Supplementary Information

Bicyclo[6.1.0]nonyne and tetrazine amino acids for Diels-Alder Reactions

Xu Li [a], Zhengkun Liu [a], Shouliang Dong [a,b,*]

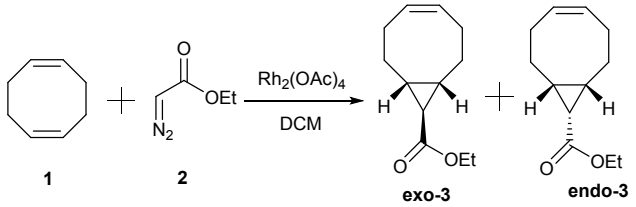
[a.] Institute of Biochemistry and Molecular Biology, School of Life Sciences, Lanzhou University, 222 Tianshui South Road, Lanzhou 730000 China. [b.] Key Laboratory of Preclinical Study for New Drugs of Gansu Province, 222 Tianshui South Road, Lanzhou 730000, China

General materials and methods

The methods utilized in this study, including the column chromatography, the TLC analysis, the reverse phase high performance liquid chromatography (RP-HPLC), the UV/vis spectra and kinetics experiments, and the fluorescence measurements, were the same as our previous report.[1] All reagents and solvents were reagent grade or were purified by standard methods before use. A431 lung cancer cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). Kidney acylase I (Ec 232-732-3, 3210 U/mg protein) from Arabidopsis thaliana (EC 3.5.5.1, 0.3 – 1.0 U/mg) was purchased from Sigma-Aldrich (Shanghai, China).

Organic synthesis

(1*R*, 8*S*, 9*r*, *Z*)-Ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (*exo*-3), and (1*R*, 8*S*, 9*s*, *Z*)-ethyl bicyclo[6.1.0]non-4-ene-9-carboxylate (*endo*-3)



$Rh_2(OAc)_4$ (221 mg, 0.5 mM) and 1, 5-cyclooctadiene (98 ml, 800 mM) were dissolved in CH_2Cl_2 (50 ml) in a round-bottom flask under the protection of nitrogen. Stirring vigorously, Ethyl diazoacetate (12.0 ml, 100 mM) was added to the solution via syringe pump over 20 h at room temperature. After stirring for another 5 h , the mixture was concentrated and the residue was purified by column chromatography on silica gel (ethyl acetate: petroleum ether = 1: 30) to afford **exo-3** and **endo-3** as colorless oils.[2]

exo-3: 10.11 g, yield 52.1%; R_f 0.32 (ethyl acetate: petroleum ether = 1: 30).

^{1}H NMR ($CDCl_3$, 300 MHz, Figure S13): δ 5.65 – 5.62 (m, 2H), 4.10 (q, J = 7.2 Hz, 2H), 2.34 – 2.04 (m, 6H), 1.59 – 1.44 (m, 4H), 1.25 (t, J = 7.2 Hz, 3H), 1.18 (t, J = 4.8 Hz, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S13): δ 174.41, 129.90, 60.22, 28.25, 27.86, 27.71, 26.63, 14.28.

ESI-MS (m/z, Figure S6): calcd. for $C_{12}H_{19}O_2$ 194.13; found $[M + H]^+$: 195.13, $[M + Na]^+$: 217.13, $[2M + Na]^+$: 411.25.

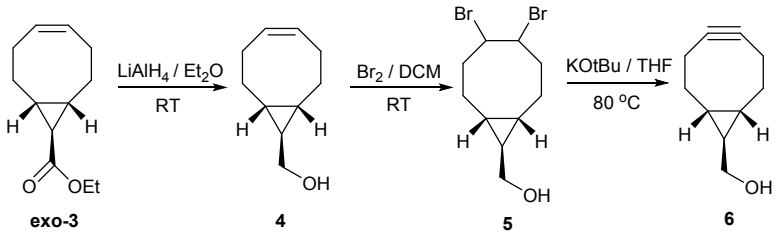
endo-3: 7.20 g, yield 37.1 %; R_f 0.25 (ethyl acetate: petroleum ether = 1: 30).

^{1}H NMR ($CDCl_3$, 300 MHz, Figure S14): δ 5.62 – 5.59 (m, 2H), 4.12 (q, J = 7.2 Hz, 2H), 2.55-2.45 (m, 2H), 2.25 – 2.16 (m, 2H), 2.10 – 2.01 (m, 2H), 1.87 – 1.79 (m, 2H), 1.70 (t, J = 8.8 Hz, 1H), 1.43 – 1.34 (m, 2H), 1.26 (t, J = 7.2 Hz, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S14): δ 172.20, 129.38, 59.64, 27.02, 24.12, 22.61, 21.16, 14.35.

ESI-MS (m/z, Figure S6): calcd. for $C_{12}H_{19}O_2$ 194.13; found $[M + H]^+$: 195.13, $[M + Na]^+$: 217.12, $[2M + Na]^+$: 411.25.

(1*R*, 8*S*, 9*s*)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol 6



Exo-3 (9.5 g, 48.9 mM) was added to a solution of 150 ml Et_2O in a round-bottom flask and stirred vigorously under a stream of nitrogen. Then $LiAlH_4$ (1.67 g, 44.1 mM) in 120 ml Et_2O was added dropwise in the flask in an ice water bath. After stirring at room temperature for 30 min, the reaction mixture was quenched by dropwise addition of ice water until the grey solid turned into white and no gas produced. Then anhydrous Na_2SO_4 was added, and the solid was filtered off and washed thoroughly with Et_2O. The excess of Et_2O was removed by rotary evaporation to afford product **4** as colorless oils, which was pure enough to be used directly in the next step without further purification.

Compound **4** (6.5 g, 42.7 mM) was added to a solution of 100 ml CH_2Cl_2 in a round-bottom flask and stirred vigorously under a stream of nitrogen. then Br_2 (2.30 ml, 44.9 mM) in 10 ml CH_2Cl_2 was added dropwise at 0 °C until the yellow color persisted. The reaction mixture was quenched with a 10% $Na_2S_2O_3$-solution, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated in vacuo to afford product **5** as a white powder. The compound **5** was so pure and can be used directly in the next step without further purification.

Compound **5** (10 g, 32.4 mM) was added to a solution of 250 ml THF in a round-bottom flask and stirred vigorously under a stream of nitrogen. Then KOtBu (11.96 g, 106.7 mM) in 100 ml Et_2O was added dropwise to the flask in an ice water bath. Then the solution was refluxed at 80 °C for 2 h. After cooling down to the rt, the mixture was quenched with saturated NH_4Cl solution, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by column chromatography on silica gel to afford **6** as a white solid.

Compound **4**: 6.96 g, yield 93.6%; R_f 0.65（ethyl acetate: petroleum ether = 1: 1）.

Compound **5**: 12.0 g, yield 92%； R_f 0.62（ethyl acetate: petroleum ether = 1: 1）.

^{1}H NMR ($CDCl_3$, 300 MHz, Figure S15): δ 4.87 – 4.79 (m, 2H), 3.52 (d, J = 7.2 Hz, 2H), 2.73 – 2.56 (m, 2H), 2.30 – 2.20 (m, 1H), 2.14 – 2.03 (m, 2H), 1.52 (s, 1H), 1.52 – 1.35 (m, 2H), 0.94 – 0.84(m, 2H), 0.70-0.65(m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S15): δ 66.61, 56.19, 53.21, 34.93, 34.79, 28.13, 24.37, 23.62, 22.48, 19.77.

ESI-MS (m/z, Figure S7): calcd. for $C_{10}H_{16}Br_2O$ 311.95; found $[M + Na]^+$: 334.94.

Compound **6**: 3.16g, yield 65%; R_f 0.55（ethyl acetate: petroleum ether = 1: 1）.

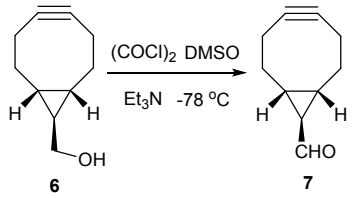
^{1}H NMR ($CDCl_3$, 300 MHz, Figure S16): δ 3.53 (d, J = 6.0 Hz, 2H), 2.44 – 2.39 (m, 3H),

2.34 – 2.23 (m, 2H), 2.21 – 2.12 (m, 2H), 1.45-1.34 (m, 2H), 0.74 – 0.61 (m, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S16): δ 98.92, 98.85, 66.89, 33.40, 27.18, 22.54, 21.47.

ESI-MS (m/z, Figure S7): calcd. for $C_{10}H_{14}O$ 150.10; found $[2M + Na]^+$: 323.19, $[3M + Na]^+$: 473.30, $[4M + Na]^+$: 623.41.

(1R, 8S, 9r)-bicyclo[6.1.0]non-4-yne-9-carbaldehyde



6 **7**

A solution of oxalyl chloride (1.95 ml, 22.8 mM) was added to 70 ml CH_2Cl_2 in a round-bottom flask and stirred vigorously under a stream of nitrogen. A solution of DMSO (3.3 ml, 45.6 mM) in 13 ml CH_2Cl_2 was added dropwise over 20 min to the flask at – 78 °C. After stirring for an additional 30 min, compound **6** (2.85 g, 19 mM) in 25 ml dichloromethane was added dropwise over 10 min to the flask at – 78 °C. After the solution was stirred at – 78 °C for an additional 60 min, triethylamine (16 ml, 114 mM) was added to the flask at – 78 °C. The reaction mixture was stirred for 60 min at – 78 °C and then allowed to warm to 10 °C for an additional hour. The reaction mixture was quenched with water, and then the two layers were separated. The aqueous layer was acidified with 1% HCl and then back-extracted with additional CH_2Cl_2. The combined organic layers were washed with 1% HCl, followed by 5% aqueous $NaHCO_3$ solution. The aqueous extracts were back-extracted with CH_2Cl_2 and the combined organic extracts were washed with NaCl and dried over $MgSO_4$. The solvent was removed by rotary evaporation to afford **7** as a white powder. The compound **7** was pure enough to be used directly in the next step without further purification.[3]

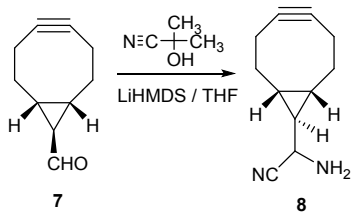
Compound **7**: 2.67 g, yield 95%; R_f 0.56 (ethyl acetate: petroleum ether = 1: 3).

^{1}H NMR ($CDCl_3$, 300 MHz, Figure S17): δ 9.11 (d, J = 5.4 Hz, 1H), 2.46 – 2.19 (m, 6H), 1.60 – 1.47 (m, 5H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S17): δ 200.98, 98.41, 36.89, 32.19, 27.42, 20.94.

ESI-MS (m/z, Figure S8): calcd. for $C_{10}H_{12}O$ 148.09; found $[M + H]^+$: 149.11.

2-amino-2-((1R, 8S, 9r)-bicyclo[6.1.0]non-4-yn-9-yl)acetonitrile



Compound **7** (2.67 g, 18.1 mM) was dissolved in anhydrous THF (95 ml) and cooled to - 40 °C. lithium bis(trimethylsilyl)amide (LiHMDS) (1.0 M solution in THF, 21.65 ml, 21.65 mM) was added dropwise. The reaction mixture was warmed to rt and stirred for 4 h. Acetone cyanohydrin (3.31 ml, 36.2 mM) was then added dropwise, and the reaction mixture was stirred at rt for 12 h and quenched by saturated aqueous $NaHCO_3$. The mixture was extracted with EtOAc and the combined organic extracts were dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica to afford **8** as a white solid.

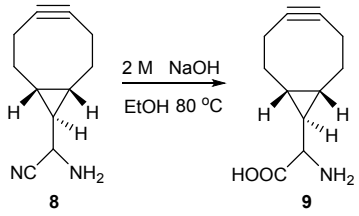
Compound **8**: 2.45 g, yield 78%, R_f 0.42（ethyl acetate: petroleum ether = 3: 1）.

^{1}H NMR ($CDCl_3$, 300 MHz, Figure S18): δ 3.64 (d, J = 6.6 Hz, 1H), 2.49 – 2.41 (m, 2H), 2.36 – 2.27 (m, 2H), 2.20 – 2.15 (m, 2H), 1.95 (s, 2H), 1.44 – 1.39 (m, 2H), 0.97 – 0.90 (m, 2H), 0.81 – 0.77 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz, Figure S18): δ 120.89, 98.50 (98.44), 46.75, 33.03 (32.99), 28.46, 22.93, 21.68, 21.14 (21.11).

ESI-MS (m/z, Figure S8): calcd. for $C_{11}H_{14}N_2$ 174.12, found $[M + H]^+$: 175.11.

2-amino-2-((1R, 8S, 9r)-bicyclo[6.1.0]non-4-yn-9-yl)acetic acid



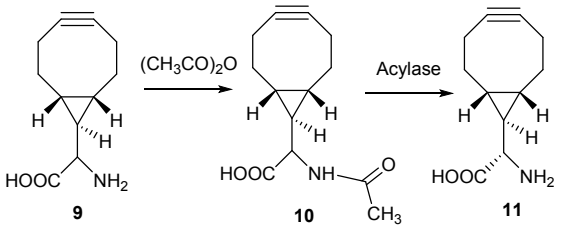
Compound **8** (2 g, 11.5 mM) was added to a solution of ethanol (30 ml) in a round-bottom flask and stirred vigorously under a stream of nitrogen. A solution of 2 M NaOH (30 ml) was added to the flask. Then the solution was refluxed at 80 °C for 2 h. After cooling down to rt, the excess ethanol was removed by rotary evaporation, and the solution was acidified to pH 2.5 with 5 M HCl in an ice water bath. Ethyl acetate was added to the solution to remove the unreacted raw materials **8**, and the two layers were separated. The aqueous layer was loaded on a Dowex 50WX8 cation exchange resin. The column was washed with a 5 × bed volume of water to keep the pH at about 5.5 and then eluted with 1.5 M NH_4OH. The eluate was concentrated and lyophilized to afford product **9** as a white powder.

Compound **9**: 850 mg, yield 38.1%; R_f 0.58 (N-butyl alcohol: glacial acetic acid: water = 4: 1: 1).

The ^{1}H NMR and ^{13}C NMR of compound **9** were identical to end-product **11**(DMSO, Figure S19)**.**

ESI-MS (m/z, Figure S9): calcd. for $C_{11}H_{15}NO_2$ 193.11, found $[M + H]^+$: 194.11.

(S)-2-amino-2-((1R, 8S, 9r)-bicyclo[6.1.0]non-4-yn-9-yl)acetic acid



Compound **9** (193 mg, 1 mM) was added to a solution of 1 M NaOH (3 ml) in a round-bottom flask, and Ac_2O (0.25 ml, 2.5 mM) was added. After being stirred vigorously at rt for 2 h under a stream of nitrogen, the solution was acidified to pH 2.5 with 1 M HCl and extracted with EtOAc. The organic layers were combined and washed with brine (2 × 5 ml) and dried over $MgSO_4$. The solvent was removed by rotary evaporation to afford **10** as colorless oils. without further purificationCompound **10** (104.5 mg, 0.5 mM) was dissolved in water (5 ml) and adjusted pH to 7.5 by the addition of NH_4OH. Then kidney acylase I (20 mg) was added and the mixture was stirred at 37 °C. 48 h later, the solution was centrifuged with 10 kDa dialysis membrane at 4000 rpm for 25 min at 10 °C. The acylase I was recovered and the solution was acidified with HCl, extracted with EtOAc. The EtOAc layer containing Ac-D-BCN amino acid was used for obtaining D-BCN amino acid. The aqueous layer was loaded on a Dowex 50WX8 cation exchange resin. The column was washed with a 5× bed volume of water to keep the pH at about 5.5 and then eluted with 1.5 M NH_4OH. The eluate was concentrated and lyophilized product **11** as a white powder.

Compound **10**: 114 mg, yield 54%; R_f 0.42 (Methanol: CH_2Cl_2 = 1: 10).

ESI-MS (m/z, Figure S9): calcd. for $C_{13}H_{17}NO_3$ 235.12, found $[M + H]^+$: 236.12, $[M + Na]^+$: 258.11.

Compound **11**: 50 mg, yield 25%; R_f 0.63（N-butyl alcohol: glacial acetic acid: water = 4: 1: 1）.

^{1}H NMR (DMSO, 300 MHz, Figure S19): δ 8.35 (s, 2H), 3.44 (d, J = 8.1 Hz, 2H), 2.50 – 2.06 (m, 4H), 1.38 – 1.21 (m, 1H), 1.12-0.88 (m, 2H), 0.70 – 0.57 (m, 1H).

^{13}C NMR (DMSO, 100 MHz, Figure S19): δ 170.70, 98.73 (98.67), 55.94, 32.48 (32.37),

24.57, 23.05 (22.87), 20.49.

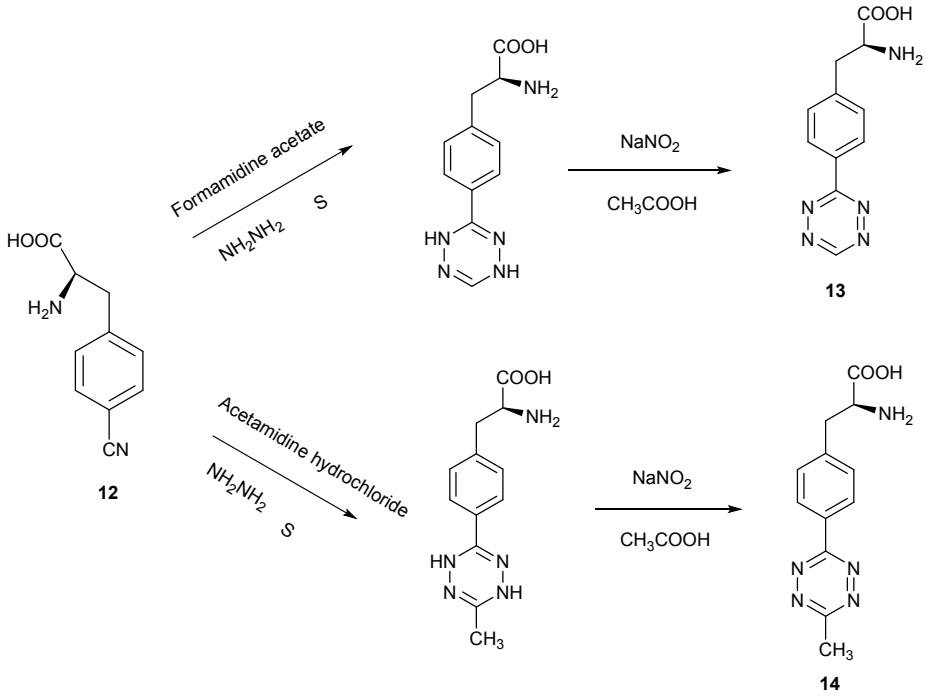
ESI-MS (m/z, Figure S10): calcd. for $C_{11}H_{15}NO_2$ 193.11, found $[M + H]^+$: 194.11.

Monitoring enantiomeric purity of BCN amino acid

Derivatization of an amino acid with FDAA produced a diastereomer referred to as DNPA-amino acid. L-BCN amino acid (1.9 mg, 7.8 μM) was placed in a 2 ml plastic tube. The tube was added with 300 μl acetone solution of FDAA (3 mg, 11 μM), the molar ratio of FDAA to amino acid 1.4: 1, followed by $NaHCO_3$ (1 M, 60 μl, 60 μM). The contents were mixed at 30 – 40 °C for one hour with frequent mixing. After cooling down to rt, HCl (2 M, 30 μl, 60 μM) was added to the reaction mixture. The DNPA-amino acid can be separated and estimated by HPLC with the linear gradient of MeCN from 5% to 95% within 20 min at a flow rate of 1 ml/min and the UV detection at 340 nm. The absorption spectra in RP-HPLC showed four peaks, and the four peaks were measured combined with mass spectrometry to confirm the purity. The results showed that the product was only found in peak 4, while no product in peak 3. It has been recognized that the peak 4 was the FDAA derivative of L-BCN amino acid. Moreover, acetone solution of FDAA was injected for HPLC as well, and the results showed that the FDAA had the same absorption spectra and retention behavior as that with peak 3. It is evident that the tetrazine-containing amino acid was L-isomer and can be used for peptide synthesis.

Analytical RP-HPLC (Figure S1): Rt 17.70 min. ESI-MS (m/z, Figure S5): calcd. for $C_{20}H_{23}N_5O_7$ 445.16; found $[M + H]^+$: 446.16.

Synthesis of tetrazine-containing amino acid 13 and 14



Compound **13** was prepared according to our previously published work about the synthesis of tetrazine derivatives.[2]

Compound **12** (475 mg, 2.5 mM), formamidine acetate (1.04 g, 10 mM), and sulfur (80 mg, 2.5 mM) were mixed in a round-bottom flask under the protection of nitrogen. Anhydrous hydrazine (3.14 ml, 100 mM) was added to the flask, and the mixture gradually turned yellow viscous after 22 h' stirring at rt. Then the yellow viscous material was dissolved with 15 ml acetic acid, and centrifuged to obtain the supernatant solution. Fully mixed sodium nitrite (863 mg, 12.5 mM) in 1.5 ml water was dropped slowly to the above supernatant solution in an ice water bath for 30 min to give a purple solution. Evaporated organic solvent out in vacuum condition as far as possible. Upon addition of ethanol to the reaction mixture, the solid was filtered off and washed thoroughly with ethanol to afford product **13** as a purple powder. The compound **13** was pure enough to be used directly in the next step without further purification.

Compound **14** was prepared by an identical procedure as described for compound **13**. Upon addition of acetamidine hydrochloride (945.4 mg, 10 mM) instead of formamidine acetate to afford product **14** as a purple powder.

Compound **13**: 268.4 mg, yield 43.8%; R_f 0.58 (N-butyl alcohol: glacial acetic acid: water = 4: 1: 1).

^{1}H NMR (DMSO, 295 K, 300 MHz, δ Figure S20): 10.62 (s, 1H), 8.47 (d, J = 8.1 Hz, 5H), 7.59 (d, J = 8.1 Hz, 2H), 4.32(s, 1H), 3.25 (d, 2H).

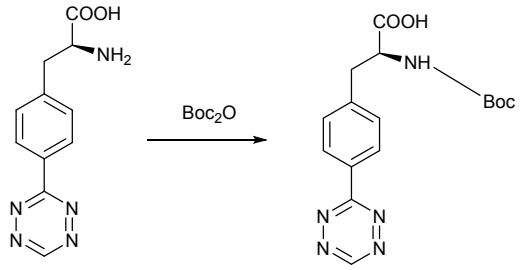
^{13}C NMR (100 MHz, DMSO-d6, Figure S20): δ 170.26, 165.36, 158.12, 140.26, 130.76, 130.59, 127.92, 52.87, 35.74.

ESI-MS (m/z, Figure S10): calcd. for $C_{11}H_{11}N_5O_2$ 245.09; found $[M + H]^+$: 246.1.

Compound **14**: 231.2 mg, yield 35.7%; R_f 0.52 (N-butyl alcohol: glacial acetic acid: water = 4: 1: 1).

ESI-MS (m/z, Figure S11): calcd. for $C_{12}H_{13}N_5O_2$ 259.11; found $[M + H]^+$: 260.11; $[2M + H]^+$: 519.22.

Synthesis of protected tetrazine-containing amino-acid 15



13 15

To a stirred ice-cold solution of compound **2** (245.2 mg, 1 mM) in a 1: 1 mixture of water and dioxane (25 ml), sodium bicarbonate (210 mg, 2.5 mM) was added followed by Boc_2O (506 mg, 1.5 mM, 1.5 eq) in dioxane (3 ml) in portions within 5 min. The mixture was stirred under nitrogen gas at rt for 8 h. Rotary evaporation to remove as much of 1, 4-dioxane, and washed the mixture twice with ether. Hydrochloric acid (1 mM) was added slowly to the solution under an ice water bath to adjust pH to 3. Then ethyl acetate was added to extract the purple product. The product was washed twice with saturated aqueous sodium chloride, and then dried over anhydrous sodium sulfate overnight. Excess ethyl acetate was removed by rotary evaporation, and purified by chromatography on a silica gel column to afford product **15** as a purple powder.

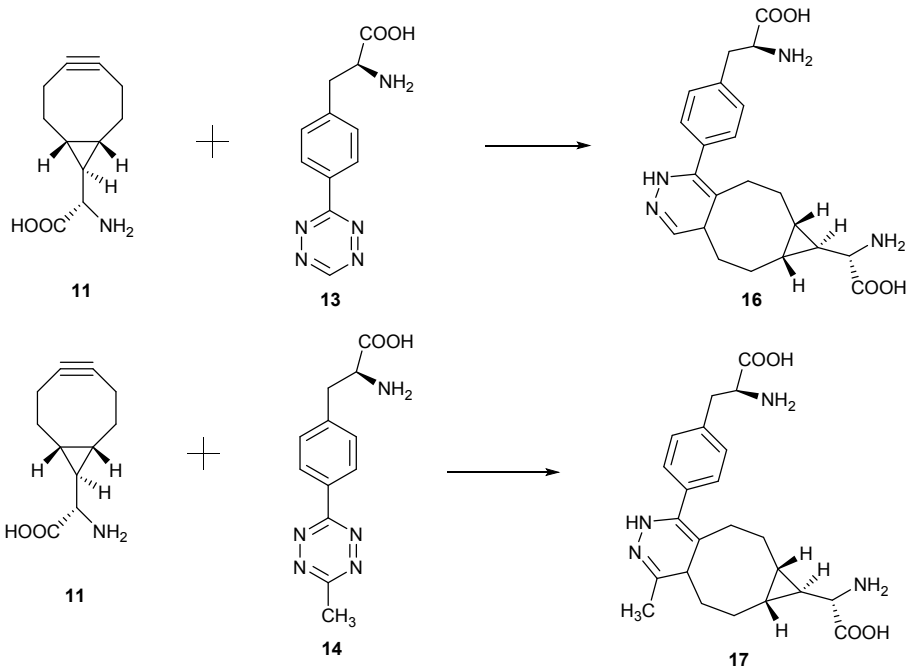
Compound **15**: 249.4 mg, yield 72.3%; R_f 0.45 (petroleum ether: ethyl acetate: acetic acid = 20: 20: 1).

^1H NMR (DMSO, 295K, 300 MHz, δ, Figure S21): 10.58 (s, 1H), 8.42 (d, J = 8.1 Hz, 2H), 7.56 (d, J = 8.1 Hz, 2H), 7.24 (d, J = 8.4 Hz, 1H), 4.24 – 4.16 (m, 1H), 3.20 – 3.14 (m, 1H), 3.01 – 2.93 (m, 1H), 2.51 (s, 1H), 1.32 (s, 9H).

^{13}C NMR (100 MHz, DMSO-d6, Figure S21): δ 173.35, 165.42, 158.03, 155.41, 143.51, 130.22, 129.94, 127.56, 78.06, 54.80, 38.36, 28.07.

ESI-MS (m/z, Figure S11): calcd. for $C_{16}H_{19}N_5O_4$ 345.14; found $[M + Na]^+$: 368.13, $[2M + Na]^+$: 713.27, $[3M+Na]^+$: 1035.42.

Kinetic experiments of the reaction between BCN and tetrazine derivatives



All kinetic experiments were conducted under pseudo first order conditions. The pseudo first order rate constants were obtained by plotting the natural log of the concentration of the limiting reactant versus time in seconds. The second order rate constants and half-lives were extrapolated from the rate measurements derived under pseudo first order conditions.

As illustrated in Scheme 1, an equal volume of the Phosphate Buffered solution (PBS) of **11** (2×10^{-4} M) and the PBS of **13** (2×10^{-5} M) was mixed into the cuvette at room temperature . The absorption spectrum of the starting material **13** under the absorption peak at 523 nm, because the product **16** and its isomers had no absorption at 523 nm, the absorbance value was recorded once every 1 seconds. An exponential curve was used for calculating the reaction rate, the calculated pseudo first order reaction constant kobs = 0.13 ± 0.01, the half-life $t_{1/2}$ = 5.4 s, and further calculations obtained second-order reaction rate constant $k_2 = 513 \pm 10\ M^{-1}{\cdot}S^{-1}$ (see Figure S3).

As illustrated in Scheme 1, an equal volume of the phosphate buffered solution (PBS) of **11** (4×10^{-3} M) and the PBS of **14** (4×10^{-4} M) was mixed into the cuvette at rt. The absorption spectrum of the starting material **14** under the absorption peak at 527 nm, compound **14** reacted with compound **11** by an identical procedure as described for compound **13.** The changes in the absorbance spectrum in 527 nM of the reaction were observed, the absorbance value was recorded once every 5 seconds. An exponential curve was used for calculating the reaction rate, the calculated pseudo first order reaction constant kobs = $5.78 \times 10^{-3} \pm 6 \times 10^{-6}$, the half-life $t_{1/2}$ = 120 s, and further calculations obtained second-order reaction rate constant $k_2 = 1.45 \pm 0.05\ M^{-1}{\cdot}S^{-1}$ (see Figure S4).

Compound **16**: yield 94.1%, RP-HPLC: Rt 4.48 min; purity 97.5%.

^{1}H NMR (DMSO, 295K, 300 MHz, δ, Figure S22): 9.04 (d, J = 2.4 Hz, 1H), 8.47 (s, 2H), 8.32 (s, 2H), 7.44 (s, 4H), 4.29 (s, 1H), 3.29-3.17 (m, 2H), 3.07-3.00 (m, 1H), 3.89-2.82 (m, 2H), 2.42 (t, J = 10.5 Hz, 2H), 2.26-2.14 (m, 2H), 1.76 – 1.38 (m, 3H), 1.06 – 0.76 (m, 4H).

^{13}C NMR (100 MHz, DMSO-d^6, Figure S22): δ 170.49, 170.38, 158.62, 158.16, 129.34, 129.15, 129.05, 118.29, 114.37, 55.33, 27.60, 24.36, 22.46, 21.96.

ESI-MS (m/z, Figure S12): calcd. for $C_{22}H_{26}N_4O_4$ 410.20; found $[M + H]^+$: 411.20.

Compound **17**: yield 92.6%, RP-HPLC: Rt 9.52 min; purity 96.2%.

ESI-MS (m/z, Figure S12): calcd. for $C_{23}H_{28}N_4O_4$ 424.20; found $[1/2M + H]^+$: 213.11; $[M + H]^+$: 425.22; $[2M + H]^+$: 849.44.

Cancer cell labeling.

The solution of **11** (0.27 mg, 0.83 μM) in DMF (1 ml) was added to Cy5.5 N-hydroxysuccinimide ester (1 mg, 0.83 μM). The reaction mixture was stirred for 16 h at rt. After being precipitated in cold ether, the crude product was purified by preparative RP-HPLC on a C_{18} column. The desired fractions containing L-BCN-Cy5.5 conjugate were collected and lyophilized to give a blue powder.

Cetuximab (5 mg) was dissolved in 10 mM $NaHCO_3$ buffer (pH = 8.4) before the addition of approximately 3 equiv 5(6)-carboxyfluorescein succinimidyl ester (0.25 mg) and 3 equiv Boc-protected tetrazine-containing amino acid. The reaction was conducted under the conditions of rt and vibration. The solution was then purified using an Amicon centrifugal filter (3 KM, Millipore) to afford the fluorophore and tetrazine modified cetuximab.[4]

To confirm the biological utility of the new BCN and tetrazine derivatives, **13** was chosen as the representative compound for validation in pretargeted labeling studies with live A431 epidermoid carcinoma cells. Since we have already demonstrated that tetrazine **13** possessed faster reaction kinetic, better serum stability, higher water solubility, and easier synthesis/purification, so we attempted to demonstrate that the tetrazine from this study was still a practical cycloaddition partner with BCN for bioorthogonal use. A431 epidermoid carcinoma cells, which overexpressed HER2/neu receptors, have been shown to be useful in pretargeted cell labeling assays with a BCN-tetrazine system.

To confirm the cycloaddition reactivity in a biological environment, A431 cells and modified antibody were incubated at 37 °C for 3 h and washed to clean the unbounded antibody of the cell surface. L-BCN-Cy5.5 conjugate reacted with the antibody, which was modified with fluorophore or tetrazine, in 10% fetal bovine serum and Hanks' balanced salt solution at 37 °C for 1 hour via IED-DA reaction (Figure 1). Then a washing step was needed to remove the unreacted L-BCN-

Cy5.5 conjugate. The unlabeled cetuximab and L-BCN-Cy5.5 conjugate or fluorophore and tetrazine modified cetuximab and unlabeled Cy5.5 were taken as the control experiments. In the end, the cellular changes were monitored by the fluorescence microscopy in rhodamine channel and near infrared channel (NIR) respectively. The results suggested that the antibody could be visualized clearly in rhodamine channel, and the covalently bound L-BCN-Cy5.5 conjugate could be monitored apparently in NIR channel, while control experiments showed no NIR fluorescence (Figure 2). This study further demonstrated that our selected BCN and tetrazine derivatives are useful in bioorthogonal labeling on par with the previously reported compounds.

References to Supplementary Information

1. Z. Ni, L. Zhou, X. Li, J. Zhang, S. Dong, PLoS One 2015, 10, e0141918.

2. M. T. Taylor, M. L. Blackman, O. Dmitrenko, J. M. Fox, *J. Am. Chem. Soc.*, 2011, 133, 9646-9649.

3. T. C. Adams, A. C. Dupont, J. P. Carter, J. F. Kachur, M. E. Guzewska, W. J. Rzeszotarski, S. G. Farmer, L. Noronha-Blob, C. Kaiser, *J. Med. Chem*., 1991, 34, 1585-1593.

4. N. K. Devaraj, R. Upadhyay, J. B. Haun, S. A. Hilderbrand and R. Weissleder, *Angew. Chem. Int. Ed.*, 2009, **48**, 7013-7016.

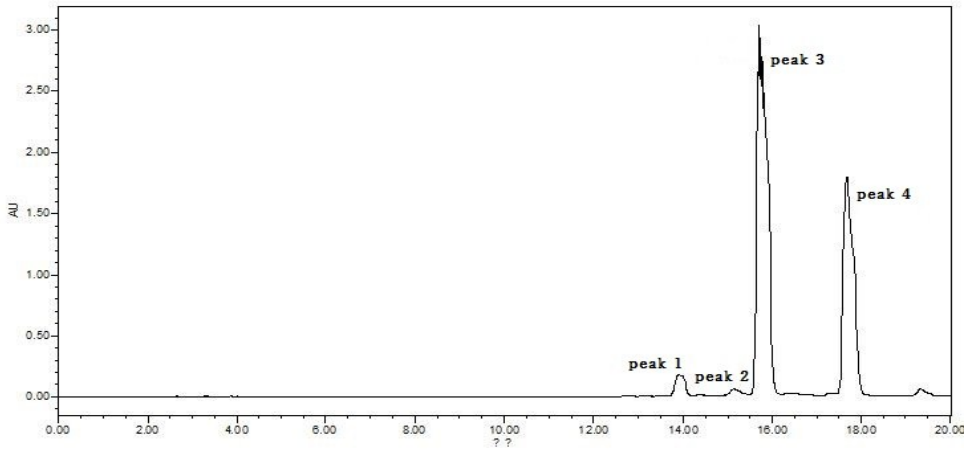


Figure S1. Derivatization of the BCN amino acid with FDAA produced a diastereomer referred to as DNPA-amino acid. The DNPA-amino acid can be separated and estimated by HPLC, and the results showed four peaks of absorption spectra in RP-HPLC.

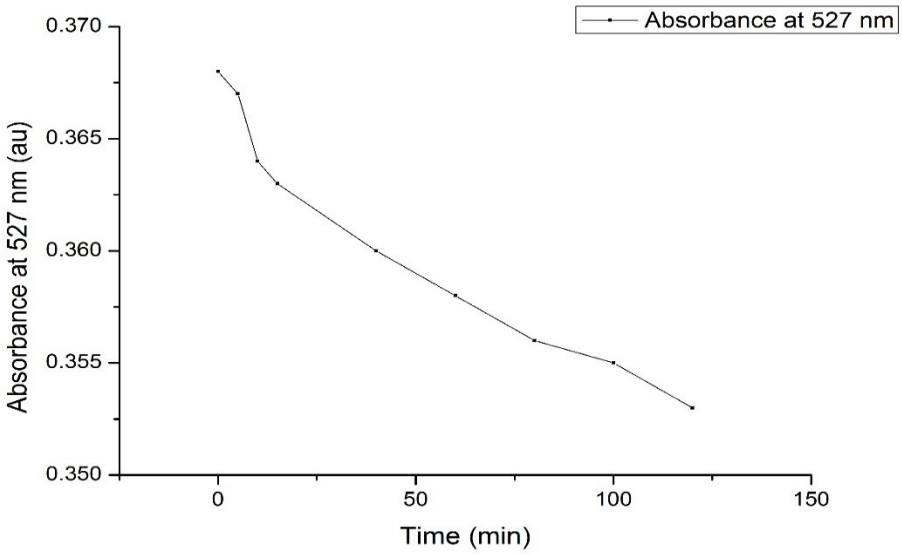


Figure S2. The stability of **14** in 20% piperidine/DMF at 20.0 ± 0.1 °C monitored at 527 nm. A 4.1% decrease in absorption was observed within 2 h.

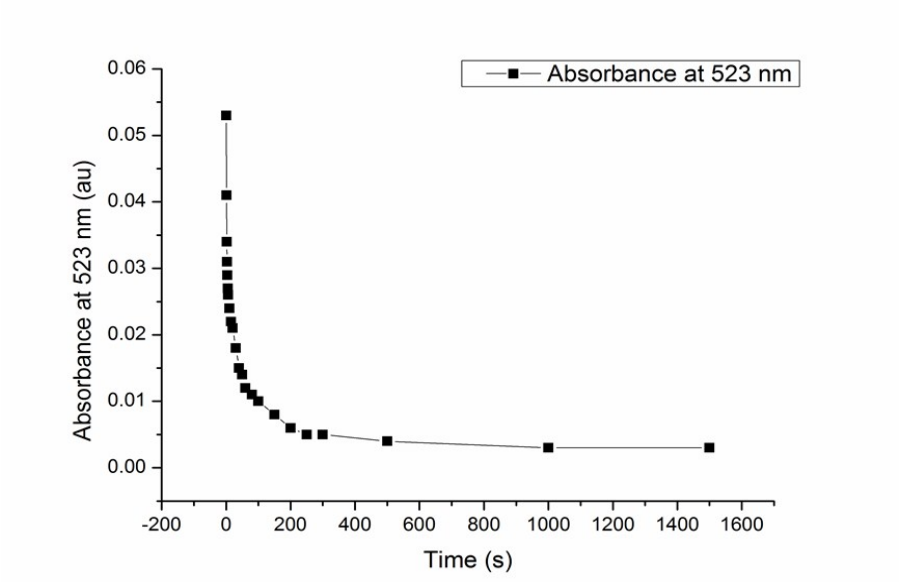


Figure S3. Kinetics of the reaction of **11** (2×10^{-4} M) with **13** (2×10^{-5} M) in deionized water, monitored by UV-vis at 523 nm. Kinetic runs were performed in triplicate (all data was presented above) and fitted to an observed first-order rate constant (k_{obs}), which was used to calculate the second order rate constant.

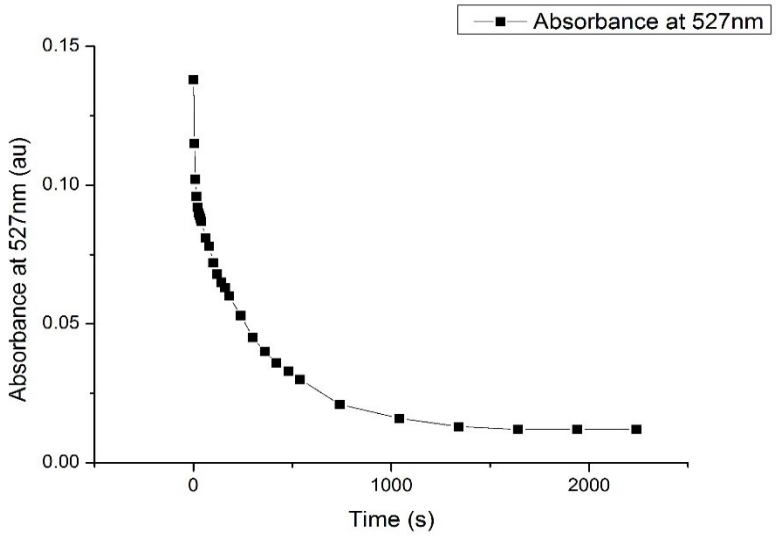
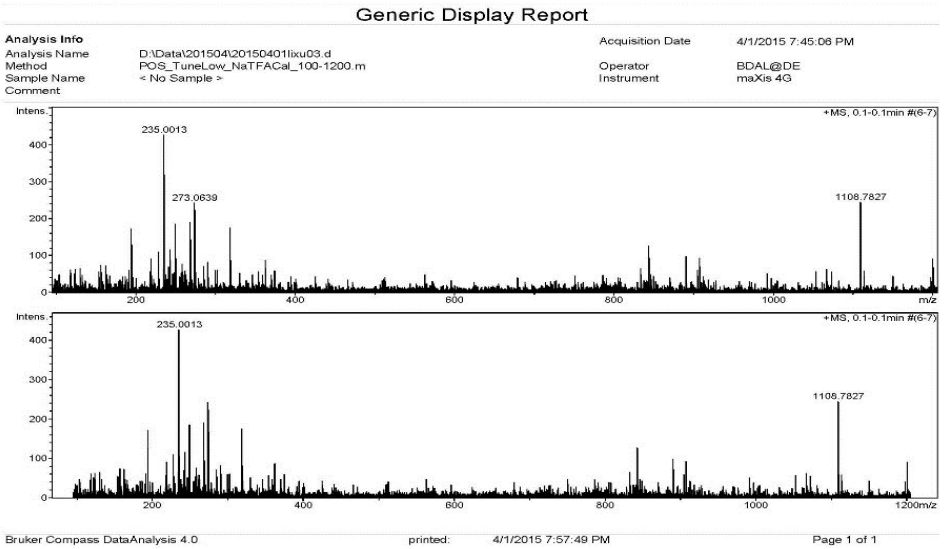


Figure S4. Kinetics of the reaction of **11** (4×10^{-3} M) with **14** (4×10^{-4} M) in deionized water, monitored by UV-vis at 527 nm. Kinetic runs were performed in triplicate (all data was presented above) and fitted to an observed first-order rate constant (kobs), which was used to calculate the second order rate constant.

A



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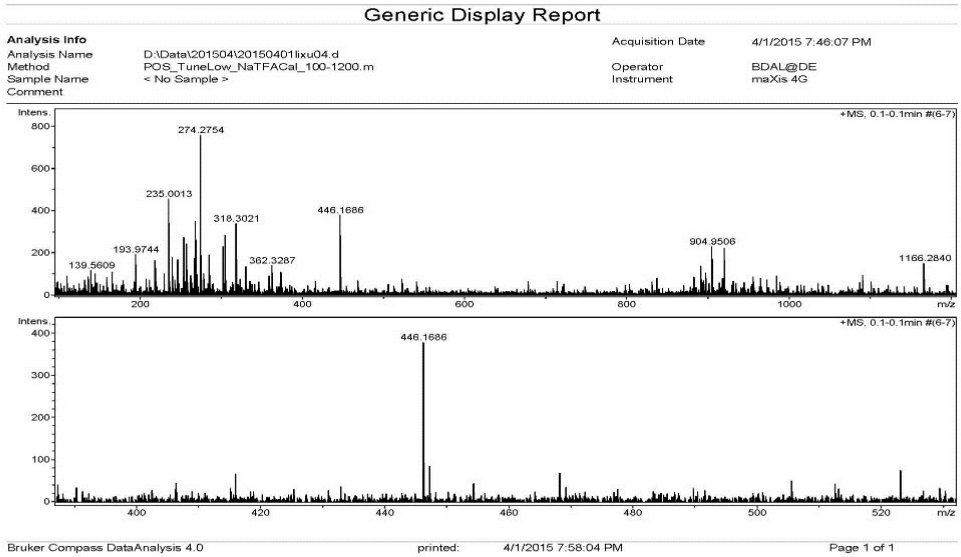
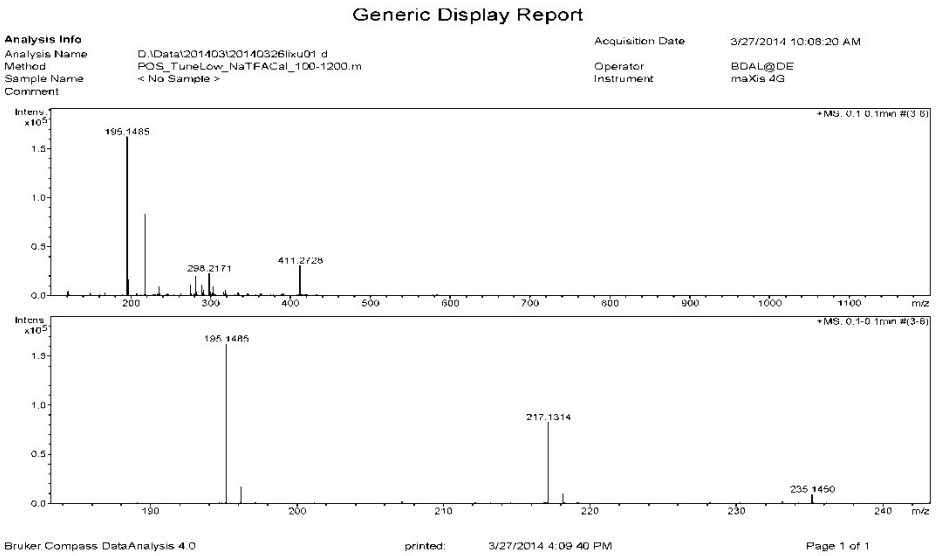


Figure S5. ESI-MS spectrum of peak 3 (**A**) and peak 4 (**B**)

A



B

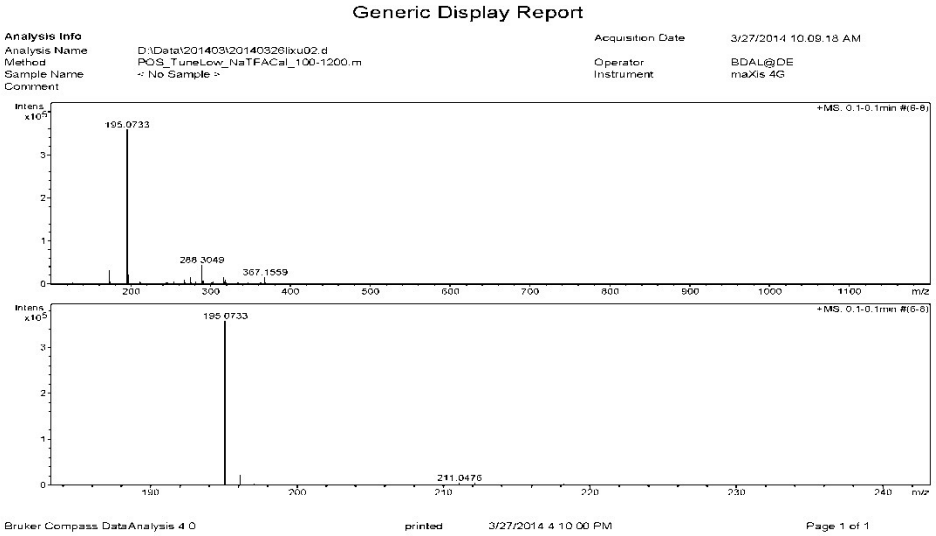
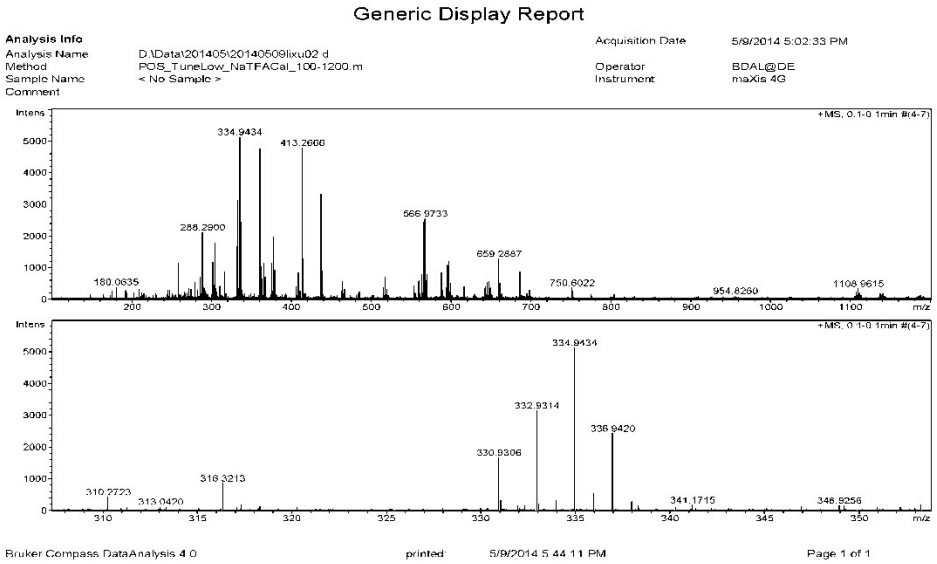


Figure S6. ESI-MS spectrum of **exo-3** (**A**) and **endo-3** (**B**)

A



B

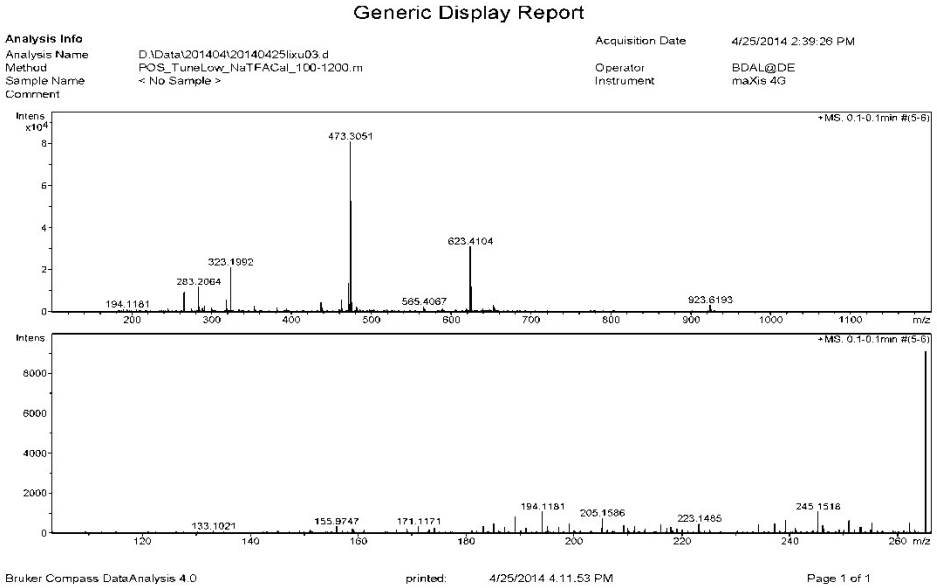
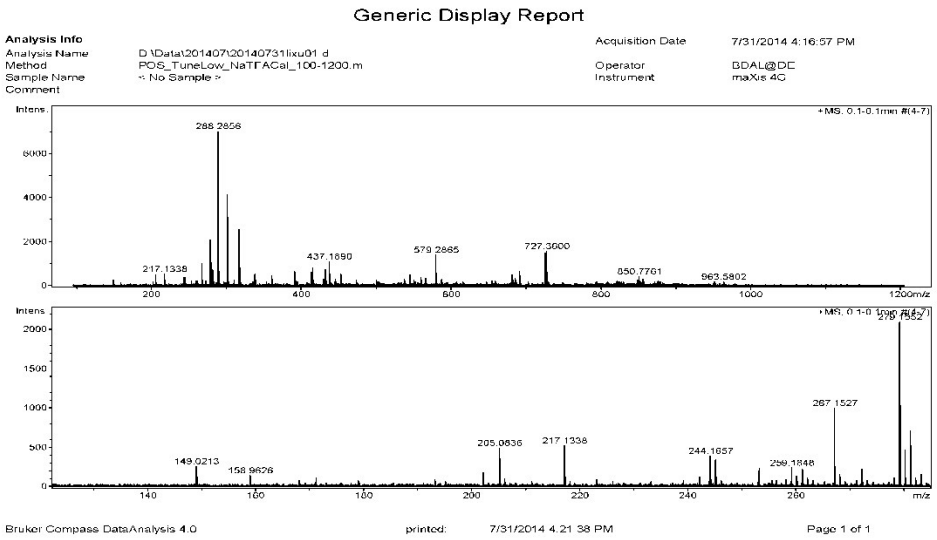


Figure S7. ESI-MS spectrum of compound **5** (**A**) and compound **6** (**B**)

A



B

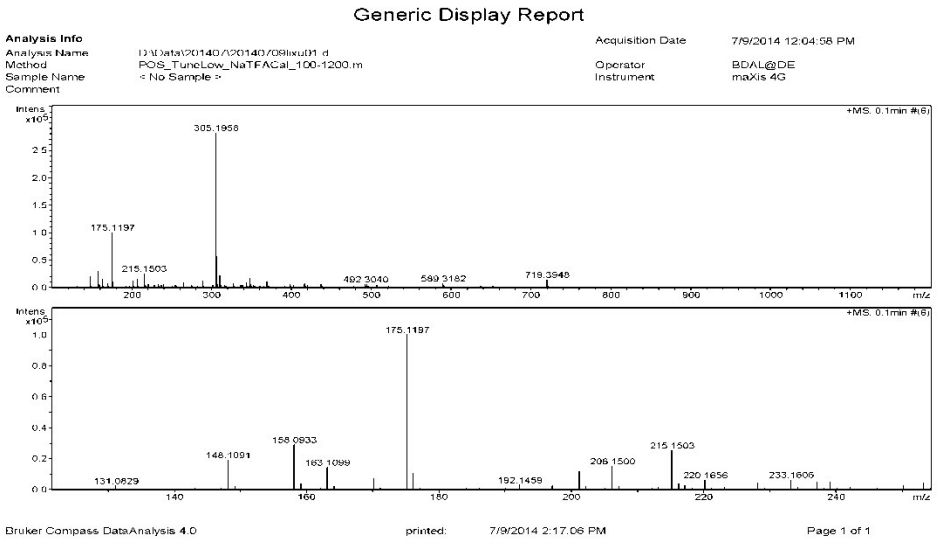
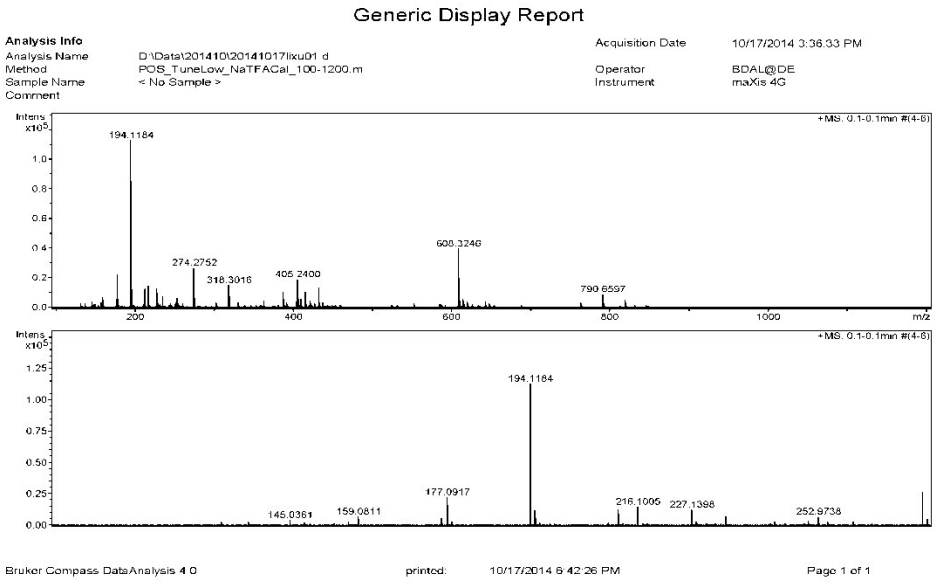


Figure S8. ESI-MS spectrum of compound **7** (**A**) and compound **8** (**B**)

A



B

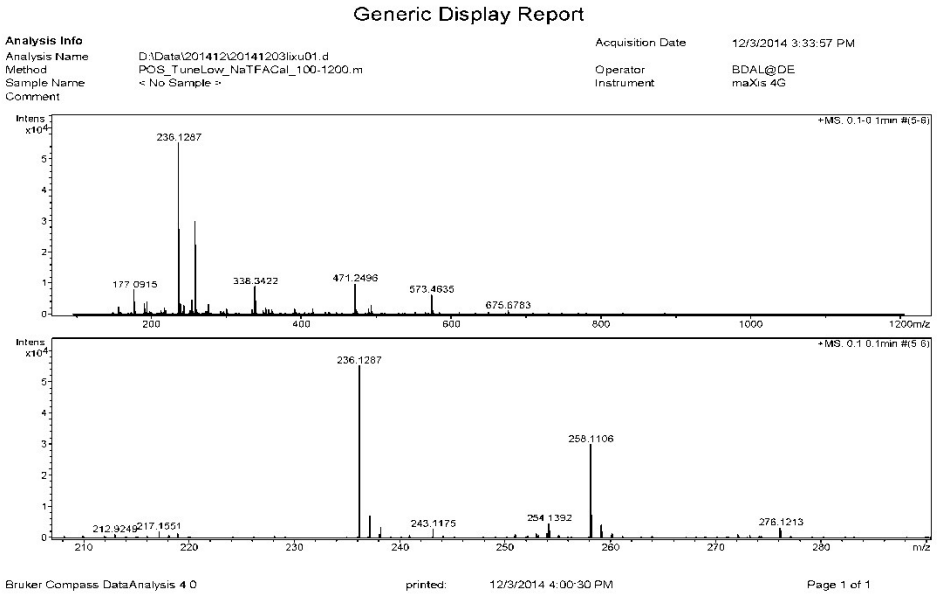
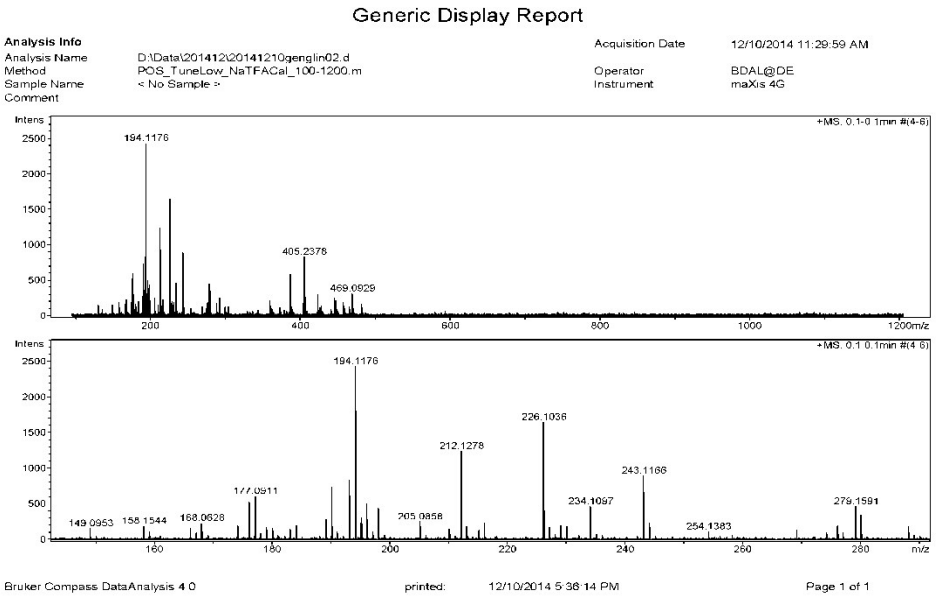


Figure S9. ESI-MS spectrum of compound **9** (**A**) and compound **10** (**B**)

A



B

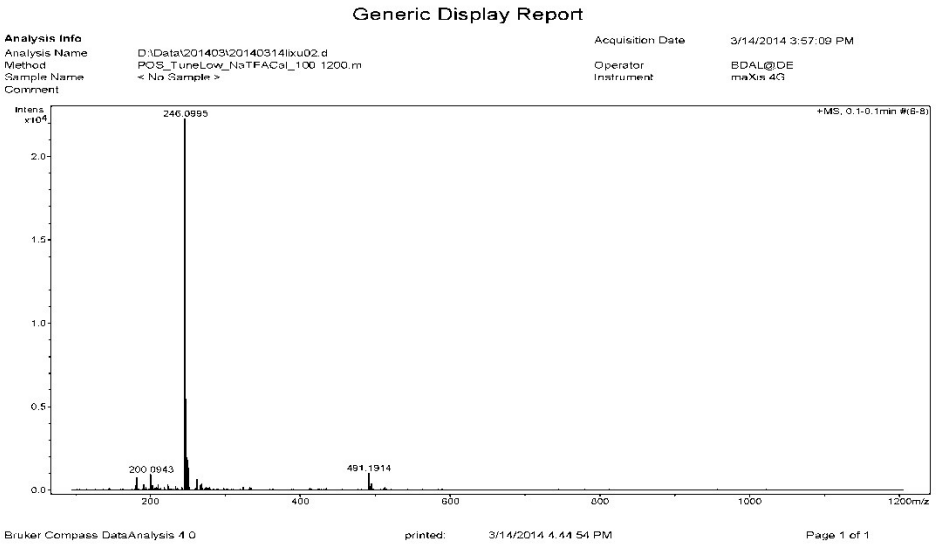
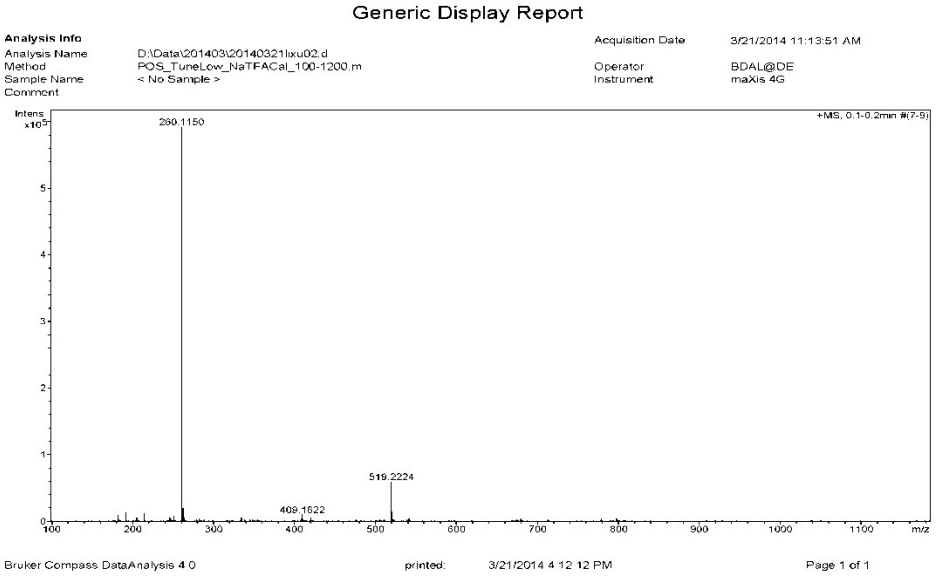


Figure S10. ESI-MS spectrum of compound **11** (**A**) and compound **13** (**B**)

A



B

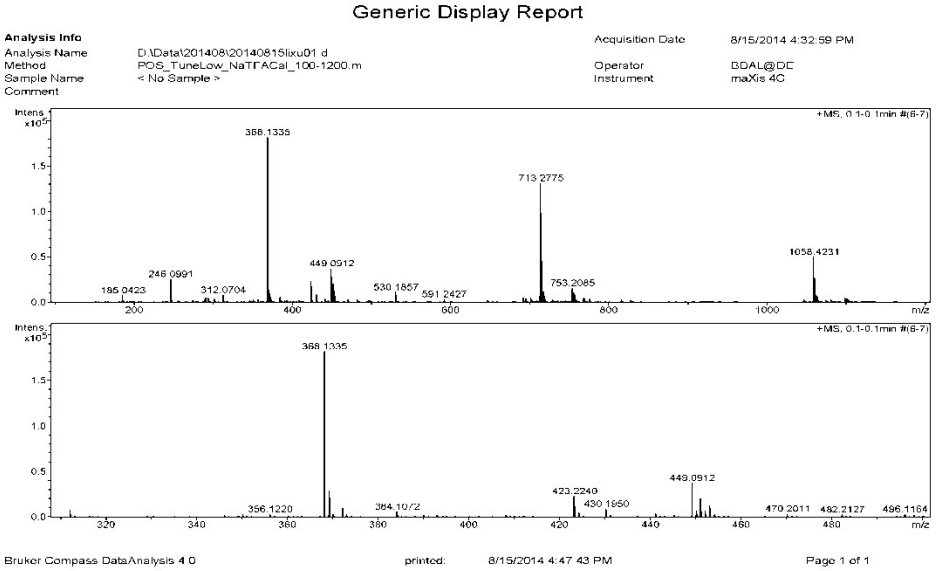
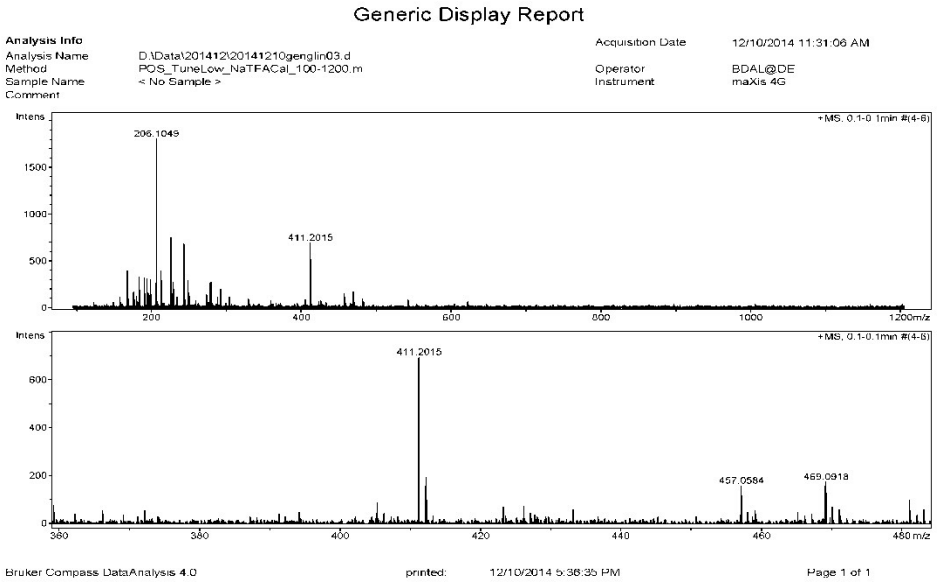


Figure S11. ESI-MS spectrum of compound **14** (**A**) and compound **15** (**B**)

A



B

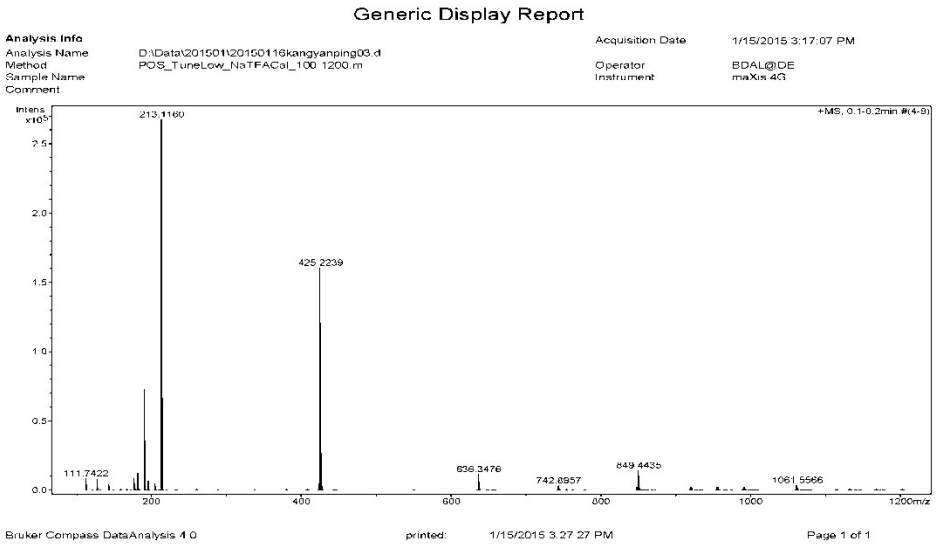
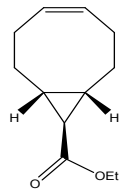
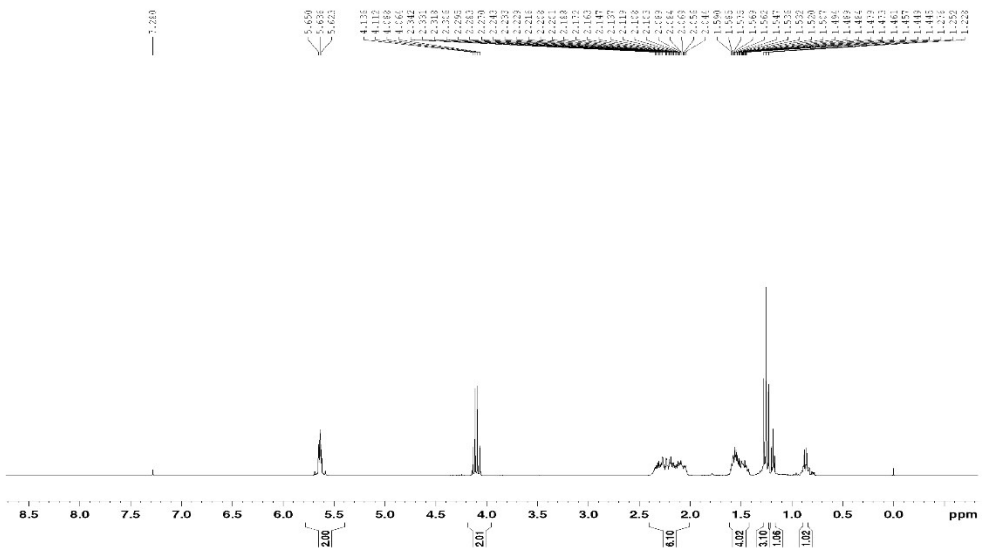


Figure S12. ESI-MS spectrum of compound **16** (**A**) and compound **17** (**B**)



A



B

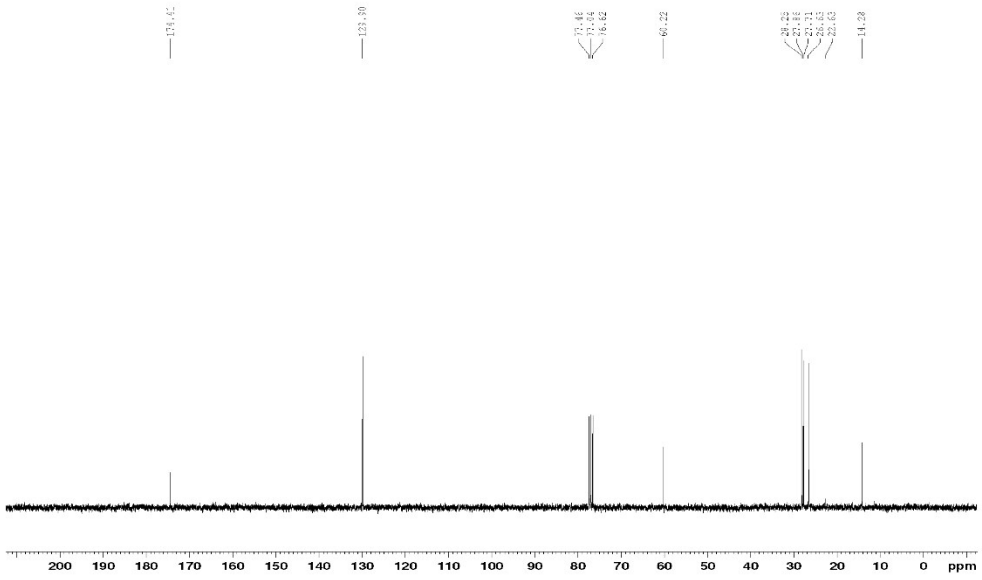
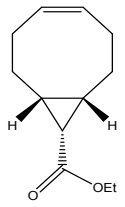
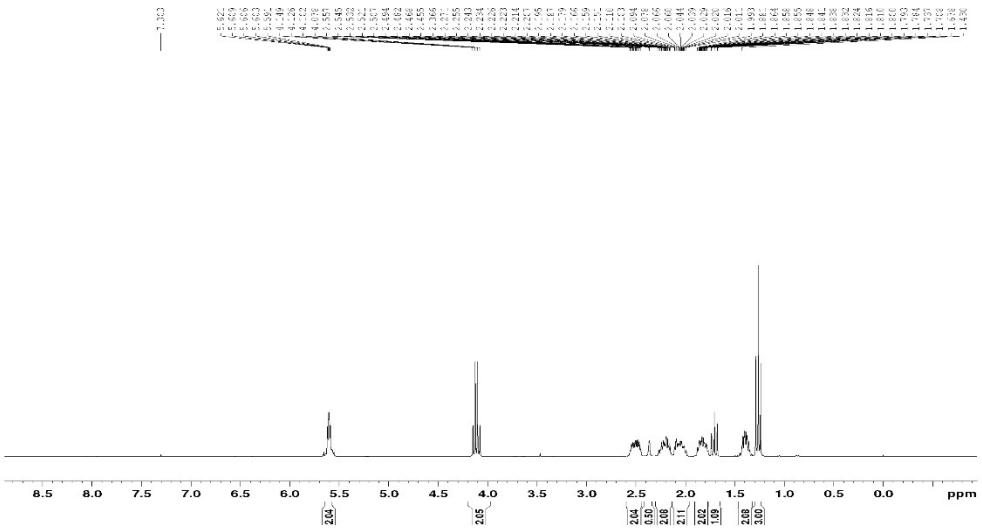


Figure S13. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of **exo-3**



A



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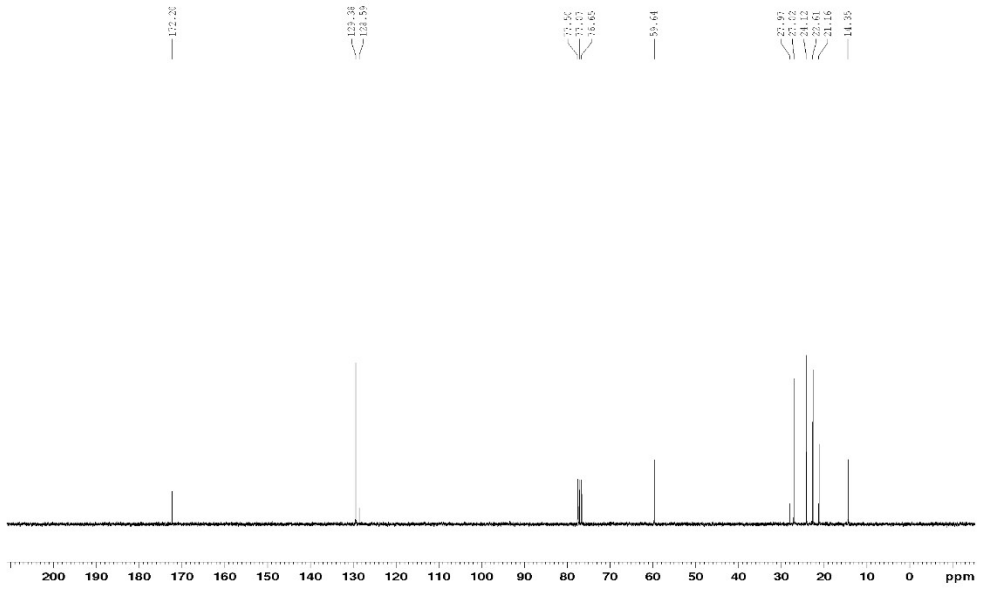
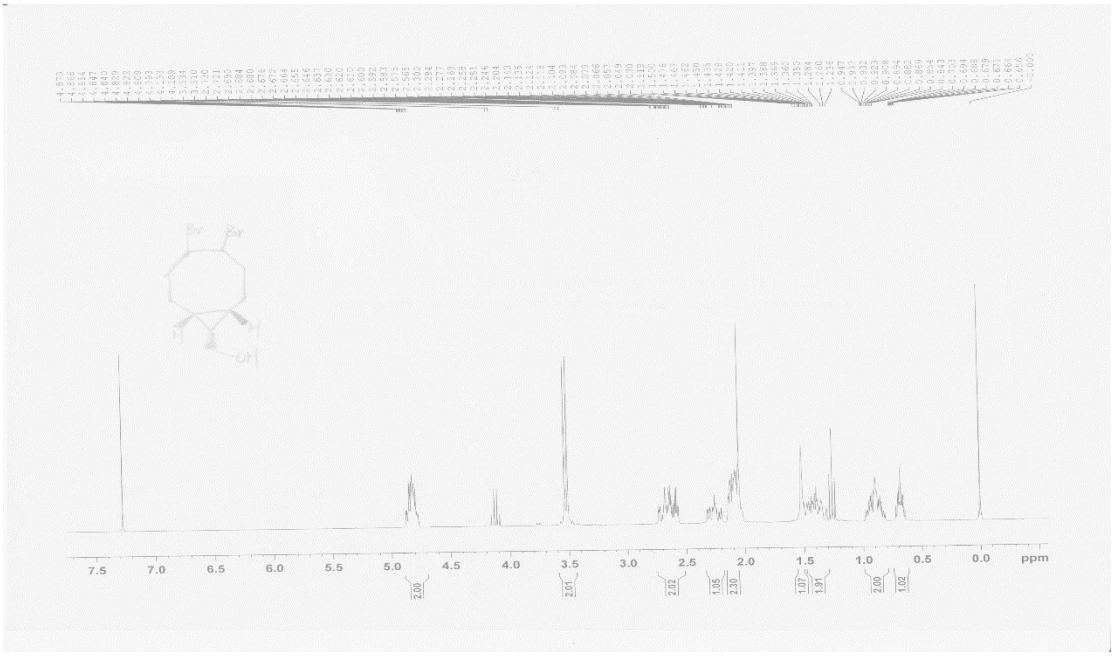


Figure S14. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of **endo-3**

A



B

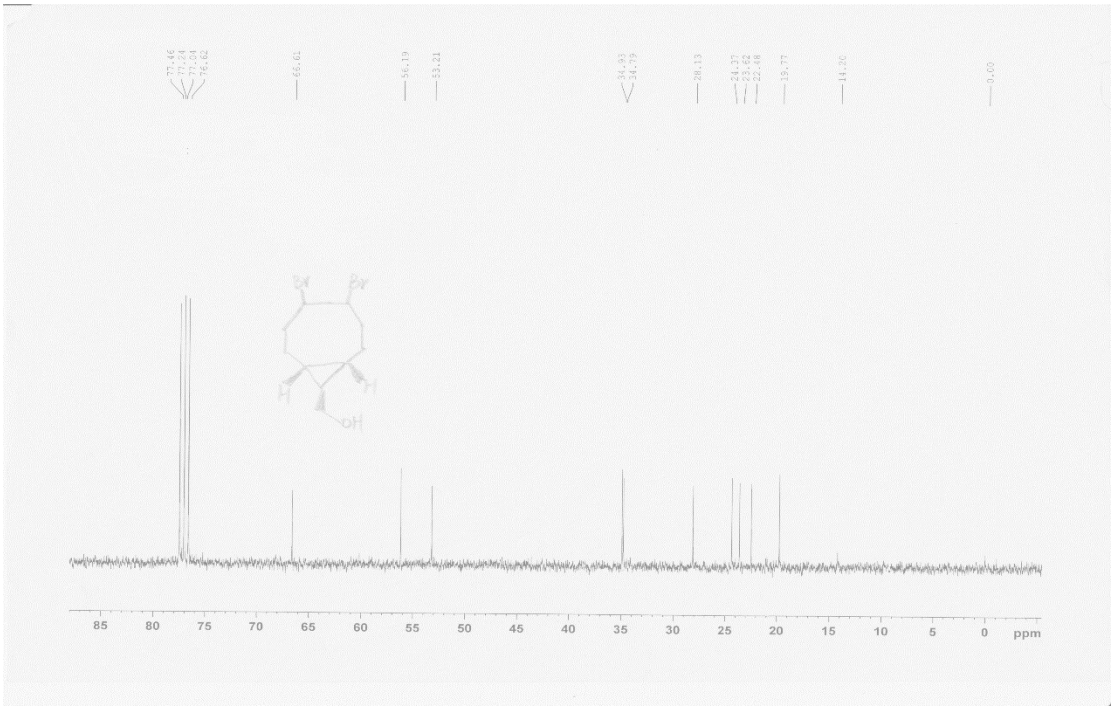
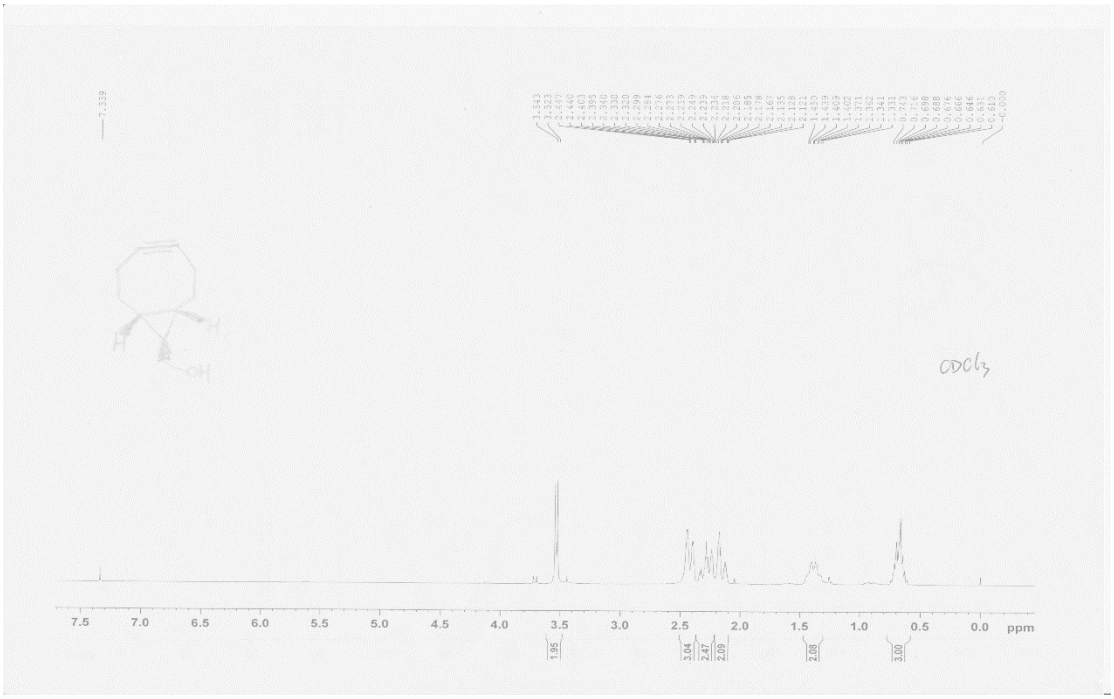


Figure S15. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **5**

A



B

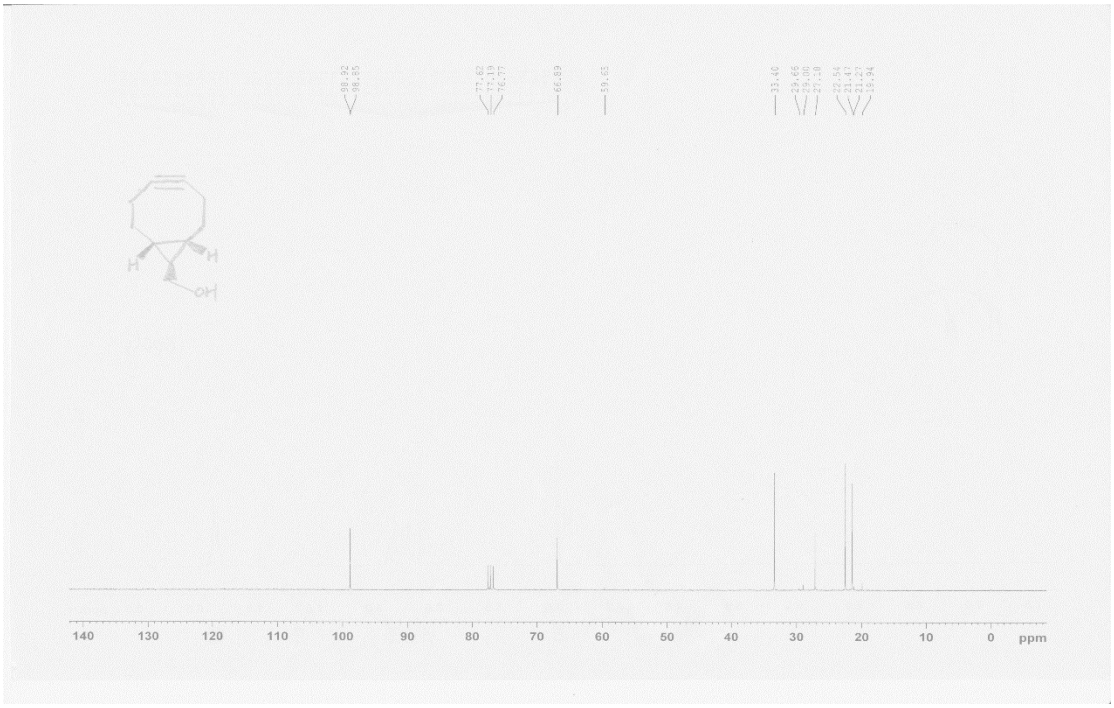
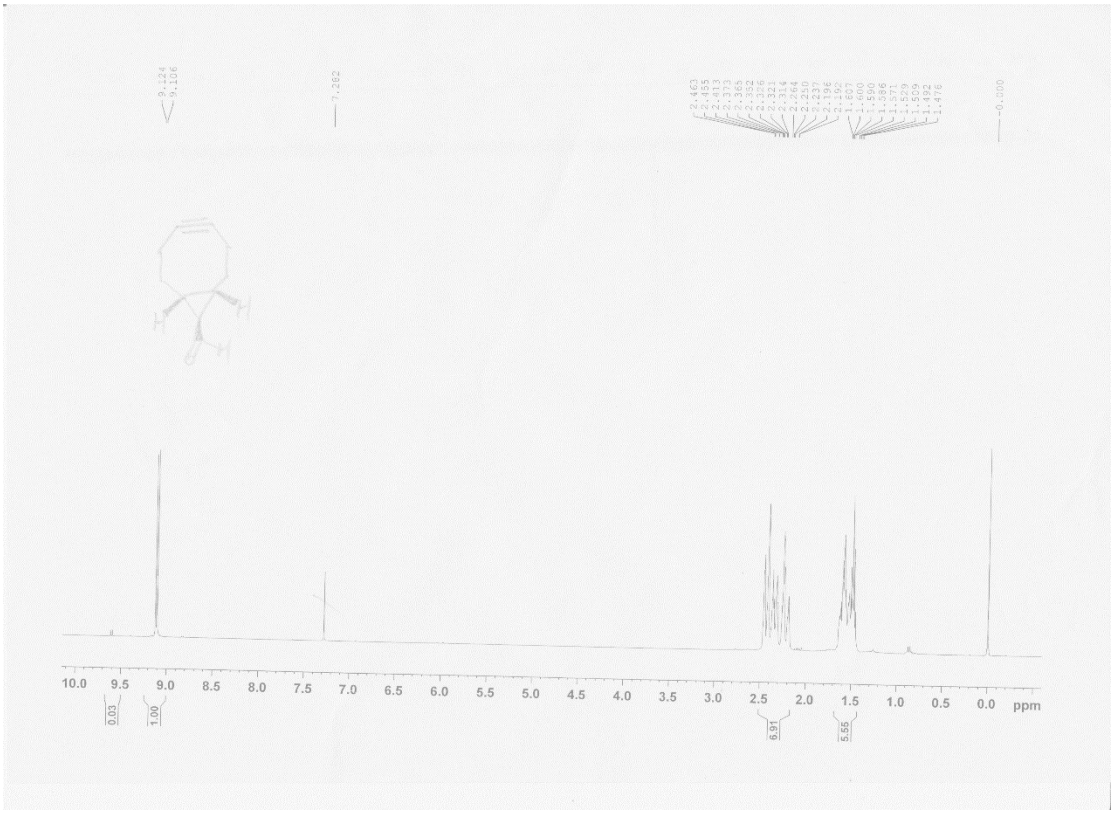


Figure S16. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **6**

A



B

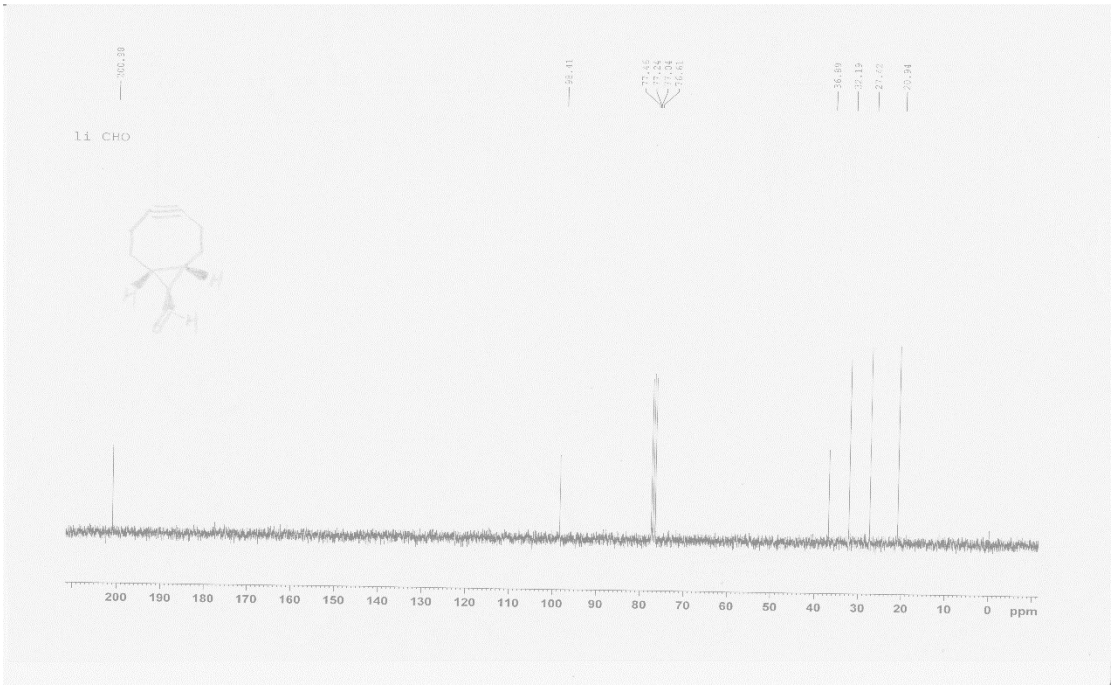
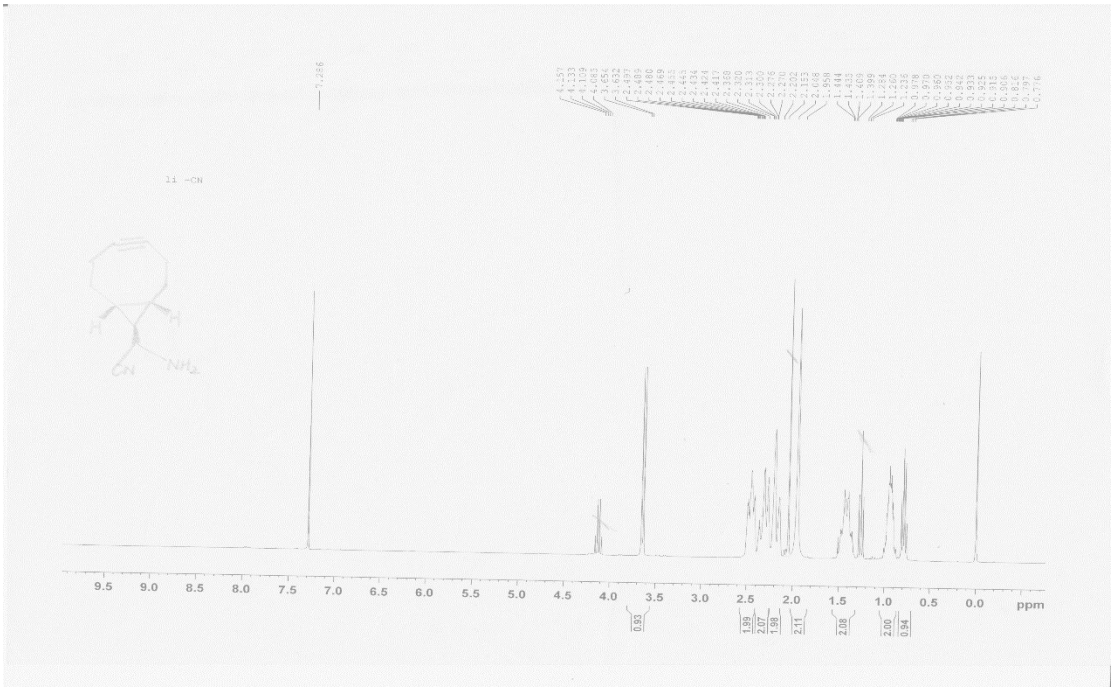


Figure S17. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **7**

A



B

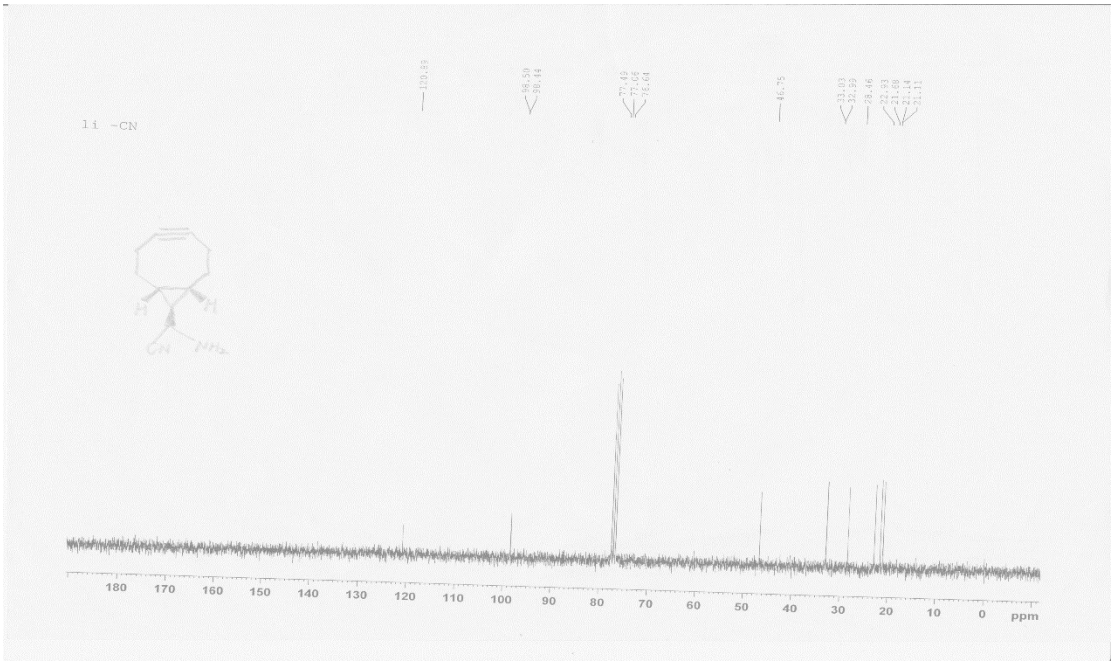
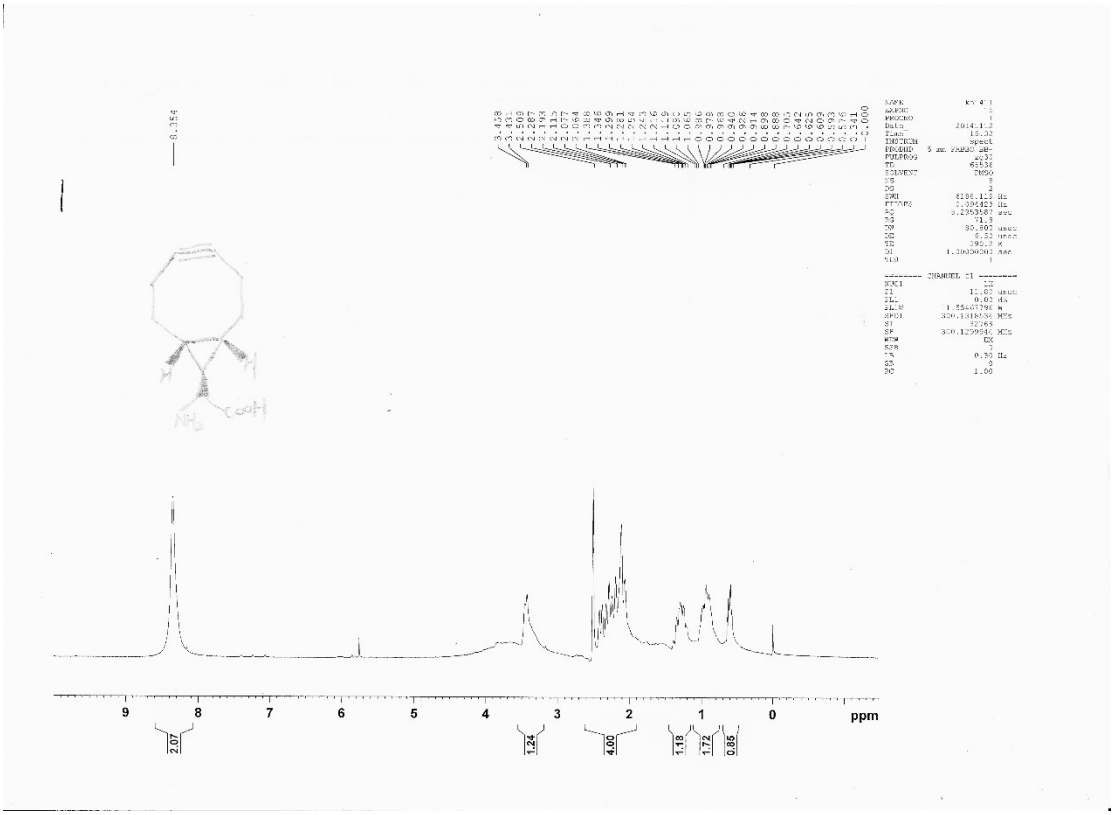


Figure S18. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **8**

A



B

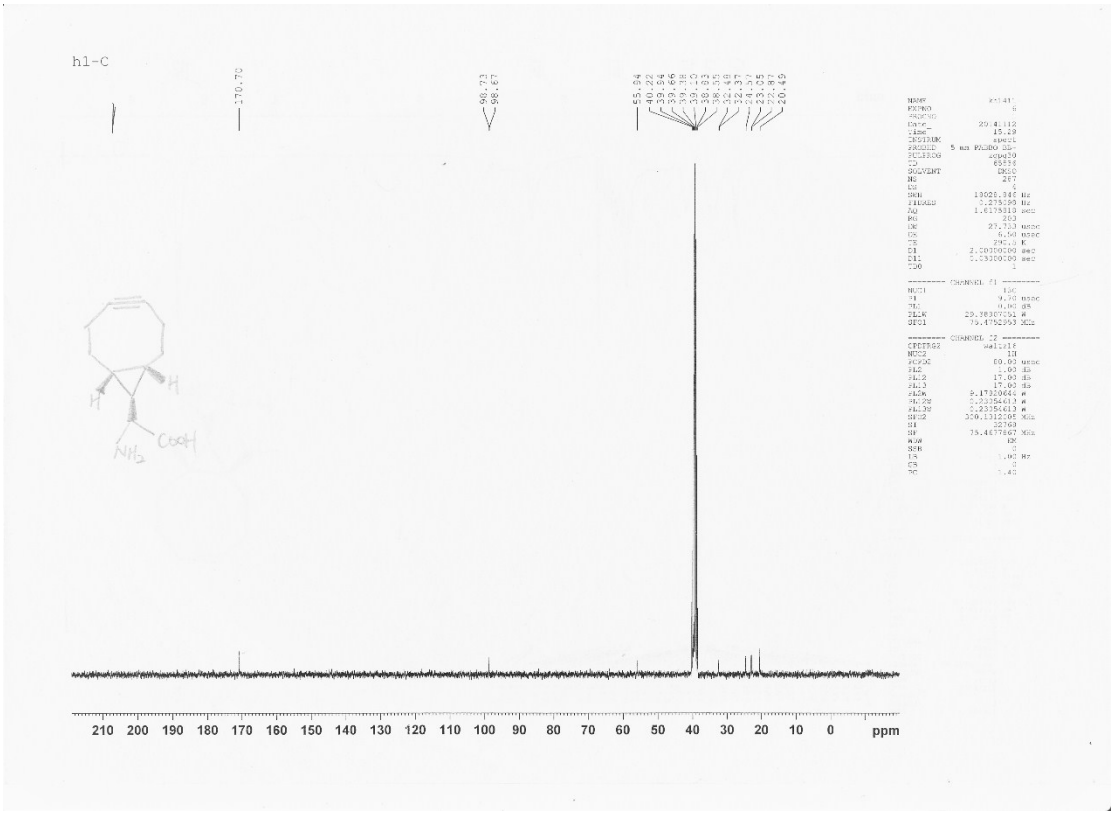
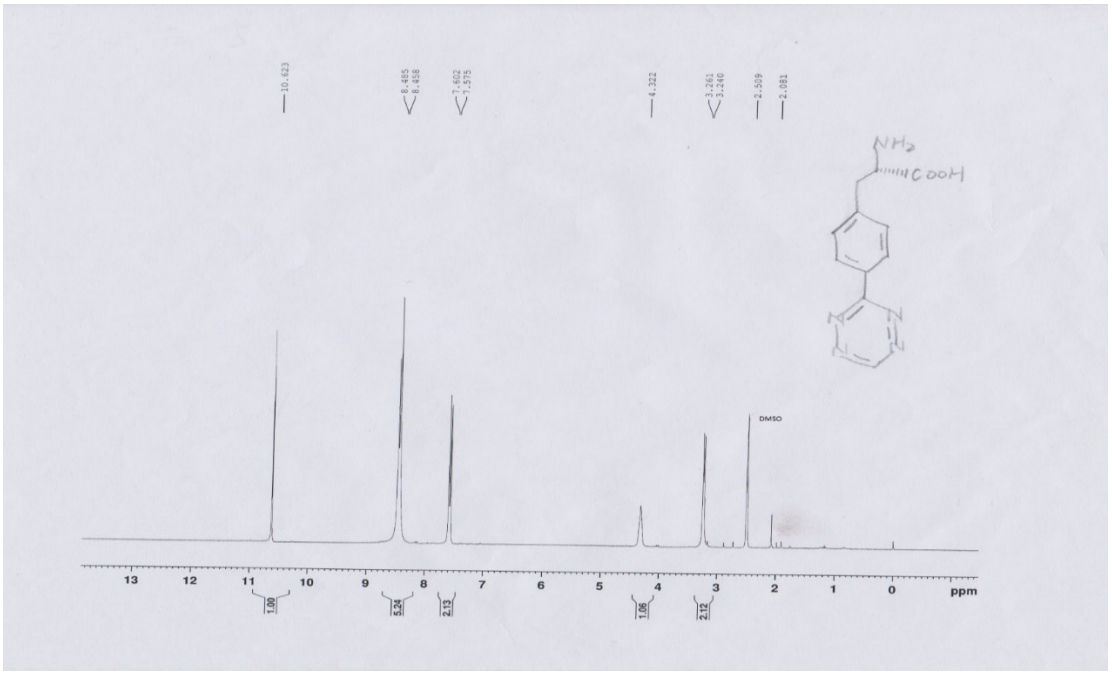


Figure S19. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **11**

A



B

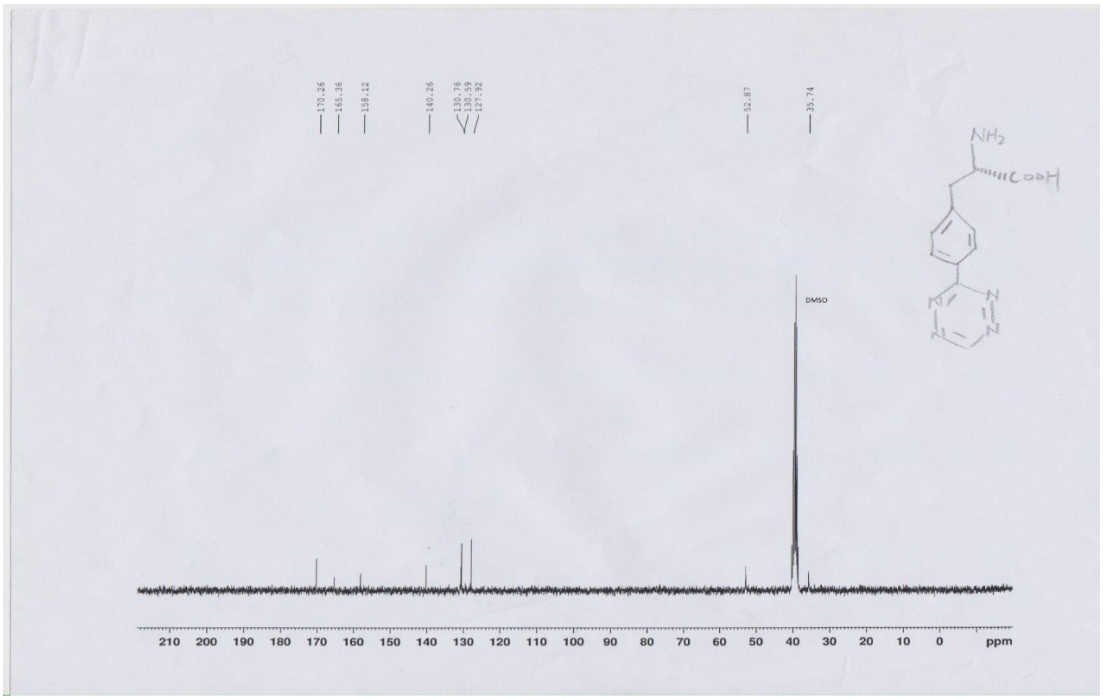
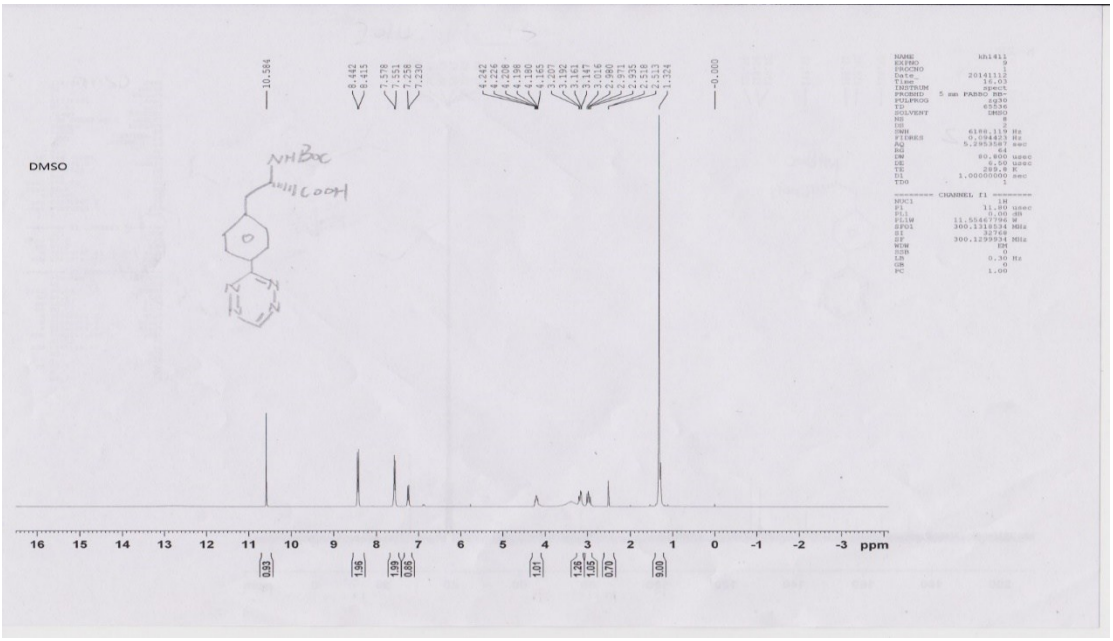


Figure S20. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **13**

A



B

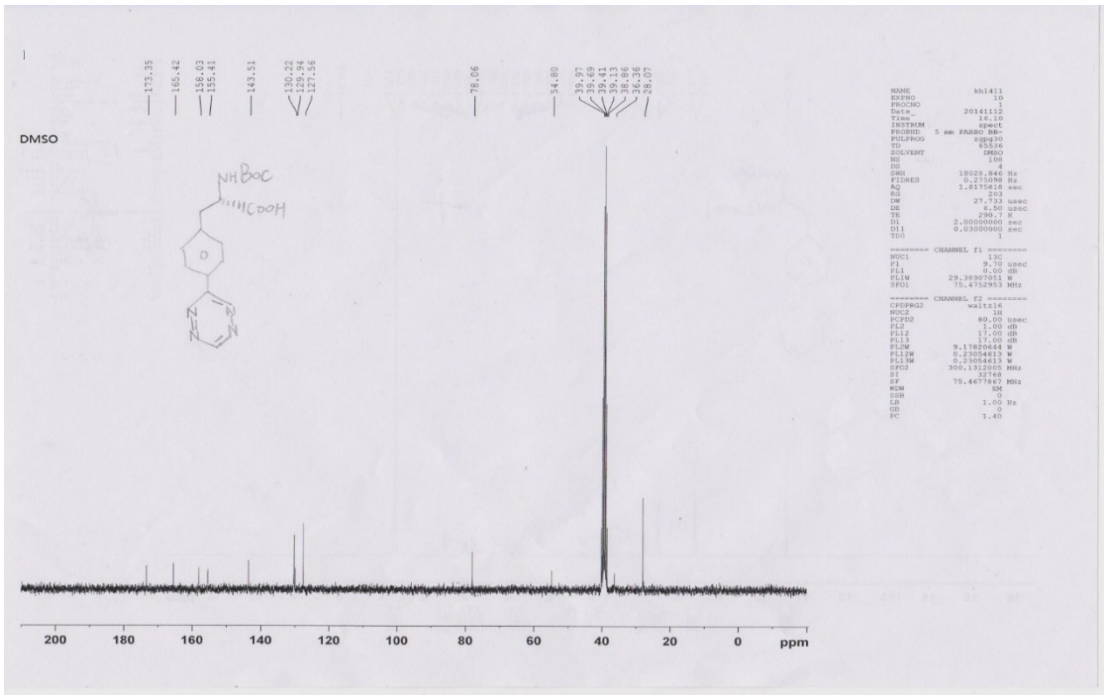
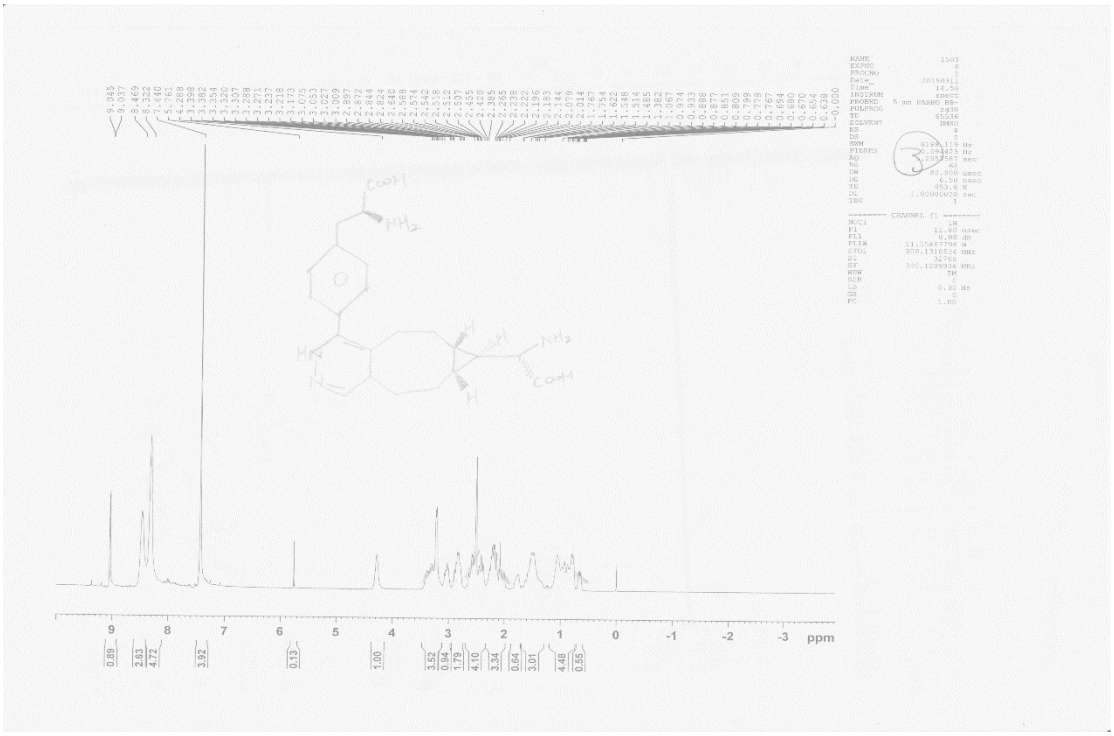


Figure S21. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **15**

A



B

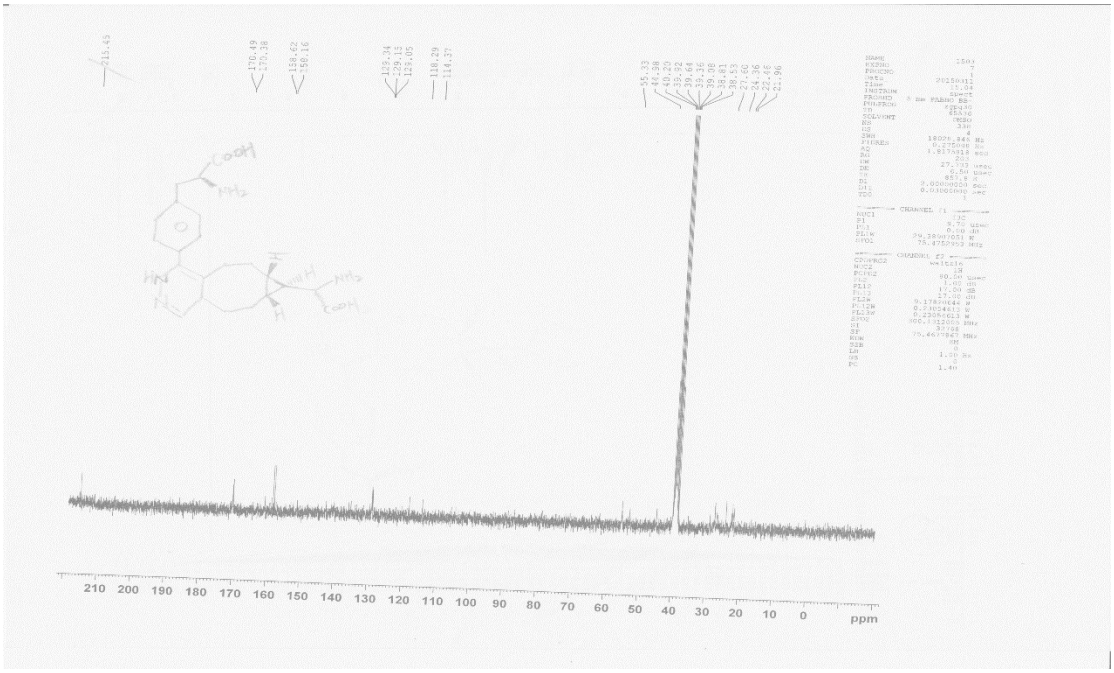


Figure S22. ^{1}H NMR spectrum (**A**) and ^{13}C NMR spectrum (**B**) of compound **16**