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Azidomethyl-ruthenocene: Facile Synthesis of a Useful Metallocene Derivative and its Application in the 'Click' Labelling of Biomolecules

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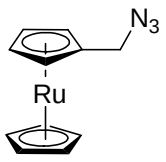
1) Materials. All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or dried over 3/4 Å molecular sieves. All preparations were carried out using standard Schlenk techniques. Hydroxymethyl-ruthenocene (**1**) was prepared from ruthenocene *via* theruthenocene-aldehyde following the literature procedure.[1, 2] Compound **3** was prepared as reported earlier.[3]

2) Instrumentation and methods. *^{1}H and ^{13}C NMR spectra* were recorded in deuterated solvents on Bruker DRX 200, 250, 400 or 600 spectrometers at 30°C. The chemical shifts, δ, are reported in ppm (parts per million). The residual solvent peaks have been used as an internal reference. The abbreviations for the peak multiplicities

are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). The abbreviations for the signal are Rc (ruthenocene), Fmoc arom (aromatic protons of the Fmoc group) and Ph (benzene ring protons). *Infrared spectra* were recorded on an ATR unit using a Bruker Tensor 27 FTIR spectrophotometer at 4 cm^{-1} resolution. Signal intensity is abbreviated br (broad), s (strong), m (medium), and w (weak). *Mass spectra* were recorded with a Bruker Esquire 6000 (ESI) and MAT 8200 (EI) spectrometers. *Elemental microanalyses* were performed using a Fisons Carlo Erba EA1108 instrument (CHNS version). Analytical RP-HPLC for the Rc-Enk conjugates **7** and **8** was carried out on a KNAUER HPLC instrument with a Varian C-18 column (250 × 8 mm) with a flow rate of 0.75 mL min^{-1}. 5% acetonitrile in water (A) and 5% water in acetonitrile (B) with 0.1% TFA were used as the eluents. The following solvent gradient was applied.

Time (min)	% A	% B
0	94	6
1	94	6
31	11	89
32	11	98
39	94	6
45	94	6

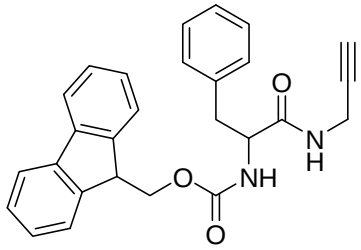
3) Synthesis and characterization data of the compounds 2, 4, 5 and 6.



2

Azidomethyl-ruthenocene (2). To a stirred solution of the hydroxymethyl-ruthenocene (**1**) (200 mg, 0.77 mmol) and diphenylphosphoryl azide (275 mg, 1 mmol) in 3 mL toluene, 1,8-diazabicyclo[5.4.0]-undec-7-en (DBU) (152 mg, 1 mmol) was added at 0°C under N_2 atmosphere and the mixture was allowed to stir 4h at the same temperature and then 12h at room temperature. The reaction mixture was concentrated. Flash column chromatography (silica gel, hexane:EtOAc 18:1→15:1) gave **2** as white solid (140 mg, 63%).

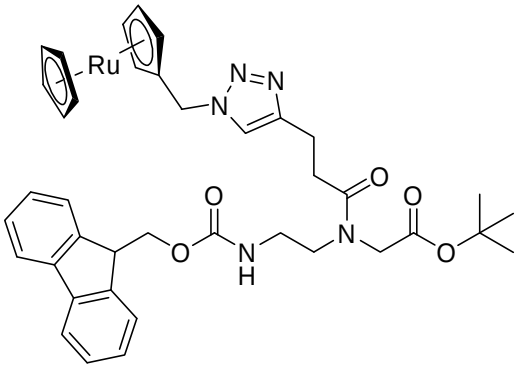
Data for **2**. ^{1}H NMR Spectrum (250MHz, CD_2Cl_2): δ 3.99 (s, 2H, Rc-C*H*$_2$-N_3), 4.61 (m, 7H, *Rc*-CH_2-N_3), 4.71 (m, 2H, *Rc*-CH_2-N_3); ^{13}C NMR Spectrum (250MHz, CD_2Cl_2): δ 50.5 (Rc-*C*H_2-N_3), 70.6 (*C*H Rc), 70.8 (*C*H Rc), 70.9 (*C*H Rc), 85.5 (*C* Rc); IR bands(ν): 3101w, 3074w, 2935w, 2101s (br, -N_3), 1464w, 1443w, 1376w, 1252m, 1241s, 1224m, 1100m, 1050s, 1036m, 1024s, 995w, 894w, 856s, 830m, 803s, 753s, 633s cm^{-1}; EI$^+$-MS: *m*/*z* (%): 287.2 (10) $[M]^+$, 259.1 (90) $[M\text{-}N_2]^+$, 245.1 (25) $[M\text{-}N_3]^+$, 232.1 (80) $[M\text{-}CH_2N_3]^+$, 167.1 (100) $[M\text{-}C_5H_5]^+$; Anal. calcd. for $C_{11}H_{11}N_3Ru$: C 46.15, H 3.87, N 14.68. Found: C 46.39, H 3.87, N 14.49.



4

Compound 4.[4] To a stirred solution of the Fmoc-L-phenylalanine (1 g, 2.58 mmol) in 30 mL DMF, HATU (1.3 g, 3.35 mmol) and DIPEA (432 mg, 3.35 mmol) were added and the mixture was allowed to stir for 30 min under an argon atmosphere. Propargylamine (142 mg, 2.58 mmol) in 5 mL DMF was added and the mixture was stirred for 48 h at room temperature. The reaction mixture was then concentrated, diluted with 250 mL of DCM and washed with distilled water and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated. Flash column chromatography (silica gel, hexane:EtOAc 1:1) gave **4** as white solid (899 mg, 82%).

Data for **4**. R_f = 0.45 (silica gel, hexane:EtOAc 1:1); ^{1}H NMR Spectrum (200MHz, DMSO-d_6): δ 2.74-2.85 (m, 1H, CH_2-C≡C*H*), 2.93-3.03 (m, 1H, CH-C*H*$_2$-Ph), 3.13 (m, 1H, CH-C*H*$_2$-Ph), 3.90 (m, 2H, C*H*$_2$-C≡CH), 4.09-4.32 (m, 4H, C*H* Fmoc, C*H*$_2$ Fmoc and C*H*-CH_2-Ph), 7.19-7.46 (m, 9H, CH-CH_2-*Ph* and Fmoc *arom*), 7.61-7.70 (m, 3H, Fmoc *arom* and N*H*), 7.88 (m, 2H, Fmoc *arom*), 8.49 (t, 1H, N*H*); ^{13}C NMR Spectrum (200MHz, DMSO-d_6): δ 28.1 (*C*H_2-C≡CH), 37.5 (CH-*C*H_2-Ph), 46.5 (*C*H Fmoc), 56.1 (*C*H-CH_2-Ph), 65.6 (*C*H_2 Fmoc), 73.1 (CH_2-C≡*C*H), 80.9 (CH_2-*C*≡CH), 120.1 (Fmoc *arom*), 125.2 (Fmoc *arom*), 126.1 (*Ph*), 127.0 (*Ph*), 127.5 (Fmoc *arom*), 127.9 (Fmoc *arom*), 129.2 (*Ph*), 138.1 (*Ph*), 140.6 (Fmoc arom), 143.7 (Fmoc arom), 155.7 (NH*C*OO), 171.2 (*C*ONH); IR bands(ν): 3288m (br), 3065w, 1690m, 1650s, 1532s, 1445w, 1389w, 1285s, 1257m, 1232s, 1081w, 1032m, 754m, 737s, 642m cm^{-1}; ESI-MS (positive detection mode): *m*/*z* (%): 447.06 (100) $[M+Na]^+$.

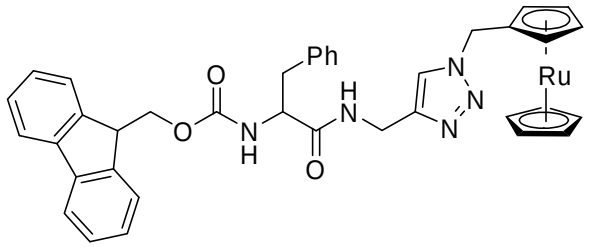


5

Compound 5. To a stirred solution of **3** (100 mg, 0.21 mmol) and **2** (120 mg, 0.42 mmol) in 16 mL THF, aqueous solution of $CuSO_4 \cdot 5H_2O$ (6 mg, 0.024 mmol in 1.5 mL water) was added followed by the addition of aqueous solution of sodium ascorbate (10 mg, 0.05 mmol in 1.5 mL water). The resulting mixture was allowed to stir at room temperature for 48h and the progress of the reaction was monitored by TLC (silica gel, EtOAc). After completion, the THF was removed and the reaction mixture was diluted with 150 mL of EtOAc. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Flash column chromatography (silica gel, EtOAc) yielded pure **5** as white sticky solid (138 mg, 86%).

Data for **5**. R_f = 0.47 (silica gel, EtOAc); ^{1}H NMR Spectrum (400MHz, CD_2Cl_2): δ 1.35 (maj) and 1.38 (min) (rotamers, s, 9H, OC(CH_3)$_3$), 2.60 (min) and 2.75 (maj) (rotamers, m, 2H, CH_2-CH_2-triazol ring), 2.98 (m, 2H, CH_2-CH_2-triazol ring), 3.31 (m, 2H, NH-CH_2-CH_2), 3.49 (m, 2H, CH_2-CH_2-N), 3.89 (maj) and 3.96 (min) (rotamers, s, 2H, N-CH_2-COOC(CH_3)$_3$), 4.23 (m, 1H, C*H* Fmoc), 4.34 (m, 2H, Fmoc-CH_2), 4.48-4.52 (m, 7H, *Rc*), 4.64 (m, 2H, *Rc*), 4.94 (maj) and 5.01 (min) (rotamers, s, 2H, Rc-CH_2-triazole ring), 5.72 (min) and 5.98 (maj) (rotamers, s, br, 1H, OCON*H*), 7.27-7.37 (m, 3H, C*H* Fmoc arom and C*H* triazol ring), 7.39-7.42 (m, 2H, Fmoc arom), 7.59-7.65 (m, 2H, Fmoc arom), 7.77-7.81 (m, 2H, Fmoc arom); ^{13}C NMR Spectrum (400MHz, CD_2Cl_2): δ 21.5 (CH_2-*C*H_2-triazol ring), 28.1 (min) and 28.2 (maj) (rotamers, OC(*C*H_3)$_3$) 32.4 (min) and 32.7 (maj) (rotamers, *C*H_2-CH_2-triazol ring), 39.8 (maj) and 39.9 (min) (rotamers, NH-*C*H_2-CH_2), 47.6 (maj) and 47.7 (min) (rotamers, *C*H Fmoc), 48.3 (maj) and 49.4 (min) (rotamers, CH_2-*C*H_2-N), 49.7 (maj) and 49.8 (min) (rotamers, Rc-*C*H_2-triazol ring), 50.1 (maj) and 51.9 (min) (rotamers, N-*C*H_2-COOC(CH_3)$_3$), 66.9 (maj) and 67.1 (min) (rotamers, Fmoc-*C*H_2O), 71.0 (maj) and 71.1 (min) (rotamers, *C*H Rc), 71.4 (*C*H Rc), 71.4 (maj) and 71.5 (min) (rotamers, *C*H Rc), 82.1 (maj) and 83.0 (min) (rotamers, *C* Rc), 85.9 (O*C*(CH_3)$_3$), 120.3 (Fmoc arom), 121.3 (*C*H triazol ring), 125.5 (Fmoc arom), 127.4 (min) and 127.5 (maj) (rotamers, Fmoc arom), 128.0 (min) and128.1 (maj) (rotamers, Fmoc arom), 141.6 (Fmoc arom), 144.5 (maj) and 144.6 (min) (rotamers, Fmoc arom), 146.9 (*C* triazol ring), 156.7 (NH*C*OO), 169.3 (min) and 169.9 (maj) (rotamers, *C*OOC(CH_3)$_3$), 172.7

(min) and 173.6 (maj) (rotamers, CH_2CON); IR bands(ν): 2930w, 1718s (br), 1647m (br), 1518w, 1449w, 1367w, 1232s (br), 1151s, 1048w, 997w, 810w, 759m, 714s cm^{-1}; ESI-MS (positive detection mode): *m*/*z* (%): 764.27 (100) $[M+H]^+$, 786.08 (25) $[M+Na]^+$, 802.02 (10) $[M+K]^+$.

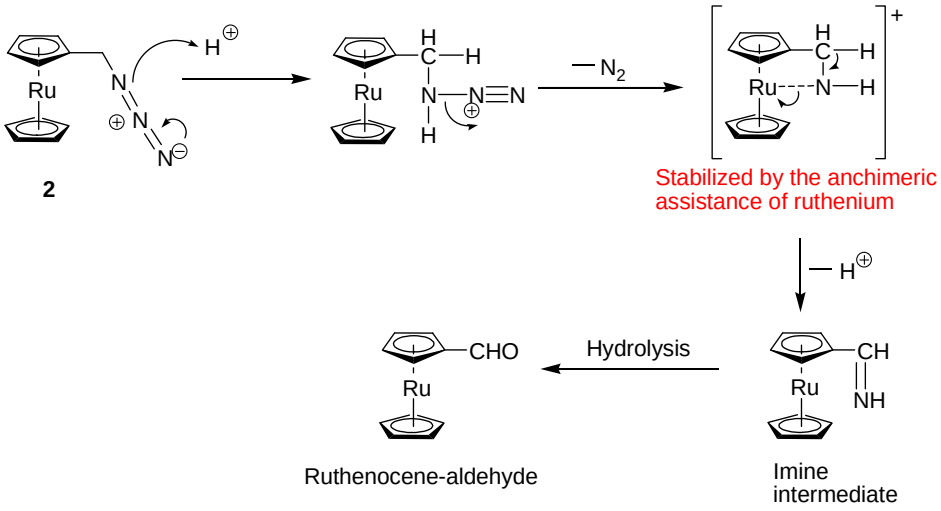


6

Compound 6. To a stirred solution of **4** (100 mg, 0.24 mmol) and **2** (138 mg, 0.48 mmol) in 32 mL THF, aqueous solution of $CuSO_4 \cdot 5H_2O$ (6.8 mg, 0.027 mmol in 2 mL water) was added followed by the addition of aqueous solution of sodium ascorbate (11.2 mg, 0.056 mmol in 2 mL water). The resulting mixture was allowed to stir at 40°C for 48h and the progress of the reaction was monitored by TLC (silica gel, EtOAc:DCM 1:1). After completion, the THF was removed and the reaction mixture was diluted with 150 mL of DCM. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. Flash column chromatography (silica gel, EtOAc:DCM 1:1) yielded pure **6** as white solid (114 mg, 69%).

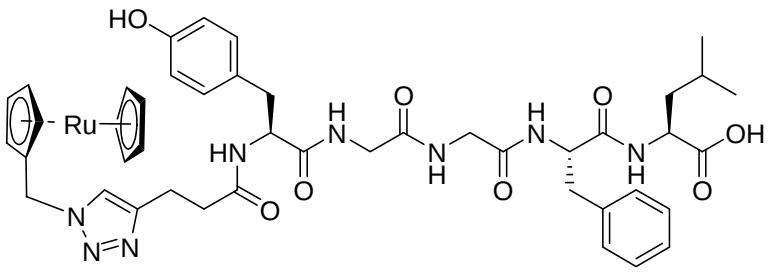
Data for **6**. R_f = 0.27 (silica gel, EtOAc:DCM 1:1); ^{1}H NMR Spectrum (400MHz, CD_2Cl_2): 2.83-3.01 (m, 2H, CH-CH_2-Ph), 4.04 (m, 1H, C*H* Fmoc), 4.17-4.35 (m, 5H, OCH_2-Fmoc, C*H*-CH_2-Ph and NH-CH_2-triazole), 4.41 (s, br, 7H, C*H* Rc), 4.55 (s, br, 2H, C*H* Rc), 4.90 (s, 2H, Rc-CH_2-triazole), 5.63 (s, br, 1H, OCON*H*) 6.97-7.44 (m, 13H, CH-CH_2-*Ph*, CON*H*, C*H* triazole and Fmoc *arom*), 7.66 (d, 2H, Fmoc *arom*); ^{13}C NMR Spectrum (400MHz, CD_2Cl_2): δ 34.1 (NH-*C*H_2-triazole), 37.7 (CH-*C*H_2-Ph), 46.4 (*C*H Fmoc), 48.9 (Rc-*C*H_2-triazole), 55.4 (*C*H-CH_2-Ph), 66.1 (*C*H_2 Fmoc), 70.0 (*C*H Rc), 70.3 (*C*H Rc), 70.4 (*C*H Rc), 84.1 (*C* Rc), 119.1 (Fmoc *arom*), 121.1 (*C*H triazole), 124.2 (Fmoc *arom*), 125.9 (*Ph*), 126.3 (*Ph*), 126.9 (Fmoc *arom*), 127.6 (Fmoc *arom*), 128.5 (*Ph*), 135.9 (*Ph*), 140.5 (Fmoc arom), 143.1 (Fmoc arom), 143.5 (*C* triazole), 155.1 (NH*C*OO), 170.2 (*C*ONH); IR bands(ν): 3289m (br), 1691s, 1648s, 1535m (br), 1449w, 1261m, 1230m, 1143w, 1037m, 953w, 808m, 738s, 668w, 620w cm^{-1}; ESI-MS (positive detection mode): *m*/*z* (%): 712.08 (10) $[M+H]^+$, 734.01 (100) $[M+Na]^+$.

4) Figure S1: Plausible mechanism for the formation of ruthenocene aldehyde from azidomethyl-ruthenocene in acidic media.



5) Synthesis, characterization data, ^{1}H NMR spectrum of 7, analytical HPLC traces and ESI-MS spectra of the Rc-triazole-peptide conjugates 7 and 8.

General Method for manual SPPS. Resin-bound enkephalin derivatives were obtained by standard Fmoc-SPPS starting from base labile Fmoc-Leu loaded TentaGel S HMBA resin (as shown in Scheme 2).The 2-Cl-trt deprotection of the Tyrosine hydroxyl group was carried with TFA 5% v/v TIS 5% v/v in CH_2Cl_2. For a complete description on manual SPPS for metallocene-peptide conjugates, see a tutorial paper by Metzler-Nolte, Mier *et al.*.[5]



Rc-Pent-Enk-COOH (**7**)

Rc-triazole-peptide conjugate 7. Dry resin bound enkephalin derivative (400 mg, load 0.24 mmol/g) with modifies *N*-terminus was taken into a fritted syringe and swollen with DMF for 1h. CuI (55 mg, 0.29 mmol) and sodium ascorbate (15 mg, 0.08 mmol) was then introduced into the syringe (from top). Afterwards, a

solution of **2** (69 mg, 0.24 mmol) in 5 mL of DMF and followed by a mixture of DIPEA (186 mg, 1.44 mmol) and 2, 6-lutidine (154 mg, 1.44 mmol) was aspired up the syringe. The mixture was shaken at room temperature for *ca.* 60h at dark. The resin was then washed with DMF (×5), CH_2Cl_2 (×5), DMF (×5) and CH_2Cl_2 (×5) successively and dried. The product was cleaved from the resin by shaking it with 7 mL of cooled (0°C) solution of a mixture of 1/3 (v/v) 1M aqueous NaOH/1, 4-dioxane for 20 minutes. The pH of the resulting solution was adjusted to 7 by addition of dilute aqueous hydrochloric acid. The solution was then lyophilized and the white solid obtained was dissolved in minimum amount of MeOH and filtered. Removal of the solvent gave **7** as off white solid. (Crude yield = 79.6 mg, 90%, trace amount of NaCl is present with the product).

Data for **7.** t_R = 24.5. ESI-MS (positive detection mode, in 50% acetonitrile and water mixture): *m*/*z* (%):923.15 (100) $[M+H]^+$. Calculated for $C_{44}H_{53}N_8O_8Ru$ ($[M+H]^+$) = 923.30. ^{1}H-NMR (600 MHz, DMSO-D_6)[6, 7]: δ = 9.31 (s, br, 1H, OH_{Tyr}), 8.68 (m, 1H, NH_{Gly}), 8.42 (m, 1H, NH_{Leu}), 8.25 (m, 1H, NH_{Phe}), 7.99 (m, 1H, NH_{Gly}), 7.82 (m, 1H, NH_{Tyr}), 7.72 (s, 1H, C*H*-triazole), 7.14-7.27 (m, 5H, H_{Ar} of Phe), 7.01 (d, 2H, ^3J = 8.1, H_{Ar} of Tyr), 6.63 (d, 2H, ^3J = 8.1, H_{Ar} of Tyr), 5.01 (s, 2H, Rc-CH_2-triazole), 4.71 (m, 2H, C_5H_4 of Rc), 4.53 (m, 1H, α-C*H* of Phe), 4.50 (m, 7H, C_5H_4 and C_5H_5 of Rc), 4.34-4.46 (m, 2H, α-C*H* of Tyr and Leu), 3.60-3.75 (m, 4H, α-CH_2 of Gly), 3.05 (m, 1H, β-CH_2 of Phe), 2.92 (m, 1H, β-CH_2 of Tyr), 2.68-2.81 (m, 4H, β-CH_2 of Phe and Tyr and CH_2-CH_2-triazole), 2.36-2.46 (m, 2H, CH_2-CH_2-triazole), 1.62 (m, 1H, γ-C*H* of Leu), 1.50 (m, 2H, β-CH_2 of Leu), 0.84 (m, 6H, CH_3 of Leu).

Figure S2. Analytical HPLC trace of 7 (crude product).

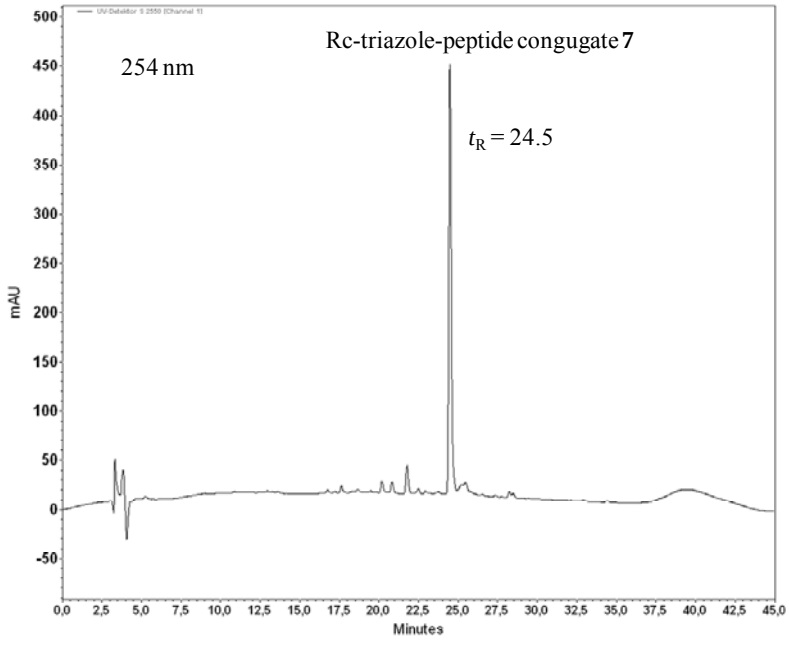


Figure S3. ESI-MS spectrum (positive detection mode) of 7. Inserts: a) experimental isotopic pattern and b) calculated isotopic pattern of $[M+H]^+$ peak of 7.

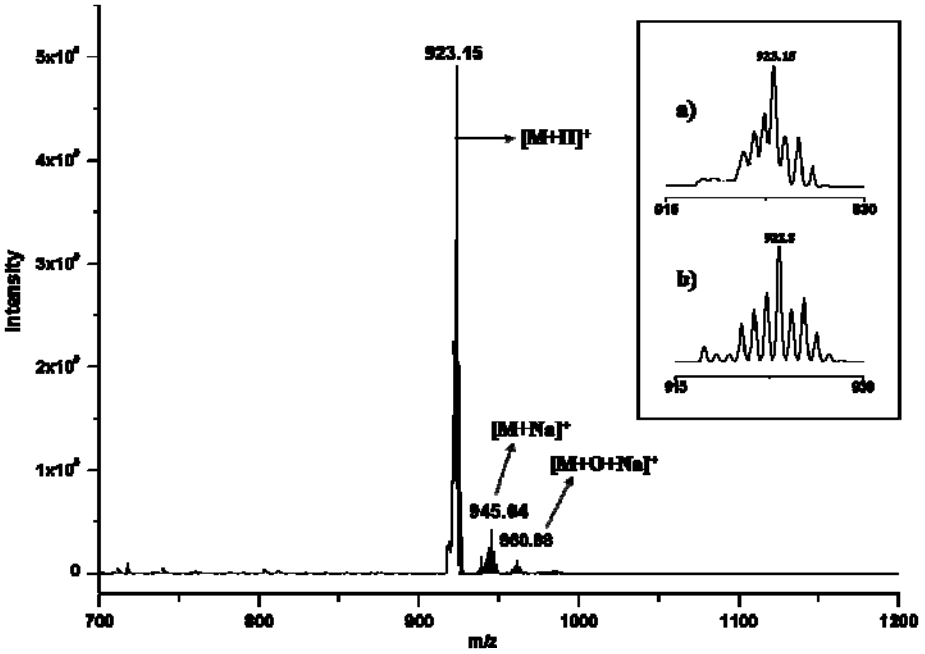
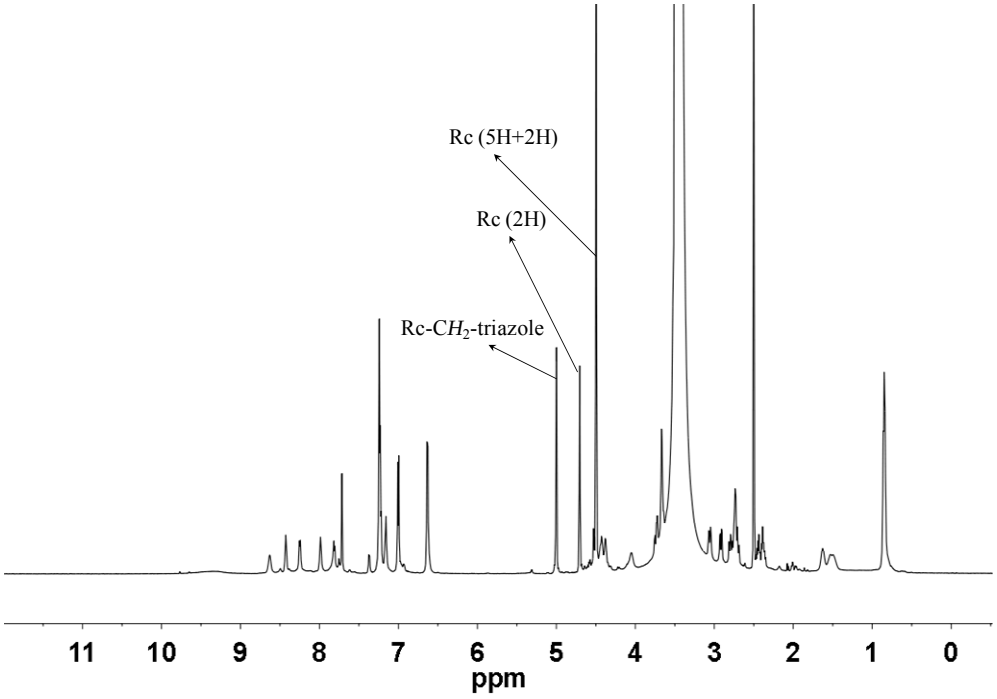
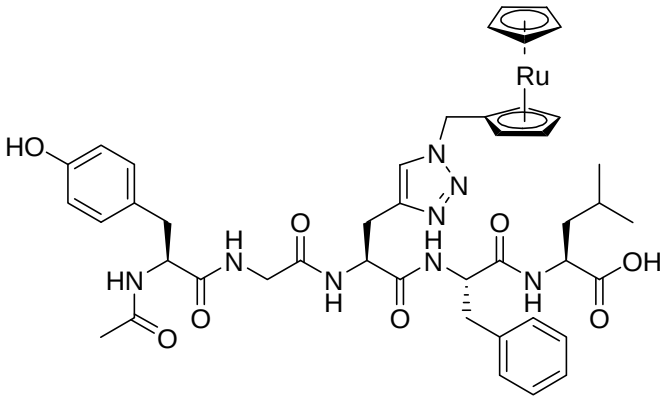


Figure S4. ^{1}H NMR spectrum of 7 in DMSO-d_6 (600 MHz).





Ac-Enk (PrGly-Rc)-COOH (**8**)

Rc-triazole-peptide conjugate 8. Similar procedure was followed but with 200 mg of the respective resin bound enkephalin derivative. All the reagents were used half of the quantities that stated above. **8** was obtained as off white solid. (Crude yield = 36 mg, 82%, trace amount of NaCl is present with the product).

Data for **8**. t_R = 24.6. ESI-MS (positive detection mode, in 50% acetonitrile and water mixture): *m*/*z* (%): 923.03 (70) $[M+H]^+$; 938.99 (100) $[M+O+H]^+$. Calculated for $C_{44}H_{53}N_8O_8Ru$ ($[M+H]^+$) = 923.30.

Figure S5. Analytical HPLC trace (crude product) of 8.

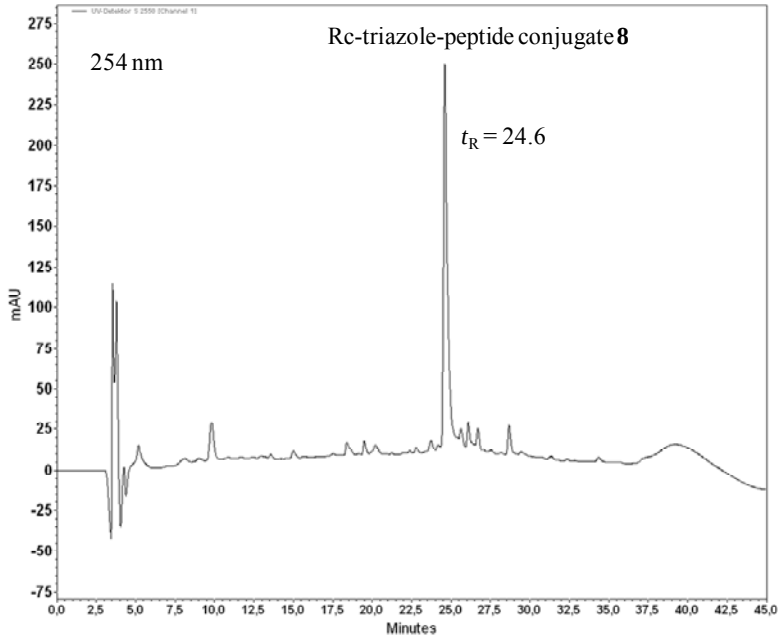
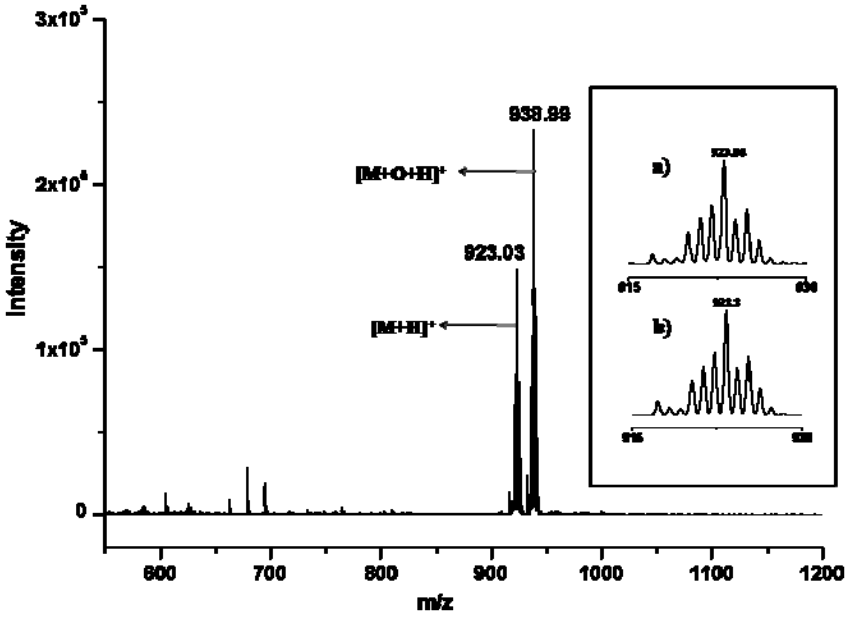
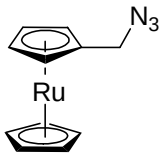


Figure S6. ESI-MS spectrum (positive detection mode) of 8. Inserts: a) experimental isotopic pattern and b) calculated isotopic pattern of $[M+H]^+$ peak of 8.

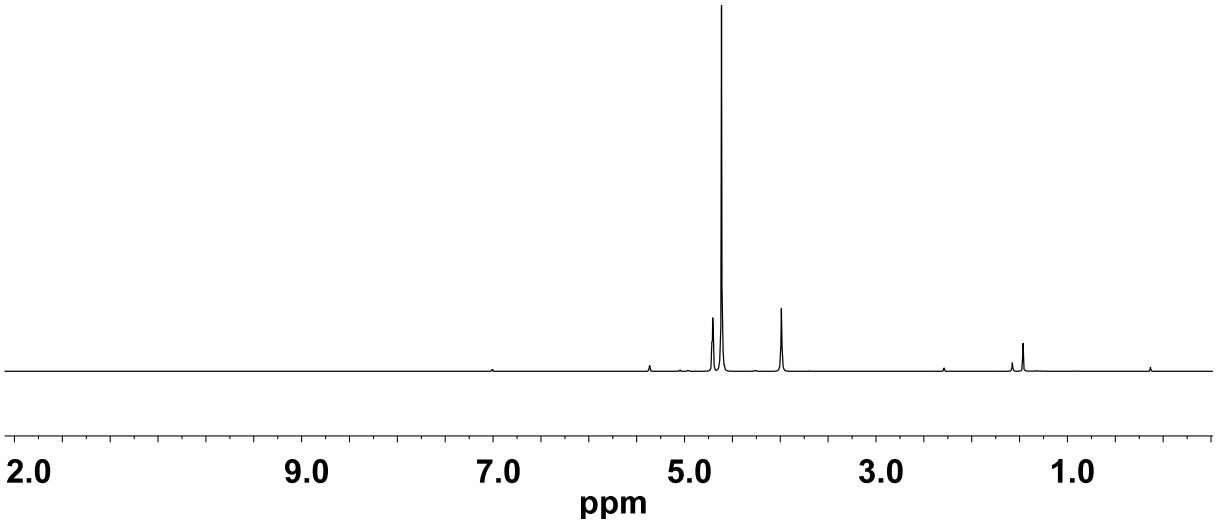


6) NMR Spectra of compounds 2, 4, 5 and 6.

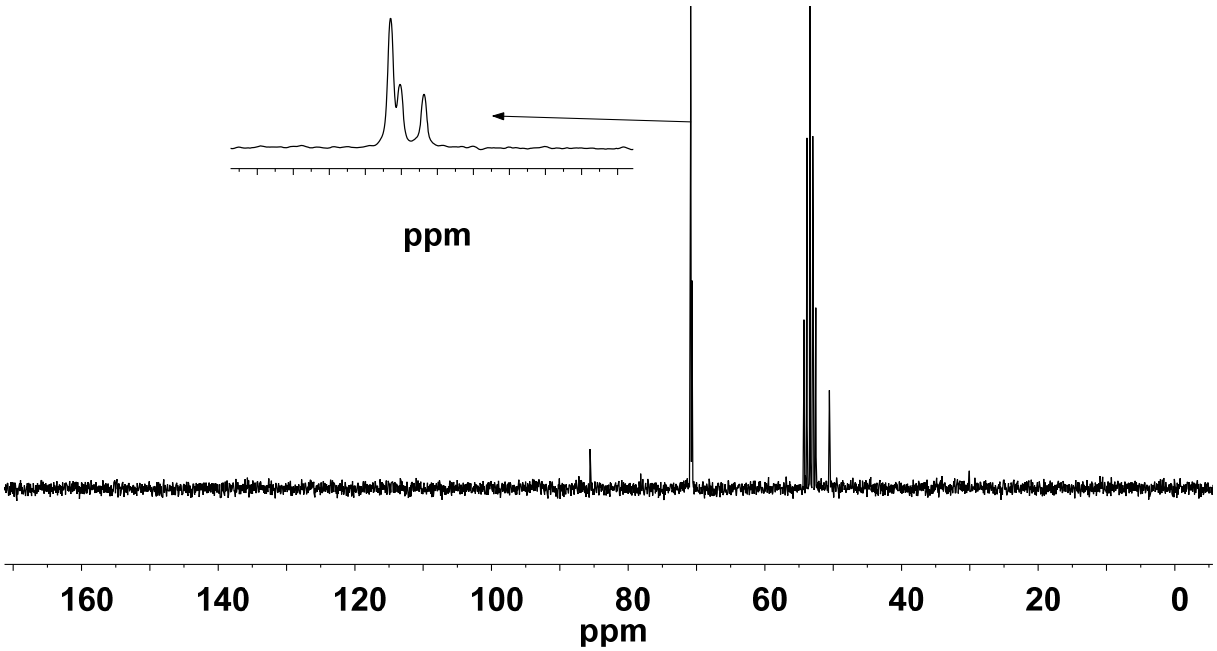


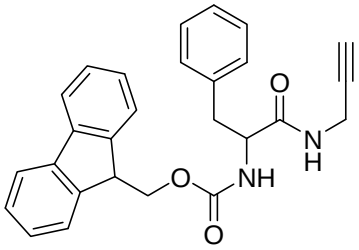
2

^{1}H NMR, 250 MHz, CD_2Cl_2



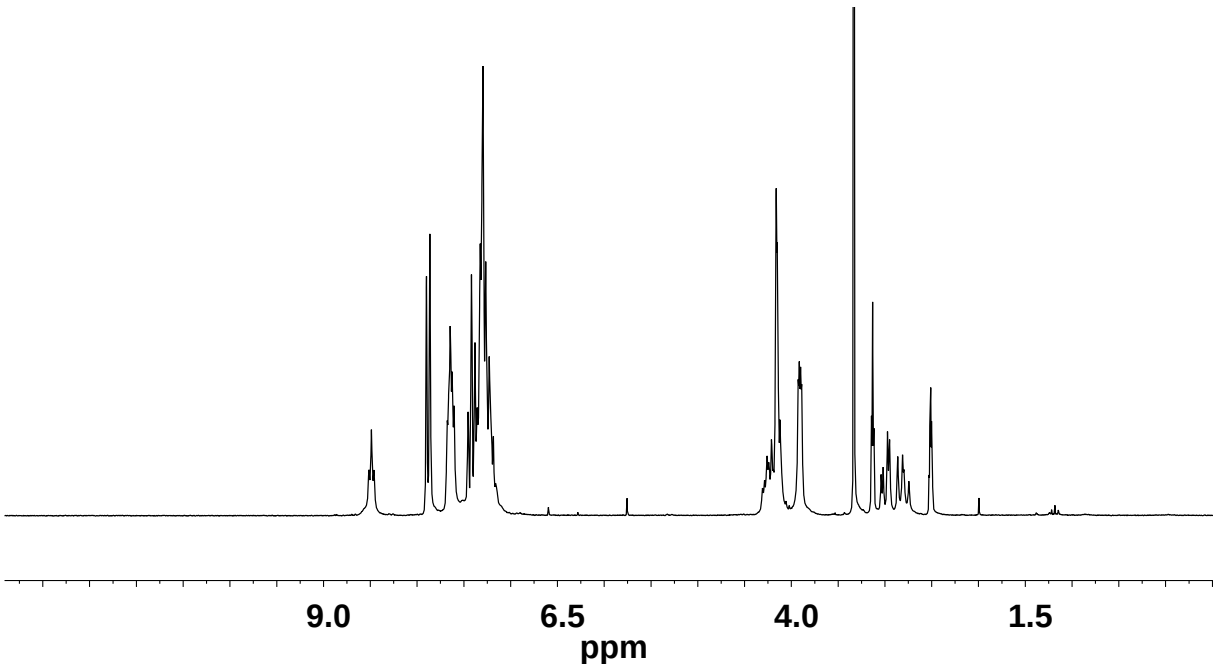
^{13}C NMR, 250 MHz, CD_2Cl_2



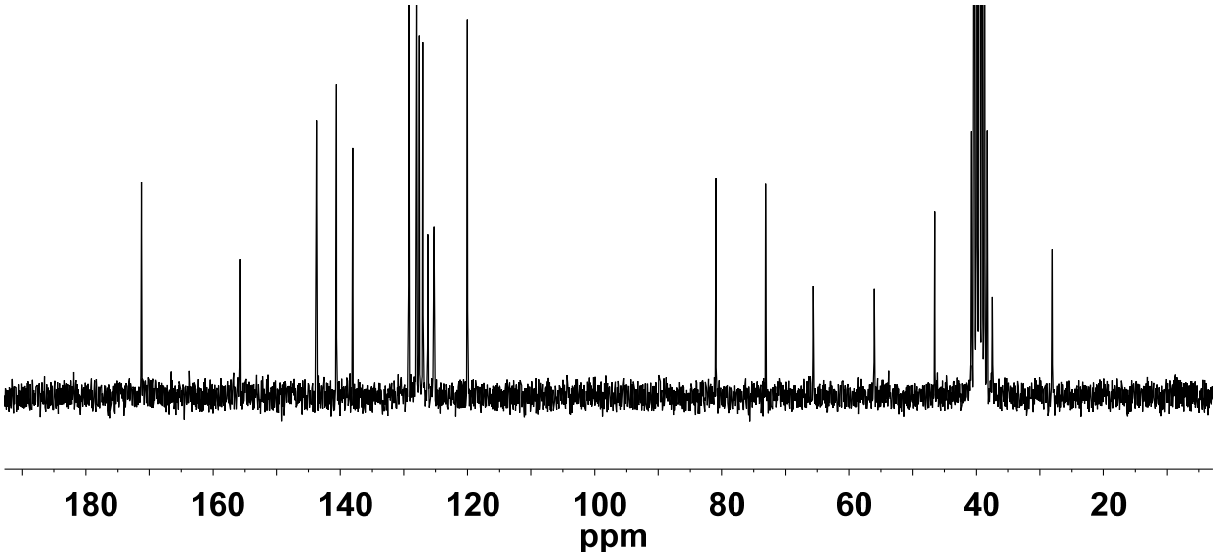


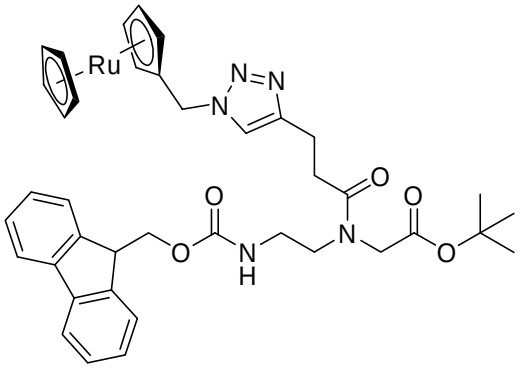
4

^{1}H NMR, 200 MHz, DMSO-d_6



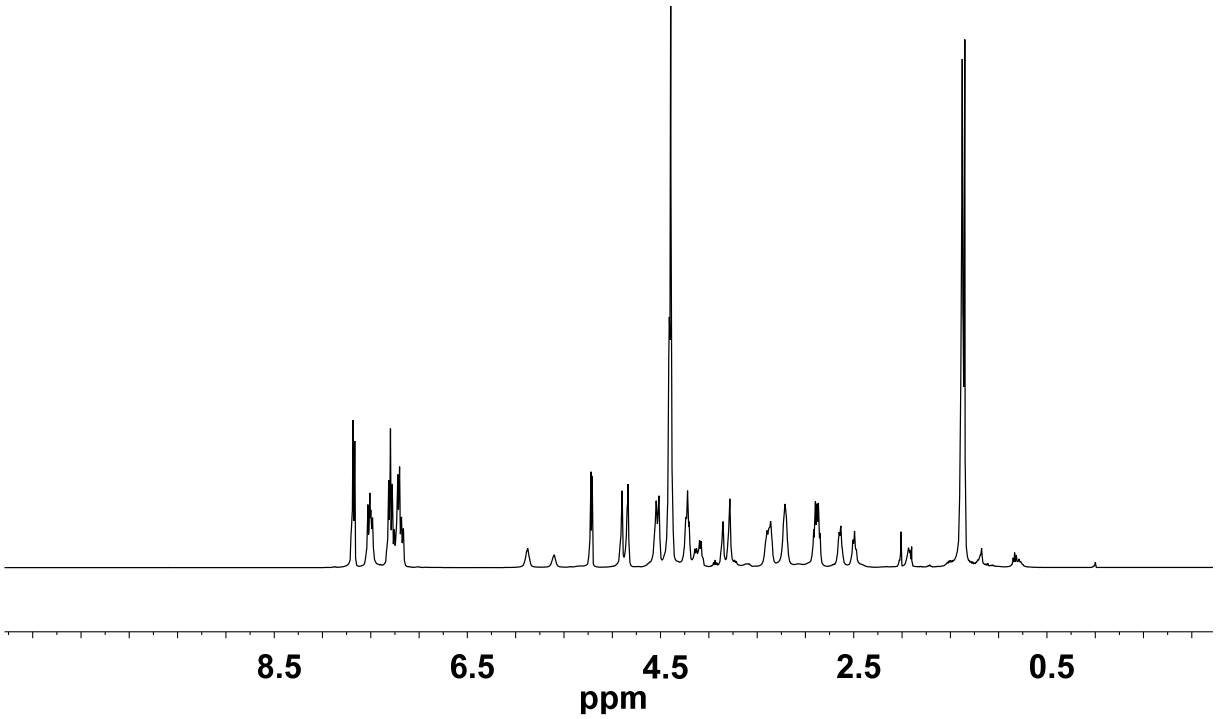
^{13}C NMR, 200 MHz, DMSO-d_6



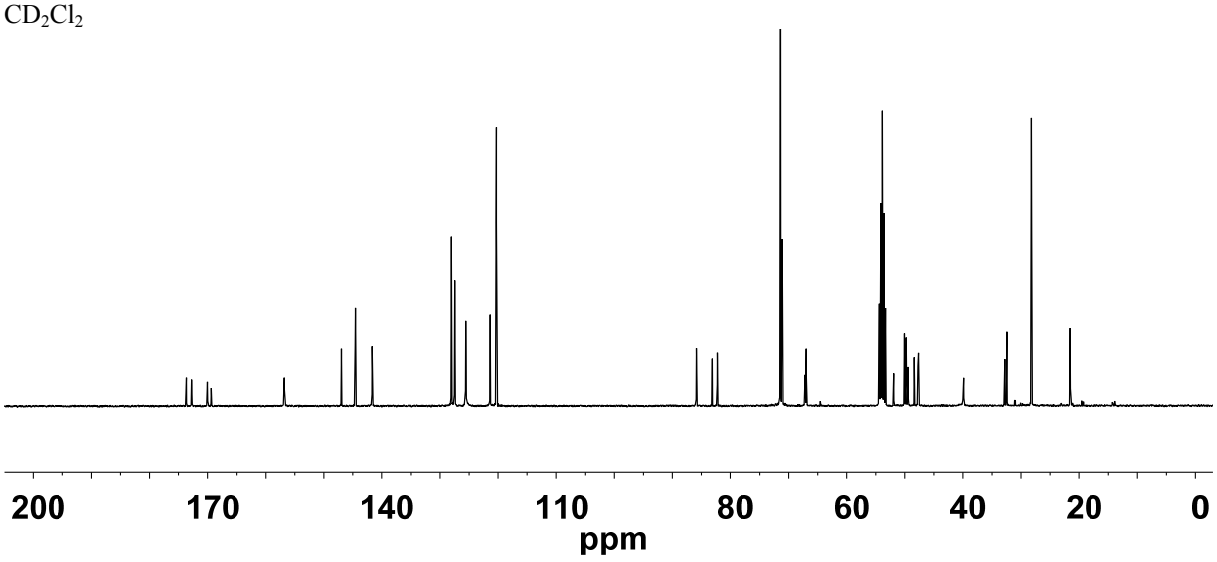


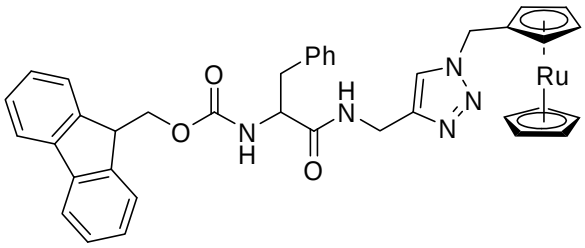
5

^{1}H NMR, 400 MHz, CD_2Cl_2



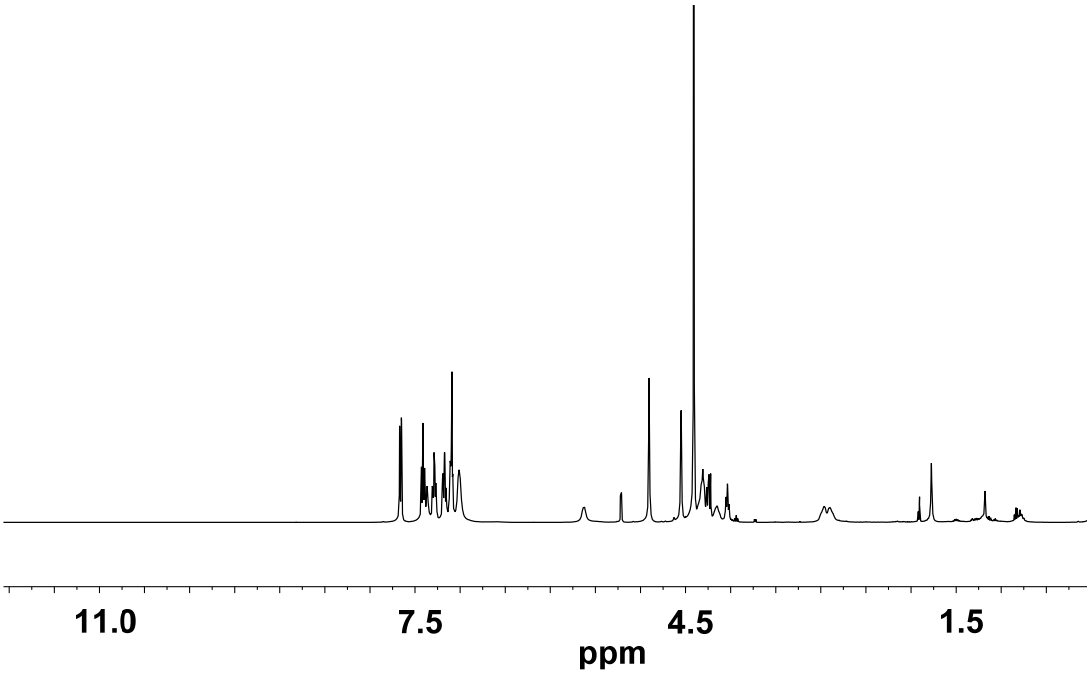
^{13}C NMR, 400 MHz,
CD_2Cl_2



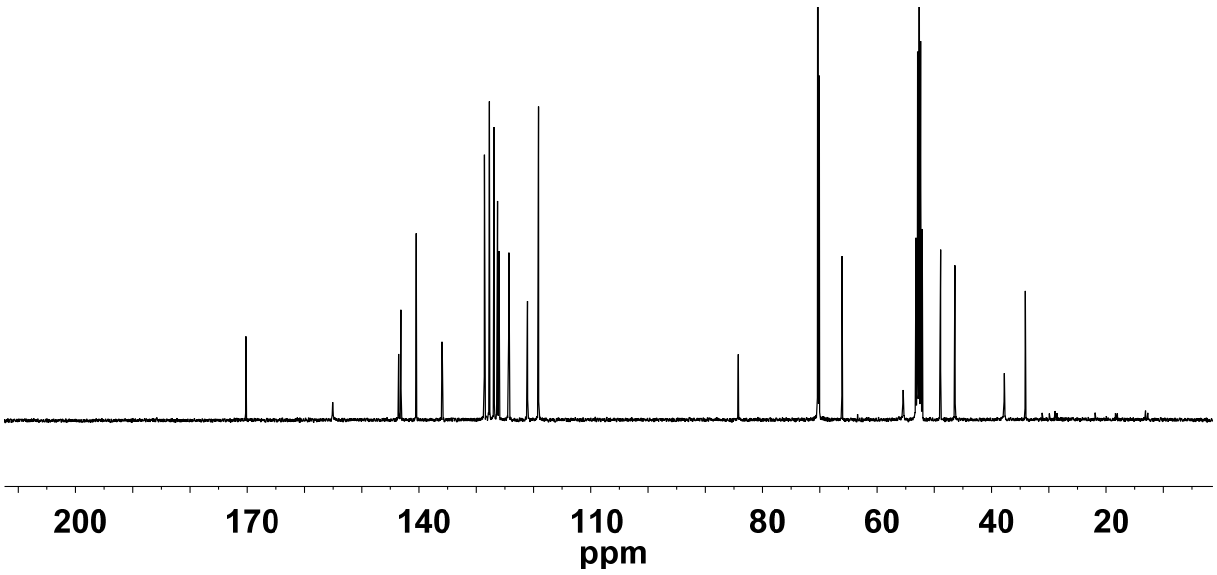


6

^{1}H NMR, 400 MHz, CD_2Cl_2



^{13}C NMR, 400 MHz, CD_2Cl_2



7) References.

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