-Mass Analysis of **9**



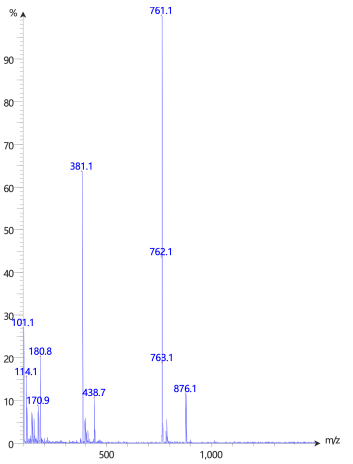
ESI-Mass Analysis of **13**



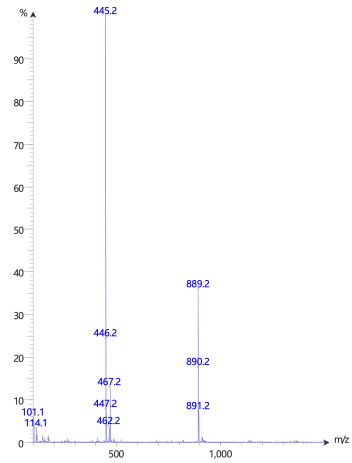
ESI-Mass Analysis of **1b**



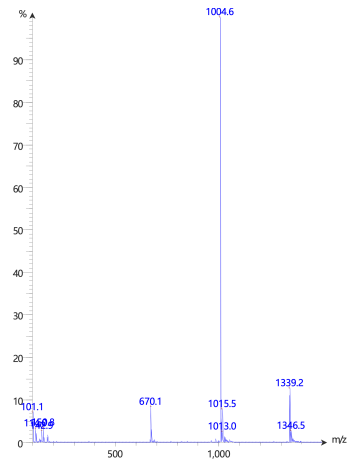
ESI-Mass Analysis of **11**



ESI-Mass Analysis of **14**



ESI-Mass Analysis of **10**



ESI-Mass Analysis of **12**

Connection of 13 with 15 using 2b.

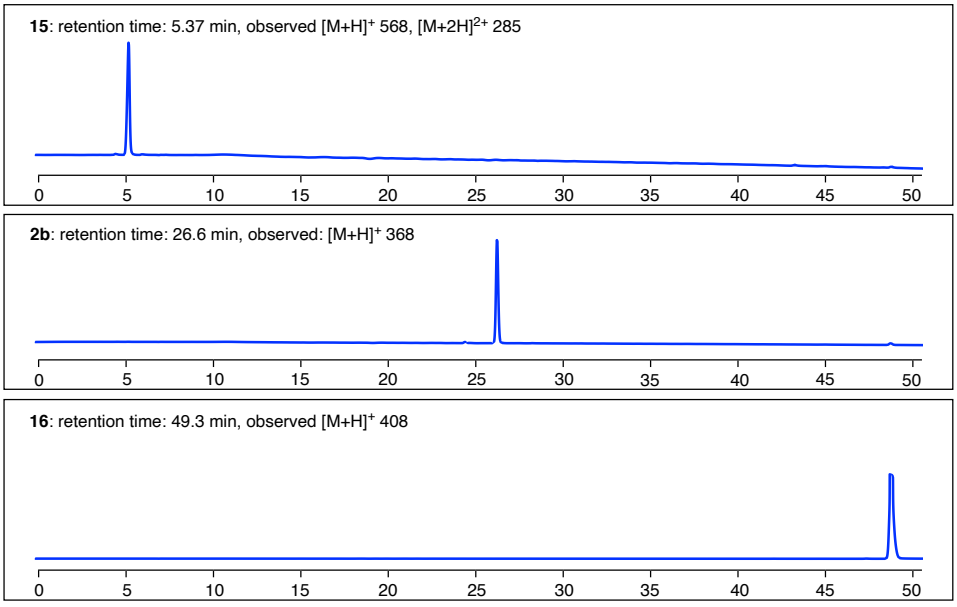
To avoid oxidation of peptide, the solvent was used after degassing by Ar bubbling.

To a solution of peptide **15** (1.66 mg, 2.93 μmol) in H_2O (50 μL)/CH_3CN (50 μL) was added *N*Ms-DACN maleimide **2b** (1.09 mg, 2.98 μmol) at 25 °C and stirred at that temperature for 1.5 h. After confirmation of consumption of all of **15** and generation of **17** was monitored by LC-MS analysis, **16** (5.40×10^{-2} M in CH_3CN, 81.3 μL, 4.39 μmol) was added to the mixture and stirred at that temperature for 36 h. Consumption of all of **17** and generation of **18** was confirmed by LC-MS analysis. Purification of the reaction mixture by preparative reverse-phase HPLC and freezing-dry afforded 2.30 mg (59%) of **18** as a white amorphous.

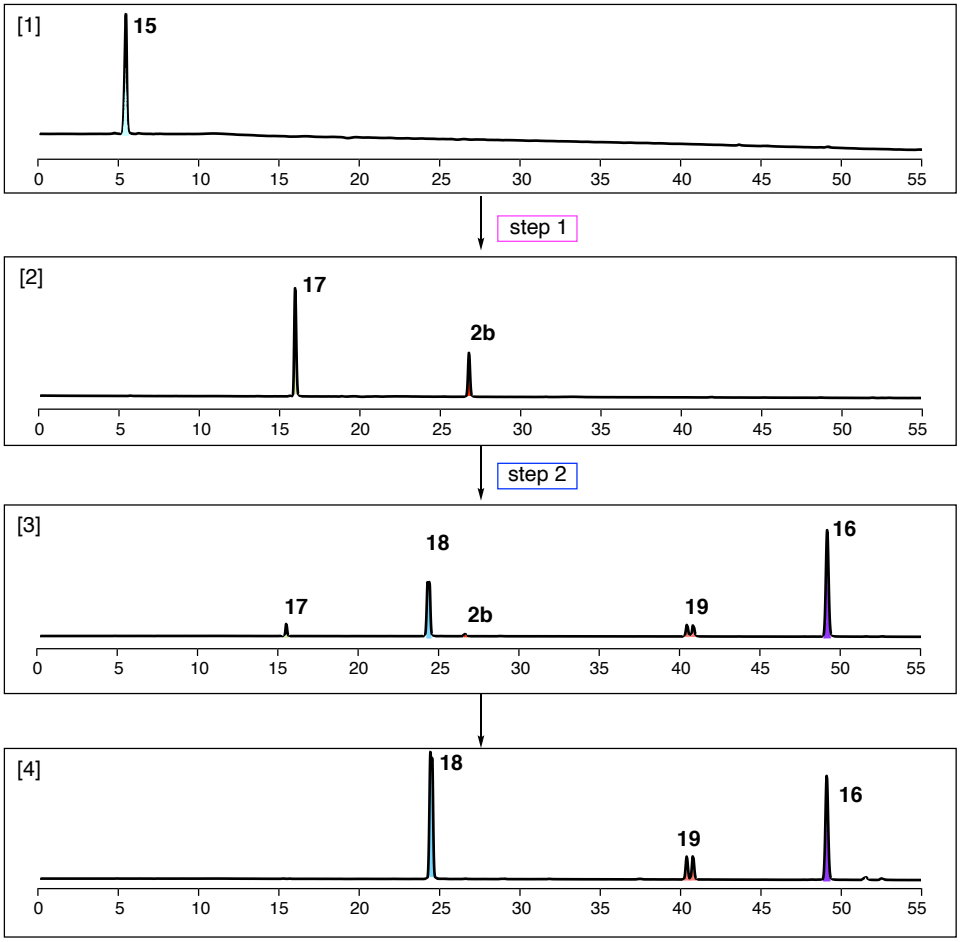
Analytical HPLC conditions: eluent: 1.0 mM formic acid aq./CH_3CN = 90:10 to 10:90 over 60 min., flow rate: 0.5 mL/min, detection: UV (220 nm), temp: 40 °C.

Preparative HPLC conditions: eluent: 1.0 mM formic acid aq./CH_3CN = 8:2, flow rate: 1.0 mL/min, detection: UV (220 nm), temp: 40 °C.

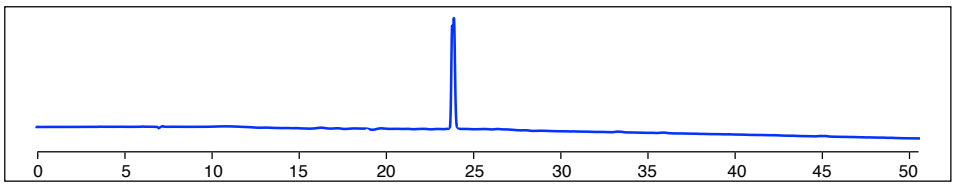
HPLC Chart of 15, 2b and 16



HPLC Analysis of Reaction Progress



HPLC Chart of 18 after Purification by Preparative Reverse-Phase HPLC



Retention Time and Observed Mass

15: retention time; 5.37 min, *m/z* 568 $[M+H]^+$, 285 $[M+2H]^{2+}$.

17: retention time; 15.9 min, *m/z* 935 $[M+H]^+$, 468 $[M+2H]^{2+}$.

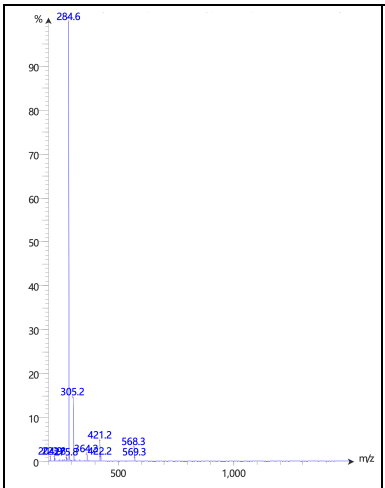
2b: retention time; 26.8 min, *m/z* 368 $[M+H]^+$.

18: retention time; 24.4 min, *m/z* 1342 $[M+H]^+$, 672 $[M+2H]^{2+}$.

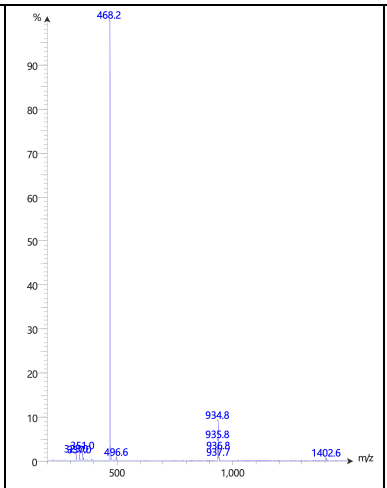
19: retention time; 40.4, 40.8 min, *m/z* 775 $[M+H]^+$, 388 $[M+2H]^{2+}$.

16: retention time; 49.2 min, *m/z* 408 $[M+H]^+$.

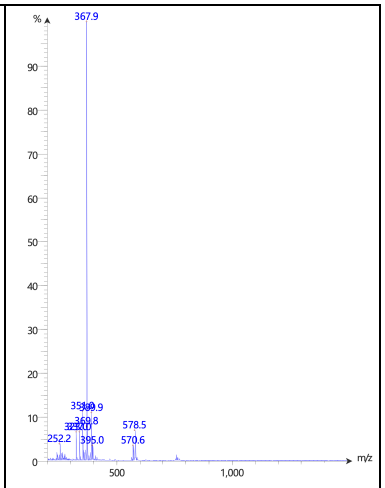
ESI-Mass Analysis



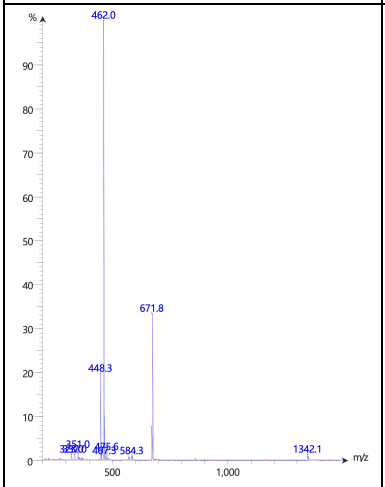
ESI-Mass Analysis of **15**



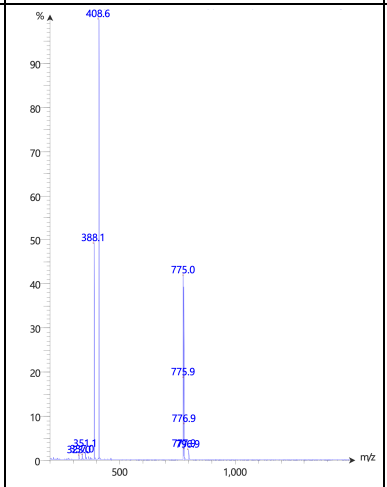
ESI-Mass Analysis of **17**



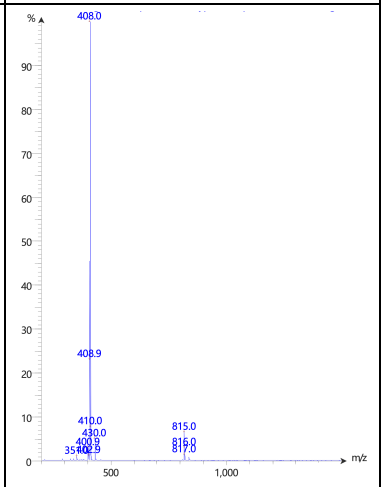
ESI-Mass Analysis of **2b**



ESI-Mass Analysis of **18**



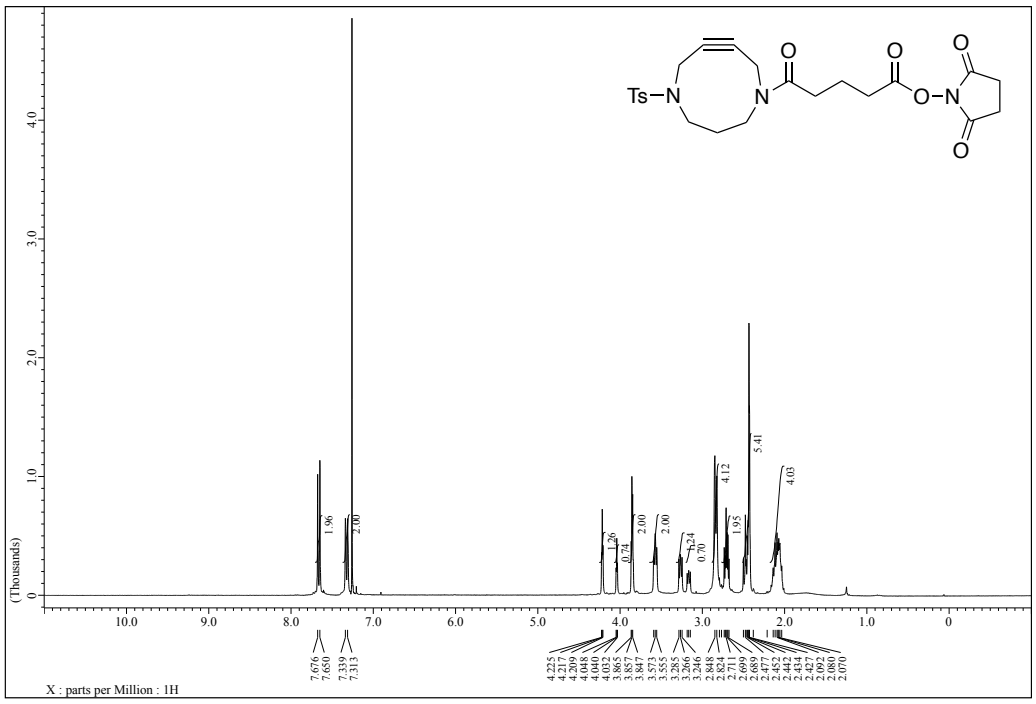
ESI-Mass Analysis of **19**



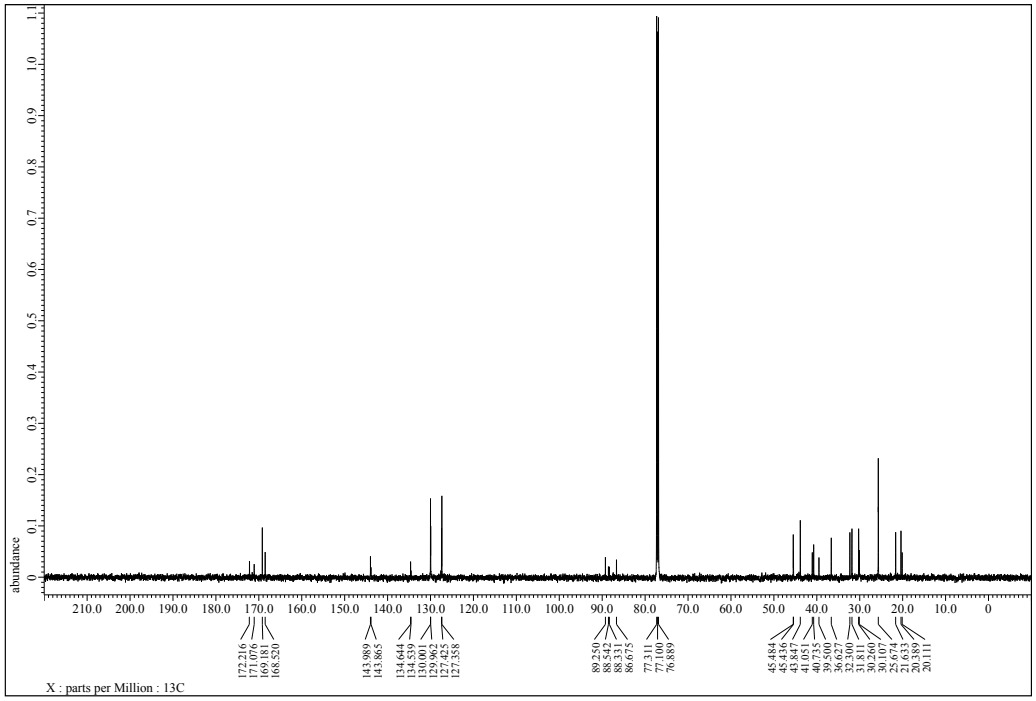
ESI-Mass Analysis of **16**

5 ^{1}H, ^{13}C NMR Spectra

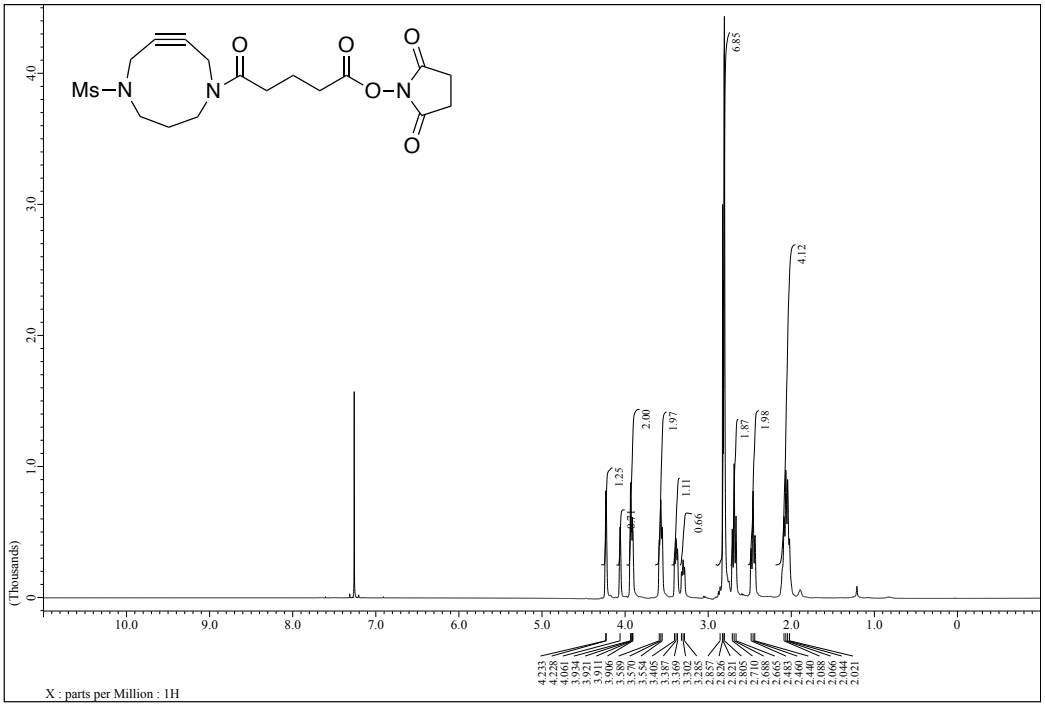
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **1a**



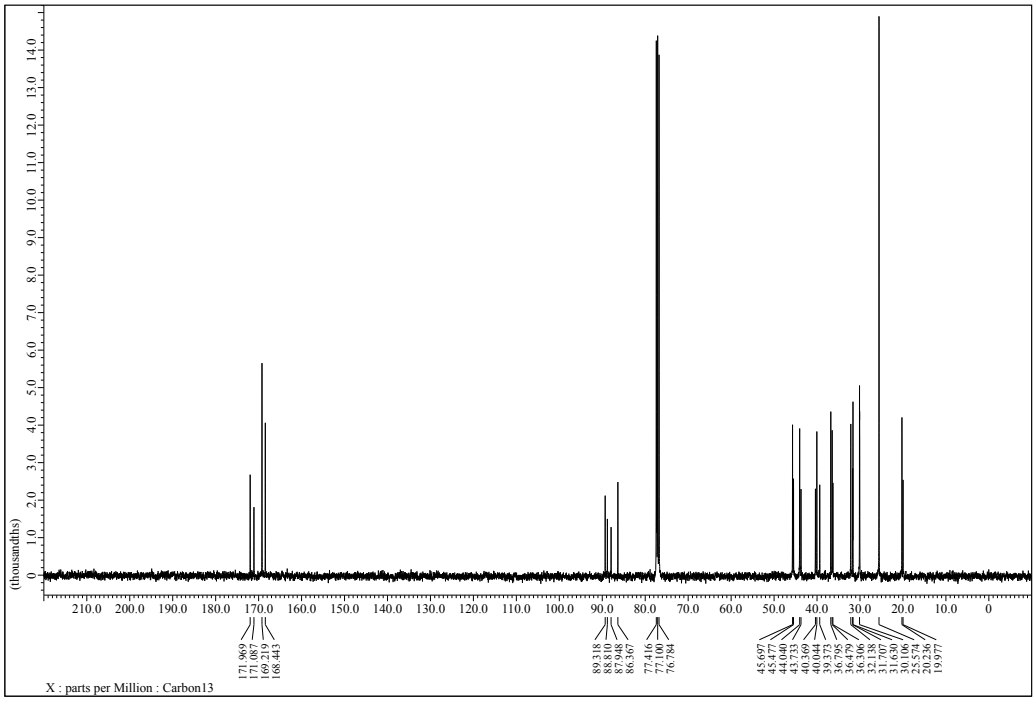
^{13}C NMR Chart (125 MHz in $CDCl_3$) of **1a**



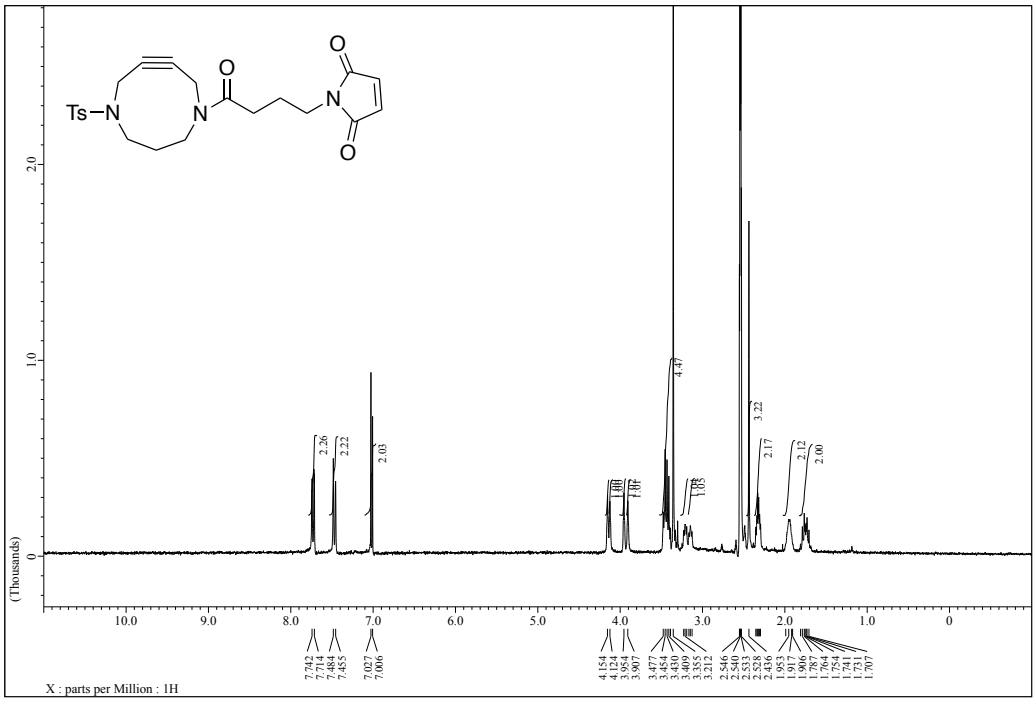
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **1b**



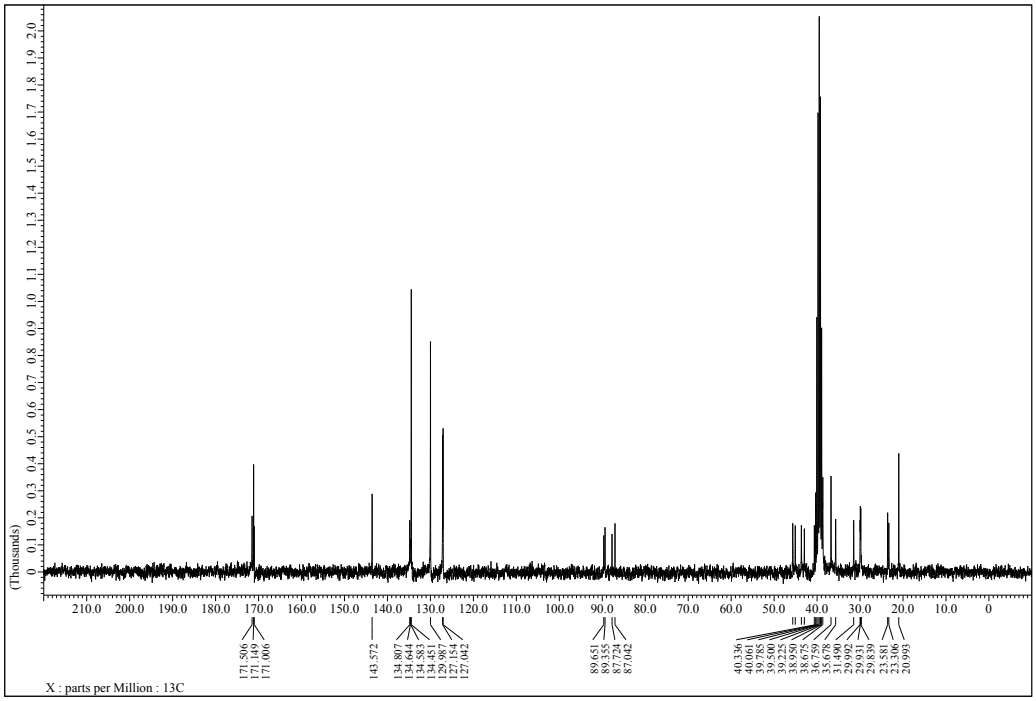
^{13}C NMR Chart (100 MHz in $CDCl_3$) of **1b**



^{1}H NMR Chart (300 MHz in DMSO-d_6) of **2a**

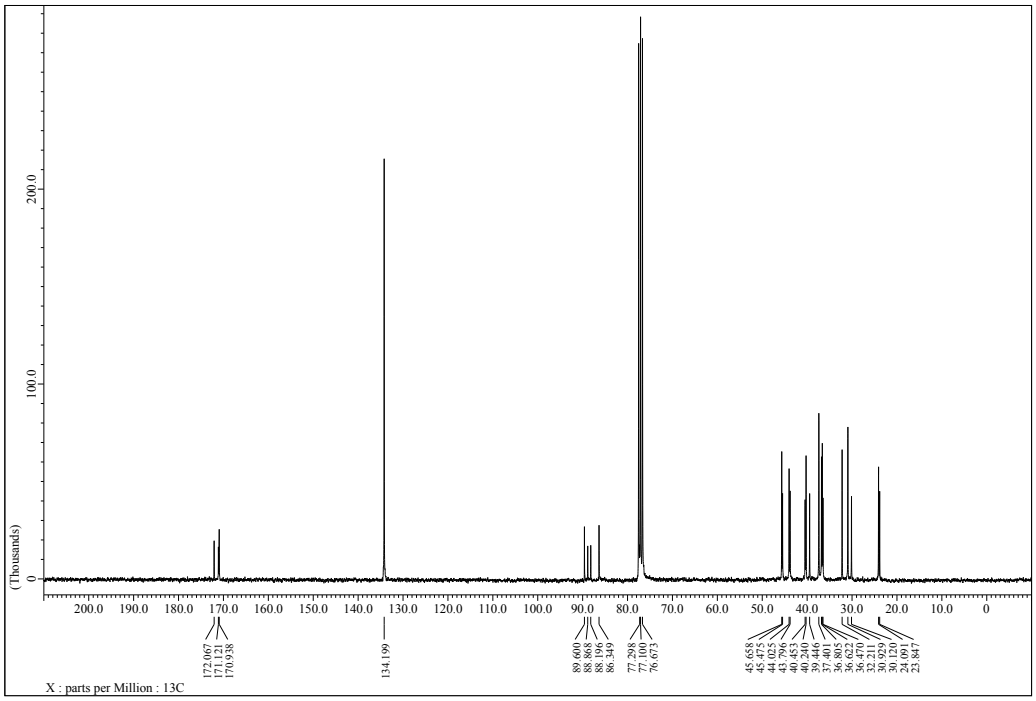


^{13}C NMR Chart (75 MHz in DMSO-d_6) of **2a**

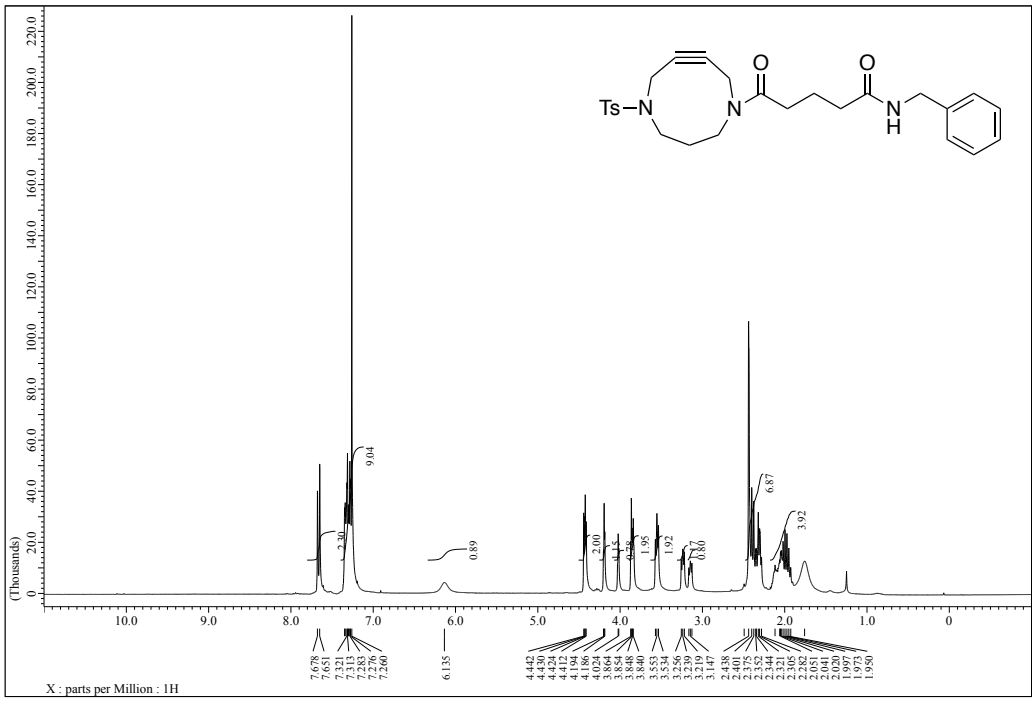


^{1}H NMR Chart (300 MHz in $CDCl_3$) of **2b**

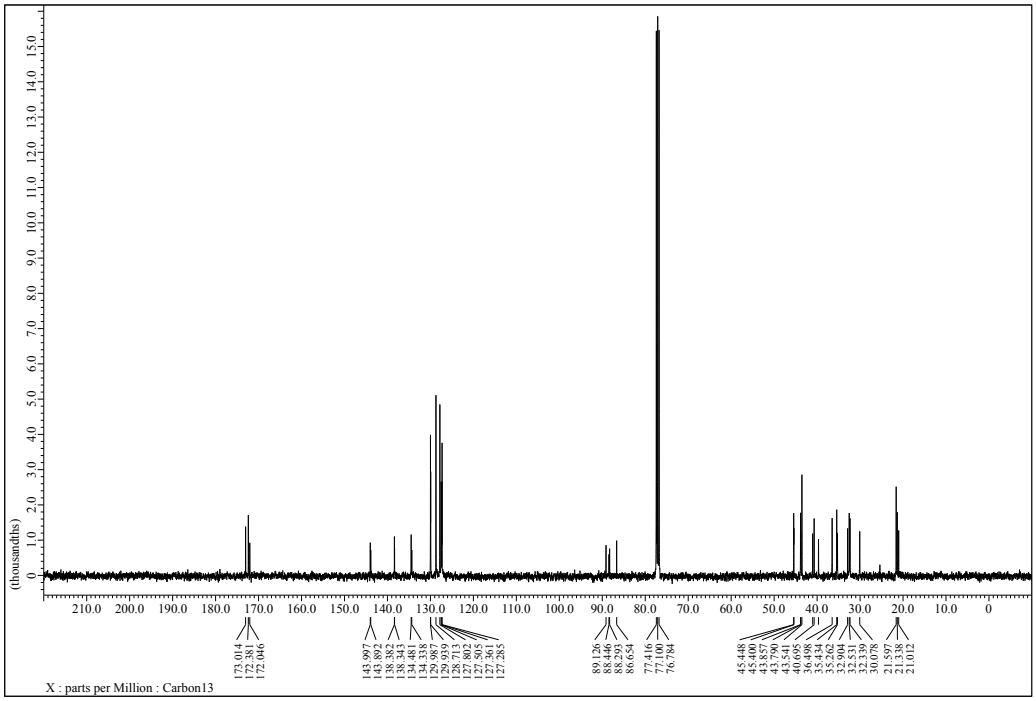
^{13}C NMR Chart (75 MHz in $CDCl_3$) of **2b**



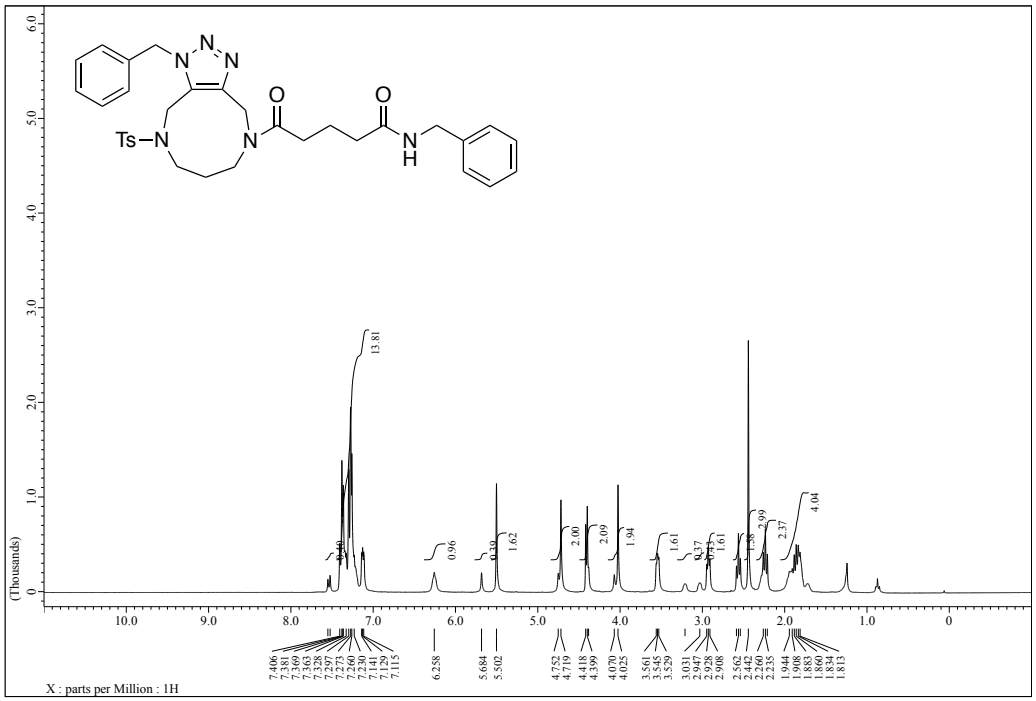
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **5a**



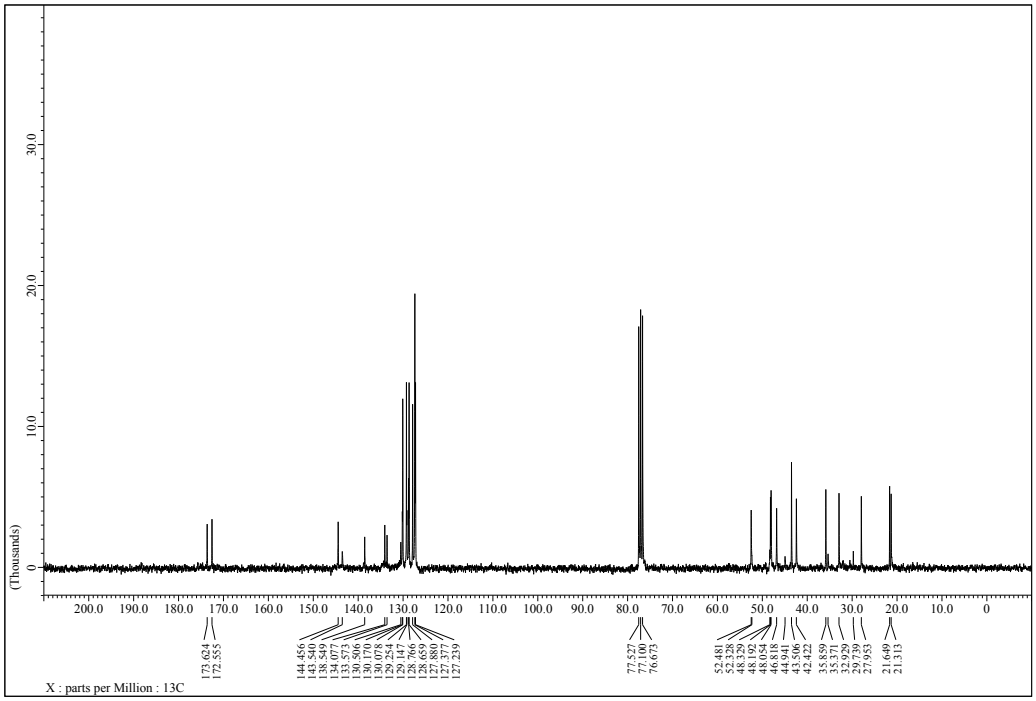
^{13}C NMR Chart (100 MHz in $CDCl_3$) of **5a**



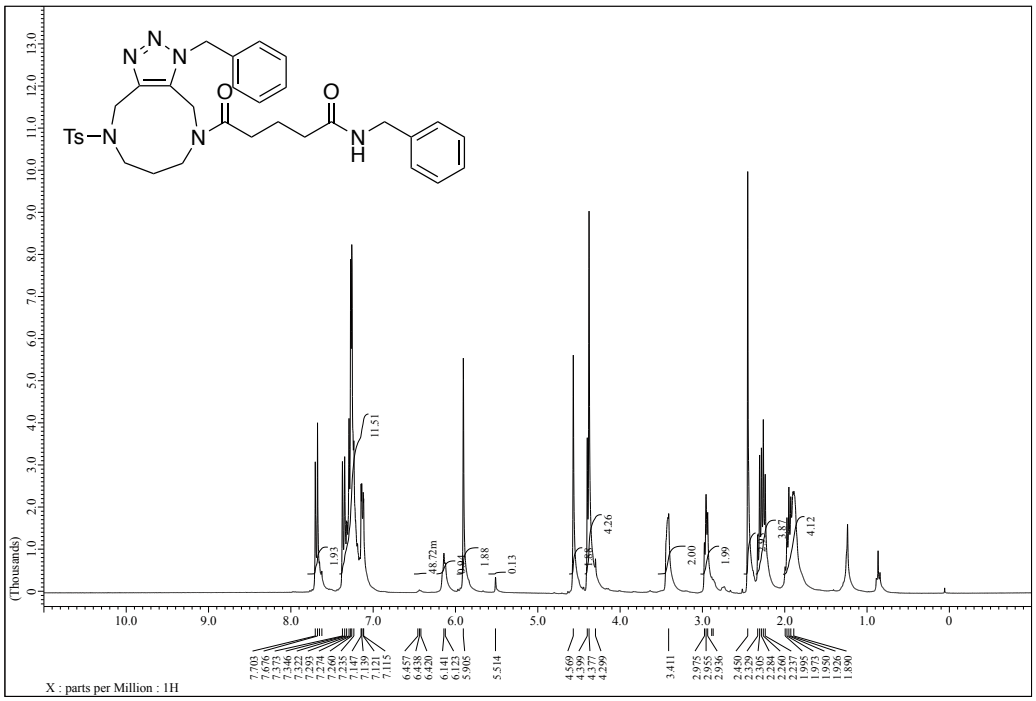
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **6a**



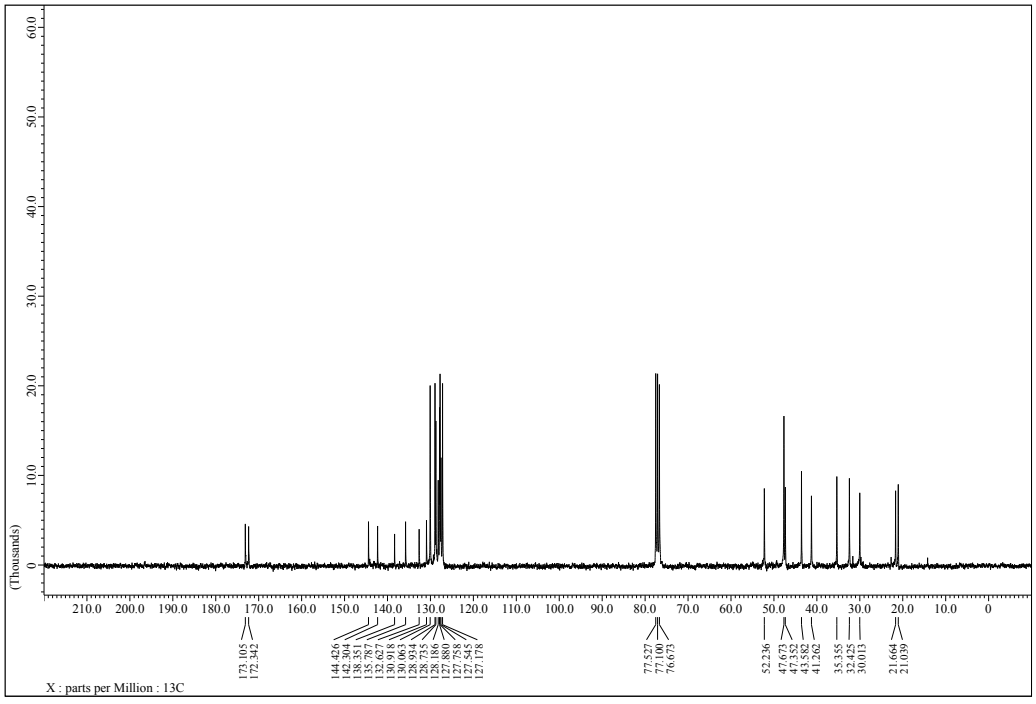
^{13}C NMR Chart (75 MHz in $CDCl_3$) of **6a**



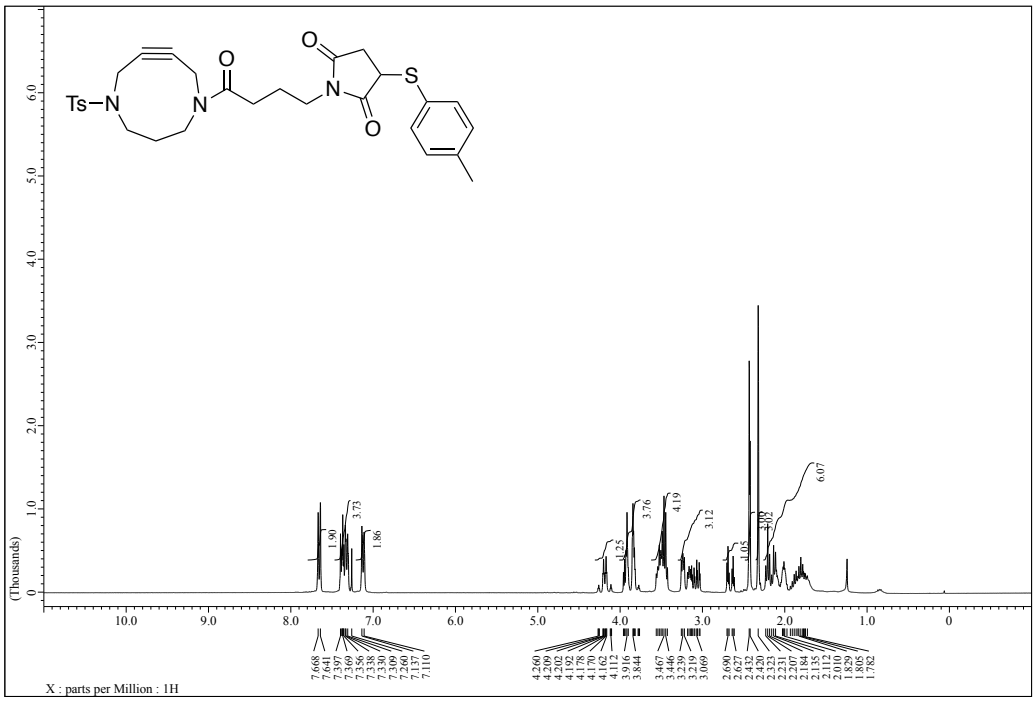
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **6a'**



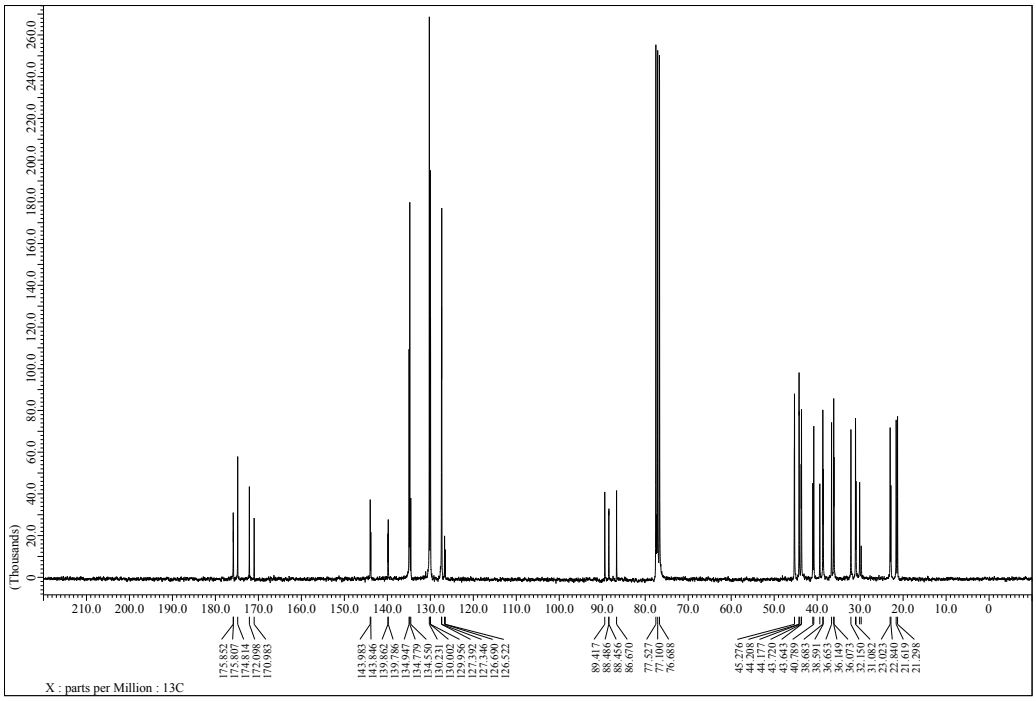
^{13}C NMR Chart (75 MHz in $CDCl_3$) of **6a'**



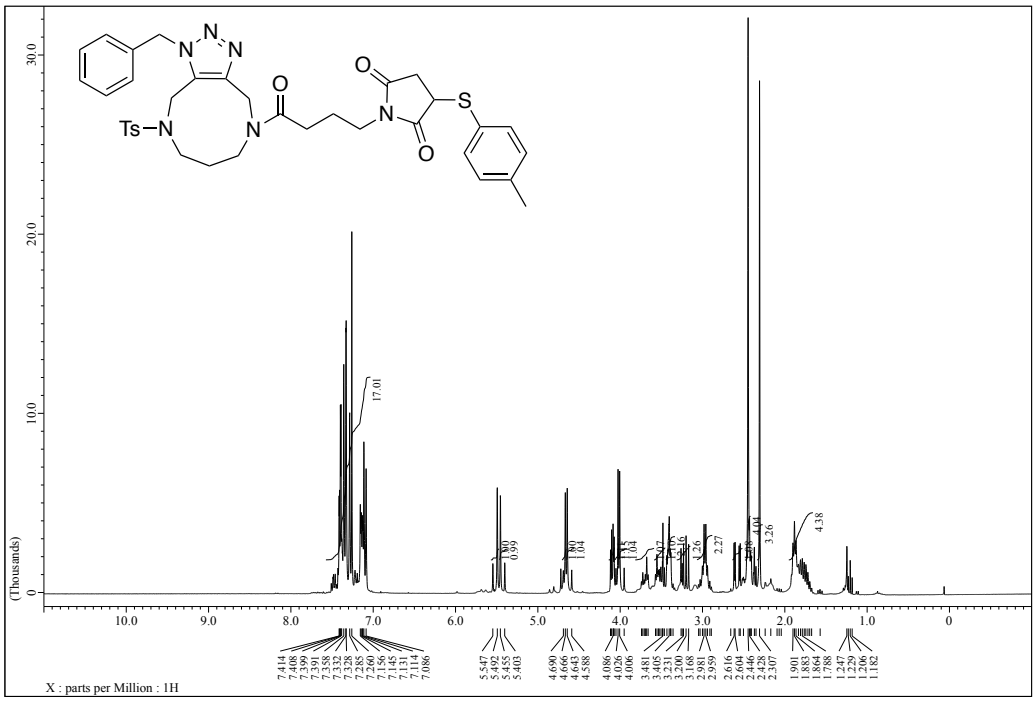
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **7a**



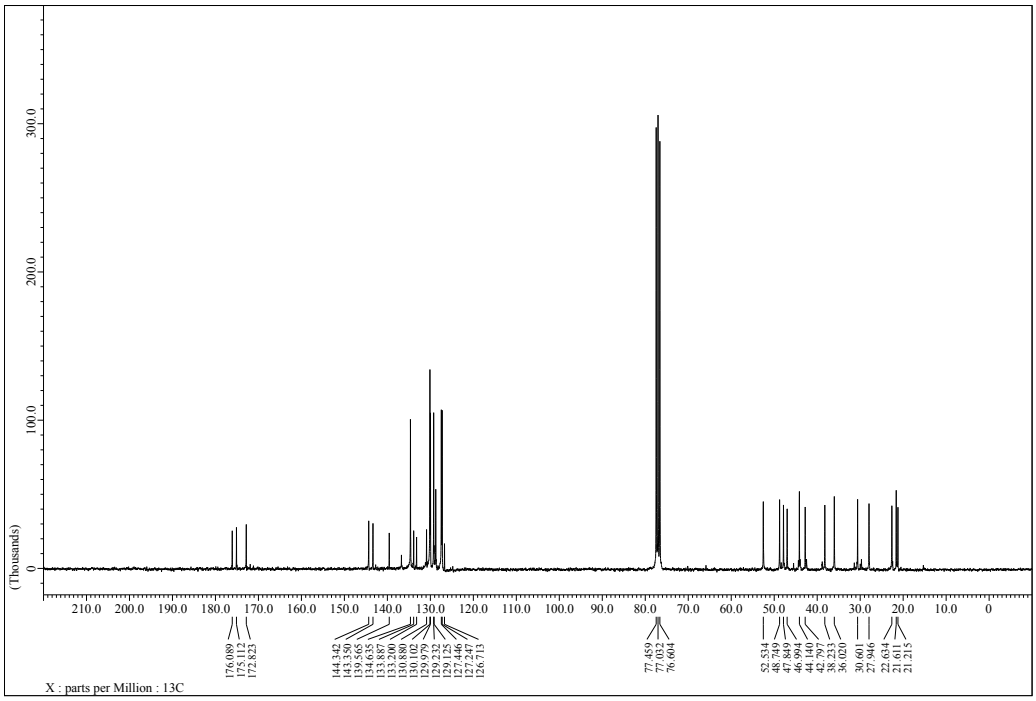
^{13}C NMR Chart (75 MHz in $CDCl_3$) of **7a**



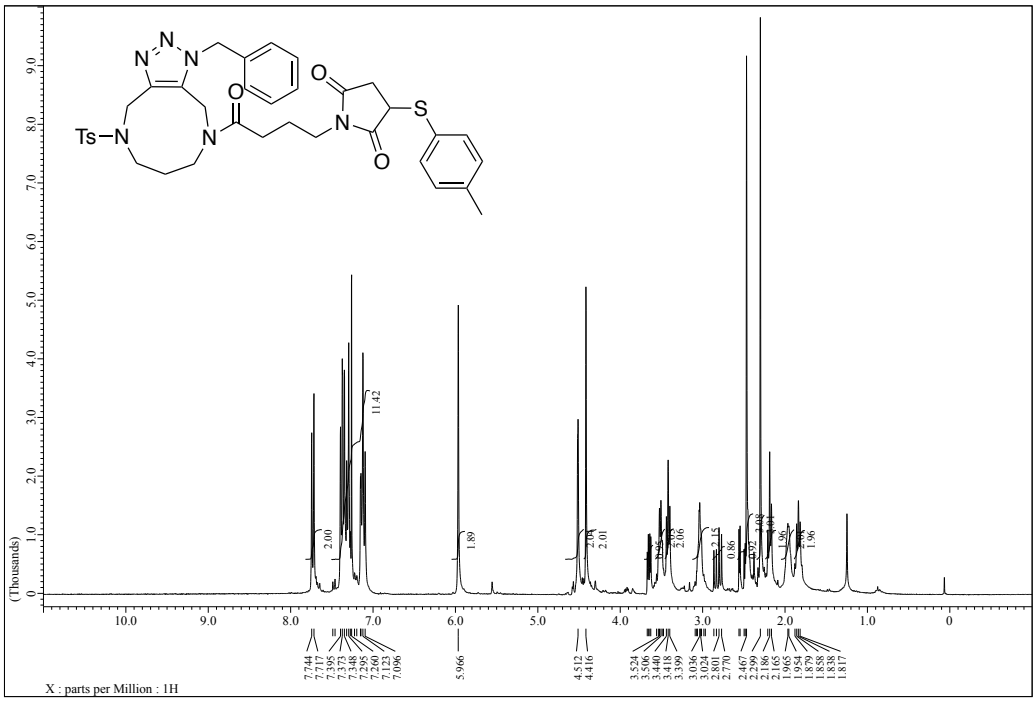
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **8a**



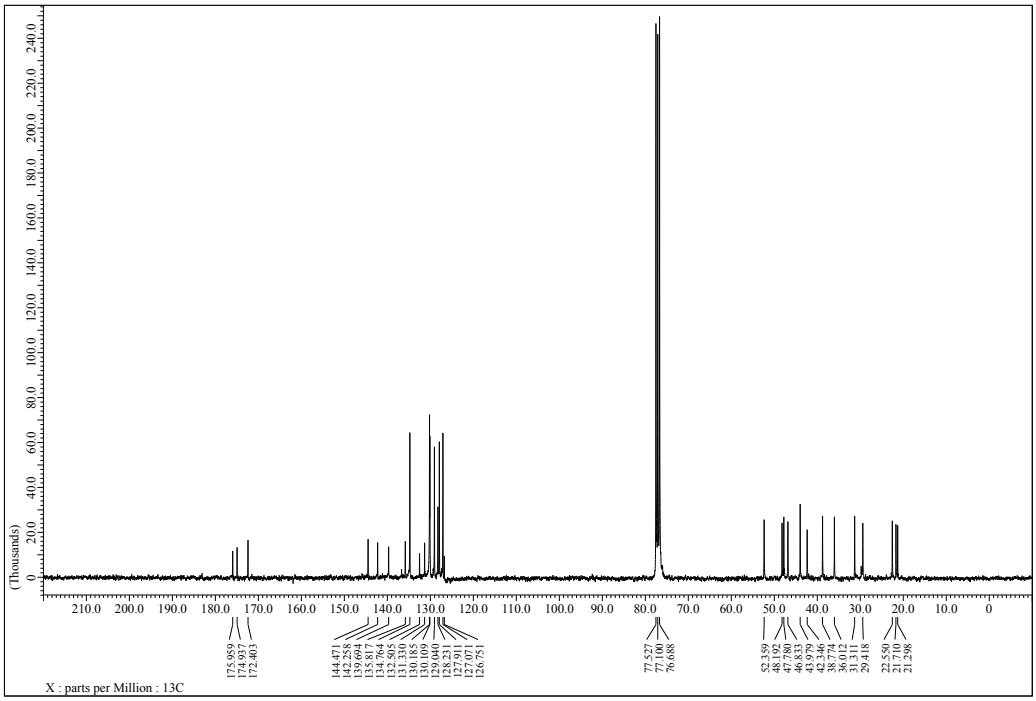
^{13}C NMR Chart (75 MHz in $CDCl_3$ of **8a**



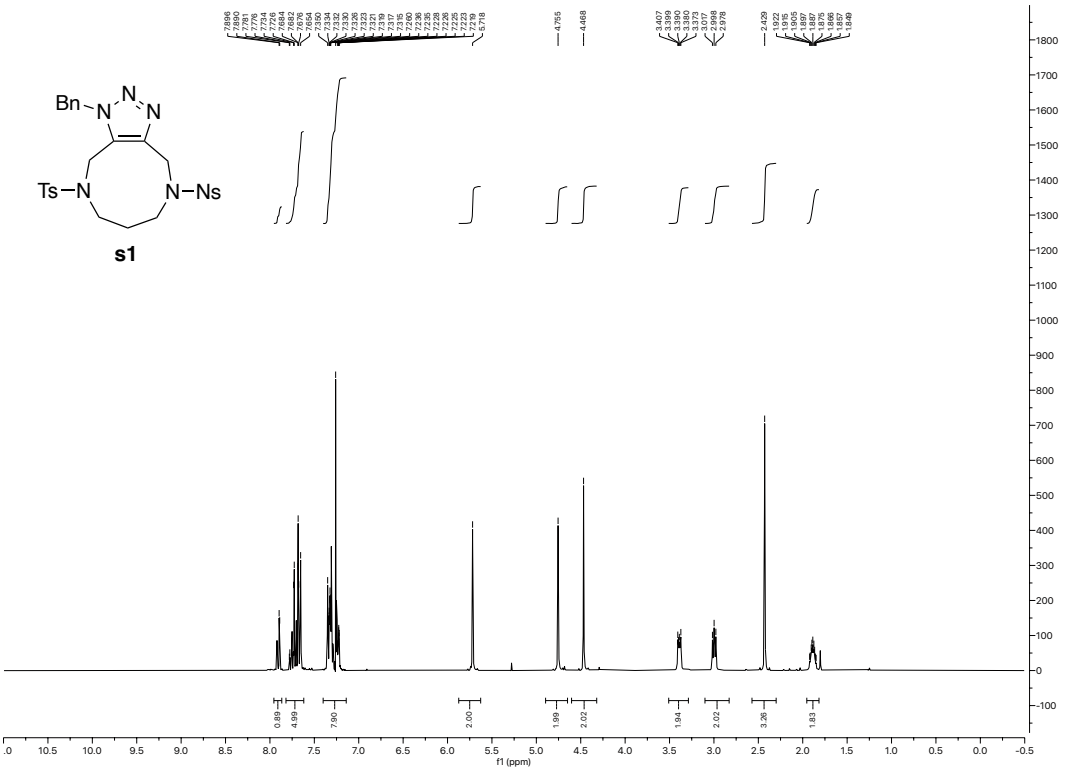
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **8a'**



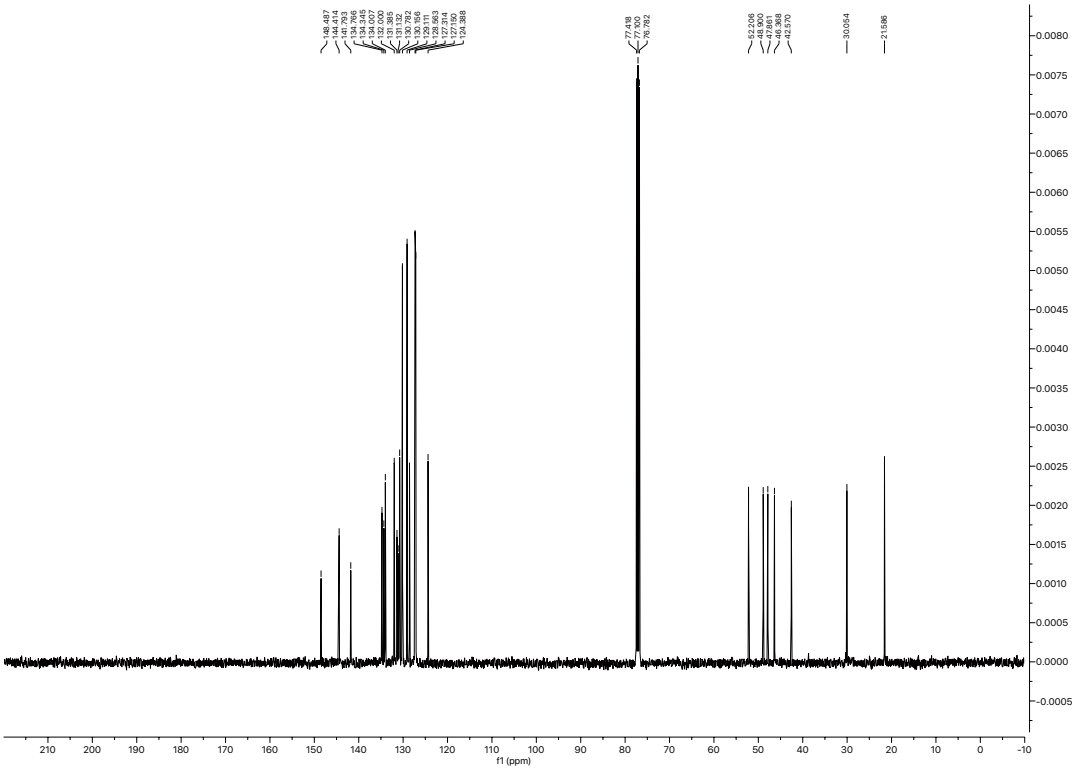
^{13}C NMR Chart (75 MHz in $CDCl_3$) of **8a'**



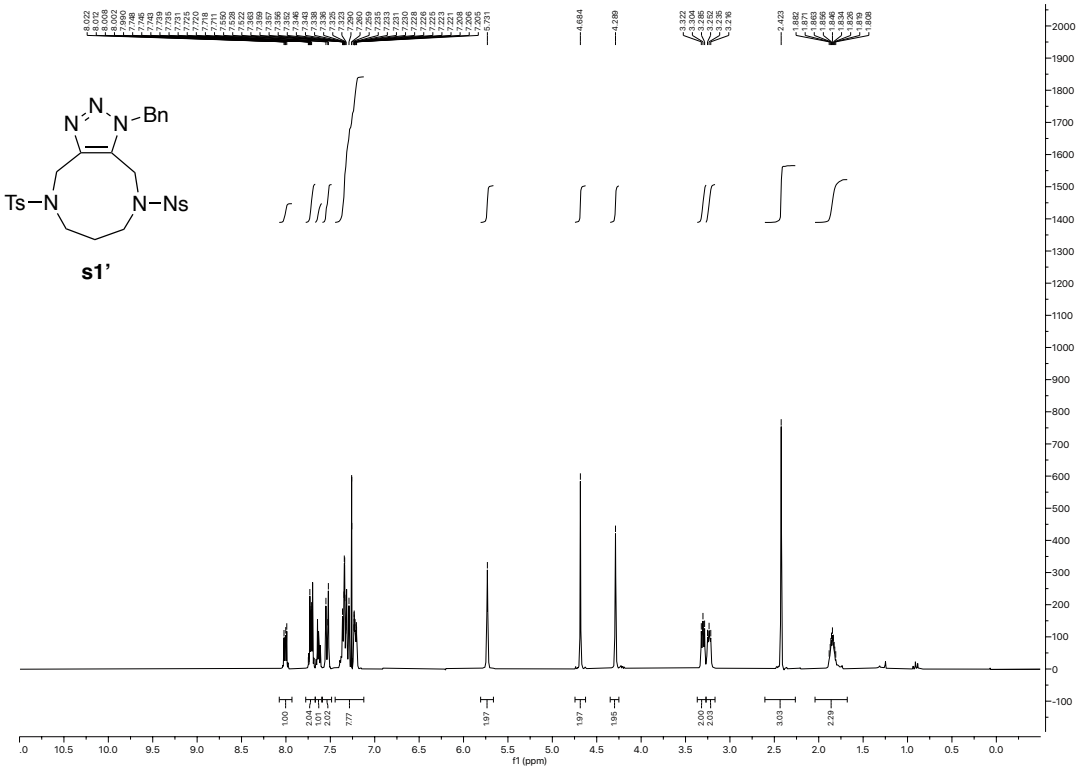
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **s1**



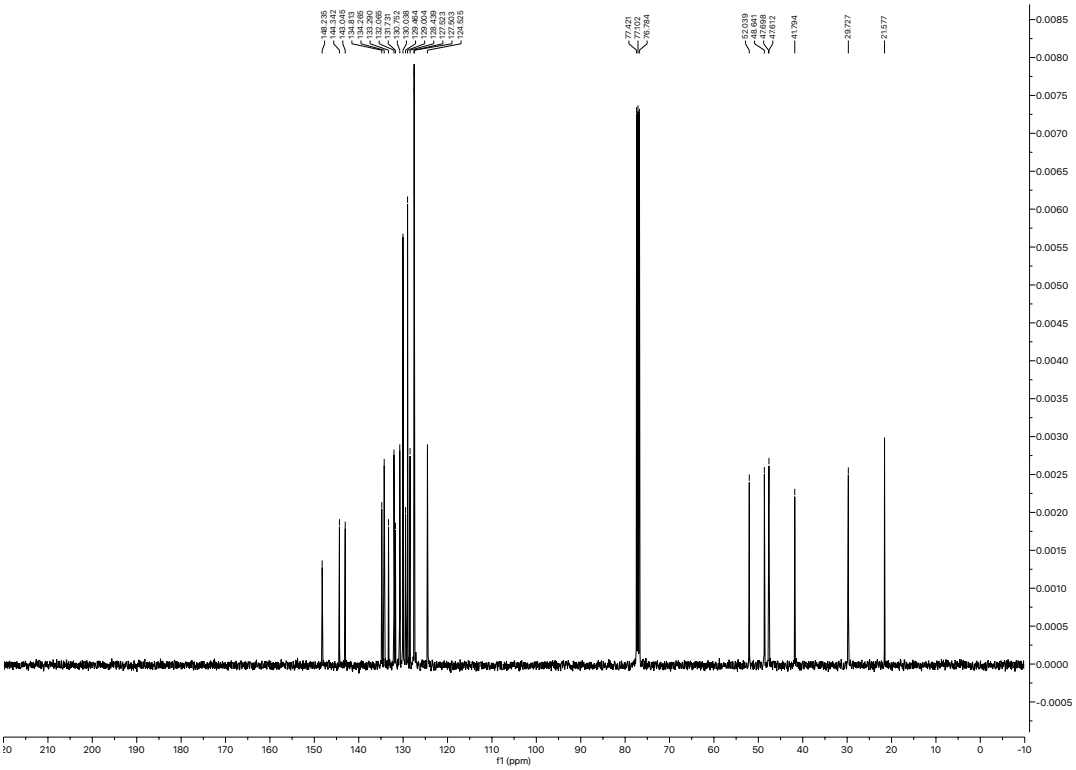
^{13}C NMR Chart (100 MHz in $CDCl_3$) of **s1**



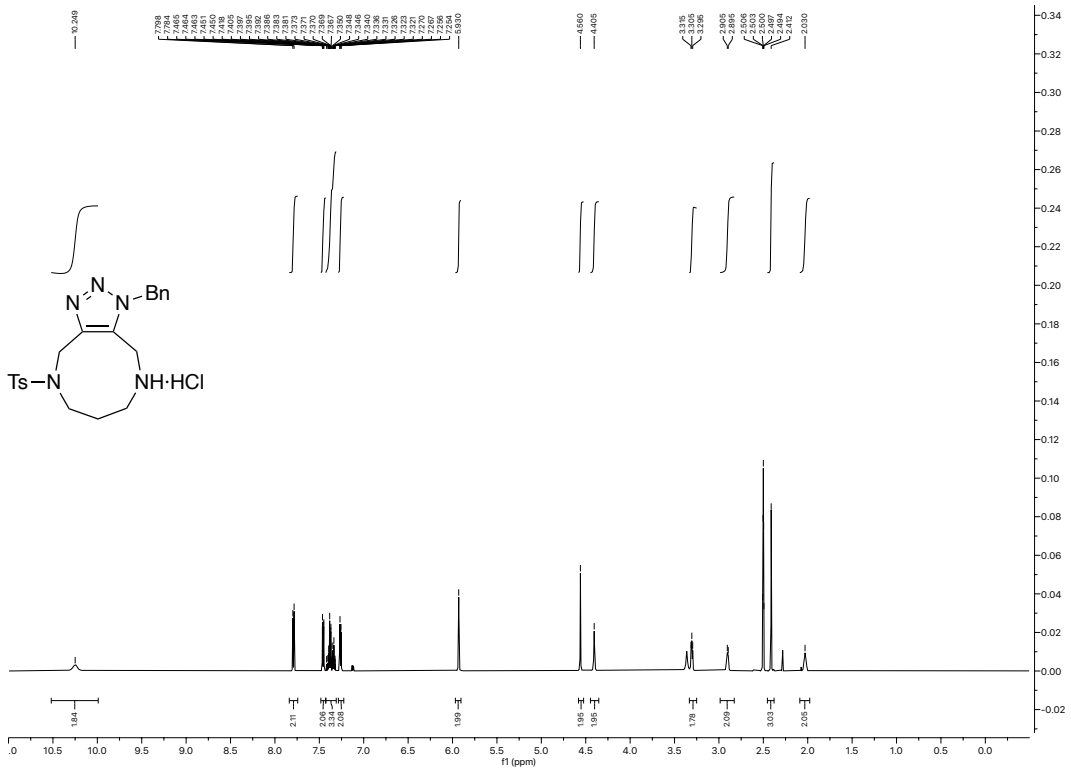
^{1}H NMR Chart (300 MHz in $CDCl_3$) of **s1'**



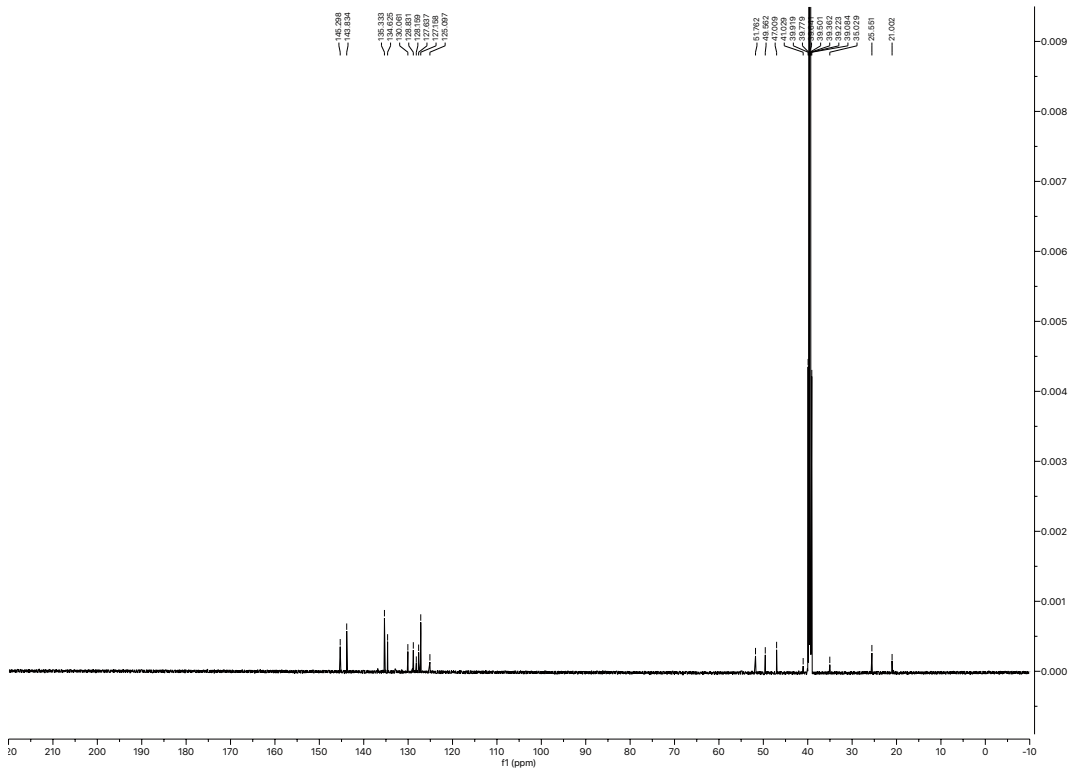
^{13}C NMR Chart (100 MHz in $CDCl_3$) of **s1'**



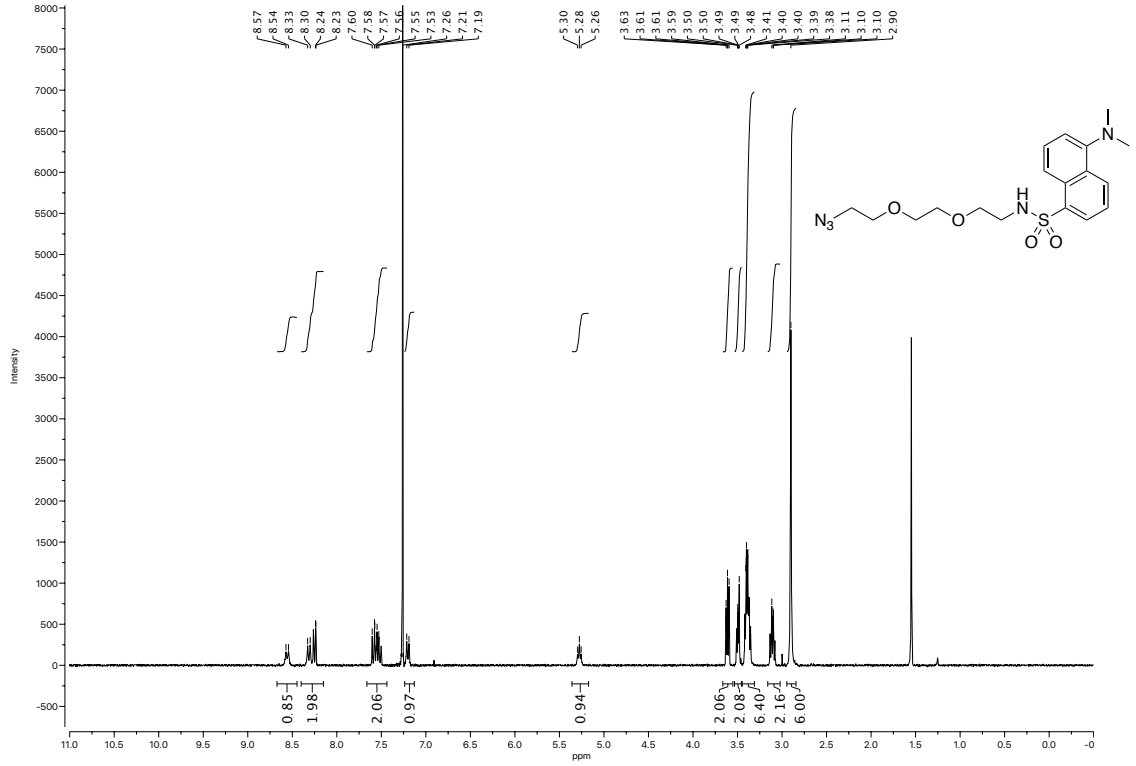
^{1}H NMR Chart (600 MHz in DMSO-d_6) of **s2'**



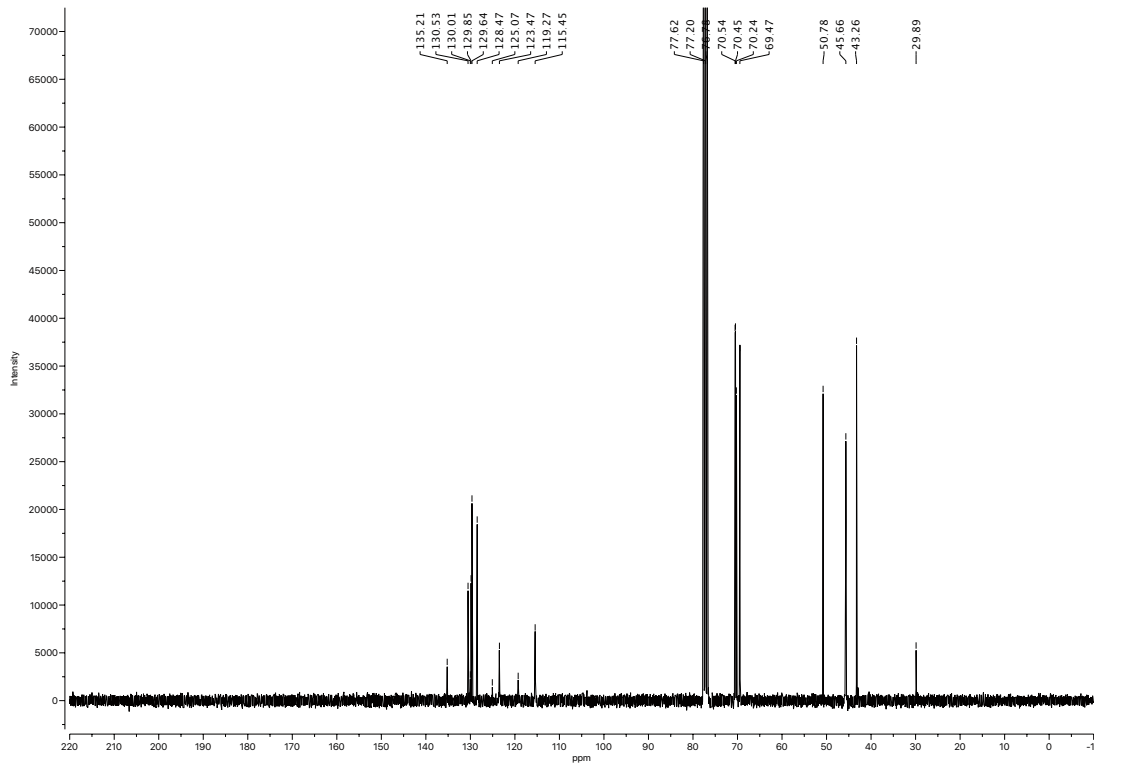
^{13}C NMR Chart (150 MHz inDMSO-d_6) of **s2'**



^{1}H NMR Chart (300 MHz in $CDCl_3$) of **16**



^{13}C NMR Chart (75 MHz in $CDCl_3$) of **16**



6. Control Experiments

Comparison of CFAAC Rate of One Pot and Stepwise Reaction using DACN-NHS Ester

One Pot Reaction

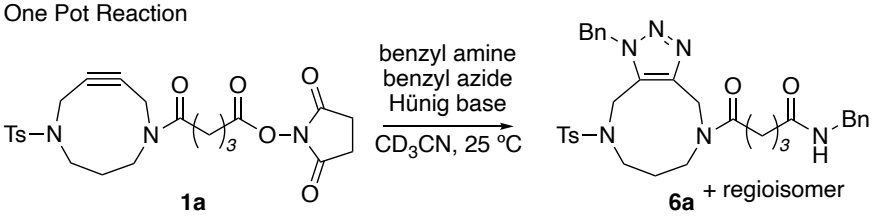
The one pot reaction of benzyl azide (1.0 eq.) and benzyl amine (1.0 eq.) with **1a** (1.0 eq.) was performed in the presence of Hünig base (1.0 eq.). All compounds were mixed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **1a** at 25 °C was monitored by ^1H NMR (600 MHz) analysis.

Stepwise Reaction

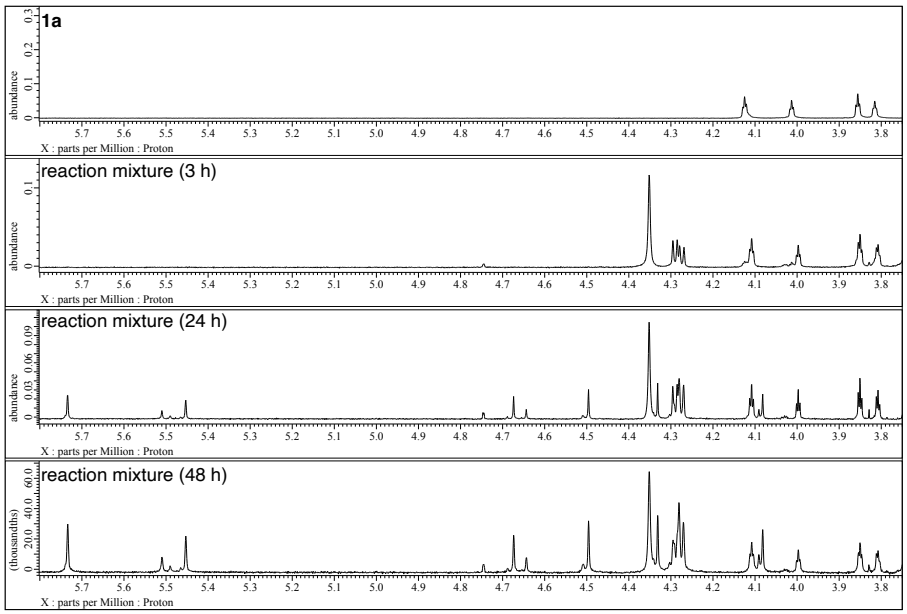
The reaction of benzyl azide (1.0 eq.) and **5a** (1.0 eq.) was performed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **5a** at 25 °C was monitored by ^1H NMR (600 MHz) analysis.

After 3 h, most of **1a** was converted into **5a** in the one pot reaction. After 24 and 48 h, the both ^1H NMR spectra of the one pot and stepwise reactions were almost identical, which means that the CFAAC rates of the reactions are similar level.

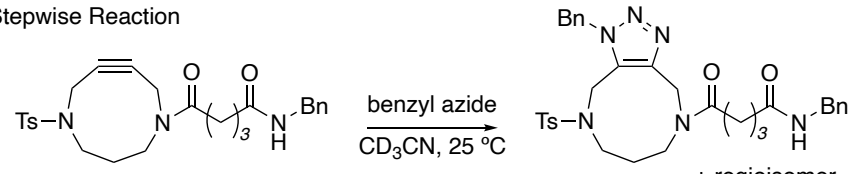
One Pot Reaction



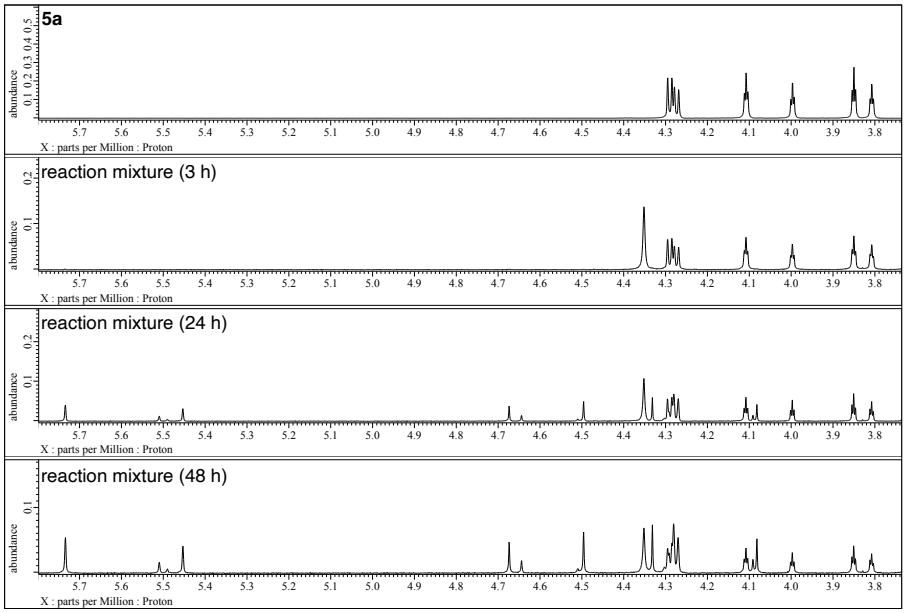
1a **6a**



Stepwise Reaction



5a **6a**



Comparison of CFAAC Rate of One-Pot Reaction and Stepwise Reaction using DACN-Maleimide

One Pot Reactioin

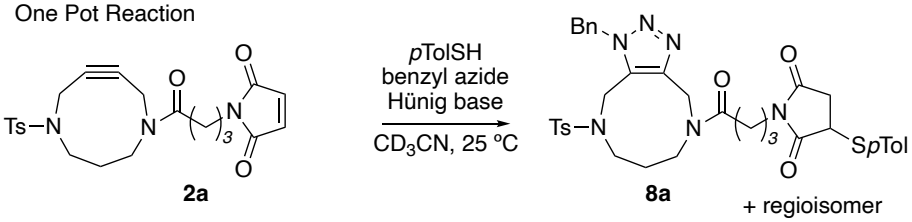
The one pot reaction of benzyl azide (1.0 eq.) with *p*-toluene thiol (1.0 eq.) using **2a** (1.0 eq.) was performed in the presence of Hünig base (1.0 eq.). All compounds were mixed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **2a** at 25 °C was monitored by ^{1}H NMR (600 MHz) analysis.

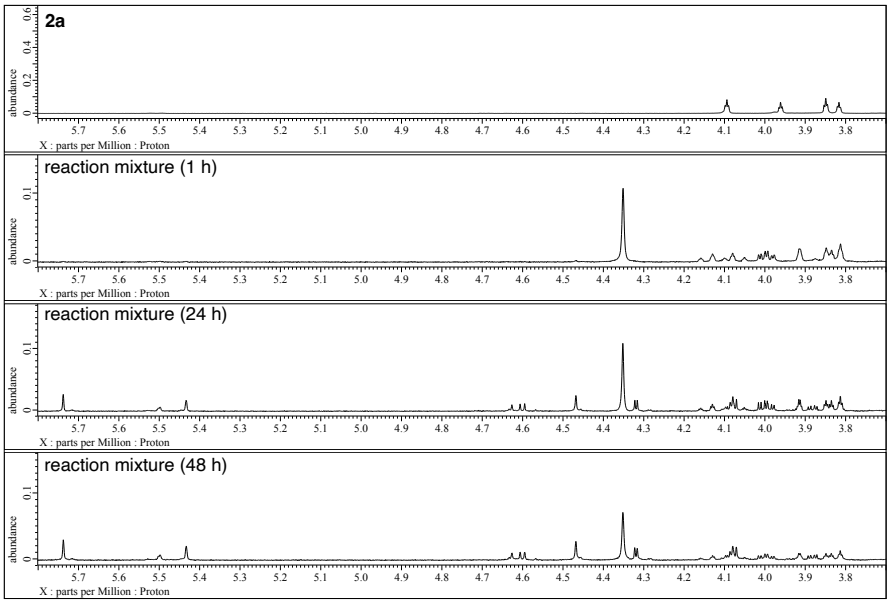
Stepwise Reaction

The reaction of benzyl azide (1.0 eq.) and **7a** (1.0 eq.) was performed in mixed in equal moles at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **7a** at 25 °C was monitored by ^{1}H NMR (600 MHz) analysis.

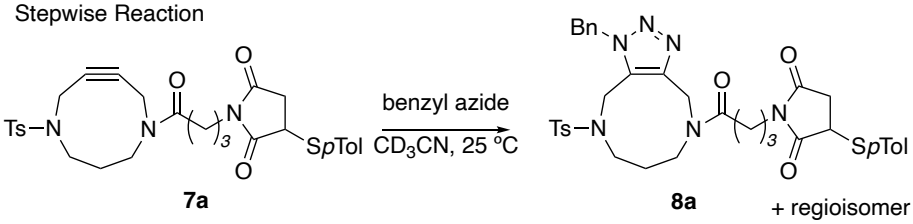
After 1 h, most of **2a** was converted into **7a** in the one pot reaction. After 24 and 48 h, the both ^{1}H NMR spectra of the one pot and stepwise reactions were almost identical, which means that the CFAAC rates of the reactions are similar level.

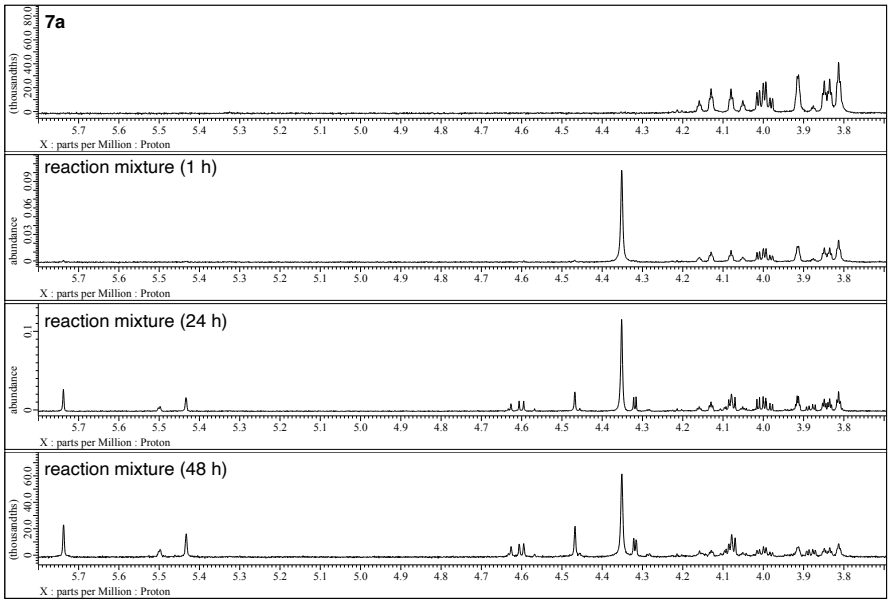
One Pot Reaction



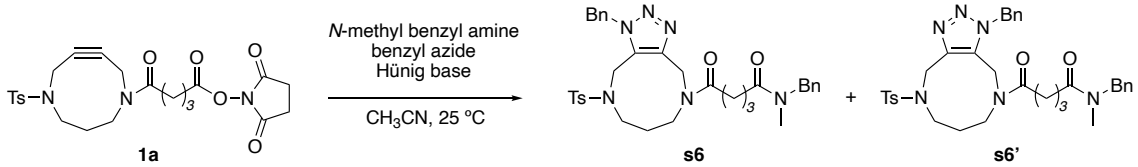


Stepwise Reaction





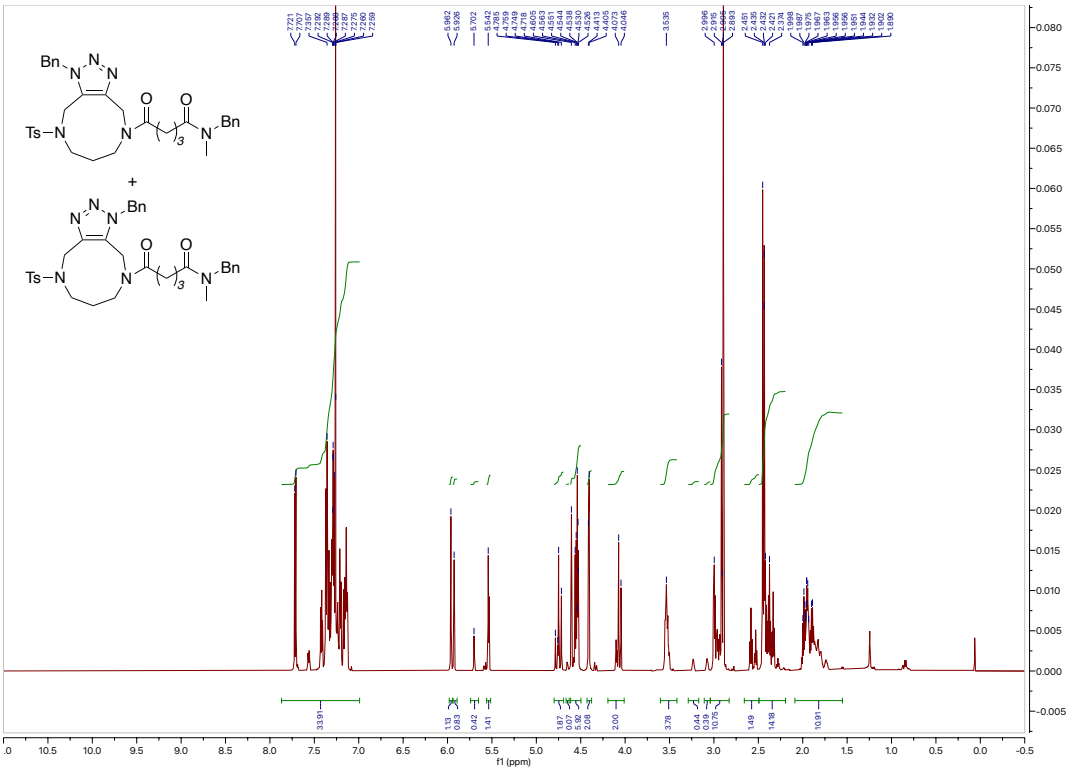
Conjugation of Azide and Secondary Amine with *N*Ts-DACN-NHS Ester



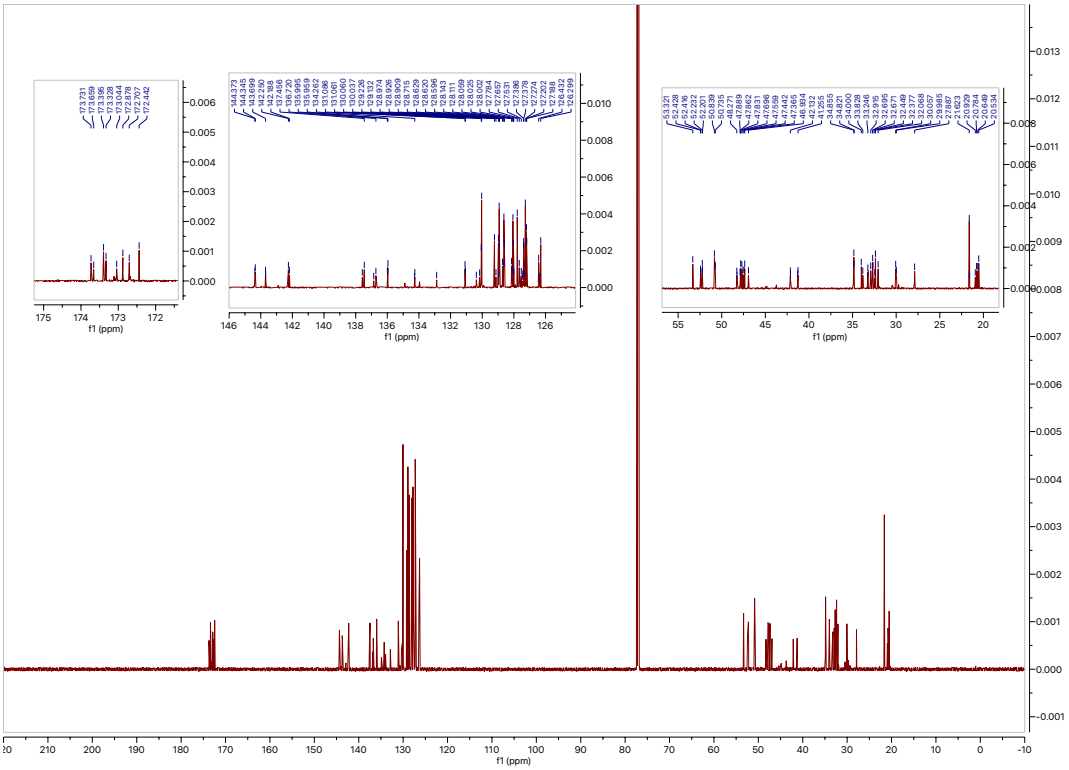
To a solution of **1a** (40.3 mg, 0.0823 mmol) in CH_3CN (4 mL) ware added benzyl azide (10.3 μL, 0.0823 mmol), *N*-methyl benzyl amine (10.6 μL, 0.0823 mmol) and Hünig's base (14.2 μL, 0.0823 mmol) at 25 ºC. After the mixture was stirred at that temperature for 50 h, the reaction was quenched with H_2O and extracted three times with AcOEt. The combined organic phase was dried over Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 46.9 mg (89%) of the regioisomere mixture of **s6** and **s6'** as colorless amorphous.

LRMS (FAB, matrix: 3-nitrobenzyl alcohol, positive): Nominal mass calcd. for $C_{34}H_{40}N_6O_4S$ $[M+H]^+$ requires *m*/*z*: 629, found *m*/*z*: 629.

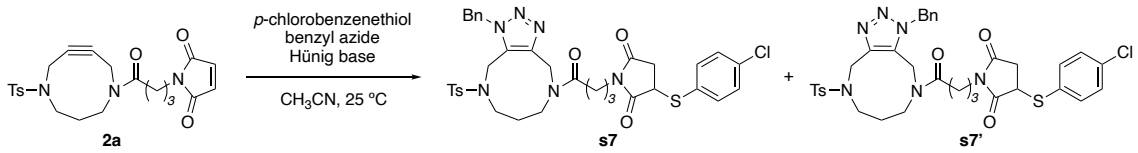
^{1}H NMR Chart (600 MHz in $CDCl_3$) of a mixture of **s6** and **s6'**



^{13}C NMR Chart (150 MHz in $CDCl_3$) of a mixture of **s6** and **s6'**



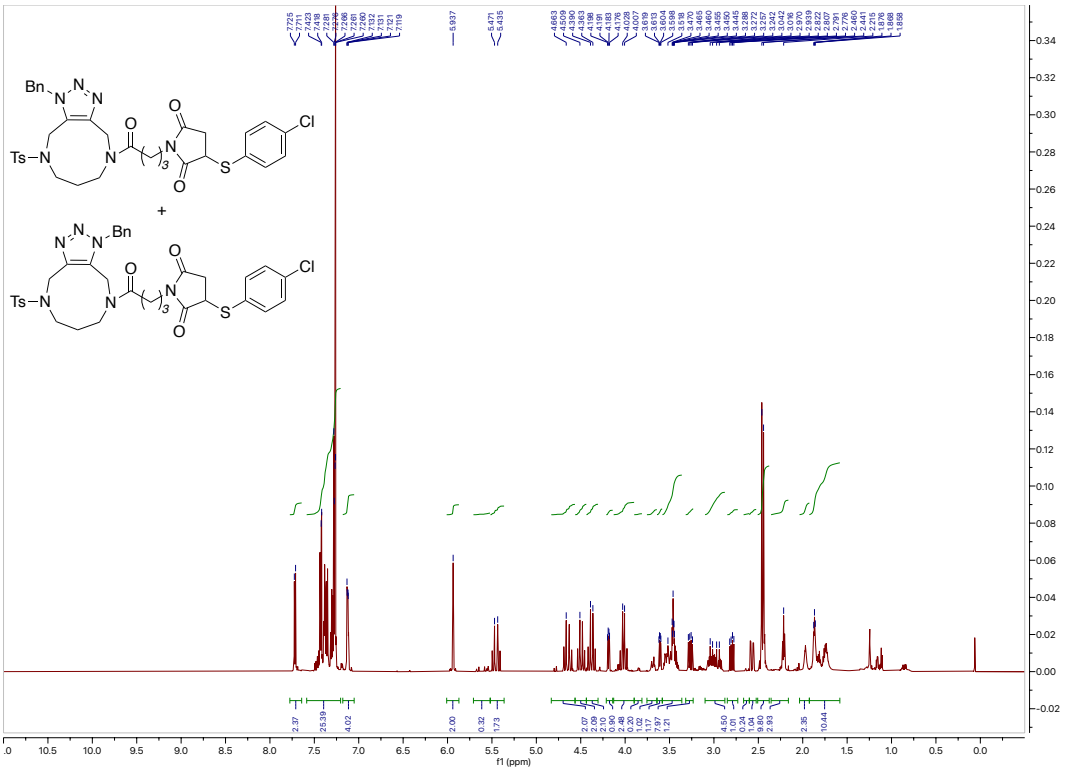
Conjugation of Azide and Electron-Withdrawing Thiol with *N*Ts-DACN-Maleimide



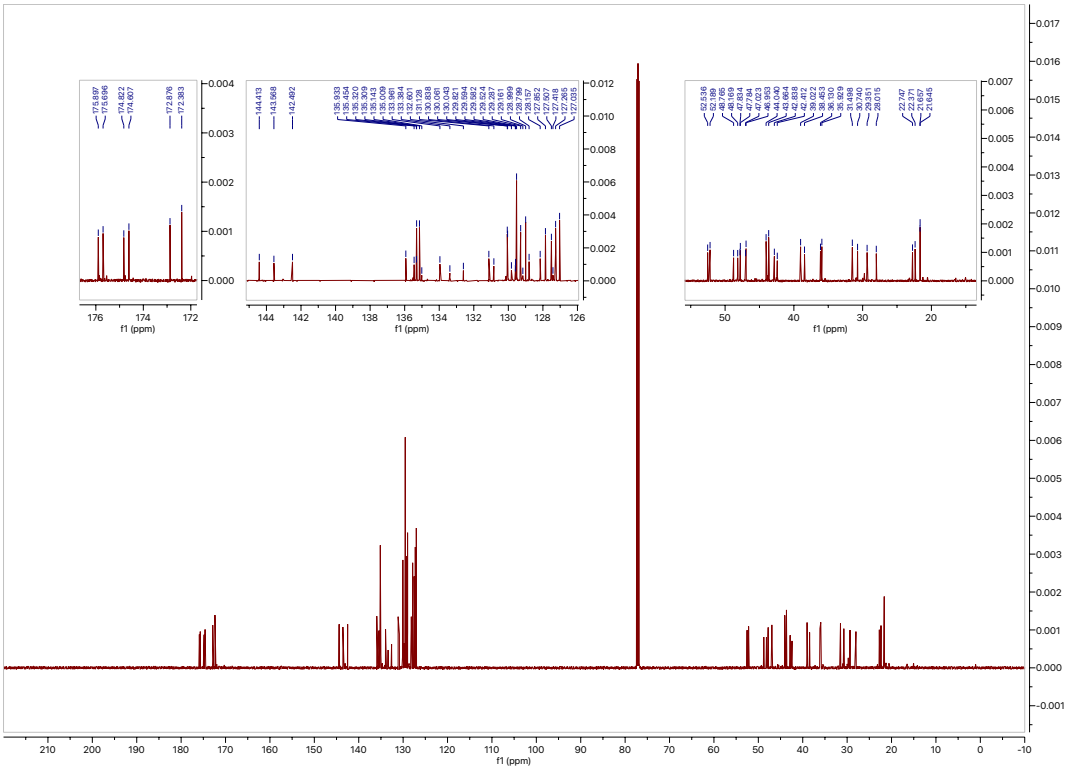
To a solution of **2a** (4.95 mg, 0.0112 mmol) in CH_3CN (0.2 mL) ware added benzyl azide (0.1 mL, 0.112 M in CH_3CN), *p*-chlorobenzenethiol (0.1 mL, 0.112 M in CH_3CN) and Hünig base 0.1 mL, 0.112 M in CH_3CN) at 25 ºC. After the mixture had been stirred at that temperature for 50 h, the reaction was quenched with H_2O and extracted three times with AcOEt. The combined organic phase was dried over Na_2SO_4, filtere, and the solvent was removed under reduced pressure. The residue was purified by preparative TLC ($CHCl_3$/MeOH = 20:1) to afford 6.8 mg (84%) of the regioisomere mixture of **s7** and **s7'** as colorless amorphous.

LRMS (FAB, matrix: 3-nitrobenzyl alcohol, positive): Nominal mass calcd. for $C_{33}H_{37}ClN_6O_5S_2$ $[M+H]^+$ requires *m*/*z*: 721, found *m*/*z*: 721.

^{1}H NMR Chart (600 MHz in $CDCl_3$) of a mixture of **s7** and **s7'**



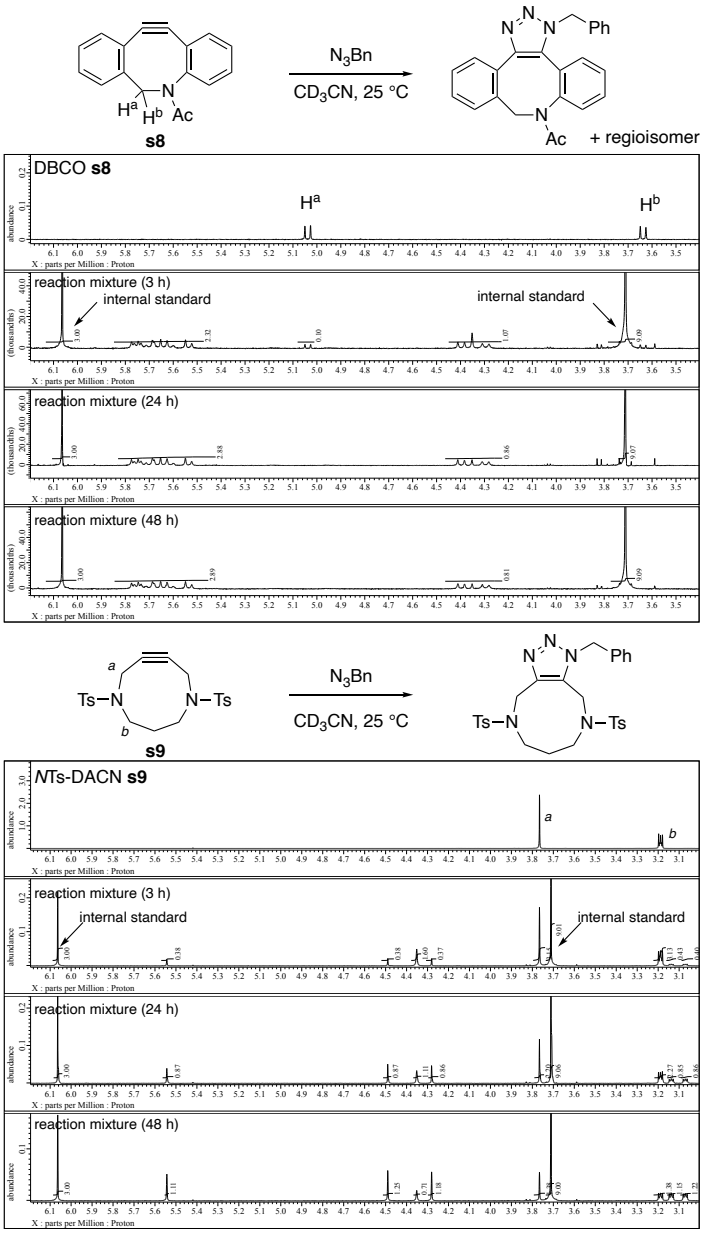
^{13}C NMR Chart (150 MHz in $CDCl_3$) of a mixture of **s7** and **s7'**



Comparison of Reactivity and Stability of DBCO Derivative and DACN

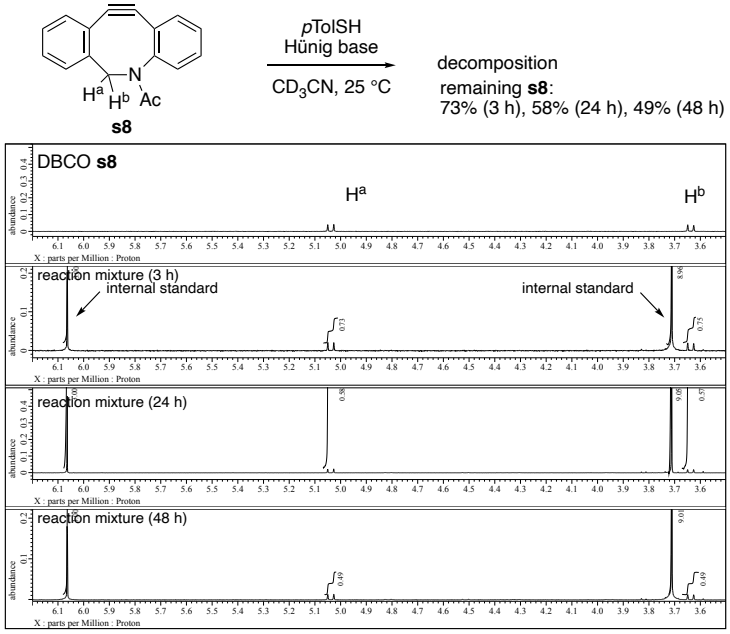
CFAAC Reactivity

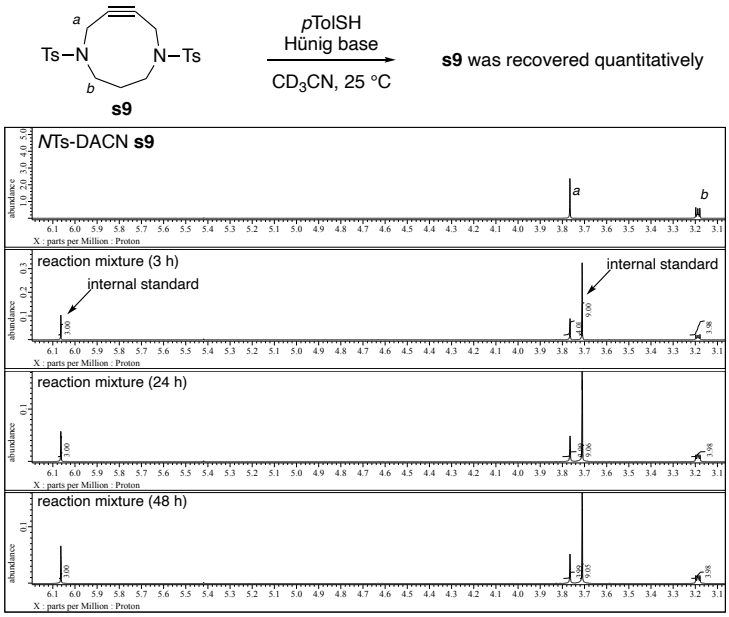
The reactions of DBCO **s8** (1.0 eq.) or *N*Ts-DACN **s9** (1.0 eq.) with benzyl azide (1.0 eq.) was performed in the presence of internal standard [1,3,5-trimethoxybenzene (1.0 eq.)]. All compounds were mixed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **s8** or **s9** at 25 °C were monitored by ^{1}H NMR (600 Mz) analysis. After 3 h, 90% of **s8** and 21% of **s9** were consumed. After 24 h, almost of all **s8** was consumed and provided 96 % (NMR yield) of triazole. On the other hand,45% of **s9** were consumed. After 48 h, 65% of **s9** was consumed and provided 63 % (NMR yield) of triazole (remainig **s9**: 35%).



Tolerance toward Thiol

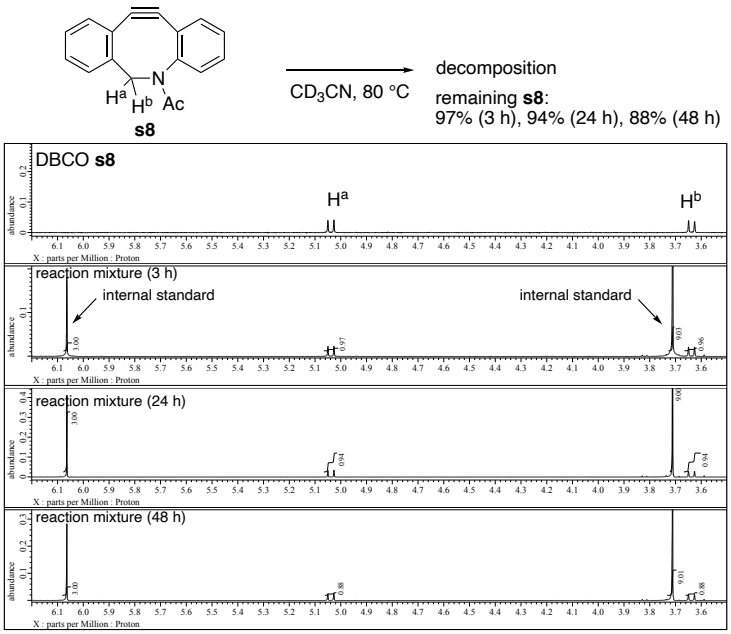
The reactions of DBCO **s8** (1.0 eq.) or *N*Ts-DACN **s9** (1.0 eq.) and *p*-toluene thiol (1.0 eq.) using Hünig's base (1.0 eq.) was performed in the presence of internal standard [1,3,5-trimethoxybenzene (1.0 eq.)]. All compounds were mixed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. Conversion of **s8** or **s9** at 25 °C were monitored by ^1H NMR (600 Mz) analysis. After 3, 24 and 48 h, 27, 42 and 51 % of **s8** was consumed respectively. On the other hand, consumption of **s9** was not observed.

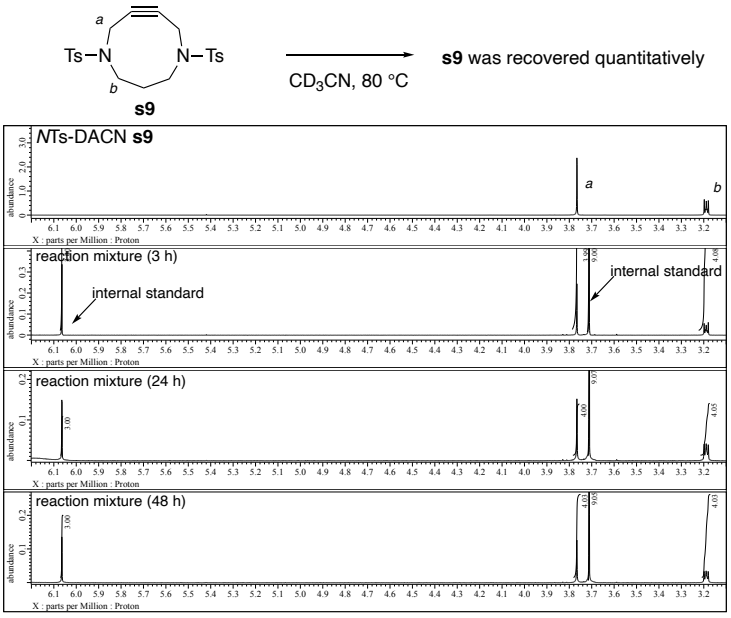




Thermal Stability

Thermal reaction of **s8** or **s9** was performed in the presence of internal standard [1,3,5-trimethoxybenzene (1.0 eq.)]. All compounds were mixed at concentration of 2.5 x 10^{-3} M in CD_3CN in NMR tubes. The NMR tubes were sealed and heated to 80 °C in oil bath. Conversion of **s8** or **s9** were monitored by ^1H NMR (600 Mz) analysis. fter 3, 24 and 48 h, 3, 6 and 12 % of **s8** was consumed respectively. On the other hand, consumption of **s9** was not observed.





7. References

(1) Y. Kawasaki, Y. Yamanaka, Y. Seto, K. Igawa and K. Tomooka, *Chem. Lett.* 2019, **48**, 495.

(2) X. Li and P. Kohli, *J. Phys. Chem. C* 2010, **114**, 6255.

(3) A. V. Dix, L. Fischer, S. Sarrazin, C. P. H. Redgate, J. D. Esko and Y. Tor, *ChemBioChem* 2010, **11**, 2302.