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Synthesis, *in vitro* inhibition effect of novel phthalocyanine complexes as carbonic anhydrase and paraoxonase enzyme inhibitors

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Abstract

The preparation and assessment of carbonic anhydrase and paraoxonase enzyme inhibition properties of 3-(2-(5-amino-4-(4-bromophenyl)-3-methyl-1H-pyrazol-1-yl)ethoxy)phthalonitrile (**2**) and its nitrogen-containing non-peripherally phthalocyanine derivatives (**3** and **4**) are reported for the first time. The new phthalonitrile and its phthalocyanine derivatives have been elucidated by FT-IR spectroscopy, ^{1}H-NMR, ^{13}C-NMR, mass and UV–vis spectroscopy. The results demonstrated that all synthesized compounds moderately inhibited carbonic anhydrase and paraoxonase enzymes. Among the compounds, the most active ones were found to be compound **4** for PON (Ki : 0.14 μM), compound 3 for hCA I (Ki : 22.52 μM) and compound 1 for hCA II (Ki : 13.62 μM).

Keywords: Phthalocyanine; Metal complex; Aminopyrazole; Carbonic anhydrase; Paraoxonase; Inhibitor

1. Introduction

Calcium-dependent enzyme paraoxonase is important for the metabolism of living things. The paraoxonase enzyme hydrolyzes aromatic carboxyl esters such as phenylacetate and naturally occurring lactone metabolites and is involved in drug metabolism. Therefore, by playing an important role in detoxification of artificial substrates, an individual can significantly alter its sensitivity to the toxicity of chemicals. hPON1 also acts as an antioxidant enzyme that is an in vivo bioscavenger [1]. Some in vitro studies have shown that PON1 can inhibit low-density lipoprotein (LDL) and high-density lipoprotein (HDL) oxidation, and low levels of oxidized lipids play a role in the onset of atherosclerosis [2]. Also, paraoxonases are members of the family of mammalian enzymes having aryldialkylphosphatase activity. The enzymatic activity of paraoxonases is very different from an organophosphate activity. By investigating this family of enzymes, there is an interest in studying genes and potential ethnic variations encoding these enzymes. In particular, research on the inhibition and selective inhibition of PON1 is crucial to shed some light on the links between reductions in the enzymatic activity of individuals with cardiovascular diseases. Evidence also shows that this family of enzymes has some role in our innate immune system. The isozyme CA I is found in many biological tissues, but the study by Gao et al [3] has shown that isozyme CA I is involved in retinal and cerebral edema, and its inhibition may be an important tool for fighting these pathologies. The CA-II is involved in several diseases, such as glaucoma, edema, epilepsy, and probably altitude sickness [4]. Many studies of carbonic anhydrase and the increase in knowledge of the therapeutic effects of this enzyme family have led to the synthesis of novel inhibitors.

Phthalocyanine (Pc) complexes which are an important family of macrocycles have many uses such as catalysts [5, 6], gas sensors [7], electrochromic devices [8], solar cells [9-11], Langmuir Blodgett films [12], liquid crystals [13], photosensitizers [14-18] and enzyme inhibitors [19, 20]. In photodynamic therapy (PDT), phthalocyanine-based complexes play a central role in the treatment of cancer by sensitizing tumor tissue to light destruction [21]. PDT is preferred to provide improved methods for the targeted tissues. In the PDT method, phthalocyanines are the most important class of photosensitizers that destroy tumor tissue without the need for surgical intervention. Especially the use of phthalocyanines as an enzyme inhibitor has attracted attention in recent years [19, 20, 22].

Among the azole compounds, pyrazole derivatives have become a crucial class in heterocyclic chemistry, especially owing to their recently valuable application. Some investigations on pyrazole derivatives has shown that pharmacologic agents containing these compounds are very important in medical chemistry [23-25]. The applications of these pyrazole derivatives in the field of chemistry

and biology have become important due to their acceptable biological and antimicrobial activities [26, 27]. In particular, due to the important pharmaceutical effects of the aminopyrazole derivatives, extensive research has been carried out to develop specific synthetic pathways for these compounds. Especially, comprehensive researches have been carried out to develop exclusive synthetic pathways for aminopyrazole derivatives due to their important pharmaceutical effects. Also, fused ring of pyridine and pyrazole gave rise to the pyrazolopyridine scaffold, which was demonstrated to exert various biological activities such as antimicrobial, anticancer, and kinase inhibitory properties [28, 29]. In particular, compounds containing the aminopyrazole group are known to be effective as an antioxidant, antitumor agent and enzyme inhibitor [25, 30, 31]. By considering these features, it has focused on the synthesis of soluble phthalocyanines substituted with aminopyrazole moiety and investigating their carbonic anhydrase and paraoxanase inhibition properties. Generally, carbonic anhydrase isoenzymes are inhibited by metal complex anions, sulfonamides, phenols and polyamines [32]. Thus, in this study, we have investigated the inhibition potential of Pcs containing amino groups against hCA I, II and PON1 isoenzymes. To the best of our knowledge, there are few studies in the literature where phthalocyanine compounds are used as paraoxonase and carbonic anhydrase enzyme inhibitors [19]. However, Arslan et al. investigated inhibition properties of axially substituted silicon phthalocyanine derivatives against hCA I and II [20]. Carbonic anhydrase and paraoxonase inhibition properties of non-peripheral chloromanganese and cobalt phthalocyanines have not been reported in the literature and this is the first investigation on non-peripherally substituted phthalocyanine complexes bearing aminopyrazole moiety as potential paraoxonase enzyme inhibitors.

2. Results and Discussion

2.1. Synthesis and characterization

Scheme 1 exhibits the synthetic pathway of phthalonitrile precursor (**2**) and its peripherally aminopyrazole-substituted chloromanganese, and cobalt phthalocyanine complexes (**3** and **4**) starting from aminopyrazole compound (**1**).

<Scheme 1>

Firstly, aminopyrazole derivative (**1**) was synthesized according to the previous study [19]. Then, compound **1** was stirred with 3-nitrophthalonitrile and anhydrous K_2CO_3 to obtain corresponding α-

substituted phthalonitrile derivative (**2**). Finally, manganese and cobalt complexes (**3** and **4**) were synthesized by utilizing corresponding metal salts in the presence of DBU catalyst.

All new complexes were elucidated by utilizing UV–Vis, FT-IR and MS spectroscopic data, all of which were coherent with the recommended structures. In the FT-IR spectrum of compound **2**; primary NH_2, aromatic C-H, aliphatic C-H, aromatic C=N, aromatic C=C, C−O−C and p-substitute benzene stretching vibrations observed at 3435–3363, 3095, 2963, 1618, 1580, 1287 and 809 cm^{-1}, respectively (Fig. S10). Also, two C≡N stretching vibrations appeared at 2242 and 2227 cm^{-1}. These nitrile stretching vibrations were not observed in the FT-IR spectra of compounds **3** and **4**. This is the strongest confirmation for the formation of phthalocyanines by cyclotetramerization of **2**. Additionally, typical vibration peaks correlate with amine groups (−NH_2) at 3538–3544 cm^{-1}, aliphatic alkyl groups (−CH) at 2872-2956 cm^{-1}, alkene groups (−C=C) at 1588–1592 cm^{-1} were distinctly observed for compounds **3** and **4** which are well-suited with the structure (Fig. S11, S12).

In the ^{1}H NMR spectrum of compound **2** (Fig. S13), 4-substituted benzene protons were observed as two doublets at 7.53 ppm (*J*=8.3 Hz) and 7.16 ppm (*J*=8.2 Hz) integrating two protons for each. Aromatic protons of benzene including nitrile groups appeared as a triplet at 7.66 ppm (*J*=8.2 Hz) and two doublets at 7.38 ppm (*J*=7.7 Hz) and 7.23 ppm (*J*=8.7 Hz) integrating one proton for each. One singlet was observed for OCH_2CH_2N protons at 4.48 ppm integrating four protons. Moreover, −NH_2 and −CH_3 protons appeared at 4.00 and 2.19 ppm as singlets, integrating for two and three protons, respectively. The ^{13}C NMR spectrum of compound **2**, having thirteen different aromatic and two nitrile carbon resonances observed at 160.95, 146.39, 143.80, 135.17, 132.52, 132.32, 130.42, 126.07, 120.14, 117.10, 116.65(x2), 115.23, 113.82, 104.92 ppm, supports the predicted structure. Also, OCH_2CH_2N and methyl carbons showed at 69.61, 46.57 and 13.28 ppm, respectively (Fig. S14). Additionally, two-dimensional ^{1}H-^{1}H COSY spectrum of compound **2** was given in Fig. 1 which also supports the proposed structure**.** ^{1}H-NMR spectrum of **3** and **4** were precluded owing to their paramagnetic nature [33].

<Fig. 1>

Mass spectra obtained by the MALDI-TOF technique confirm the proposed structures of the compounds. In addition, the metal complexes obtained were found to be sufficiently stable under MALDI-MS conditions without significant disintegration. The molecular ion peaks were observed at m/z: 423.38 $[M+H]^+$ for **2**, m/z: 1746.95 $[M\text{-}CI+2H]^+$ for **3**, m/z: 1748.20 $[M]^+$ for **4**.

2.2. Ground state electronic absorption spectra and aggregation studies

Absorption spectroscopy is the first and most widely used method to determine the absorption properties of phthalocyanine complexes. These complexes demonstrate typical electronic spectra with two absorption regions. One absorption occurs in the UV region at 300-350 nm (B band) and the other in the visible region at 600-700 nm (Q-band) [34-35]. As it is expected that the UV-Vis absorption spectra of the green-colored non-peripherally substituted metallo phthalocyanine complexes in THF exhibited two main peaks, the characteristic ligand centered π-π* transitions of a monomeric phthalocyanines **3** and **4** with the B-band and Q-band maxima at 398, 756 nm (log ε = 5.07 and 5.01) and 361, 702 nm (log ε = 5.06 and 5.11), respectively (Fig. 2).

<Fig. 2>

2.3. Biochemical results

The compound (**1**), phthalonitrile derivative (**2**) and non-peripherally aminopyrazole-substituted chloromanganese, and cobalt phthalocyanine complexes (**3** and **4**) were synthesized and tested for their inhibitory activity against carbonic anhydrase isozymes (hCA I and II) and paraoxonase enzyme (PON1). All the compounds showed a moderate inhibitory potency against these enzymes. The inhibition values of the synthesized compounds against CAs and PON1 were shown in Table 1. The data in Table 1 and the structure-activity relationships obtained from these data are summarized below:

(1) It was determined that the synthesized compounds have K_i values of 0.14-24.01 μM for PON, 22.52-39.28 μM for esterase activity of hCA I and 13.62-33.67 μM for esterase activity of hCA II. Among the compounds, the most active ones were found to be compound **4** for PON (K_i : 0.14 μM), compound **3** for hCA I (K_i : 22.52 μM) and compound **1** for hCA II.

(2) The slow, off-target cytosolic isoform hCA I was inhibited by the synthesized compounds. The chemical structure of the substituent and size of the compounds affects the CA inhibitions. The bulky phthalocyanine compounds **3** and **4** exhibited higher inhibitory activity (Ki=22.52 and 30.21 μM) than the precursors **1** and **2** (Ki=39.28 and 38.12 μM) probably due to steric effects. Additionally, because they contain multiple amine groups, compounds **3** and **4** have a better inhibition effect. The amino substituents on the phthalocyanine ring may be involved in making hydrogen bonds with the active site as observed in classical CA polyamine inhibitors [20].

(3) In case of hCA II inhibition, compound **1** was found to be more effective (Ki=13.62 μM). This can be attributed to the hydroxyl group of compound **1** which may bind to the active site of hCA II enzyme by making hydrogen bonds as observed in classical CA phenolic inhibitors [36, 37]. Moreover, compound **1** exhibited markedly higher inhibitory activity against hCA II (Ki=13.62 μM) with respect to hCA I (Ki=39.28 μM). These data indicate that compound **1** is a specific inhibitor for hCA II.

(4) For PON1 as a key enzyme in the body, the synthesized compounds had IC_{50} values of 0.17–25.44 μM and Ki values of 0.14-24.01 μM (Table 1). Among them, compound **4** (IC_{50}=0.17 μM, Ki=0.14 μM) showed the highest inhibitory activity against PON1. When the compounds reported in this study are compared with the literature, it can be stated that peripherally aminopyrazole-substituted cobalt phthalocyanine complex (**4**) can be considered as strong PON1 inhibitors while chloromanganese complex (**3**), aminopyrazole compound (**1**) and its phthalonitrile derivative (**2**) are weaker inhibitors [38-43]. It is remarkable that the cobalt-containing phthalocyanine compound is about 100 times more effective than the chloromanganese-containing phthalocyanine. And this can be explained by further studies on the effects of different phthalocyanine complexes on PON1 enzyme activity.

(5) In addition, as seen in Table 1, inhibition types for CAs and PON1 were generally noncompetitive, whereas only compound **2** inhibited hCA I in a competitive manner.

<Table 1>

3. Experimental

The used materials, equipments, preparation of haemolysate, carbonic anhydrase I and II isoenzymes purification and inhibition, measurement of paraoxonase inhibitory activity parameters were supplied as supporting information.

3.1. Synthesis

*3.1.1. 3-(2-(5-amino-4-(4-bromophenyl)-3-methyl-1H-pyrazol-1-yl)ethoxy)phthalonitrile (**2**)*

Compound **1** (1.71 g, 5.78 mmol) and 3-nitrophthalonitrile (1g, 5.78 mmol) were added to 15 mL anhydrous dimethylformamide and reacted at 40 °C under inert atmosphere. Excess potassium carbonate (3.19 g, 23.12 mmol) was added to the reaction mixture in four portions every six hours. After two days, the reaction mixture was cooled, and poured into 200 mL of ice–water mixture. The

precipitate was filtered and washed with small amount of ethanol and filtered again to dryness. The crude solid product was further purified by column chromatography on silica gel using ethanol/chloroform (1/15) mixture. Yellow-colored compound was soluble in tetrahydrofuran, chloroform, dichloromethane, and N, N-dimethylformamide. Yield: 59%, mp 193-194 °C. Anal. calcd. for $C_{20}H_{16}BrN_5O$: C, 56.89; H, 3.82; Br, 18.92; N, 16.58; O, 3.79 %. Found: C, 56.77; H, 3.74; N, 16.70. FT-IR (υ_{max}/cm^{-1}): 3435–3363 (primery N-H), 3095 (Aromatic C-H), 2963 (Aliphatic. C-H), 2242-2227 (C≡N), 1618 (C=N), 1580 (C=C), 1287 (C−O−C), 809 (p-substitue benzene). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.66 (1H, t, *J*=8.2 Hz), 7.53 (2H, d, *J*=8.3 Hz), 7.38 (1H, d, *J*=7.7 Hz), 7.23 (1H, d, *J*=8.7 Hz), 7.16 (2H, d, *J*=8.2 Hz), 4.48 (4H, s), 4.00 (2H, br, s), 2.19 (3H, s) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 160.95, 146.39, 143.80, 135.17, 132.52, 132.32, 130.42, 126.07, 120.14, 117.10, 116.65(x2), 115.23, 113.82, 104.92, 69.61, 46.57, 13.28 ppm. MALDI-MS *m/z*: 423.38 $[M+H]^+$.

3.1.2. General procedures for phthalocyanine compounds

0.100 g compound **2** (2.36 mmol), 0.038 g $MnCl_3$ and 0.025 g $CoCl_2$ (0.110 mmol of each metal salts), catalytic amount of DBU (0.05 mL) and 2-dimethylaminoethanol (3 cm^3) were mixed in sealed glass tube. The reaction mixture was refluxed at 140 °C for 12 hours under a nitrogen atmosphere. The synthesized green product was filtered after it was cooled and precipitated by adding ether. The crude product was washed with methanol and then was further purified by column chromatography using silica gel (THF/methanol-100/1). At last, the product was dried in *vacuo* at 90 °C.

*3.1.2.1. Synthesis of chloromanganese (III) phthalocyanine (**3**)*

Yield: 22%. Anal. calcd. for $C_{80}H_{64}Br_4ClMnN_{20}O_4$: C, 54.00; H, 3.63; Br, 17.96; Cl, 1,99; Mn, 3.09; N, 15.74; O, 3.60 %. Found: C, 54.08; H, 3.57; N, 15.98. FT-IR (υ_{max}/cm^{-1}): 3538 (w, -NH_2), 1584 (m, Ar), 3079 (w, Ar-H), 2877-2953 (Aliphatic C-H), 1592 (m, C=C), 1275 (s, R-O-Ar). UV-vis (THF): λ_{max}/nm (log ε): 756 (5.01), 530 (4.51), 398 (5.07). MALDI-MS *m/z*: 1747.05 $[M-CI+2H]^+$.

*3.1.2.2. Synthesis of cobalt (II) phthalocyanine (**4**)*

Yield: 37%. Anal. calcd. for $C_{80}H_{64}Br_4CoN_{20}O_4$: C, 54.97; H, 3.69; Br, 18.28; Co, 3.37; N, 16.03; O, 3.66 %. Found: C, 54.86; H, 3.58; N, 16.13. FT-IR ($\upsilon_{max}/cm^{-}1$) 3544 (w, -NH_2), 1576 (m, Ar),

3088 (w, Ar-H), 2872-2956 (Aliphatic C-H), 1588 (m, C=C), 1246 (s, R-O-Ar). UV-vis (THF): λ_{max}/nm (log ε): 702 (5.11), 361 (5.06). MALDI-MS *m/z*: 1748.15 $[M]^+$.

4. Conclusion

In conclusion, phthalonitrile precursor (**2**) and its non-peripherally substituted chloromanganese and cobalt phthalocyanine complexes (**3** and **4**) with aminopyrazole groups prepared for the first time. The synthesized compounds were tested for their inhibitory activity against paraoxonase enzyme (PON) and CA isozymes hCA I and II. All the compounds showed a moderate inhibitory potency against these enzymes. Pcs containing amino groups may be crucial to give directions to further studies.

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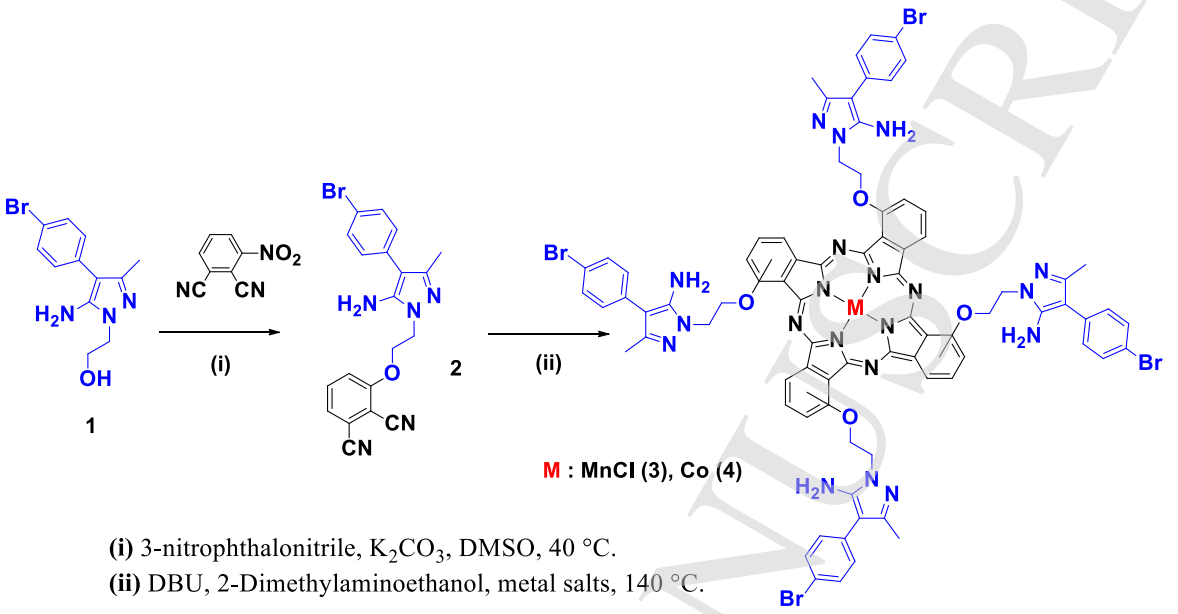

(i) 3-nitrophthalonitrile, K_2CO_3, DMSO, 40 °C.
(ii) DBU, 2-Dimethylaminoethanol, metal salts, 140 °C.

Scheme 1. The preparation of phthalonitrile **2** and its phthalocyanines (**3** and **4**)

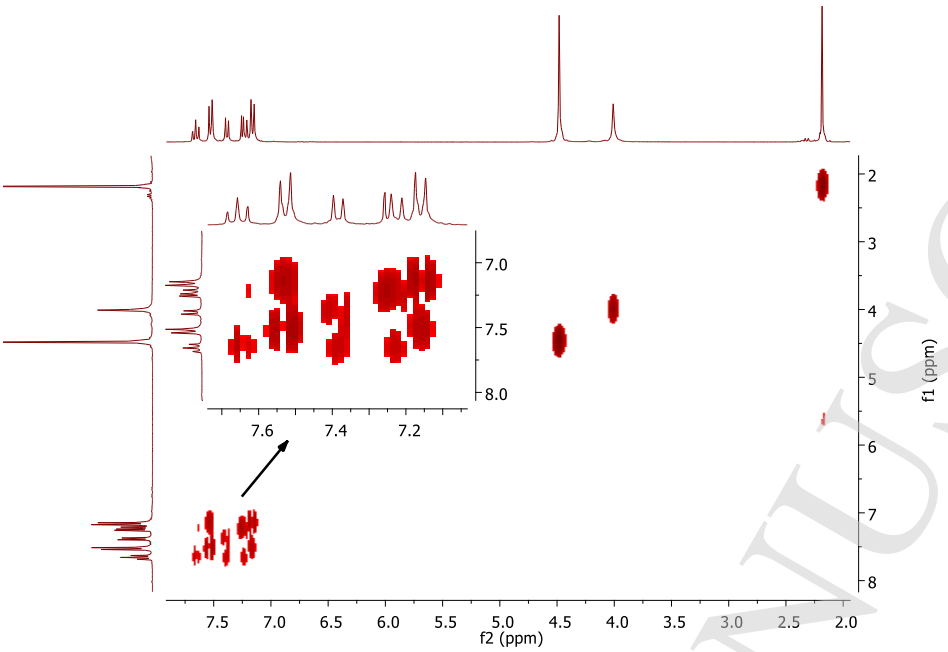

Fig. 1. Two-dimensional ^{1}H-^{1}H COSY spectrum of compound **2**

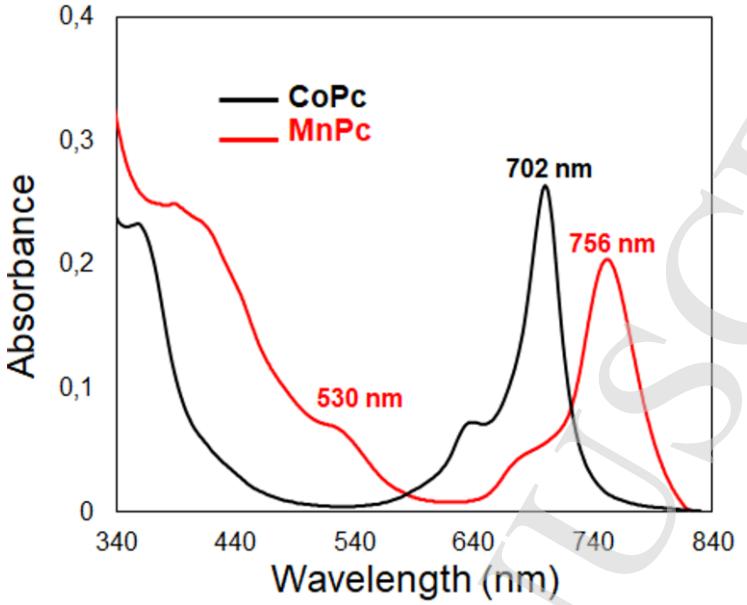

Fig. 2. Absorption spectra of phthalocyanine complexes **3** and **4** in THF (~ 2 x 10^{-6} mol dm^{-3})

Table 1. IC_{50} and Ki values (μM) of compounds (1-4) on hCA I, hCA II and PON1 enzymes

Compounds	hCA I				hCA II				PON		
	IC_{50}(μM) (Hydratase)	**IC_{50}(μM) (Esterase)**	**Ki(μM) (Esterase)**	**Type of Inhibition**	**IC_{50}(μM) (Hydratase)**	**IC_{50}(μM) (Esterase)**	**Ki(μM) (Esterase)**	**Type of Inhibition**	**IC_{50}(μM)**	**Ki(μM)**	**Type of Inhibition**
1.	60.26	39.93	39.28	Non-competitive	18,17	49.61	13.62	Non-competitive	12.42	14.65	Non-competitive
2.	43.33	41.37	38.12	Competitive	33.98	38.79	32.36	Non-competitive	25.44	24.01	Non-competitive
3.	39.83	47.30	22.52	Non-competitive	44.76	44.70	33.67	Non-competitive	14.42	13.22	Non-competitive
4.	30.78	44.62	30.21	Non-competitive	35.13	42.42	28.70	Non-competitive	0.17	0.14	Non-competitive

J. Porphyrins Phthalocyanines

SUPPLEMANTARY MATERIAL

Synthesis, *in vitro* inhibition effect of novel phthalocyanine complexes as carbonic anhydrase and paraoxonase enzyme inhibitors

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Materials

All reagents and solvents were of reagent grade quality and were obtained from commercial suppliers. The homogeneity of the products was tested in each step by TLC. The solvents were stored over molecular sieves. All solvents were dried and purified as described by Perrin and Armarego [1].

Equipment

IR spectra were recorded on a Thermo Scientific iS10 FT-IR (ATR sampling accessory) spectrophotometer and electronic spectra on a Shimadzu UV-2450 and 2600 UV-vis spectrophotometer. ^{1}H-NMR and ^{13}C NMR spectra were recorded on Agilent VNMRS 300 MHz and the spectrum was referenced internally by using the residual solvent resonances and chemical shifts were reported relative to Me_4Si as internal standard. Mass spectra were measured on a Micromass Quatro LC/ULTIMA LC-MS/MS spectrometer and MALDI-MS of complexes were obtained in dihydroxybenzoic acid as MALDI matrix using nitrogen laser accumulating 50 laser shots using Bruker Microflex LT MALDI-TOF mass spectrometer Bremen, Germany. Elemental analyses were performed in TÜBİTAK Marmara Research Centre.

Enzym Inhibition Parameters

Preparation of haemolysate and purification from blood red cells

Blood samples (25 mL) from healthy human volunteers were collected. They were centrifuged at 1000 g for 20 min at 4 ^{0}C and the supernatant was removed. The packed erythrocytes were washed three times with 0.9 % NaCl and then were haemolysed in cold water. The pH of the haemolysate was adjusted to pH 8.5 with solid Tris-base. The 25 mL haemolysate was applied to an affinity column containing Sepharose 4B-ethylene diamine-4-isothiocyanato-

benzenesulfonamide [2]. CA isozymes were then eluted with 0.1 M NaCl / 25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), which recovered hCA-I and II, respectively.

Hydratese activity assay

CA activity was measured by the Maren method based on the determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO_2 hydration [3]. The assay solution was 0.5 M Na_2CO_3 / 0.1 M $NaHCO_3$ (pH 10.0) and Phenol Red as the pH indicator was added. CO_2-hydratase activity (enzyme units (EU)) was calculated by using the equation t_0-tc/tc where t_0 and tc are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

For the inhibition studies of the synthesized compounds different concentrations of these compounds were added to the enzyme. Activity % values of CA for different concentrations of each compound were determined by regression analysis using Microsoft Office 2000 Excel. CA enzyme activity without a sample was accepted as 100 % activity. For the compunds having an inhibition effect, the inhibitor concentration causing up to 50 % inhibition (IC_{50} values) was determined from the graphs.

Esterase activity assay

CA activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenyl-acetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (Biotek Power Wave XS) according to the method described in the literature [4]. Inhibitory effects of the compounds (1-4) on enzyme activities were tested under in vitro conditions; *Ki* values were calculated from Lineweaver–Burk [5] graphs.

Purification of Paraoxonase from Human Serum by Hydrophobic Interaction Chromatography

Human serum was isolated from 40 ml fresh human blood and put into a dry tube. The blood samples were centrifuged at 3000 rpm for 15 min and the serum was removed. Firstly, serum paraoxonase was obtained via ammonium sulphate precipitation (60-80 %). The precipitate was accumulated by centrifugation at 15000 rpm for 40 min, and resolved in 100 mM Tris–HCl buffer (pH 8.0). Then, for the purification of human serum paraoxonase we used hydrophobic interaction chromatographic gel-(Sepharose 4B, L-tyrosine 1-napthylamine) [6]. The column was equilibrated with 0.1 M of a Na_2HPO_4 buffer (pH 8.00) including 1 M ammonium sulphate. The paraoxonase was eluted with an ammonium sulphate gradient using 0.1 M Na_2HPO_4 buffer with and without ammonium sulphate (pH 8.00).

Paraoxonase Enzyme Assay

Paraoxonase enzyme activity was quantified spectrophotometrically using paraoxon substrate by the method identified Gan et al. **[7**]. The reaction was determined for 1 min at 37 ºC via appearance of *p*-nitrophenol at 412 nm in a Biotek automated recording spectrophotometer. The final substrate concentration during enzyme assay was 2 mM, and all rates were measured in duplicate and corrected for the non-enzymatic hydrolysis. Paraoxonase enzyme unit was defined as the quantity of enzyme that hydrolysis of 1 μmol of p-nitro phenol. A molar extinction coefficient (ε) of 17,100 $M^{-1}cm^{-1}$ for p-nitro phenol at pH 8.0 in 100 mM Tris–base buffer was used for the calculation.

***in vitro* Kinetic Studies**

For the kinetic studies of carbazole β-lactams derivatives, different concentrations were added to the enzyme activity. Paraoxonase activity with compounds was assayed by following the hydration of paraoxon. Activity % values of paraoxonase for five or more different

concentrations of each β-lactam compound were determined by regression analysis using Microsoft Office 2010 Excel. Control enzyme activity without β-lactam was 100 % and the activity of each compound increase ratio. For the β-lactams having a positive modulation effect, the modulation effect was determined from the graphs [Figure 3].

Total Protein Determination

The absorbance at 280 nm was used to monitor the protein in the column effluents and ammonium sulphate precipitation. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford, [8] with bovine serum albumin as a standard.

SDS Polyacrylamide Gel Electrophoresis

After purification of the human paraoxonase1 (hPON1), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was applied in two different acrylamide concentrations, 10 % and 3 % for the running and stacking gel, respectively, consisting of 0.1 % SDS according to Laemmli [9]. A 20 mg sample was added to the electrophoresis composition. Gel was stayed overnight in 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol and 10 % acetic acid, then detained by fast changing the same solvent, without dye. The electrophoretic figure was photographed an image of the gel as seen Figure S1.

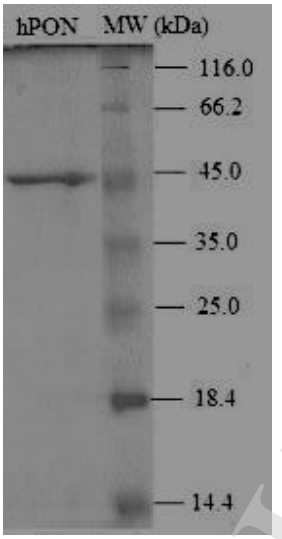

Figure S1. SDS-PAGE of human serum paraoxonase1. The poled fractions from affinity chromatography were analyzed by SDS-PAGE (12% and 3%) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 contained 5 μ L of various molecular mass standards: 3-galactosidase, (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), restriction endonuclease (25.0), 3-lactoglobulin (18.4), lysozyme (14.4). Only one protein-staining band was detectable on Line 2.

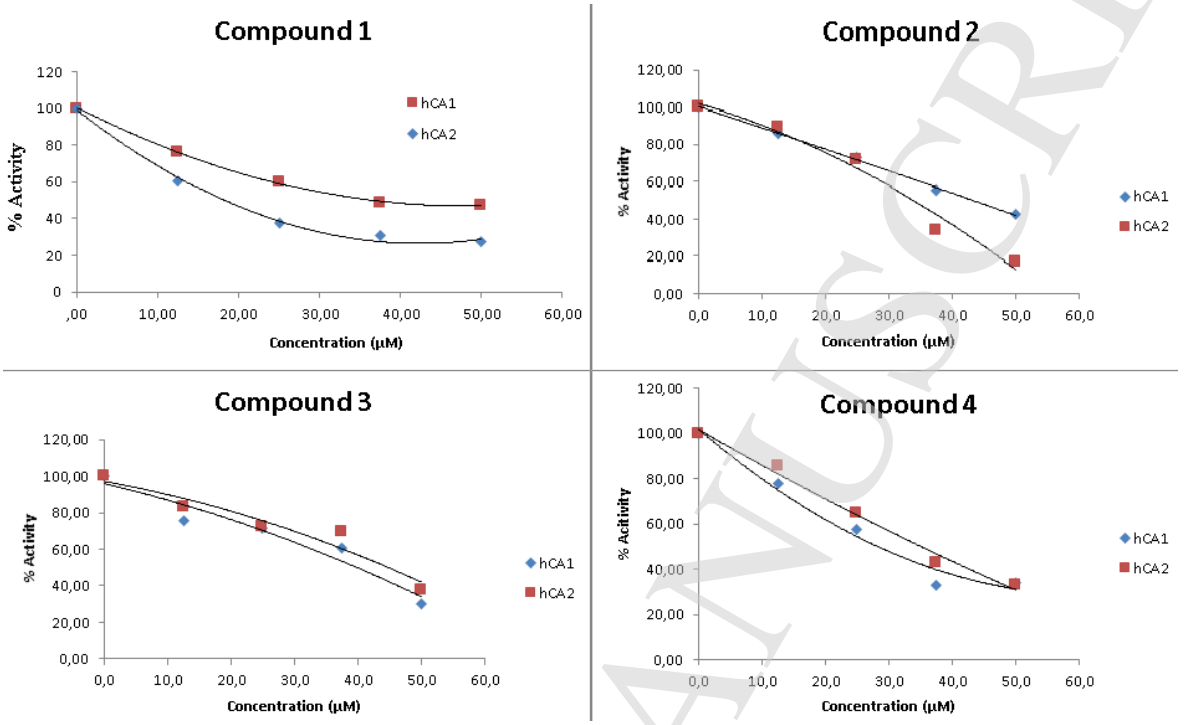

Figure S2. IC_{50} of compound 1-4 for hCA I and hCA II isoenzymes (hydratase activity)

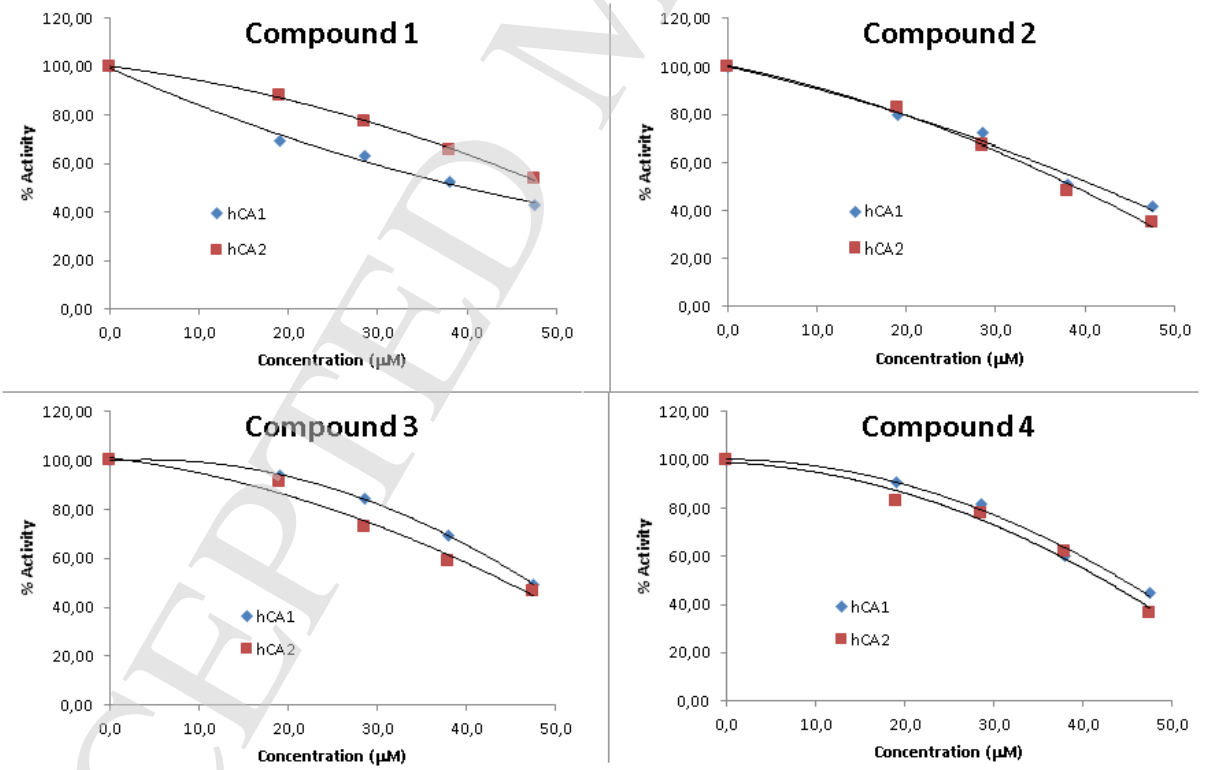

Figure S3. IC_{50} of compound 1-4 for hCA I and hCA II isoenzymes (esterase activity)

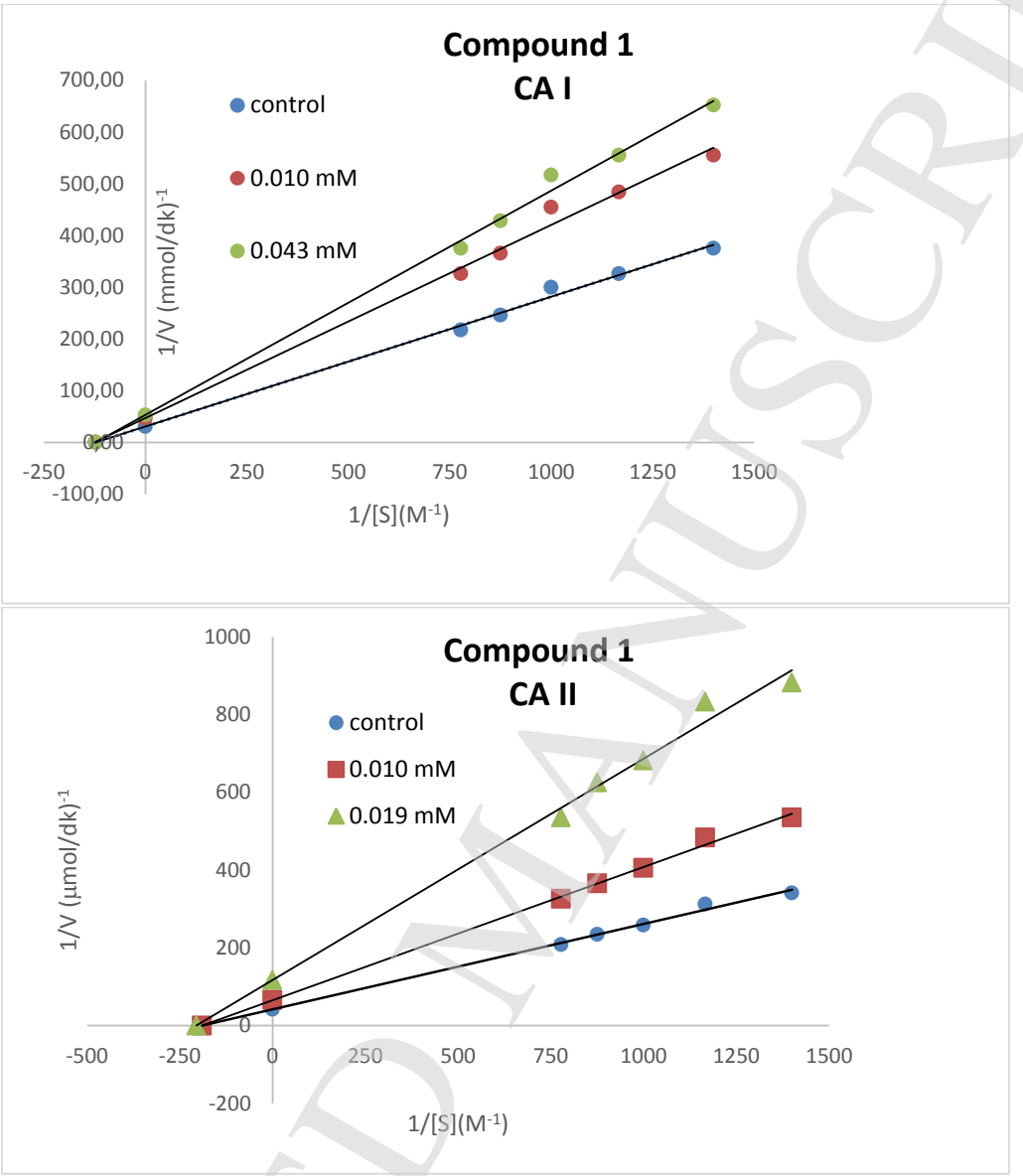

Figure S4. Lineweaver–Burk graphs esterase activity of hCA I and hCA II isoenzymes for compound 1

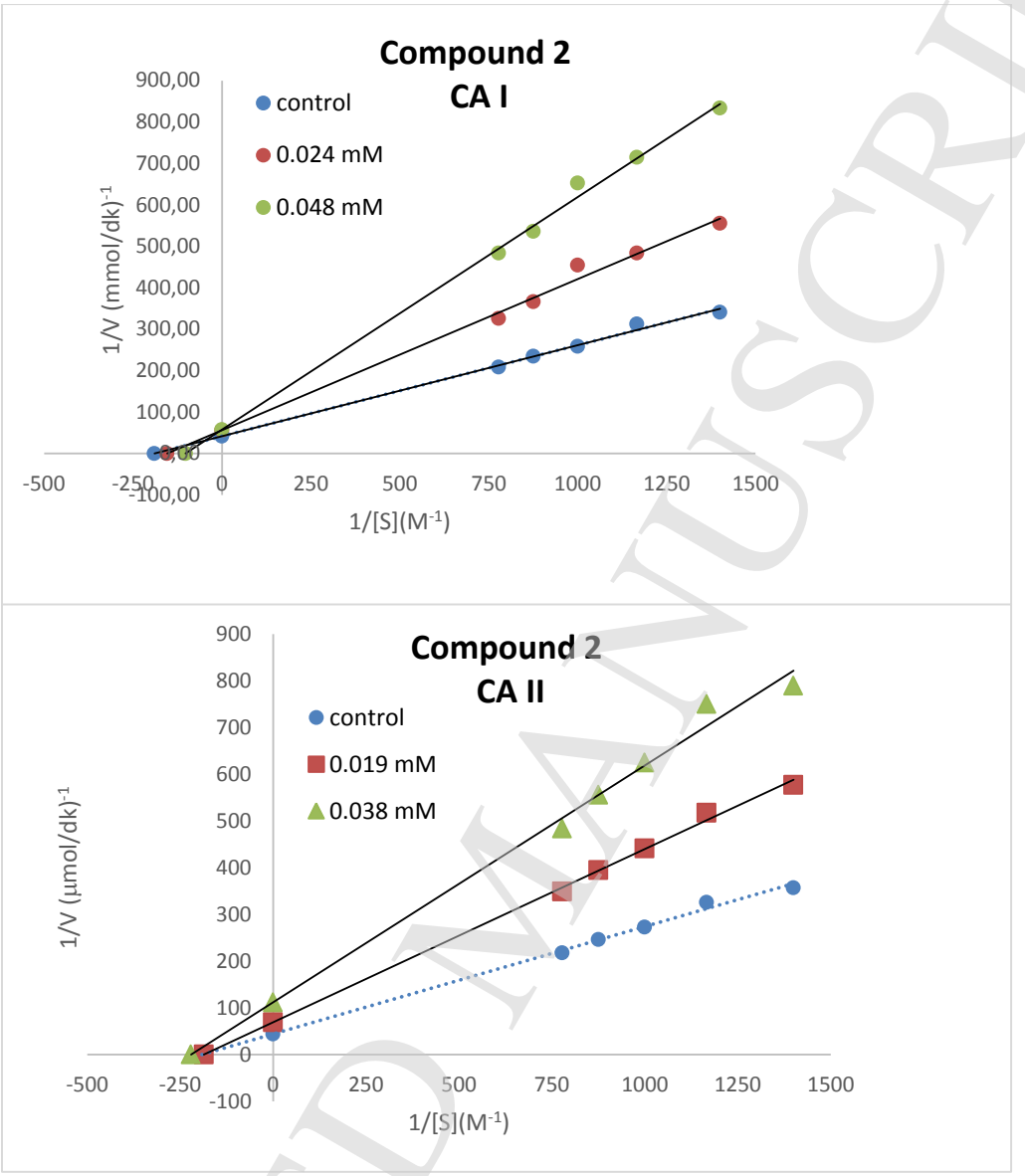

Figure S5. Lineweaver–Burk graphs esterase activity of hCA I and hCA II isoenzymes for compound 2

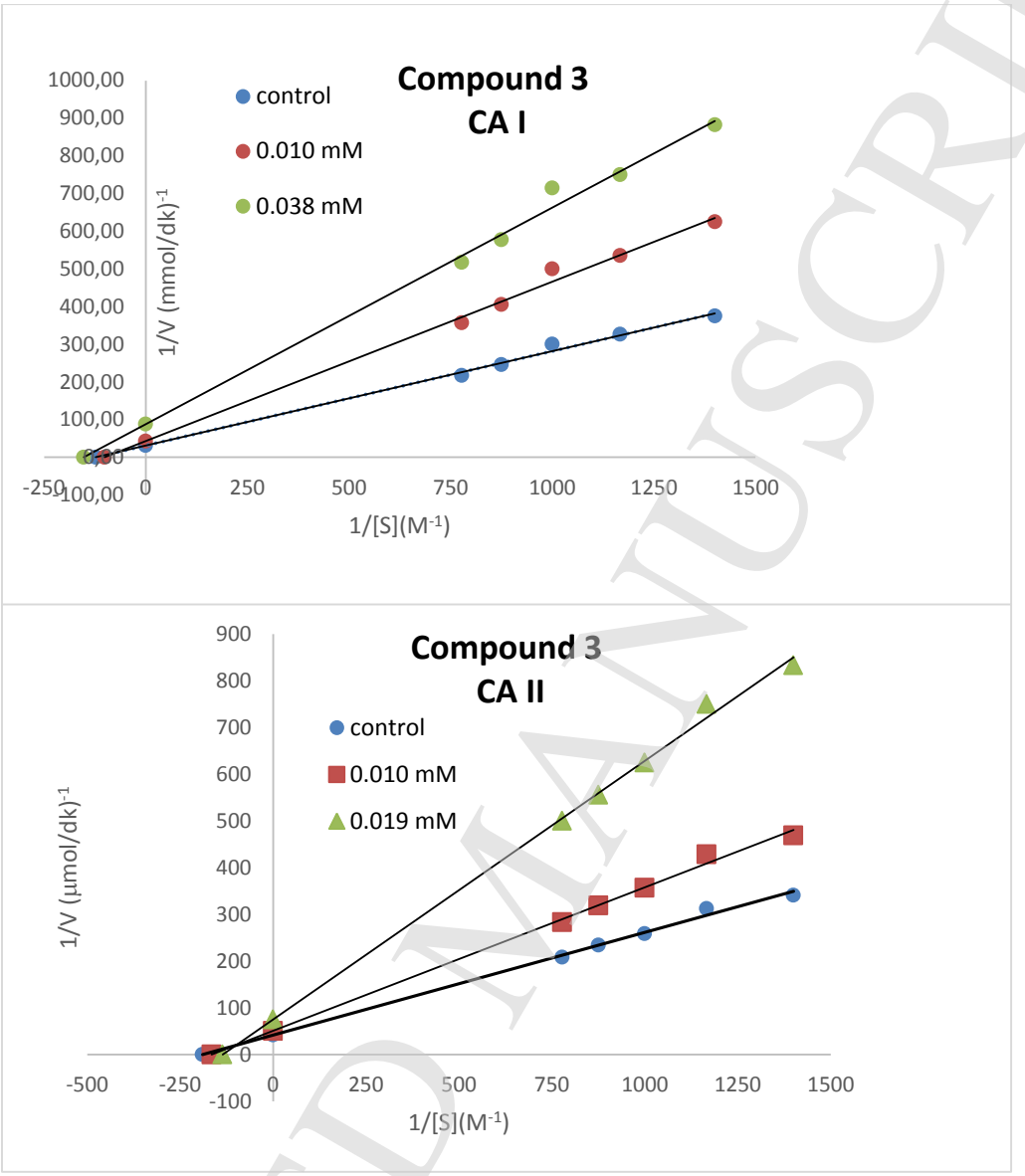

Figure S6. Lineweaver–Burk graphs esterase activity of hCA I and hCA II isoenzymes for compound 3

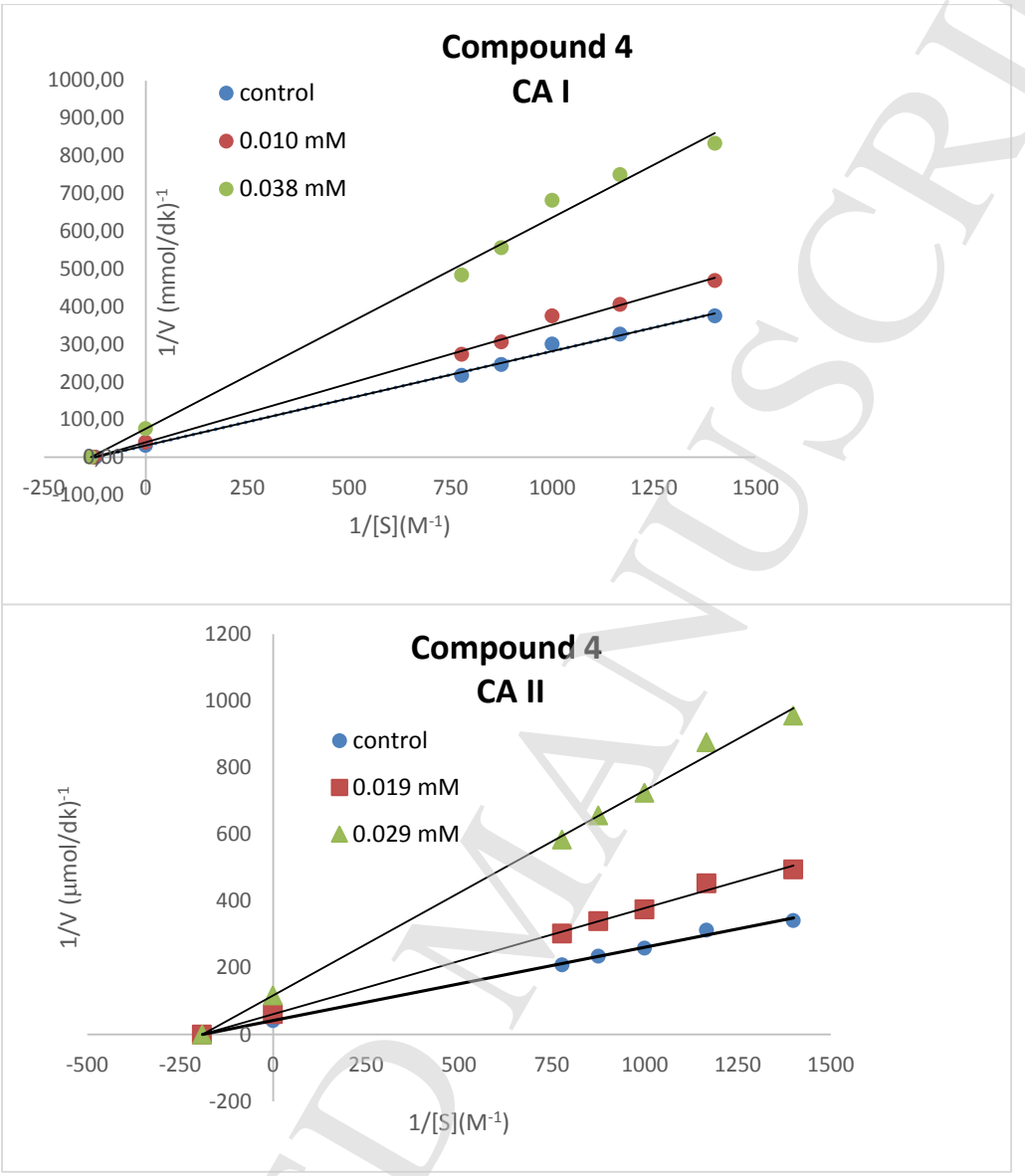

Figure S7. Lineweaver–Burk graphs esterase activity of hCA I and hCA II isoenzymes for compound 4

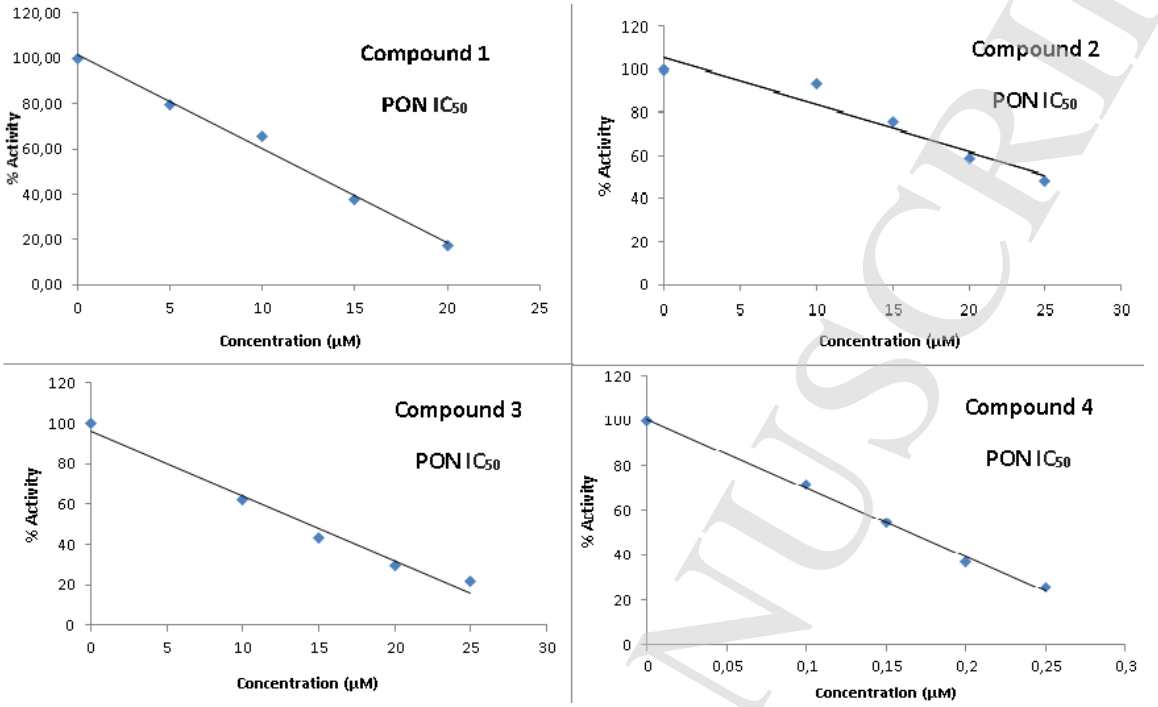

Figure S8. IC_{50} of compound 1-4 for paraoxonase1 enzymes

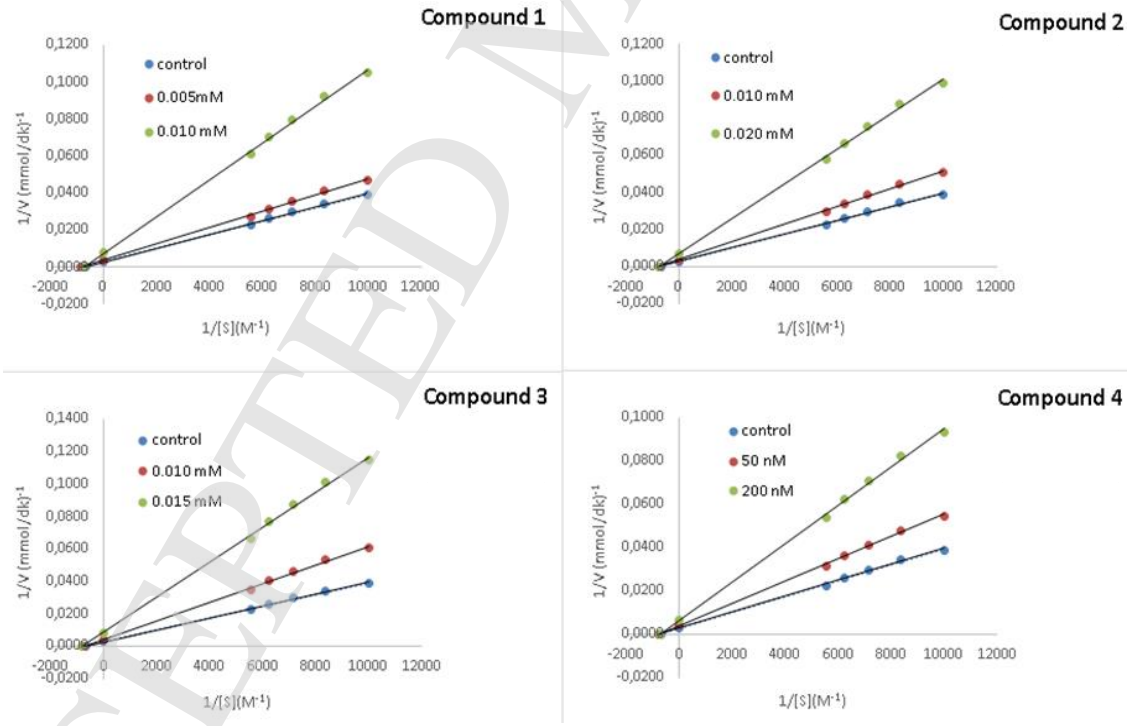

Figure S9. Lineweaver–Burk graphs of paraoxonase1 enzyme for compound 1-4.

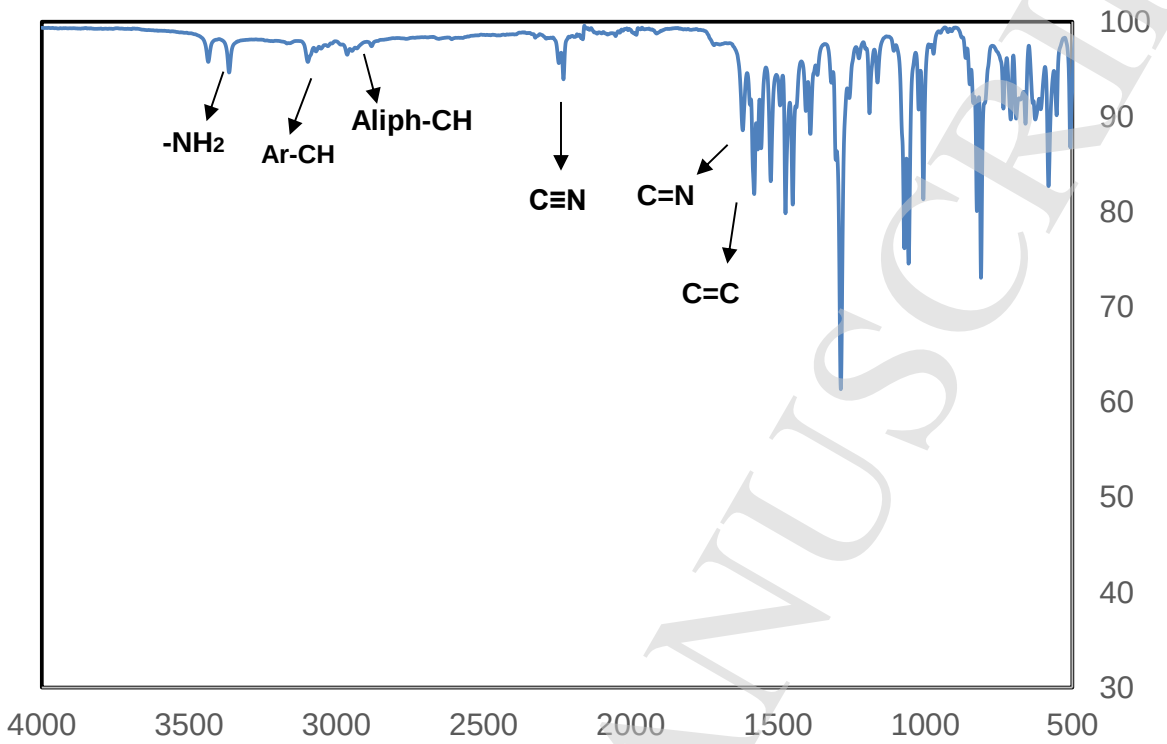

Figure S10. FT-IR spectrum of compound **2**.

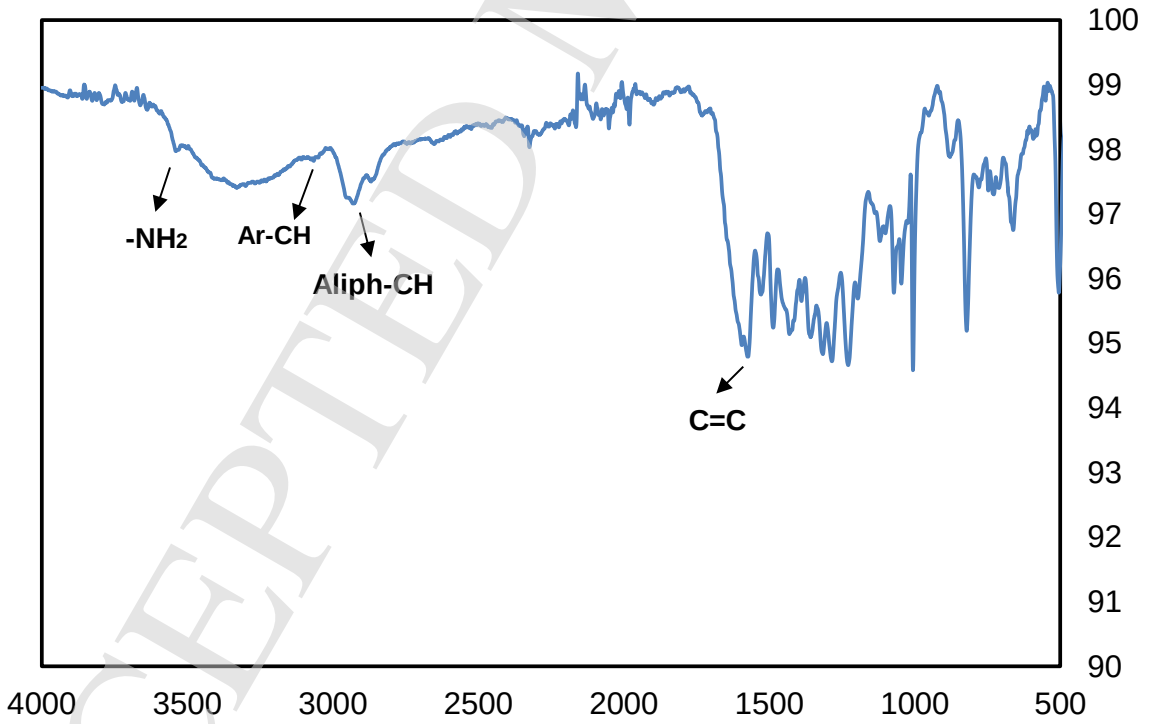

Figure S11. FT-IR spectrum of manganese complex **3**.

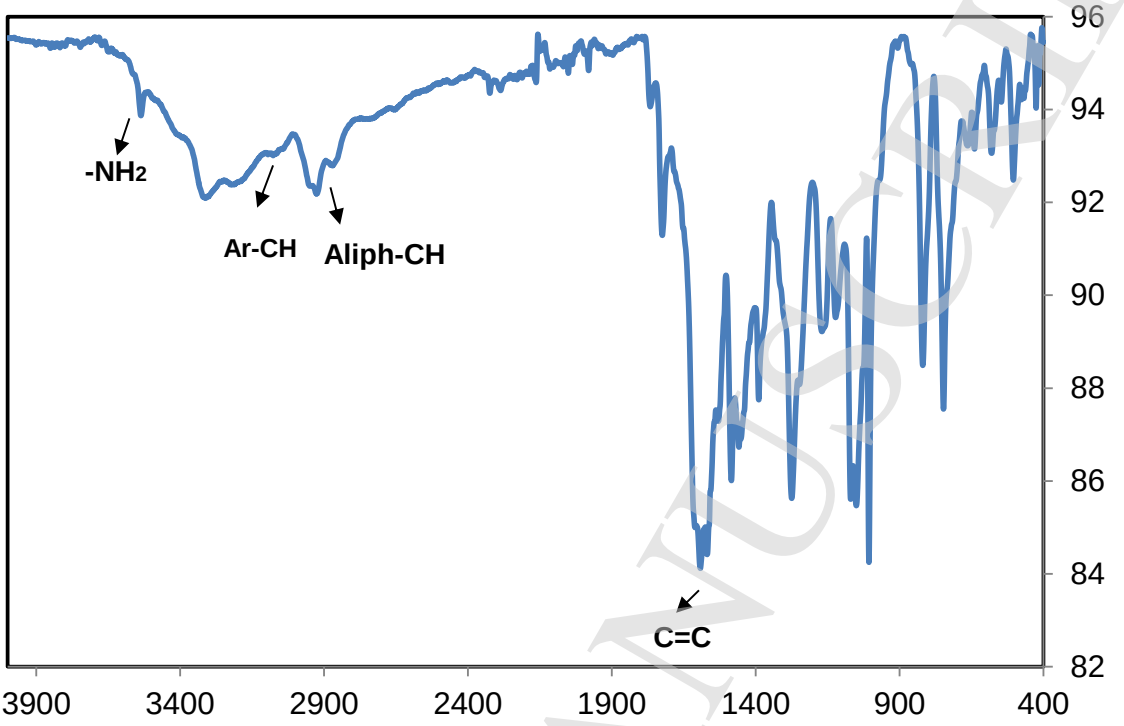

Figure S12. FT-IR spectrum of cobalt complex **4**.

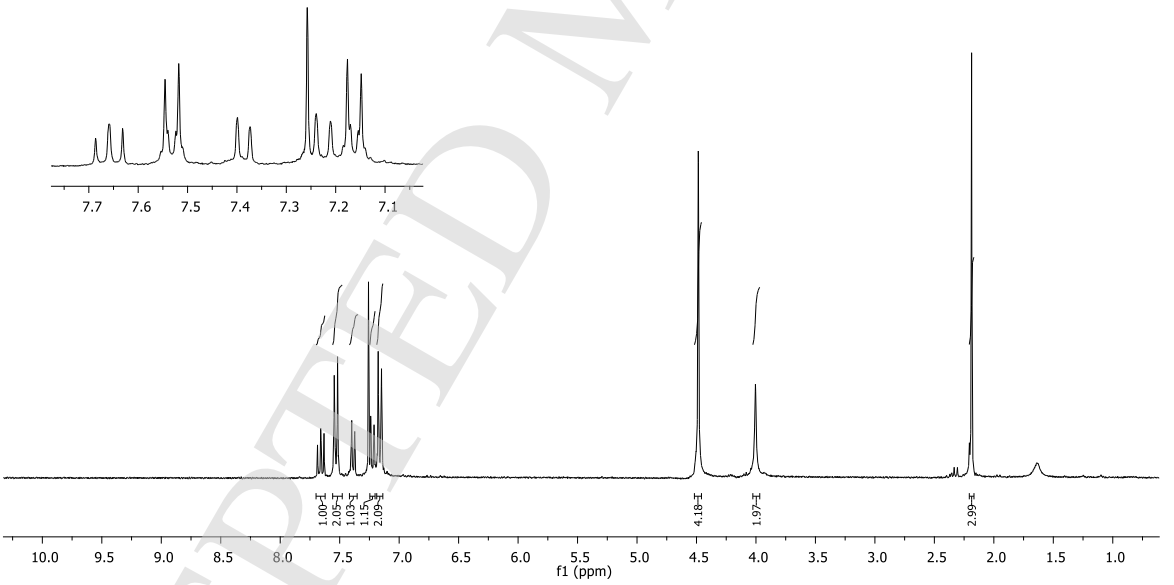

Figure S13. ^{1}H-NMR spectrum of compound **2** in $CDCI_3$.

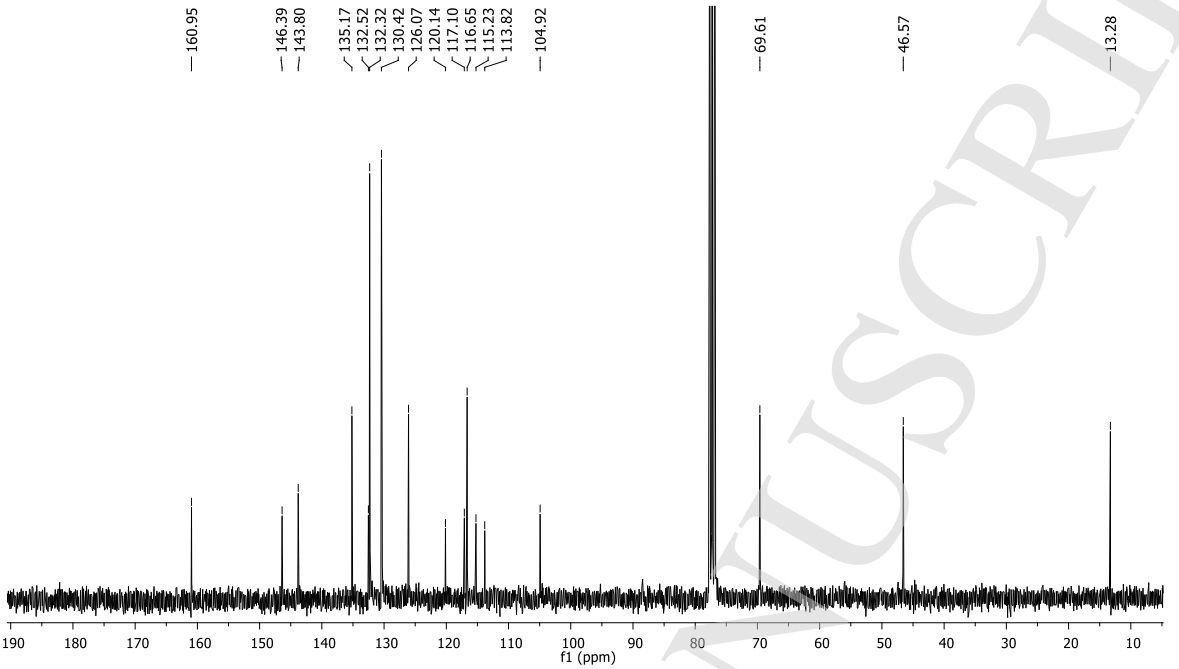

Figure S14. ^{13}C-NMR spectrum of compound **2** in $CDCI_3$.

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