Supplementary Information

Intramolecular Catalysis of Hydrazone Formation of Aryl-Aldehydes via ortho-Phosphate Proton Exchange

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1. Reagents

The molecules 7-hydrazino-4-methyl coumarin (CH), the salicylaldehyde hydrazone of CH, and 3-formyltyrosine (fY) were prepared as described in Banerjee *et al.* (2010). Salicylaldehyde (SAL) and other chemical reagents were purchased from Acros or Sigma-Aldrich. The coumarin-based fluorophore (7-diethylaminocoumarin-3-carbohydrazide, DCCH) and Texas-Red Hydrazide were obtained from Invitrogen.

2. Syntheses

^{1}H and ^{13}C NMR spectra were recorded on instruments operating at a frequency of 600 MHz. ^{1}H NMR spectra were referenced to $CDCl_3$ (7.26 ppm). ^{13}C NMR spectra were referenced to the $CDCl_3$ (77.00 ppm). Chemical shift multiplicities are reported as s= singlet, d = doublet, t = triplet, q = quartet and m = multiplet. . Reactions were monitored by thin layer chromatography using TLC plastic sheets, silica gel 60 $F_{254.}$ Column chromatography was performed using Baker silica gel 60-200 mesh or 200-400 mesh. Absorption spectra of all compounds were obtained by using a Hewlett-Packard 8453 diode array absorption

spectrophotometer. Fluorescence emission spectra for these molecules were measured using Spex FluoroMax-3 spectrofluorometer.

2-Formylphenyldihydrogen phosphate (3, SA-P): This procedure[1] is modified from Silverberg *et al*., 1996.

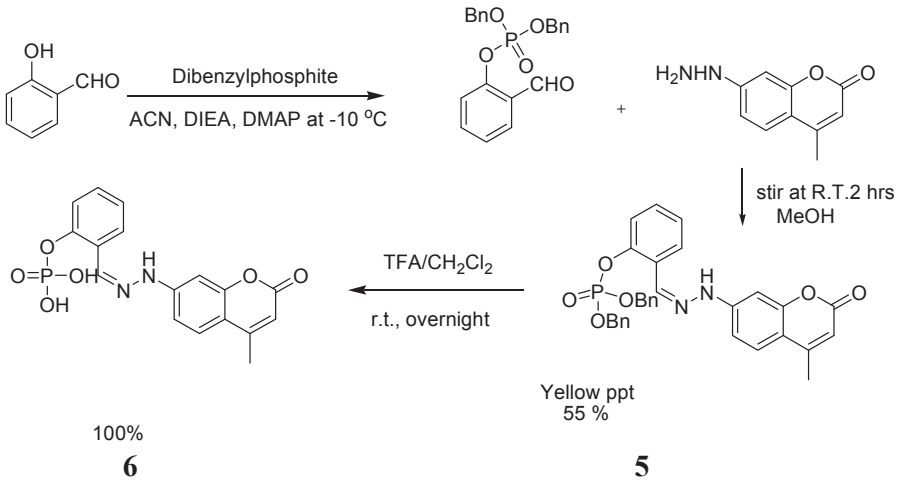
A dried three-neck flask with a stir bar inside was fitted with septa and N_2 (g) was allowed to flow through. After 15 minutes, 1.3 g of salicylaldehyde (10.6 mmol) was added to the setup, along with 35 mL of acetonitrile. The solution was cooled to -10 °C. Next, 0.74 mL (21 mmol) N,N-diisopropylethylamine and 0.024 g (1.06 mmol) N,N dimethylaminopyridine were added. Carbon tetrabromide (3.51 g, 10.6 mmol) was added to the solution, which turned yellow upon the addition. After the contents had been stirred for 2 minutes, benzyl phosphite (3.39 mL, 15.37 mmol) was added dropwise and the temperature of the solution was monitored closely to make sure it did not rise above -10 °C. After an hour (reaction completion was monitored by TLC, 4:1 ethyl acetate: hexane), 0.5 M potassium phosphate solution was added (32 mL KH_2PO_4/100 mL acetonitrile) to quench the reaction. The solution was allowed to warm to room temperature and was extracted three times with ethyl acetate. After removal of the solvent, the solid was purified by column chromatography (same conditions as TLC) to yield 3.26 g (80 %) of the dibenzyl SA-P product.

^{1}H NMR ($CDCl_3$): δ *ppm* 5.17 (s, 2H), 5.18 (s, 2H), 7.26 (t, 1H, J = 7.50 Hz), 7.42 (d, 1H, J = 8.28 Hz), 7.52 (t, 1H, J = 7.68, 8.04 Hz), 7.84 (d, 1H, J = 7.68 Hz), 10.20 (s, 1H).

Deprotection procedure: Dibenzyl SA-P (3 g, 7.82 mmol) was dissolved in 40 mL of methanol along with 0.5 g palladium catalyst. The mixture was allowed to stir as the flask and solution were purged with H_2 (g). The solution was purged three times with an outlet and after this, the outlet was removed and pressure in the flask was allowed to build. The reaction progressed for an hour and was monitored by TLC (4:1 ethyl acetate: hexanes) for completion. After the elapsed time, the solution was filtered to remove the palladium. After

concentration of the solution, it was noted that the product had a dark tint to it, so the compound was redissolved in methanol and passed through celite to remove the excess palladium. After concentration of the solutions, 1.36 g (86 %) of a orange solid of (SA-P, **3**) resulted.

Scheme S1: Synthesis of hydrazone of CH and SA-P



Synthesis of Compound 5: Coumarin hydrazine[2] (100 mg, 0.44 mmol) was added to dibenzyl-SA-P (0.162 g, 0.44 mmol) in methanol. The reaction was stirred at room temperature for 2 hrs. Completion of the reaction was monitored by TLC. The precipitate was filtered and washed with excess methanol and dried. Yellow product was obtained (130 mg, Yield 55%).

^{1}H NMR (d-DMSO): *δ ppm* 2.36 (s, 3H), 5.17 (s, 2H), 7.26 (s, 2H), 5.20 (s, 2H), 6.07 (s, 1H), 6.99-7.05 (m, 2H), 7.25-7.39 (m, 14H), 7.62 (d, 1H), 8.23 (s, 1H), 11.09 (s, 1H).

^{13}C NMR (d-DMSO): *δ ppm* 18.0, 69.6, 97.9, 109.3, 109.5, 111.8, 120.6, 125.6, 125.9, 126.5, 126.6, 127.9, 128.5, 128.6, 129.7, 133.7, 135.5, 135.6, 147.7, 147.8, 148.3, 158.4, 155.1, 160.4.

Synthesis of Compound 6 (deprotection): Compound 5 was added to 1:1 (TFA:CH_2Cl_2) and stirred at room temperature for overnight. Solvent was removed to yield actual product (60 mg, Yield 100%).

^{1}H NMR (d-DMSO): *δ ppm* 2.35 (s, 3H), 6.05 (s, 1H), 6.95-7.06 (m, 2H), 7.14-7.39 (m, 3H), 7.60 (d, 1H), 8.01 (d, 1H), 8.26 (s, 1H), 11.09 (s, 1H). ^{13}C NMR (d-DMSO): *δ ppm* 18.0, 97.8, 109.3, 111.6, 120.9, 124.4, 125.4, 126.5, 126.7, 129.6, 135.1, 148.6, 149.4, 149.5, 153.6, 155.2, 160.5. MS (ESI) M^+ 373.08 found 374.

3. Kinetics of CH with SA-P vs fY:

Kinetics of hydrazone formation were measured by absorption difference spectroscopy at 25 °C in an HP 8453 absorption spectrophotometer or an HP 8452A absorption spectrophotometer equiped with Olis software. Equal volumes of each component were placed in a dual chambered absorption cuvette. The sample was blanked and data collection commenced. The sample was then removed from the instrument, rapidly mixed by inversion, and replaced into the sample holder. Spectra were collected as a function of time until a plateau was reached. Data were fit as a pseudo-first order reaction to a single exponential equation using SigmaPlot.

All CH-fY data were collected in under pseudo-first order conditions with fY in excess (325 μM fY, 32.5 μM CH). Data for CH-SP-P data for pH 6-9 were collected using the same concentrations. At pH <6, the reaction of CH-SA-P was too fast to collect kinetic data under the same conditions. Therefore the concentrations of the solutions were lowered to 17.5 □M CH and 175 μM SA-P for the reactions at pH 4 and 5. Data for the reaction of SA-P and CH at pH 6 and 7 were also collected. The apparent second order rate constants calculated for SA-P and CH at pH 6 and pH 7 at the difference concentrations were the same within experimental error.

Buffers used: 0.1 M sodium phosphate for pH 6-8; 0.1 M sodium citrate, pH 4-5.

Sample absorption difference spectra kinetic data: 17.5 μM CH and 175 μM SA-P

Data collected at 1.7 sec intervals. Spectra shown are every 17 sec for clarity

Figure S1: Absorption difference spectra of the reaction of 17.5 μM **CH and 175** μM **SA-P in 0.1 M sodium phosphate, pH 6.** The spectra were collected at 1.7 sec intervals. For clarity, in this illustration, every 10th spectrum is plotted.

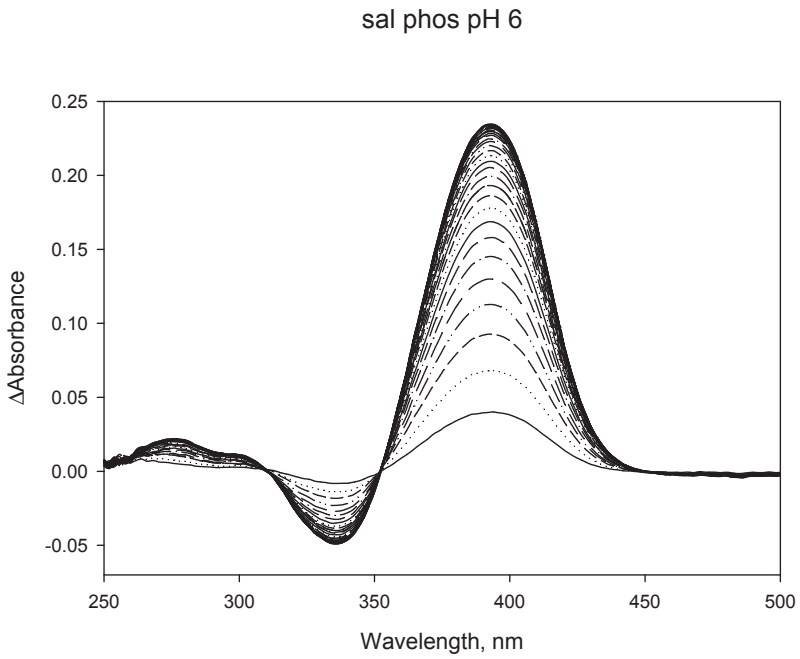
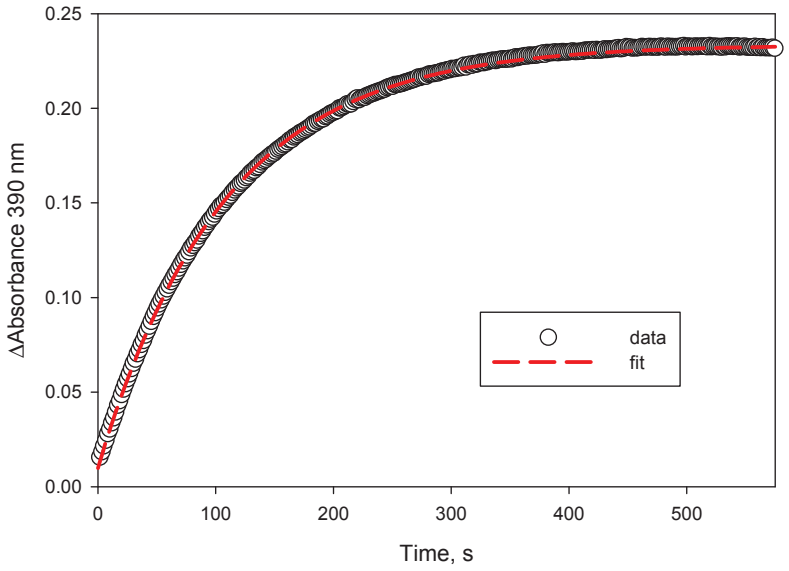
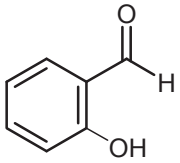


Figure S2: Data from Figure S1 (Absorbance at 390 nm) fit as a single pseudo-first order reaction

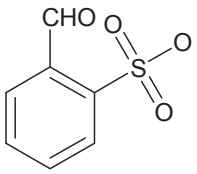


Kinetics for hydrazone formation of model compounds

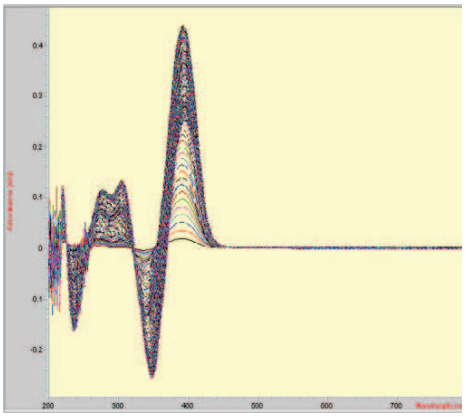
Nucleophile	Rate ($M^{-1}min^{-1}$)		pKa	Electrophile
	pH = 4	pH = 7		
$PhNHNH_2$	786	45	5.3	
$PhCONHNH_2$	52	2	3.0	
$PhCH_2NHNH_2$	3	15	7.0	

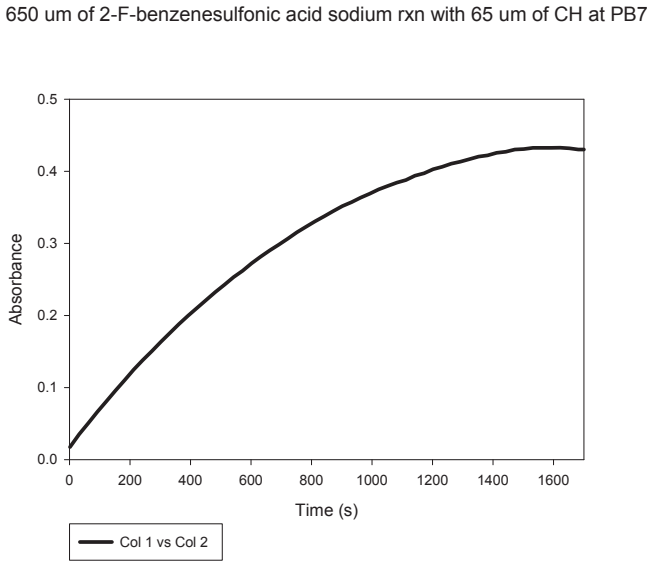
Note: Pseudo First-Order Rate Constants for Hydrazone Formation Between 3f-Tyr (3-formyl-Tyrosine) and some other hydrazine-containing probes (including with DCCH) were also studied and discussed in detail (Blanden et al. *Bioconjug. Chem*. **2011**)[3]

Kinetics of 650 µM of 2-formylbenzenesulfonic acid with 65 µM of Coumarin hydrazine (CH)



2-formylbenzenesulfonic acid



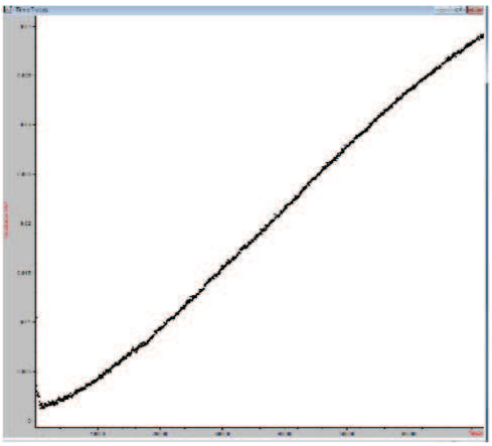


Kinetics of 650 µM of SAL-phosphate (SA-P)/Salicylaldehyde (SAL) with 65 µM of Texas Red Hydrazide in PME7 at RT.

Hydrazide/Aldehyde Couple	k_2 ($M^{-1}min^{-1}$)
	pH = 7
Texas Red Hydrazide with SAL	very slow (2 days)
Texas Red Hydrazide with SA-P	31

Kinetics of 450 µM of Benzaldehyde/ with 45 µM of Coumarin Hydrazide 7 in PBS 7 at RT.

Hydrazide/Aldehyde Couple	k_2 ($M^{-1}min^{-1}$)
	pH = 7
Coumarin hydrazide 7 with benzaldehyde	very slow (2 days)



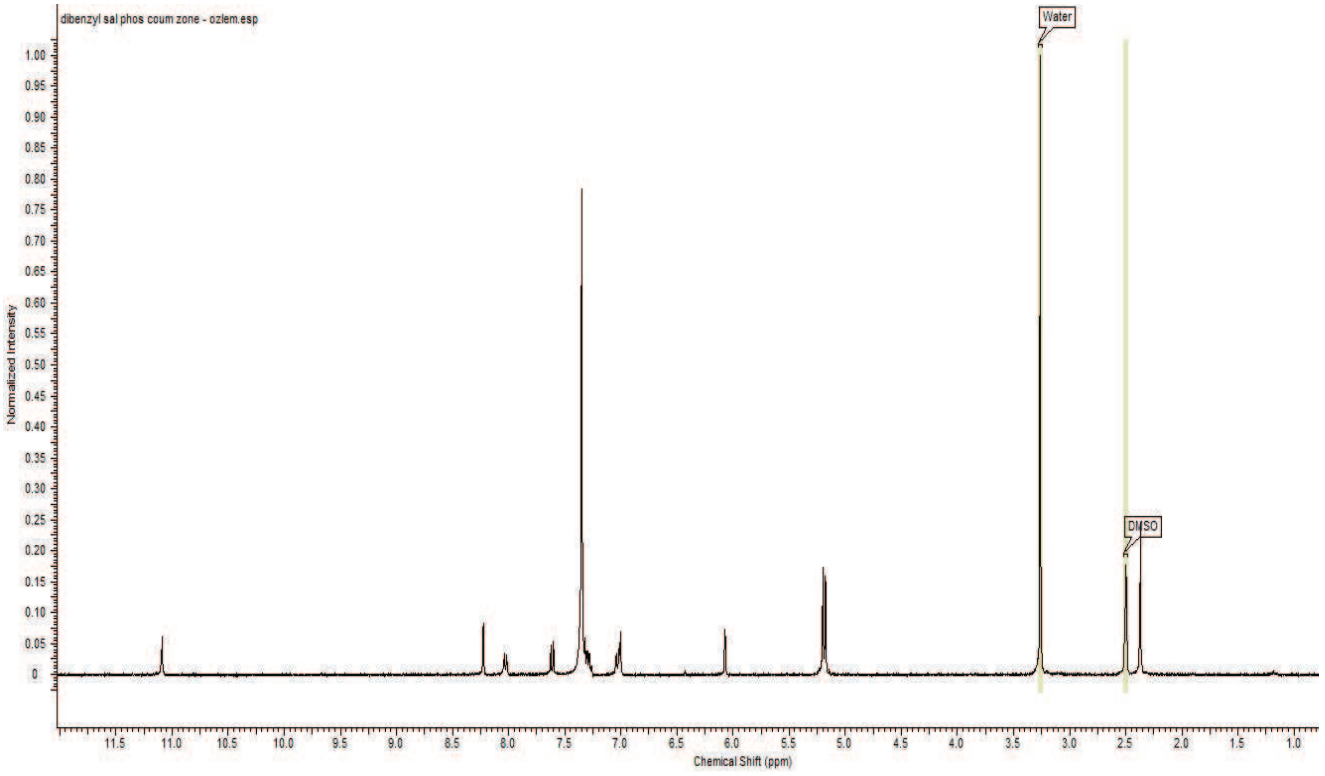
Difference spectrum of the coumarin hydrazide 7 reaction with benzaldehyde.

References:

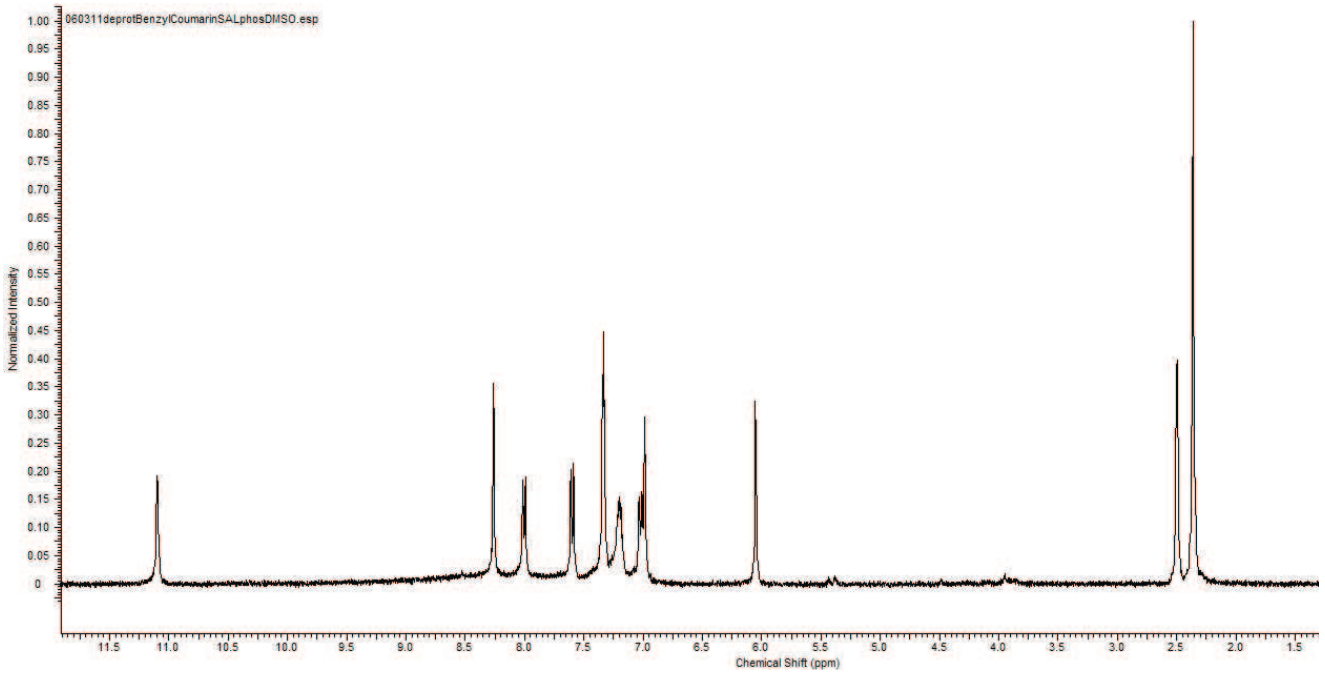
1. Silverberg, J.L.; Dillon, L.J.; Vemishetti, P., A simple, rapid and efficient protocol for the selective phosphorylation of phenols with dibenzyl phosphite. *Tet. Lett.* **1996**, 37 (6), 771-774.
2. Banerjee, A.; Panosian, T. D.; Mukherjee, K.; Ravindra, R.; Gal, S.; Sackett, D. L.; Bane, S., Site-specific orthogonal labeling of the carboxy terminus of alpha-tubulin. *ACS Chem. Biol.* **2010,** *5* (8), 777-785.
3. Blanden AR, Mukherjee K, Dilek O, Bane SL. *Bioconj. Chem*. **2011**, 22, 1954.

NMR Spectra:

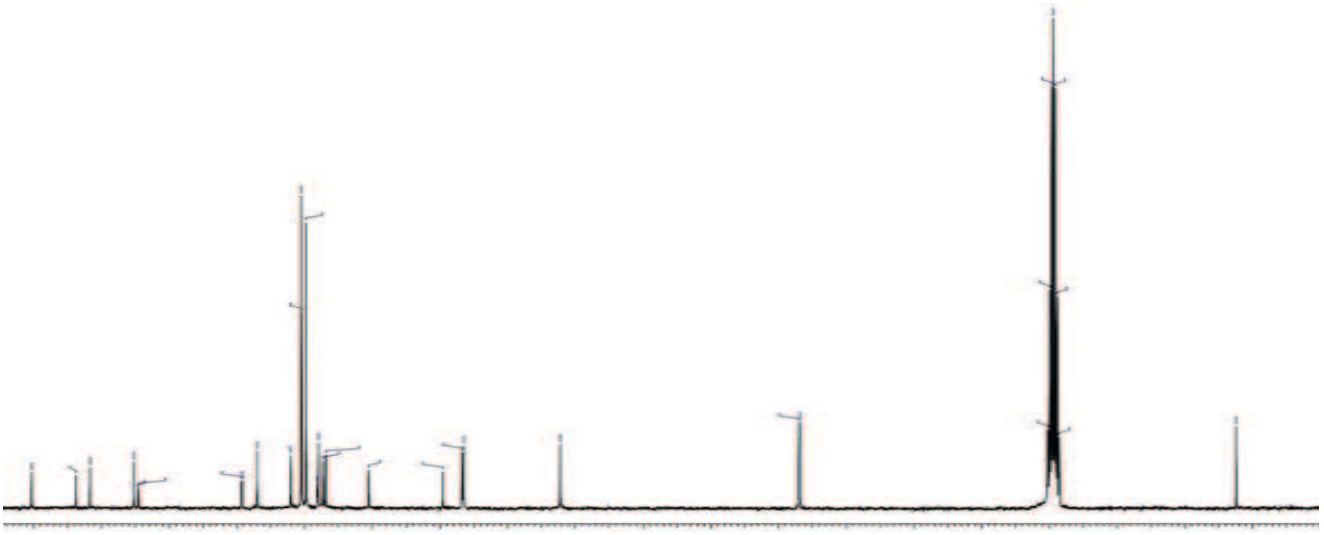
^{1}H –NMR Spectrum of Compound **5**



^{1}H –NMR Spectrum of Compound **6**



^{13}C –NMR Spectrum of Compound **5**



^{13}C –NMR Spectrum of Compound **6**