Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary Information

Universal activator of microRNAs identified from photoreaction products

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General

Nuclear magnetic resonance spectra were recorded on a Bruker DPX 300 at 300MHz with $CDCl_3$ as solvent. Chemical shifts are reported in δ (ppm) with the solvent resonance as the internal standard (chloroform, H, 7.263 ppm; C, 77.66, 77.23, 76.81 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br s = broad), coupling constant in Hz, integration, and assignment. Elemental analyses were done on a Elementar Vario

MICRO analyzer. MTT assay was tested on Bio-Rad (680) Micro plate spectrophotometer. Modulus Luminometer used for luciferase assay was from Turner BioSystems (9200-002). Quantitative RT-PCR was carried out on an Applied Biosystems 7300 Real-Time PCR System. Benzene (AR grade) was dried with sodium and distilled. MTT powder was purchased from Sigma-Aldrich. TRIzol Reagent and Lipofectamine 2000 reagent were purchased from Invitrogen. TaqMan miRNA probes, AMV reverse transcriptase, dNTP mixture, Taq transcriptase, RRI and oligo (dT) were purchased from TaKaRa. Eva green dye was purchased from Invitrogen. The stem-loop RT primers were synthesized by Invitrogen. Other materials were commercially available.

Experimental procedures

Cells, and Antibodies: Mouse myoblast cell C2C12, human hepatocellular carcinoma cell HepG2, human breast cancer MCF-7 were cultured with DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin and streptomycin (GIBCO) in a humidified atmosphere with 5% CO_2. Anti-Ago2 and anti-TRBP monoclonal antibody were purchased from Abcam, anti-Dicer monoclonal antibody were purchased from Cell Signaling. Anti-GAPDH and anti-α-tubulin were purchased from Santa Cruz Biotechnology.

Plasmid Construction: A system to screen miRNA inhibitor and activator based on the pMIR-REPORT (Applied Biosystem) reporter plasmid was constructed. This construct consists of an exprimental firefly luciferase reporter vector and an associated β-gal report control plasmid. The predicted miRNA target sequence was inserted into 3'UTR of the firefly luciferase gene between the SpeI and HindⅢ restriction sites. We selected miR-1/122/21/214/25/26b/34a/126 as the target miRNA, The plasmid without target sequence of miRNA was taken as a control.

Transfection: Plasmid transfection into C2C12, HepG2 and MCF-7 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. All transfections were performed in triplicate for statistical analysis. The cells were incubated for 6 h followed by the replacement of transfection media (OPTI-MEM) with normal media (DMEM with 2% FBS), and the compounds were added at the same time. After 48 h incubation, the cells were lysed and assayed with a Luciferase assay Kit (E4550, Invitrogen) using a turner biosystem instrument luminometer with a measurement time of 1s and a delay time of 2s.

Cell toxicity assay: Cell toxicity was examined by MTT assay. Cells were cultured in 96-well plates

(10^4 cells / well). After 12 h, replace the 10% FBS DMEM media with the 2% one. Then the cells were treated with complexes. After incubation of cells for up to 48 h, 20μL MTT solution (5mg/mL) was added to each well. The cells were incubated for another 4 h, the mixtures containing the medium and MTT were removed and the formazan crystals were dissolved in 150 μL DMSO/well. The absorbance of each well at 490 nm was determined by analysis with a micro plate Spectrophotometer, The percent growth inhibitory rate of treated cells was calculated by $(A_{tested} - A_{media\ control})/(A_{drug\text{-}free\ control} - A_{media\ control}) \times 100\%$, where A is the mean value calculated using the data from three replicate tests.

RNA Isolation and Quantitative RT-PCR: After cells were incubated with compounds (10 μM) or DMSO (1‰ final DMSO concentration) for 48 h (2% FBS DMEM, 5% CO_2). The media was removed, and total RNA was extract using TRIzol Regent (Invitrogen) according to the manufacturer's instruction. For analysis of mature miRNA, quantitative RT-PCR was carried out using TaqMan miRNA probes (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. Briefly, 1μg of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (TaKaRa; Dalian, China) and a stem-loop RT primer (Applied Biosystems). The procedure is 16 °C, 30 min; 42 °C, 30 min; 85 °C, 5 min. Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Real-Time PCR System. The procedure is 95 °C, 5 min; followed by 40 cycles of 95 °C, 15 s; 60 °C, 60 s. After the reaction, the level of miRNA was determined using threshold cycles (C_T) and copy number. The data were then normalized to the DMSO control. For quantitative RT-PCR analysis of pre-miRNA or some gene, 1μg total RNA was reverse to cDNA with oligo (dT) and thermoscript, Quantitative Real-Time PCR using Eva green dye (Invitrogen). The sequence of the sense and antisense primers were as follows: pre-miR-1 (5'-AAACATACTTCTTTATATGCCCA-3', 5'-TACATACTTCTTTACATTCCATAGC-3'); pre-miR-122 (5'-GGAGTGTGACAATGGTGTTT G-3', 5'-TTTAGTGTGATAATGGCGTTTG-3'); pre-miR-21 (5'-GCTTATCAGACTGATGTTGA CTG-3', 5'-CAGCCCATCGACTGGTG-3'); pre-miR-214 (5'-CCTGGCTGGACAGAGTGG-3', 5'-TACAGGTGAGCGGATGTT-3'); pre-miR-25 (5'-TGAGAGGCGGAGACTTGG-3', 5'-TCAGAC CGAGACAAGTGCAA-3'); pre-miR-26b (5'-TTCAAGTAATCCAGGATAGGCTGT-3', 5'-CAAG TAATGGAGAACAGGCTG-3'); pre-miR-34a (5'-TGGCAGTGTCTTAGCTGGTTG-3', 5'-GGCAG TATACTTGCTGATTGCTT-3') pre-miR-126 (5'-TATTACTTTTGGTACGCGCTG-3',5'-GCGCAT TATTACTCACGGTAC-3')

Western blotting: Samples of cultured cells after treatment with compound were lysed in a buffer

containing cell lysis buffer (Biocam), 1×protease inhibitor (Complete, Roche) and 1×PMSF (Phenylmethanesulfonyl fluoride, Amresco) . The cell extract was used in western blot analysis with anti-Ago2, anti-Dicer, anti-TRBP anti-GAPDH and α-tubulin monoclonal antibody. ***Immunoprecipitation Analysis:*** Immunoprecipitation assays were performed according to the manufacturer's instructions. After treatment with compound for 48 h, Cells were resuspended in IP buffer (20 mM Tri-HCl (PH=7.5), 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 0.5 mM dithiothreitol (DTT), 1 mM NaF, 1 × protease inhibitor and 1 × PMSF). Mouse monoclonal anti-AGO2 antibody (2 μg) was used to immunoprecipitate RNA-binding proteins. After purification, immunoprecipitated RNA was analyzed by quantitative RT-PCR using TaqMan miRNA probes.

Scheme 1. Photoreactions leading to compound **3-5** in the compound block.

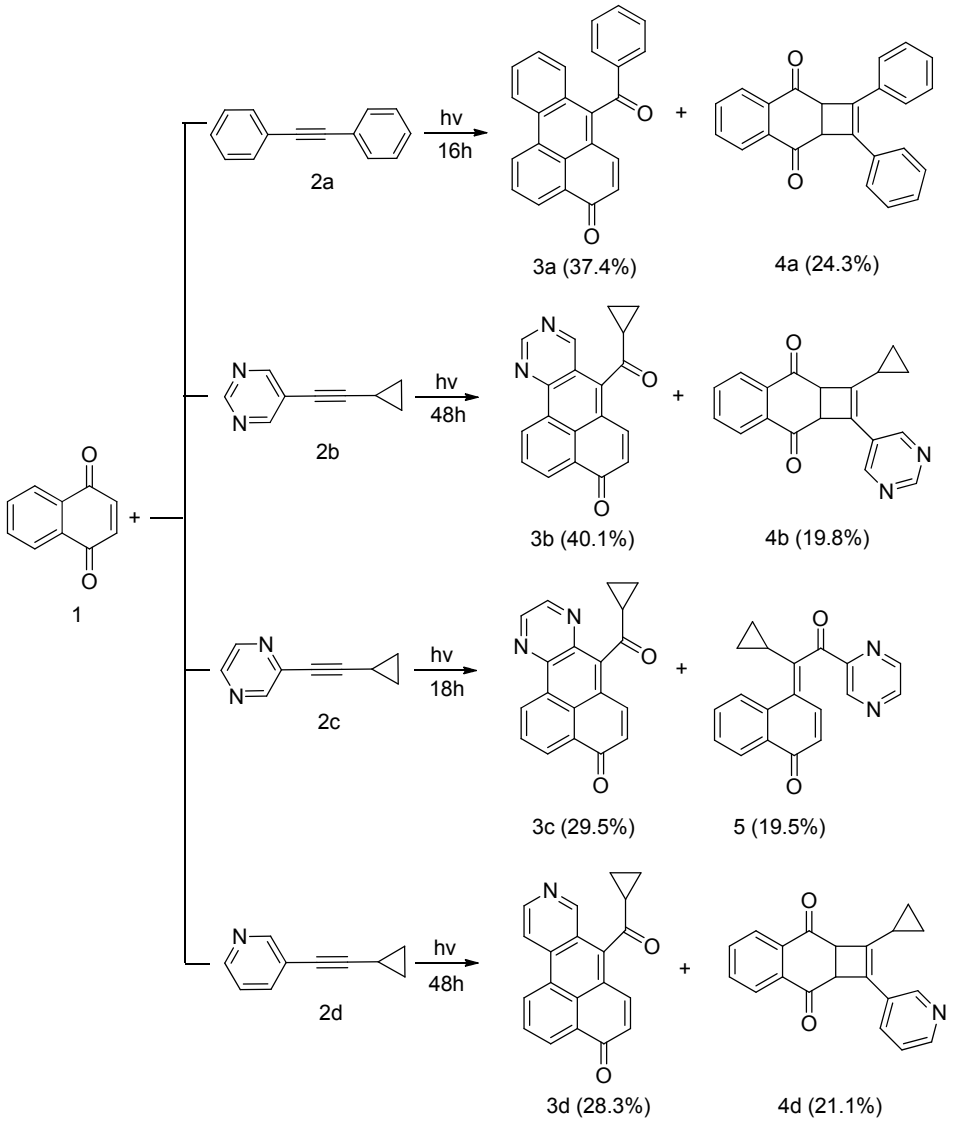


Figure 1. MTT assay on the cytotoxicity of **3a** in C2C12, HepG2 and MCF-7 cell. Cells were incubated with **3a** (10, 20 and 30μM) for 48 h, then the cell viability was tested by MTT assay. Values are mean ± s.d. for triplicate samples after normalization to DMSO control. Compound **3a** showed no obvious toxicity at concentrations under 10μM. Due to solubility limit, cytotoxicity of **3a** at concentrations higher than 30μM was not measured.

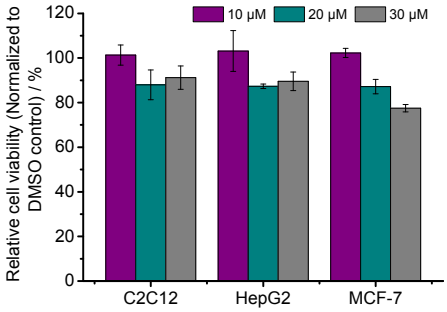


Table 1. Cellular assay results on **3-5** with representative structure in the compound block. The concentration of the compounds used to treat the cells was the highest concentration under which the compound could dissolve well and showed no obvious cytotoxicity on cells. Relative luciferase level on C2C12 cells transfected with luciferase reporter for miR-1 and empty vector, relative luciferase level on HepG2 cells transfected with luciferase reporter for miR-122 and empty vector.

Compound	Concn. (μM)	Relative Fluc.		Relative Fluc.	
		empty vector	miR-1	empty vector	miR-122
3b	3	0.64 ± 0.04	0.62 ± 0.04	0.94 ± 0.01	0.87 ± 0.07
3c	1	0.97 ± 0.08	1.18 ± 0.07	0.90 ± 0.06	0.86 ± 0.04
3d	5	0.53 ± 0.08	0.47 ± 0.11	1.04 ± 0.18	1.05 ± 0.19
4a	10	2.11 ± 0.22	2.03 ± 0.21	1.01 ± 0.21	1.00 ± 0.11
4b	10	1.48 ± 0.06	1.75 ± 0.24	2.19 ± 0.26	1.80 ± 0.26
4d	10	2.0 ± 0.01	1.83 ± 0.36	0.99 ± 0.12	1.15 ± 0.14
5	5	1.30 ± 0.11	1.24 ± 0.15	1.17 ± 0.23	1.27 ± 0.01

Figure 2. Dose dependence of the regulation activity on miR-214/25/126/34a of **3a** (Determined by luciferase assay on MCF-7 reporter cells).

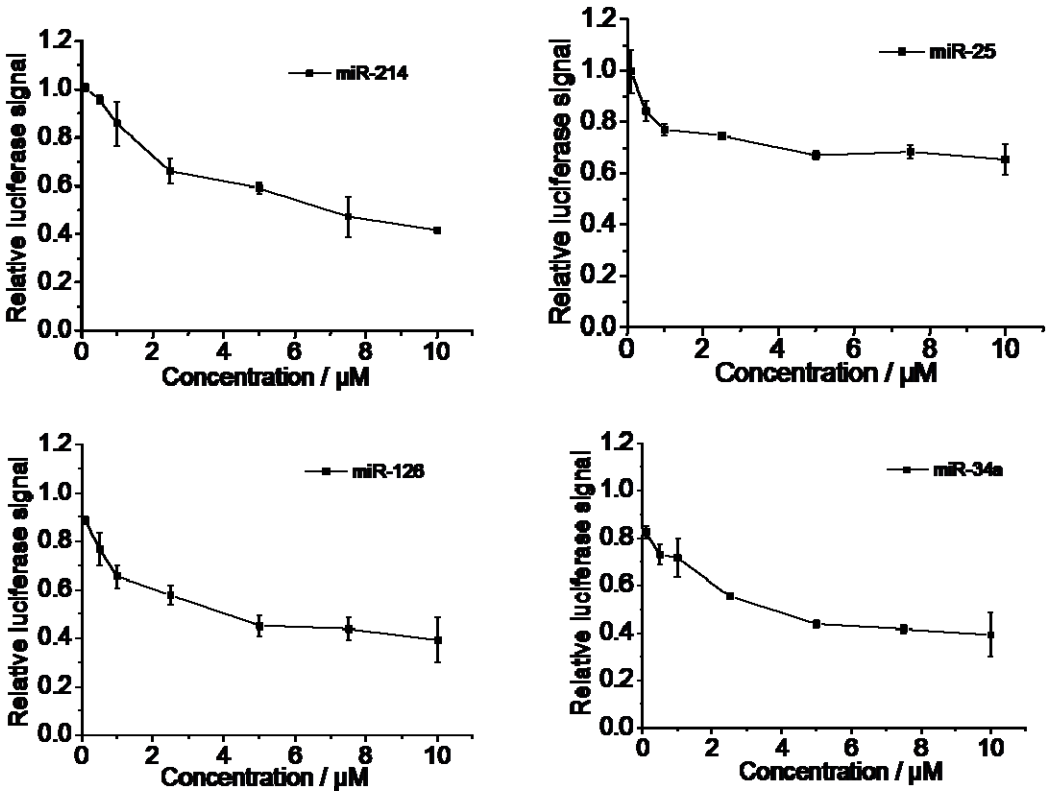


Figure 3. Cellular Ago2 expression level and amount of miRNAs associated with Ago2 upon treatment of compound **3a** (10μM). (A): Western blot assay was used to detect the Ago2 protein level after treatment with **3a** in MCF-7 cell. (B): The quantification of the Ago2 protein expression in (A). (C): IP assay was used to detect the miRNA level after treatment with **3a**. Values are mean ± s.d. for triplicate samples. The results indicatd that **3a** showed no effect on Ago2 protein expression and did not change the amount of Ago2-associated miRNAs.

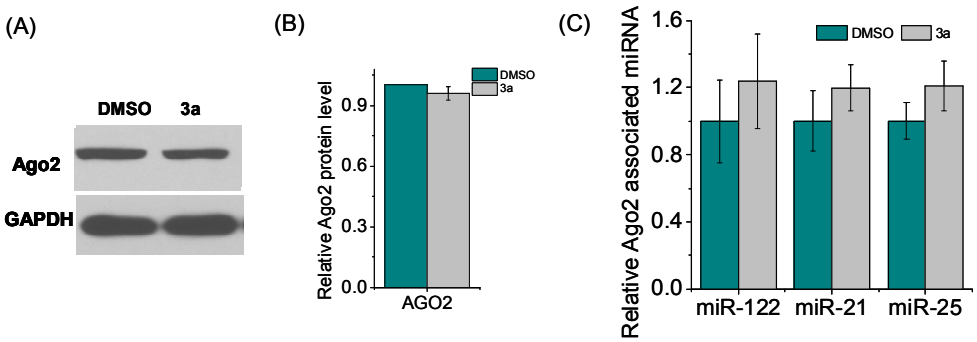
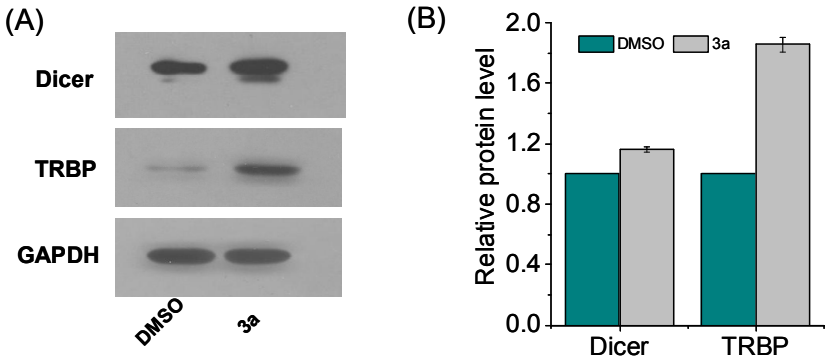


Figure 4. Cellular expression level of Dicer and TRBP with or without treatment of compound **3a** (10μM). (A) Western blot assay was used to test the protein level of Dicer and TRBP in MCF-7 cell after treatment with **3a**. (B) The quantification of the protein leve in (B). Values are mean ± s.d. for triplicate samples. Compound **3a** showed no influence on Dicer expression, but significant upregulation on TRBP protein level was observed in cells treated with **3a**.



The preparation of 3-5 with representative structure and compound characterization data

Photolysis of 1 with 2a. A solution of **1** (158 mg, 1 mmol) and **2a** (356 mg, 2 mmol) in benzene (100 mL) was irradiated with light of wavelength >400 nm under Ar atmosphere for 16 h to reach a 64.1% conversion of **1** Solvent was removed under reduced pressure, the residue was separated by Flash column chromatograph on silica gel (petroleum ether/ethyl acetate) to give pure analytic samples of **3a** (37.4%) and **4a** (24.3%).

7-benzoyl-*4H*-benzo[*de*]anthracen-4-one (3a): yellow crystal; m.p.: 208 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.05 (d, *J* = 7.9 Hz, 1H), 8.80 (d, *J* = 8.5 Hz, 1H), 8.70 (dd, *J* = 7.5, 0.7 Hz, 1H), 7.98 (t, *J* = 7.9 Hz, 1H), 7.92 (d, *J* = 7.4 Hz, 2H), 7.82–7.76 (m, 1H), 7.72–7.54 (m, 4H), 7.48 (t, *J* = 7.7 Hz, 2H), 6.69 (d, *J* = 10.0 Hz, 1H)；^{13}C NMR (75 MHz, $CDCl_3$): δ 198.2, 185.1, 141.9, 138.3, 137.4, 134.8, 131.3, 130.3, 130.1 (2C), 129.8, 129.7, 129.5, 129.2 (2C), 129.1, 128.9, 128.4, 128.1(2C), 127.9, 126.5, 123.4, 123.1; MS m/z (EI): 334 (100), 306 (27), 305 (38), 257 (38), 201 (29), 200 (46), 105 (41), 77(35)**;** Anal. Calcd. for: $C_{24}H_{14}O_2$: C 86.21, H 4.22; Found: C 86.04, H 4.32%.

1,2-diphenylcyclobuta[b]naphthalene-3,8(*2aH,8aH*)-dione (4a): pale yellow crystal; m.p.: 170–171 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 8.04–8.00 (m, 2H),7.74–7.71 (m, 2H), 7.63–7.60 (m, 4H), 7.36–7.30 (m, 6H), 4.48 (s, 2H)；^{13}C NMR (75 MHz, $CDCl_3$): δ 195.7 (2C), 140.9, 134.5 (3C), 134.1, 133.2, 129.0 (3C), 128.6 (4C), 127.6 (3C), 127.0 (4C), 50.7 (2C); MS m/z (EI): 336 (100), 308 (34), 307 (89), 231 (22). 202 (27), 178 (66)**;** Anal. Calcd. for: $C_{24}H_{16}O_2$: C 85.69, H 4.79; Found: C 85.75, H 4.94%.

Photolysis of 1 with 2b. A solution of **1** (158 mg, 1 mmol) and **2b** (288 mg, 2 mmol) in benzene (100 mL) was irradiated with light of wavelength >400 nm under Ar atmosphere for 48 h to reach a 55.4% conversion of **1** Solvent was removed under reduced pressure, the residue was separated by Flash column chromatograph on silica gel (petroleum ether/ethyl acetate) to give pure analytic samples of **3b** (40.1%) and **4b** (19.8%).

7-(cyclopropanecarbonyl)-*4H*-naphtho[*1,β-gh*]quinazolin-4-one (3b): pale yellow crystal; m.p.: 235–236 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.60–9.56 (m, 2H), 9.41 (s, 1H), 8.79 (dd, *J* =7.4, 1.6 Hz, 1H), 8.03 (td, *J* = 7.7, 1.9 Hz, 1H), 7.87 (dd, *J* = 10.1, 1.2 Hz 1H), 6.84 (dd, *J* =10.1, 1.2 Hz, 1H), 2.53–2.45 (m, 1H), 1.69–1.65 (m, 2H), 1.42–1.36 (m, 2H)； ^{13}C NMR (75 MHz, $CDCl_3$): δ 205.2, 184.2, 157.6, 157.1, 151.0, 140.6, 136.9, 132.5, 131.2, 130.8, 129.7, 129.6, 129.1, 128.8, 123.7, 119.9, 25.7, 15.1 (2C); MS m/z (EI): 300 (100), 272 (35), 259 (78). 231 (48), 204 (67), 176 (37); Anal. Calcd. for: $C_{19}H_{12}N_2O_2$: C 75.99, H 4.03, N 9.33%; found: C 76.78, H 4.11, N 9.14%.

1-cyclopropyl-2-(pyrimidin-5-yl)cyclobuta[b]naphthalene-3,8(*2aH,8aH*)-dione (4b): brown crystal; m.p.: 134–135 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.01 (s, 1H), 8.90 (s, 2H), 8.01–7.94 (m, 2H), 7.75–7.67 (m, 2H), 4.29 (d, *J* = 3.8 Hz, 1H), 3.99 (d, *J* = 3.8 Hz, 1H), 1.80–1.73 (m, 1H), 1.08–0.76 (m, 4H)； ^{13}C NMR (75 MHz, $CDCl_3$): δ 195.4, 194.8, 157.0, 154.2 (2C), 150.6, 134.8, 134.7, 134.0, 133.8, 133.7, 127.8, 127.5, 127.3, 50.7, 48.2, 12.5, 7.0, 6.5; MS m/z (EI): 302 (64), 274 (22), 273 (25), 259 (44), 159 (58), 144 (100), 76 (23); Anal. Calcd. for: $C_{19}H_{14}N_2O_2$: C 75.48, H 4.67, N 9.27%; found: C 75.61, H 4.64, N 9.24%.

Photolysis of 1 with 2c. A solution of **1** (158 mg, 1 mmol) and **2c** (288 mg, 2 mmol) in benzene (100 mL) was irradiated with light of wavelength >400 nm under Ar atmosphere for 48 h to reach a 45.2% conversion of **1** Solvent was removed under reduced pressure, the residue was separated by Flash column chromatograph on silica gel (petroleum ether/ethyl acetate) to give pure analytic samples of **3c** (29.5%) and **5** (19.5%).

7-(cyclopropanecarbonyl)-4H-naphtho[1,8-fg]quinoxalin-4-one (3c): pale yellow crystal; m.p.: 246–247 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.52 (dd, *J* = 8.1, 0.9 Hz, 1H), 9.00 (d, *J* = 3.2 Hz, 2H), 8.77 (dd, *J* = 7.4, 0.9 Hz, 1H), 8.04 (t, *J* = 7.8 Hz, 1H), 7.82 (d, *J* = 10.1 Hz, 1H), 6.86 (d, *J* = 10.1 Hz, 1H), 2.52–2.44 (m, 1H), 1.64–1.55 (m, 2H), 1.30–1.23 (m, 2H)； ^{13}C NMR (75 MHz, $CDCl_3$): δ 205.9, 184.6, 145.3, 145.2, 143.2, 142.3, 140.3, 137.5, 131.4, 131.1, 130.4, 129.7, 129.6, 128.8, 127.9, 125.8, 25.3, 13.4 (2C); MS m/z (EI): 272 (100), 244 (37), 231 (28), 176 (29); Anal. Calcd. for: $C_{19}H_{12}N_2O_2$: C 75.99, H 4.03, N 9.33%; found: C 76.16, H 3.81, N 9.17%.

4-(1-cyclopropyl-2-oxo-2-(pyrimidin-2-yl)ethylidene)naphthalen-1(4H)-one (5): brown crystal; m.p.: 132–133 ˚C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.37 (s, 1H), 8.81 (d, *J* = 2.2 Hz, 1H), 8.67–8.66 (m, 1H), 8.56 (d, *J* = 8.1 Hz,

1H), 8.30 (d, *J* = 7.7 Hz, 1H), 7.68(td, *J* = 7.6, 1.3 Hz, 1H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.13 (d, *J* = 10.3 Hz, 1H), 6.30 (d, *J* = 10.2 Hz, 1H), 2.40–2.30 (m, 1H), 1.18–1.11 (m, 2H), 0.80–0.74 (m, 2H)；^{13}C NMR (75 MHz, $CDCl_3$): δ 196.4, 184.4, 151.5, 148.4, 147.9, 144.8, 144.6, 144.3, 140.5, 132.6, 131.4, 130.5, 128.8, 128.2, 127.1, 126.6, 15.6, 10.4 (2C); MS m/z (EI): 302 (76), 273 (58), 195 (100), 167 (35), 165 (86), 152 (64), 139 (23), 79 (31); Anal. Calcd. for: $C_{19}H_{14}N_2O_2$: C 75.48, H 4.67, N 9.27%; found: C 75.31, H 4.47, N 9.04%.

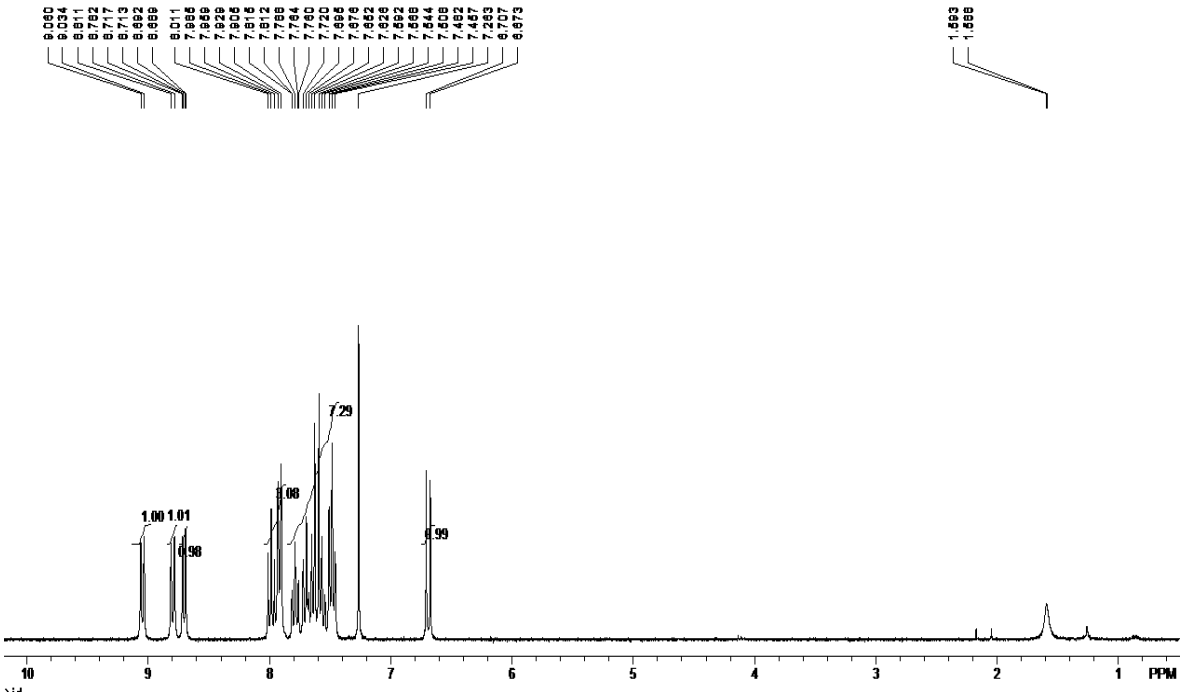
Photolysis of 1 with 2d. A solution of **1** (158 mg, 1 mmol) and **2d** (286 mg, 2 mmol) in benzene (100 mL) was irradiated with light of wavelength >400 nm under Ar atmosphere for 48 h to reach a 78.9% conversion of **1** Solvent was removed under reduced pressure, the residue was separated by Flash column chromatograph on silica gel (petroleum ether/ethyl acetate) to give pure analytic samples of **3d** (28.3%) and **4d** (21.1%).

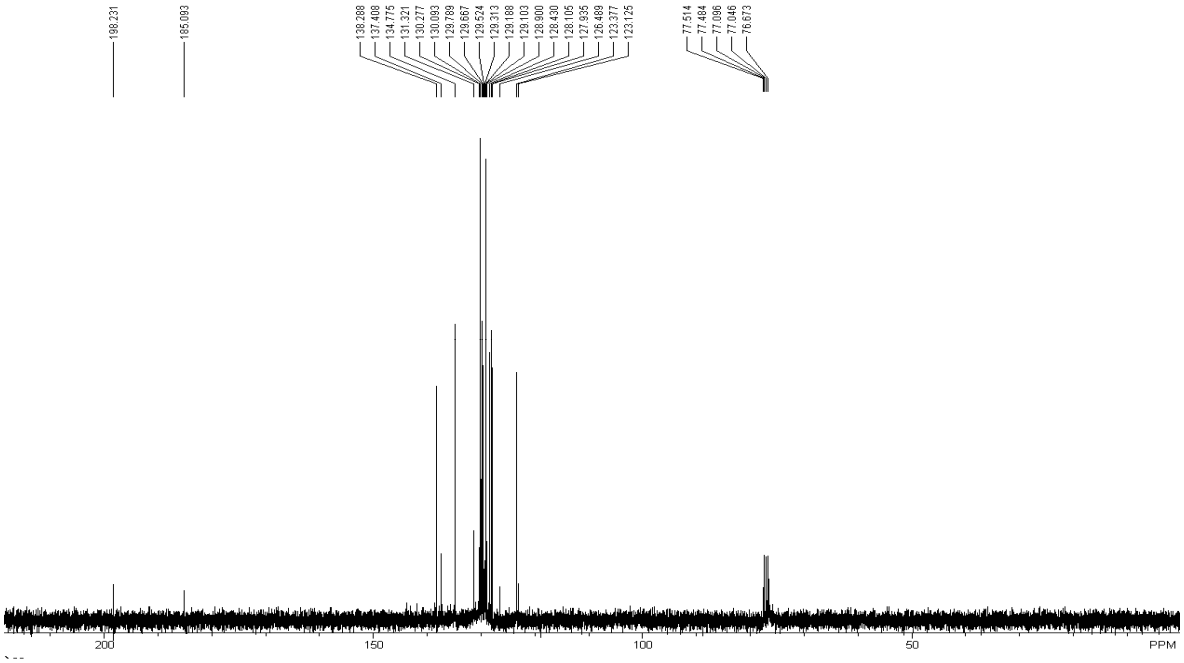
7-(cyclopropanecarbonyl)-4H-naphtho[1,8-fg]isoquinolin-4-one (3d): pale yellow crystal; m.p.: 184–185 °C; ^{1}H NMR (300MHz, $CDCl_3$): δ 9.65 (d, *J* = 8.1 Hz, 1H), 9.10 (d, *J* = 3.0 Hz, 1H), 8.75 (d, *J* = 7.4 Hz, 1H), 8.23 (d, *J* = 8.4 Hz, 1H), 8.02 (t, *J* = 7.8 Hz, 1H), 7.85 (d, *J* = 10.1 Hz, 1H), 7.63 (dd, *J* = 8.3, 4.4 Hz, 1H), 6.84 (d, *J* = 10.7 Hz, 1H), 2.51–2.43 (m, 1H) 1.64–1.61 (m, 2H), 1.38–1.32 (m, 2H)；^{13}C NMR (75 MHz, $CDCl_3$): δ 206.8, 184.9, 151.2, 147.2, 142.8, 137.6, 134.6, 130.8, 130.6(2C), 130.5, 129.7, 128.5, 128.0, 122.9, 122.6, 122.5, 25.4, 14.5 (2C); MS m/z (EI): 299 (100), 271 (92), 230 (85), 202 (48), 201 (22), 175 (23); Anal. Calcd. for: $C_{20}H_{13}NO_2$: C 80.25, H 4.38, N 4.68%; found: C 82.42, H 4.54, N 4.84%.

1-cyclopropyl-2-(pyridin-3-yl)cyclobuta[b]naphthalene-3,8(2aH,8aH)-dione (4d): brown crystal; m.p.: 154–155 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.86 (s, 1H), 8.47 (d, *J* = 4.6 Hz, 1H), 8.04 (dd, *J* = 7.3, 1.4 Hz, 1H), 8.00–7.96 (m, 1H), 7.89–7.85 (m, 1H), 7.80–7.69 (m, 2H), 7.31–7.26 (m, 1H), 4.31 (d, *J* = 3.8 Hz, 1H), 3.99 (d, *J* = 3.8 Hz, 1H), 1.89–1.80 (m, 1H), 1.07–0.75(m, 4H)；^{13}C NMR (75 MHz, $CDCl_3$): δ 196.1, 195.5, 148.5, 147.9, 147.7, 139.2, 137.4, 134.6, 134.5, 133.8, 133.4, 129.1, 127.7, 127.4, 123.4, 50.2, 48.5, 12.2, 6.5, 6.2; MS m/z (EI): 301 (76), 272 (45), 258 (36), 244 (23), 159 (31), 143 (100), 142 (34), 76 (29); Anal. Calcd. for: $C_{20}H_{15}NO_2$: C 79.72, H 5.02, N 4.65%; found: C 80.02, H 4.88, N 4.75%.

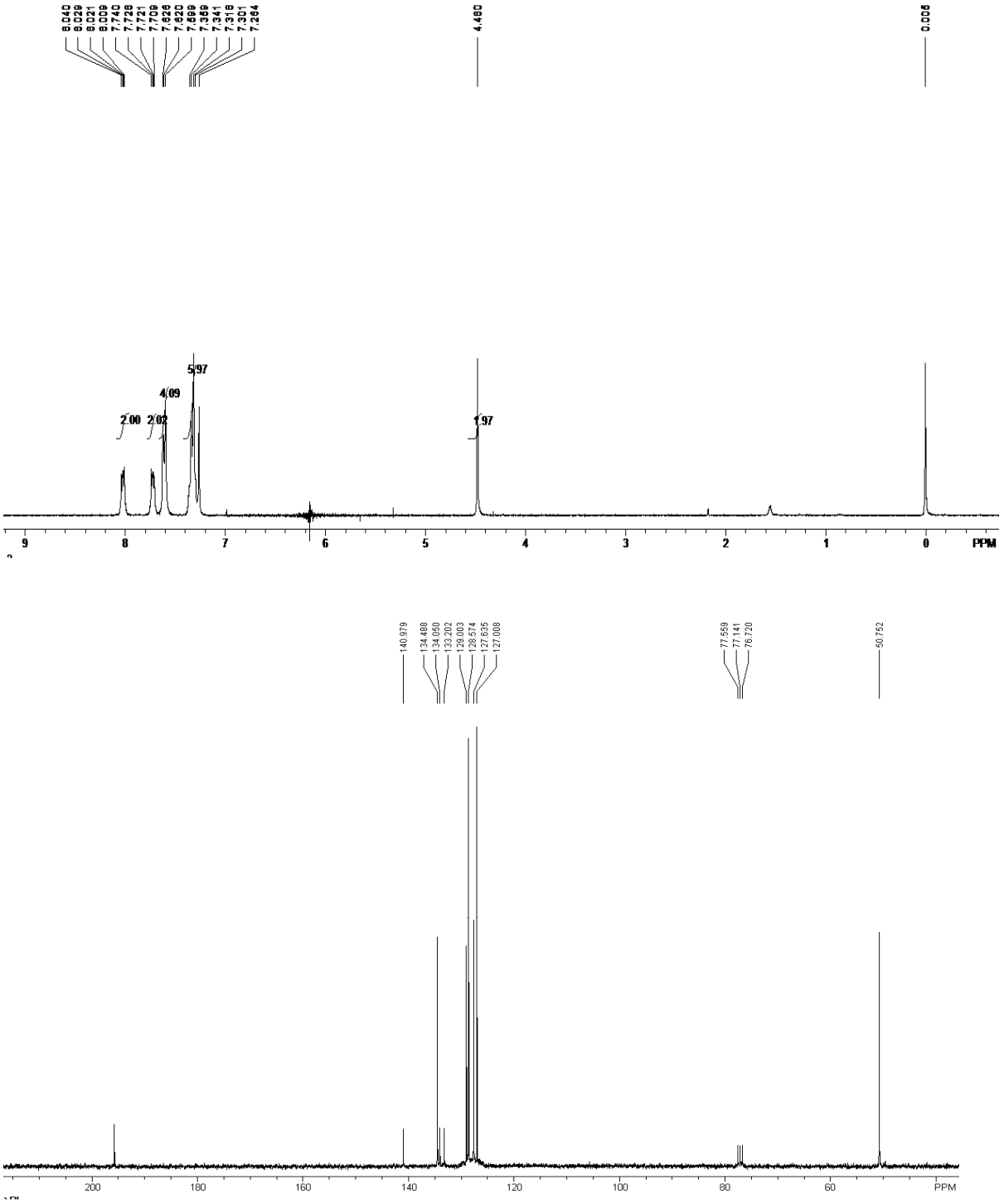
Copies of ^{1}H NMR and ^{13}C NMR spectra

^{1}H NMR and ^{13}C NMR of **3a**:





^{1}H NMR and ^{13}C NMR of **4a**:



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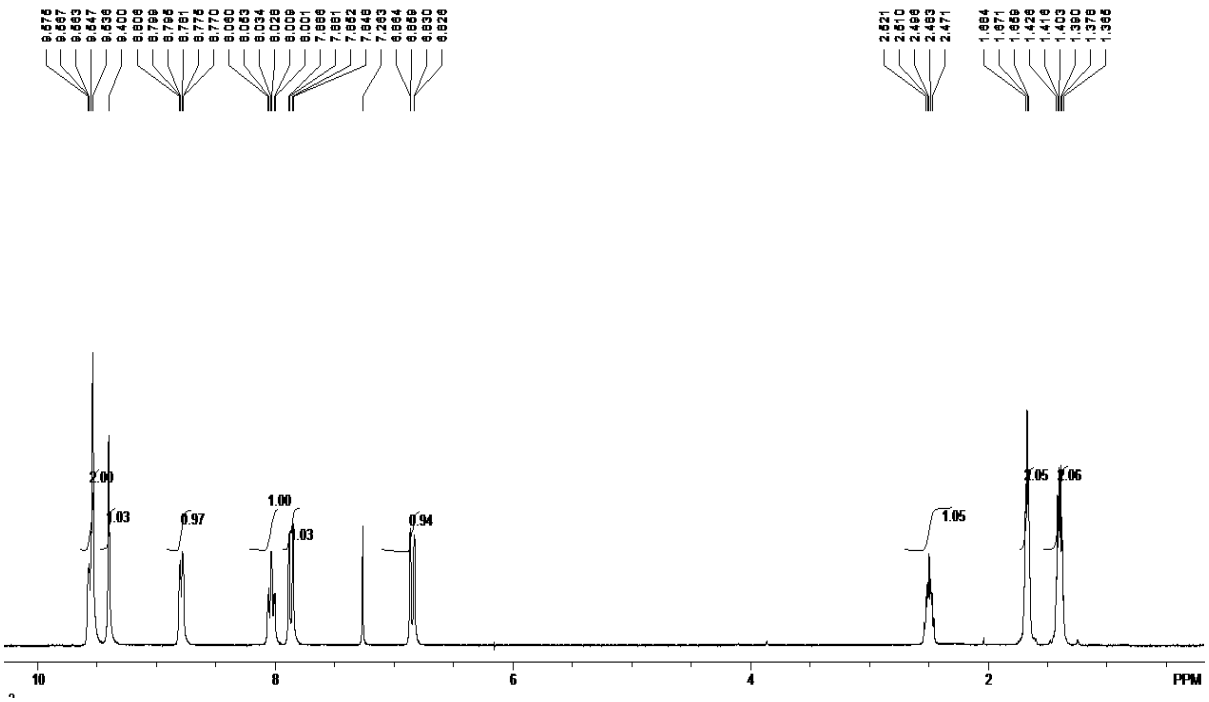
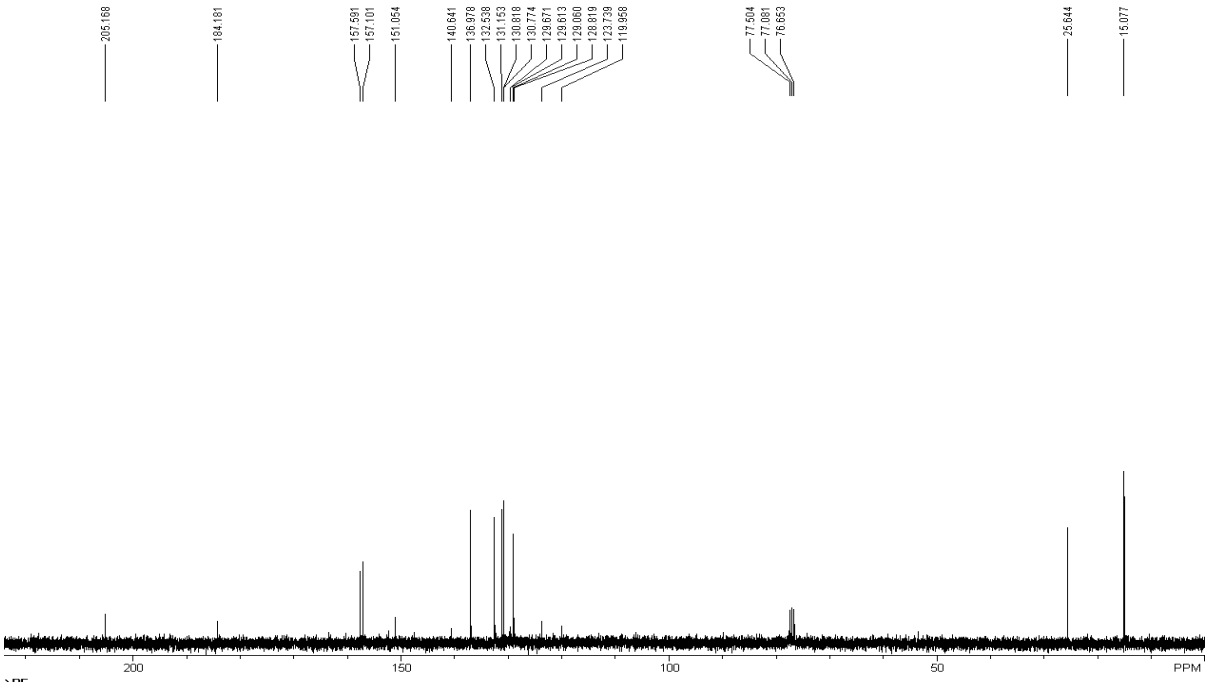
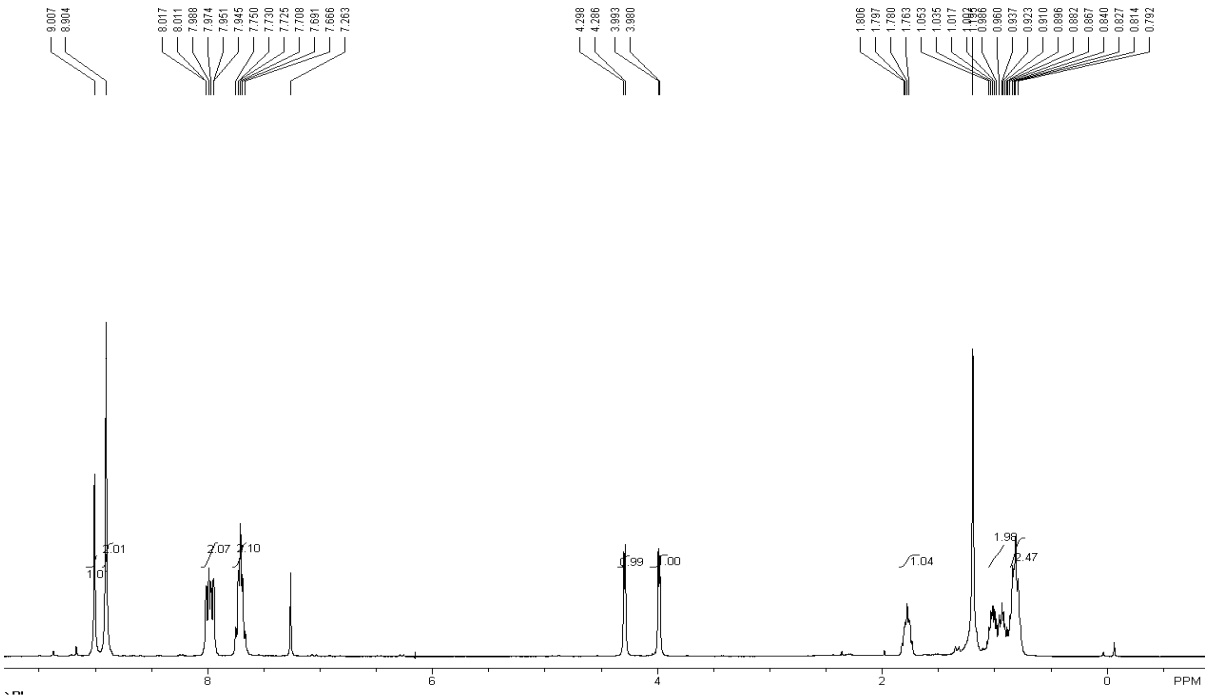
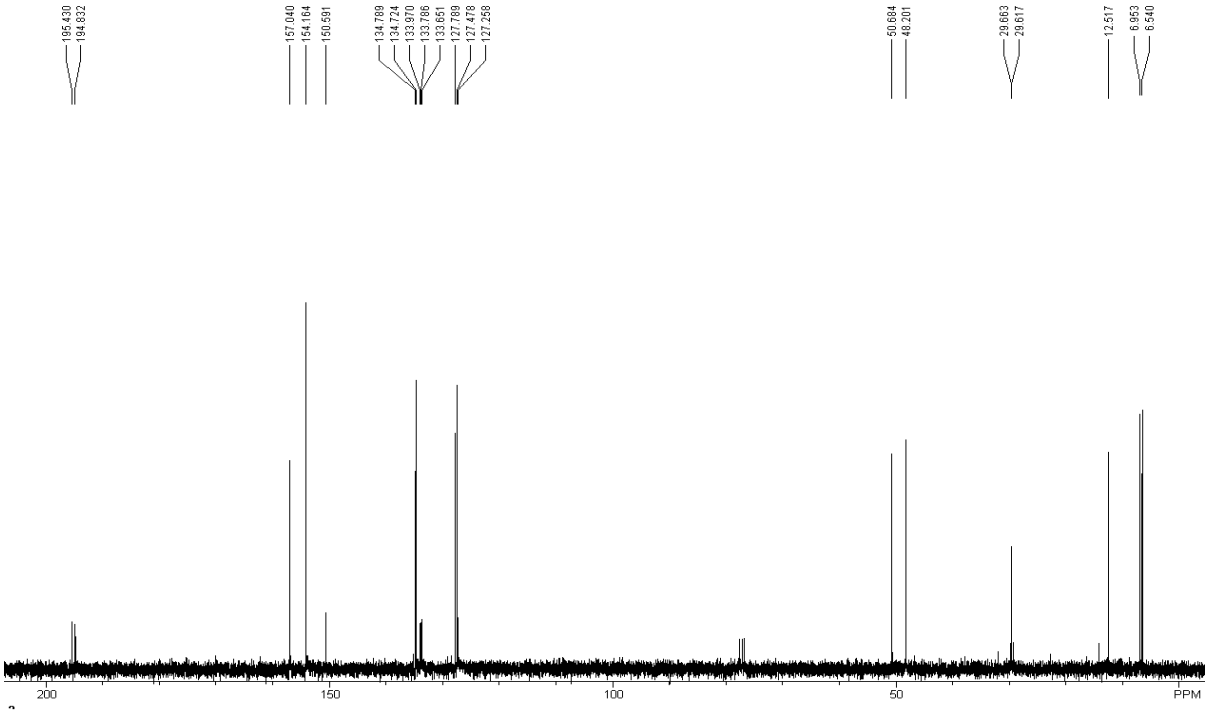


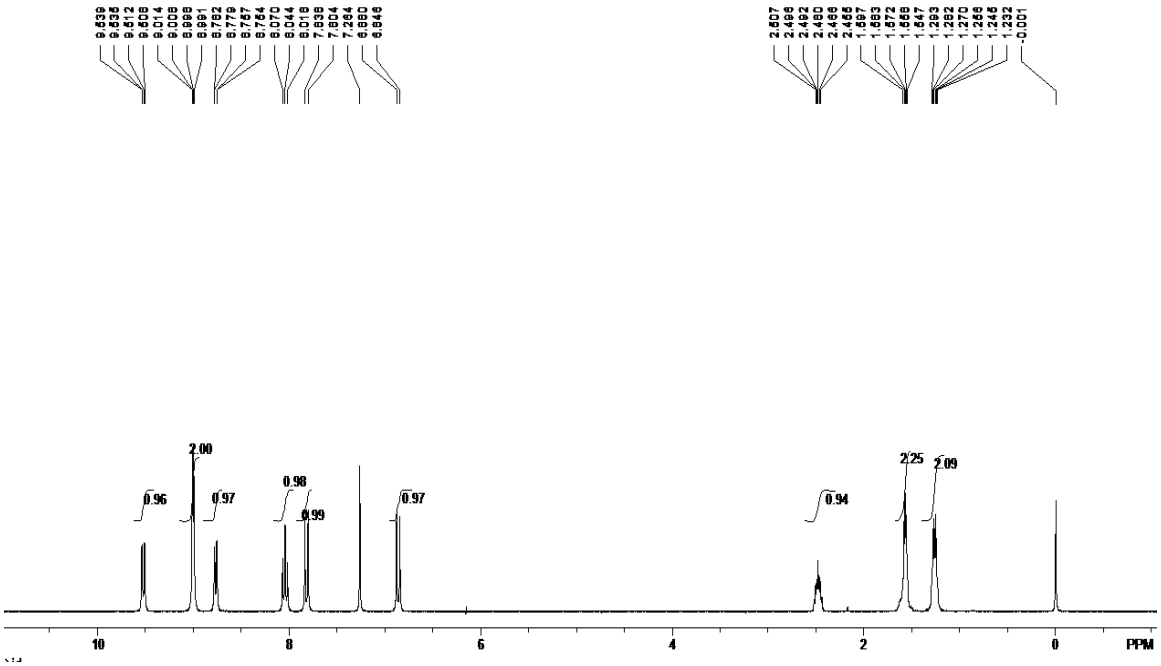


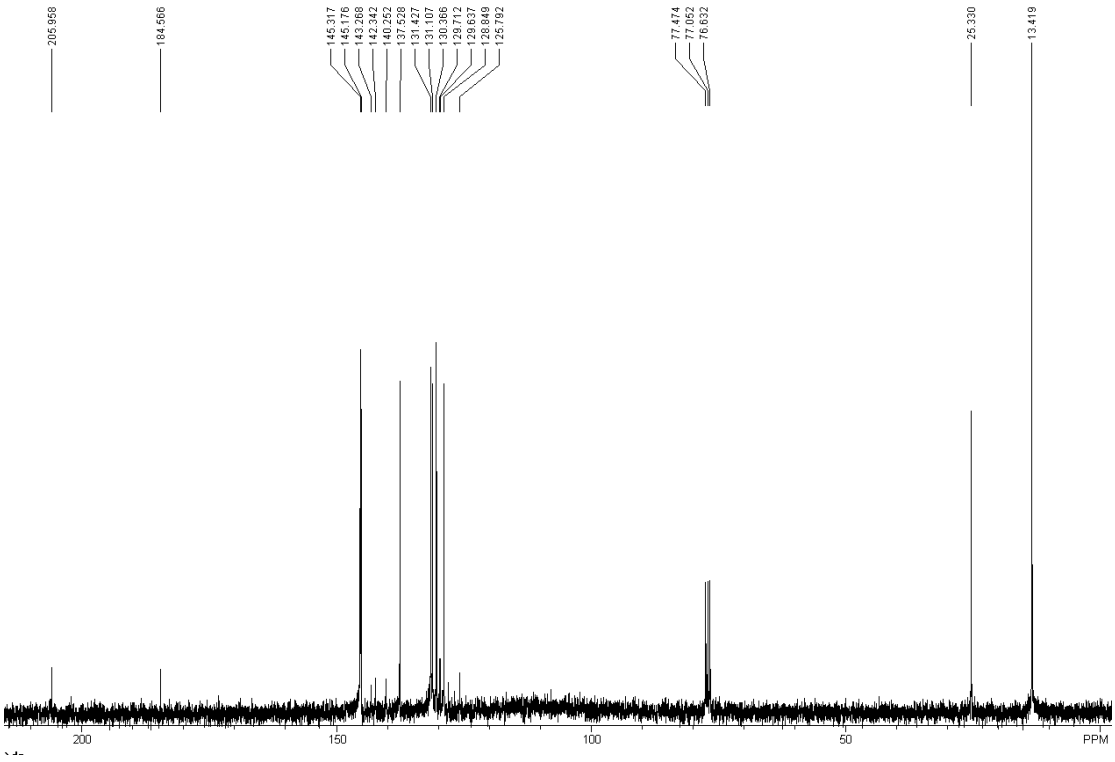
^{1}H NMR and ^{13}C NMR of **4b**:



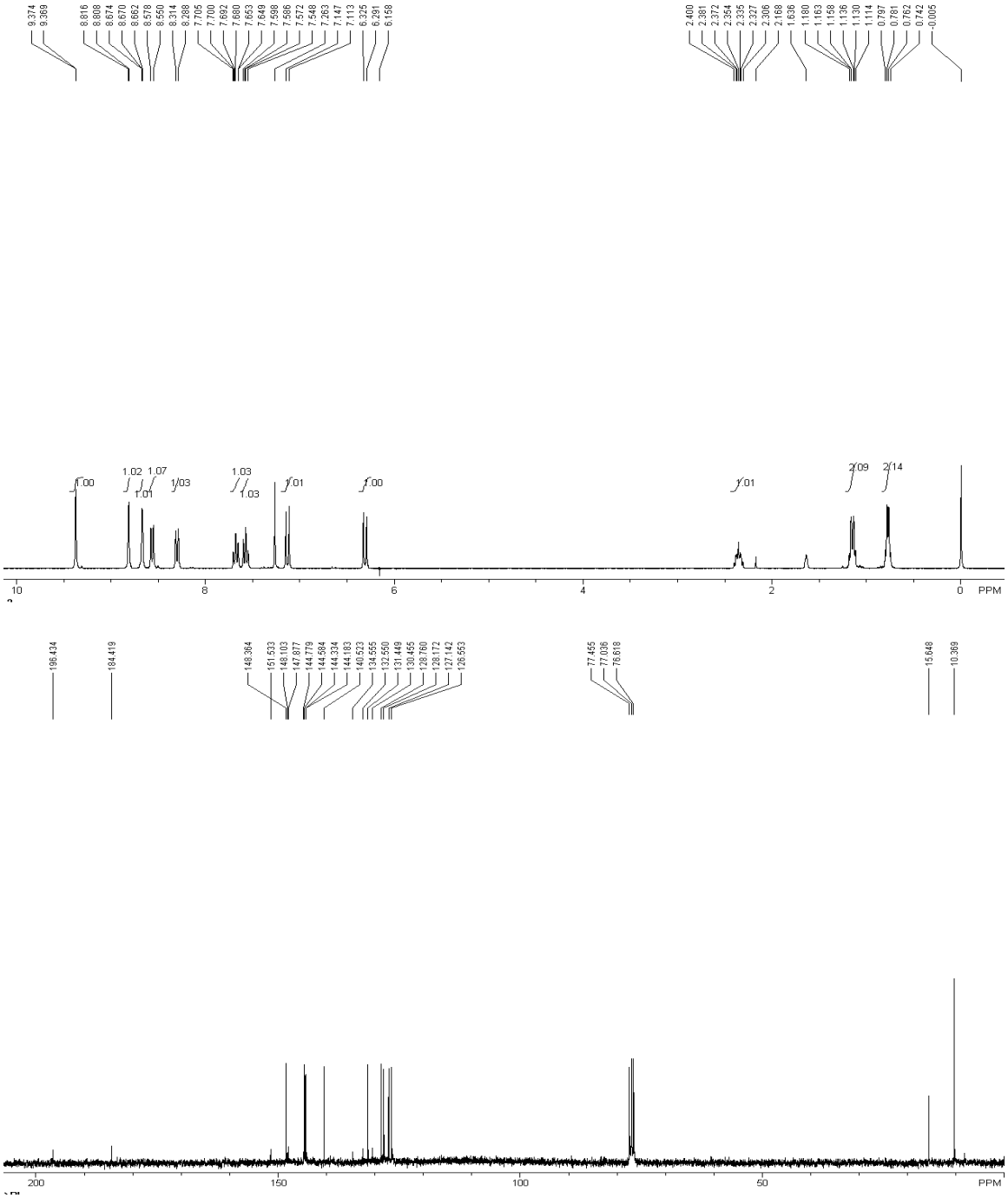


^{1}H NMR and ^{13}C NMR of **3c**:

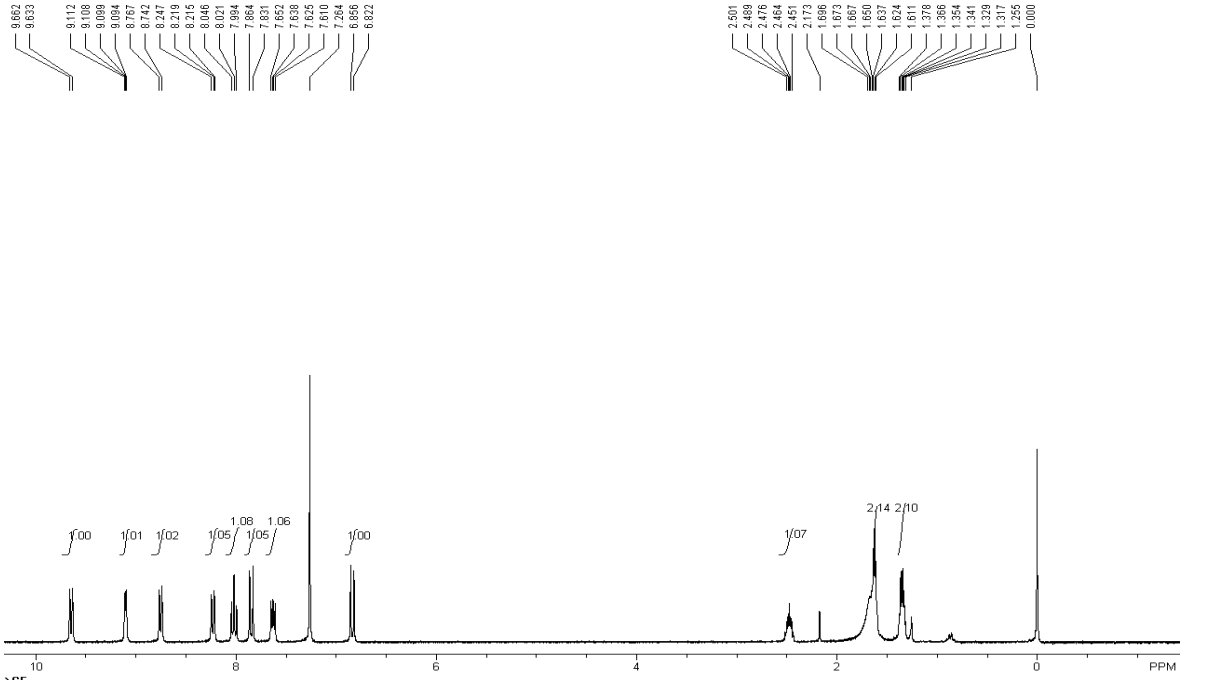


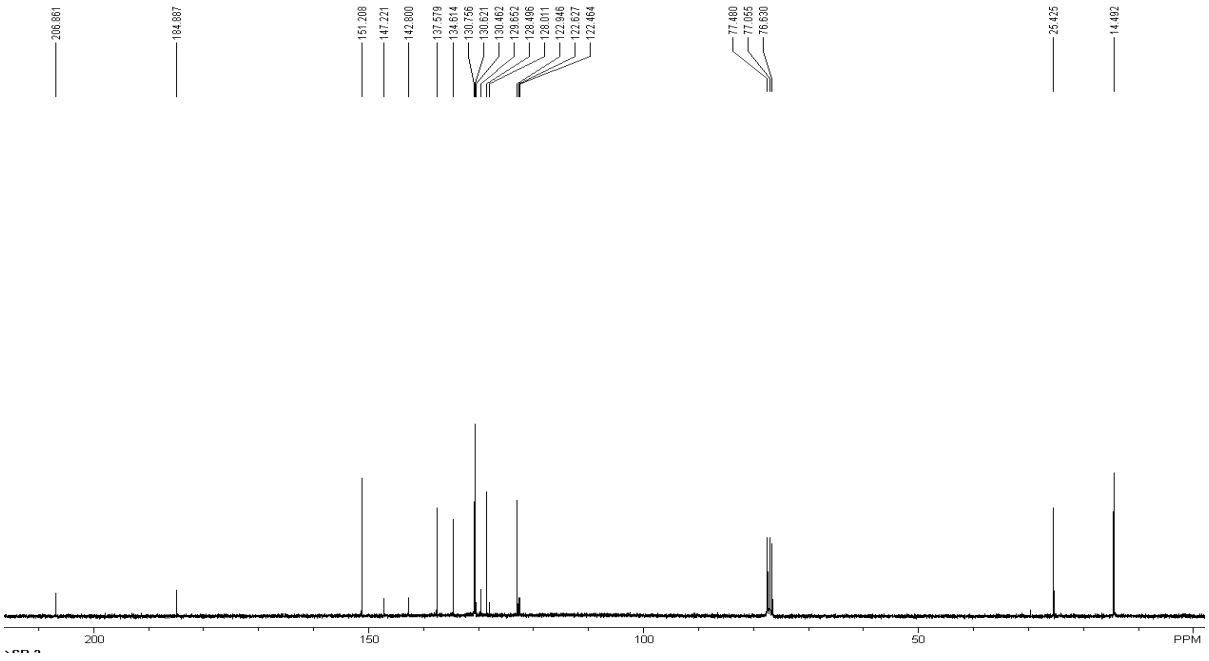


^{1}H NMR and ^{13}C NMR of **5**:



^{1}H NMR and ^{13}C NMR of **3d**:





^{1}H NMR and ^{13}C NMR of **4d**: