SYNTHESIS AND ANTIOXIDANT ACTIVITY OF CONJUGATED OLIGO-AROMATIC COMPOUNDS CONTAINING A 3,4,5-TRIMETHOXYBENZYL SUBSTITUENT

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FACULTY OF SCIENCE
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ABSTRACT

The main aim of the thesis was the synthesis, characterization and investigation of new synthetic compounds bearing the well-known 3,4,5-trimethoxybenzyloxy free radical scavenger group. The compounds are aimed as potential antioxidants to reduce free radical-induced cell damage. Theoretical calculations obtained by DMOL3 based on density functional theory (DFT) were used to rationalize the antioxidant activities. The theoretical investigation emphasized on a hydrogen atom transfer (HAT) mechanism. Therefore, the bond dissociation energy (BDE) for the most active (weakest) X-H bond (where X= O, N, S) in each molecule was compared.

The key compound, 4-(3,4,5-trimethoxybenzyloxy)benzohydrazide, was synthesized by reacting hydrazine hydrate with ethyl 4-(3,4,5-trimethoxybenzyloxy) benzoate. It was converted into series of hydrazone and thiosemicarbazide derivatives. Besides, reaction of the hydrazide with carbon disulfide furnished 5-(4-(3,4,5-trimethoxybenzyloxy) phenyl)-1,3,4-oxadiazole-2-(3H)-thione, which was subsequently converted into a series of s-alkylated derivatives. Treatment of the thiosemicarbazide with 4N NaOH led to a series of 1,2,4-triazoles, for a thiosemicarbazide with an electron-withdrawing nitro- group, which led to the hydrolysis of the thiosemicarbazide instead. All synthesized compounds were characterized by IR, NMR and mass spectral analyses.

The free radical scavenging activities of the synthesized compounds were investigated by DPPH and FRAP assays, using ascorbic acid and BHT as references. The DPPH radical scavenging activity, which is applicable to reaction based on both HAT and single electron transfer (SET), depended on the type and the position of substituents of the compounds at the varied aromatic group.

Hydrazone derivatives with a hydroxyl group at the para position and additional electron donating groups (EDGs) at the ortho position of the phenyl ring of the aldehyde component, showed high free radical scavenging activities. In contrast, electron withdrawing groups (EWGs) decreased the activity.

Some of the compounds exhibited potent antioxidant activities that were equal to or better than the reference compound BHT. One of the 1,3,4,-oxadizole derivatives exhibited a strong scavenging effect on the DPPH radical, due to the exchangeable proton between the –NH and –SH groups in the oxadiazole ring. However, S-alkylation of this compound eliminated this activity. The thiosemicarbazides exhibited stronger inhibitory effects on the DPPH radical than the reference compounds, ascorbic acid and BHT. The most active compound bore a strong electron withdrawing nitro group at the para position of the phenyl group. Cyclization of the thiosemicarbazides reduced the radical scavenging abilities.

The FRAP assay showed different trends than the DPPH radical scavenging results. Some of the compounds exhibited high activities in the FRAP assay while showing low activity in the DPPH assay. High FRAP activities were obtained for acid hydrazide as well as one of the hydrazone derivatives. Most of the investigated compounds exhibited low activities in the FRAP assay.

Thiosemicarbazides were highly active in both antioxidant assays, i.e. DPPH and FRAP. Therefore they deserve attention in the synthesis of bioactive compounds.

ABSTRAK

Tujuan utama tesis ini adalah sintesis, pencirian dan penyiasatan sebatian sintetik yang mengandungi kumpulan 3,4,5-trimethoxybenzyloxy yang terkenal sebagai agen perencat radikal bebas. Sebatian ini berpotensi bertindak sebagai antioksidan untuk mengurangkan kerosakan sel yang disebabkan oleh radikal bebas. Pengiraan secara teori yang diperolehi daripada DMOL3 berdasarkan teori fungsi ketumpatan (DFT) telah digunakan untuk merasionalkan aktiviti antioksidan. Siasatan teori lebih menekankan kepada mekanisme perpindahan atom hidrogen (HAT). Oleh itu, tenaga penceraian ikatan (BDE) untuk ikatan XH yang paling aktif (paling lemah) (di mana X = O, N, S) dalam setiap molekul dibandingkan..

Sebatian utama, 4- (3,4,5-trimethoxybenzyloxy) benzohydrazide, telah disintesis oleh reaksi hidrazin hidrat dengan etil 4- (3,4,5-trimethoxybenzyloxy) benzoate. Ia telah ditukar menjadi siri hydrazone dan thiosemicarbazide derivatif. Selain daripada itu, reaksi hydrazide dengan karbon disulfida dilengkapi 5- (4- (3,4,5-trimethoxybenzyloxy) phenyl) -1,3,4-oxadiazole-2- (3H) -thione, yang kemudiannya ditukar ke dalam satu siri derivatif S-alkylated. Rawatan thiosemicarbazide dengan 4N NaOH membentuk satu siri 1,2,4-triazoles, untuk thiosemicarbazide, yang mengandungi kumpulan nitro yang boleh mengeluarkan elektron, yang sebaliknya mengakibatkan hidrolisis thiosemicarbazide. Semua sebatian yang disintesis telah dicirikan melalui IR, NMR dan analisis jisim spektrum.

Aktiviti memerangkap radikal bebas oleh sebatian yang disintesis telah dikaji menggunakan asai DPPH dan FRAP, menggunakan asid askorbik dan BHT sebagai rujukan. aktiviti memerangkap radikal DPPH, yang berasaskan kedua-dua tindak balas HAT dan pemindahan elektron tunggal (SET), adalah bergantung kepada jenis dan kedudukan gantian sebatian aromatik di kumpulan yang berbeza-beza.

Derivatif Hydrazone dengan kumpulan hidroksil pada kedudukan para dan kumpulan penderma elektron tambahan (EDGs) pada kedudukan orto cincin phenyl komponen aldehid, menunjukkan aktiviti memerangkap radikal bebas yang tinggi. Sebaliknya, kumpulan yang mengeluarkan elektron (EWG) menurunkan aktiviti tersebut. Sebahagian daripada sebatian yang disintesis menunjukkan aktiviti antioksidan yang sama atau lebih baik daripada sebatian rujukan, BHT. Salah satu derivatif 1,3,4-oxadiazole mempamerkan kesan memerangkap yang kuat ke atas radikal DPPH diakibatkan oleh saling-tukar proton di antara kumpulan -NH dan SH di dalam gelang oxadiazole tersebut. Walau bagaimanapun, S-alkylation sebatian ini menghapuskan aktiviti ini.

Thiosemicarbazides menunjukkan kesan perencatan yang lebih kukuh terhadap radikal DPPH berbanding sebatian rujukan, asid askorbik dan BHT. Sebatian yang paling aktif mempunyai kumpulan nitro yang bekeupayaan tinggi mengeluarkan elektron, di kedudukan para kumpulan phenyl. Pensiklikan thiosemicarbazides mengurangkan kebolehan memerangkap radikal.

Cerakinan FRAP menunjukkan trend yang berbeza daripada keputusan aktiviti memerangkap radikal DPPH. Sebahagian daripada sebatian mempamerkan aktiviti FRAP yang tinggi manakala aktiviti DPPH adalah rendah. Aktiviti FRAP yang tinggi telah diperolehi untuk asid hydrazide dan juga salah satu daripada derivatif hydrazone. Sebahagian besar sebatian yang di kaji menunjukkan aktiviti FRAP yang rendah. Thiosemicarbazides adalah sangat aktif dalam kedua-dua esei antioksidan, iaitu DPPH dan FRAP. Oleh itu sebatian ini berhak mendapat perhatian dalam sintesis sebatian bioaktif.

ACKNOWLEDGEMENTS

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LIST OF SYMBOLS AND ABBREVIATIONS

AA	:	Ascorbic acid
ABTS	:	Azino-Bis-Ethylbenzthiazoline-Sulfonic Acid
BDE	:	Bond dissociation energy (enthalpy change)
BHA	:	Butylated hydroxyanisole
BHT	:	Butylated hydroxytoluene
CAT	:	Catalase
CoAH	:	Co-antioxidant
COX	:	Cyclooxygenase enzyme
CO-RMs	:	Carbon monoxide-releasing molecules
DFT	:	Density functional theory
DPPH	:	Radical 2,2-diphenyl-1-picrylhydrazyl
HOMO	:	Highest occupied molecular orbital
EDG	:	Electron donating group
EWG	:	Electron withdrawing group
FDA	:	Food and Drug Administration
FRAP	:	Ferric ion reducing antioxidant power assay
FRS	:	Free radical scavengers
GPx	:	Glutathione Peroxidase
HREIMS	:	High resolution electron ionization mass spectroscopy
HAT	:	Hydrogen atom transfer
IC_{50}	:	Half maximal inhibitory concentration
IP	:	Ionization potential
LUMO	:	Lowist unoccupied molecular orbital
MPAO	:	Multipotent antioxidants

NMR	:	Nuclear magnetic resonance
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
ORAC	:	Oxygen Radical Absorption Capacity
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
RT	:	Room temperature
SAR	:	Structure-activity relationship
SD	:	Spin density
SOD	:	Superoxide Dismutase
SET	:	Single electron transfer
SOMO	:	Semi-occupied orbital
TBHQ	:	Tertiary butyl hydroquinone
TEAC	:	Trolox-equivalent antioxidant capacity assay
TMP	:	Trimethoprim
TMDC	:	Trimethoxy-2`,5`-dihydroxychalcone
α-TOH	:	α-Tocopherol
TPTZ	:	Tripyridyltriazine

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CHAPTER 1: INTRODUCTION

1.1 Free radicals

Free radicals are one of the main causes of many pathological conditions, such as several degenerative and chronic diseases. A free radical is formed when a covalent bond between entities is broken and one of the binding electron remains with each newly formed fragment. These species with unpaired electrons are highly reactive and can take part in chemical reactions and play an important role in many chemical processes, including those involved in human physiology.

Free radicals are typically formed as a result of normal cellular metabolism. If not immediately deactivated, free radicals can cause a chain reaction of oxidation to occur, causing damage to normal cells including lipids, DNA and proteins. Thousands of free radical reactions can occur within seconds of the initial reaction. Free radicals capture electrons from other substances in order to neutralize themselves (Krishnamurthy & Wadhwani, 2012).

1.2 The effect of ROS/RNS Species in Human Body

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are radicals which are produced as by-products from the cellular redox process, which have gained a lot of interest because of their active role in many diseases (Venkat Ratnam, Ankola, Bhardwaj, Sahana, & Ravi Kumar, 2006). Studies have shown that their numbers increase when cells are exposed to harmful environmental influences (Drew & Leeuwenburgh, 2002), such as pollutants (Sandström, Nowak, & Van Bree, 2005), sunlight, radiation (Wan, Ware, Zhou, Donahue, Guan, & Kennedy, 2006), emotional stress, smoking (Agarwal, 2005), excessive alcohol (Wu & Cederbaum, 2003), and some drugs (Toler,

2004). Free radicals can also be produced endogenously such as during electron transport in the mitochondrial electron transport chain, and as part of the immune system (Das & Vasudevan, 2005) Figure 1.1, summarized some of the common sources of free radicals in the body.

The source of ROS generation for different diseases settings are of great interest. Despite the existence of these multiple sources, a large number of studies in the last decade indicate that a major source of ROS involved in redox signaling is a family of complex enzymes, namely NADPH oxidases (Shah & Channon, 2004).
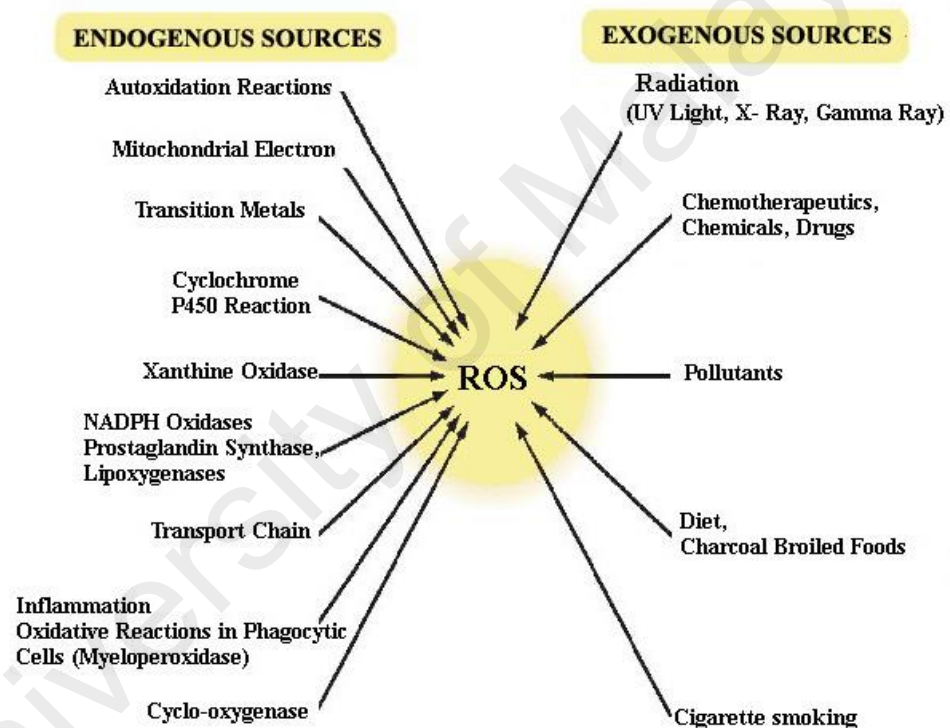
Figure 1.1. Endogenous and exogenous sources of free radicals
("physrev.physiology.org," ; "www.intechopen.com,")

Most cells produce ROS such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^\bullet OH$) and peroxyl radical. Non-radical molecules like hydrogen peroxide (H_2O_2), can be converted into free radicals by transition metals. Reactive nitrogen species (RNS) mainly include nitric oxide, nitrogen dioxide and the potent oxidant peroxynitrite (Süzen, 2007),

2

Table 1.1 lists some of the major ROS and RNS. Depending on their concentration, ROS are well known for playing a double role as both deleterious and beneficial species (Cooper, Vollaard, Choueiri, & Wilson, 2002). The delicate balance between their two opposite effects is crucial in preventing oxidative damage.

Table 1.1. List of ROS and RNS species

Reactive oxygen species (ROS)		Reactive nitrogen species (RNS)	
Radicals	Non- radicals	Radicals	Non- radicals
Hydroxyl $OH^{\bullet}$	Hypochlorous acid $HOCl$	Nitric oxide $^{\bullet}NO$	Peroxynitrite $OONO^{-}$
Superoxide $O_2^{\bullet -}$	Hydrogen peroxide H_2O_2	Nitrogen dioxide $NO_2^{\bullet}$	Peroxynitrous acid $ONOOH$
Nitric oxide NO	Peroxynitrite $ONOO^{-}$		Nitroxyl anion NO^{-}
Peroxyl $RO_2^{\bullet}$	Singlet oxygen $^{-1}O_2$		Nitryl chloride NO_2Cl
Lipid peroxyl LOO	Lipid peroxide $LOOH$		Nitrosyl cation NO^{+}
Alkoxyl $RO^{\bullet}$	Ozone O_3		Dinitogen trioxide N_2O_3
Hydroperoxyl $ROOH^{\bullet}$			Nitrous acid HNO_2

The beneficial effects of these reactive species occur at low/moderate concentrations and involve normal physiological reaction and cellular responses. For example, carbon monoxide-releasing molecules (CO-RMs) are, in general, transition metal carbonyl complexes that liberate controlled amounts of CO. In animal models, CO-RMs are bactericides that kill both Gram positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The CO-RMs induce the generation of ROS, which contribute to the antibacterial activity of these compounds (Tavares, Nobre, & Saraiva, 2012).

At high concentration, free radicals can produce oxidative stress that can be harmful. The radicals can effect key organic substances such as lipids, proteins, and DNA, causing oxidation and damage to these biomolecules, which may ultimately cause a variety of

degenerative diseases (Pham-Huy, He, & Pham-Huy, 2008), such as ageing (Nivas, Gaikwad, & Chavan, 2010), carcinogenesis (Schroeder & Adams, 1941; Waris & Ahsan, 2006), coronary heart disease and many other health problems related to advancing age (Cadenas & Davies, 2000; Uchida, 2000). The inflammatory nature of rheumatoid arthritis implies that a state of oxidative stress may also exist in these diseases (Remans, Van Oosterhout, Smeets, Sanders, Frederiks, Reedquist, Tak, Breedveld, & Van Laar, 2005; Surapaneni & Venkataramana, 2007).

The human body is able to counterbalance ROS, generated from both endogenous and exogenous sources, by various antioxidant defence mechanisms and eliminate excess ROS immediately from the cell by cellular enzymatic and non-enzymatic processes (Apel & Hirt, 2004).

1.3   Antioxidants

To protect the cells and organ systems of the body against ROS, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Percival, 1998). Antioxidants are reducing substances.

At low concentration compared to that of oxidizable substrates, they can delay or prevent oxidation caused by ROS and RNS. Alternatively, the antioxidants can also prevent ROS and RNS from being formed. Antioxidants act through several mechanisms including as radical chain reaction inhibitors, metal chelators, antioxidant enzyme cofactors and oxidative enzyme inhibitors (Süzen, 2007).

In cases where free radical attack is uncontrollable and cell damage cannot be renovated, oxidative damage to cells and tissues can occur, leading to, for example, growth of cancerous cells, lipid peroxidation of cell membranes and protein oxidation. Therefore, the existence of non-enzymatic antioxidants as well as enzymatic antioxidants

in biological systems is important in preventing the cumulative effects of oxidative damage, which is crucial to cellular and organismal survival (Bagchi & Puri, 1998).

Enzymatic antioxidants on the other hand, act by converting ROS into a less reactive form. These enzymes are made up of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Superoxide dismutase (SOD) which is an important endogenous antioxidant enzyme, can exist in several common forms; SOD1 is located in the cytoplasm, SOD2 in the mitochondria, while SOD3 is extracellular. SOD catalyses the disputation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (Sen & Chakraborty, 2011). Catalase (CAT) is a common and efficient enzyme found in the cell and function to convert hydrogen peroxide molecules to water and oxygen. Glutathione peroxidase (GPx) is present in the cytoplasm of the cells and protect the cells against oxidative injury by converting H_2O_2 into water, Figure 1.2.
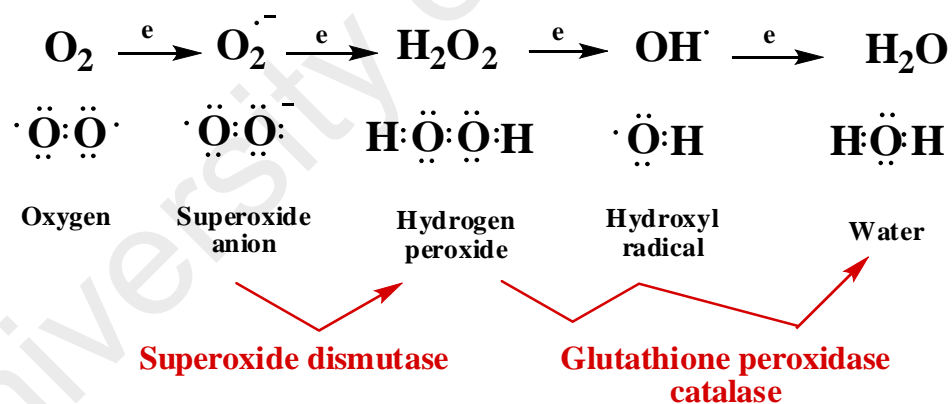
Figure 1.2. Formation and elimination of ROS
(Apel & Hirt, 2004)

Non-enzymatic antioxidants include vitamins (A, C, E, K), minerals (zinc, selenium), antioxidant cofactors (coenzyme Q), carotenoids, organosulfur compounds, and polyphenols (flavonoids and phenolic acids) (Mukai, Tokunaga, Itoh, Kanesaki, Ohara, Nagaoka, & Abe, 2007; Venkat Ratnam, Ankola, Bhardwaj, Sahana, & Ravi Kumar, 2006). These antioxidants stabilize free radicals before they can attack cellular

components. They act either by reducing the energy of the free radicals or by donating some of their electrons for its use, thereby causing it to be stable in either form. In addition, they may also interrupt the oxidizing chain reaction to minimize damage caused by free radicals. These are often some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ10 are produced in human body and they can also be derived from dietary sources. Polyphenols are the major class of antioxidants which are derived from diet. Figure 1.3. outlines the classification of antioxidants (Venkat Ratnam, Ankola, Bhardwaj, Sahana, & Ravi Kumar, 2006).
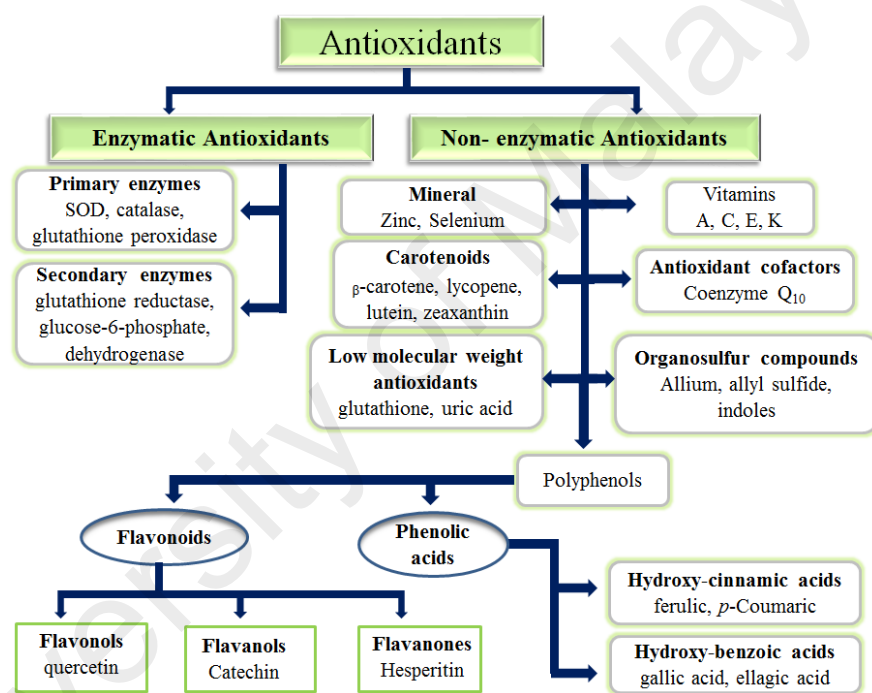
Figure 1.3. Classification of antioxidants

The glutathione system (glutathione, GPx, and GR) is a key defence against hydrogen peroxide. The major antioxidant role of glutathione and its related enzymes in the cell is to intercept the oxidant before it can react with DNA (Deffie, Alam, Seneviratne, Beenken, Batra, Shea, Henner, & Goldenberg, 1988). On the other hand, phospholipase enzymes can repair lipids by catalysing the cleavage of peroxidized fatty acid side chains from the membrane, and replacing them with new, undamaged fatty acids (Shigenaga, Hagen, & Ames, 1994).

In general, antioxidants can be classified into two classes, namely natural and synthetic antioxidants.

1.3.1   Natural Antioxidants

Antioxidants which exist in plants are classified as natural antioxidants. A wide range of phytochemicals contain antioxidant properties, such as α-tocopherol and polyphenolic compounds which comprised of amongst others, cinnamic derivatives, flavanoids and coumarins (Dillard & German, 2000; Key, Allen, Spencer, & Travis, 2002; Rimm, Stampfer, Ascherio, Giovannucci, Colditz, & Willett, 1993; Zhang, Hunter, Forman, Rosner, Speizer, Colditz, Manson, Hankinson, & Willett, 1999) (Figure 1.4 and 1.5). Their effects include the ability to scavenge primary and secondary radicals, to inhibit free radical induced membrane damages, and the potential to bind iron that can prevent radical formation (Amorati, Pedulli, Cabrini, Zambonin, & Landi, 2006; Silva, Borges, Guimarães, Lima, Matos, & Reis, 2000).

Figure 1.4. Selected natural compounds with antioxidant activities

Generally, diet containing various vegetables and fruits, that are rich with antioxidants has been associated with various beneficial effects on human health (Cao, Booth, Sadowski, & Prior, 1998; Lotito & Frei, 2006). They can reduce the risk of oxidative damage, hence providing protection against related diseases such as cancer, diabetes and heart diseases (Nivas, Gaikwad, & Chavan, 2010; Sandoval, Okuhama, Zhang, Condezo, Lao, Angeles, Musah, Bobrowski, & Miller, 2002; Schroeder & Adams, 1941). However, some phenolic compounds can be harmful when consumed in large amounts (Schrier, 2007). One of the negative side effects is attributed to the ability of various phenolic compounds to bind and precipitate proteins (Bravo, 1998).

Gallic acid
(1.5)

Protocatechuic acid
(1.6)

Apigenin
(1.7)

Caffeic acid
(1.8)

Luteolin
(1.9)

Tyrosol
(1.10)

Hydroxytyrosol
(1.11)

Eugenol
(1.12)
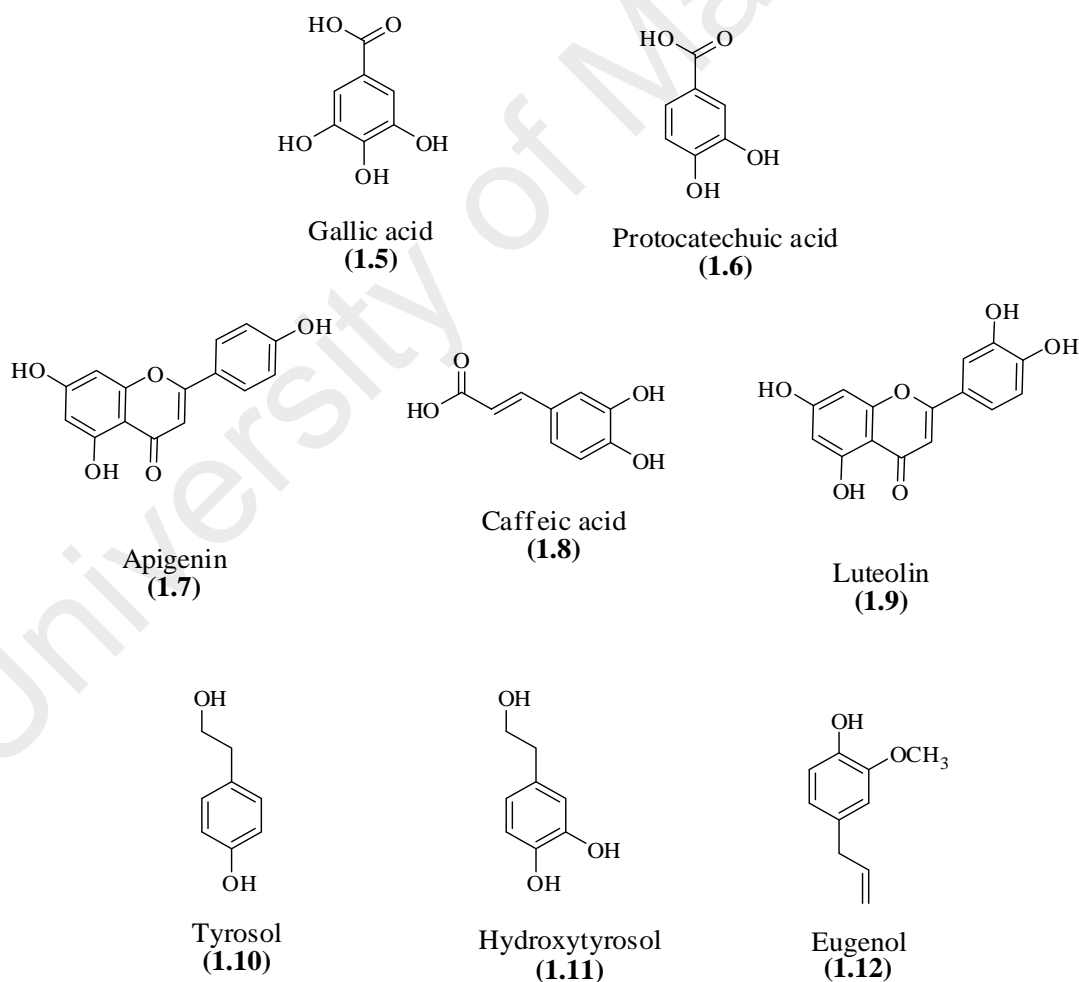
Figure 1.5. Some natural phenolic compounds

Vitamin E plays an important role in a wide spectrum of biochemical and physiological processes (Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). It is a lipophilic antioxidant, which can donate one or two H- atoms. As shown in scheme 1.1, the first H-atom transfer from vitamin E originates from the phenolic hydroxyl group does not affect the structure of vitamin E (Fuller, 2004). The free radical of vitamin E is unreactive due to resonance stabilization. The activity of vitamin E is lost when it neutralizes a free radical, but it can be regenerated by another antioxidant such as vitamin C (Shao, Chu, Lu, & Kang, 2008; Zhou, Wu, Yang, & Liu, 2005). A second H-atom can be donated without the first proton having been replenished leading to the irreversible breakage of the chromanol ring (Davies, Sevanian, Muakkassah-Kelly, & Hochstein, 1986; Fuller, 2004).
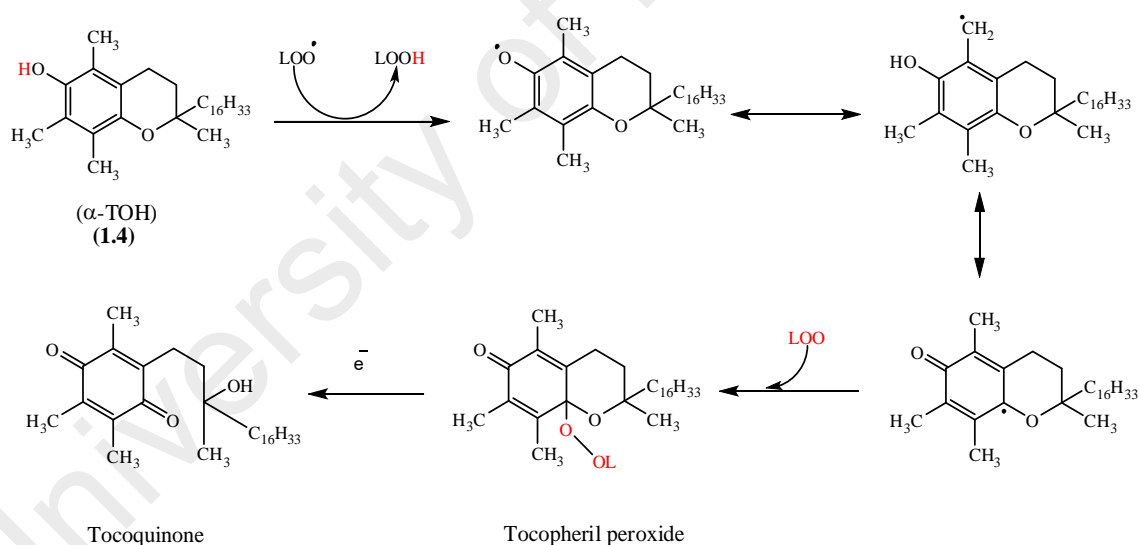
Scheme 1.1. Radical scavenging mechanism of α-TOH
(Gülçin, 2012)

Ascorbic acid is a hydrophilic antioxidant, which is an effective reductant and can terminate radical chain reactions by transfer of a single electron (Niki, 1987). A donation of one electron by ascorbate provides a resonance stabilized semi-dehydro ascorbate

radical. Ascorbic acid behaves as a vinylogous carboxylic acid where the electrons in the double bond, hydroxyl group lone pair, and the carbonyl double bond form a conjugated system. Donation of a second electron results in dehydroascorbate, which is unstable and breaks down to oxalic and threonic acid. With addition of glutathione, dehydroascorbate can be reconverted to ascorbic acid (Diplock, 1994; Meister, 1994) (Scheme 1.2).

Ascorbic Acid
(1.13)

Semi-dehydroascorbate radical
with electron delocalized
between three O atoms

Dehydroascorbate
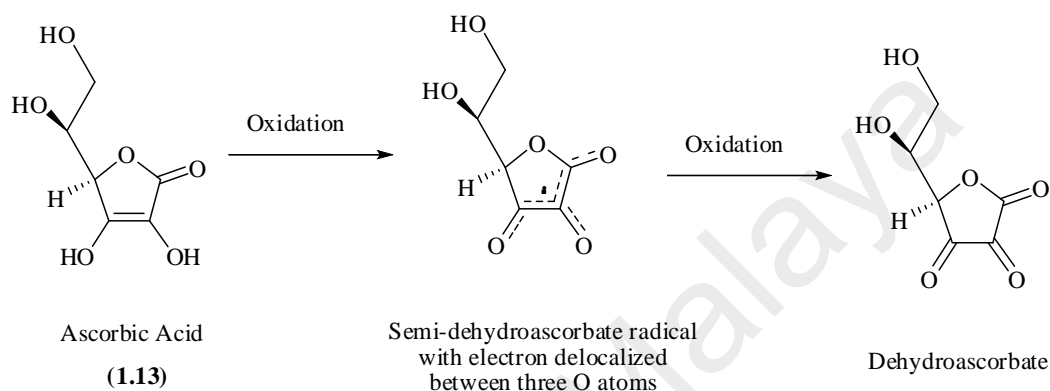
Scheme 1.2. Oxidation states of ascorbic acid
(Diplock, 1994; Meister, 1994)

1.3.2   Synthetic Antioxidants

Synthetic antioxidants have a wide range of use especially as preservative to prevent lipid peroxidation in the food and edible oil industry (Hilton, 1989; Hussain, Babic, Durst, Wright, Flueraru, Chichirau, & Chepelev, 2003). These antioxidants, which are chemically synthesized, act mainly via two different pathways and are known as primary and secondary antioxidants (Venkatesh, 2011).

1.3.2.1   Primary Antioxidants

Primary antioxidants prevent the formation of free radicals and can be classified into free radical terminators, oxygen scavengers and chelating agents. Radical terminators include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and gallates. Important examples of oxygen scavengers include sulphites, glucose oxidase and ascorbyl palmitate, while chelating agents, which are the ligand of heavy, metals such as iron and copper.

Secondary aromatic amines as primary antioxidants react with peroxyl radicals to form stable hydroperoxides by donating their hydrogen atom, as shown in Figure 1.6.
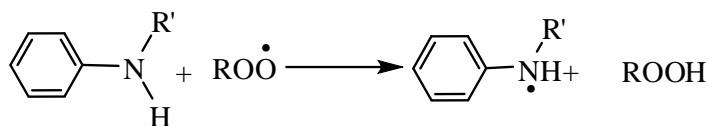
Figure 1.6. An example of a primary antioxidant acting through hydrogen donation

Polyphenols are the most widely studied compounds among the many classes of primary antioxidants in biochemical systems (Burton, Doba, Gabe, Hughes, Lee, Prasad, & Ingold, 1985; Denisov & Denisova, 1999; Halliwell, 1999; Noguchi & Niki, 2000). Phenolic antioxidants (ArOH) have recently attracted increasing interest in the pharmaceutical and food industries (Richelle, Tavazzi, & Offord, 2001). They are substances that possess an aromatic ring bearing one or more hydroxyl substituents (Packer & Sies, 2001), which can effectively quench and terminate lipid oxidation (Lodovici, Guglielmi, Casalini, Meoni, Cheynier, & Dolara, 2001). Flavonoids are the largest sub-group of phenolic compounds, covering both extracts from plants like green tea leaves, and synthetic derivatives.

They show very potent pharmacological activities, including antioxidant behaviours (Li, Liu, Zhang, & Yu, 2008; Stevenson & Hurst, 2007). They can be classified into sub classes, as shown in Figure 1.7.

Flavones
(1.14)

Flavanonols
(1.15)

Flavanones
(1.16)

Flavonols
(1.17)

Flavanols
(1.18)

Isoflavones
(1.19)
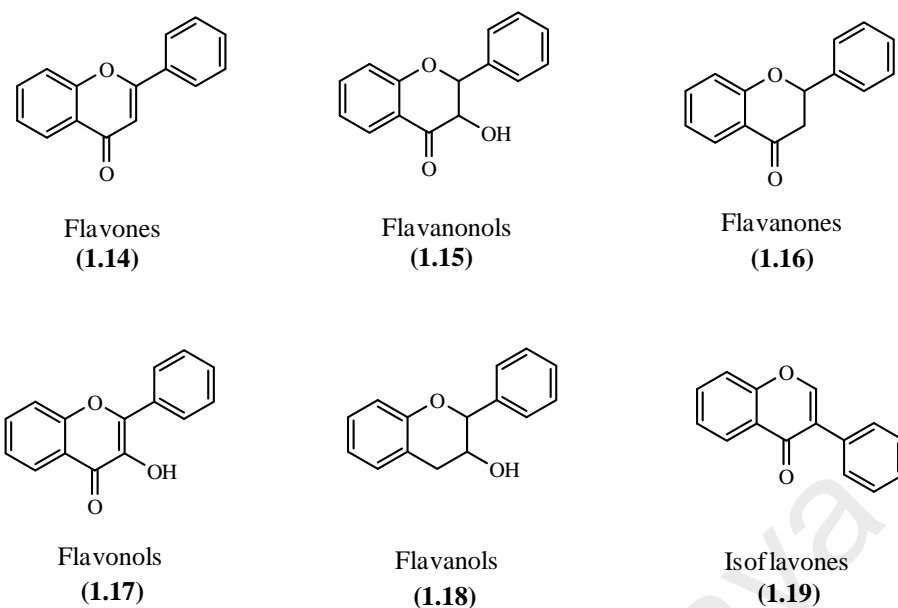
Figure 1.7. Chemical Structures of different sub classes of flavonoids

Chalcones (1,3-diarylprop-2-en-1-ones), Figure 1.8, are open-chain (bicyclic) flavonoids, defined by the presence of two aromatic rings A and B that are joined by a three-carbon α,ß-unsaturated carbonyl system. These compounds are key to many flavonoids and display a wide range of biological activity (Batovska & Todorova, 2010; Ni, Meng, & Sikorski, 2004). Hydroxyl substituted chalcones and cyclic chalcone analogues have been investigated for their antioxidant activity and have attracted much attention (Guzy, Vašková-Kubálková, Rozmer, Fodor, Mareková, Poškrobová, & Perjési, 2010; Kozlowski, Trouillas, Calliste, Marsal, Lazzaroni, & Duroux, 2007; Miranda, Stevens, Ivanov, McCall, Frei, Deinzer, & Buhler, 2000; Vogel & Heilmann, 2008).
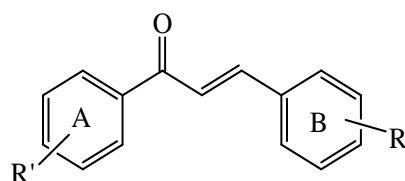
Figure 1.8. General chemical structure of chalcones

1.3.2.2 Secondary antioxidants

Secondary antioxidants such as thiodipropionic acid and dilauryl thiodipropionate, are different from chain-breaking antioxidants. They are not involved in reactions with radicals or donation of electrons, like chain-breaking antioxidants, but react with lipid peroxides (LOOH) through non-radical processes and convert them into stable end products like alcohols. Thiols (RSH), such as cysteine and gluthathione, sulphides (R-S-R), such as methionine and 3,3`-thiodipropionic acid, and free amino groups of proteins (R-NH$_2$) react with lipid peroxides to form stable products according to the reaction given below (Pokorný, Yanishlieva, & Gordon, 2001).

$$RSH + LOOH \longrightarrow R\text{-}S\text{-}S\text{-}R + LOH + H_2O \qquad 1.1$$

$$R\text{-}S\text{-}R + LOOH \longrightarrow R\text{-}SO\text{-}R + LOH \qquad 1.2$$

$$R\text{-}NH_2 + LOOH \longrightarrow R\text{-}N\,(OH)\,L + H_2O \qquad 1.3$$
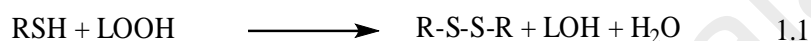
1.4 Secondary Aromatic Amine as antioxidant

Amines have received more attention than the other functional groups in organic chemistry (Brown, 1994). Due to their interesting physiological activities, in particular secondary amines are extremely important pharmacophores in numerous biologically active compounds, especially in the area of drug discovery (Insaf & Witiak, 1999; Salvatore, Yoon, & Jung, 2001).

Aromatic amines and their derivatives, which are structurally similar to phenolic derivatives, can easily transfer their amine hydrogen to peroxyl radicals (Lucarini, Pedrielli, Pedulli, Valgimigli, Gigmes, & Tordo, 1999). The inhibition rate constant (k_{inh}) of the hydrogen transfer reaction of phenolic and aromatic amines are about 4 or 5 orders of magnitude larger than the propagation rate constant (k_p), as shown in Scheme 1.3. Therefore, the antioxidants are action as inhibitors even at low concentrations, and the phenolic or amine antioxidant is able to suppress oxidative chains (Minisci, 1997(27)).

Propagation	ROO$^\bullet$ + RH	$\xrightarrow{k_p}$	ROOH + R$^\bullet$			k_p = 0.1-50 M^{-1}s^{-1}

Propagation   ROO$^\bullet$ + RH $\xrightarrow{k_p}$ ROOH + R$^\bullet$ k_p = 0.1-50 M^{-1}s^{-1}

Inhibition $\begin{cases} \text{ROO}^\bullet + \text{ArO-H} \xrightarrow{k_{inh}} \text{ROOH} + \text{ArO}^\bullet \qquad k_{inh} = 10^4\text{-}10^6 \text{ M}^{-1}\text{s}^{-1} \\ \text{ROO}^\bullet + \text{Ar}_2\text{N-H} \xrightarrow{k_{inh}} \text{ROOH} + \text{Ar}_2\text{N}^\bullet \qquad k_{inh} = 10^5 \text{ M}^{-1}\text{s}^{-1} \end{cases}$

Diamagnatic products $\begin{cases} \text{ArO}^\bullet + \text{ROO}^\bullet \longrightarrow \text{Products} \\ \text{Ar}_2\text{N}^\bullet + \text{ROO}^\bullet \longrightarrow \text{Products} \end{cases}$
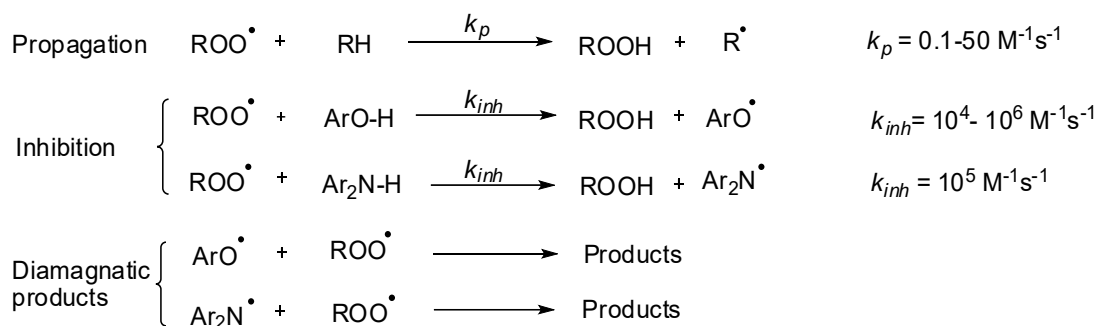
Scheme 1.3. k_p and K_{inh} of phenol and aromatic amine

1.5 Hindered Phenol as Antioxidants

The variation of phenolic antioxidant structures is important for their physical properties, resulting in differences in their antioxidant activity. Phenolic compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) represent the major families of both natural and synthetic antioxidants. Their antioxidant activity is due to the labile hydrogen atom of the phenolic OH that can be easily abstracted by peroxyl radicals sustaining the autoxidative chain reaction, and forming a relatively unreactive aryloxyl radical.

Hindered phenols, such as BHA and BHT, in which the phenolic ring contains *tert-*butyl substituents in each ortho position to the phenolic hydroxyl group, are extremely effective as primary antioxidants (Reische, Lillard, & Eitenmiller, 1998). BHA is a highly fat-soluble, antioxidant which is used widely in bulk oils as well as oil-in-water emulsions (Eskin & Przybylski, 2000). It is known as an effective co-antioxidant (CoAH) and for regeneration of other antioxidants such as BHT and α-TOH (de Guzman, Tang, Salley, & Ng, 2009; Preedy & Watson, 2006).

BHA comes in two isomers, which are; 2-*tert*-butyl-4-methoxyphenol and 3-*tert*-butyl-4-methoxyphenol, (Figure1.9, **I** and **II**), respectively. Isomer (**I**) is generally considered to be a better antioxidant than isomer (**II**) (Buck & Edwards, 1997).

I   (1.20)   II
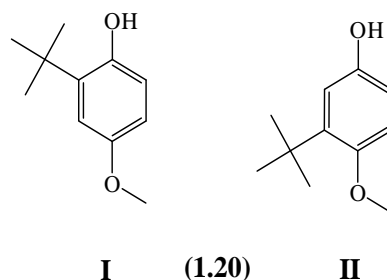
Figure 1.9. BHA isomers (I and II)

BHT is one of the most commonly used antioxidants in food containing fats, petroleum products and rubber (Dacre, 1961). It is recognized as safe for use in foods by the Federal Register in 1977 (Stuckey, 1972). BHT and its derivatives have become attractive antioxidants (Amorati, Lucarini, Mugnaini, & Pedulli, 2003).

Butylated hydroxytoluene
(BHT)
(1.21)

BHT radical-ends chain reaction

Unreactive radical because tert-butyl groups block access (steric hindrance)
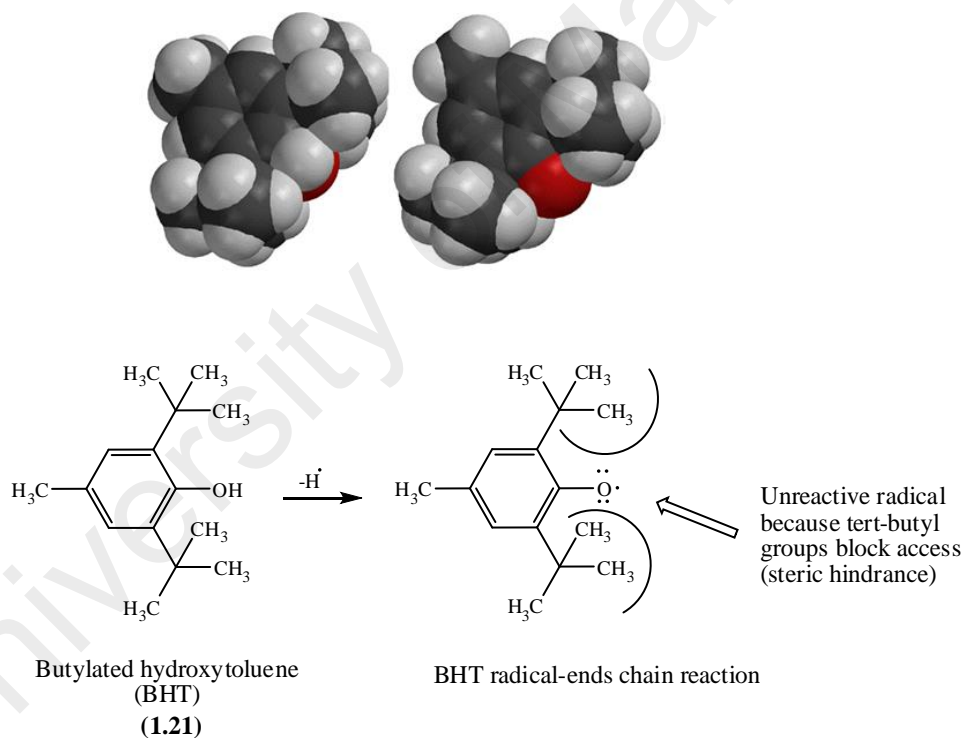
Figure 1.10. Steric hindrance effects on stabilization of phenolic antioxidants
(Ariffin, Rahman, Yehye, Alhadi, & Kadir, 2014)

It has been reported that electron-donating substituents, such as methyl and tert-butyl at the 2,4,6-positions, increase the primary antioxidant activity of phenols (Kajiyama & Ohkatsu, 2001). This is due to the stabilization of the phenoxyl radical by inductive and hyperconjugative effects. In BHT, the *di-tert*-butyl groups at the ortho

15

position provide enough steric hindrance to minimize undesirable reactions, such as pro-oxidation, and make a significant contribution to the stability of free radicals (Fukumoto & Mazza, 2000; Ross, Barclay, & Vinqvist, 2003) (Figure 1.10).

1.6   Solubility of Antioxidants

Physically, antioxidants can be categorized into two groups based on their solubility, which are hydrophilic and lipophilic antioxidants. Hydrophilic antioxidants are water-soluble. Examples are ascorbic acid and most polyphenolic compounds. Lipophilic antioxidants, on the other hand, are only lipid-soluble. Typical examples are vitamin E and carotenoids. The difference between these two types of free radical scavengers (FRS) in food is referred to as the antioxidant polar discrepancy, as shown in Figure 1.11. It is based on the observation that non-polar FRS are more effective in emulsified oils than polar FRS, whilst polar FRS are more effective than non-polar FRS in bulk oils (Alamed, 2008; McClements & Decker, 2000). The key to this fact is the ability of the FRS to accumulate in a medium where lipid oxidation is most common. Polar FRS concentrate at oil-air or oil-water interfaces in bulk oils, where most oxidation occurs due to the high concentrations of oxygen and prooxidants. Non-polar FRS accumulate in the lipid phase and at the oil-water interface in emulsions, where interactions occur between hydroperoxides at the droplet surface and prooxidants in the aqueous phase (Decker, 1998).
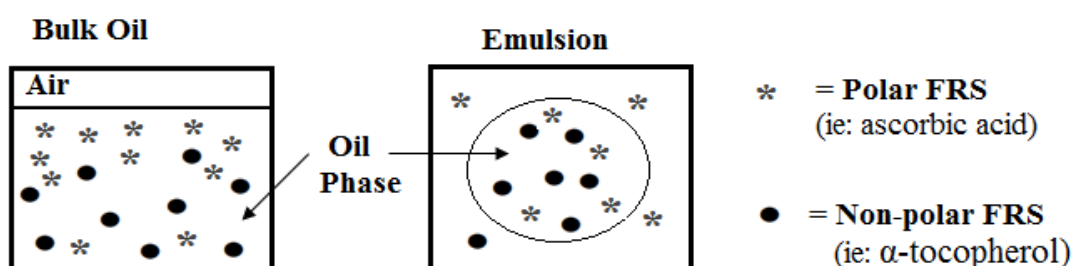
Figure 1.11. Physical location of hydrophilic and hydrophobic free radical scavengers in bulk oils and oil-in-water emulsions

Many di-*tert*-butylphenol bicyclic derivatives are highly lipophilic, resulting in generally poor aqueous solubility. Otherwise, addition of ionizable functionality to the heterocyclic ring (hydrophilic) linked to the phenol ring would reduce their lipophilicity and support in absorption and elimination of these compounds.

1.7   Mechanism of action of antioxidants

Free radical scavenging capacity of substituted phenols (ArOH) is generally attributed to the liberation of hydrogen atom from their phenolic hydroxyl group to a peroxyl radical (ROO$^{\bullet}$) at a much faster rate than that of radical propagation (Leopoldini, Russo, & Toscano, 2011; Trouillas, Marsal, Siri, Lazzaroni, & Duroux, 2006). Nitrogen and sulfur analogs may provide alternatives for labile hydrogen in some other antioxidants (esp. ArXH, with X = O, N, S) (Netto, Chae, Kang, Rhee, & Stadtman, 1996; Padmaja, Rajasekhar, Muralikrishna, & Padmavathi, 2011).

There are controversies in the literature over the specific mechanistic pathway for antioxidant compounds, especially polyphenols. Several articles stated that phenols could donate hydrogen atoms from the phenolic group (Barclay, Edwards, & Vinqvist, 1999; Khopde, Priyadarsini, Venkatesan, & Rao, 1999). Phenolic antioxidants are excellent candidates as free radical scavengers due to resonance bond delocalization of the unpaired electron by the aromatic ring that stabilizes the radical (Shahidi, Janitha, & Wanasundara, 1992).

Antioxidants (ArXH) can deactivate radicals (R$^{\bullet}$) via several mechanisms, such as Hydrogen Atom Transfer (HAT), Proton-Coupled Electron Transfer (PCET), Electron Transfer–Proton Transfer (ET–PT) or Sequential ET–PT (SET–PT), Sequential Proton-Loss-Electron Transfer (SPLET), and Single Electron Transfer (SET) (Anouar, Raweh, Bayach, Taha, Baharudin, Di Meo, Hasan, Adam, Ismail, Weber, & Trouillas, 2013). In this study only the two major mechanisms, i.e. HAT and SET, will be considered.

1. In hydrogen atom transfer (HAT), a phenolic H-atom is transferred in a single step.

$$\text{ArX-H} \; + \; \text{R}^{\bullet} \longrightarrow \text{ArX}^{\bullet} \; + \; \text{R-H} \qquad 1.4$$

2. Single electron transfer (SET), involves two steps; the initial formation of a radical cation from ArXH by electron transfer [eq.1.5] is followed by rapid deprotonation of ArXH$^{+\bullet}$ [eq.1.6], which compensates the charge of the initial anion R^{-} [eq.1.7] (Bakalbassis, Lithoxoidou, & Vafiadis, 2006).

$$\text{ArXH} \; + \; \text{R}^{\bullet} \longrightarrow \text{ArXH}^{+\bullet} \; + \; \text{R}^{-} \qquad 1.5 \quad \text{(electron transfer)}$$

$$\text{ArXH}^{+\bullet} \longrightarrow \text{ArX}^{\bullet} \; + \; \text{H}^{+} \qquad 1.6 \quad \text{(deprotonation)}$$

$$\text{R}^{-} \; + \; \text{H}^{+} \longrightarrow \text{RH} \qquad 1.7 \quad \text{(hydroperoxide formation)}$$
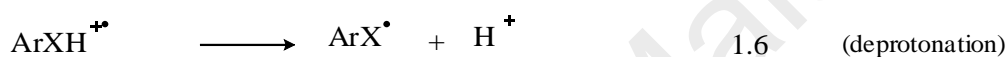
The final result is usually the same, regardless of the mechanism. However, kinetics and potential for side reactions are different. HAT reaction mechanism dominating in a given system will be determined by antioxidant structure and properties, solubility and solvent system (Prior, Wu, & Schaich, 2005). Polar aprotic solvents reduce the rate of many ArXH/R reactions. This has been explained based on hydrogen bonding of ArXH to the solvent. The strong bonding of the hydrogen prevents the HAT. Only the free (non-hydrogen-bonded) fraction of ArXH is capable of transferring an H-atom to R$^{\bullet}$ (Barclay, Edwards, & Vinqvist, 1999; Foti, Barclay, & Ingold, 2002).

It has been reported that electron-donating substituents, such as alkyl or alkoxyl at the ortho positions to the phenolic group, increase the primary antioxidant activity of phenols by increasing the rate of hydrogen atom transfer from the phenolic group (Nishida & Kawabata, 2006; Rao, Swamy, Chandregowda, & Reddy, 2009). This is due to the lowering of the bond dissociation enthalpy (*BDE)* of the phenol O-H group (Lucarini,

Pedulli, & Cipollone, 1994), and the stabilization of the phenoxyl radical by inductive and hyperconjugative effects.

1.8   Assay methods for antioxidant activities

Analytical methods are available to assess antioxidant activities of natural and synthetic compounds in foods or biological systems (Gupta, Sharma, Lakshmy, Prabhakaran, & Reddy, 2009; Lanigan & Yamarik, 2002; Wang, Chu, Chu, Choy, Khaw, Rogers, & Pang, 2004). These methods must be selected in relation to the characteristic (advantages and disadvantages) of a particular method. The characteristic such as targeting information (chemical parameters, total antioxidant capacity), sensitivity, and cost, should be considered to determine the most useful methods for a specific situation.

Generally, antioxidant activity assays can be categorized into two types according to the reaction mechanism involved, which are assays based on H-atom transfer reactions and the other is based on single electron transfer (Turrens, 2003). Free radical scavenging assays that are commonly used to evaluate antioxidant activity *in vitro* are: ABTS or 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), which is radical cation scavenging assay (Cano, Alcaraz, Acosta, & Arnao, 2002). Oxygen Radical Absorption Capacity (ORAC), (Ou, Hampsch-Woodill, & Prior, 2001) (FRAP) Ferric Reducing Antioxidant Power assay (Benzie & Strain, 1996) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assay (Blois, 1958).

In the present study, the antioxidant activities of synthesized compounds were tested *in vitro* using DPPH and FRAP assays, the two most common antioxidant assays (Ahmad, Mehmood, & Mohammad, 1998; Kuş, Ayhan-Kılcıgil, Özbey, Kaynak, Kaya, Çoban, & Can-Eke, 2008).

1.8.1   DPPH radical scavenging assay

2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) radical scavenging assay is often used as a quick and reliable parameter to investigate the antioxidant activities of phenolic compounds (Göktürk Baydar, Özkan, & Yaşar, 2007; Rice-Evans, Miller, & Paganga, 1996; Van Acker, Tromp, Griffioen, Van Bennekom, Van Der Vijgh, & Bast, 1996). DPPH is a stable free radical that can accept a hydrogen radical or an electron to become a stable molecule and anion, respectively (Blois, 1958). Due to the delocalization of the unpaired electron over the entire structure, DPPH is a relatively stable free radical (Figure 1.12) (Ionita, 2005).

(**1.22**)

Figure 1.12. The structure of the free stable radical of DPPH
(Ionita, 2005)

DPPH induces dehydrogenation on phenols, and changes into DPPH-H, as shown in Figure 1.13.

DPPH$^\bullet$ DPPH-H

Figure 1.13. DPPH reactions with antioxidant (ArXH)

In methanolic medium, the odd electron configuration in DPPH shows a strong absorption band at 515 nm, which is reflected in the purple colour of the solution. The molecular absorbance short to short wavelength upon radical scavenging, resulted in a colour change from deep purple to yellow (Eklund, Långvik, Wärnå, Salmi, Willför, & Sjöholm, 2005; Sharma & Bhat, 2009). This behaviour enables a simple photometric measurement of the remaining DPPH concentration. However, the DPPH radical scavenging ability strongly depends on the geometric accessibility of the radical trapping site.

The presence of steric hindrance may prevent a test compound from reaching the radical site of DPPH, resulting in a low activity (Faria, Calhau, de Freitas, & Mateus, 2006). Nevertheless, it has been proven that the DPPH assay is suitable for measuring HAT type antioxidant behaviour in an effective way (Goupy, Dufour, Loonis, & Dangles, 2003; Goupy, Hugues, Boivin, & Amiot, 1999). HAT is the key step in the radical chain reaction. It was found to be the preferred and dominant mechanism of antioxidant action for polyphenols under neutral to acidic conditions and in non-protic solvents (Bors, Heller, Michel, & Saran, 1990; Zhang, Sun, Zhang, & Chen, 2000). Scheme 1.4, shows the eugenol-DPPH radical scavenging effect by the HAT mechanism.

(1.23)
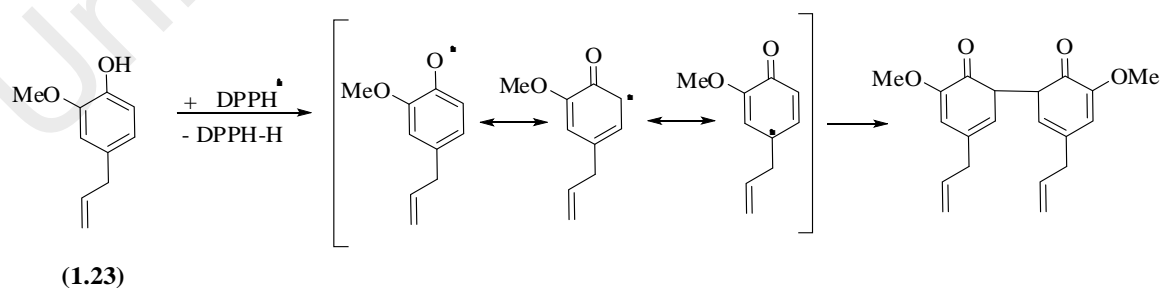
Scheme 1.4. Eugenol – DPPH radical scavenging by HAT mechanism

(Bortolomeazzi, Verardo, Liessi, & Callea, 2010)

1.8.2   Ferric Reducing Antioxidant Power assay (FRAP)

Reductant and oxidant are chemical terms, whereas antioxidant and pro-oxidant have meaning in the context of a biological system. An antioxidant that can effectively reduce pro-oxidants may not be able to sufficient reduce Fe^{3+}. An antioxidant is a reductant, but a reductant is not necessarily an antioxidant (Prior & Cao, 1999).

The FRAP assay is a simple, versatile and low-cost test that is commonly used to evaluate the antioxidant activities of synthetic and natural products (Benzie & Strain, 1996). It measures the reducing capacity of Fe^{3+} ions in acidic medium (Benzie & Strain, 1999), based on the reduction of the ferric tripyridyltriazine $[Fe(TPTZ)_2]^{3+}$ complex to ferrous tripyridyltriazine $[Fe(TPTZ)_2]^{2+}$ (Figure 1.14) by the transfer of a single electron from an antioxidant compound, according to the equation below:

$$[Fe\,(TPTZ)_2]^{3+} +\ ArH \xrightarrow{\ +e\ } [Fe\,(TPTZ)_2]^{2+} +\ Ar^{\cdot +} \qquad 1.8$$

Fe³⁺-TPTZ
(1.24)

Fe²⁺-TPTZ
(1.25)
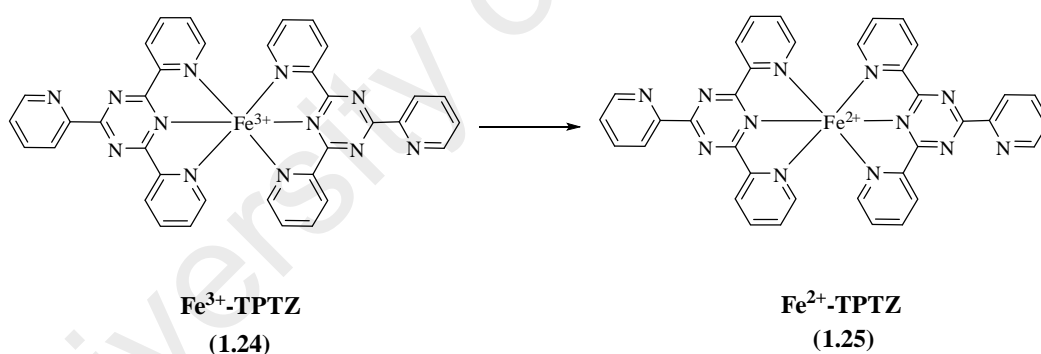
Figure 1.14. Chemical structures of compounds bearing methoxy group

The FRAP assay utilizes the SET mechanism to determine the capacity of an antioxidant in the reduction of an oxidant. It is strongly solvent dependent. The expected solvent effect for SET is an increase with increasing polarity and hydrogen bonding ability of the solvent (J. S Wright, E. R. Johnson, & G. A. DiLabio, 2001).

Upon reduction, the ferric colour changes (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005), and the degree of the colour change can be correlated with the

concentration of antioxidants in the sample. It is also used as an indicator to track the end point of the reaction (Forman, Maiorino, & Ursini, 2010). However, the reducing capacity of a sample is not directly related to its radical scavenging capability.

In order to accurately measure the total reducing power, certain condition must apply;(1) the redox potential of the antioxidant must be sufficed to reduce $[Fe(TPTZ)_2]^{3+}$ (thermodynamic requirement), (2) the reaction rate must be sufficiently fast to enable completion of the reaction within the 4-min reaction time (kinetic requirement), (3) the oxidized antioxidant ArXH and its secondary reaction products should have no absorption at 595 nm, the maximum absorption of $[Fe(TPTZ)_2]^{2+}$. These conditions are sometimes difficult to meet. In particular, not all antioxidants are able to reduce ferric ions at the required fast rate (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002).

1.9   Methoxy groups in drug molecular structures

Methoxy groups on aromatic systems have been extensively investigated for their well-known antioxidant effect (Marinho, Pedro, Pinto, Silva, Cavaleiro, Sunkel, & Nascimento, 2008). These groups are important in cytotoxic and microtubule-binding agents used for cancer chemotherapy (Ağırtaş, Cabir, & Özdemir, 2013; Odlo, Fournier-Dit-Chabert, Ducki, Gani, Sylte, & Hansen, 2010). For instance, structure activity relationship (SAR) studies on combretastatin A-4 (CA-4), which is an anti-tumuor drug from the combretastatin group (Nam, Kim, You, Hong, Kim, & Ahn, 2002), have shown that the 3,4,5-trimethoxy substitution on ring A is important for its cytotoxic activity (Medarde, Maya, & Pérez-Melero, 2004; Odlo, Hentzen, Fournier dit Chabert, Ducki, Gani, Sylte, Skrede, Flørenes, & Hansen, 2008). More recently, 2,4,5-trimethoxy chalcones and their analogues 2,4,5-trimethoxy-2`,5`-dihydroxychalcone (TMDC), have shown superior DPPH radical scavenging activities, Figure 1.15 (Shenvi, Kumar, Hatti, Rijesh, Diwakar, & Reddy, 2013).

CA-4
(1.26)

TMDC
(1.27)
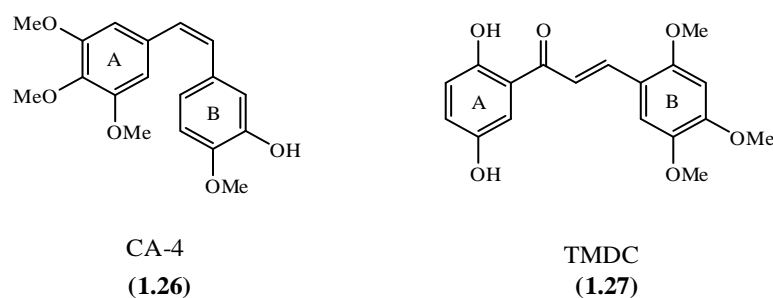
Figure 1.15. Chemical structures of compounds bearing methoxy group

With widespread usage of antibiotics in medicinal, agricultural, and livestock industries, increasing amounts of antibiotic contaminants are being discharged into the aquatic environment, which can subsequently be introduced to drinking water sources. Although typically present at low concentrations, the antibiotic contaminants in water sources may pose human health and ecological risks attributable to their potential to foster prevalence and proliferation of antibiotic resistance in bacterial species (Jury, Khan, Vancov, Stuetz, & Ashbolt, 2011). Another example of methoxy group in aromatic system is Trimethoprim (TMP), as shown in Figure 1.16, has been commonly used in combination with sulfamethoxazole (a sulfonamide antibiotic), known as cotrimoxazole, to reduce the antibiotic resistance of sulfamethoxazole since 1969. TMP is among the most frequently detected antibiotics in the environment, and it has been detected in municipal wastewater effluent at concentrations of several hundred nanograms per liter (Göbel, Thomsen, McArdell, Joss, & Giger, 2005; Gulkowska, Leung, So, Taniyasu, Yamashita, Yeung, Richardson, Lei, Giesy, & Lam, 2008; Lin, Yu, & Lateef, 2009; Lindberg, Wennberg, Johansson, Tysklind, & Andersson, 2005) and in surface water bodies at concentrations from 10 to several hundred nanograms per liter (Christian, Schneider, Färber, Skutlarek, Meyer, & Goldbach, 2003; Glassmeyer, Furlong, Kolpin, Cahill, Zaugg, Werner, Meyer, & Kryak, 2005; Kolpin, Furlong, Meyer, Thurman, Zaugg, Barber, & Buxton, 2002; Roberts & Thomas, 2006).
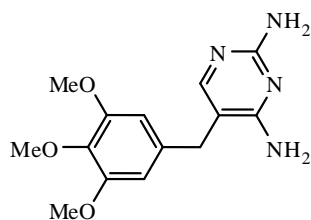
TMP

(1.27)

Figure 1.16. Chemical structures of compound Trimethoprim

The reactivity of TMP to chlorine dioxide (ClO_2) which is one of the common water disinfection oxidants resides mostly in its diaminopyrimidinyl moiety at neutral to alkaline pH, but centres in its trimethoxybenzyl moiety at acidic conditions. Overall, transformation of TMP by ClO_2 can be expected under typical ClO_2 disinfection conditions (Wang, He, & Huang, 2011). TMP also is used in the treatment of *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) (Cody, Luft, Pangborn, Gangjee, & Queener, 2004).
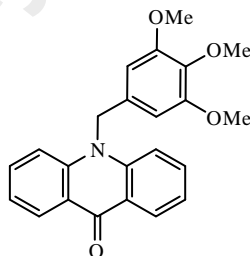
(1.28)

Figure 1.17. Chemical structures of compound acridinone derivative

Compound 10-(3,4,5-dimethoxy)benzyl-9(10H)-acridinone (Figure 1.17), which is one of the acridinone derivatives, was tested for its in vitro antitumor activities against CCRF-CEM leukemia cells. This compound showed highly activity with IC_{50} at about 1.5 µM. Assay-based antiproliferative activity study using CCRF-CEM cell lines

revealed that the acridone group and the substitution pattern on the benzene unit had significant effect on cytotoxicity activities (Gao, Jiang, Tan, Zu, Liu, & Cao, 2008).

1.10 Biological activities of hydrazones

Imine and hydrazone compounds are a class of organic compounds containing the azomethine or carbon nitrogen (HC=N) double bond. They were first prepared by the German chemist Hugo Schiff and are today known as Schiff base (Moffett & Rabjohn, 1963, 4). Aromatic hydrazones are more stable and more easily synthesized than the ones derived from aliphatic aldehydes, which readily polymerize (Engel, Yoden, Sanui, & Ogata, 1985). Hydrazones are some of the most widely used organic compounds. They are used as pigments and dyes, catalysts, inter-mediates in organic synthesis, and as polymer stabilizers (Dhar & Taploo, 1982). They have also been shown to exhibit a broad range of biological activities, such as analgesic (Gökçe, Utku, & Küpeli, 2009), anti-inflammatory (Sondhi, Dinodia, & Kumar, 2006), anticancer (Terzioglu & Gürsoy, 2003), including antitumoral (Mladenova, Ignatova, Manolova, Petrova, & Rashkov, 2002; Walsh, Meegan, Prendergast, & Al Nakib, 1996), antifungal (Al-Amiery, Al-Majedy, Abdulreazak, & Abood, 2011; Panneerselvam, Nair, Vijayalakshmi, Subramanian, & Sridhar, 2005; Singh, Barwa, & Tyagi, 2006; Sridhar, Saravanan, & Ramesh, 2001), antibacterial (Abu-Hussen & A., 2006; Karthikeyan, Prasad, Poojary, Subrahmanya Bhat, Holla, & Kumari, 2006), antimalarial, antimicrobial (Sharma, Parsania, & Baxi, 2008), and antipyretic properties (Dhar & Taploo, 1982; Przybylski, Huczynski, Pyta, Brzezinski, & Bartl, 2009). The combination of hydrazones with other functional groups can lead to compounds with unique physical and chemical characteristics (Xavier, Thakur, & Marie, 2012). They are considered important intermediates for the synthesis of heterocyclic compounds (Banerjee, Mondal, Chakraborty, Sen, Gachhui, Butcher, Slawin, Mandal, & Mitra, 2009). Imine or

azomethine groups are present in various natural, and non-natural biologically active compounds. It has been shown that these groups are critical to their biological activities (Mishra, Kale, Singh, & Tiwari, 2009; Przybylski, Huczynski, Pyta, Brzezinski, & Bartl, 2009). For example, the chemically-synthesized hydrazones that are shown in Figure 1.18, showed good bio availability and significant antioxidant activities.
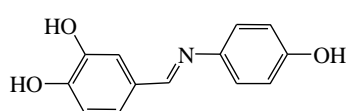
4-((4-hydroxyphenylimino)
methyl)benzene-1,2-diol
(1.29)
(Cheng et al., 2010)
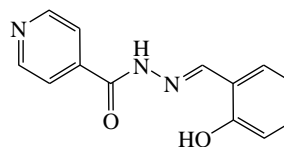
N-(4-hydroxybenzylidene)-2-
methoxy benzohydrazide
(1.30)
(Hruskova et al., 2011)
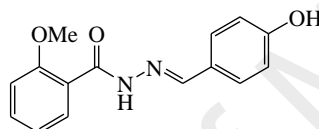
N-(4-hydroxybenzylidene)-2-
methoxybenzohydrazide
(1.31)
(Anouar et al., 2013)

Figure 1.18. Examples of antioxidant hydrazones
(Cheng, Tang, Luo, Jin, Dai, Yang, Qian, Li, & Zhou, 2010), (Hruskova, Kovarikova, Bendova, Haskova, Mackova, Stariat, Vavrova, Vavrova, & Simunek, 2011), (Anouar, Raweh, Bayach, Taha, Baharudin, Di Meo, Hasan, Adam, Ismail, Weber, & Trouillas, 2013)

1.11   The significance of five membered heterocyclic ring

Heterocycles are an important class of compounds. Their numbers enormous, making up more than half of all known organic compounds, and the number is increasing rapidly. Accordingly the literature on the subject is very vast. Heterocycles are present in a wide variety of drugs (Bräse, Gil, Knepper, & Zimmermann, 2005), most vitamins and many natural products. Also the major components of biological molecules, such as DNA and RNA, and many biologically active compounds are heterocyclic. Besides biological

activity, Most heterocycles possess important applications in materials science, such as dyestuff, fluorescent sensor, brightening agents, information storage, plastics, and analytical reagents (Fei & Gu, 2009). Moreover, they also act as organic conductors, semiconductors, molecular wires, photovoltaic cells, and organic light-emitting diodes (OLEDs) (Facchetti, 2010; Perepichka & Perepichka, 2009). It has been reported that certain compounds bearing 1,3,4-oxadiazole/thiadiazole and 1,2,4-triazole nucleus possess significant biological activity (Mullican, Wilson, Conner, Kostlan, Schrier, & Dyer, 1993; Tozkoparan, Gökhan, Aktay, Yeşilada, & Ertan, 2000).

1.12   Biological activities of 1,3,4-oxadiazole

The capability of the 1,3,4-oxadiazole nucleus to undergo a variety of chemical reactions has made them important building blocks in synthesis of compounds with potential use in medicinal chemistry. Documented data suggest that 1,3,4-oxadiazoles possess strong antiallergic, urease inhibitory, cytotoxic, muscle relaxant, photo and electron luminescence effects. A majority of oxadiazole derivatives are known to exhibit antioxidant ability. Examples of some of these compounds are shown in Figure 1.19.
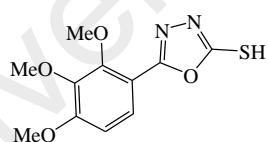
5-(2,3,4-Trimethoxyphenyl)-
[1,3,4]-oxadiazole-2-thiol
(1.32)
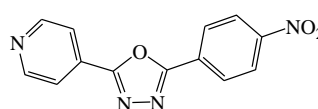
4-[5-(4-Nitrophenyl)-
[1,3,4 -oxadiazole] -2-yl]pyridine
(1.33)
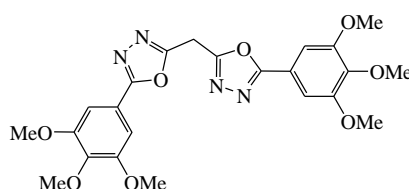
Bis (5-(3,4,5-trimethoxyphenyl)-
1,3,4-oxadiazol-2-yl)methane

(1.34)

Figure 1.19. Examples of 1,3,4-oxadiazole derivatives that can act as antioxidants

1.13   Biological Activities of Thiosemicarbazide

Thiosemicarbazides are convenient precursors, which have been extensively utilized in heterocyclic synthesis. In terms of biological activity, thiosemicarbazides are useful intermediates and subunits for the development of molecules with pharmaceutical potential. Within the last few years, an increased interest in the chemistry of thiosemicarbazide has developed. Thiosemicarbazides exhibit various biological activities including, antiviral, anticancer, antibacterial, antifungal and antimalarial (Garoufis, Hadjikakou, & Hadjiliadis, 2009; Pandeya, Sriram, Nath, & DeClercq, 1999; Pingaew, Prachayasittikul, & Ruchirawat, 2010; Zhang, Qian, Zhu, Yang, & Zhu, 2011). A selection of thiosemicarbazide, which exhibit antioxidant activities, are shown in Figure 1.20.
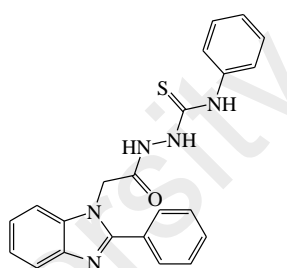
N-phenyl-2-(2-(2-phenyl-1H-
benzo[d]imidazole-1-yl) acetyl)
hydrazinecarbothioamide

(1.35)
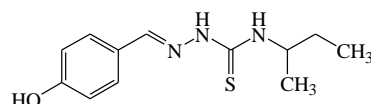
(2E)-2-(4-Hydroxybenzylinene)-N-
(Propan-2-yl) hydrazinecarbothioamide

(1.36)
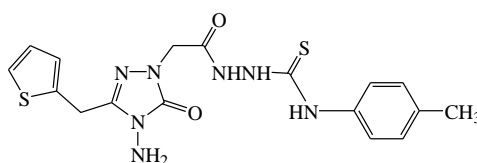
2-(2-(4-amino-5-oxo-3-(thiophen-2-ylmethyl)-
4,5-dihydro-1H-1,2,4-triazol-1-yl)acetyl)-N-(4-
methylphenyl)hydrazinecarbothioamide

(1.37)

Figure 1.20. Thiosemicarbazide derivatives as antioxidant

1.14   Biological Activity of 1,2,4-Triazole

The synthesis of 1,2,4-triazole derivatives and investigation of their chemical and biological behaviour have gained interest in recent decades for potential medicinal application. The 1,2,4-triazole is by far the most widely studied isomer. These compounds possess various medicinal properties, such as hypoglycaemic (Anton-Fos, Garcia-Domenech, Perez-Gimenez, Peris-Ribera, Garcia-March, & Salabert-Salvador, 1994), antimicrobial (Patel, Khan, & Rajani, 2010), anti-inflammatory (Turan-Zitouni, Kaplancikli, Ozdemir, Chevallet, Kandilci, & Gumusel, 2007), anticonvulsant (Gülerman, Rollas, Kiraz, Ekinci, & Vidin, 1997), antitubercular (Suresh Kumar, Rajendraprasad, Mallikarjuna, Chandrashekar, & Kistayya, 2010), and antidepressant activities (Kane, Dudley, Sorensen, & Miller, 1988). There are several drugs available, which contain triazole rings, such as the antifungal drugs, displayed in Figure 1.21.

Fluconazole
(1.38)

Iitraconazole
(1.39)
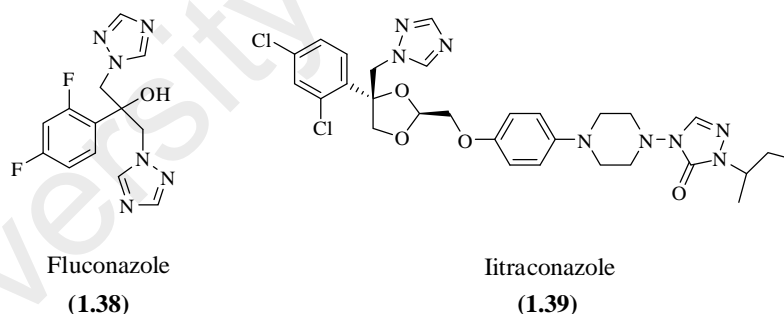
Figure 1.21. Drugs containing triazole ring

A series of 1-(3,4,5-trimethoxyphenyl)-5-aryl-1,2,4-triazoles (Figure 1.22), were synthesized as *cis*-restricted combretastatin analogues and evaluated for antiproliferative activity, inhibitory effects on tubulin polymerization, cell cycle effects, and apoptosis induction.

(1.40)

R= alkyl, alkoxy, halogen
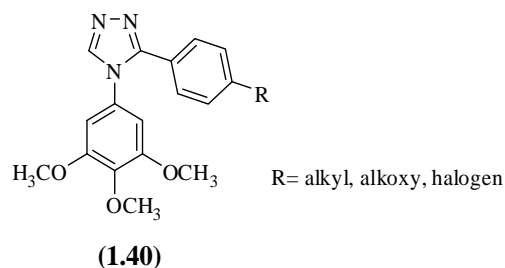
Figure 1.22. Synthesis of 1-(3,4,5-trimethoxyphenyl)-5-aryl-1,2,4-triazoles compounds as cis-restricted combretastatin analogues

Their activity exceeded or was comparable with combretastatin, implying their potential anti-cancer effects.(Romagnoli, Baraldi, Cruz-Lopez, Lopez Cara, Carrion, Brancale, Hamel, Chen, Bortolozzi, Basso, & Viola, 2010) 1,2,4-triazole also possess urease inhibitory action and antioxidant activities (Khan, Ali, Hameed, Rama, Hussain, Wadood, Uddin, Ul-Haq, Khan, Ali, & Choudhary, 2010). Example as is shown in Figure 1.23.

3-(4-chlorophenyl)-4-(2,4-dimethylphenyl)-
1H-1,2,4-triazole-5(4H)-thione

(1.41)
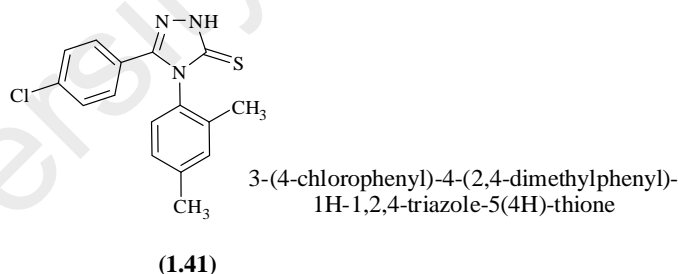
Figure 1.23. 1,2,4-Triazole as an antioxidant

This research work involves synthesis and investigation of antiradical potential of a series of compounds bearing 3,4,5-trimethoxybenzyloxy, (Figure 1.24).
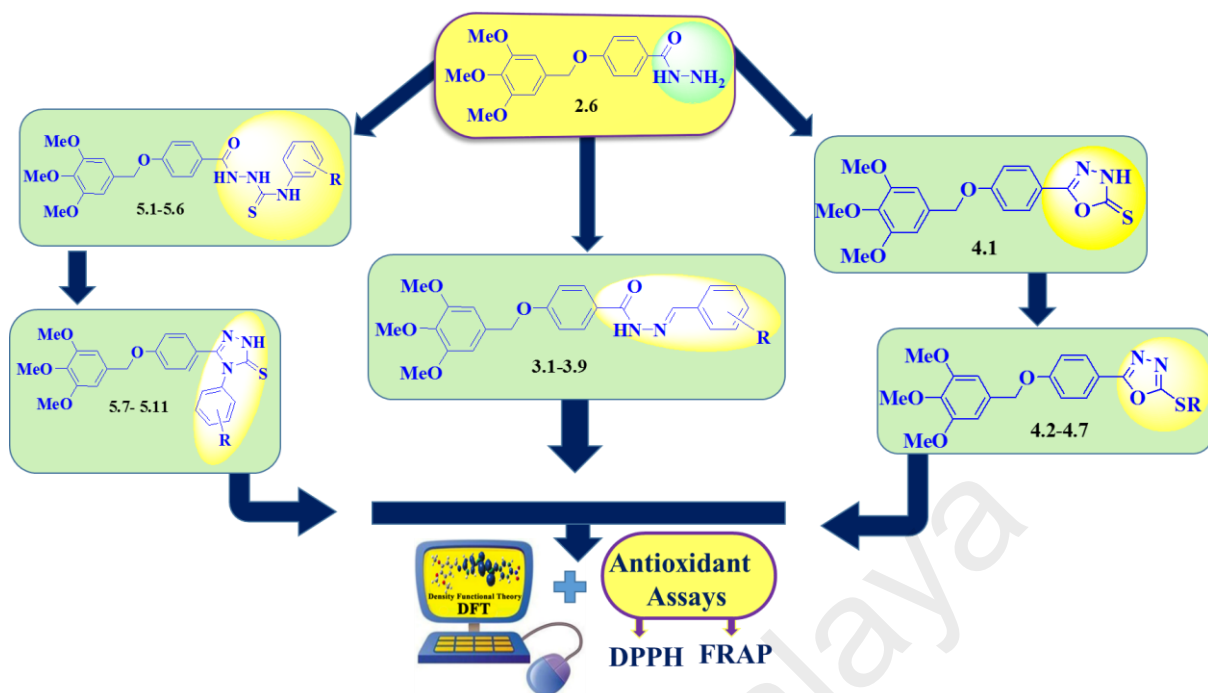
Figure 1.24. Thesis graphical abstract of the synthesized compounds

1.15   Optimization of antioxidant activity by theoretical study

Due to the rapid advancement in computer technology, theoretical calculations have gained popularity amongst the scientific community. Density functional theory (DFT) is one of the most widely used methods for calculations of the structure of atoms, molecules, crystals, surfaces, and their interactions. The basic principle behind the DFT is that the energy of a molecule can be determined from the electron density instead of a wave function.
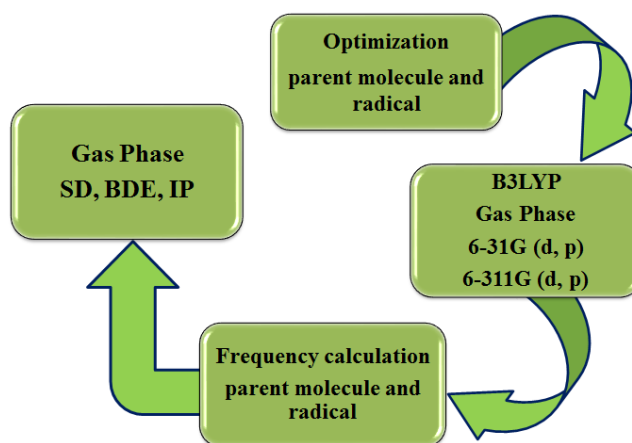
Figure 1.25. Steps involved in the computational method

These calculations are based on the Hohenberg-Kohn theorem, according to which, the electron density can be used to determine all properties of a system. The efficiency of the computational approach depends on the level of the applied theory. Figure 1.25, described the DFT theory steps. The antioxidant abilities of compounds can be related to a calculated spin density (SD) of related radical species (Leopoldini, Pitarch, Russo, & Toscano, 2004). A more delocalized spin density in the radical reflects high stability, thus favouring the radical formation.(Parkinson, Mayer, & Radom, 1999) Therefore, lower SD values indicate better antioxidants. For the HAT mechanism, the bond dissociation enthalpy (BDE) of the hydrogen is a good primary indicator for antioxidant reactivity (DiLabio, Pratt, LoFaro, & Wright, 1999). The antioxidant activity reflected in the calculated BDE values is often attributed to π electron delocalization, leading to the stabilization of the radicals obtained after H-abstraction. It is assumed that if π electron delocalization exists in the parent molecule, it also exists in the corresponding radical (Trouillas, Marsal, Siri, Lazzaroni, & Duroux, 2006).

The ease of the homolytic cleavage of the X-H bond, reflecting the efficacy of an antioxidant, can be estimated by calculating the bond dissociation enthalpy (BDE) of the respective X-H bond were (X = O, N, S, C). Lower BDEs indicate weaker X-H bond, from which the hydrogen is more easily extracted (Lucarini, Pedrielli, Pedulli, Cabiddu, & Fattuoni, 1996).

Another factor influencing antioxidant reactivity is the molecular orbital of the radicals, consisting of the highest occupied molecular orbital (HOMO) and the lowest occupied molecular orbital (LUMO). The HOMO energy which indicates electron-giving ability is suitable for representing the free radical scavenging potential of the compounds, since the process to impede the auto-oxidation may involve not only the H-atom abstraction but also electron-transfer (Burton & Wiemers, 1985; DiLabio, Pratt, LoFaro, & Wright, 1999). According to SET mechanism, the ionization potential (IP) is the most

significant energetic factor for scavenging activity evaluation, which is related to the π electron delocalization (Trouillas, Marsal, Siri, Lazzaroni, & Duroux, 2006), whereby a lower IP value will result in an easier electron transfer (Rojano, Saez, Schinella, Quijano, Vélez, Gil, & Notario, 2008).

1.16   Study Objectives

The oxidative stress which is occur by free radical, causing damage to normal cells including lipids, DNA and proteins and lead to cellular injury and many serious diseases. It is well known, the antioxidant compounds are protect the cells as well as the organ systems of the body against free radical. Therefore, in this study a new series of potential antioxidant bearing 3,4,5-trimethoxybenzyl moiety have been synthesized, as shown in Figure 1.24. The objectives for this project are to:

1. Design, synthesize and characterize multipotent antioxidants (MPAO) containing a Schiff base or a five membered heterocyclic ring, which are 1,3,4-oxadiazole and 1,2,4-triazole.
2. Verify and evaluate the antioxidant activities of the synthesized compounds using *in vitro* DPPH and FRAP antioxidant assays.
3. Rationalize the antioxidant behaviour of selective synthesized compounds based on theoretical calculation.

2.1   Introduction

This chapter deals with synthesis and characterization of the starting materials, the ester ethyl 4-(3,4,5-trimethoxybenzyloxy)benzoate and the acid hydrazide 4-(3,4,5-trimethoxybenzyloxy)benzohydrazide. The structures were determined by IR, (^{1}H, ^{13}C) NMR and MS (HREIMS).

2.2   Synthesis of the acid hydrazide 2.6

The first target compound, 3,4,5-trimethoxybenzyl bromide (**2.2**), was prepared according to a procedure found in the literature (Mabe, 2006). Compound **2.2** was easily obtained by reaction of 3,4,5-trimethoxybenzyl alcohol (**2.1**) with phosphorous tribromide in Et$_2$O, (Scheme 2.1).
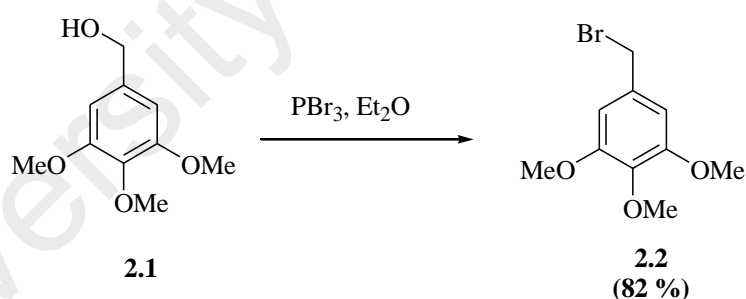
Scheme 2.1: Preparation of compound 2.2

In organic synthesis, Fischer esterification is one of the most common methods (Joseph, Sahoo, & Halligudi, 2005; Yamamoto, Shimizu, & Hamada, 1996). Esterification of 4-hydroxybenzoic acid (**2.3**) was achieved using a large excess of absolute ethanol to shift the equilibrium towards the product formation. The ester, ethyl 4-hydroxybenzoate (**2.4**) was obtained in 96 % yield (Scheme 2.2).
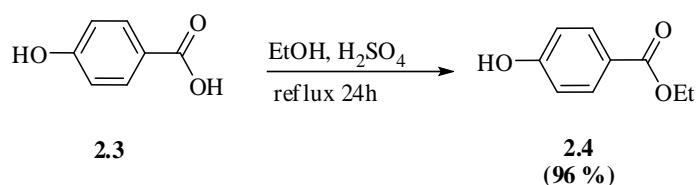
Scheme 2.2: Synthesis of ester 2.4

Compound ethyl 4-(3,4,5-trimethoxybenzyloxy)benzoate (**2.5**) was synthesized according to the **Williamson ether synthesis**, by refluxing compounds **2.2** and **2.4** in acetone in the presence of anhydrous K_2CO_3. Reacting the compound **2.4** with the base K_2CO_3 generates the phenolic anion to react with benzyl halide **2.2**, which give the final compound **2.5**. The formation of compound **2.5** is demonstrated in Scheme 2.3.
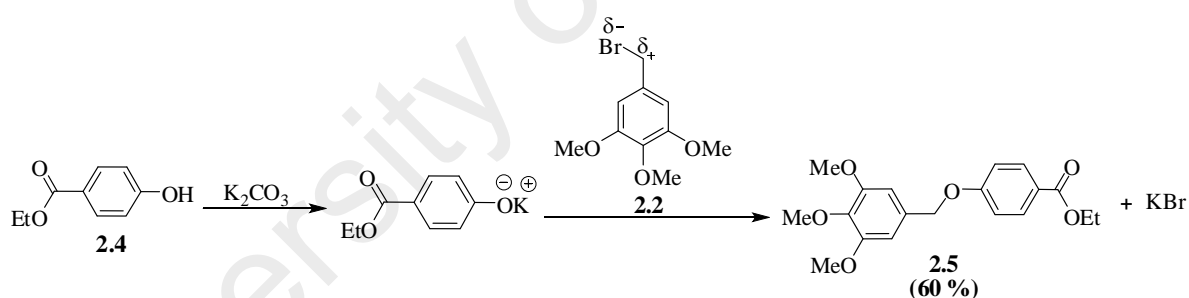
Scheme 2.3: Synthesis of the ester 2.5

The choice of solvent is extremely important for reactions with highly reactive halides. Alcohols are often be used as solvents in many reactions. Unfortunately, ethanol reacts quickly with benzyl bromide (**2.2**) under basic conditions to give a substitution product **2.2a**, as shown in Scheme 2.4. DMF can be used as an effective solvent in this reaction. However, the product **2.5** was obtained in better yield and higher purity in acetone than in DMF.
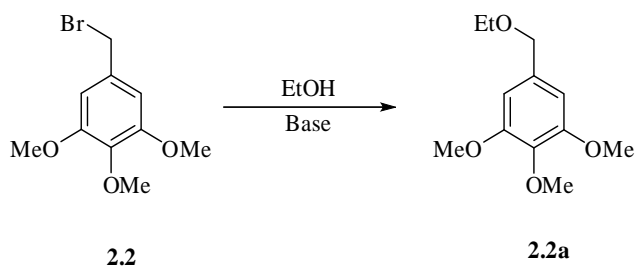
Scheme 2.4: Reaction of ethanol with compound 2.2

Hydrazide reactions are of great importance in organic synthesis. They are employed as a key intermediate for synthesis of five membered heterocyclic compounds and many other target compounds and are useful building blocks for the assembly of various heterocyclic rings. The direct hydrazination of esters with hydrazine hydrate in alcohol is the most commonly reported synthetic approach for hydrazides (Carvalho, da Silva, de Souza, Lourenço, & Vicente, 2008; Küçükgüzel, Mazi, Sahin, Öztürk, & Stables, 2003). Ester **2.5** then reacted with hydrazine hydrate (85%) in absolute ethanol under reflex. The conversion of compound **2.5** to the corresponding acid hydrazide **2.6** obtained in a very good yield (87%), as shown in Scheme 2.5.
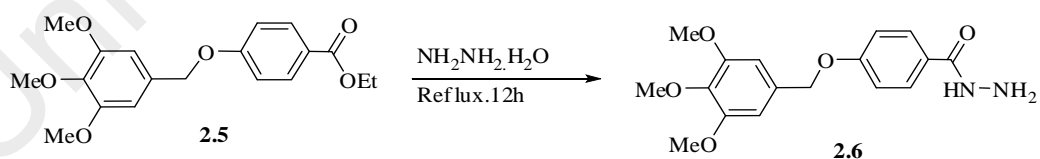
Scheme 2.5. Synthesis of compound 2.6

The structures of the synthesized compounds **2.5-2.6** were elucidated by spectroscopic methods. The IR spectra displayed a C=O stretching absorption of the ester (**2.5**) at 1714 cm^{-1} and the hydrazide (**2.6**) at 1676 cm^{-1}, respectively . The presence of NH and NH$_2$ was reflected in absorption at 3272 and 3250 cm^{-1}.

The ¹H-NMR spectrum of compound **2.5** exhibited signals originating from the ethyl ester and methoxy groups at 1.30 & 4.27 ppm and 3.67 & 3.79 ppm, respectively. Conversion of the ester **2.5** into the key intermediate **2.6** led to the disappearance of signals belonging to the ethoxy group. Instead, new signals reflecting the hydrazide structure appeared at 4.43 ppm (2H) and 9.61 ppm (1H). The ¹³C NMR spectra of **2.5** confirmed the presence of the ethyl group at 14.7 and 60.8 ppm. The carbonyl group in **2.5** and **2.6** appeared in the region of 165.8 and 166.0 ppm, respectively, whereas the carbons of methoxy groups were found at 56.4 and 60.5 ppm for **2.5-2.6**. The HREIMS spectra of **2.5** and **2.6** as shown in Table 2.1.

Table 2.1: HREIMS of molecular ion peaks of compounds 2.5 and 2.6

Compound	HREIMS m/z $[M]^+$	Theo. Mass	Composition
2.5	347.1455	347.1488	$C_{19}H_{22}O_6$
2.6	333.1459	333.1450	$C_{17}H_{20}N_2O_5$

Antioxidant activities of ethyl-4-(3,4,5-trimethoxybenzyloxy)benzoate and 4-(3,4,5-trimethoxybenzyloxy)benzohydrazide, were determined using DPPH and FRAP assays. The data are presented in chapter 3 together with the hydrazone compounds.

CHAPTER 3: SYNTHESIS AND ANTIOXIDANT ACTIVITY OF HYDRAZONE

DERIVATIVES (3.1-3.9)

3.1   Introduction

This chapter discusses hydrazones, i.e. the synthesis and structural elucidation of derivatives **3.1-3.9**. The synthesized compounds have been characterized by IR, NMR and mass spectral analyses. The antioxidant activities of the synthesized compounds were experimentally verified by DPPH and FRAP assays and correlated with a theoretical study.   In organic chemistry, hydrazones and their derivatives constitute a versatile class of compounds, due to desirable characteristics like ease of preparation, increased hydrolytic stability relative to azomethine, and tendency toward crystallinity (Belskaya, Dehaen, & Bakulev, 2010). Hydrazones have an imine group, containing two connected nitrogen atoms of different natures.

The C=N double bond is conjugated with a lone electron pair of the terminal nitrogen atom. The structural fragments mentioned above are mainly responsible for the physical and chemical properties of hydrazones (Figure 3.1). The two nitrogen atoms of the hydrazones are nucleophilic, but the amino type nitrogen is more reactive. The carbon atom of hydrazone group has both electrophilic and nucleophilic character (Belskaya, Dehaen, & Bakulev, 2010). Thus, hydrazones have the capability to react with electrophilic and nucleophilic reagents (Brehme, Enders, Fernandez, & Lassaletta, 2007).
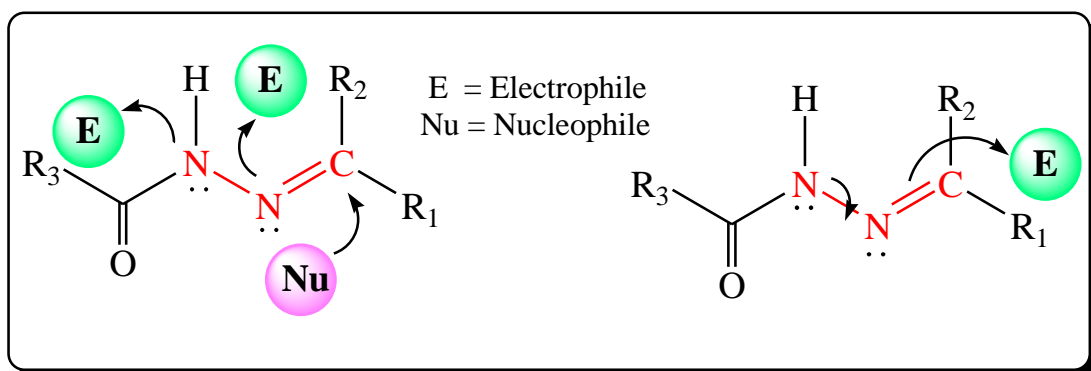
Figure 3.1. Classification of active centres of the hydrazones

The most general and oldest method for the synthesis of hydrazones is the condensation of hydrazines (primary amines) with carbonyl compounds (aldehydes or ketones).(Brehme, Enders, Fernandez, & Lassaletta, 2007) The mechanism of the hydrazone formation is shown in Scheme 3.1. Due to the ease of their application, hydrazones are widely used in the preparation of dyes and pharmaceuticals.
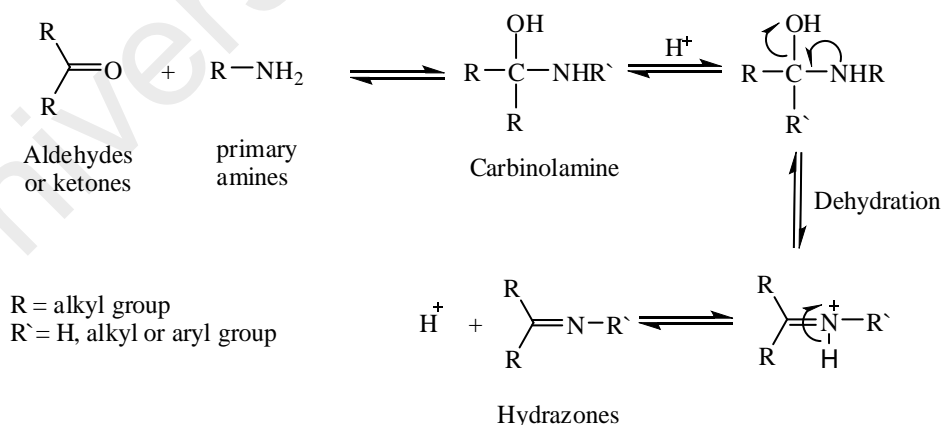
Scheme 3.1. The mechanism of formation of the hydrazone

3.2 SAR and the rational design of antioxidant hydrazones

The generic structure of the new hydrazones, as illustrated in Figure 3.2, contains of a well-known free radical scavenger, the 3,4,5-trimethoxybenzyl group (ring A). The active group -CO-NHN=C- enables resonance of the two adjacent aromatic rings B and C, leading to multiple resonance structures, which allows this functional group to act as an electron donating group to enhance the radical scavenging activity. The imine group of the hydrazone that contains a lone pair of electrons might be used to form a covalent bond with a biological target (Al-Amiery, Al-Majedy, Ibrahim, & Al-Tamimi, 2012).

It has been reported that electron-donating substituents at the 2,4,6-positions, such as alkyl or alkoxyl groups (e.g. : t-Bu or methoxy), increase the primary antioxidant activities of phenols (Kajiyama & Ohkatsu, 2001) by increasing the rate of hydrogen atom transfer from the phenolic group (Nishida & Kawabata, 2006; Rao, Swamy, Chandregowda, & Reddy, 2009). This is due to the lowering of the bond dissociation enthalpy (*BDE)* of the phenol O-H group (Lucarini, Pedulli, & Cipollone, 1994) and the stabilization of the phenoxyl radical by inductive and hyperconjugative effects as well as the methoxy group resonance effect. Due to these resonance-based stabilizing effects, hydrazones with a phenolic hydroxyl group at the para position on ring C with additional EDGs at the ortho-position can exhibit potent antioxidant activities.
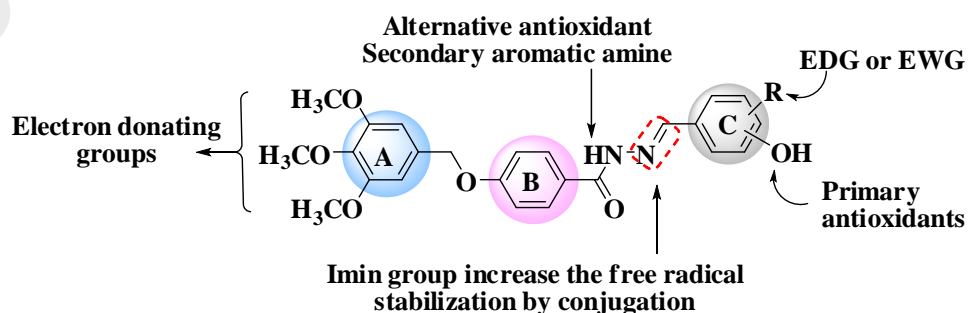
Figure 3.2. SAR analysis of synthesized hydrazones

3.3   Synthesis of the hydrazone derivatives 3.1-3.9

The treatment of the acid hydrazide **2.6** with various aromatic aldehydes in absolute ethanol, provided the hydrazone derivatives **3.1-3.9** in good yields. The reaction mechanism of the synthesized compounds is illustrated in Scheme 3.2, whereas the synthetic procedure is outlined in Scheme 3.3.
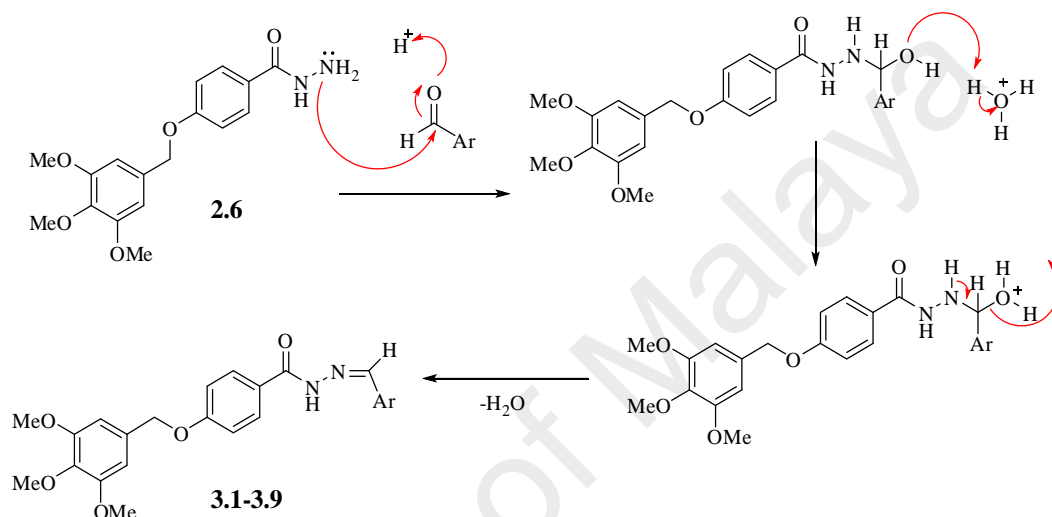
Scheme 3.2. Reaction mechanism of the synthesis of hydrazone derivatives 3.1-3.9
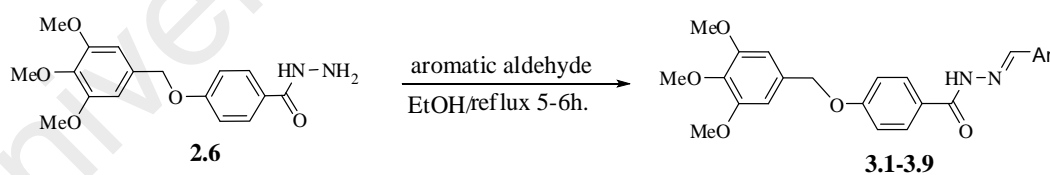
NO	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9
Ar	(81%)	(74%)	(72%)	(85%)	(72%)	(74%)	(80%)	(81%)	(83%)

Scheme 3.3. Synthesis of hydrazone derivatives

The structures of the hydrazone derivatives **3.1-3.9** were determined using NMR (^{1}H, ^{13}C), IR and MS (HREIMS). The ^{1}H-NMR spectrum for all compounds showed a singlet peak in the region between 11.40 and 12.40 ppm due to the amide NH as well as another singlet peak between 8.20 and 8.90 ppm reflecting an upfield shift of the aldehyde peak due to the formation of the hydrazone.

At the same time, the signal for the amino group of hydrazide **2.6** disappeared, thus confirming the presence of the imine in the hydrazone compounds. It is important to note that some of hydrazone compounds may be obtained as *E/Z*- geometric isomers (the symbols *Z* and *E* correspond, respectively, to the *cis* and *trans* arrangement of the 'principal' substituents relative to the double bond) (Brokaite, Mickevicius, & Mikulskiene, 2006). The literature indicates different conformers for hydrazones. Images as shown in Figure 3.3.
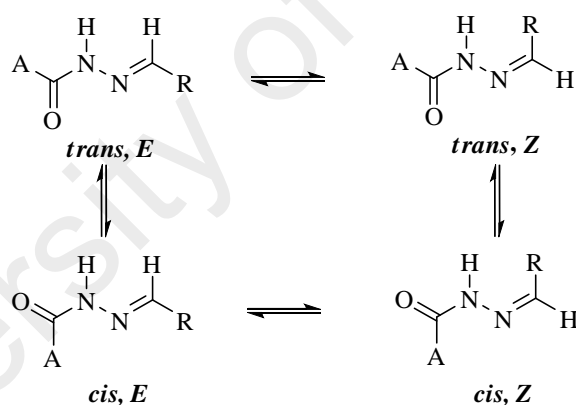
Figure 3.3. *E/Z* isomerization of N-acylhydrazones
(Demirbas, Karaoglu, Demirbas, & Sancak, 2004)

The stereochemistry of the hydrazone is favourably E (Landge, Tkatchouk, Benítez, Lanfranchi, Elhabiri, Goddard, & Aprahamian, 2011; Tobin, Hegarty, & Scott, 1971). This configuration was confirmed for compound **3.1** and **3.8**, based on chemical shift of the *o*-hydroxy groups. Protons of phenolic groups for compounds **3.2, 3.3, 3.4, 3.7**, and **3.9** appear as a singlet peak in the region between 7.39 – 9.90 ppm, whereas the proton of phenolic groups in compounds **3.1** and **3.8** were shifted further downfield at 12.80 and

12.02 respectively. The shifted in chemical for **3.1** and **3.8** is believed due to hydrogen bonding between the OH group and the N atom of the imine, as shown in Figure 3.4. Based on this observation we believe that all of the hydrazones, **3.1-3.9**, have *trans* (*E*) configurations at the imine double bonds. This observations are in agreement with the data reported by *Levrand et al* (Levrand, Fieber, Lehn, & Herrmann, 2007).
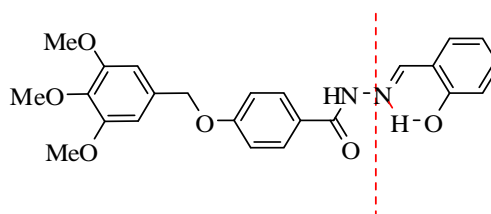
Figure 3.4. Intramolecular H-bond formation of compound 3.8

The ^{13}C NMR spectra of **3.1-3.9** showed the presence of the C=N group at the region of 147.0-148.0 ppm. The methoxy groups appeared at 56.4 and 60.5 ppm, respectively. The HREIMS data for compounds **3.1-3.9** as shown in Table 3.1.

Table 3.1: HREIMS of molecular ion peaks of compounds 3.1-3.9

Compound	HREIMS m/z [M]$^+$	Theo. Mass	Composition
3.1	549.0721, 551.0250	549.0419, 551.0398	$C_{24}H_{22}BrClN_2O_6$
3.2	549.2959	549.2952	$C_{32}H_{40}N_2O_6$
3.3	497.1920	497.1914	$C_{26}H_{28}N_2O_8$
3.4	481.1973	481.1965	$C_{26}H_{28}N_2O_7$
3.5	511.2089	511.2070	$C_{27}H_{30}N_2O_8$
3.6	533.1894	533.1889	$C_{27}H_{30}N_2O_8$
3.7	459.1533	459.1523	$C_{27}H_{30}N_2O_8$
3.8	459.1529	459.1523	$C_{24}H_{24}N_2O_6$
3.9	459.1529	459.1523	$C_{24}H_{24}N_2O_6$

3.4 In vitro antioxidant activities

In the present study, the antioxidant activities of synthesized compounds **2.5** and **2.6** and their hydrazone derivatives **3.1-3.9** were tested *in vitro* by using DPPH and FRAP, the two most common antioxidant assays (Ahmad, Mehmood, & Mohammad, 1998; Kuş, Ayhan-Kılcıgil, Özbey, Kaynak, Kaya, Çoban, & Can-Eke, 2008), as mentioned in chapter 1.

3.4.1 DPPH free radical scavenging activities

The DPPH radical scavenging ability strongly depends on the geometric accessibility of the radical trapping site. Steric hindrance may prevent a test compound from reaching the radical site of DPPH, resulting in low activity (Faria, Calhau, de Freitas, & Mateus, 2006). The DPPH assay is based on either a hydrogen atom transfer (HAT) or a single electron transfer (SET) mechanism. The effects of different concentrations of the test compounds on the DPPH radical scavenging activities, which were compared to standard antioxidants (ascorbic acid (AA) and BHT), are displayed in Figure 3.5. These results gave rise to the subsequent calculation of IC_{50} values (as presented in Table 3.2) and the maximum inhibition of the DPPH radical by the synthesized compounds at a concentration of 125 μg/mL (shown in Figure 3.6).
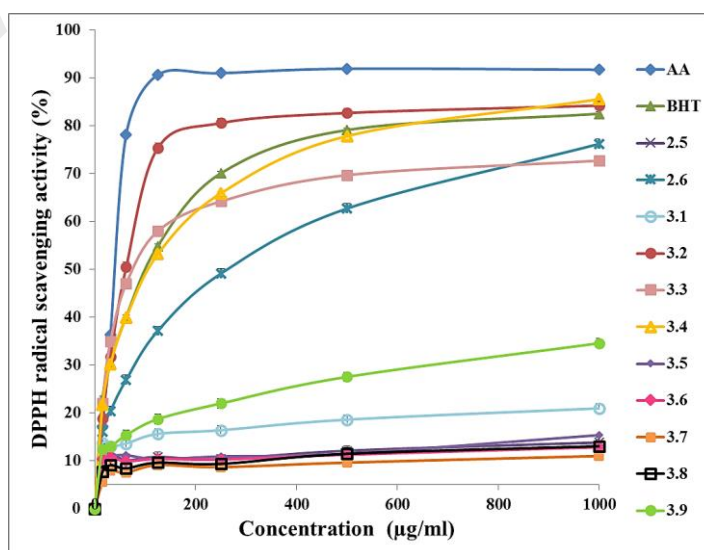
Figure 3.5. DPPH radical-scavenging activities of the synthesized compounds in comparison with positive controls (BHT and ascorbic acid)

IC$_{50}$ values could only be determined in four of the compounds, whereas the remaining compounds did not reach 50% inhibition of the DPPH radicals within the concentration range investigated in this study. For the DPPH assay, a dominant HAT mechanism is assumed. Preferred hydrogen abstraction sites are phenolic hydroxyl groups, preferably with conjugation to the hydrazone, as the latter could stabilize the resulting radical by additional resonance structures. Polar aprotic solvents reduce the rates of many ArXH/R reactions, which has been explained by observation that most of the molecules of ArXH are hydrogen bonded to the solvent, and these species are unable to react by HAT with R$^{\bullet}$. Only the free (non-hydrogen-bonded) fraction of ArXH is capable of transferring a H-atom to R$^{\bullet}$ (Barclay, Edwards, & Vinqvist, 1999; Foti, Barclay, & Ingold, 2002).

Table 3.2: IC$_{50}$, DPPH radical scavenging maximum inhibition and FRAP values of the synthesized compounds

Compounds	*DPPH IC$_{50}$* (*μg/mL*)	(*μM*)*	*Radical scavenging maximum inhibition %*	*FRAP μM*
5	NA	NA	10.8 ± 0.3	27 ± 7
6	264 ± 2	794 ± 2	37.2 ± 0.2	3514 ± 29
3.1	NA	NA	15.6 ± 0.2	125 ± 15
3.2	62 ± 0.5	113 ± 0.5	75.3 ± 0.3	1096 ± 30
3.3	75 ± 1	151 ± 1	57.9 ± 0.1	2087 ± 8
3.4	106 ± 0.4	221 ± 0.4	53.3 ± 0.3	1844 ± 20
3.5	NA	NA	10.4 ± 0.4	1609 ± 12
3.6	NA	NA	10.4 ± 0.2	1236 ± 7
3.7	NA	NA	9.3 ± 0.1	1819 ± 17
3.8	NA	NA	9.6 ± 0.1	26 ± 8
3.9	NA	NA	18.7 ± 0.2	2562 ± 9
AA	40 ± 0.1	227 ± 0.1	90.6 ± 0.2	3056 ± 94
BHT	99.7 ± 0.8	454 ± 0.8	54.8 ± 0.4	2836 ± 13

Results are expressed as a mean ± standard deviation (n = 3). DPPH radical scavenging activities are expressed as IC$_{50}$ concentrations of the compounds (μg/mL) required to inhibit 50% of the radicals and the maximum inhibition values. NA = did not reach 50% inhibition of the DPPH radicals at the concentrations used in this study). FRAP, ferric reducing antioxidant power.* IC$_{50}$ concentrations of the compounds (μM).

A comparison of the previous plots (in µg/mL) with the molar concentration plots in µM (see appendix page 209, Figure. D 1) display no significant differences with the series, owing to similar molecular weight. However, the data become more favorable in comparison with standard antioxidant compounds, which have lower molecular weight.

Based on the structures and antioxidant activities, the hydrazone antioxidants in this study can be sorted into three classes.

The first class consists of **3.2**, **3.3** and **3.4,** which exhibited potent antioxidant activities that were equal to or better than that of the reference compound BHT. All of these compounds contain a phenolic hydroxyl group at the para position to the hydrazone and additional EDGs at the ortho position to this hydroxyl group. The high activity can be explained by the resonance-based stabilizing effects of the EDG as well as the hydrazone on the phenoxy-radical.
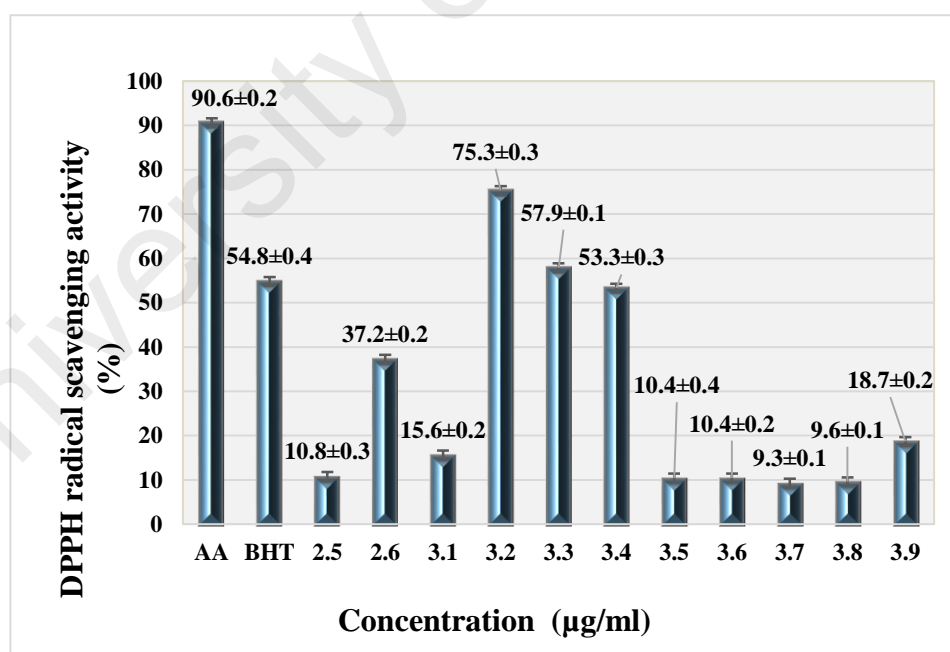
Figure 3.6. The maximum DPPH radical-scavenging activities of the synthesized compounds at 125 µg/mL concentration, in comparison with positive controls (BHT and ascorbic acid).

The order of activity, **3.2 ~ 3.3 > 3.4** with IC_{50} values of 62, 75 and 106 μg/mL, respectively, is in line with this explanation, as the latter only contains one stabilizing EDG ortho to the radical, whereas the former two compounds involve two such groups. In addition, the higher activity of compound **3.2** is due to an enhanced steric hindrance effect provided by the ortho tertiary butyl group, which stabilizes the phenolic antioxidant radical (Ariffin, Rahman, Yehye, Alhadi, & Kadir, 2014; Çelik, Özyürek, Güçlü, & Apak, 2010). A mechanistic explanation for the scavenging of the DPPH free radical by the compounds is proposed in (Scheme 3.4).
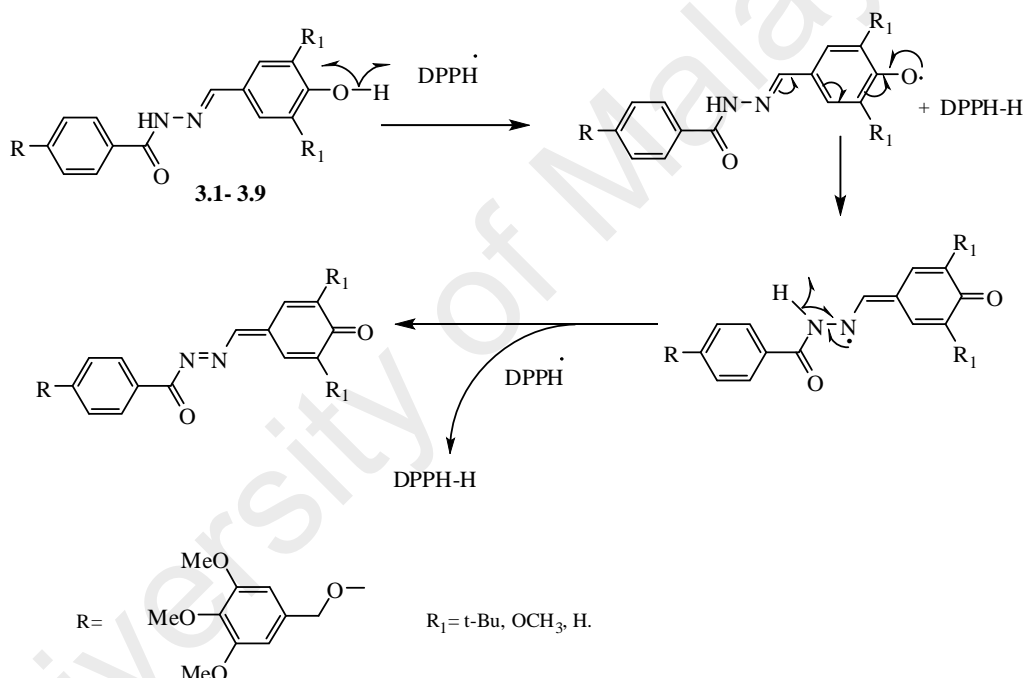
Scheme 3.4. Proposed reactions for scavenging of DPPH· by hydrazone derivatives

The second class covers compounds **3.7**, **3.8** and **3.9**. Like in the first class a phenolic hydroxyl group is present, but without additional EDGs or EWGs. The DPPH radical scavenging activities for this class are substantially lower than those in the first class. None of the compounds reached 50% inhibition of the DPPH radicals. The sequence of the DPPH radical scavenging activities is **3.9** > **3.8** > **3.7**. The sequence indicates a higher

activity for the hydroxyl groups in the para and ortho positions to the hydrazone compared to the meta analogue **3.7**. This finding is in line with resonance stabilizing effects (Liu & Wu, 2009). The para substituted **3.9** was more active than **3.8** with ortho substitution. It is postulated that an intra-molecular hydrogen bond of the phenolic hydrogen with the hydrazone nitrogen in a 6-membered ring disfavours the DPPH scavenging activity of the ortho substituted phenol due to the enhanced binding of the phenolic hydrogen atom (Jorgensen, Cornett, Justesen, Skibsted, & Dragsted, 1998), as displayed in Figure 3.4. The introduction of substituents with dominant polarization based electron withdrawing effects over potential electron donating effects due to resonance slightly increased the antioxidant activity (Bakalbassis, Lithoxoidou, & Vafiadis, 2006), as seen by the comparison of compounds **3.1** and **3.2**. The third class consists of compounds **3.5** and **3.6**, which are practically inactive. Neither of these compounds contains a phenolic hydroxyl group, but they may abstract a hydrogen atom at the amide to form resonance stabilized radicals (Anouar, Raweh, Bayach, Taha, Baharudin, Di Meo, Hasan, Adam, Ismail, Weber, & Trouillas, 2013).

3.4.2 DFT study of the DPPH radical scavenging activities

The antioxidant abilities of the hydrazone compounds can be related to a calculated spin density (SD) of related radical species (Leopoldini, Pitarch, Russo, & Toscano, 2004). A more delocalized spin density in the radical stabilizes the latter, thus favouring radical formation (Parkinson, Mayer, & Radom, 1999). Therefore, lower SD values indicate better antioxidants.

For the HAT mechanism, the easiness of the homolytic cleavage of the X-H bond, reflecting the efficacy of an antioxidant, can be estimated by calculating the bond dissociation enthalpy (BDE) of the respective O-H or N-H bond. Lower BDEs indicate weaker O-H or N-H bonds, from which the hydrogen is more easily extracted (Lucarini,

Pedrielli, Pedulli, Cabiddu, & Fattuoni, 1996). The presence of substituents, including both EWG and EDG, in aromatics and their respective position affects the SD and BDE values (Liu & Wu, 2009). To evaluate the reactivity of the NH and OH groups for HAT, the spin densities of the respective radical species of the hydrazone compounds were determined. The results for these two separate calculations are summarized in Figure 3.7.

The results, as shown in Table 3.3, indicated higher stabilities for the oxygen radicals of almost all of the compounds. An exception, however, was compound **3.1**, for which the spin density of the N-radical was slightly below that of the O-radical. Thus the experimental data are in agreement with the calculated data. As explained earlier, this could be due to an intramolecular hydrogen bond of the phenolic hydrogen with the hydrazone nitrogen as shown in Figure 3.4.
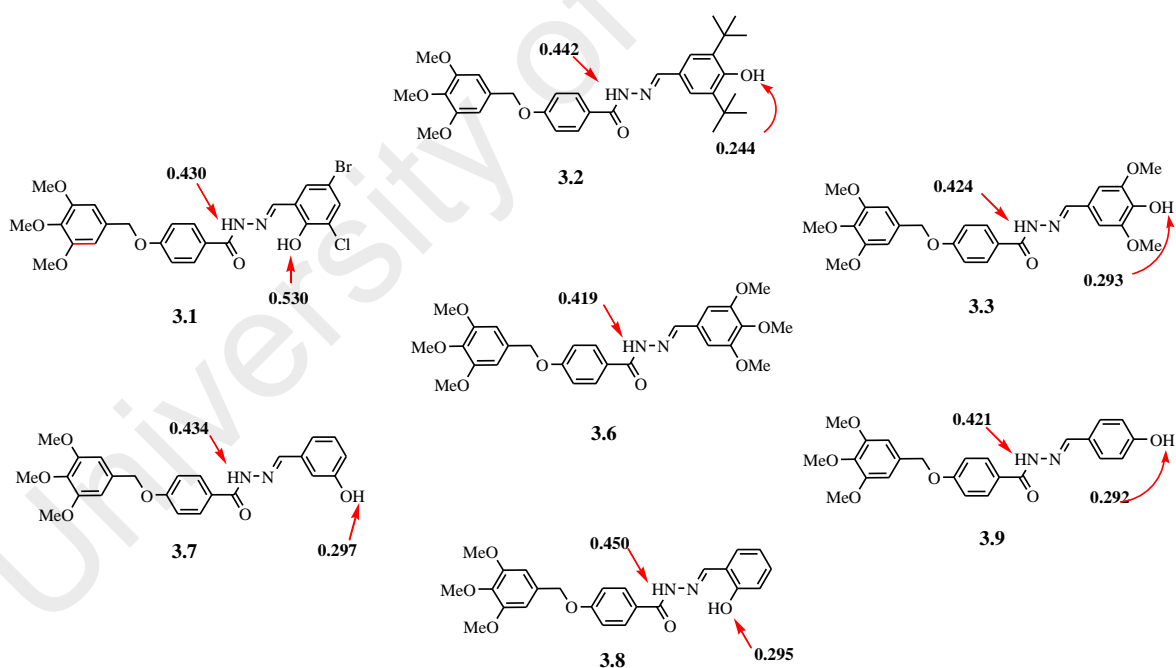
Figure 3.7. Spin densities for selected compounds; the structures represent two independently calculated radicals obtained by abstraction of a hydrogen atom from the OH or the NH group

As shown in Figure 3.6, the OH group of compound **3.2** exhibits the lowest spin density. This is in agreement with its experimental DPPH scavenging activity. However, the spin density for compound **3.3** do not match the experimental results. We could not find a logical explanation for the observation at the moment. The SD for the oxygen radicals of most of these compounds were similar and reflect the experimental DPPH scavenging activities.

Nonetheless, the relative activities of **3.7-3.9** are in agreement with the spin densities. Another important parameter to be considered is the BDE. The antioxidant activity reflected in the calculated BDE value (Mayer, Parkinson, Smith, & Radom, 1998), is often attributed to π electron delocalization, leading to the stabilization of the radicals obtained after H-abstraction. It is assumed that if π electron delocalization exists in the parent molecule, it also exists in the corresponding radical (Trouillas, Marsal, Siri, Lazzaroni, & Duroux, 2006). Therefore, a close relationship between the BDE and spin density is to be expected. BDE values were calculated for the homolysis of N-H and O-H bonds. The data are shown in Table 3.3. As expected, they reflect the spin density trends.

Table 3.3. Calculated properties for the antioxidant compounds

Compounds	BDE (kcal/mol)		Spin Density	
	N-H	O-H	N-radical	O-radical
3.1	24.45	24.62	0.43	0.53
3.2	20.84	20.84	0.44	0.24
3.3	21.87	21.82	0.42	0.29
3.6	25.36	-	0.42	-
3.7	26.22	26.17	0.43	0.30
3.8	25.98	26.05	0.45	0.30
3.9	22.85	22.97	0.42	0.29
AA	-	16.56	-	-
BHT	-	20.80	-	-

Based on the BDE values a comparison was made between compounds **3.8** and **3.9** to determine the influence of the position of the hydroxyl group at ring C (aldehyde component). Compound **3.9** with the hydroxyl group at the para position has a lower BDE compared to the ortho analogue **3.8**, which is in agreement with the higher experimental antioxidant activity for the para compound. This effect is attributed to an intramolecular hydrogen bond for **3.8**. The intramolecular hydrogen bond increases the value of the BDE are in agreement with previous studies (Amorati, Lucarini, Mugnaini, & Pedulli, 2003; Ariffin, Rahman, Yehye, Alhadi, & Kadir, 2014), because the formation of the radical requires not only the splitting of the OH-bond but also the cleavage of the intramolecular hydrogen bond. The calculated atomic distance of approximately 1.46 Å between the hydrazone nitrogen and the hydroxyl hydrogen confirms the hydrogen bonding, as displayed in Figure 3.8.
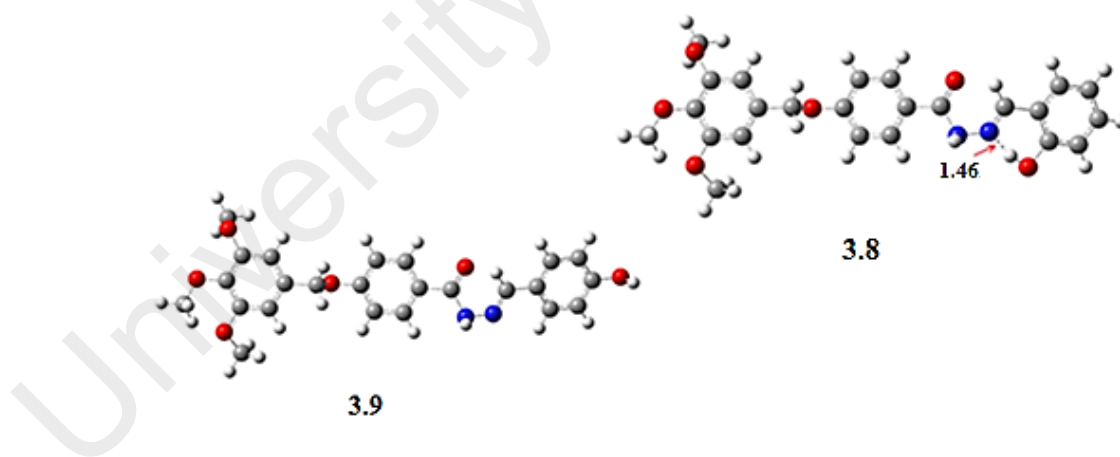
Figure 3.8. Optimized three-dimensional structures of compounds 3.8 and 3.9

3.4.3   Ferric reducing antioxidant power (FRAP) activity

As mentioned in chapter 1, the FRAP assay measures the reducing capacity of Fe^{3+} ions, according to equation (1.8).

$$[Fe\,(TPTZ)_2]^{3+} + Ar \xrightarrow{\;+e\;} [Fe\,(TPTZ)_2]^{2+} + Ar^{\bullet +} \qquad 1.8$$
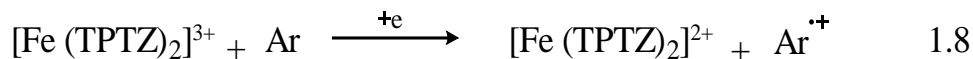
The FRAP assay utilizes an SET mechanism to determine the capacity of an antioxidant in the reduction of an oxidant. The ferric colour changes to blue (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005), and the degree of the colour change can be correlated with the concentration of antioxidants in the sample. However, the reducing capacity of a sample is not directly related to its radical scavenging capability. For the SET mechanism, a strong solvent dependence is expected. The expected solvent effect for SET is an increase in activity with an increase in the polarity and hydrogen bonding ability of the solvent (J. S Wright, E. R. Johnson, & G. A. DiLabio, 2001).

The FRAP values of the tested compounds, as displayed in Table 3.2, showed a different trend than the DPPH radical scavenging results. Some of the compounds are inactive in the DPPH assay but exhibit high activities in the FRAP assay (Figure 3.9). With the exception of compounds **2.5**, **3.1** and **3.8** (FRAP values below 150 μM), all of the investigated compounds exhibited ferric reducing activities above 1000 μM. Compound **2.6** which was only mildly active in the DPPH assay, showed the highest ferric reducing activity, while compound **3.2**, which was highly active in the DPPH assay, showed an unusually low FRAP value.

The most significant parameter for the evaluation of the antioxidant activity is the ionization potential (IP), which is related to the π electron delocalization (Trouillas, Marsal, Siri, Lazzaroni, & Duroux, 2006), whereby a lower IP value will result in an easier electron transfer (Rojano, Saez, Schinella, Quijano, Vélez, Gil, & Notario, 2008). Nevertheless, the IP values showed poor correlation with the FRAP results.
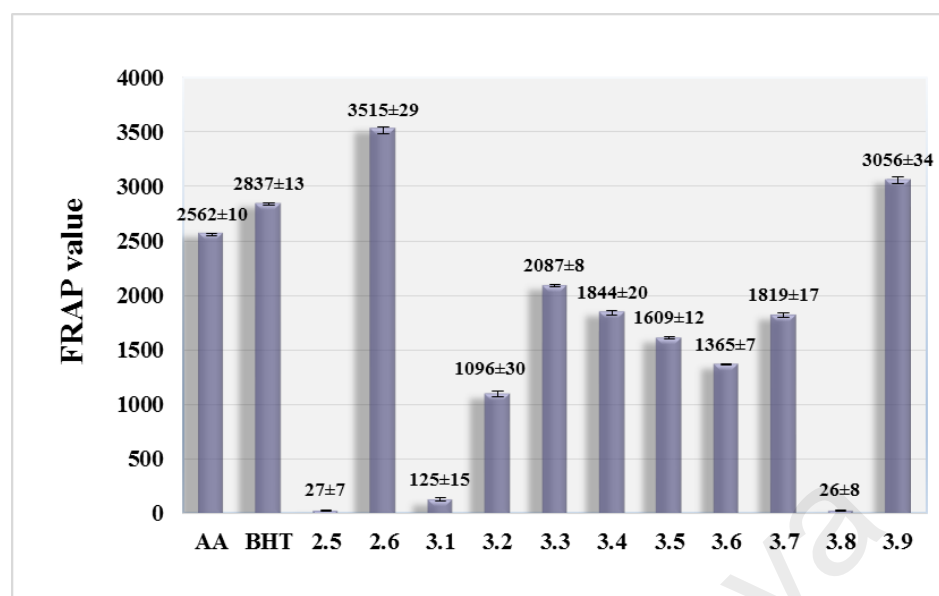
Figure 3.9. Ferric reducing antioxidant power (FRAP)

3.4.4 Correlation analysis

A Pearson correlation analysis was performed to investigate the relationship between the DPPH radical scavenging and ferric reducing activities of the compounds, as shown in Figure 3.10. It was expected that the antioxidant activities of the compounds in both assays would show similar trends and that the values of both could, therefore, be correlated. However, the data revealed only a moderate positive correlation between the two antioxidant assays, indicating that compounds with high DPPH radical scavenging activities did not necessarily show high ferric reducing activities and vice versa. *Y. Unver et al* reported a similar observation for 1,2,4-triazole derivatives (Ünver, Sancak, Celik, Birinci, Küçük, Soylu, & Burnaz, 2014). The different results probably reflect differences in the reaction mechanisms of the two assays. Moreover, the DPPH radical scavenging activity may be affected by steric issues, whereas for the FRAP assay, solvent issues may apply.
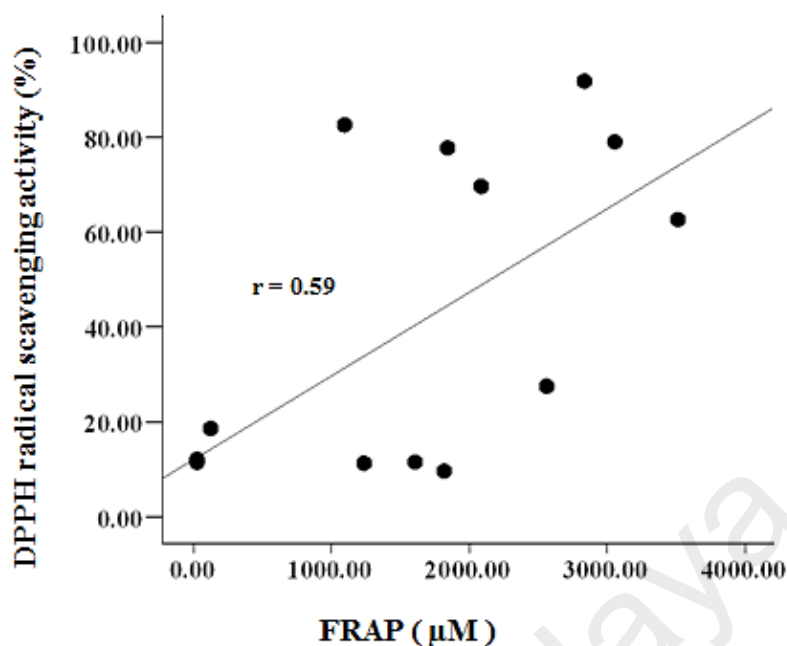
Figure 3.10. Pearson correlation analysis between the DPPH radical scavenging activities and ferric reducing activities of the synthesized compounds

In HAT, the OH group in the C ring of the active hydrazones is considered the most reactive site for radical scavenging activity. However, a SET process does not require such a specific group, as an aromatic system is already sufficient to enable the electron transfer. Therefore, SET reactions based on the hydrazones **3.1-3.9** may originate either from the AB-ring-system or ring C.

In fact, the contour plots of the HOMO for different hydrazine compounds **3.1-3.9** suggest different reactivity sites for SET. For example, the HOMO of compound **3.7**, which exhibited a low activity in the DPPH assay but a high activity in FRAP, suggests SET at trimethoxylated ring A, whereas for compound **3.2**, the electron density is concentrated on ring C, as shown in Figure 3.10. For compound **3.9**, which showed the highest FRAP activity, both ring systems appear to be potentially active for SET.
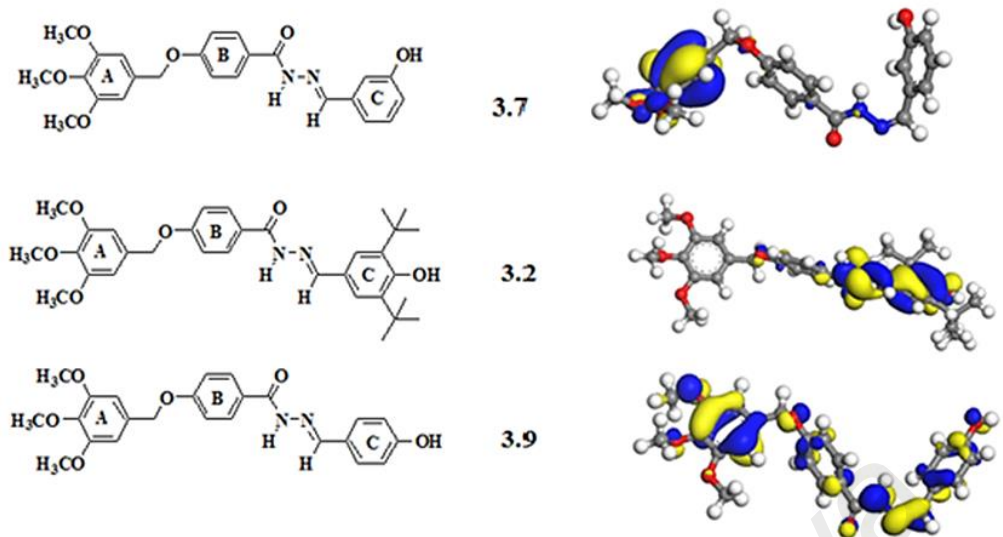
Figure 3.11. Density distributions of the HOMOs for selected hydrazones

CHAPTER 4: SYNTHESIS OF 1,3,4-OXADIAZOLE AND BIS 1,3,4-OXADIAZOLE DERIVATIVES (4.1-4.7)

4.1   Introduction

This chapter deals with the synthesis of a series of hybrid molecules possessing 1,3,4-oxadiazoles and the determination of their antioxidant properties. All structures were confirmed by IR, NMR and mass spectral analyses.

Compounds possessing a 1,3,4-oxadiazole group have ability to undergo various chemical reactions to give the desired structures, making them important for molecule planning and design (Dolman, Gosselin, O'Shea, & Davies, 2006). 1,3,4-oxadiazole heterocycles have gained considerable interest in medicinal chemistry as bioisosteres of amides and esters. Their pharmacological activity is mediated by hydrogen bonding interactions with receptors (Guimarães, Boger, & Jorgensen, 2005; Rahman, Mukhtar, Ansari, & Lemiere, 2005). Moreover, these compounds exhibit potentially interesting physical properties, such as electro-optical behaviour, luminescence and electrochemical properties (Huda & Dolui, 2010; Kim, 2008; Wang, Li, Lin, Hu, Chen, & Mu, 2009). Oxadiazole and their derivatives can be considered as simple five-membered heterocycles containing two nitrogen atoms and one oxygen. The oxadiazole exist in different isomeric forms, such as (1,2,3-), (1,2,4-), (1,2,5-) and 1,3,4-oxadiazole as shown in Figure 4.1 (Kumar, Jayaroopa, & Kumar, 2012).
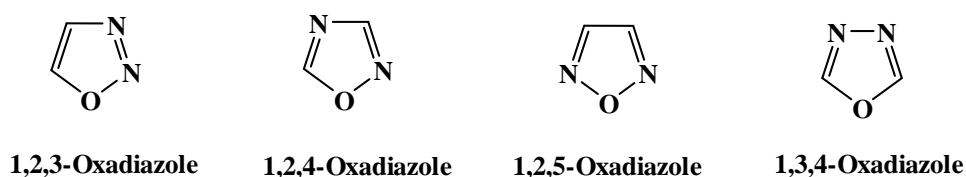
Figure 4.1. Isomeric forms of oxadiazole

The literature reports several conventional methods for the synthesis of 1,3,4-oxadiazoles compounds, which have received special attention since 1955. In this study the synthesis of 1,3,4-oxadiazoles is based on either the intermolecular condensation of acid hydrazides with carboxylic acids in the presence of cyclizing reagents, such as phosphorus oxychloride, or the reaction of aryl acid hydrazides with carbon disulfide in the presence of alcoholic KOH. Both pathways produced 1,3,4-oxadiazole in good yield, as shown in Scheme 4.1.
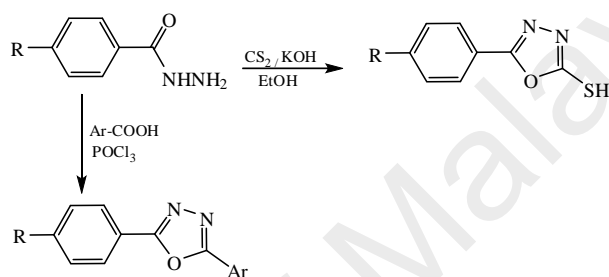
Scheme 4.1. Synthesis of 1,3,4-oxadiazole

4.2 SAR and the rational design of antioxidant 1,3,4-oxadiazole

Figure 4.2 shows the generic structure of the tested radical scavenger, and coupling of 1,3,4-oxadiazole and the 3,4,5-trimethoxybenzyl group (ring A).The later acts as an electron donating group to enable radical-scavenging activity based on an SET mechanism, as displayed in Scheme 4.8. Figure 4.2 only shows the NH-tautomer, the exchangeable hydrogen atom at the thiourea group. On the other hand, this hydrogen atom may be used to form a covalent bond with a biological target and gain rise to additional radical scavenging activity based on HAT.
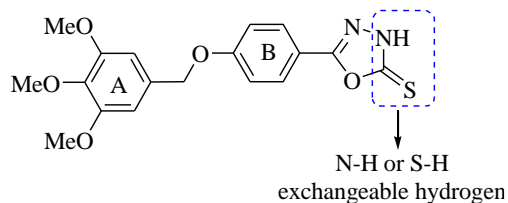
Figure 4.2. SAR analysis of synthesized 1,3,4-oxadiazole

4.3 Synthesis of 1,3,4-oxadiazole and its derivatives

As shown in Scheme 4.2, the treatment of the acid hydrazide **2.6** with carbon disulfide in the presence of KOH and 95% ethanol under reflux gave compound 5-(4-(3,4,5-trimethoxybenzyloxy) phenyl)-1,3,4-oxadiazole -2-(3H) -thione (**4.1**) (Shafiee, Naimi, Mansobi, Foroumadi, & Shekari, 1995). The mechanism (Tomi, Al-Qaisi, & Al-Qaisi, 2011) for the reaction is outlined in Scheme 4.3.
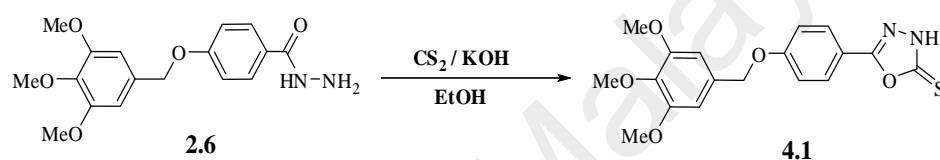
Scheme 4.2. Synthesis of 1,3,4-oxadiazole 4.1
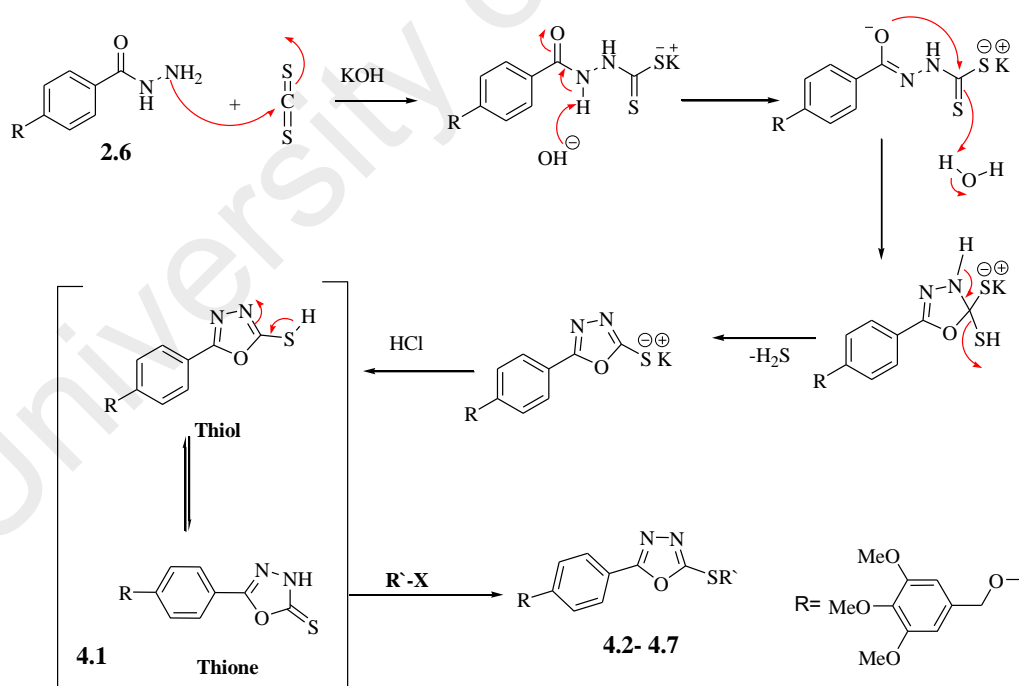
Scheme 4.3. Proposed mechanism for synthesis of compound 4.1 and (4.2-4.7)

Aryl acid hydrazides (**Rc-f**) were synthesized by refluxing of substituted ethyl benzoates with hydrazine hydrate (85%) in ethanol. Reaction of the latter (**Rc-f**) with

chloroacetic acid in presence of phosphorus oxychloride under reflux gave 2-chloromethyl-5-aryl-1,3,4-oxadiazole (**4c-f**) (Padmavathi, Reddy, Reddy, & Mahesh, 2011), as shown in Scheme 4.4.

R= c = Br, e = CH$_3$
    d = Cl, f = OCH$_3$
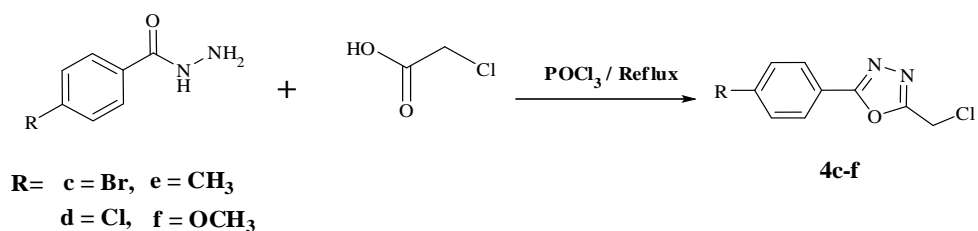
Scheme 4.4. Synthesis 2-chloromethyl-5-aryl-1,3,4-oxadiazole (Rc-f)

A reaction mechanism for this synthesis of 1,3,4-oxadiazole was proposed by Bentiss and Lagrenee. Scheme 4.5 applies for the synthesis of **4c-f** (Bentiss & Lagrenee, 1999). No oxadiazole was found when only one equivalent of POCl$_3$ was applied. The reactions requires at least two equivalents. According to Bentiss and Lagrenee the dichlorophosphate ion ($^-$OPOCl$_2$) is a better leaving group than the chloride ion Cl$^-$ (Smith & March, 2007).
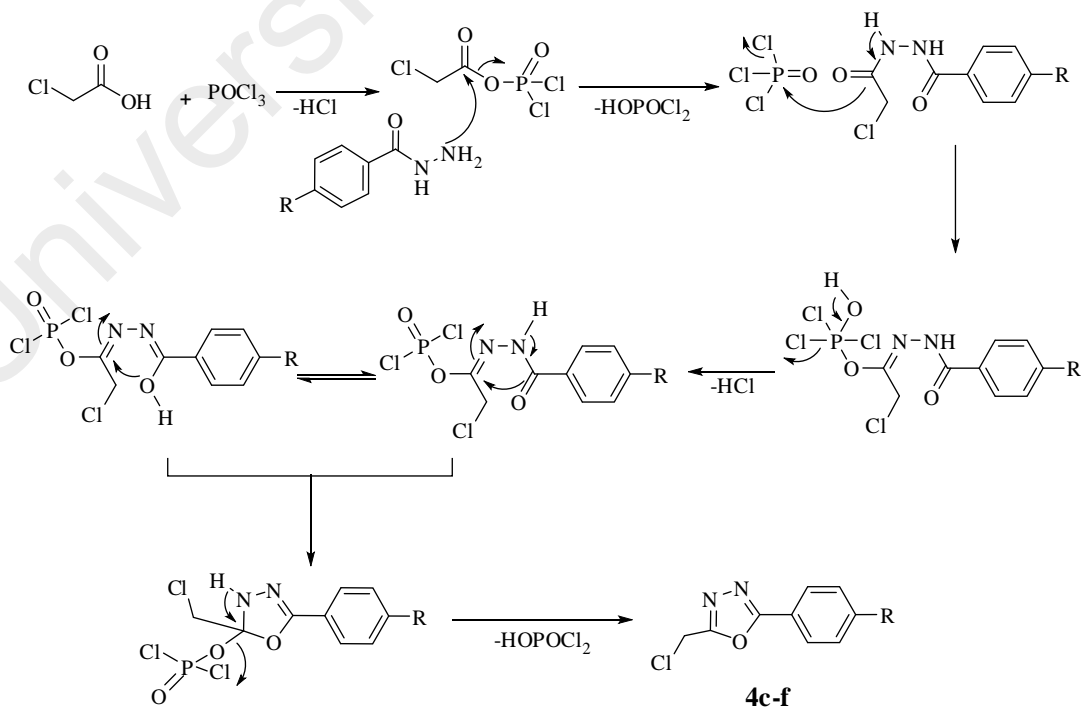
Scheme 4.5. Proposed mechanism of synthesis 4c-f

Alkylation of compound **4.1** with alkyl halides in alkali medium (K_2CO_3) under reflux (Almasirad, Vousooghi, Tabatabai, Kebriaeezadeh, & Shafiee, 2007) formed the S-alkylated products **4.2-4.7**. Application of simple alkyl halides (**4a-b**) led to the mono - 1,3,4-oxadiazoles **4.2-4.3**, while the 2-chloromethyl-5-aryl-1,3,4-oxadiazoles (**4c-f**) provided the bis-1,3,4-oxadiazoles **4.4-4.7**. The reaction are displayed in Scheme 4.6.

Scheme 4.6. Synthesis of 1,3,4-oxadiazole derivatives 4.2-4.7

Two tautomers can be associated with the compound **4.1** based on the thiol-thione equilibrium shown in Figure 3.2. One tautomer usually predominates (Koparır, Çetin, & Cansız, 2005). In this case the thione form was observed, as indicated by the IR spectrum of compound **4.1**, showing a sharp absorption band at 3173 cm^{-1} representing the NH group. The corresponding NH proton appeared at 14.6 ppm in ^{1}H NMR spectrum while the thio methane carbonyl (C=S) was found at 177.6 ppm in the ^{13}C NMR spectrum. No signal representing a hydrazide structure was observed.

Figure 4.3. Thiol – thione tautomers of 1,3,4-oxadiazole

All the 1,3,4-oxadiazole derivatives **4.2-4.7** were confirmed by the appearance of S−CH$_2$ characteristic singlet peak between 4.5 and 5.1 ppm in their ^{1}H NMR and the corresponding carbon peak between 26 and 49 ppm in their ^{13}C NMR spectrum. Moreover, the structures of target compounds **4.1-4.7** were confirmed by HREIMS spectra, as tabulated in Table 4.1. All the compounds are obtained in good yields.

Table 4.1: HREIMS of molecular ion peaks of compounds 4.1-4.7

Compound	*HREIMS m/z [M]$^+$*	*Theo. Mass*	*Composition*
4.1	375.1018	375.1007	$C_{18}H_{18}N_2O_5S$
4.2	565.0407, 567.0390	565.0399, 567.0378	$C_{25}H_{23}BrN_2O_5S$
4.3	593.0362, 595.0339	593.0348, 595.0327	$C_{26}H_{23}BrN_2O_6S$
4.4	633.0425, 635.0402	633.0408, 635.0387	$C_{27}H_{23}BrN_4O_6S$
4.5	589.0927, 591.0905	589.0913, 591.0884	$C_{27}H_{23}ClN_4O_6S$
4.6	569.1471	569.1459	$C_{28}H_{26}N_4O_6S$
4.7	585.1420	585.1408	$C_{28}H_{26}N_4O_7S$

4.4 In vitro antioxidant activities

The synthesized compounds were investigated and evaluated based on their antioxidant activities by using DPPH and FRAP assays. These assays refer to different reaction mechanisms, i.e. HAT and SET, as described in chapter 3. In the DPPH assay a hydrogen atom transfers from the antioxidant to the DPPH radical, and the efficiency of their transfer depends on the structure of the antioxidant and the substituent. The FRAP assay, on the other hand, measures the reducing capacity of Fe^{3+} ions due to the single electron transfer. All activities recorded in comparison to ascorbic acid (AA) and butylated hydroxytoluene (BHT) as standard antioxidants.

4.4.1   DPPH free radical scavenging activities

Antioxidant activities of the compounds are related with their hydrogen radical donating ability to DPPH radical to form stable DPPH molecules (Eklund, Långvik, Wärnå, Salmi, Willför, & Sjöholm, 2005; Sharma & Bhat, 2009). This leads to a corresponding decrease in absorbance and changing sample solution colour from purple to yellow. The effects of different concentrations of the test compounds **4.1-4.7** on the DPPH radical scavenging activities are displayed in Figure 4.4, giving rise to the subsequent calculation of IC_{50} values and maximum inhibition, as shown in Table 4.2.
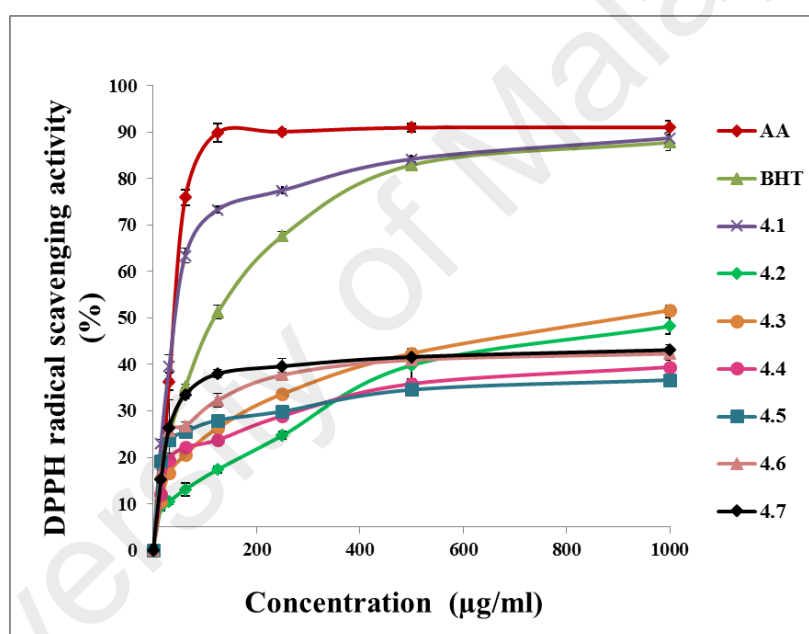
Figure 4.4. DPPH radical-scavenging activities of the synthesized compounds in comparison with positive controls (BHT and ascorbic acid

Compound **4.1** exhibited a strong scavenging effect on the DPPH radical, with an IC_{50} value of 45 ± 2 µg/mL and 73.3 ± 0.7 % maximum inhibition. This inhibition is higher compared to BHT with 94.0 ± 0.6 µg/mL and 56.9 ± 0.4 % maximum inhibition. The good radical scavenging activity of compound **4.1** is attributed to the exchangeable hydrogen in the oxadiazole moiety. The removal of the H-atom based on HAT mechanism stabilizes the radical due to conjugation of the primary radical, thus lowering its energy.

Table 4.2. IC$_{50}$, DPPH radical scavenging maximum inhibition and FRAP values of the synthesized compounds

Compounds	DPPH IC$_{50}$ (µg/mL)	IC$_{50}$ (µM)*	Radical scavenging maximum inhibition %	FRAP µM
4.1	45 ± 2	120 ± 2	73 ± 1	1688 ± 12
4.2	NA	NA	17 ± 0.7	158 ± 32
4.3	NA	NA	26 ± 0.8	238 ± 27
4.4	NA	NA	24 ± 1	256 ± 5
4.5	NA	NA	28 ± 0.9	193 ± 14
4.6	NA	NA	32 ± 2	268 ± 13
4.7	NA	NA	38 ± 0.8	157 ± 7
AA	42 ± 2	239 ± 2	90 ± 2	2231 ± 8
BHT	119 ± 2	541 ± 2	51 ± 1	1274 ± 24

Results are expressed as a mean ± standard deviation (n = 3). DPPH radical scavenging activities are expressed as IC$_{50}$ concentrations of the compounds (µg/mL) required to inhibit 50% of the radicals and the maximum inhibition values. NA = not active (did not reach 50% inhibition of the DPPH radicals at the concentrations used in this study). FRAP, ferric reducing antioxidant power. *as IC$_{50}$ concentrations of the compounds (µM).

Owing to similar molecular weight of the compounds in this series, a comparison of the previous plots (in µg/mL) with the molar concentration plots in µM (see appendix page 209, Figure D 2) display no significant differences, except that the data become more favorable in comparison with standard antioxidant compounds, which have lower molecular weight.

A proposed mechanism for the scavenging of the DPPH free radical by the compound **4.1** is presented in Scheme 4.7.
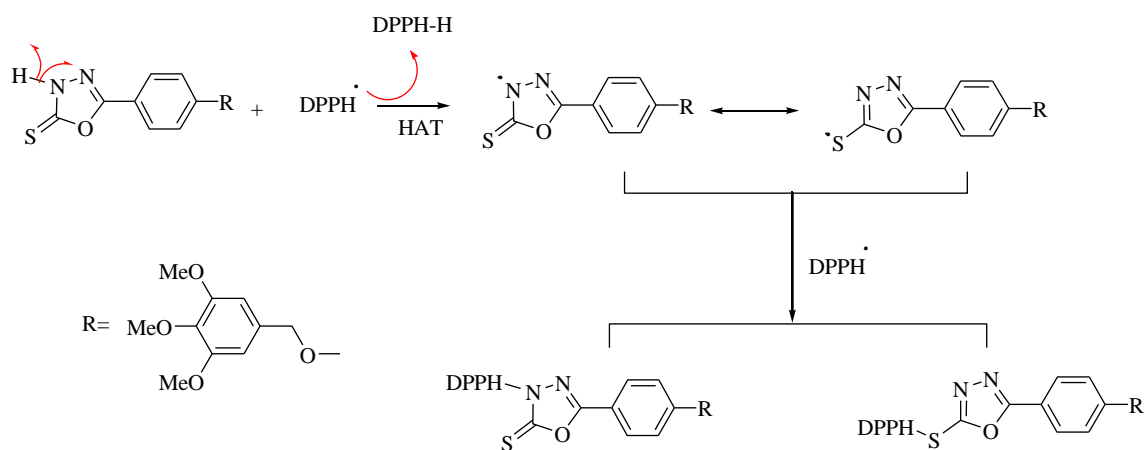
Scheme 4.7. HAT proposed mechanism for scavenging of DPPH• by 1,3,4-oxadiazole

An evidence was reported to describe the proposed mechanism (Dong, Venkatachalam, Narla, Trieu, Sudbeck, & Uckun, 2000), in which both thiol and thione tautomeric forms are responsible for the antioxidant activity. In contrast, S-alkylation eliminated this activity. The interaction of oxadiazole derivatives with the DPPH free radical indicates their free radical scavenging ability. Compounds **4.2 -4.7** did not reach 50% inhibition of the DPPH radicals within the concentration range investigated in this study. The S-alkylation of compound **4.1** virtually eliminated the free radical scavenging activities. This indicates a critical role of the exchanging proton for the antioxidant behaviour of the compound, thus suggesting its involvement bond on a HAT mechanism. Previous studies have already stated the importance of the non S-alkylated thioamide (Velkov, Balabanova, & Tadjer, 2007), and suggesting by antioxidant activities for thiourea group (Ozdem, Alicigüzel, Ozdem, & Karayalcin, 2000). It is assumed that the low remaining radical scavenging activities upon S-alkylation originates from a SET mechanism, related to Scheme 4.8.
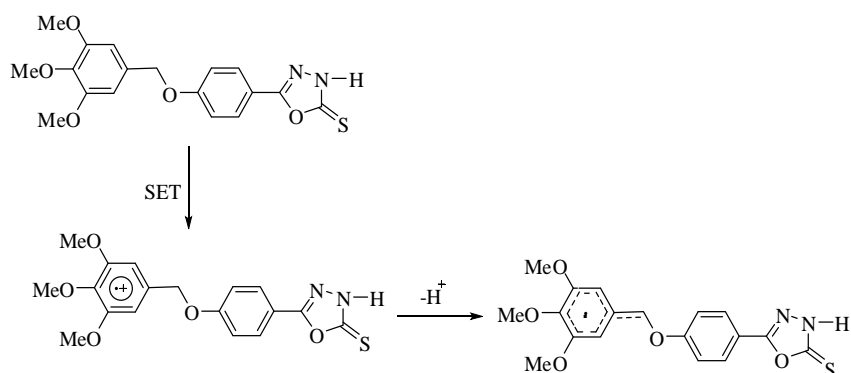
Scheme 4.8: SET proposed mechanism for 1,3,4-oxadiazole

Figure 4.5 showed the maximum inhibition of DPPH radical-scavenging activities of the synthesized compounds **4.2-4.7** at 125 µg/mL concentration. The DPPH free radical scavenging activity of these compounds can arise either from methylene CH_2 group attached to 3,4,5-trimethoxy phenyl ring or from the CH_2 near oxadiazole moiety.

A reactive free radical can undergo electron transfer or abstract H-atom from either of these two sites.(Indira Priyadarsini, MAITY, Naik, Sudheer Kumar, Unnikrishnan, Satav, & MOHAN, 2003; Jovanovic, Steenken, Boone, & Simic, 1999) Compounds **4.2-4.7** did not reach 50% inhibition of the DPPH radicals within the concentration range investigated in this study. This indicates that hydrogen abstraction from the -CH_2 group in these compounds by DPPH is not a favourable process. The order of DPPH radical scavenging activities based on maximal inhibition, of the oxadiazole derivatives **4.2-4.7** were **4.7** > **4.6** > **4.5** > **4.3** > **4.4** > **4.2**. Compounds **4.3** was more active than compound **4.2**, which both bear the *p*-bromo phenyl. This is due to the presence of the carbonyl substituent that enhanced the activity of compound **4.3**. Despite increasing the aromaticity in the substituent side of the synthesized compounds **4.4- 4.7**, the majority of these compounds showed low interaction with the DPPH radical. However, the substitution of EDGs or EWGs groups in the aromatic ring affected DPPH˙ scavenging activities for the synthesized compounds.
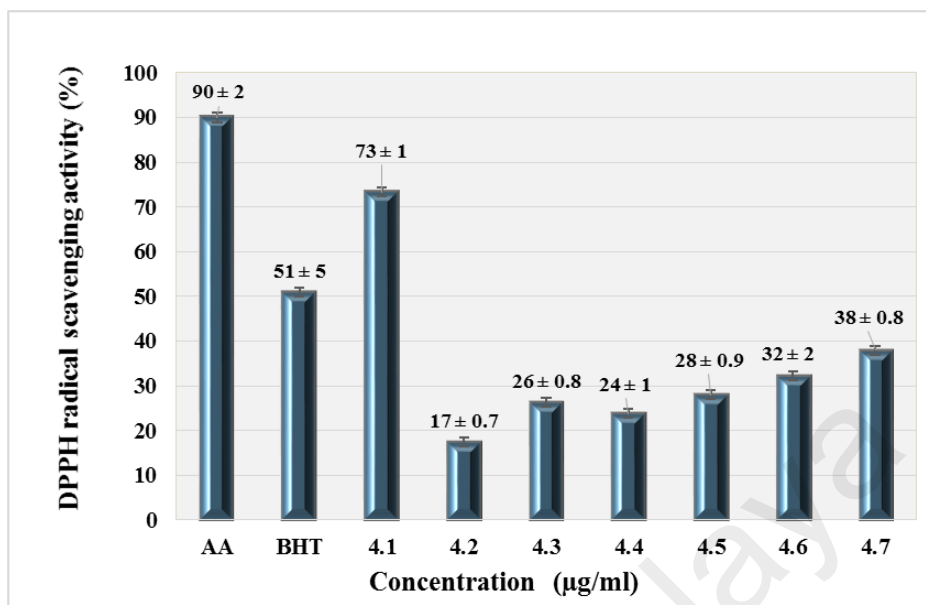
Figure 4.5. The maximum DPPH radical-scavenging activities of the synthesized compounds at 125 μg/mL concentration, in comparison with positive controls (BHT and ascorbic acid).
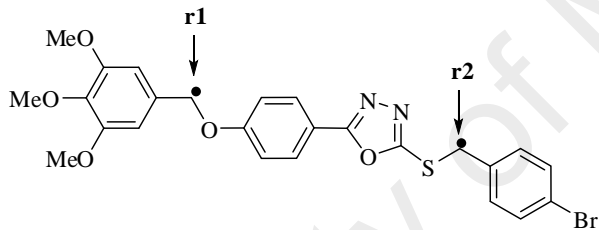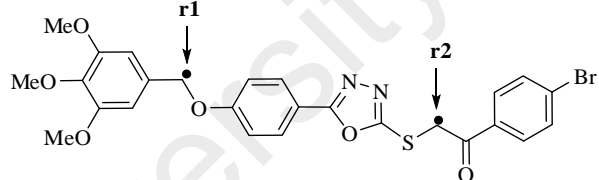
In compounds **4.6** and **4.7** the presence of EDGs, i.e. the methyl and methoxy groups on the phenyl ring at para position might favour antiradical activity, whereas the remaining compounds with EWGs showed less antiradical activities. The results from the above experiments thus confirm that the antioxidant activities are more pronounced in oxadiazole compound **4.1**, which contain the NH group compared to the one in which have S-alkylation. This may be due to the presence of identical NH group, which can easily donate the hydrogen compared to the CH_2 group.

4.4.2 DFT study of the DPPH radical scavenging activities of oxadiazole

Further confirmed by DFT calculations, compared the H-abstraction from the two methylene groups -CH_2 in oxadiazole derivatives in the absence of either OH or NH groups. Computational studies was carried out on compounds **4.2** and **4.3** in order to compare the abstraction energy of H atom between the–CH_2 groups in different sites

(Table 3). BDE for each –CH radical position is achieved by the difference between total energy of parent molecules with total energy of free radical molecules. Table 3 shows the figure of radical molecules in which the H atom has been abstracted at two positions, **r1** and **r2**. The BDE of **4.2-r2** is slightly lower than BDE for **4.2-r1** showing that the abstraction of H atom in **r2** position gives rise to a more stable radical condition than **r1**. This is probably due to the phenyl ring attached to the bromine atom, creating an electron withdrawing group and thus, increasing the radical stability at the **r2** position.

Table 4.3 : Calculated properties for compound 4.2 and 4.3

Compound		BDE (kcal/mol)	Spin density
	4.2-r1	279	0.985
	4.2-r2	268	0.983
	4.3-r1	273	0.921
	4.3-r2	274	0.979

For compound **4.2** the bond lengths for H-CH (**r1**) and H-CH (**r2**) were calculated. The values are 1.095 Å and 1.099 respectively, and the total energy for **4.2**-**r2** was higher than **4.2**-**r1**, which showed that the hydrogen abstraction in **4.2**-**r2** to form radical is more favourable compared to **4.2**-**r1**. As shown in Table 4.3, the dissociation processes are calculated and the energy required to break the H-CH (**r1**) and H-CH (**r2**) bonds are predicted to be 279 kcal/mol and 268 kcal/mol respectively, confirming that the H-CH (**r2**) was weaker.

However, bond lengths for H-CH (**r1**) and H-CH (**r2**) for compound **4.3** are 1.096 Å and 1.082 Å respectively, which means the bond distance of H-CH (**r2**) is shorter and the process of H abstraction is possibly occurs more easily in H-CH (**r1**) than H-CH (**r2**). The energy of the radicals is 0.95 kcal/mol, suggesting that in compound **4.3**, the H-CH (**r1**) is more stable than the H-CH (**r2**), showing that the abstraction of the radical in **r1** is more preferred than in **r2**. The BDE values of **4.3-r1** is lower than **4.3-r2**, confirming that the H-CH (**r1**) bond is weaker and the spin density of the H-CH (**r1**) and H-CH (**r2**) are 0.921 and 0.979, respectively, showed that the abstraction of the H from H-CH (**r1**) is possible compared to H-CH (**r2**) (Lucarini, Pedrielli, Pedulli, Cabiddu, & Fattuoni, 1996).

The HOMO energy which characterizes the ability of electron-giving is appropriate representation of free radical scavenging efficiency of the compounds. This is because the process to inhibit auto-oxidation may include the electron transfer besides the abstraction of the H-atom (Nagaoka, Kuranaka, Tsuboi, Nagashima, & Mukai, 1992). On the other hand, the atomic sites characterized by high density of the HOMO distribution are very sensitive to the attack of free radicals and other reactive agents. The more delocalized the HOMO orbital , the more numerous the electron sites, and the more redox reactions will occur (Mikulski, Górniak, & Molski, 2010).

The HOMO distribution for compounds **4.3-4.7** (Figure 4.6) is a useful visualization to support the explanation of the hydrogen abstraction above. The delocalization of electron distribution surrounding position **r1**, the trimethoxybenzyl site, which give a sign that the probability of electron distribution is stable in this region, and the favourable mechanism for these compounds may be the SET mechanism.
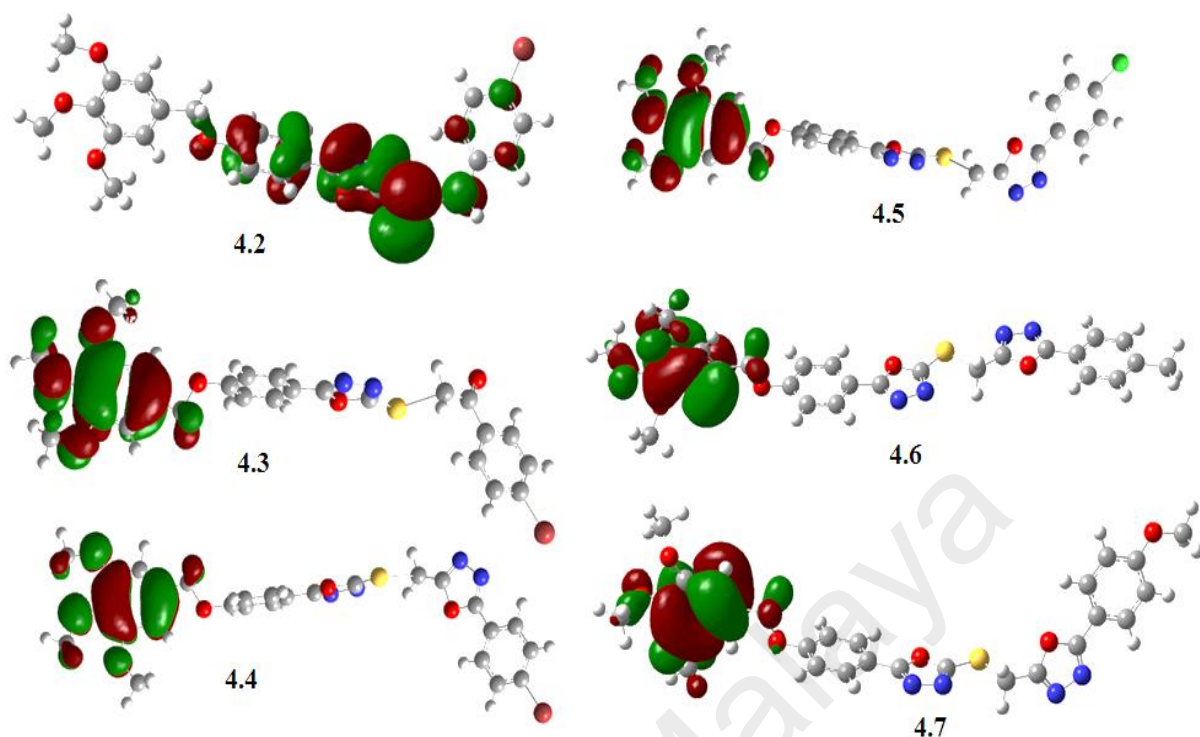
Figure 4.6. HOMO distribution for compounds 4.2-4.7

4.4.3   Ferric reducing antioxidant power (FRAP) activities

The FRAP assay measures the antioxidant ability to reduce ion Fe^{+3} (Kim & Lee, 2009; Morales, Martin, Açar, Arribas-Lorenzo, & Gökmen, 2009). The mechanism of FRAP assay is completely by electron transfer rather than a mixture of SET and HAT. Therefore, in combination with other methods, FRAP can be very useful to distinguish dominant mechanisms for different antioxidants. Electron-donating ability is determined by the one-electron oxidation potential of the parent antioxidants, expressed by definition as the reduction potential of the corresponding radicals. As shown in Figure 4.7, compound **4.1** has higher reducing potential than BHT, results are represented in Table 4.2. The FRAP results are in agreement with those obtained from the antioxidant activities determined by the DPPH radical scavenging assay. These results indicate higher activity with compound **4.1** and much lower activity with the remaining compounds **4.2-4.7**.
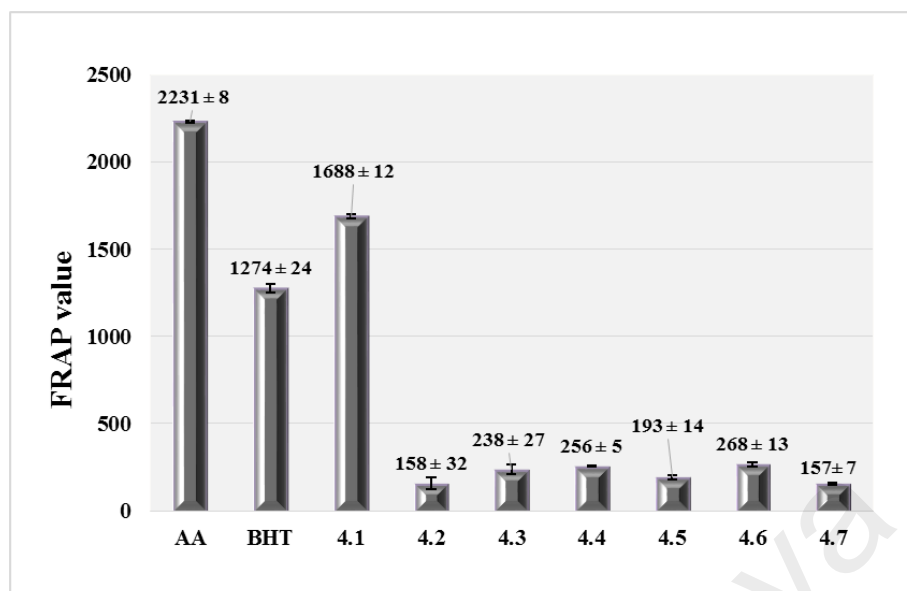
Figure 4.7. FRAP values for compounds 4.1- 4.7

4.4.4 Correlation analyses

Pearson correlation analyses were performed to predict the relationship between the two antioxidant assays (Figure 4.8). FRAP values showed a similar trend with DPPH scavenging results. The trend of these two antioxidant assays was revealed to be a very strong significant positive correlation (r= 0.97), indicating that the compounds which showed high DPPH radical scavenging activity, also showed high ferric reducing power activity. Thus, it clearly showed that the SET mechanism is the proposed mechanism in DPPH and FRAP assays (Bakalbassis, Lithoxoidou, & Vafiadis, 2006). (Equation 1.5-1.7, Chapter 1).
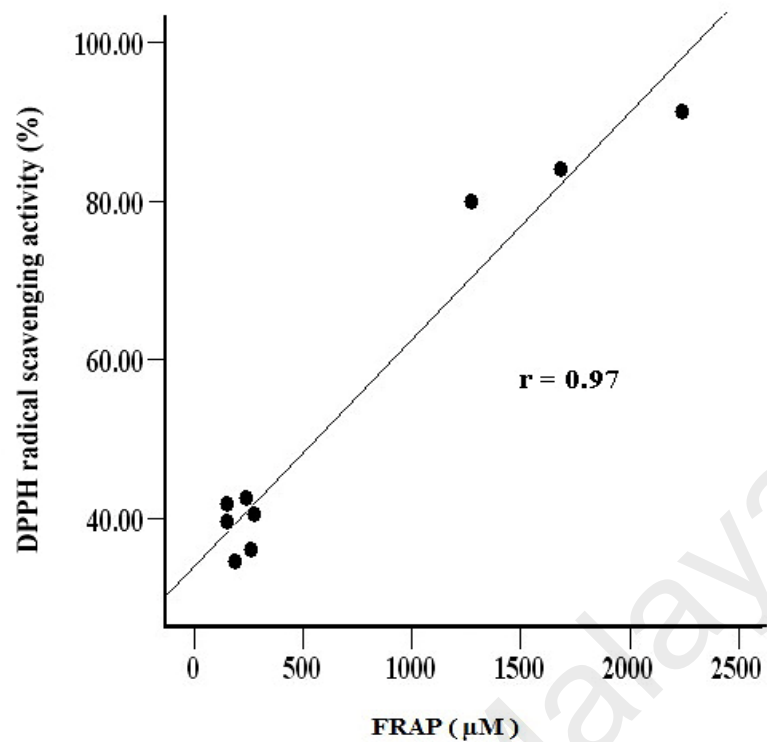
Figure 4.8. Pearson correlation analysis between the DPPH radical scavenging activities and ferric reducing activities of synthesized compounds

CHAPTER 5: SYNTHESIS OF ARYLTHIOSEMICARBAZIDES (5.1-5.6) AND 1,2,4-TRIAZOLE-5(4*H*)-THIONES (5.7-5.11)

5.1   Introduction

Thiosemicarbazide derivatives are interesting and versatile intermediates for the synthesis of important heterocycles such as triazoles, thiadiazoles, oxadiazoles and thiazolidinones (Dolman, Gosselin, O'Shea, & Davies, 2006; Mobinikhaledi, Foroughifar, Kalhor, Ebrahimi, & Fard, 2010). These compounds belong to the large group of thiourea derivatives, which have biological activities dependent on the parent aldehyde or ketone moiety (Du, Guo, Hansell, Doyle, Caffrey, Holler, McKerrow, & Cohen, 2002). The conjugated N-N-S tridentate ligand system of a thiosemicarbazide (-NH-NH-CS-NH-) seems essential for various biological activities (Hu, Yang, Pan, Xu, & Ren, 2010), and has therefore received considerable pharmaceutical interest (Hu, Zhou, Xia, & Wen, 2006). Bases are essential for many chemical reactions. The factors affecting the acidity of arylthiosemicarbazides will be analyzed by studying the effects of C=O and C=S on the acidity of the three N-H groups in arylthiosemicarbazides.

Triazoles, a group of heterocycles, are known as chemotherapeutic agents. They can be synthesized by base-catalyzed intramolecular dehydrative cyclization of thiosemicarbazides. In 2010 Imtiaz *et al* (Khan, Ali, Hameed, Rama, Hussain, Wadood, Uddin, Ul-Haq, Khan, Ali, & Choudhary, 2010), found the triazole-5-thione derivatives to be relatively more active than the isomeric thiadiazole derivatives (Figure 5.1). This is due to the presence of the thiourea system in the triazole ring. The experimentally determined antioxidant activity of hydroxyphenylurea derivatives exhibited 10 times higher antioxidant activity than α-TOH (Zhang, 2005).
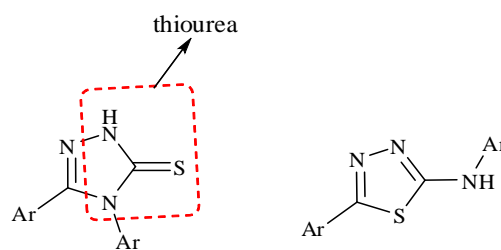
Figure 5.1. 1,2,4-Triazole and 1,3,4-thiadiazole derivatives

5.2 Structure-Activity Relationship of arylthiosemicarbazides

The structure-activity relationship (SAR) study for drug molecules should reveal structural features that benefit their ability to interact with the biological target. Drug molecules consist of various components known as functional groups, which indicate their biological activity. Each individual group in a designed structure can serve to provide one or more specific functions. In this study, the new thiosemicarbazides were designed to have multipotent antioxidants (MPAO) built into one structure. This feature can be strongly correlated to C=O and C=S bonds, likewise, and the presence of three amide NH groups. The structures of arylthiosemicarbazides and 1,2,4-triazoles are shown in (Figure 5.2) and (Figure 5.3) respectively.
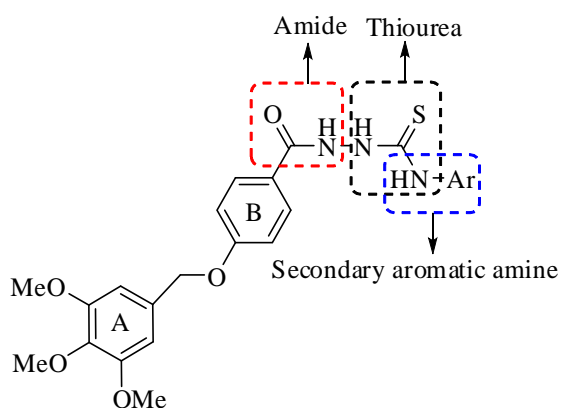
Figure 5.2. Rational design of arylthiosemicarbazides

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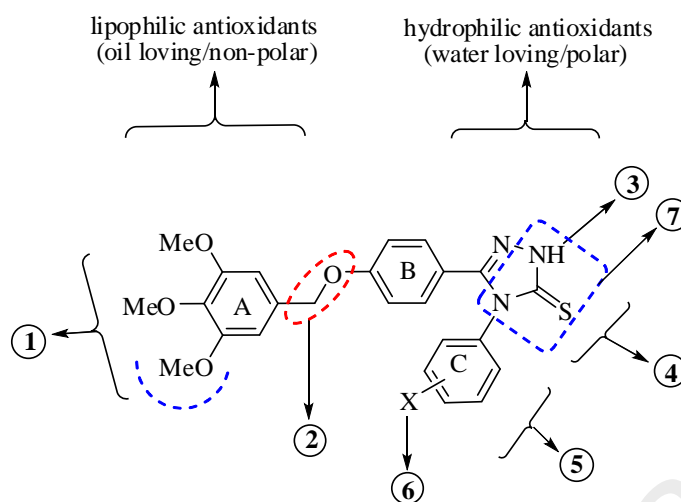
① 3,4,5-trimethoxybenzyl group, a well-known free radical scavenger
② -CH$_2$O- is electron donating group stabilize the ring
③ N-H or S-H exchangeable proton
④ Triazole, inhibitor ring
⑤ Substituted phenyl to increase the free radical stabilization
⑥ EDGs or EWGs substituents
⑦ Thiourea (free radical scavenger system)

Figure 5.3. Rational design of 1,2,4-triazole-5(4*H*)-thiones

5.3 Results and discussion

5.3.1 Synthesis

5.3.1.1 Synthesis of the arylthiosemicarbazide derivatives 5.1-5.6

Out of the amino groups from acid hydrazide, only the hydrazide–NH$_2$ can react with carbonyl compounds (Demirbas, Karaoglu, Demirbas, & Sancak, 2004). It was observed that the polarity of the solvent used in the reaction plays a very important role in controlling the reactivity and the reaction pathway of the compound. Use of a polar solvent affects on the acid hydrazide reactivity, hence the arylthiosemicarbazides (**5.1-5.6**) were successfully formed in very good yields (86-93%) when the acid hydrazide (**2.6**) was exposed to suitable arylisothiocyanates in the presence of absolute ethanol at RT according to synthetic Scheme 5.1.
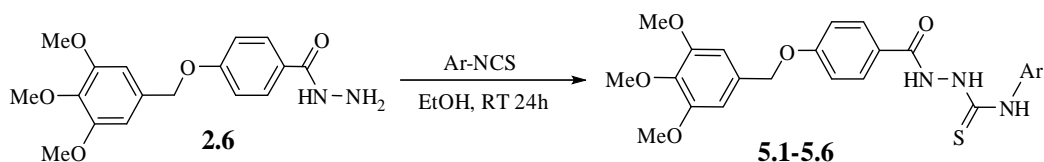
NO.	5.1	5.2	5.3	5.4	5.5	5.6
Ar	(Cl)	(OCH₃)	(OCH₃)	(CH₃)	(CH₃)	(NO₂)
	89 %	91 %	93 %	86 %	90 %	92 %

Scheme 5.1. The synthesis of arylthiosemicarbazide derivatives 5.1-5.6

All arylthiosemicarbazide compounds **5.1-5.6** showed NH stretching bands between 3321 cm^{-1} and 3136 cm^{-1} in the IR spectra. The C=O stretching band was nicely separated from C=C bands at 1659-1665 cm^{-1}. A strong band at 1227-1238 cm^{-1} was assigned to the C=S stretching (Dziewońska, 1967), while no stretching band for a S-H vibration was observed near 2550 cm^{-1}, thus confirming the thiourea tautomers.

The ^{1}H-NMR spectrum of compounds **5.1-5.6** exhibited signals originating from the methoxy groups at 3.67 and 3.79 ppm. The signal due to the primary amino group of hydrazide **2.6** disappeared. Three singlet peaks for amide -NH of the thiosemicarbazide were found. Between 9.20 and 9.75 ppm, the NH of the thio amide detected, whereas the other two singlets at 9.67 - 9.81 and 10.35 - 10.51 ppm were attributed to the (thio) hydrazides. Both appeared as a broad peaks, which may indicate the formation of intramolecular hydrogen bonding due to the formation of thioxo, enol and thio-enol forms as shown in Figure 5.4 (Akinchan, West, Yang, Salberg, & Klein, 1995; Jampílek, Doležal, Kuneš, Raich, & Liška, 2005).
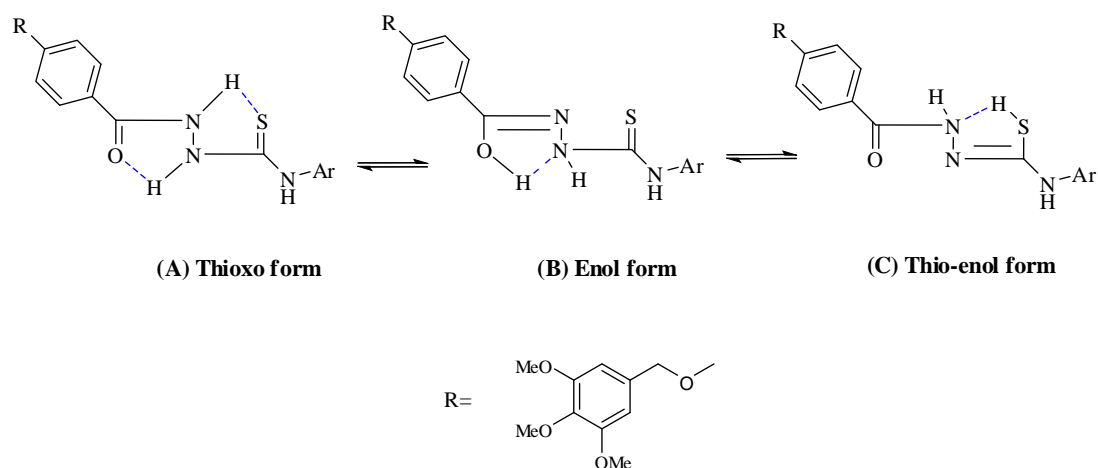
Figure 5.4. Intramolecular hydrogen-bonded forms of 5.1-5.6

In the ^{13}C NMR spectra, additional signals belonging to the C=S group were found at 181.0 -181.6 ppm. The HREIMS spectra of **5.1-5.6**, confirmed the identity of the compounds. Details are given in Table 5.1.

Table 5.1. HREIMS of molecular ion peaks of compounds 5.1-5.6

Compound	HREIMS m/z $[M]^+$	Theo. Mass	Composition
5.1	524.1019	524.1012	$C_{24}H_{24}ClN_3O_5S$
5.2	520.1514	520.1507	$C_{25}H_{27}N_3O_6S$
5.3	520.1522	520.1507	$C_{25}H_{27}N_3O_6S$
5.4	504.1570	504.1558	$C_{25}H_{27}N_3O_5S$
5.5	504.1571	504.1558	$C_{25}H_{27}N_3O_5S$
5.6	535.1257	535.1252	$C_{24}H_{24}N_4O_7S$

5.3.1.2 Structural factors that affect basicity of arylthiosemicarbazide

Polarity and resonance effect are two major factors well-known to influence the basicity of amines. Electron pair delocalization is probably the major factor in reducing the basicity of arylthiosemicarbazides. Thus, the increase in the electron pair delocalization causes a reduction of basicity and nucleophilicity of the corresponding compounds. Thiosemicarbazide has two amino groups ($-NH_2$), which are structurally related to the NH_2 of hydrazides.(Demirbas, Karaoglu, Demirbas, & Sancak, 2004) Of the two, only one amino group (NH-1) is reactive (Figure 5.5) (Kesel, 2011).
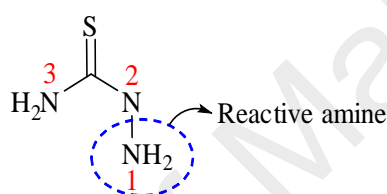
Figure 5.5. Active amino group in semicarbazide

The thiosemicarbazide compounds have two carbonyl groups, C=O and C=S, which are structurally similar but exhibit significantly different reactivities. The C=O bond is much more polar than the C=S bond due to the smaller difference in electronegativity for carbon (2.50) and sulfur (2.58) compared to carbon and oxygen (3.44). Hence, the C=O bond exhibits more polarization, with a partial negative charge on the oxygen (nucleophilic) and a partial positive charge on the carbon (electrophilic).

In agreement with the electronegativity difference between O and S, it should be noted that the charge on the thiocarbonyl carbon is considerably smaller than that at the carbonyl carbon. Therefore, the thiocarbonyl carbon should have reduced electrophilicity, which contributes to its reduced reactivity toward nucleophiles.

On the other hand, thioamides are known to have larger rotational barriers than amides. The barrier arises from the interaction of the NH with the adjacent C=O or C=S group as shown in Figure 5.6 (Hadad, Rablen, & Wiberg, 1998; Wiberg & Wang, 2011).
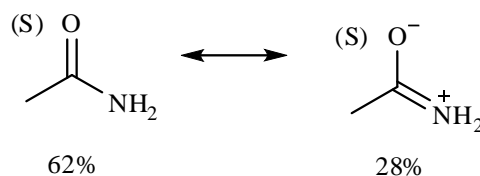
Figure 5.6. Amide resonance
(Kemnitz & Loewen, 2007)

The longer C=S bond also leads to a bond that is more polarizable by neighbouring groups. For instance, electron-withdrawing groups on the thiocarbonyl carbon atom reduce the electron density on sulphur significantly, and can reverse the expected polarity of the C=S bond, resulting in a preferred nucleophilic attack on the sulphur rather than the carbon. In general, the degree of conjugation in thioamide is considerably higher than in an amide. Both, the amide and the thioamide functional groups, withdraw electron density from the conjugated system. However, the thioamide is the stronger π-electron attractor (Velkov, Balabanova, & Tadjer, 2007; Wiberg & Wang, 2011), as shown in Figure 5.7.
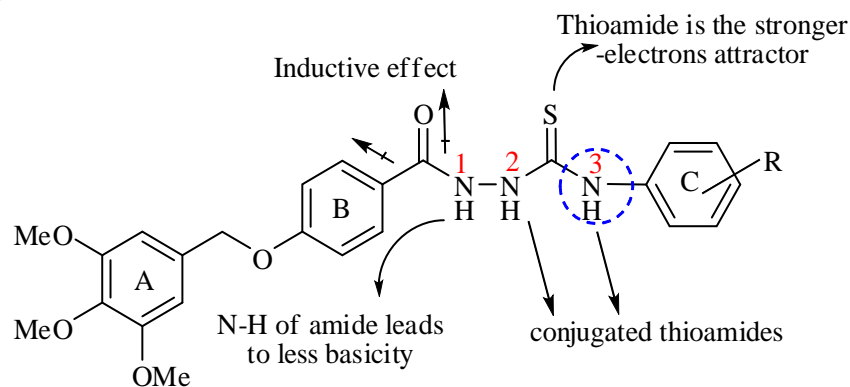
Figure 5.7. SAR of arylthiosemicarbazide

The C=S bond is almost 50 kcal/mol weaker than the C=O bond (Velkov, Balabanova, & Tadjer, 2007; Wiberg & Wang, 2011). This not only increases the reactivity of the C=S group but also completely changes the equilibrium of the tautomer between the thione and thiol forms of these compounds. The thiol is often more stable, resulting in a thione tendency to rearrange into the tautomeric thiol form (Figure 5.8).
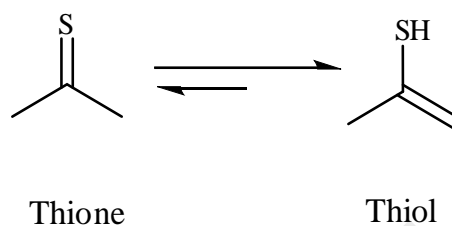
Figure 5.8. Tautomeric equilibrium of thione and thiol forms

As shown in Figure 5.7, the amino group (NH-3) of the thiosemicarbazide shows the lone pair of electrons on the nitrogen to be delocalized between two neighbouring groups; the thione and adjacent phenyl ring. Therefore, NH-3 group is less basic (more acidic) than either NH-2 or NH-1. Similarly, NH-2 is expected to be less basic than NH-1.

5.3.1.3 Synthesis of the 1,2,4-triazole derivatives 5.7-5.11

Refluxing arylthiosemicarbazide compounds **5.1-5.6** for 5-6 hours under strongly basic conditions with 4 M sodium hydroxide and ethanol resulted in the formation of the cyclized compound 4-(4-aryl)-3-(4-(3,4,5-trimethoxybenzyloxy)-phenyl)-1H-1,2,4-triazole-5-(4H)-thione (**5.7-5.11**) (Tsotinis, Varvaresou, Calogeropoulou, Siatra-Papastaikoudi, & Tiligada, 1997) in high yields (75 - 81%). An exception was compound **5.6**, which cleaved to give the corresponding acid 4-(3,4,5-trimethoxybenzyloxy)benzoic acid (**5.12**, 69%) instead of the triazole. The reaction sequences is outlined in the Scheme 5.2.
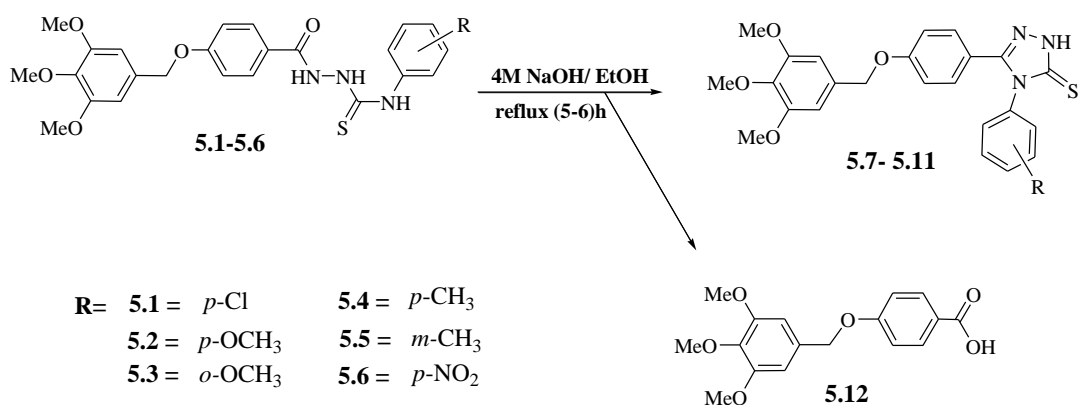
Scheme 5.2. Synthesis of 1,2,4- triazoles (5.7-5.11) and compound 5.12

The proposed mechanism for the base-catalysed intramolecular dehydrative cyclization of arylthiosemicarbazides (**5.1-5.6**) is illustrated in Scheme 5.3 (Cretu, Barbuceanu, Saramet, & Draghici, 2010). The NH-3, which is located between the thione and phenyl ring (C) is more acidic (less basic) than NH-1 and NH-2 as explained previously. The reaction is initiated by the nucleophilic attack of the nitrogen anion on the carbon of the amide carbonyl. This is followed by dehydration to obtained a five membered aromatic ring, the 1,2,4-triazole
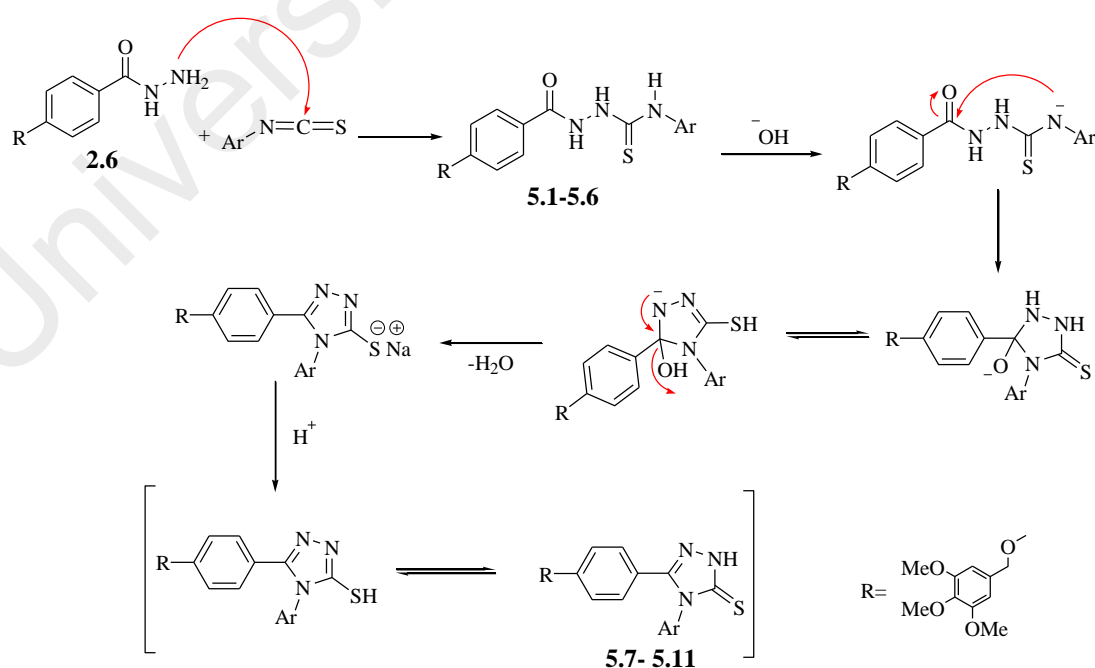
Scheme 5.3. Proposed mechanism for the formation of 1,2,4-triazole-5(4H)-thiones (5.7-5.11)

The conversion of arylthiosemicarbazide compounds (**5.1-5.6**) into 1,2,4-triazole-5(4H)-thiones in basic media, was monitored by the disappearance of the C=O peak in the IR spectra. A signal at 14.0 -14.1 ppm in the ^{1}H NMR spectra of compounds **5.7- 5.11** indicated the -NH, while the C=S group was observed at 168.8 - 169.1 ppm in the ^{13}C NMR spectra. The results of the HREIMS analyses of **5.7- 5.11** and compound **5.12** are given in Table 5.2.

Table 5.2. HREIMS of molecular ion peaks of compounds 5.7- 5.11 and 5.12

Compound	HREIMS m/z [M]$^+$	Theo. Mass	Composition
5.7	506.0915, 508.0901	506.0907, 508.0878	$C_{24}H_{22}ClN_3O_4S$
5.8	502.1408	502.1402	$C_{25}H_{25}N_3O_5S$
5.9	502.1416	502.1402	$C_{25}H_{25}N_3O_5S$
5.10	486.1467	486.1453	$C_{25}H_{25}N_3O_4S$
5.11	486.1469	486.1453	$C_{25}H_{25}N_3O_4S$
5.12	341.1001	341.0995	$C_{17}H_{18}O_6$

5.3.1.4 Synthesis of compound 5.12

Treatment of arylthiosemicarbazide compounds with strong base (4M sodium hydroxide) resulted in cyclization to 1,2,4-triazoles, except for compound **5.6**. The strong electron withdrawing nitro group in the compound **5.6** led to the hydrolysis of the semicarbazide, giving the corresponding acid **5.12** instead. Sodium hydroxide is a strong base and can also react as a nucleophile. Based on that, several mechanisms can explain the cleavage of **5.6** to compound **5.12**. The first mechanism proposes the deprotonation of the thionamide -NH. The low reactivity of the anion due to resonance delocalization of the nitro group limits the nucleophilicity of the thionamide anion, resulting in failure

of the cyclization. Instead hydrolysis of thiosemicarbazide by another hydroxide ion occurs, leading to the cleaved compound **5.12,** as shown in Scheme 5.4.
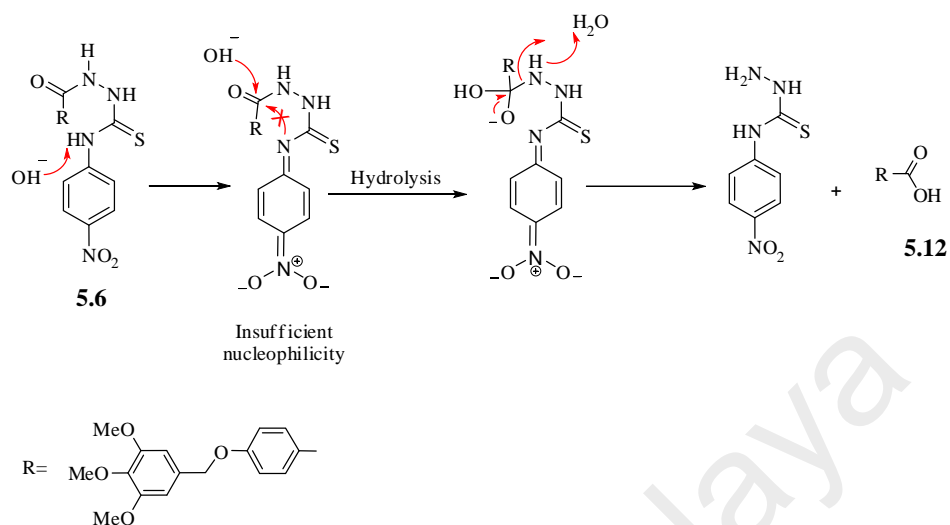
Scheme 5.4. Proposed mechanism of attack of nucleophile on triazole ring

Another possible mechanism proposes the intermediary formation of a 2-amino-1,3,4-oxadiazole (**5.12b**) by desulfurative cyclization (Yang, Lee, Kwak, & Gong, 2012). The latter undergoes hydrolysis due to the harsh condition, i.e. reflux and strong base. The nucleophile (OH⁻) attacks the iso-urea carbonyl of the 1,3,4-oxadiazole ring, which has low π electron density, to induce the ring cleavage (Somani & Shirodkar, 2011), as shown in Scheme 5.5.
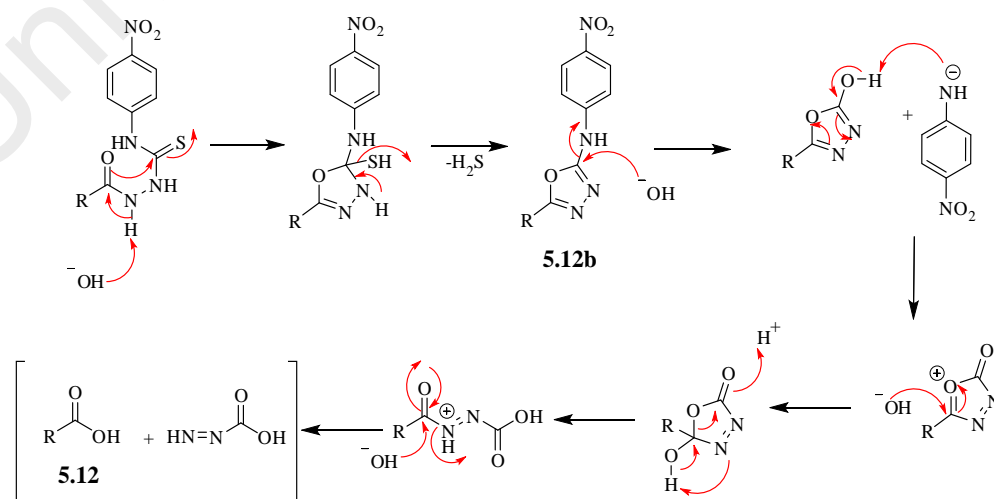
Scheme 5.5. Proposed mechanism of attack of nucleophile on oxadiazole ring

The last mechanism considered for the hydrolysis of **5.6** is initiated by a nucleophilic attack of the amide anion on the electrophilic carbon of the phenyl ring, leading to a spiro-bicycle with a heterocyclic five membered ring. This reaction is followed by nucleophilic attack of hydroxide on the iso-urea carbonyl, resulting in hydrolysis to yield compound **5.12**, (Scheme 5.6).
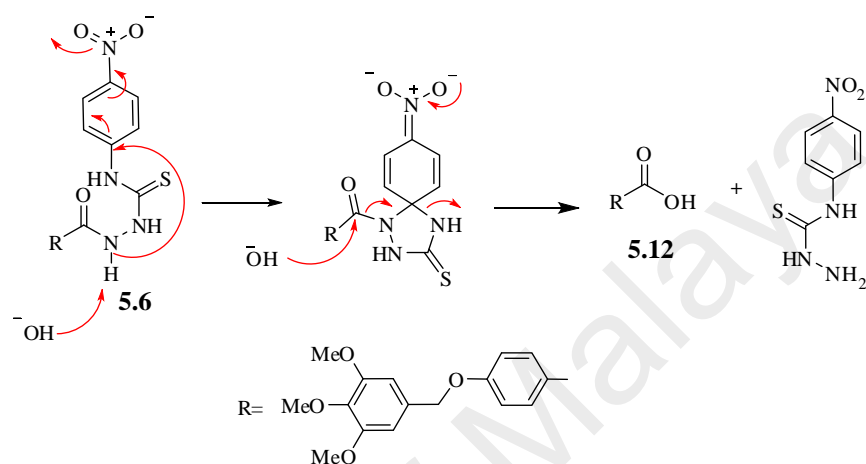
Scheme 5.6. Nucleophilic attack on carbon carbonyl

In the attempt to identify the route of hydrolysis for compound **5.6**, a series of reactions involving strong and weak base treatment has been performed, as outline in Scheme 5.7.
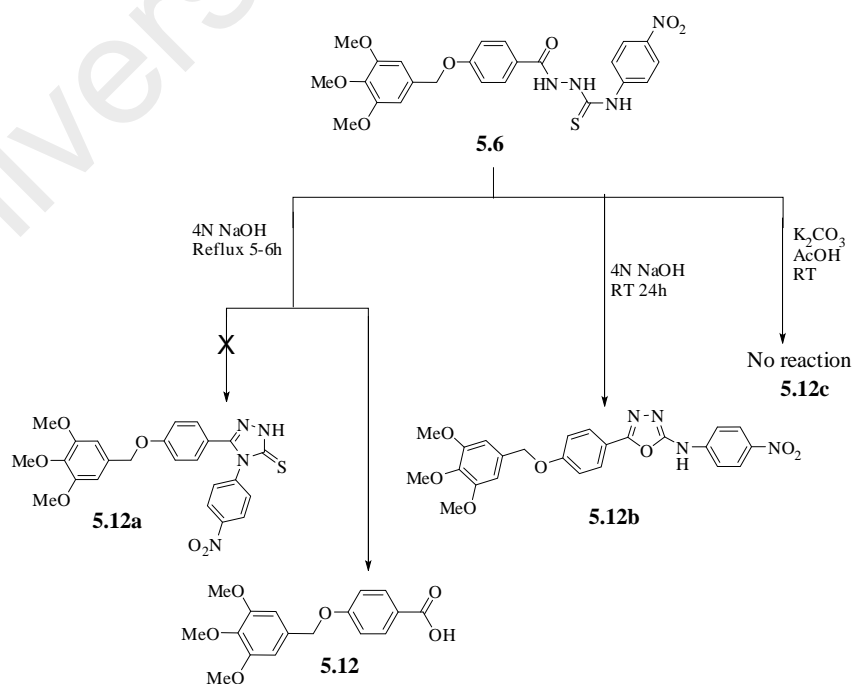
Scheme 5.7. Influence of different condition reaction on compound 5.6

Compound **5.6** was treated under different conditions to investigate the influence of the latter. Reaction of compound **5.6** with strong base (4M NaOH) under reflux, led to the hydrolysis product **5.12** instead of the targeted compound **5.12a**. Application of mild conditions, i.e. anhydrous K_2CO_3 at RT for 24 hours, did not furnish any cyclization products. Instead the NMR spectra supported the presence of compound **5.6,** indicating no reaction. When the reaction was done with strong base (4M NaOH) at RT for 24 hours, cyclization occurred. Instead of the targeted triazole (**5.12a**), the compound 4-nitrophenyl-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazol-2-amine (**5.12b**), was obtained in about 60% (Scheme 5.8).
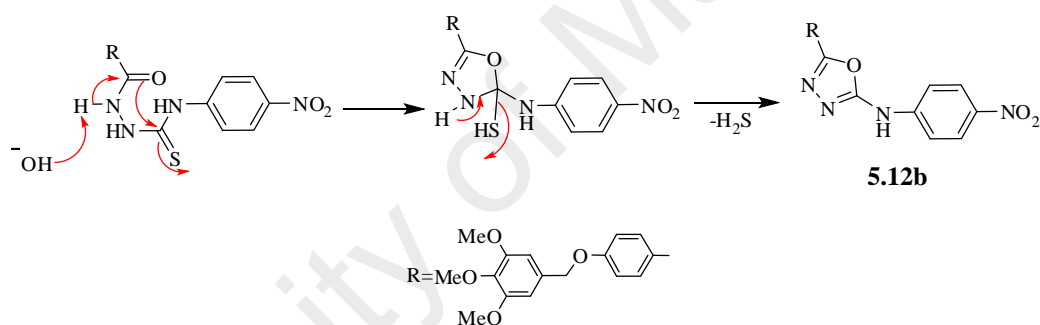
Scheme 5.8. Desulfurative cyclization reaction of compound 5.6

The reaction output matches previously reported cyclization of thiosemicarbazides to 2-amino-1,3,4-oxadiazole, which applied desulfurating agents, such as methyl iodide,(Fülöp, Semega, Dombi, & Bernath, 1990) ethyl bromoacetate (Küçükgüzel, Kocatepe, De Clercq, Şahin, & Güllüce, 2006), and mercury oxide (AboulWafa, 1984), instead of alkali. Another interesting approach for the cyclization reaction of arylthiosemicarbazide in oxidation by iodine in ethanol at room temperature in presence of alkali (Mavandadi & Pilotti, 2006) (Scheme 5.9).
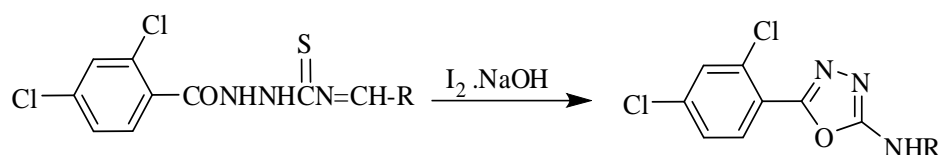
Scheme 5.9. Synthesis of 2- amino- 1,3,4-oxadiazole
(Somani & Shirodkar, 2011)

The ^{1}H NMR spectrum of the cleaved compound **5.12** exhibited a signal at 12.62 ppm belonging to hydroxyl group and the ^{13}C NMR a peak at 167.4 ppm belonging to C=O group. In case of compound **5.12b** the ^{1}H NMR spectra showed two singlet peaks at 9.05 and 10.85 ppm. One of them is due to the amino NH, while the second peak maybe due to presence of H_2O. Better crystals were obtained for the initial precipitation of **5.12b** performed in presence of water, compared to the recrystallization from an anhydrous solvent confirms association of the compound with water. The ^{13}C NMR spectra of **5.12b** confirmed the presence of the C=N at 160.84 and 165.12 ppm, respectively, whereas, the signal of the carbonyl group for compound **5.6** disappeared. The MS spectra of **5.12b** is shown in Table 5.3.

Table 5.3. MS of molecular ion peaks of compound 5.12b

Compound	MS m/z [M]$^+$	Theo. Mass	Composition
5.12b	479.1	478.1488	$C_{24}H_{22}N_4O_7$

5.3.1.5   DFT study for anion formation of compound 5.6

The assumption is that thiosemicarbazides, when donating protons, can form a structure stabilized by delocalization of electrons throughout the molecule. Table 5.4 lists the calculated values of the total energy (E_t) for anion of compound **5.6** at different positions. As shown in Scheme 5.10, the highest E_t value for the anion of **5.6-N1** suggests

that the abstraction of the proton in **5.6-N1**, i.e. the amide, is more favourable than the alternative positions, referring to **5.6-N2** and **5.6-N3**. The increase in the electron density of **N1** reduces the **N1–H** bond strength and increases the proton donating ability. Thus, the E_t of **N–H** in the **5.6-N2** position should be less than that of **5.6-N1** position.
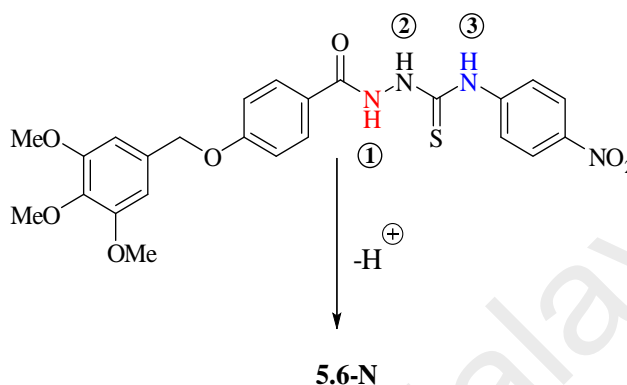
Scheme 5.10. Abstraction of proton in compound 5.6 at different position

Table 5.4. Calculated total energy of compounds 5.6 anions

Entries Anion	5.6-N1	5.6-N2	5.6-N3
E total (kcal/mol)	304.40	304.33	304.22

5.3.2 In vitro free radical scavenging activities

5.3.2.1 DPPH free radical scavenging activities

The synthesized compounds **5.1-5.12** were tested against DPPH at different concentrations (Figure 5.9) and compared with standard antioxidants ascorbic acid (AA) and BHT, as standard antioxidants. All results are presented as IC_{50} values and maximum radical scavenging and FRAP in Table 5.5. The maximum inhibition of the DPPH radical by the synthesized compounds **5.1-5.12** at a concentration of 125 µg/mL is shown in Figure 5.10.
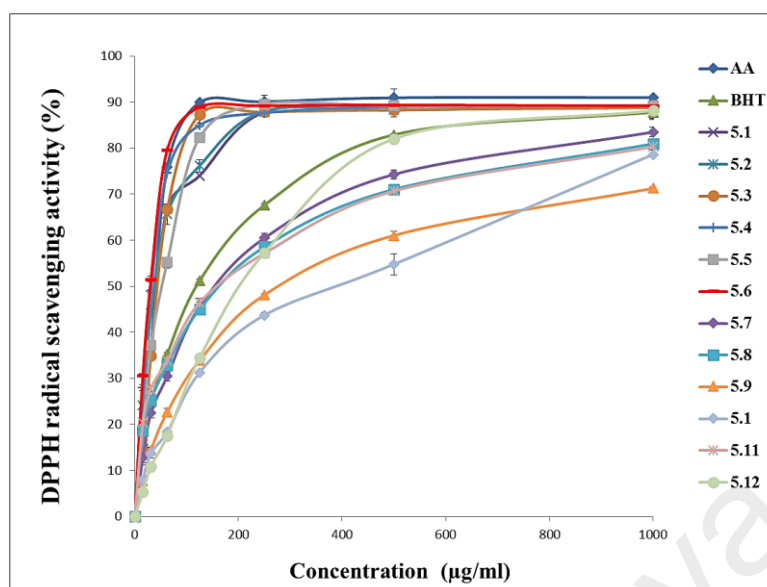
Figure 5.9. DPPH radical-scavenging activities of compounds 5.1-5.12

Table 5.5. DPPH radical scavenging activity and FRAP values of the synthesized compounds

Compounds	DPPH IC$_{50}$		Radical scavenging maximum inhibition %	FRAP μM
	(μg/mL)	(μM)*		
5.1	42 ± 1	84 ± 1	74 ± 1	1140 ± 33
5.2	38 ± 1	77 ± 1	76 ± 3	2476 ± 64
5.3	43 ± 1	87 ± 1	87 ± 2	1566 ± 26
5.4	32 ± 1	67 ± 1	85 ± 2	1905 ± 42
5.5	52 ± 2	108 ± 2	82 ± 2	2519 ± 103
5.6	29 ± 2	57 ± 2	89 ± 2	838 ± 38
5.7	154 ± 11	318 ± 11	46 ± 2	629 ± 13
5.8	162 ± 4	338 ± 4	45 ± 1	189 ± 18
5.9	274 ± 3	571 ± 3	34 ± 2	634 ± 22
5.10	157 ± 3	339 ± 3	31 ± 1	213 ± 29
5.11	389 ± 2	839 ± 2	46 ± 1	365 ± 25
5.12	201 ± 14	632 ± 14	34 ± 1	370 ± 10
AA	42 ± 2	239 ± 1.5	90 ± 1	2231 ± 87
BHT	119 ± 2	541 ± 2.1	51 ± 1	1274 ± 44

Results are expressed as a mean ± standard deviation (n = 3). DPPH radical scavenging activities are expressed as IC$_{50}$.concentrations of the compounds (μg/mL) required to inhibit 50% of the radicals. FRAP, ferric reducing antioxidant power. * IC$_{50}$.concentrations of the compounds in μM.

A comparison of the previous plots (in μg/mL) with the molar concentration plots in μM (see appendix page 209, Figure. D 3) display no significant differences with the series, owing to similar molecular weight. However, the data become more favorable in comparison with standard antioxidant compounds, which have lower molecular weight.

The higher free radical scavenging activities of thiosemicarbazide compounds **5.1-5.6** (Figure 5.10), could be attributed to the presence of an NH group from the aromatic amines in the thiosemicarbazides, which can donate a hydrogen atom via a HAT mechanism leading to neutralization of the DPPH radical (Nguyen, Le, & Bui, 2013). The resulting free radical was stabilized by delocalization of the odd electron into the aromatic ring (Nicolaides, Fylaktakidou, Litinas, & Hadjipavlou-Litina, 1998).
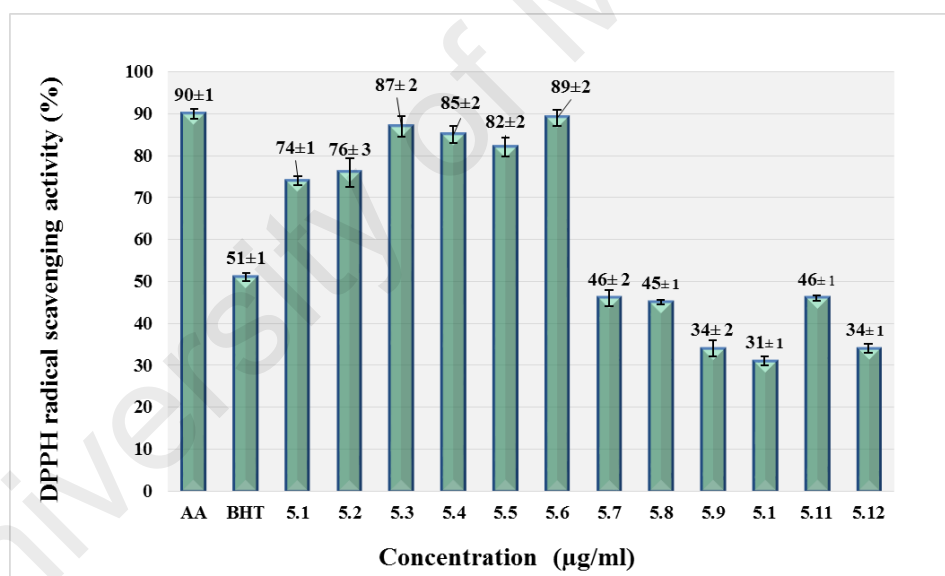
Figure 5.10. The maximum DPPH radical-scavenging activities of compounds 5.1-5.12 at 125 μg/mL concentration

The DPPH radical scavenging potential of thiosemicarbazide compounds **5.1-5.6** can be explained according to the proposed mechanism in Scheme 5.11 (Kopylova, Gasanov, & Freidlina, 1981). A reaction of two DPPH molecules is not possible due to their steric hindrance (Brand-Williams, Cuvelier, & Berset, 1995). The scavenging mechanism showed that aryl thiourea in thiosemicarbazides is able to neutralize two DPPH radicals.
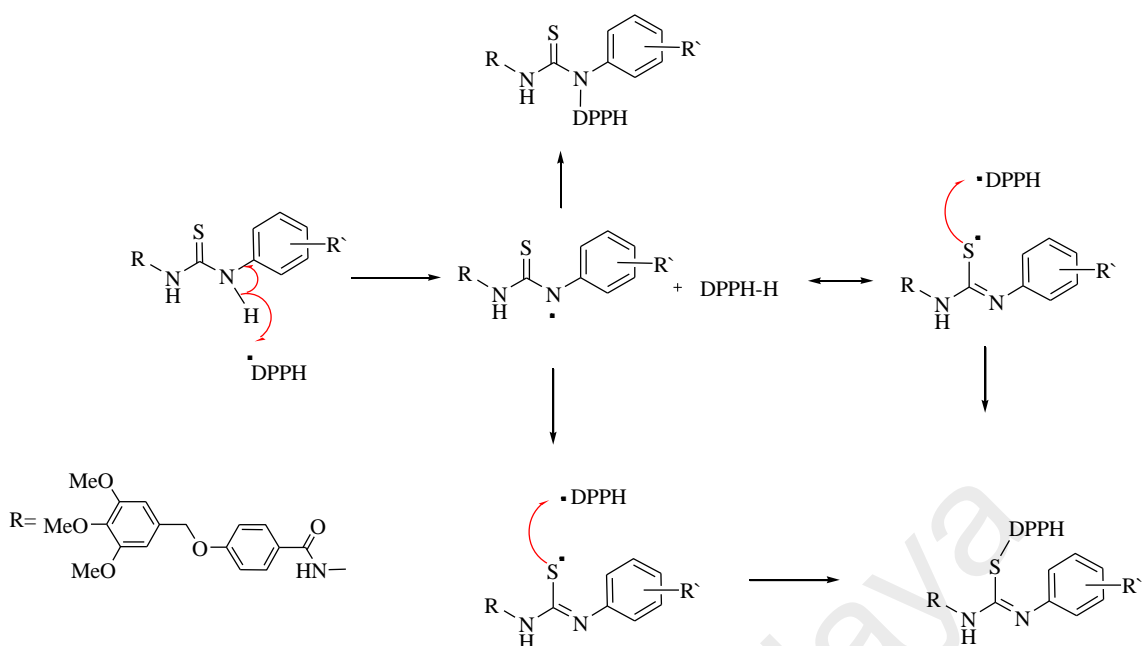
Scheme 5.11. Proposed mechanism for scavenging of DPPH· by thiosemicarbazide derivatives 5.1-5.6

Support for the proposed mechanism is provided by a previous report that described the antioxidant activities of aromatic amine derivatives and five-membered heterocyclic amines (Lucarini, Pedrielli, Pedulli, Valgimigli, Gigmes, & Tordo, 1999). The aromatic amines form an important class of antioxidants, which is similar to phenolic derivatives (Nishiyama, Yamaguchi, Fukui, & Tomii, 1999). They can easily transfer their amine hydrogen to peroxyl radicals, making them good H-donors (Lucarini, Pedrielli, Pedulli, Cabiddu, & Fattuoni, 1996; Minisci, 1997(27)).

Another evidence can be found in the antioxidant activity of phenethyl-5-bromopyridyl thiourea, which exists in both thiol and thione form. The thiol has been shown to exhibit antiradical activities and all phenethyl-5-bromopyridyl thiourea compounds exhibited antioxidant activities (Figure 5.11, **I**). The thiol-thione tautomerism has been evaluated on implications on the antioxidant activities.

The results using S-alkylated derivatives indicated that an unalkylated thiourea group is critical for antioxidant activities and that S-alkylation virtually eliminated this activity (Figure 5.11, **II**). This result suggests that the thiol group (II) is responsible for the

antioxidant activities due to its favourable electron-donating characteristics (Dong, Venkatachalam, Narla, Trieu, Sudbeck, & Uckun, 2000).
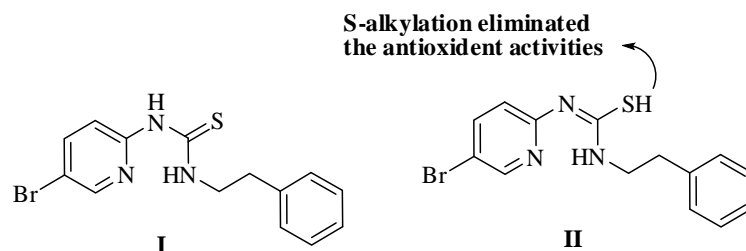
Figure 5.11. Thiol-thione tautomerism and S-alkylation in phenethyl-5-bromo-pyridyl thiourea

As can be seen from Table 5.5, the thiosemicarbazides **5.1-5.6**, exhibited stronger inhibitory effects in both antioxidant assays than the triazoles **5.7-5.12**. All thiosemicarbazides achieved >50% inhibition of the DPPH radical. The order of the activity follows **5.6** > **5.4** > **5.2** > **5.1** > **5.3 > 5.5**. These activities are comparable to the antioxidant references ascorbic acid (AA)(Chen, Xie, Nie, Li, & Wang, 2008; Sharma & Bhat, 2009) and butylated hydroxytoluene (BHT) (Kuş, Ayhan-Kılcıgil, Özbey, Kaynak, Kaya, Çoban, & Can-Eke, 2008; Sankaran, Kumarasamy, Chokkalingam, & Mohan, 2010; Yehye, Rahman, Ariffin, Hamid, Alhadi, Kadir, & Yaeghoobi, 2015).

Amongst them, compound **5.6** with IC_{50} 29 ± 2 and maximal inhibition of 89 ± 2 % demonstrated a higher activity. The presence of a strong electron-withdrawing group, such as NO_2 on the phenyl ring, has a great impact on the activity. The electron withdrawing effect polarizes the π-electrons of the phenyl ring, which enhances the acidity of the conjugated -NH group, exceeding the acidity of the other -NH groups in the compound. Therefore the thioamide hydrogen can be abstracted to form a structure stabilized by delocalization of electrons throughout the molecule, as shown in Scheme 5.12.
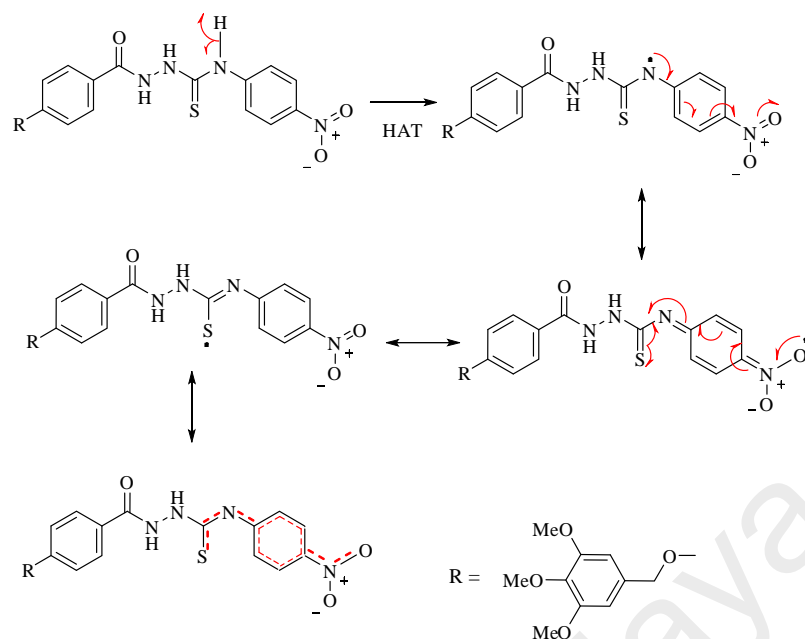
Scheme 5.12. Delocalization of the electron in the radical of compound 5.6

Compounds **5.6**, **5.4**, **5.2** and **5.1**, with substitution on the phenyl ring in para position the activity in the para position, regardless if electron donating or withdrawing, was more active than the ortho and meta substituted compounds **5.3** and **5.5**. Increase of the activity followed the order (para > ortho > meta) depending on the position of the substituent (Lawandy, Shehata, & Younan, 1996).

The *p*-methoxy substituents showed higher activity than the ortho-analog. This is in agreement with other reports describing a decrease in radical scavenging activities of phenol (or aniline) upon substitution in *o*-position. The reason is an intra-molecular hydrogen bonding between the -NH and the oxygen atom of *o*-methoxy group (Kajiyama & Ohkatsu, 2001), which strengthens the binding of the NH-hydrogen to the molecule, which reduces the DPPH scavenging activity (Jorgensen, Cornett, Justesen, Skibsted, & Dragsted, 1998). This H-bond increases the energy needed to abstract the hydrogen atom from a phenolic hydroxyl group (Amorati, Lucarini, Mugnaini, & Pedulli, 2003) . A similar effect is expected for -NH group of compound **5.3** based on the hydrogen bonding shown in Figure 5.12.
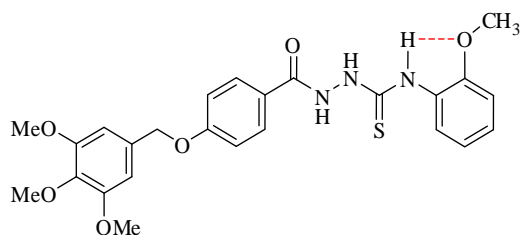
Figure 5.12. Formation of intramolecular hydrogen bond in compounds 5.3

On the other hand, the 1,2,4-triazole compounds **5.7-5.11**, as well as the hydrolyzed compound **5.12** exhibited only moderate to low DPPH radical scavenging activities, comparable with BHT as listed in Table 5.5 and Figure 5.9 and 5.10. The activities decrease in the order **5.7** > **5.10** > **5.8** > **5.12** > **5.9 > 5.11**. Compound **5.7** which bears *p*-halogen substituent (*p*- chloro) phenyl group with IC$_{50}$ 154 ± 11 µg/mL and 46 ± 2 % inhibition, shows higher inhibition than other compounds. The compounds with electron donating substituents, such as *p*-CH$_3$ (**5.10**) and *p*-OMe (**5.8**), showed higher antioxidant activities than the remaining compounds **5.12**, **5.9** and **5.11** (Ariffin, Rahman, Yehye, Alhadi, & Kadir, 2014). The stereoelectronic effects of *p*-methoxy stabilize the aryloxyl or arylaminyl radical through the p-type lone-pair orbital on the para heteroatom as reported in the literature (Ariffin, Rahman, Yehye, Alhadi, & Kadir, 2014; Ingold & Burton, 1992; J. S Wright, E. R. Johnson, & G. A. DiLabio, 2001).

5.3.2.2 DFT study of the DPPH radical scavenging activities of thiosemicarbazide

In order to rationalize the experimental observations, density functional theory (DFT) calculations have been carried out to estimate the actual energetics for different reactions, and evaluate the stability of radicals generated by H-abstraction from NH groups in the synthesized compounds. Bond dissociation enthalpies (BDEs) have also been calculated at B3LYP theory level for the respective H atom elimination paths.

The calculations applied gas-phase conditions. The two main factors determining free radical scavenging activity of antioxidants are the strength of the bond for the hydrogen atom and the electron donating ability of the antioxidants. Lower BDEs as associated with higher antioxidant activity (Lucarini, Pedrielli, Pedulli, Cabiddu, & Fattuoni, 1996).

Table 5.6 lists the calculated BDEs for the studied compound **5.6**. Comparing the BDE values suggests abstraction of a H in **r1,** i.e. at the thioamide group. For this radical, the p-type orbital of nitrogen atom at the nitro group delocalizes the unpaired electron, have stabilizing the radical and lowering the BDE. As expected, the decrease in the electron density at **N1** for **5.6-r1** is reflected in the low spin density. The density distributions of the SOMOs, spin densities and BDE of the respective radical species of compound **5.6** in different position were determined separately. The results for these separate calculations are summarized in Figure 5.13.

Table 5.6. Calculated properties for compound 5.6

Compound	*BDE (kcal/mol)*	*Spin Density*
5.6-r1	32.4	0.395
5.6-r2	33.6	0.445
5.6-r3	34.0	0.789
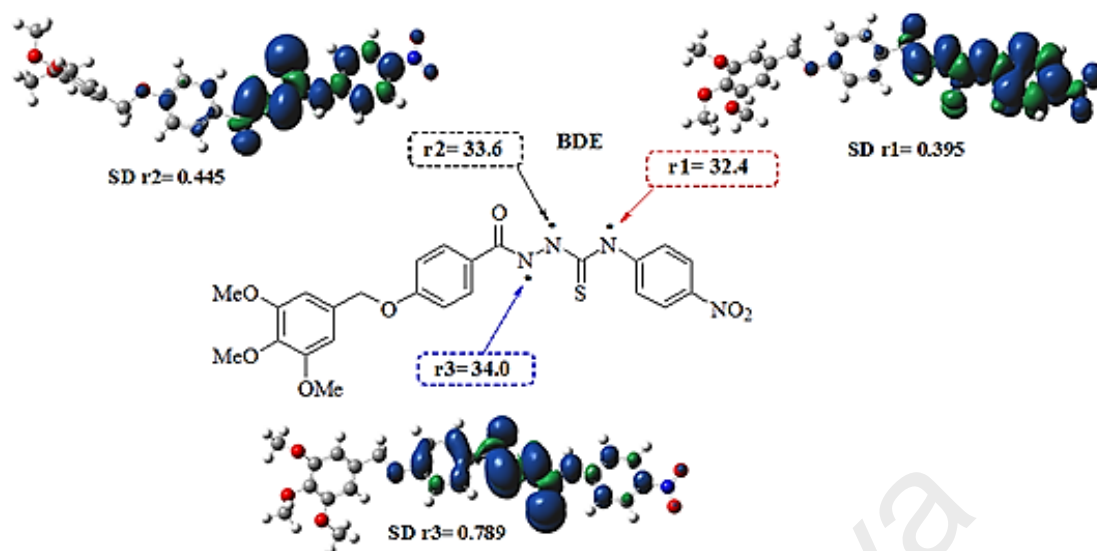
Figure 5.13. Density distributions of the SOMOs, BDE and SD calculated on the r1-N, r2-N and r3-N atoms in their radicals of compound 5.6

The BDE in **5.6-r2** increased due to the involvement of the hydrogen in intramolecular hydrogen bonding with the hydrazide carbonyl, which increases energy needed for H abstraction. The same applies for **5.6-r3** based on the thiourea carbonyl. In fact, the position of **r3** requires even more energy for the abstraction of the H.

5.3.2.3 Ferric reducing antioxidant power (FRAP) activities

The FRAP values, shown in Table 5.5 (page 86) and Figure 5.14 showed a similar trend with the DPPH assay, indicating higher activities for thiosemicarbazides **5.1-5.6**, and lower activity for the triazoles **5.7-5.11** as well as compound **5.12**. However, the FRAP activity of compound **5.6** is low, in contrast to its high activity in the DPPH scavenging test, which may be an indication of structural dependence of the antioxidation reaction in DPPH assay. The activity order for the thiosemicarbazides followed **5.5** > **5.2** > **5.4** > **5.3** > **5.1 > 5.6**.
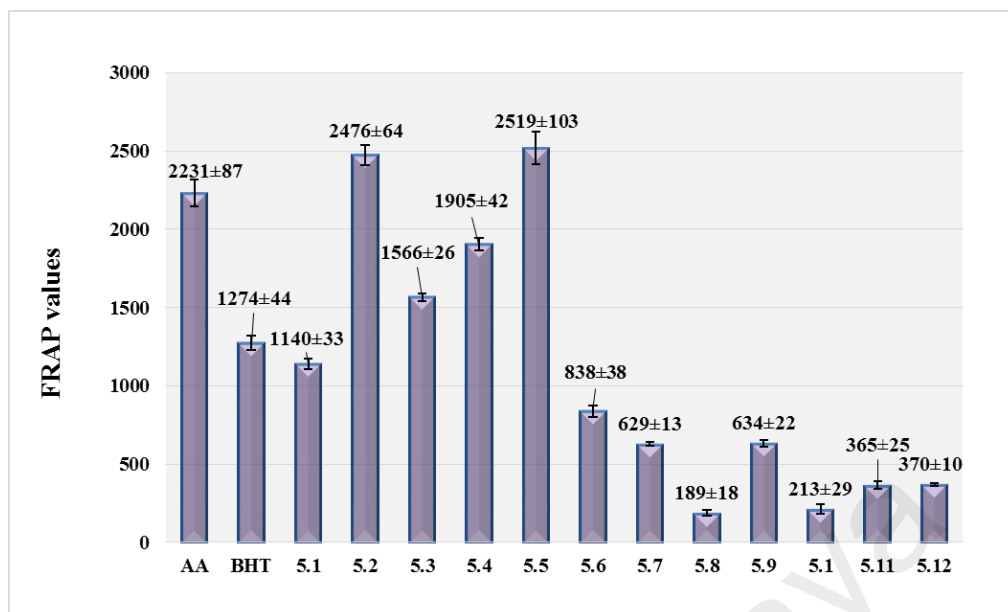
Figure 5.14. FRAP values for compounds 5.1-5.12

Compounds **5.5** and **5.2** bearing *m*-methyl and *p*-methoxy groups with values 2519 ± 13 and 2476 ± 6 μM respectively, were highly active compared to ascorbic acid and BHT. The compounds **5.4** and **5.3** with *p*-methyl and *o*-methoxy groups, with values 1905 ± 4.2 and 1566 ± 3 μM respectively, showed higher activity than BHT. On the other hand, compounds **5.1** and **5.6** with electron withdrawing substituents showed much lower activities. The results clearly indicate higher activities for the thiosemicarbazide compounds and a significant drop of the activity upon conversion to the triazole thiols **5.7-5.11**. This might be attributed to the disappearance of the hydrogen atom on the nitrogen of the thiosemicarbazide structure, and to the less resonance structures.

5.3.3   Correlation analysis

To investigate the relationship between the DPPH radical scavenging and ferric reducing activities of compounds **5.1-5.12**, a Pearson correlation analysis was performed (Kong, Mat-Junit, Aminudin, Ismail, & Abdul-Aziz, 2012). There was no significant correlation between the two assays for thiosemicarbazides and triazoles compounds. This is might be due to the relatively small differences between statistical parameters (Amić & Lučić, 2010; James S Wright, Erin R Johnson, & Gino A DiLabio, 2001). Instead scatter plot was performed as shown in Figure 5.15. Similar trend was found between both assays for thiosemicarbazides, while differences have seen between the results of triazole compounds. It is possible that the differences in the reaction mechanisms of the two assays are reflected in different results.
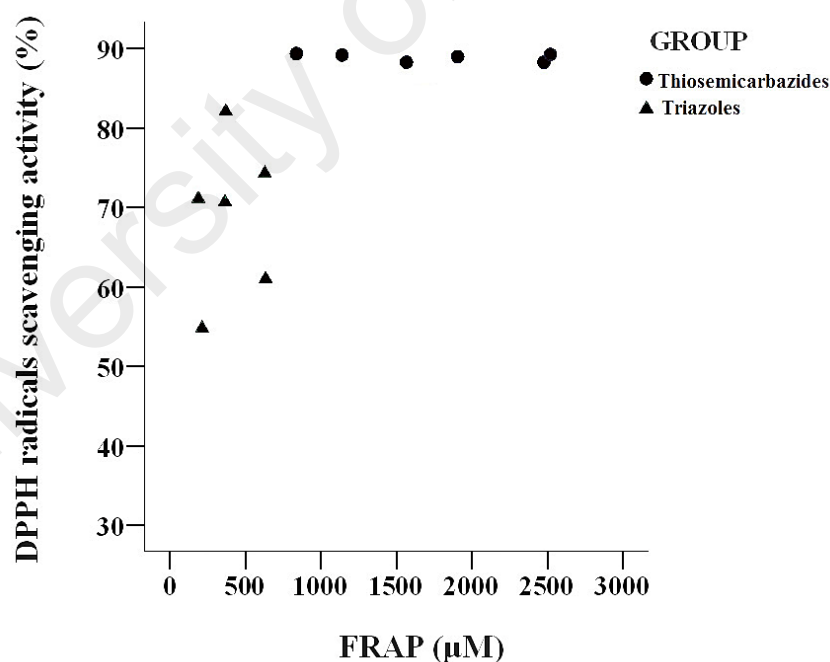

Figure 5.15. Scatter plot between the DPPH radical scavenging activities and ferric reducing activities of synthesized compounds

CHAPTER 6: CONCLUSION

In an effort to generate compounds bearing the 3,4,5-trimethoxy benzyl moiety as free radical scavenging agents, three series of derivatives of 3,4,5-trimethoxy benzyl hydrazine were prepared and the antioxidant behaviour was evaluated by DPPH and FRAP assays. In order to rationalize the experimental observations, DFT calculations have been carried out to explain differences in the radical-scavenging abilities of the compounds. The findings can be summarized as follows:

1. The core intermediate for all compounds, i.e. 4-(3,4,5-trimethoxybenzyloxy) benzohydrazide (**2.6**) was obtained in a 3-steps synthesis in more than 50% overall yield from commercial resources.

2. Reaction of the key intermediate, acid hydrazide **2.6** with various aromatic aldehydes furnished the corresponding hydrazone in good yield.

3. Treatment of hydrazide **2.6** with CS_2 and KOH provided the 1,3,4-oxadiazole thione in high yield. Reaction of the later with alkyl halide provided a series of S-alkylated oxadiazoles.

4. Hydrazide **2.6** reacted with thio isocyanates to form a series of arylthiosemicarbazides. The later was cyclized with strong base (4M NaOH) in good yield to form a series of 1,2,4-triazoles. However, the presence of a nitro group in para position of the semicarbazide prevented the cyclization. The nitro substituted thiosemicarbazide was investigated on its reaction with bases under various conditions:

   a) Refluxing with strong base (4M NaOH) led to hydrolysis of the thiosemicarbazide instead of the cyclization.

   b) Mild conditions, i.e. treatment with K_2CO_3 at RT for 24 hours, did not lead to any reaction.

c) Reaction of with strong base (4M NaOH) at RT for 24 h lead to a cyclization in about 60%. However, instead of the 1,2,4-triazoles a 1,3,4-oxadiazole was obtained.

5. The DPPH scavenging activity of tested hydrazones showed that the EDGs near to para phenolic hydroxyl possessed a higher antioxidant activity than EWGs.

6. Electron withdrawing substituents enhance the antioxidant activities in thiosemicarbazide compounds.

7. Besides stereoelectronic effect of substituents hydrogen bonding effect the antioxidant activity. For hydrazones para substitution was more active than ortho substitution. This is believed to originate from an intra-molecular hydrogen bond while disfavours the DPPH scavenging activity of the ortho hydroxy substituent.

8. S-alkylation of thio 1,3,4-oxadiazoles practically eliminated the antioxidant activity, which indicated that an unalkylated thioamide group (with ionizable proton) is a good free radical scavenger.

9. The FRAP values show a similar trend with DPPH radical scavenging results indicating higher activities with thiosemicarbazides derivatives. The results revealed that thiosemicarbazides are excellent hydrogen and electron donor compounds, and exhibit promising antioxidant activities and deserve attention in the synthesis of bioactive compounds.

10. Pearson correlation analysis was performed to investigate the relationship between the DPPH radical scavenging and ferric reducing activities of the synthesized compounds. The data revealed :

a) A strong positive correlation between the two antioxidant assays for the 1,3,4-oxadiazole derivatives. This reflect similarity in the reaction mechanism which probably SET mechanism.

b) A moderate positive correlation for the hydrazones, and for thiosemicarbazides and their cyclized analogs 1,2,4-triazoles, indicating that the compound with high DPPH radical scavenging activities did not necessarily show high ferric reducing activities and vice versa.

11. Computational studies using DMOL3 based on DFT.1, were correlated with antioxidant activities for the synthesized compounds. The calculation include several parameter that determined the antioxidant activity, such as spin density (SD), Bond dissociation energy (BDE), ionization energy (IP).

For future studies, I suggest to evaluate the anti-inflammatory or anticancer activity of the synthesized compounds particularly the thiosemicarbazides, furthermore, a kinetic study for the synthesized compounds can be applied. A new series of potential antioxidants could be obtained by reaction of hydrazones with sodium acetate and in glacial acetic acid to obtain oxadiazoles (Scheme 6.1). Investigation of the later could help to understand the difference between the hydrazones and their cyclized oxadiazole analogs on the scavenging of free radicals.
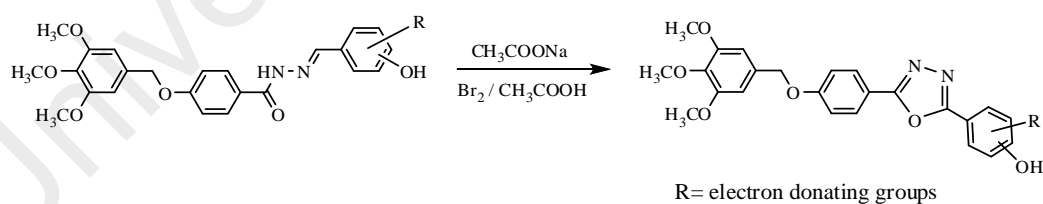

**Figure 6.1. Proposed structure for future study**

CHAPTER 7: EXPERIMENTAL DETAILS

7.1   General

Chemicals and solvents were obtained from Sigma-Aldrich and Merck used without purification. NMR spectra were measured on a Bruker 400 MHz FT-NMR spectrometer (Bruker, Falladen, Switzerland) using DMSO-d_6 as solvent. Chemical shifts are reported in (ppm) and coupling constants (J) in Hz. The abbreviations s = singlet, d = doublet, dd = double doublet, t = triplet, q = quadruplet, m = multiplet and bs = broad signal were used throughout. HRESI mass spectra were recorded at the Department of Chemistry at the University of Singapore on a MAT 95 x1-T spectrometer at 70 eV (Agilent Technologies, Santa Clara, CA, USA). Melting points were determined using a Mel-Temp II melting point apparatus (laboratory devices Inc, Holliston, USA) and are uncorrected. The experimental method for all the IR data by use ATR, and the IR spectra were recorded on a RX1 FT-IR spectrometer (Perkin-Elmer, Waltham, MA, USA). The absorbance of the reaction mixtures in the DPPH and FRAP assays were determined by UV spectroscopy using a Power Wave X340, BioTek Inc, Instrument (Winooski, VT, USA). Thin layer chromatography (Silica gel TLC) applied Si-60 plates from Merck, and were developed with UV light and in an iodine chamber.

7.2 Experimental details

7.2.1 3,4,5-Trimethoxybenzyl bromide (2.2) (Mabe, 2006)
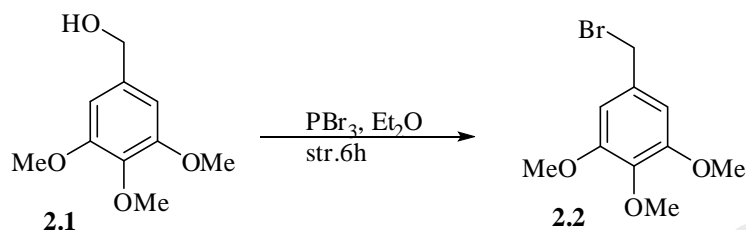
PBr$_3$ (2.2 g, 11 mmol) was added dropwise to a solution of 3,4,5-trimethoxybenzyl alcohol (2.1) (2.0 g, 10 mmol) in Et$_2$O at -40°C. The mixture was stirred at -40°C for 6 hours, then washed once with water (30 ml) and extracted with Et$_2$O. The combined extract was dried over anhydrous sodium sulphate and solvent was removed under reduced pressure. Pale yellow solid was obtained and used for further reactions without purification. The product yield (2.13 g, 82%), m.p. 70–73 °C (lit.72.3-73.4 °C) (Mareike C. Holland, Jan Benedikt Metternich, Constantin Daniliuc, W. Bernd Schweizer, & Gilmour, 2015). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 3.66 (s, 3 H), 3.78 (s, 6 H), 4.70 (s, 2 H), 6.77 (s, 2 H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 33.9, 56.4, 60.5, 106.7, 133.6, 137.9, 153.3.
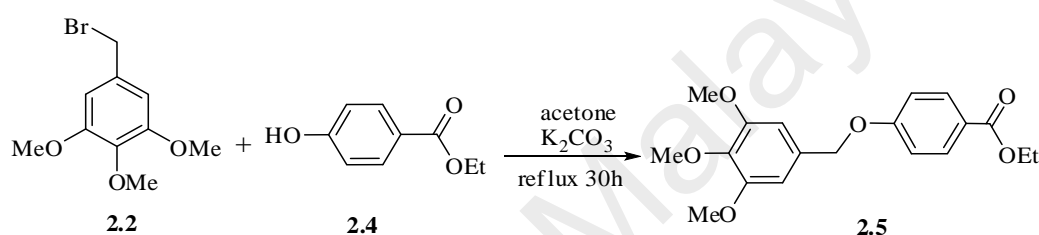
7.2.2 Ethyl 4-hydroxybenzoate (2.4)
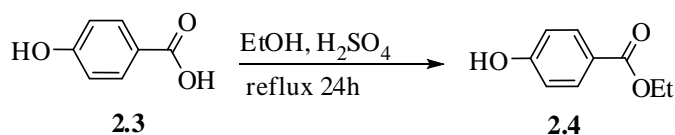
A mixture of 4- hydroxylbenzoic acid (2.3) (2.0 g, 14.0 mmol), absolute ethanol (50 ml) and sulphuric acid (2-3 mL) was refluxed for 24 hours. After cooling, ethanol was evaporated under reduced pressure, aq. NaHCO$_3$ (3%, 10 mL) was added, the solution

was extracted with ethyl acetate, filtered and dried over anhydrous sodium sulphate. The crude product, obtained after concentration under reduced pressure, was recrystallized from ethanol to give white crystals (2.23 g, 96%) m.p. 111-113°C (lit.110-113 °C) (Fabrizio Carta, 2013). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 1.27 (t, J = 7.1 Hz, 3H), 4.23 (q, J = 7.1 Hz, 2H), 6.86 (d, J = 8 Hz, 2H), 7.82 (d, J = 8 Hz, 2H), 10.30 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 14.6, 60.4, 115.7, 121.1, 131.8, 162.4, 166.0.

### 7.2.3   Ethyl 4-[(3,4,5-trimethoxybenzyl)oxy]benzoate (2.5)

A mixture of compound **2.2** (1.0 g, 3.8 mmol), anhydrous K$_2$CO$_3$ (0.53 g, 3.8 mmol) and ethyl 4-hydroxybenzoate (**2.4**) (0.63 g, 3.8 mmol) in acetone (25 mL) was refluxed for 30 hours. Acetone was evaporated under reduced pressure and the resulting brown oil was extracted with three portions (30 mL) of ethyl acetate against 5% NaOH. The combined organic layer were washed with water (30 mL) and dried over anhydrous Na$_2$SO$_4$. After evaporation of the solvent the product **2.5** was crystallized from methanol to give colourless crystals (0.80 g, 61%). m.p. 77-80°C. IR, cm^{-1}: ν = 3066 (CH$_{Ar}$), 2941 (CH$_{aliph.}$), 1714 (C=O), 1462 (C=C), 1233 (C-O), 1020 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 1.30 (t, J = 6.9 Hz, 3H, CH$_3$), O-CH$_3$ [3.66 (s, 3H), 3.78 (s, 6H)], 4.27 (q, J = 6.9 Hz, 2H, CH$_2$), 5.09 (s, 2H, O-CH$_2$), Ar.H [6.79 (s, 2H), 7.14 (d, J = 8 Hz, 2H), 7.93 (d, J = 8 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 14.7 (1C, **CH$_3$**), (3C, O-**CH$_3$**) [56.4, 60.5], 60.8 (1C, **CH$_2$**), 70.3 O-**CH$_2$**, Ar.C [105.9, 115.2, 122.8, 131.6, 132.4, 137.7, 153.4, 162.7], 165.8 (ester C=O). HREIMS. C$_{19}$H$_{22}$O$_6$, m/z= 347.1455 (M+H$^+$). Calcd. 347.1488.

**7.2.4**   4-(3,4,5-Trimethoxybenzyloxy)benzhydrazide (**2.6**)
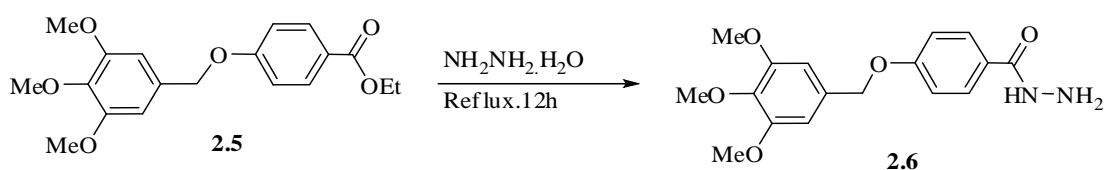
Hydrazine hydrate (2 mL, 0.4 mmol, 85%) was added to a solution of compound **2.5** (1.0 g, 2.9 mmol) in absolute ethanol (3 mL). The solution was refluxed for 24 hours. After that, ethanol was evaporated under reduced pressure and a small amount of water (2-3 mL) was added to precipitate the hydrazide **2.6**, which was filtered and dried in vacuum to give a shiny white solid (0.83 g, 87%). m.p. 106-108°C. IR, cm^{-1}: ν = 3272, 3250 (m, NH, NH$_2$), 3003 (CH$_{Ar}$), 2941 (CH$_{aliph.}$), 1676 (C=O), 1236 (C-O), 1120 (C-N), 1020 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.78 (s, 6H)], 4.43 (s, 2H, NH-N$\underline{H_2}$), 5.07 (s, 2H, O-CH$_2$), Ar.H [6.79 (s, 2H), 7.07 (d, J = 8 Hz, 2H), 7.81 (d, J = 8 Hz, 2H)], 9.60 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (3C, O-**C**H$_3$) [56.4, 60.5], 70.1 (O-**C**H$_2$), Ar.C [105.8, 114.8, 126.2, 129.2, 132.7, 137.6, 153.4, 161.0], 166.0 (hydrazide C=O). HREIMS. C$_{17}$H$_{20}$N$_2$O$_5$, m/z = 333.1459 (M+H$^+$). Calcd. 333.1450.

7.2.5   General procedure for the synthesis of the hydrazones of 4-(3,4,5-trimethoxy-benzyloxy)benzohydrazide (**3.1-3.9**)
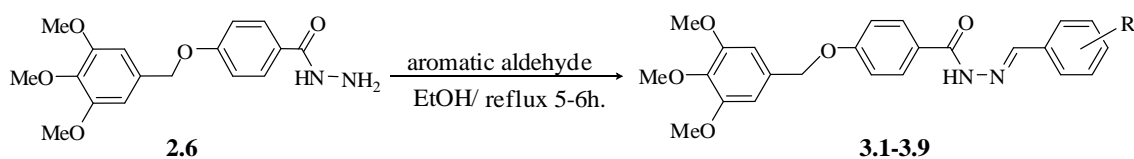
A solution of the required aldehyde (1.2 mmol) in absolute ethanol (15 mL) was added to a solution of compound **2.6** (0.40 g, 1.2 mmol) in absolute ethanol (10 mL) and the mixture was refluxed for 5-6 hours. The solution was concentrated to half of its volume under reduced pressure and subsequently poured on crushed ice. The product was precipitated, dried and finally recrystallized from ethanol.

7.2.5.1   N-(5-Bromo-3-chloro-2-hydroxybenzylidene)-4-(3,4,5-tri-methoxybenzyl-oxy)benzohydrazide **(3.1)**
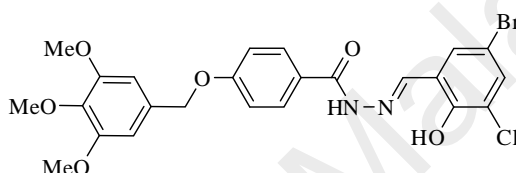

White solid (0.53 g, 81%), m.p. 235-236°C. IR, cm^{-1}: ν = 3315 (OH$_{phenol}$), 3211 (NH), 2936 (CH$_{Ar}$), 2834 (CH$_{aliph}$), 1642 (C=O), 1495 (C=C), 1238 (C-O), 1120 (C-N), 1023 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.12 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 7.20 (d, J = 8 Hz, 2H), 7.69 (d, J = 2.4 Hz, 1H), 7.72 (d, J = 2.4 Hz, 1H), 7.96 (d, J = 8 Hz, 2H)], 8.52 (s, 1H, CH), 12.41, 12.80 (s, 1H, NH, OH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (3C, O-**C**H$_3$) [56.4, 60.5], 70.3(O-**C**H$_2$), Ar.C [105.9, 111.2, 115.2, 120.9, 123.7, 124.8, 129.7, 130.3, 132.5, 133.3, 137.7, 147.0 (C=N), 153.4, 153.7, 162.0], 162.9 (C=O). HREIMS, C$_{24}$H$_{22}$BrClN$_2$O$_6$, m/z = 549.0271, 551.0250 (M+H$^+$). Calcd. 549.0419, 551.0398.

**7.2.5.2**   N-(3,5-Di-tert-butyl-4-hydroxybenzylidene)-4-(3,4,5-tri-methoxybenzyl-oxy)benzohydrazide (**3.2**)
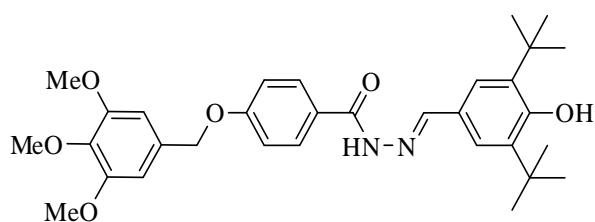
White solid (0.49 g, 74%). m.p. 237-238ºC. IR, cm^{-1}: ν = 3606 (OH$_{phenol}$), 3234 (NH), 2941 (CH$_{Ar}$), 2833 (CH$_{aliph.}$), 1644 (C=O), 1596 (C=N), 1495 (C=C), 1231 (C-O). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 1.42 (s, 18H, 2 × t-Bu), O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 7.15 (d, J = 8 Hz, 2H)], 7.39 (s, 1H, OH), Ar.H [7.47 (s, 2H), 7.90 (d, J = 8 Hz, 2H)], 8.39 (s, 1H, CH), 11.49 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 30.6 (6C, 2 × C(**CH$_3$**)$_3$), 35.0 (2C, 2× **C**(CH$_3$)$_3$), (3C, O-**C**H$_3$) [56.4, 60.5], 70.2 (O-**C**H$_2$), Ar.C [105.8, 115.0, 124.3, 126.2, 126.4, 129.8, 129.9, 132.6, 137.7, 139.7, 149.3 (C=N), 153.4, 156.5, 161.4], 162.7 (C=O). HREIMS, C$_{32}$H$_{40}$N$_2$O$_6$, m/z = 549.2959, (M+H$^+$). Calcd. 549.2952.

7.2.5.3   N-(4-Hydroxy-3,5-dimethoxybenzylidene)-4-(3,4,5-trimethoxybenzyloxy) benzohydrazide (**3.3**)
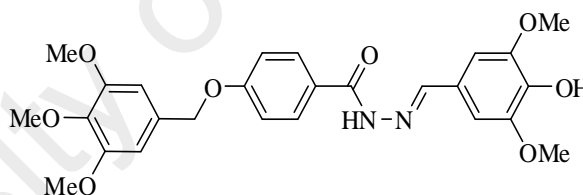
White solid (0.43 g, 72 %). m.p. 159-160ºC. IR, cm^{-1}: ν = 3480 (OH$_{phenol}$), 3227 (NH), 2941 (CH$_{Ar}$), 2833 (CH$_{aliph}$), 1642 (C=O), 1585, 1593 (C=N), 1238 (C-O). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.68 (s, 3 H), 3.79 (s, 6 H), 3.83 (s, 6 H)], 5.11 (s, 2 H, O-CH$_2$), Ar.H [6.81 (s, 2H), 6.99 (s, 2H), 7.15 (d, J = 8 Hz, 2H), 7.91 (d, J = 8 Hz, 2H)], 8.35 (s, 1H, CH), 8.88 (s, 1H, OH), 11.60 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (5C, O-**C**H$_3$) [56.4, 56.5, 60.5], 70.2 (O- **C**H$_2$), Ar.C [105.0, 105.8, 115.0, 125.2, 126.3, 129.9, 132.6, 137.6, 138.3, 148.4 (C=N), 148.6, 153.4, 161.4], 162.9 (C=O). HREIMS, C$_{26}$H$_{28}$N$_2$O$_8$, m/z = 497.1920, (M+H$^+$). Calcd. 497.1914.

7.2.5.4   N-(3-Ethoxy-4-hydroxybenzylidene)-4-(3,4,5-trimethoxybenzyloxy)
benzohydrazide (**3.4**)
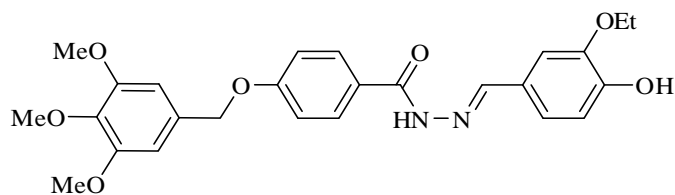
White solid (0.49 g, 85%). m.p. 176-177ºC. IR, cm^{-1}: ν = 3434 (OH$_{phenol}$), 3219 (NH),
2941 (CH$_{Ar}$), 2833 (CH$_{aliph.}$), 1645 (C=O), 1594 (C=N), 1242 (C-O). ^{1}H NMR (400 MHz,
DMSO-d$_6$) δ, ppm: 1.37 (t, J = 6.8 Hz, 3H, CH$_3$), O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)],
4.08 (q, J = 6.8 Hz, 2H, CH$_2$), 5.11 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 6.86 (d, J = 8 Hz,
1H), 7.08 (d, J = 8 Hz, 1H), 7.15 (d, J = 8 Hz, 2H), 7.30 (s, 1H), 7.90 (d, J = 8 Hz, 2H)],
8.32 (s, 1H, CH), 9.43 (s, 1H, OH), 11.53 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$)
δ, ppm: 15.2 (1C, **CH$_3$**), (3C, O-**CH$_3$**) [56.4, 60.5], 64.4 (1C, **CH$_2$**), 70.2 (O-**CH$_2$**), Ar.C
[105.8, 110.8, 115.0, 116.0, 122.4, 126.3, 129.9, 132.6, 137.7, 147.6, 148.3 (C=N), 149.6,
153.4, 161.4], 162.8 (C=O). HREIMS, C$_{26}$H$_{28}$N$_2$O$_7$, m/z = 481.1973, (M+H$^+$). Calcd.
481.1965.

**7.2.5.5**   N-(2,3,4-Trimethoxybenzylidene)-4-(3,4,5-trimethoxybenzyl-oxy)benzo-
hydrazide (**3.5**)
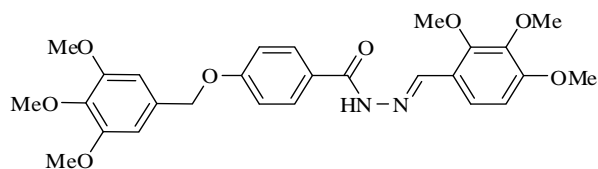
White solid (0.44 g, 72%). m.p. 189-190ºC. IR, cm^{-1}: ν = 3207 (NH), 2941 (CH$_{Ar}$),
2841 (CH$_{aliph.}$), 1644 (C=O), 1595 (C=N), 1239 (C-O). ^{1}H NMR (400 MHz, DMSO-d$_6$)
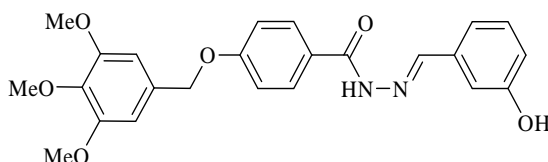δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.72 (s, 3H), 3.79 (s, 6H), 3.85 (s, 6 H)], 5.11 (s, 2H, O-

CH$_2$), Ar.H [6.64 (bs, 1H), 6.81 (s, 2H), 6.91 (bs, 1H), 7.16 (d, *J* = 8 Hz, 2H), 7.92 (d, *J* = 8 Hz, 2H)], 8.39 (bs, 1H, CH), 11.71 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (6C, O-*C*H$_3$) [56.37, 56.4, 60.5, 60.6], 70.2 (O-*C*H$_2$), Ar.C [104.7, 105.8, 115.0, 126.2, 130.0, 130.4, 132.6, 137.6, 139.6, 147.7 (C=N), 153.4, 153.7, 161.5], 163.0 (C=O). HREIMS, C$_{27}$H$_{30}$N$_2$O$_8$, m/z = 511.2089, (M+H$^+$), Calcd. 511.2070.

7.2.5.6 N-(3,4,5-Trimethoxybenzylidene)-4-(3,4,5-trimethoxybenzyl-oxy)benzo-hydrazide (**3.6**)
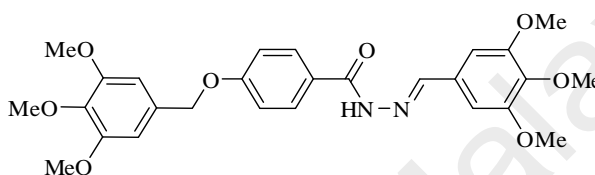
White solid (0.45 g, 74%). m.p. 182-183ºC. IR, cm^{-1}: ν = 3215 (NH), 2941 (CH$_{Ar}$), 2841 (CH$_{aliph.}$), 1639 (C=O), 1594 (C=N), 1239 (C-O). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.71 (s, 3H), 3.79 (s, 6H), 3.84 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 7.04 (s, 2H), 7.15 (d, *J* = 8 Hz, 2H), 7.91 (d, *J* = 8 Hz, 2H)], 8.37 (bs, 1H, CH), 11.74 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (6C, O-*C*H$_3$) [56.4, 56.4, 60.5, 60.6], 70.2 (O-*C*H$_2$), Ar.C [104.7, 105.8, 115.0, 126.1, 130.0, 130.4, 132.6, 137.6, 139.6, 147.7 (C=N), 153.4, 153.7, 161.5], 163.1 (C=O). HREIMS, C$_{27}$H$_{30}$N$_2$O$_8$, m/z = 533.1894, (M+Na$^+$), Calcd. 533.1889.

7.2.5.7 N-(3-Hydroxybenzylidene)-4-(3,4,5-trimethoxybenzyloxy)benzohydrazide (**3.7**)

White solid (0.42 g, 80%). m.p. 98-99ºC. IR, cm^{-1}: ν = 3404 (OH$_{phenol}$), 3232 (NH), 2941 (CH$_{Ar}$), 2833 (CH$_{aliph.}$), 1646 (C=O), 1593 (C=N), 1239 (C-O), 692 (meta-sub.).^{1}H
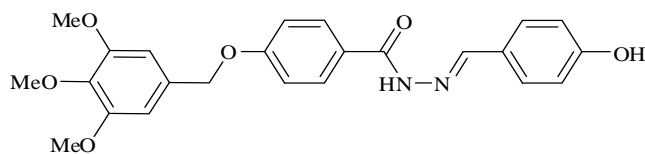
NMR (400 MHz, DMSO-d_6) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 6.83 (dd, J = 8 Hz, J = 1.8 Hz, 1H), 7.10 (d, J = 8 Hz, 1H), 7.15 (d, J = 8 Hz, 2H), 7.19 ($_{bs}$, 1H), 7.25 (t, J = 8 Hz, 1H), 7.90 (d, J = 8 Hz, 2H), 8.34 (s, 1H, CH), 9.69 (s, 1H, OH), 11.69 (bs, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: (3C, O-CH$_3$) [56.4, 60.5], 70.2 (O-CH$_2$), Ar.C [105.8, 113.1, 115.0, 117.8, 119.2, 126.0, 130.0, 130.4, 132.6, 136.1, 137.6, 147.8 (C=N), 153.4, 158.1, 161.5], 163.0 (C=O). HREIMS, C$_{24}$H$_{24}$N$_2$O$_6$, m/z = 459.1533, (M+Na$^+$). Calcd. 459.1523.

**7.2.5.8**   N-(2-Hydroxybenzylidene)-4-(3,4,5-trimethoxybenzyl oxy)benzohydrazide (**3.8**)
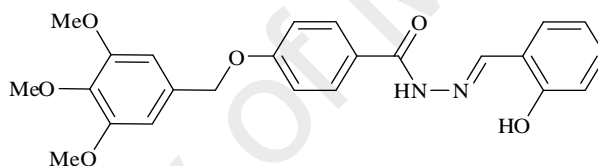
White solid (0.44 g, 83%). m.p. 115-116ºC. IR, cm^{-1}: ν = 3496 (OH$_{phenol}$), 3218 (NH), 2919 (CH$_{Ar}$), 2840 (CH$_{aliph}$), 1636 (C=O), 1606 (C=N), 1239 (C-O), 761 (ortho-sub.). ^{1}H NMR (400 MHz, DMSO-d_6) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 6.91 – 6.95 (m, 2H), 7.17 (d, J = 8 Hz, 2H), 7.30 (t, J = 7.2 Hz, 1H), 7.52 (d, J = 7.2 Hz, 1H), 7.94 (d, J = 8 Hz, 2H), 8.62 (s, 1H, CH), 11.39 (s, 1H, OH), 12.02 (s, 1H, NH).^{13}C NMR (100 MHz, DMSO-d_6) δ, ppm: (3C, O-CH$_3$) [56.4, 60.5], 70.2 (O-CH$_2$), Ar.C [105.8, 115.1, 116.9, 119.1, 119.8, 125.4, 130.1, 131.7, 132.6, 137.6, 148.4 (C=N), 153.4, 157.9, 161.8], 162.8 (C=O). HREIMS, C$_{24}$H$_{24}$N$_2$O$_6$, m/z = 459.1529, (M+Na$^+$). Calcd. 459.1523.

7.2.5.9   N-(4-Hydroxybenzylidene)-4-(3,4,5-trimethoxybenzyloxy)benzohydrazide (**3.9**)

White solid (0.63 g, 84%). m.p. 227-228ºC. IR, cm^{-1}: ν = 3383 (OHphenol), 3302 (NH), 2925 (CHAr), 2837 (CHaliph.), 1647 (C=O), 1598 (C=N), 1250 (C-O), 841 (para-sub.). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.10 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 6.84 (d, J = 8 Hz, 2H), 7.14 (d, J = 8 Hz, 2H), 7.55 (d, J = 8 Hz, 2H), 7.90 (d, J = 8 Hz, 2H)], 8.34 (s, 1H, CH), 9.90 (s, 1H, OH), 11.52 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: (3C, O-*C*H$_3$) [56.4, 60.5], 70.2 (O-*C*H$_2$), Ar.C [105.8, 115.0, 116.2, 125.9, 126.3, 129.2, 129.9, 132.6, 137.6, 148.0 (*C*=N), 153.4, 159.8, 161.4], 162.7 (*C*=O). HREIMS, C$_{24}$H$_{24}$N$_2$O$_6$, m/z = 459.1529, (M+Na$^+$). Calcd. 459.1523.

7.2.6   General procedure for the synthesis of 5-(4-(3,4,5-trimethoxybenzyloxy) phenyl)-1,3,4-oxadiazole-2-(3H)-thione (**4.1**)
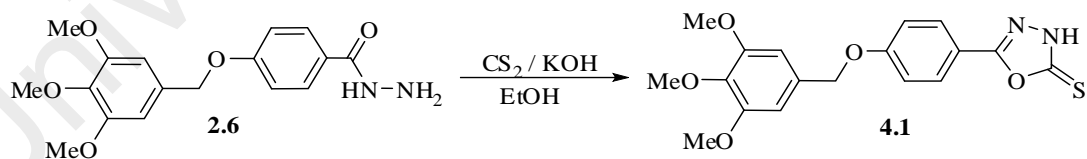
A mixture of compound **2.6** (1.20 g, 3.6 mmol), anhydrous potassium hydroxide (0.6 g, 4.3 mmol), carbon disulfide (0.91 Ml, 15.5 mmol) and absolute ethanol (15 mL) was refluxed for 24 hours. The solvent was removed in vacuum and the residue was poured on 100 mL ice water and acidified with 5% HCl. The precipitate was collected, washed with water, dried and crystallized from ethanol to give white solid (1.16 g, 86%), m.p. 198-200°C. IR, cm^{-1}: ν = 3173 (NH), 2967 (CH$_{Ar}$), 2841 (CH$_{aliph}$.), 1599 (C=N), 1070

(O-CH$_3$), 1130 (C-N). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.12 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 7.21 (d, J = 8.5 Hz, 2H), 7.83 (d, J = 8.5 Hz, 2H)], 14.60 (bs, 1H, NH).^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [56.4, 60.5], 70.3 (O-CH$_2$), Ar.C [105.9, 115.4, 116.1, 128.4, 132.3, 137.7, 153.4, 161.0], 161.8 (C=O), 177.6 (C=S). HREIMS, C$_{18}$H$_{18}$N$_2$O$_5$S, m/z = 375.1018, (M+H^{+}). Calcd. 375.1007.

7.2.7 General procedure for the synthesis of 5-aryl-2-(chloromethyl)-1,3,4-oxadiazoles (**4c-f**)

R= c = Br, e = CH$_3$
d = Cl, f = OCH$_3$
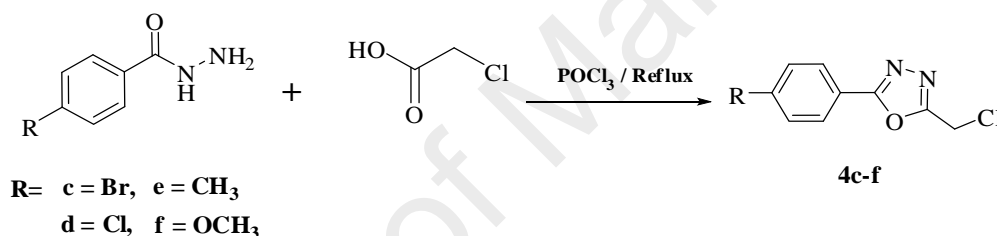
A mixture of aryl acid hydrazide (10 mmol), chloroacetic acid (1.0g, 10 mmol) and POCl$_3$ (7 mL, 73 mmol) was refluxed for 6 hours. The reaction mixture was poured onto crushed ice. The resulting precipitate was filtered, washed with saturated sodium bicarbonate solution and then with water, dried and recrystallized from ethanol.(Padmavathi, Reddy, Reddy, & Mahesh, 2011; Zhang, Wang, Xuan, Fu, Jing, Li, Liu, & Chen, 2014)

**7.2.7.1** 5-(4-Bromophenyl)-2-(chloromethyl)-1,3,4-oxadiazole (**4c**)
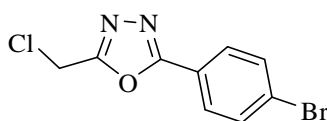
Pale red solid (2.10 g, 77%), m.p. 180-184°C (lit. 187-188 °C) (Cao, Wei, Zhao, Li, Huang, & Qian, 2005) . ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 5.13 (s, 2H, CH$_2$), Ar-H [7.81 (d, J = 8.2 Hz, 2H), 7.94 (d, J = 8.2 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 33.7 (CH$_2$), Ar-C [122.5, 126.5, 129.1, 133.1], (C=N) [163.5, 164.8].

**7.2.7.2**   2-(Chloromethyl)-5-(4-chlorophenyl)-1,3,4-oxadiazole (**4d**)
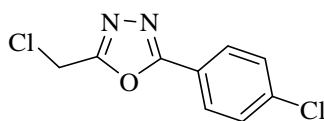
Pale brown solid (1.83 g, 80%), m.p. 71-76°C (lit. 82-83°C).(Zhang, Wang, Xuan, Fu, Jing, Li, Liu, & Chen, 2014) ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 5.15 (s, 2H, CH$_2$), Ar-H [7.68 (d, J = 8.3 Hz, 2H), 8.02 (d, J = 8.3 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 33.7 (CH$_2$), Ar-C [122.2, 128.9, 130.2, 137.6], (C=N) [163.5, 164.7].

7.2.7.3   2-(Chloromethyl)-5-p-tolyl-1,3,4-oxadiazole (**4e**)
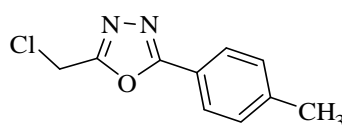

Pale pink solid (1.79 g, 86%), m.p. 104-106°C (lit. 116-118°C).(Zhang, Wang, Xuan, Fu, Jing, Li, Liu, & Chen, 2014) ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 2.38 (s, 3H, CH$_3$), 5.12 (s, 2H, CH$_2$), Ar-H [7.39 (d, J = 8.1 Hz, 2H), 7.88 (d, J = 8.1 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 21.5 (CH$_3$), 33.7 (CH$_2$), Ar-C [120.5, 127.1, 130.5, 143.1], (C=N) [163.1, 165.5].

7.2.7.4   2-(Chloromethyl)-5-(4-methoxyphenyl)-1,3,4-oxadiazole (**4f**)
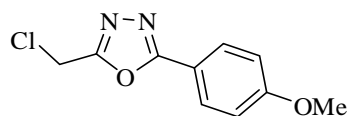

Pink solid (2.0 g, 93%), m.p. 91-94°C (lit. 92-93°C).(Zhang, Wang, Xuan, Fu, Jing, Li, Liu, & Chen, 2014) ^{1}H NMR (400 MHz, DMSO-d6) δ, ppm: 3.85 (s, 3H, OCH3), 5.11 (s, 2H, CH2), Ar-H [7.13 (d, J = 8.9 Hz, 2H), 7.93 (d, J = 8.9 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d6) δ, ppm: 33.8 (CH2), 56.0 (OCH3), Ar-C [115.4, 115.6, 129.0, 162.7] (C-O), (C=N) [162.8, 165.4].

7.2.8   General procedure for the synthesis of 2-(4-arylthio)-5-(4-(3,4,5-trimethoxy benzyl oxy)phenyl)-1,3,4-oxadiazole (**4.2 -4.7**)
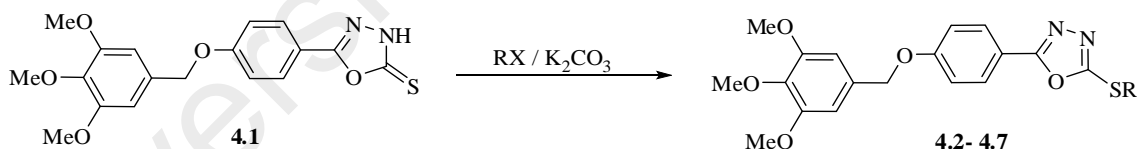
Compound **4.1** (0.50 g, 1.3 mmol) was dissolved in acetone (25 mL) and anhydrous potassium carbonate (0.18 g, 1.3 mmol) was added, followed by Alkyl halide (0.3 g, 1.3 mmole). The mixture was refluxed for 24 hours and the solvent was removed in vacuum. Water was added and mixture extracted with ethyl acetate. The organic layer was washed with water and dried over sodium sulfate, filtered, and concentrated in vacuum. The precipitate was recrystallized from methanol.

7.2.8.1 2-(4-Bromobenzylthio)-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazole (**4.2**)
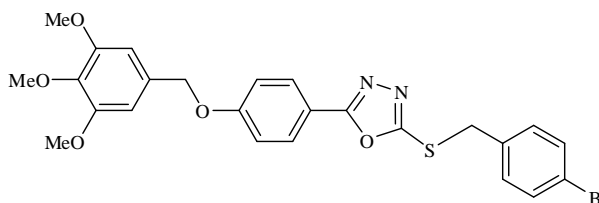
White solid (0.53 g, 75%), m.p. 104-106°C. IR, cm^{-1}: ν = 2937 (CH$_{Ar}$), 2832 (CH$_{aliph.}$), 1591 (C=N), 1069 (O-CH$_3$), 528 (C-Br). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 4.54 (s, 2H, S-CH$_2$), 5.11 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 7.21 (d, J = 8.6 Hz, 2H), 7.43 (d, J = 8.2 Hz, 2H), 7.53 (d, J = 8.2 Hz, 2 H), 7.89 (d, J = 8.6 Hz, 2 H)].^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 35.6 (S-CH$_2$), O-CH$_3$ [56.4, 60.5], 70.3(O-CH$_2$), Ar.C [105.9, 116.0, 116.1, 121.4, 128.7, 131.7, 131.9, 132.4, 136.8, 137.7, 153.4, 161.7], (C=N) [162.8, 165.7]. HREIMS, C$_{25}$H$_{23}$BrN$_2$O$_5$S, m/z = 565.0407, 567.0390 (M+Na$^+$). Calcd. 565.0399, 567.0378.

7.2.8.2 5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1-(4-Bromophenyl)-2-thioethyl-one-1,3,4- oxadiazole (**4.3**)
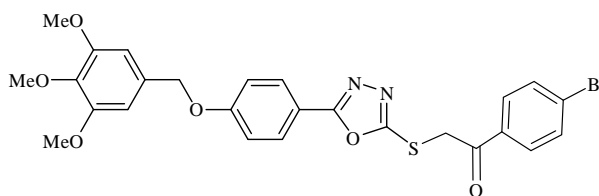
White solid (0.57 g, 77%), m.p. 120-122°C. IR, cm^{-1}: ν = 2944 (CH$_{Ar}$), 2838 (CH$_{aliph.}$), 1594 (C=N), 1607 (C=O), 1070 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.78 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), 5.14 (s, 2H, S-CH$_2$), Ar.H [6.80 (s, 2H), 7.21 (d, J = 8.8 Hz, 2H), 7.80 (d, J = 8.5 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H),

8.00 (d, J = 8.5 Hz, 2H)].^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 49.1 (S-*C*H$_2$), O-*C*H$_3$ [56.4, 60.5], 70.3(O-*C*H$_2$), Ar.C [105.9, 115.9, 116.1, 128.6, 128.7, 130.9, 132.4, 132.5, 134.5, 137.7, 153.4, 161.6, 162.8, 165.5], 192.6 (*C*=O). HREIMS, C$_{26}$H$_{23}$BrN$_2$O$_6$S, m/z = 593.0362, 595.0339 (M+Na$^+$). Calcd. 593.0348, 595.0327.

7.2.8.3   2-(5-(4-Bromophenyl)-1,3,4-oxadiazole-2-yl)methylthio)-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazole (**4.4**)
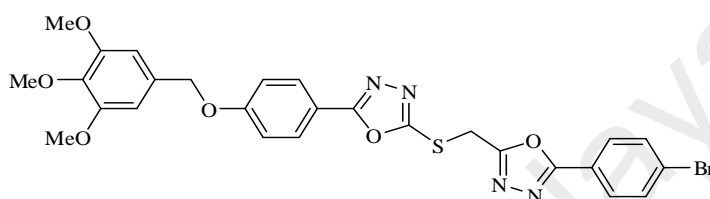
Pale reddish brown solid (0.54 g, 68%), m.p. 130-132°C. IR, cm^{-1}: ν = 2925 (CH$_{Ar}$), 2818 (CH$_{aliph.}$), 1591 (C=N), 1124 (O-CH$_3$), 525 (Br-C). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 4.95 (s, 2H, S-CH$_2$), 5.11 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 7.21 (d, J = 8.2 Hz, 2H), 7.66 (d, J = 8.4 Hz, 2H), 7.89 - 7.95 (m, 4H)].^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 26.4 (S-*C*H$_2$), O-*C*H$_3$ [56.4, 60.5], 70.3 (O-*C*H$_2$), Ar.C [105.9, 115.8, 116.1, 122.7, 126.3, 128.8, 132.3, 133.1, 137.7, 153.4, 161.5], (*C*=N) [161.8, 164.0, 164.4, 166.1]. HREIMS, C$_{27}$H$_{23}$BrN$_4$O$_6$S, m/z = 633.0425, 635.0402 (M+Na$^+$). Calcd. 633.0408, 635.0387.

7.2.8.4   2-(5-(4-Chlorophenyl)-1,3,4-oxadiazole-2-yl)methylthio)-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazole (**4.5**)
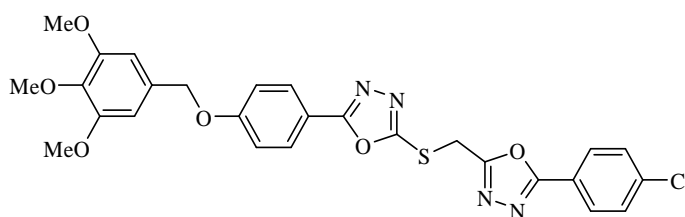
Pale brown solid (0.51 g, 69%), m.p. 140-142°C. IR, cm⁻¹: ν = 2926 (CH_Ar), 2833 (CH_aliph.), 1591 (C=N), 1125 (O-CH₃), 698 (Cl-C). ¹H NMR (400 MHz, DMSO-d₆) δ, ppm: O-CH₃ [3.67 (s, 3H), 3.79 (s, 6H)], 4.95 (s, 2H, S-CH₂), 5.11 (s, 2H, O-CH₂), Ar.H [6.81 (s, 2H), 7.21 (d, *J* = 8.8 Hz, 2H), 7.66 (d, *J* = 8.5 Hz, 2H), 7.89 - 7.95 (m, 4H). ¹³C NMR (100 MHz, DMSO-d₆) δ, ppm: 26.4 (S-*C*H₂), O-*C*H₃ [56.4, 60.5], 70.3 (O-*C*H₂), Ar.C [105.9, 115.8, 116.1, 122.4, 128.7, 128.82, 130.1, 132.3, 137.4, 137.7, 153.4, 161.5], (*C*=N) [161.8, 163.9, 164.3, 166.1]. HREIMS, C₂₇H₂₃ClN₄O₆S, m/z = 589.0927, 591.0905 (M+Na⁺). Calcd. 589.0913, 591.0884.

7.2.8.5 2-(5-(4-Methylphenyl)-1,3,4-oxadiazole-2-yl)methylthio)-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazole (**4.6**)
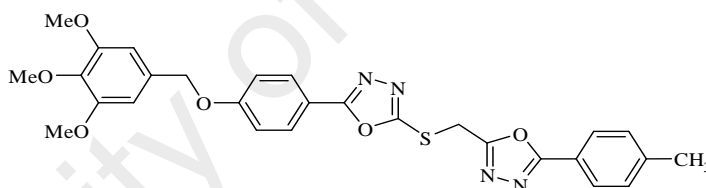
White solid (0.50 g, 71%), m.p. 128-130°C. IR, cm⁻¹: ν = 2939 (CH_Ar), 2827 (CH_aliph.), 1598 (C=N), 1121 (O-CH₃). ¹H NMR (400 MHz, DMSO-d₆) δ, ppm: 2.38 (s, 3H, CH₃), O-CH₃ [3.66 (s, 3H), 3.78 (s, 6H)], 4.92 (s, 2H, S-CH₂), 5.11 (s, 2H, O-CH₂), Ar.H [6.80 (s, 2H), 7.21 (d, *J* = 8.9 Hz, 2H), 7.37 (d, *J* = 8.2 Hz, 2H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.91 (d, *J* = 8.9 Hz, 2H)]. ¹³C NMR (100 MHz, DMSO-d₆) δ, ppm: 21.6 (*C*H₃), 26.4 (S-*C*H₂), O-*C*H₃ [56.4, 60.5], 70.3 (O-*C*H₂), Ar.C [105.9, 115.8, 116.1, 120.7, 126.9, 128.8, 130.5, 132.3, 137.7, 142.9, 153.4, 161.5], (*C*=N) [161.8, 163.4, 165.1, 166.2]. HREIMS, C₂₈H₂₆N₄O₆S, m/z = 569.1471, (M+Na⁺). Calcd. 569.1459.

7.2.8.6 2-(5-(4-Methoxyphenyl)-1,3,4-oxadiazole-2-yl)methylthio)-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazole (**4.7**)
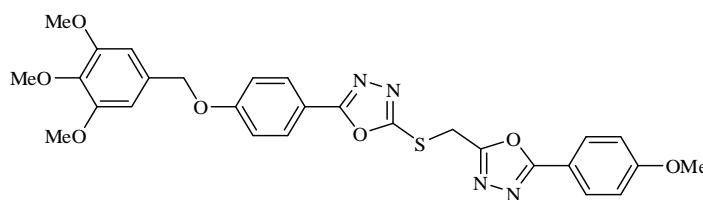
White solid (0.51 g, 70%), m.p. 118-120°C. IR, cm^{-1}: ν = 2950 (CH$_{Ar}$), 2833 (CH$_{aliph.}$), 1596 (C=N), 1125 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.66 (s, 3 H), 3.78 (s, 6 H), 3.84 (s, 3 H)], 4.91 (s, 2 H, S-CH$_2$), 5.11 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2 H), 7.11 (d, J = 8.7 Hz, 2H), 7.21 (d, J = 8.7 Hz, 2H), 7.86 (d, J = 8.6 Hz, 2H), 7.91 (d, J = 8.6 Hz, 2H)]. ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 26.4 (S-CH$_2$), O-CH$_3$ [56.0, 56.4, 60.5], 70.3 (O-CH$_2$), Ar.C [105.9, 115.4, 115.8, 115.82, 116.1, 128.8, 128.86, 132.3, 137.7, 153.4, 161.5, 161.8], (C=N) [162.6, 163.1, 165.0, 166.2]. HREIMS, C$_{28}$H$_{26}$N$_4$O$_7$S, m/z = 585.1420, (M+Na$^+$). Calcd. 585.1408.

7.2.9 General procedure for the synthesis of N-(4-aryl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.1-5.6**)
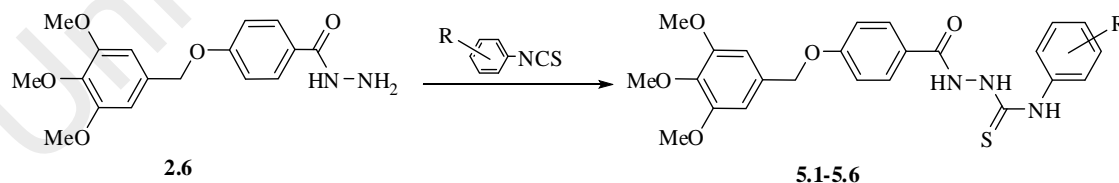
To a stirred solution of compound **2.6** (0.40 g, 1.2 mmol), absolute ethanol (15 ml), aryl isothiocyanate (1.2 mmol) was added. The reaction mixture was heated to 50°C for 1h, then stirred for 24 hours at room temperature. The precipitate was collected by filtration, washed with cold absolute ethanol, dried in vacuum and purified using an appropriate solvent.

**7.2.9.1** N-(4-Chlorophenyl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.1**)
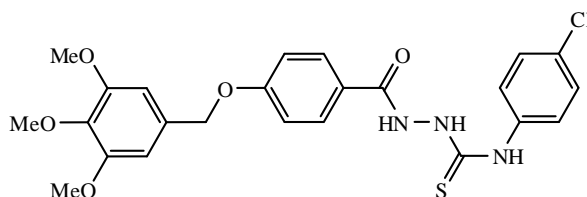
White solid purified from ethanol (0.53 g, 89%), m.p. 180-186°C. IR, cm⁻¹: ν = 3320 (NH), 3215 (NH), 3136 (NH), 1661 (C=O), 1591 (C=C), 1230 (C=S), 1122 (O-CH₃). ¹H NMR (400 MHz, DMSO-d₆) δ, ppm: O-CH₃ [3.66 (s, 3H), 3.78 (s, 6H)], 5.11 (s, 2H, O-CH₂), Ar.H [6.80 (s, 2H), 7.12 (d, *J* = 8.7 Hz, 2H), 7.37 (d, *J* = 8.7 Hz, 2H), 7.50 (d, 2H), 7.93 (d, *J* = 8.6 Hz, 2H)], 9.75 (bs, 1H, NH), 9.81 (bs, 1H, NH), 10.40 (bs, 1H, NH). ¹³C NMR (100 MHz, DMSO-d₆) δ, ppm: O-*C*H₃ [56.4, 60.5], 70.1(O-*C*H₂), Ar.C [105.8, 114.8, 125.3, 128.0, 128.3, 129.5, 130.3, 132.6, 137.6, 138.8, 153.4, 161.6], 166.0 (*C*=O), 181.6 (*C*=S). HREIMS, C₂₄H₂₄ClN₃O₅S, m/z = 524.1019, (M+Na⁺). Calcd. 524.1012.

7.2.9.2 N-(4-Methoxyphenyl)-2-(4-(3,4,5-trimethoxybenzyloxy)   benzoyl)hydrazine carbothioamide (**5.2**)
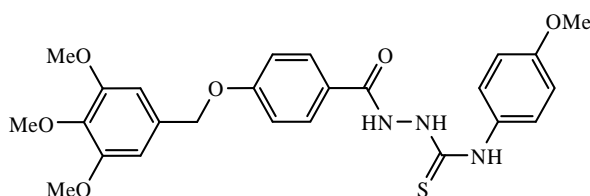
White solid purified from ethanol (0.54 g, 91%), m.p. 160-162°C. IR, cm⁻¹: ν = 3321 (NH), 3222 (NH), 3182 (NH), 1665 (C=O), 1597 (C=C), 1234 (C=S), 1124 (O-CH₃). ¹H NMR (400 MHz, DMSO-d₆) δ, ppm: O-CH₃ [3.65 (s, 3H), 3.74 (s, 3H), 3.77 (s, 6H)],

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5.10 (s, 2H, O-CH$_2$), Ar.H [6.79 (s, 2H), 6.89 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 8.7 Hz, 2H), 7.28 (d, J = 8.7 Hz, 2H), 7.93 (d, J = 8.7 Hz, 2H)], 9.55 (bs, 1H, NH), 9.67 (bs, 1H, NH), 10.35 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [55.7, 56.4, 60.5], 70.1(O-CH$_2$), Ar.C [105.8, 113.6, 114.7, 125.4, 128.1, 130.3, 132.5, 132.6, 137.5, 153.4, 157.2, 161.5], 166.0 (C=O). HREIMS, C$_{25}$H$_{27}$N$_3$O$_6$S, m/z = 520.1514, (M+Na$^+$). Calcd. 520.1507.

7.2.9.3 N-(2-Methoxyphenyl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.3**)
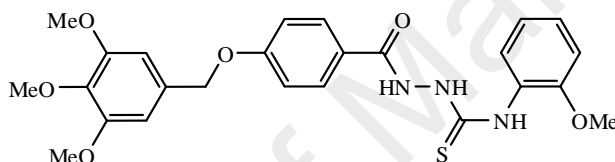
White solid purified from Methanol: CHCl$_3$ (0.55 g, 93%), m.p. 123-125°C. IR, cm^{-1}: ν = 3319 (NH), 3260 (NH), 3139 (NH), 1662 (C=O), 1595 (C=C), 1227 (C=S), 1125 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.73 (bs, 3H), 3.78 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 6.95 (t, 1H), 7.03 (d, J = 8 Hz, 1H), 7.05 (s, 1H), 7.14 (d, J = 8 Hz, 2H), 7.92 (d, J = 8 Hz, 2H) 8.00 (s, 1H)], 9.20 (bs, 1H, NH), 9.77 (bs, 1H, NH), 10.51 (bs, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [56.2, 56.4, 60.5], 70.1(O-CH$_2$), Ar.C [105.8, 111.9, 115.0, 120.3, 125.1, 125.8, 126.5, 128.3, 130.2, 132.6, 137.6, 153.4, 161.7], 166.7 (C=O), 181.0 (C=S). HREIMS, C$_{25}$H$_{27}$N$_3$O$_6$S, m/z = 520.1522, (M+Na$^+$). Calcd. 520.1507.

7.2.9.4 N-(*p*-Tolyl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.4**)
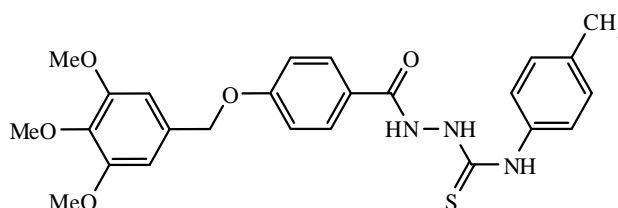
White solid purified from ethanol (0.49 g, 86%), m.p. 165-167°C. IR, cm^{-1}: ν = 3321 (NH), 3220 (NH), 3163 (NH), 1662 (C=O), 1593 (C=C), 1234 (C=S), 1124 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 2.28 (s, 3H, CH$_3$), O-CH$_3$ [3.66 (s, 3H), 3.78 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.79 (s, 2H), 7.12 - 7.14 (m, 4H), 7.30 (d, *J* = 8.8 Hz, 2H), 7.91 (d, *J* = 8.8 Hz, 2H)], 9.59 (s, 1H, NH), 9.71 (bs, 1H, NH), 10.36 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d6) δ, ppm: 21.0 (CH$_3$), O-*C*H$_3$ [56.4, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.8, 114.8, 125.4, 126.4, 128.9, 130.3, 132.6, 134.6, 137.2, 137.6, 153.4, 161.6], 166.0 (*C*=O). HREIMS, C$_{25}$H$_{27}$N$_3$O$_5$S, m/z= 504.1570, (M+Na$^+$). Calcd. 504.1558.

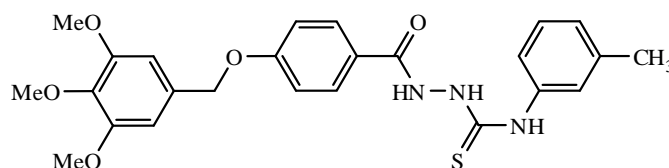**7.2.9.5** N-(m-Tolyl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.5**)

White solid purified from ethanol (0.52 g, 90%), m.p. 89-101°C. IR, cm^{-1}: ν = 3321 (NH), 3220 (NH), 3170 (NH), 1663 (C=O), 1597 (C=C), 1238 (C=S), 1127 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 2.29 (s, 3H, CH$_3$), O-CH$_3$ [3.66 (s, 3H), 3.78 (s, 6H)], 5.11 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 6.98 (d, *J* = 7 Hz, 1H), 7.13 (d, *J* = 8 Hz, 2H), 7.20 - 7.22 (m, 2H), 7.37 (d, *J* = 6 Hz, 1H), 7.93 (d, *J* = 8 Hz, 2H), 9.61 (bs, 1H,

NH), 9.72 (bs, 1H, NH), 10.37 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 21.4 (*C*H$_3$), O-*C*H$_3$ [56.4, 60.5], 70.1(O-*C*H$_2$), Ar.C [105.8, 114.8, 123.5, 125.4, 126.1, 126.9, 128.2, 130.3, 132.6, 137.6, 139.6, 153.4, 161.6], 166.0 (*C*=O), 181.6 (*C*=S). HREIMS, C$_{25}$H$_{27}$N$_3$O$_5$S, m/z = 504.1571, (M+Na$^+$). Calcd. 504.1558.
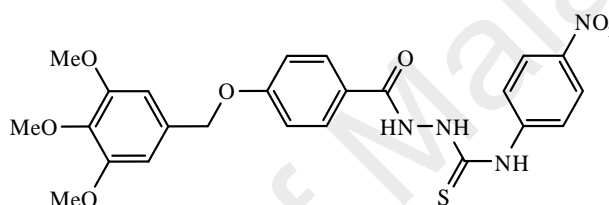
7.2.9.6 N-(4-Nitrophenyl)-2-(4-(3,4,5-trimethoxybenzyloxy)benzoyl)hydrazine carbothioamide (**5.6**)

Light yellow solid purified from methanol (0.57 g, 92%), m.p. 196-199°C. IR, cm^{-1}: ν = 3319 (NH), 3190 (NH), 3153 (NH), 1659 (C=O), 1593 (C=C), 1232 (C=S), 1123 (O-CH$_3$), 1505, 1330 (NO$_2$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.66 (s, 3H), 3.78 (s, 6H)], 5.12 (s, 2H, O-CH$_2$), Ar.H [6.80 (s, 2H), 7.14 (d, *J* = 8.5 Hz, 2H), 7.90 - 7.95 (m, 4H), 8.21 (d, *J* = 8.7 Hz, 2H)], 10.11 (bs, 2H, NH), 10.50 (bs, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-*C*H$_3$ [56.4, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.8, 114.9, 124.0, 125.1, 125.3, 129.3, 130.3, 132.6, 137.6, 146.2, 153.4, 161.7], 166.1 (*C*=O), 181.3 (*C*=S). HREIMS, C$_{24}$H$_{24}$N$_4$O$_7$S, m/z = 535.1257, (M+Na$^+$). Calcd. 535.1252.

7.2.10 General procedure for the synthesis of 4-(4-aryl)-3-(4-(3,4,5-trimethoxybenzyl oxy)phenyl)-1H-1,2,4-triazole-5-(4H) -thione (**5.7-5.11**)
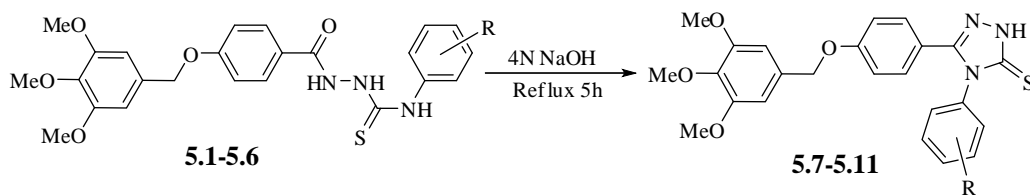
A mixture of arylthiosemicarbazide **5.1-5.6** (1.1 mmol) and sodium hydroxide solution (4 M, 20 ml) in 25 mL absolute ethanol, was refluxed for 5-6 hours. After cooling, 100 ml ice water was added. The solution pH was adjusted to 5-6 using diluted hydrochloric acid. The precipitate was filtered, washed with cold water, dried and recrystallized from suitable solvent.

7.2.10.1 4-(4-Chlorophenyl)-3-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1H-1,2,4-triazole-5-(4H)-thione (**5.7**)
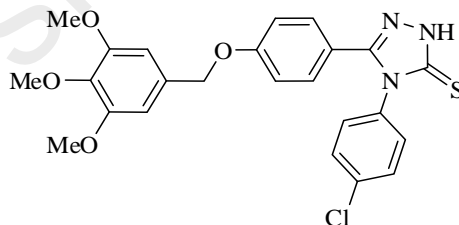
White solid purified from ethanol (0.31 g, 81%), m.p. 226-229°C. IR, cm^{-1}: ν = 3105 (NH), 1611 (C=N), 1330 (C-N), 1225 (C=S), 1125 (O-CH$_3$), 702 (C-Cl). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.65 (s, 3H), 3.76 (s, 6H)], 5.00 (s, 2H, O-CH$_2$), Ar.H [6.75 (s, 2H), 7.01 (d, J = 8.8 Hz, 2H), 7.26 (d, J = 8.8 Hz, 2H), 7.40 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.6 Hz, 2H)], 14.08 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-*C*H$_3$ [56.3, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.9, 115.3, 118.4, 129.9, 130.4, 131.2, 132.5,

134.0, 134.4, 137.6, 150.9 (*C*=N), 153.4, 160.3], 168.8 (*C*=S). HREIMS, $C_{24}H_{22}ClN_3O_4S$, m/z = 506.0915, 508.0901 (M+Na$^+$). Calcd. 506.0907, 508.0878.

7.2.10.2 4-(4-Methoxyphenyl)-3-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1H-1,2,4-triazole-5-(4H)-thione (**5.8**)
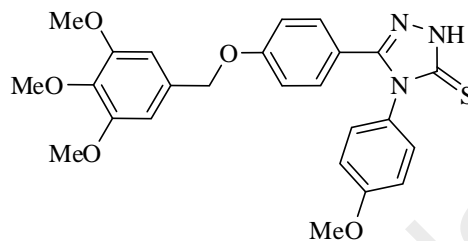
White solid purified from ethanol : CHCl$_3$ (0.30 g, 77%), m.p. 220-221$^°$C. IR, cm^{-1}: ν = 3109 (NH), 1608 (C=N), 1329 (C-N), 1228 (C=S), 1122 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.65 (s, 3H), 3.75 (s, 6H), 3.80 (s, 3H)], 4.99 (s, 2H, O-CH$_2$), Ar.H [6.74 (s, 2H), 6.99 (d, *J* = 8 Hz, 2H), 7.03 (d, *J* = 8 Hz, 2H), 7.24-7.27 (m, 4H)], 13.99 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-*C*H$_3$ [55.9, 56.3, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.9, 114.9, 115.3, 118.7, 127.7, 130.2, 130.4, 132.5, 137.6, 151.1 (*C*=N), 153.4, 160.0, 160.2], 169.1 (*C*=S). HREIMS, $C_{25}H_{25}N_3O_5S$, m/z = 502.1408, (M+Na$^+$). Calcd. 502.1402.

7.2.10.3 4-(2-Methoxyphenyl)-3-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1H-1,2,4-triazole-5-(4H)-thione (**5.9**)
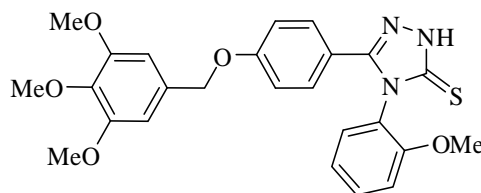
White solid purified from ethyl acetate (0.29 g, 75%), m.p. 239-242$^°$C. IR, cm^{-1}: ν = 3271 (NH), 1614 (C=N), 1329 (C-N), 1242 (C=S), 1122 (O-CH$_3$). ^{1}H NMR (400 MHz,

DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.57 (s, 3H), 3.64 (s, 3H), 3.74 (s, 6H)], 4.98 (s, 2H, O-CH$_2$), Ar.H [6.73 (s, 2H), 6.97 (t, *J* = 8 Hz, 1H), 7.08 (d, *J* = 8 Hz, 1H), 7.24 (d, *J* = 8 Hz, 2H), 7.40 (d, *J* = 8 Hz, 1H), 7.48 (t, *J* = 8.8 Hz, 1H)], 13.98 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-*C*H$_3$ [56.2, 56.3, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.9, 113.4, 115.3, 119.0, 121.4, 123.6, 129.4, 131.0, 131.8, 132.5, 137.6, 151.4 (*C*=N), 153.4, 155.0, 160.2], 169.1 (*C*=S). HREIMS, C$_{25}$H$_{25}$N$_3$O$_5$S, m/z = 502.1416, (M+Na$^+$). Calcd. 502.1402.

7.2.10.4 4-(p-Tolyl)-3-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1H-1,2,4-triazole-5-4H)-thione (**5.10**)
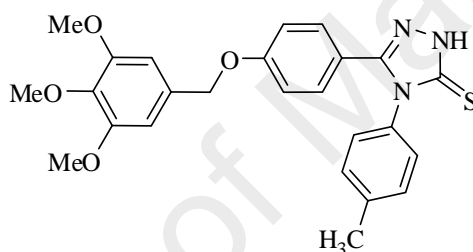
White solid purified from ethanol (0.30 g, 81%), m.p. 103-107°C. IR, cm^{-1}: ν = 3174 (NH), 1609 (C=N), 1332 (C-N), 1232 (C=S), 1123 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 2.37 (s, 3H, CH$_3$), O-CH$_3$ [3.67 (s, 3H), 3.76 (s, 6H)], 4.97 (s, 2H, O-CH$_2$), Ar.H [6.71 (s, 2H), 6.95 (d, *J* = 8 Hz, 2H), 7.18 (d, *J* = 8 Hz, 2H), 7.24 (d, *J* = 8 Hz, 2H), 7.28 (d, *J* = 8 Hz, 2H)], 13.97 (bs, 1H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 21.2 (*C*H$_3$), O-*C*H$_3$ [56.3, 60.5], 69.9 (O-*C*H$_2$), Ar.C [105.8, 114.9, 122.2, 128.9, 129.0, 129.5, 132.8, 135.6, 137.1, 137.5, 150.4 (*C*=N), 153.3, 158.6], 168.9 (*C*=S). HREIMS, C$_{25}$H$_{25}$N$_3$O$_4$S, m/z = 486.1467, (M+Na$^+$). Calcd. 486.1453.

**7.2.10.5** 4-(m-Tolyl)-3-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1H-1,2,4-triazole-5-(4H) -thione (**5.11**)
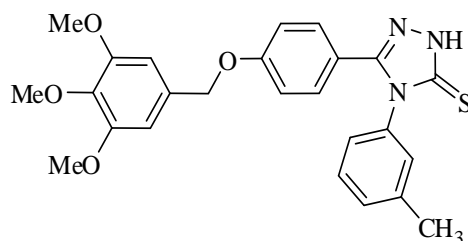
White solid purified from methanol (0.27 g, 74%), m.p. 187-190°C. IR, cm^{-1}: ν = 3110 (NH), 1609 (C=N), 1328 (C-N), 1227 (C=S), 1124 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: 2.30 (s, 3H, CH$_3$), O-CH$_3$ [3.64 (s, 3H), 3.74 (s, 6H)], 4.97 (s, 2H, O-CH$_2$), Ar.H [6.73 (s, 2H), 6.97 (d, J = 8 Hz, 2H), 7.10 (d, J = 7 Hz, 1H), 7.16 (s, 1H), 7.25 (d, J = 8 Hz, 2H), 7.30 (d, J = 7 Hz, 1H), 7.37 (dd~t, J = 7 Hz, 1H)], 14.04 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: 21.2 (*C*H$_3$), O-*C*H$_3$ [56.3, 60.5], 70.1 (O-*C*H$_2$), Ar.C [105.8, 115.3, 118.6, 126.2, 129.5, 129.6, 130.2, 130.6, 132.5, 135.1, 137.6, 139.4, 150.9 (*C*=N), 153.3, 160.2], 168.9 (*C*=S). HREIMS, C$_{25}$H$_{25}$N$_3$O$_4$S, m/z = 486.1469, (M+Na$^+$). Calcd. 486.1453.

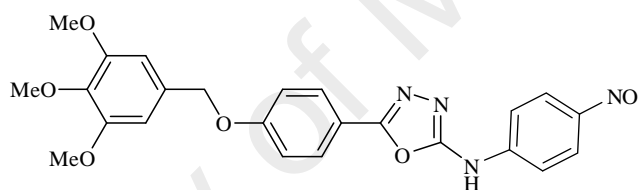**7.2.10.6** 4-(3,4,5-trimethoxybenzyloxy)benzoic acid (**5.12**)
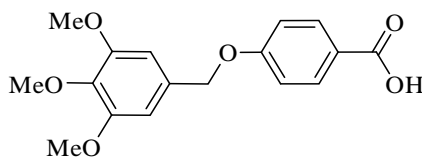
A mixture of arylthiosemicarbazide **5.6** (1.1 mmol) and sodium hydroxide solution (4 M, 20 ml) in 25 mL absolute ethanol, was refluxed for 5-6 hours. After cooling, 100 ml ice water was added. The solution pH was adjusted to 5-6 using diluted hydrochloric acid. The precipitate was filtered, washed with cold water, dried and recrystallized from

ethanol. The hydrolysis of compound **5.6** giving the corresponding acid **5.12**. White solid purified from methanol (0.18 g, 69%), m.p. 146-148°C. IR, cm^{-1}: ν = 2938 (OH, m), 1680 (C=O), 1241 (C=C), 1126 (O-CH$_3$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.66 (s, 3H), 3.78 (s, 6H)], 5.09 (s, 2H, O-CH$_2$), Ar.H [6.79 (s, 2H), 7.10 (d, J = 8.8 Hz, 2H), 7.90 (d, J = 8.8 Hz, 2H)], 12.62 (bs, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d6) δ, ppm: O-CH$_3$ [56.4, 60.5], 70.2 (O-CH$_2$), Ar.C [105.9, 115.1, 123.6, 131.8, 132.5, 137.7, 153.4, 162.4], 167.4 (C=O). HREIMS, C$_{17}$H$_{18}$O$_6$, m/z = 341.1001, (M+Na$^+$). Calcd. 341.0995.

7.2.10.7 4-nitrophenyl-5-(4-(3,4,5-trimethoxybenzyloxy)phenyl)-1,3,4-oxadiazol-2-amine (**5.12b**)

A mixture of arylthiosemicarbazide **5.6** (1.1 mmol) and sodium hydroxide solution (4 M, 20 ml) in 25 mL absolute ethanol, was stirred at RT for 24 hours. The precipitate was filtered, washed with cold water, dried and recrystallized from 95% ethanol. The precipitate is the corresponding acid **5.12** from the hydrolysis of compound **5.6**. Yellow solid purified from ethanol 95% (0.35 g, 62%), m.p. 185-189°C. IR, cm^{-1}: ν = 3293 (NH), 2993 (CH$_{Ar}$), 2832 (CH$_{aliph.}$), 1590 (C=N), 1329, 1505 (C-NO$_2$). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [3.67 (s, 3H), 3.79 (s, 6H)], 5.09 (s, 2H, O-CH$_2$), Ar.H [6.81 (s, 2H), 7.15 (d, J = 8.8 Hz, 2H), 7.79 (d, J = 8.8 Hz, 2H), 7.98 - 8.05 (m, 4H), 9.05 (s, 1H), 10.85 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ, ppm: O-CH$_3$ [56.4, 60.5], 70.2 (O-CH$_2$), Ar.C [105.8, 115.3, 116.8, 125.4, 127.7, 128.5, 132.7, 137.6, 138.3, 149.5, 153.4, 159.6], (C=N) [160.8, 165.1]. MS, C$_{24}$H$_{22}$N$_4$O$_7$, m/z = 479.10, (M+H$^+$). Calcd. 479.15.

7.2.11   Analysis of the antioxidant activities

7.2.11.1 DPPH radical scavenging activities

The DPPH free radical scavenging assay was performed according to the method of Brand-Williams (Benzie & Strain, 1996) with slight modifications. The reaction mixture was prepared by mixing 195 µl of a 100 µM methanolic DPPH solution with 50 µl of the test compounds at different concentrations (0-1000 µg /mL). The test compounds were initially dissolved in dimethyl sulfoxide (DMSO). BHT and ascorbic acid were used as positive controls and were run in parallel in a 96-well plate. After 30 min of incubation in the dark at room temperature, the absorbance of the reaction mixture was determined at 515 nm. The colour of the reaction mixture changed from purple to yellow as a result of the decreased absorbance. The radical scavenging activity was calculated by the following equation:

$$\text{scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the DPPH radical without a sample or standard; and A_1 is the absorbance of the DPPH radical with a sample or standard. IC_{50} values which represent the efficient concentration of the standards and samples that inhibit 50% of the DPPH radicals were calculated and expressed in µg/mL (Shenvi, Kumar, Hatti, Rijesh, Diwakar, & Reddy, 2013; Wang, Xue, An, Zheng, Dou, Zhang, & Liu, 2015). The IC_{50} value were calculated in another unit in µM, as shown in Figure C 19 page 207.

7.2.11.2 Ferric reducing antioxidant power activity (FRAP)

FRAP was determined according to the method described by Benzie and Strain (Benzie & Strain, 1996) with slight modifications. Three reagents were initially prepared as follows: 300 mM acetate buffer (pH = 3.6), 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$. Fresh FRAP working solution was prepared by mixing

the acetate buffer with the TPTZ solution in 20 mM $FeCl_3$ in a ratio of 10:1:1 (v/v/v), respectively. Five microliters of the samples or standards was mixed with 300 µl of the FRAP reagent were run in parallel in a 96-well plate, followed by a 30 min incubation period at $37°C$. Thereafter, the absorbance of the coloured product was measured at 595 nm. The results were calculated, based on a calibration curve plotted using iron sulfate ($FeSO_4$) (0-1 mM).

7.2.12   Theoretical calculations

The calculations for the hydrazones were performed using DMOL3 (Accelrys Inc.) based on DFT.1, 2 The electron correlation was treated by the spin polarized generalized gradient approximation (GGA) and was applied with the exchange functional of Burke3 and correlation functional of Perdew and Ernzerhof (Becke, 1992; Perdew, Burke, & Ernzerhof, 1996).

The hydrazone molecules and the free radicals generated after H-abstraction from the hydroxyls were modelled in Materials Visualizer and were initially optimized by the "smart minimization" method in Discover. Then, calculations in the gas phase employed the DFT method provided by DMOL3. The energy optimization was performed and the structural properties were obtained at the Generalized Gradient Approximation (GGA) level, with the spin unrestricted approach and PBE/DNP method. The calculations for the thiosemicarbazides, triazoles and oxadiazoles were performed using Gaussian program 09.(Frisch, Trucks, Schlegel, Scuseria, Robb, Cheeseman, Scalmani, Barone, Mennucci, Petersson, Nakatsuji, Caricato, Li, Hratchian, Izmaylov, Bloino, Zheng, Sonnenberg, Hada, Ehara, Toyota, Fukuda, Hasegawa, Ishida, Nakajima, Honda, Kitao, Nakai, Vreven, Montgomery Jr., Peralta, Ogliaro, Bearpark, Heyd, Brothers, Kudin, Staroverov, Kobayashi, Normand, Raghavachari, Rendell, Burant, Iyengar, Tomasi, Cossi, Rega, Millam, Klene, Knox, Cross, Bakken, Adamo, Jaramillo, Gomperts, Stratmann, Yazyev,

Austin, Cammi, Pomelli, Ochterski, Martin, Morokuma, Zakrzewski, Voth, Salvador, Dannenberg, Dapprich, Daniels, Farkas, Foresman, Ortiz, Cioslowski, & Fox, 2009; Henry, Varano, & Yarovsky, 2008) The geometry of each molecule and radical in the gas-phase was optimized using DFT method with UB3LYP functional (Becke, 1993) without any constraints. The calculations were performed using B3LYP/6-311G (d,p) level of theory to perform the most reliable optimization of the geometrical parameters of these compounds and to calculate physical descriptors characterizing their antioxidant ability. It particular, the homolytic bond dissociation enthalpy (BDE), HOMO orbital distribution and spin density.

7.2.13 Statistical analysis

All analyses were performed in triplicates. Results were expressed as a means ± standard deviation. A Pearson correlation test was utilized to study the relationship between the antioxidant activities of the synthesized compounds in the DPPH and FRAP antioxidant assays. Correlation is significant at the 0.05 level. The data were statistically analysed using the SPSS statistical software, version 15 (SPSS Inc., Chicago, Illinois, USA).

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Kareem, H. S.; Ariffin, A.; Nordin, N.; Heidelberg, T.; Abdul-Aziz, A.; Kong, K. W.; Yehye, W. A., Correlation of antioxidant activities with theoretical studies for new hydrazone compounds bearing a 3,4,5-trimethoxy benzyl moiety. *Eur. J. Med. Chem.* **2015**.(published).

2. Kareem, H. S.; Nordin, N.; Heidelberg, T.; Abdul-Aziz, A.; Ariffin, A., Conjugated oligo-aromatic compounds bearing a 3,4,5-Trimethoxy moiety: Investigation of their antioxidant activity correlated with a DFT study. Molecules 2016, 21 (2), 224. (published).

3. The 27th International Symposium on Chemical Engineering (ISChE 2014) Kuala Lumpur, Malaysia, Antioxidant activities of new Schiff base compounds containing 3,4,5-trimethoxy benzyl group correlates with theoretical studies, December 6-7, 2014, Kuala Lumpur,Malaysia.

APPENDIX A: NMR (^{1}H AND ^{13}C) SPECTRUM FOR THE SYNTHESIZED COMPOUNDS
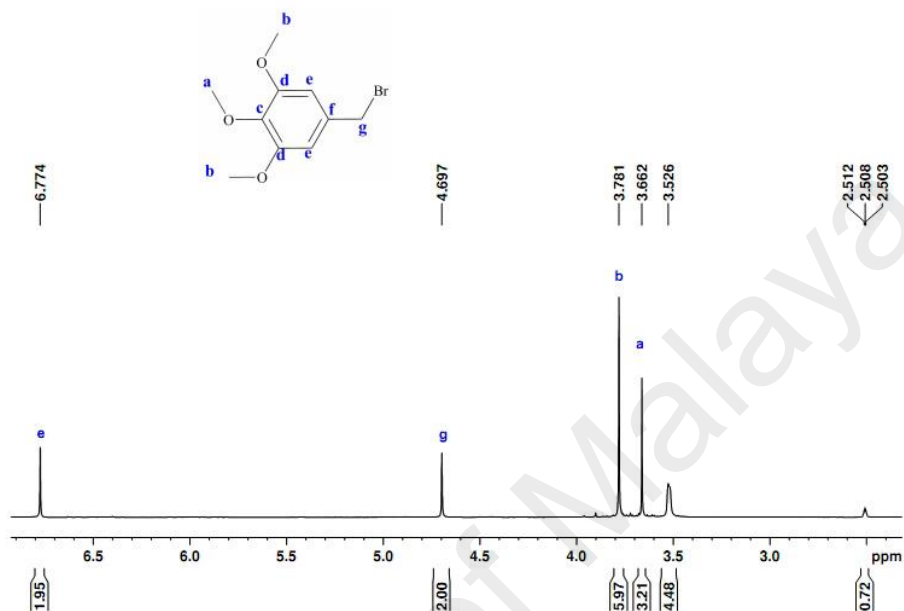
Figure A 1: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 2.2
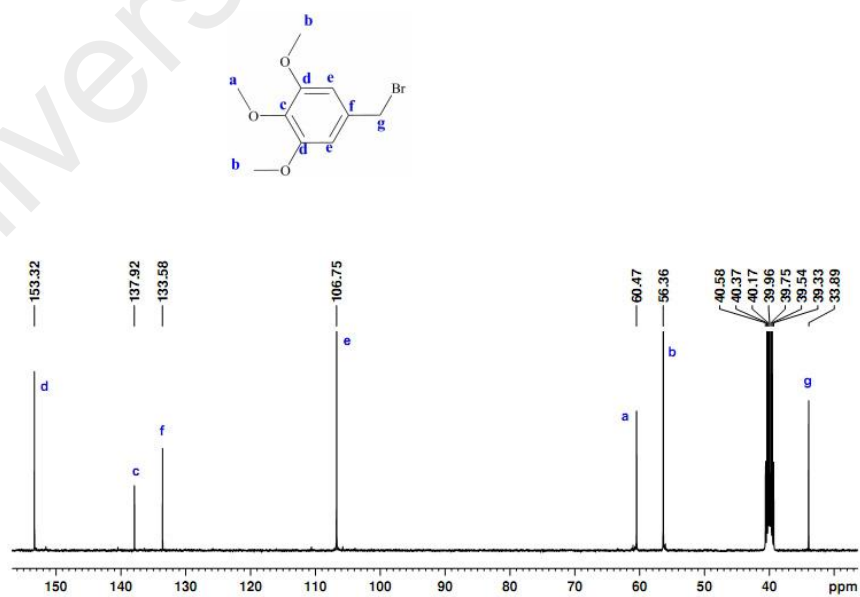
Figure A 2: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 2.2
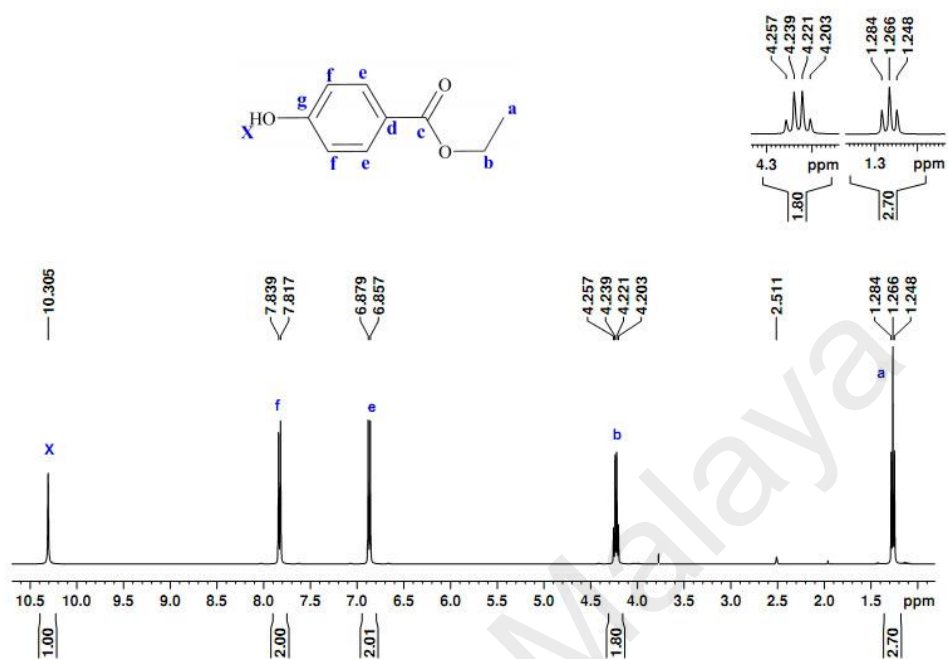
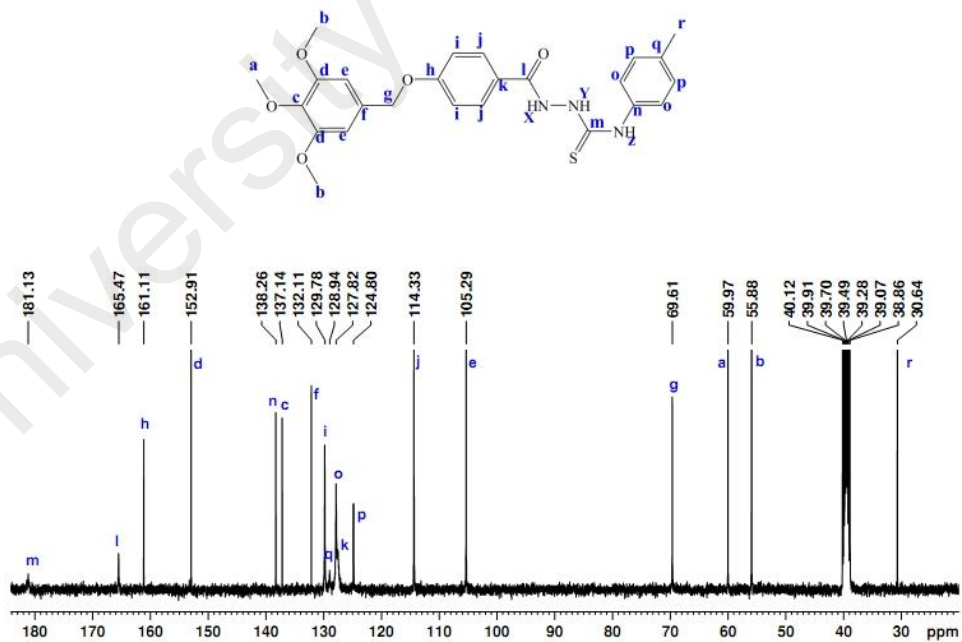**Figure A 3: ¹H spectrum (DMSO-d₆, 400MHz) of 2.4**
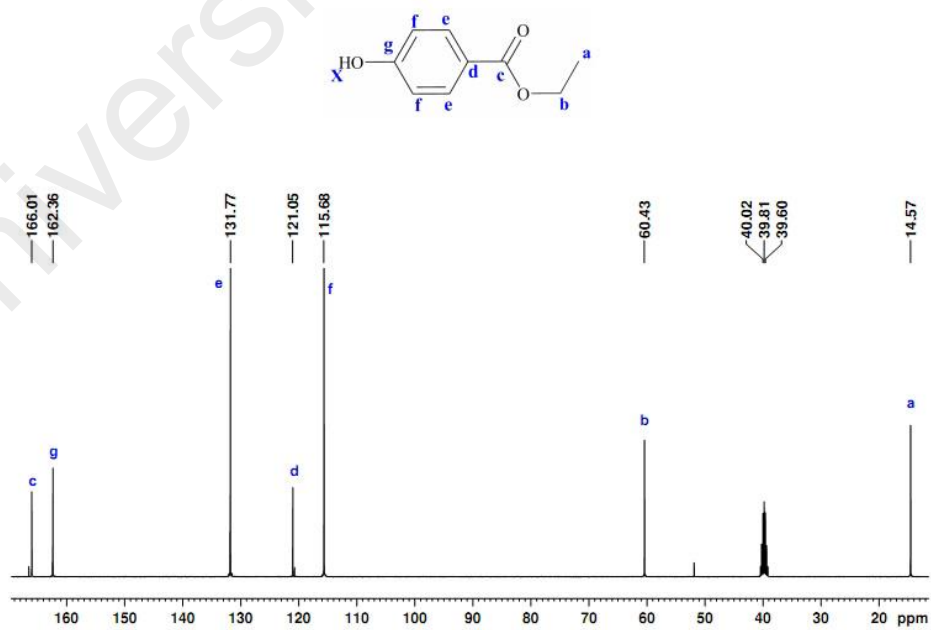
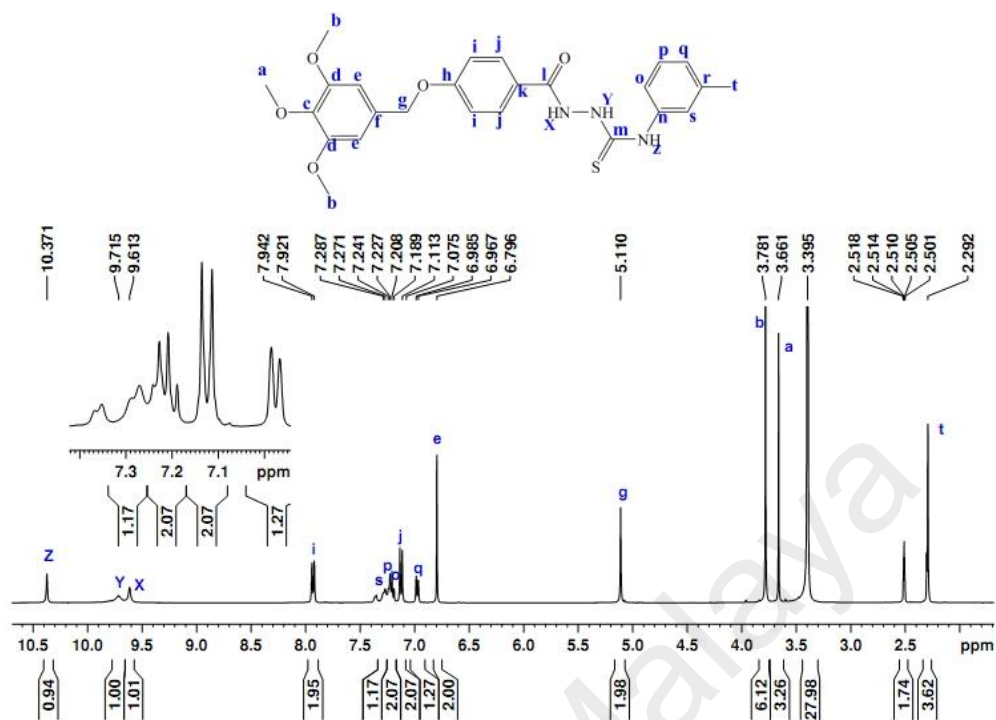**Figure A 4: ¹³C spectrum (DMSO-d₆, 100MHz) of 2.4**

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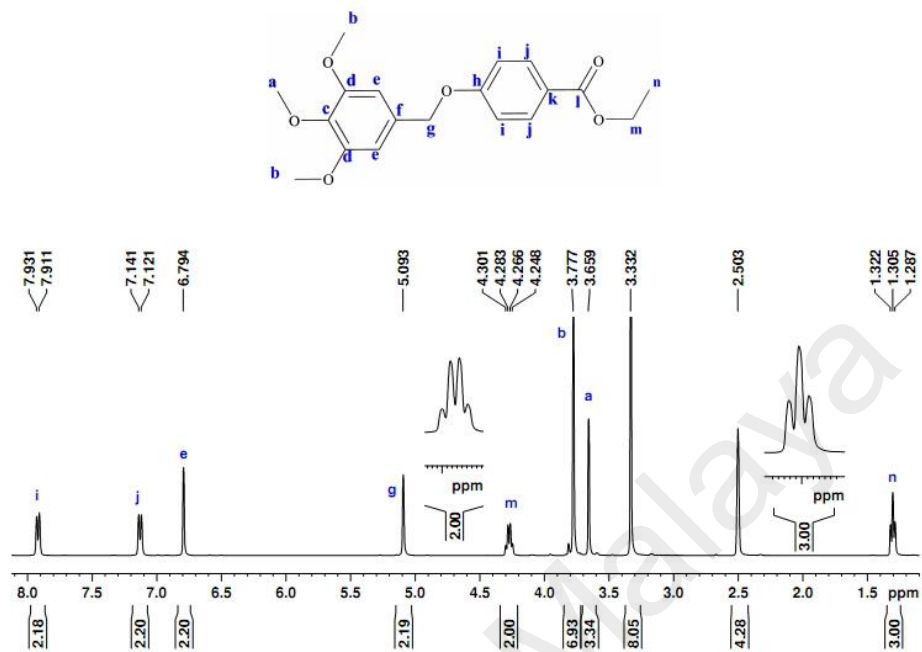
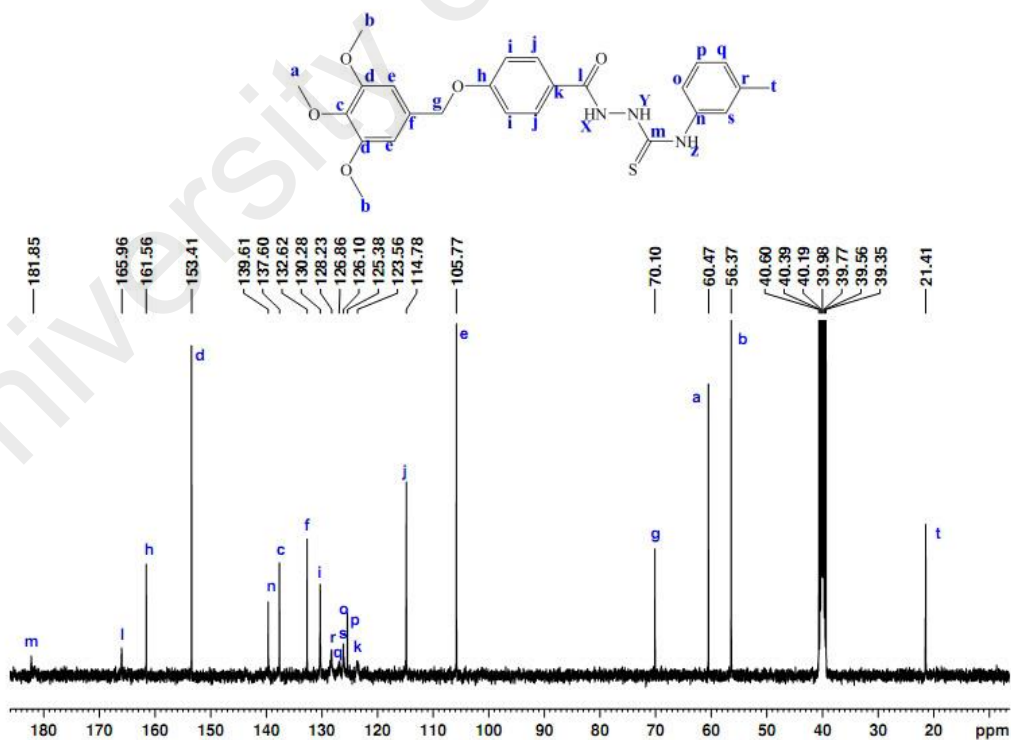**Figure A 5: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 2.5**
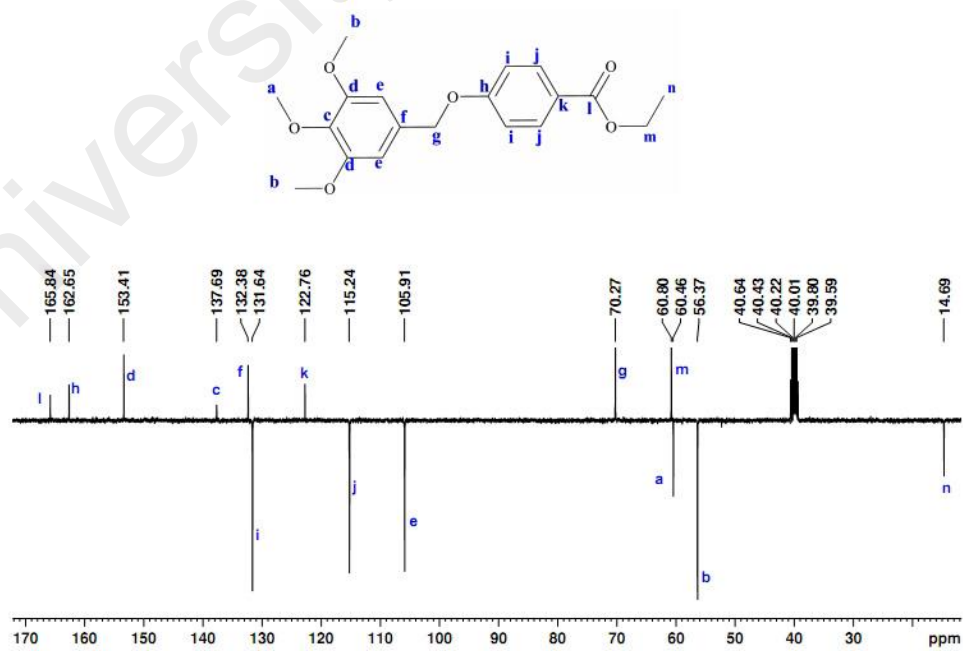
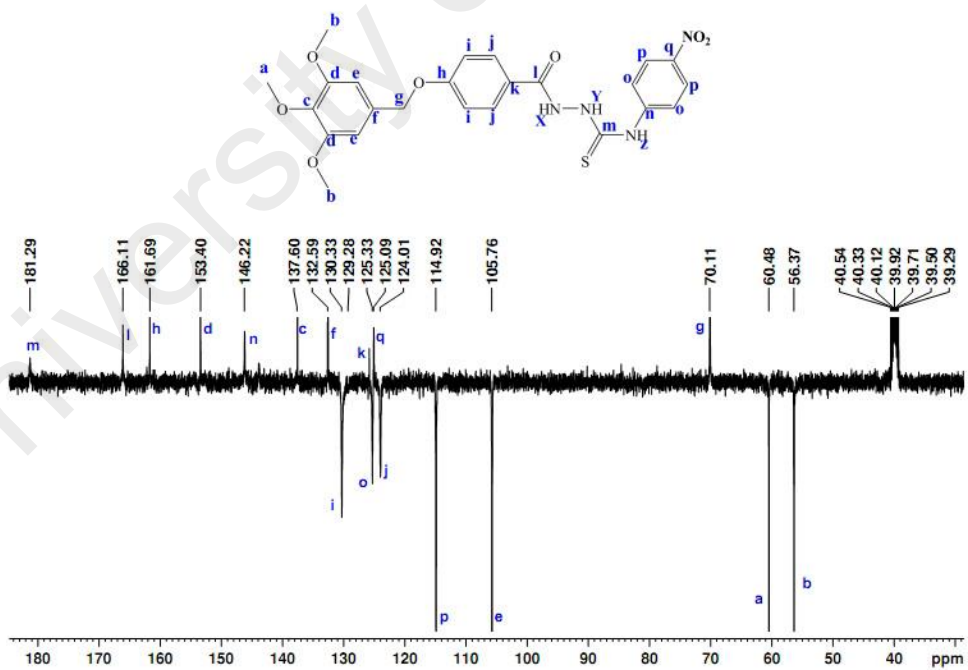**Figure A 6: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 2.5**

155
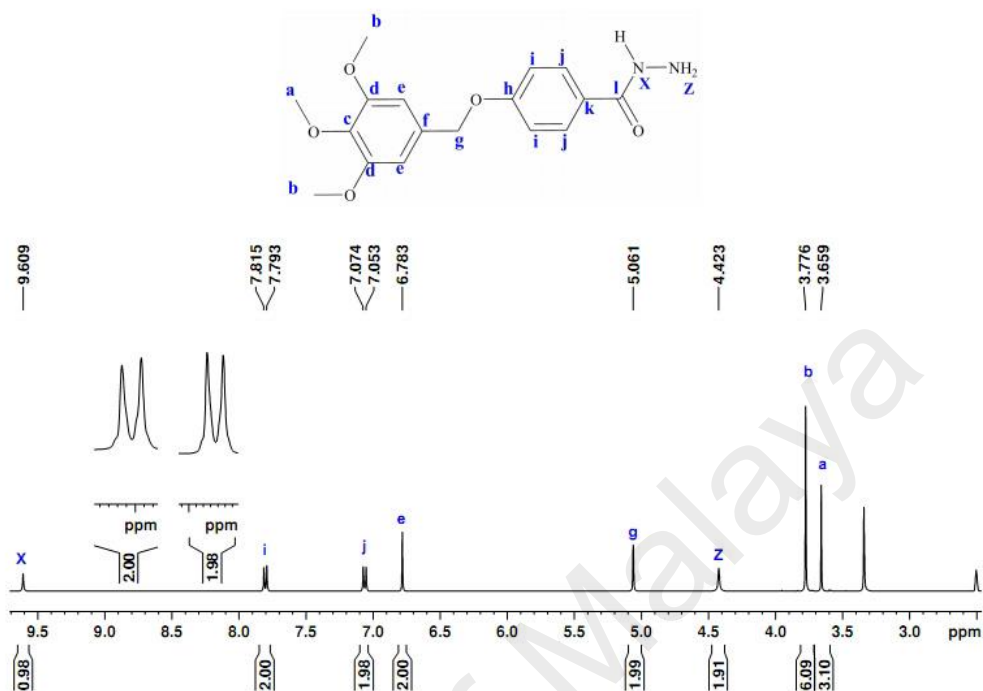
Figure A 7: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 2.6
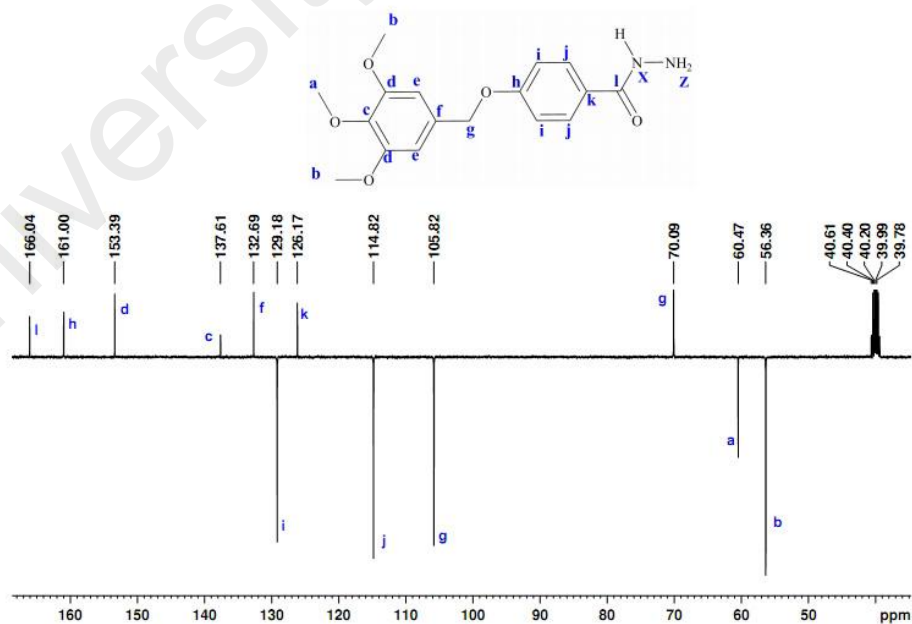
Figure A 8: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 2.6
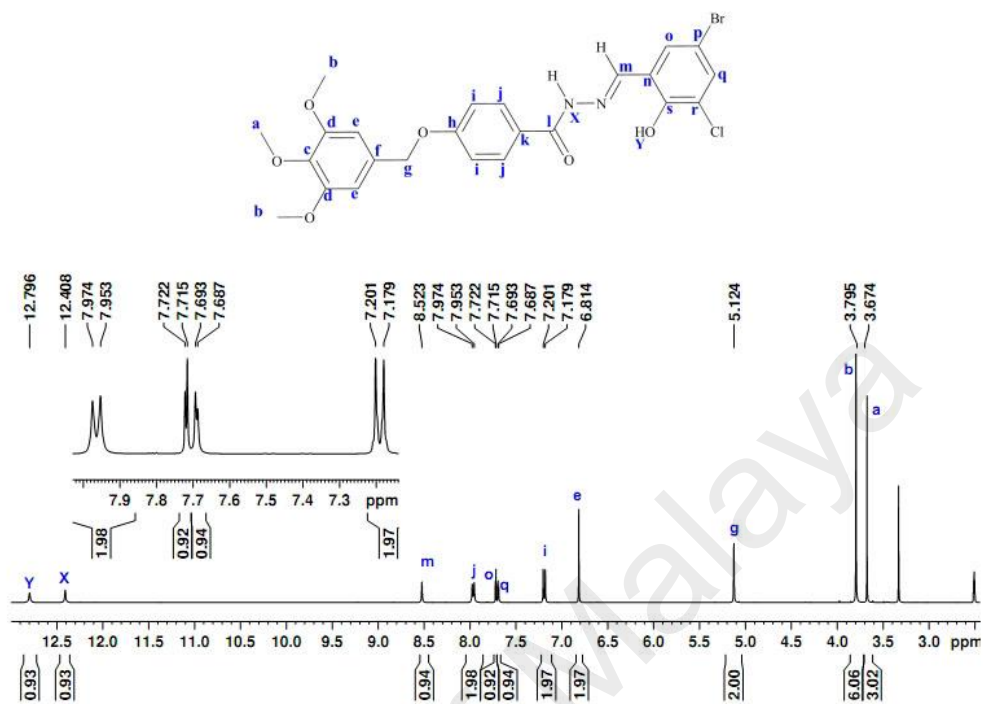
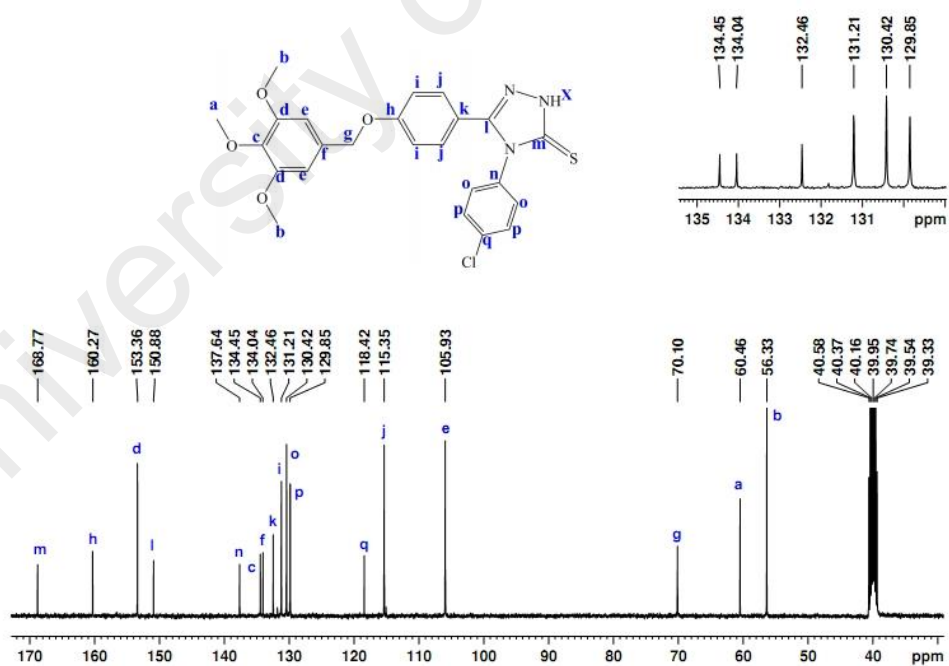**Figure A 9: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 3.1**
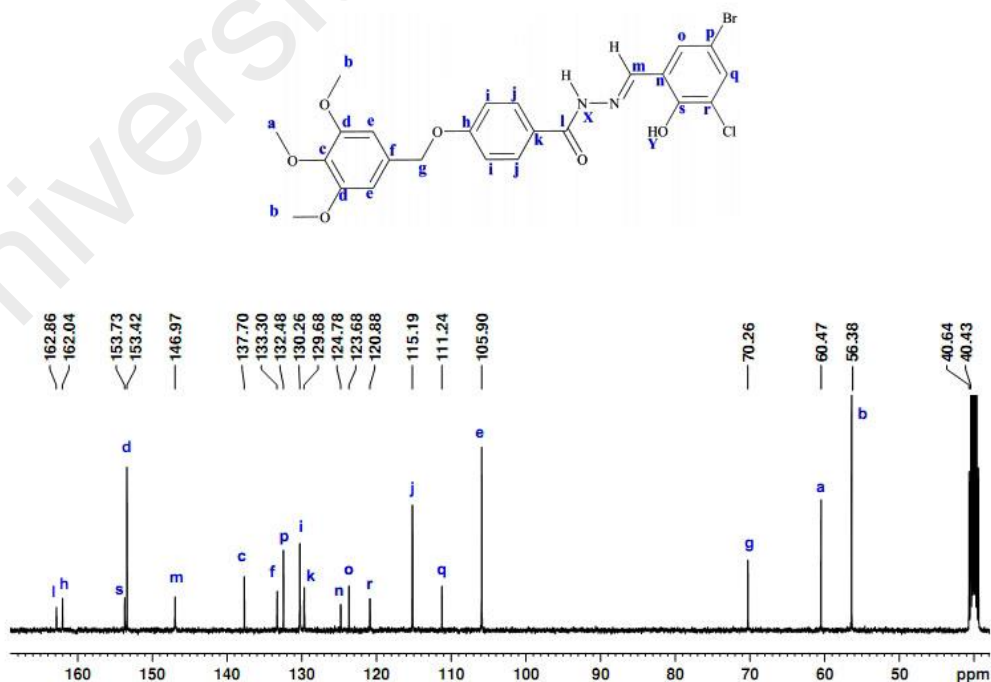
Figure A 10: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 3.1

157
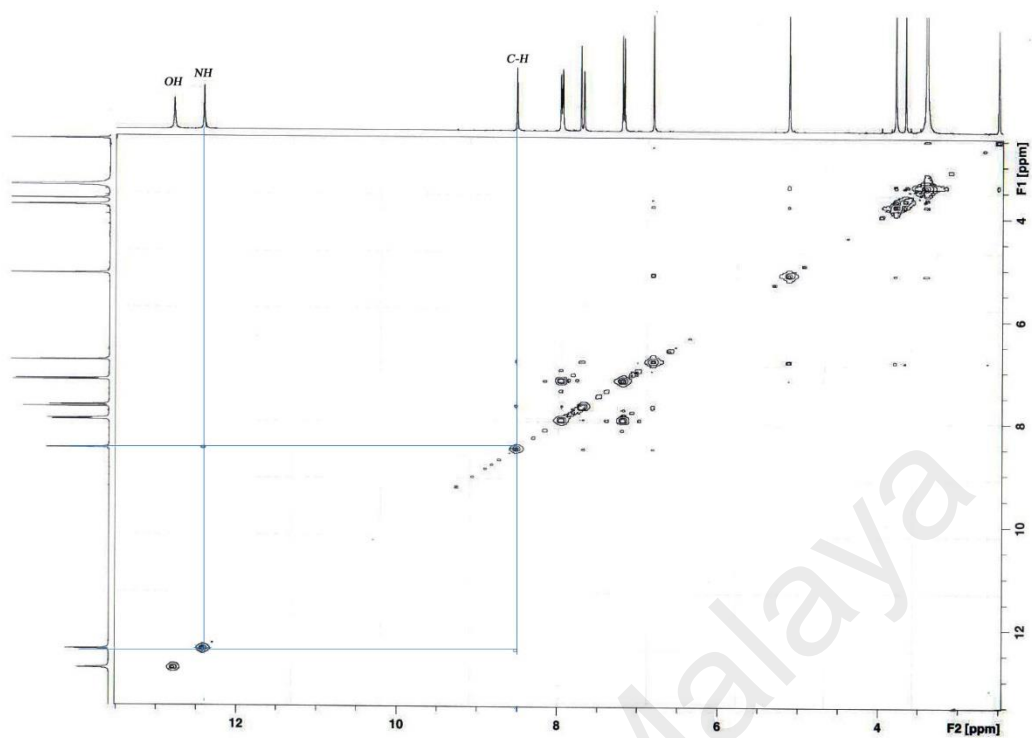
Figure A 11: COSY spectrum (DMSO-d$_6$, 400MHz) of 3.1
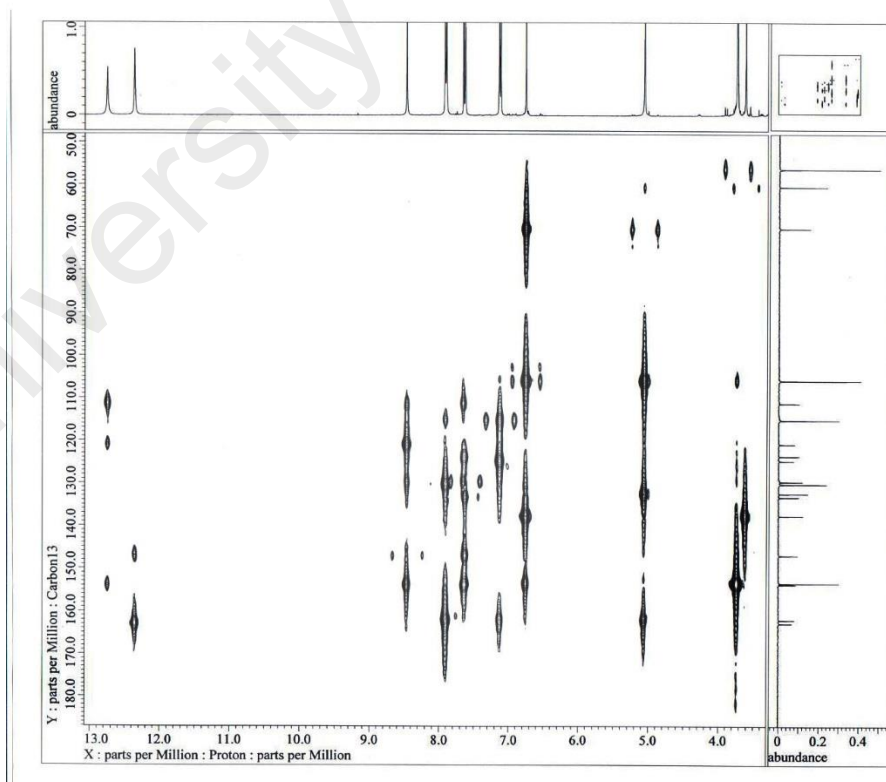
Figure A 12: HMBC spectrum (DMSO-d$_6$, 400MHz) of 3.1
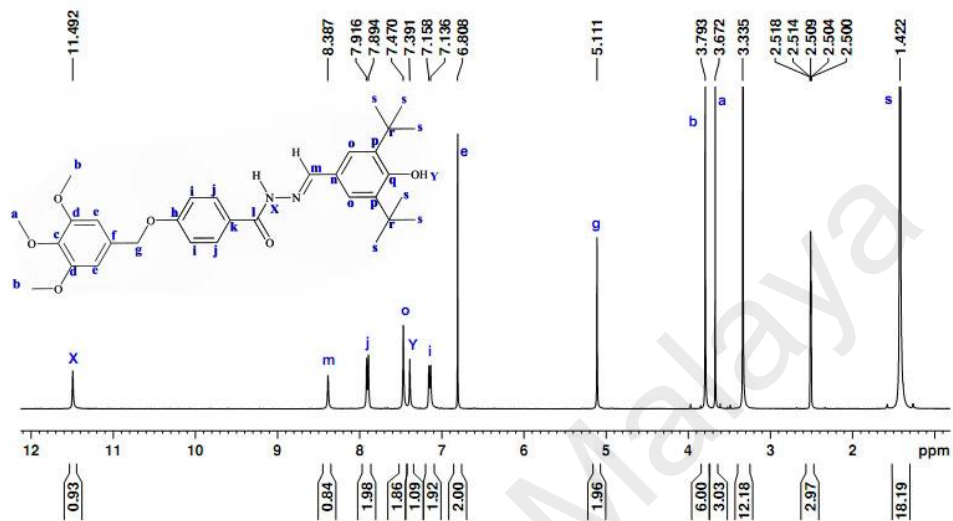
Figure A 13: ¹H spectrum (DMSO-d6, 400MHz) of 3.2
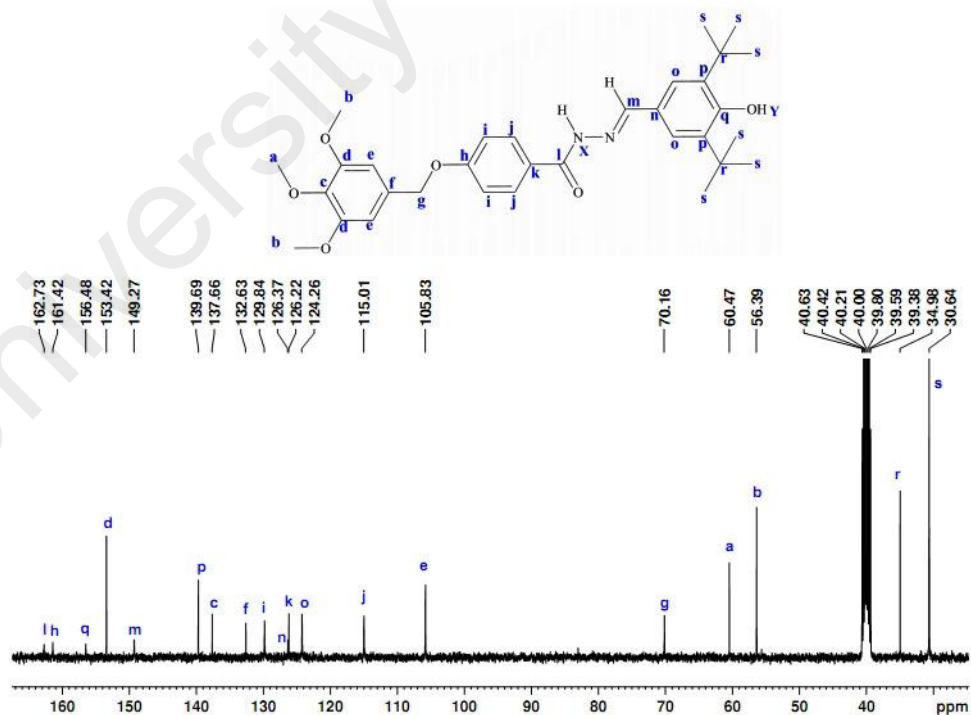
Figure A 14: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.2
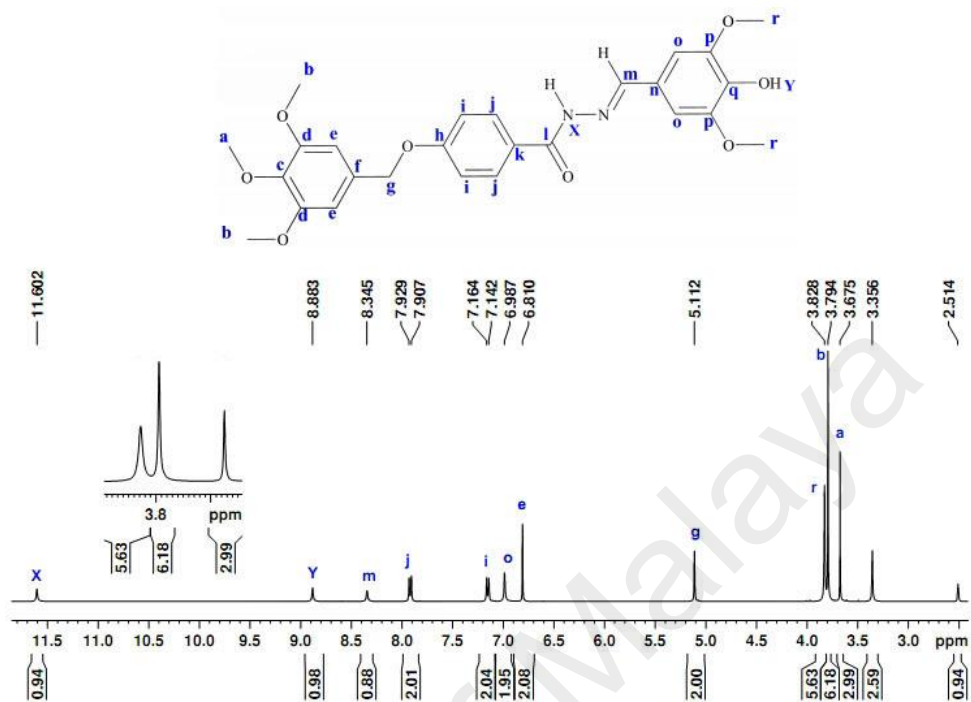
Figure A 15: ¹H spectrum (DMSO-d₆, 400MHz) of 3.3
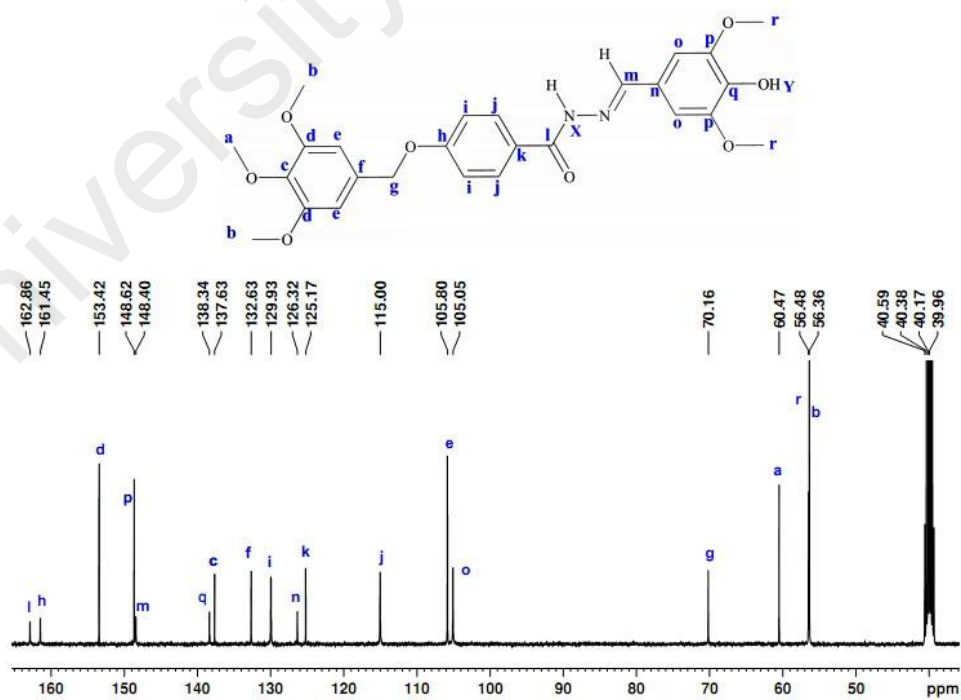
Figure A 16: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.3

160
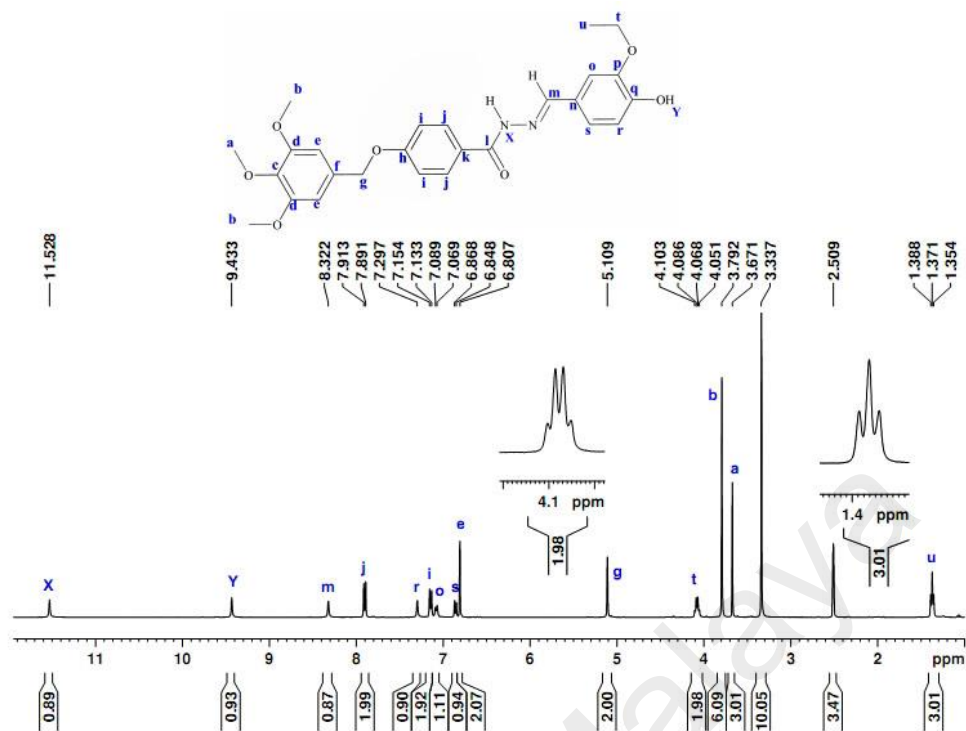
Figure A 17: ¹H spectrum (DMSO-d₆, 400MHz) of 3.4
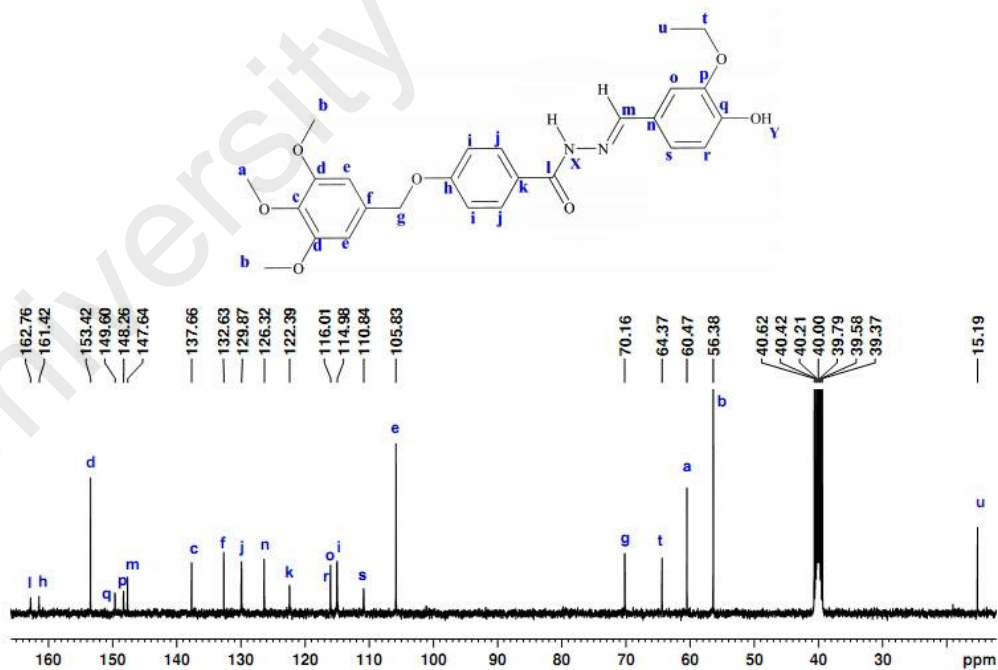
Figure A 18: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.4
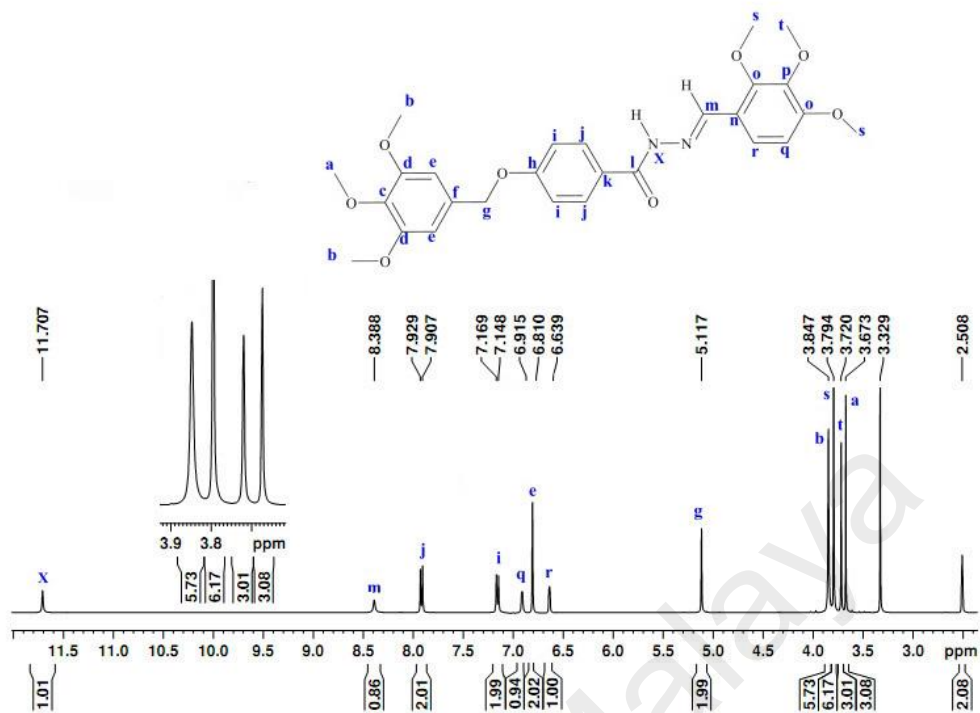
Figure A 19: ¹H spectrum (DMSO-d₆, 400MHz) of 3.5
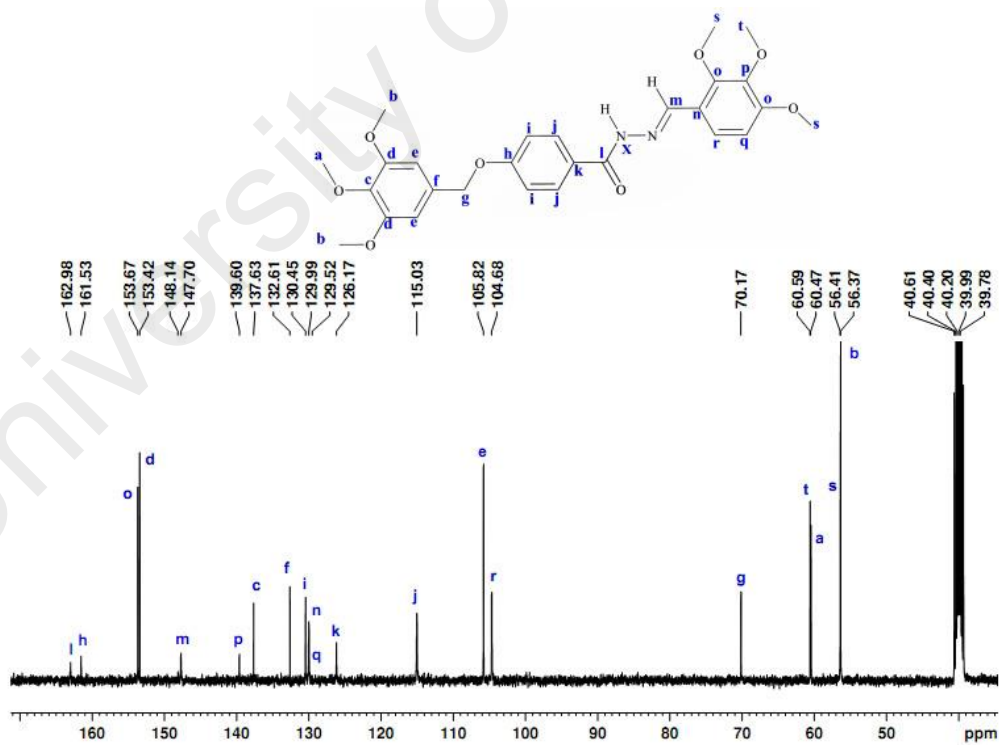
Figure A 20: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.5

162
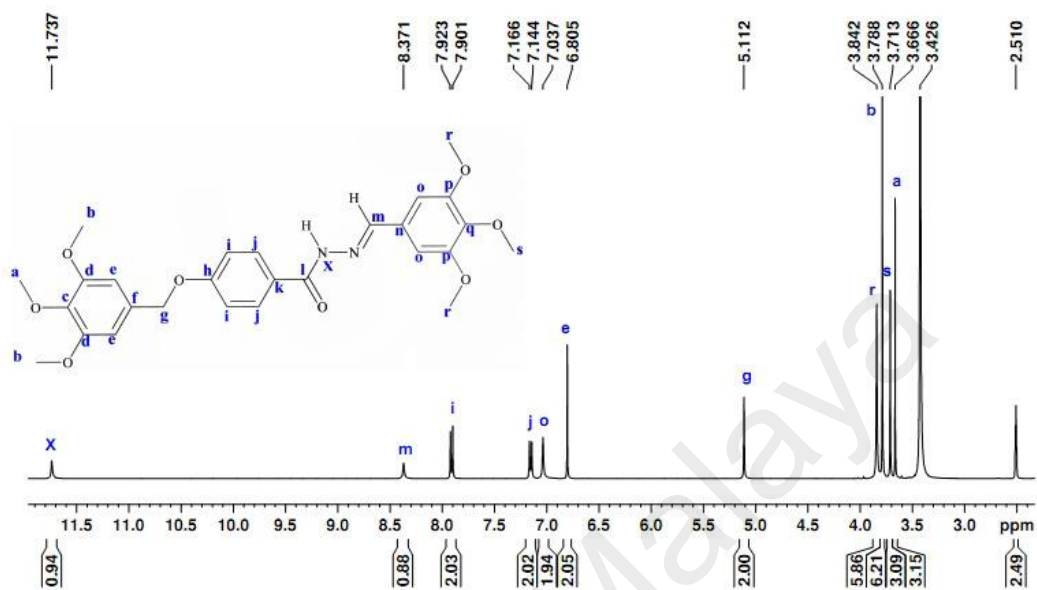
Figure A 21: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 3.6
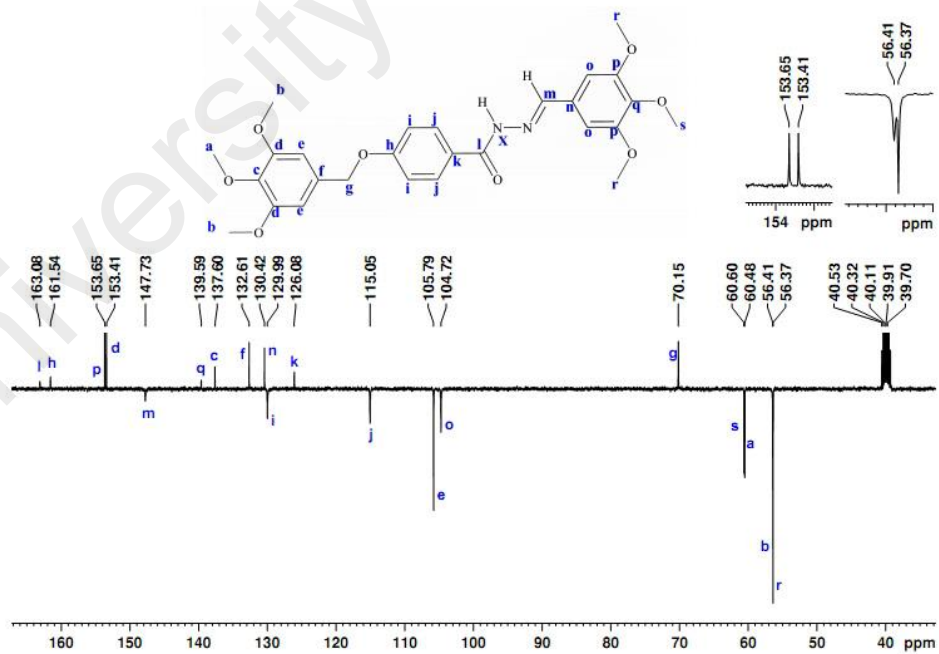
Figure A 22: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 3.6

163
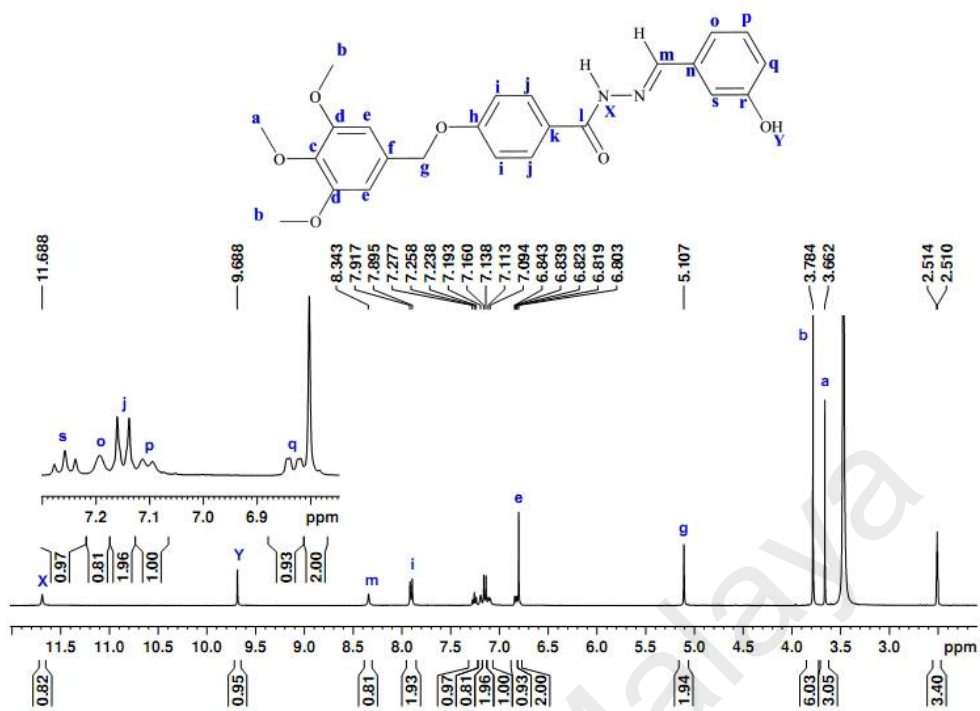
Figure A 23: ¹H spectrum (DMSO-d₆, 400MHz) of 3.7
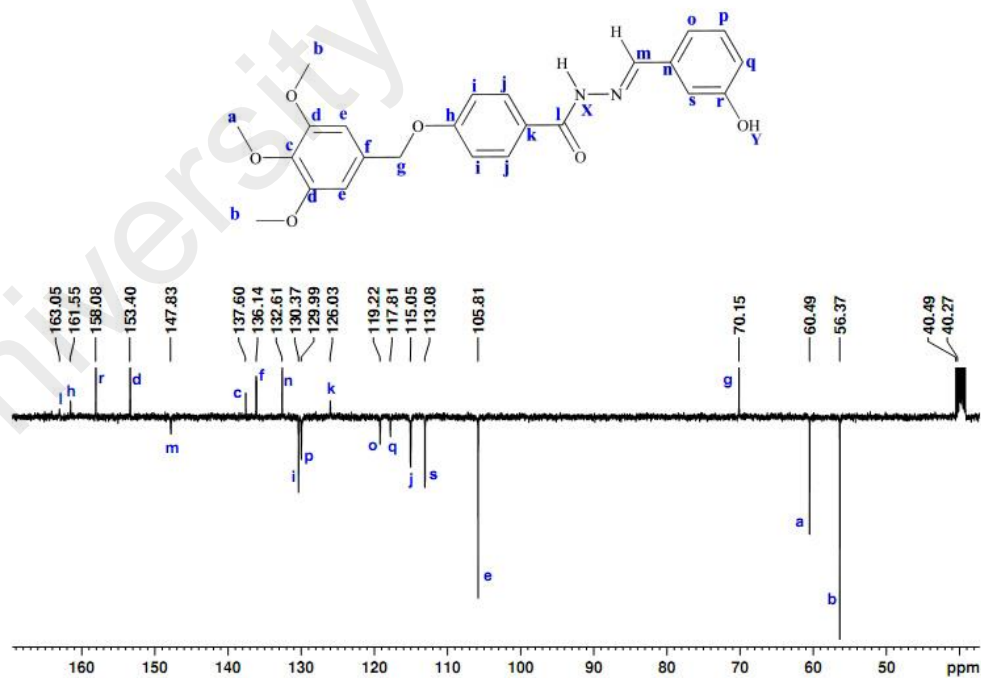
Figure A 24: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.7

164
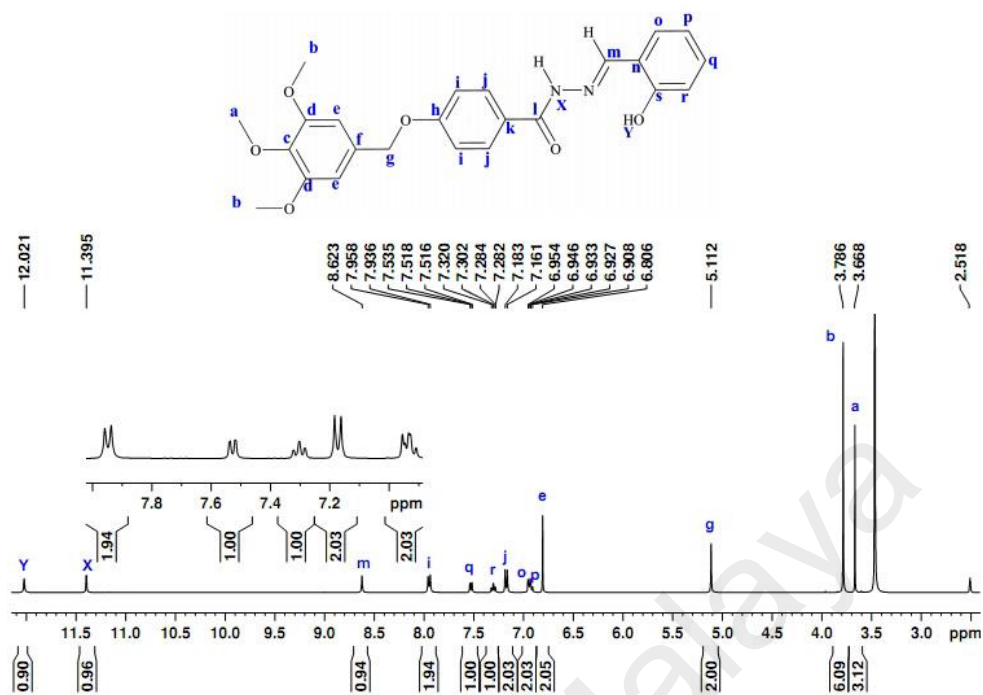
Figure A 25: ¹H spectrum (DMSO-d₆, 400MHz) of 3.8
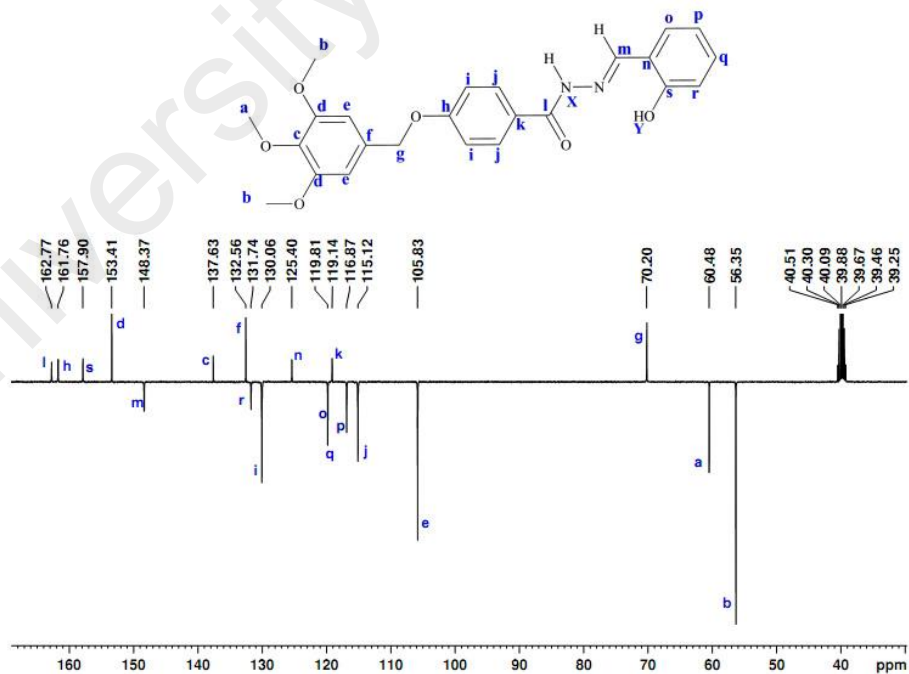
Figure A 26: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.8
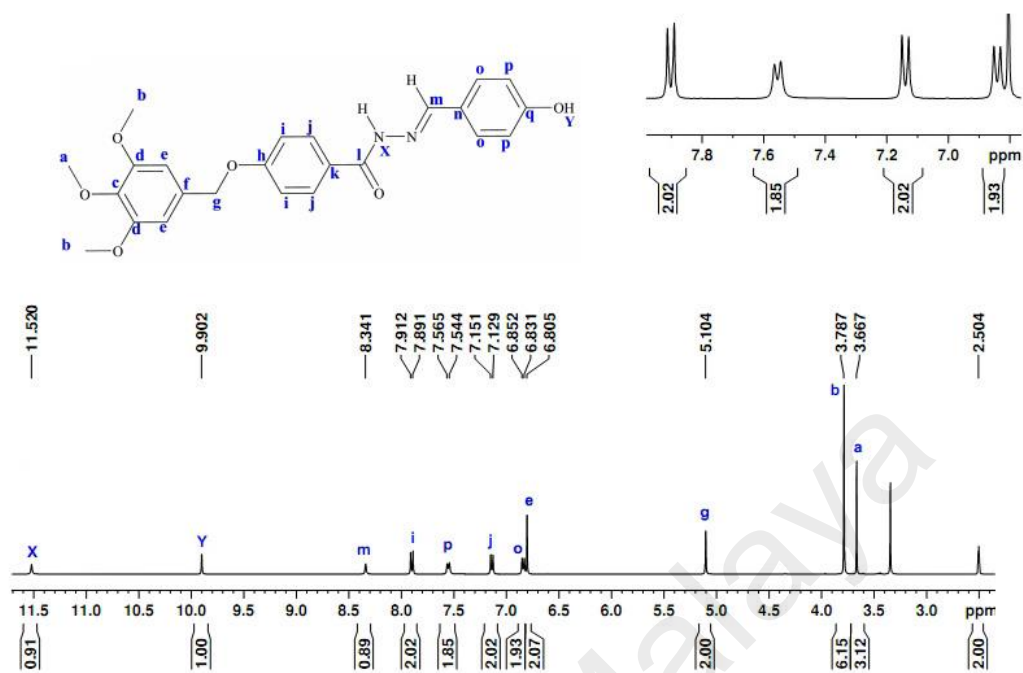
Figure A 27: ¹H spectrum (DMSO-d₆, 400MHz) of 3.9
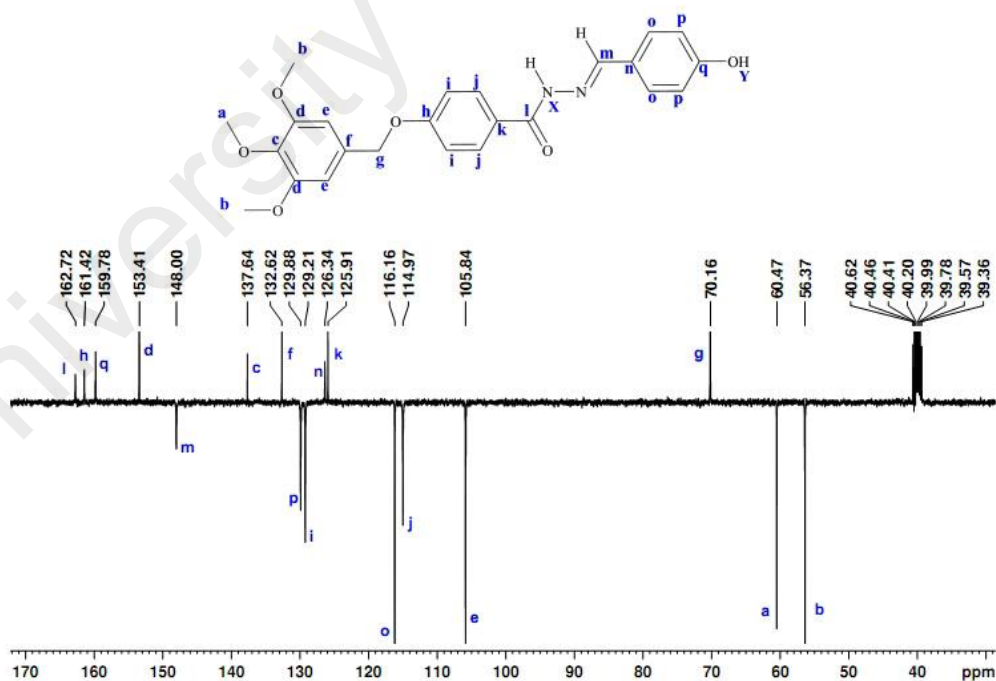
Figure A 28: ¹³C spectrum (DMSO-d₆, 100MHz) of 3.9
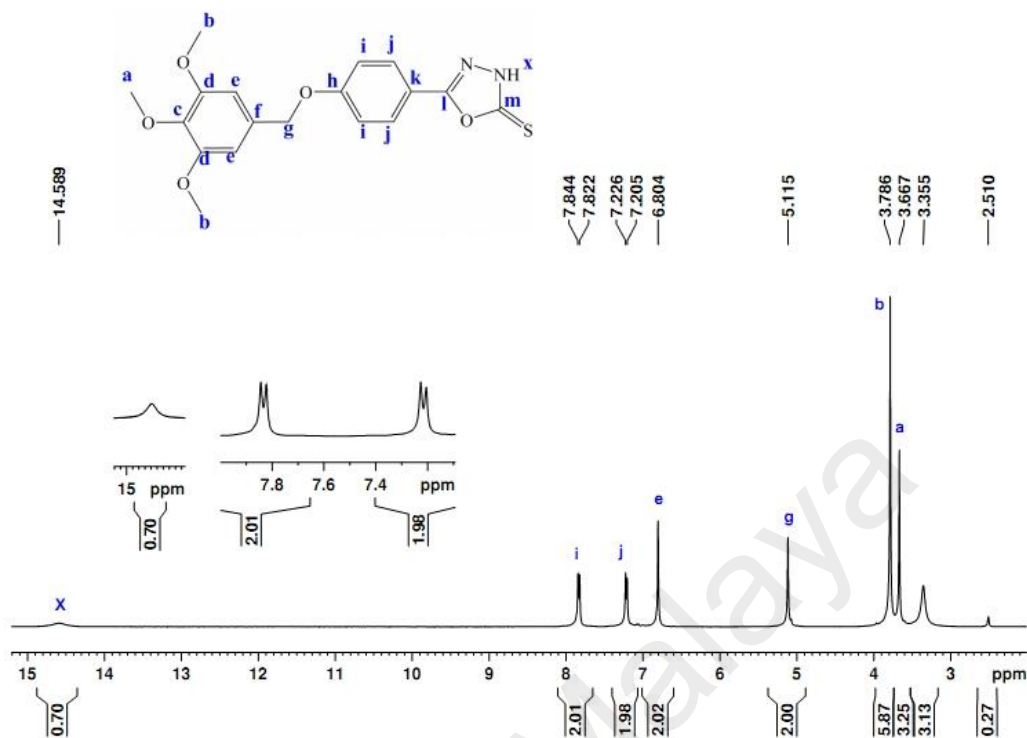
Figure A 29: ¹H spectrum (DMSO-d₆, 400MHz) of 4.1
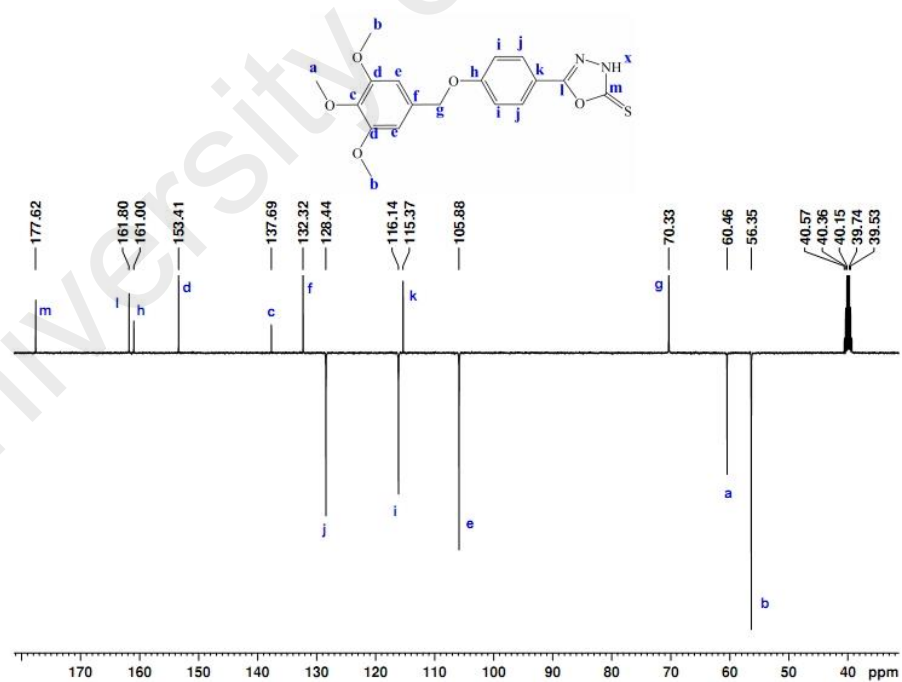
Figure A 30: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.1

167
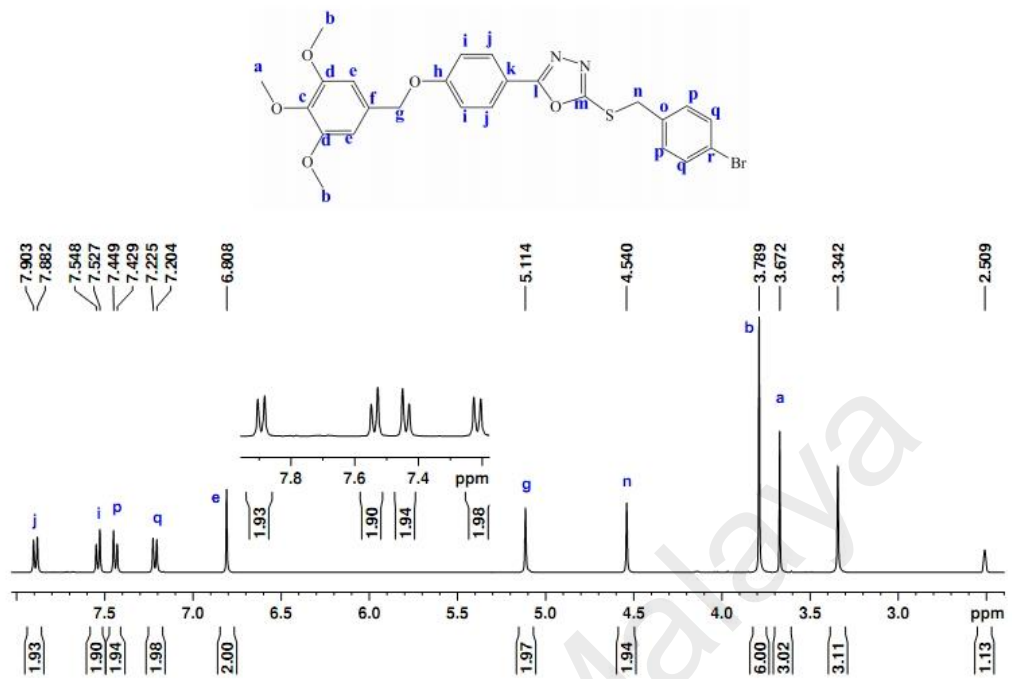
Figure A 31: ¹H spectrum (DMSO-d₆, 400MHz) of 4.2
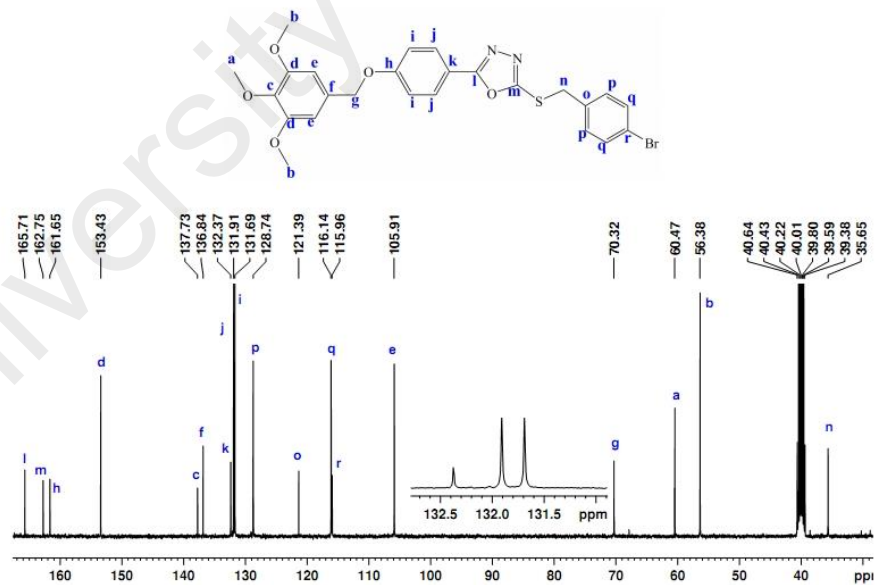
Figure A 32: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.2

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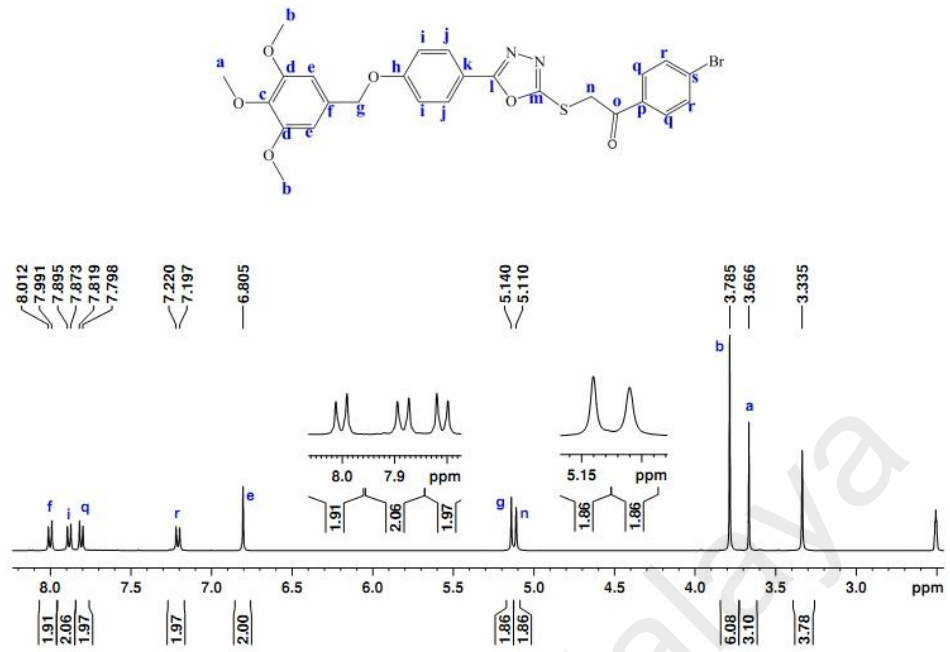
Figure A 33: ¹H spectrum (DMSO-d₆, 400MHz) of 4.3
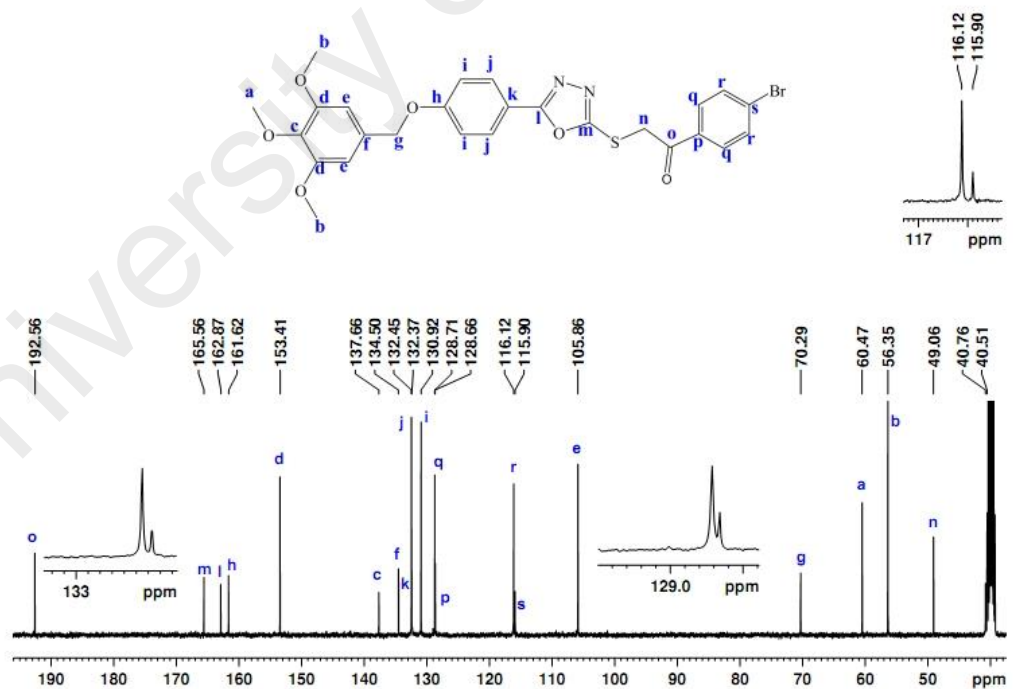
Figure A 34: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.3
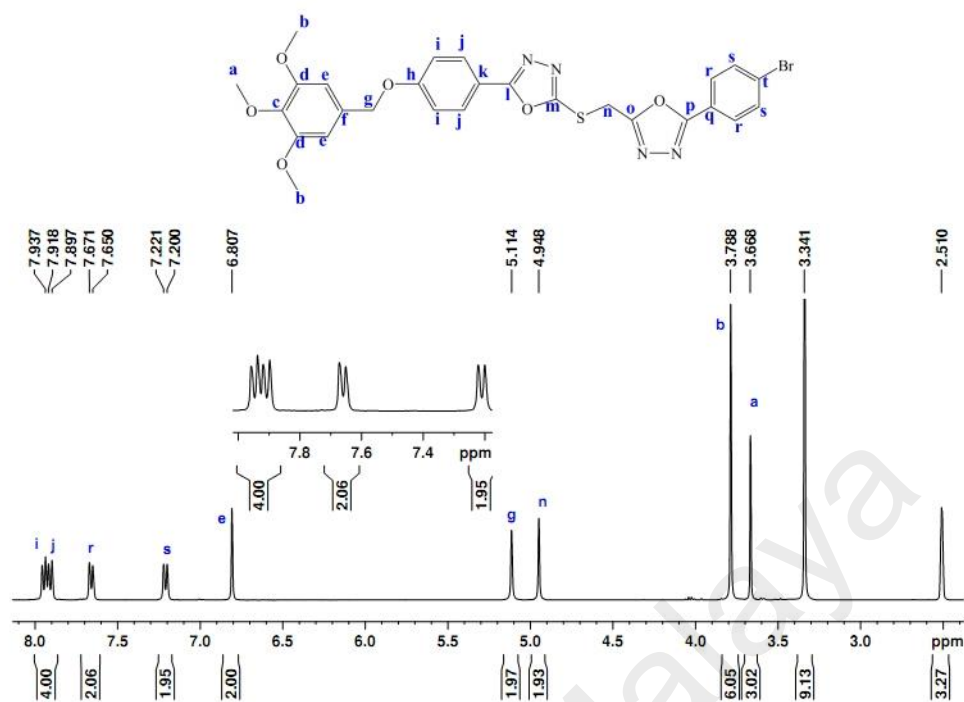
Figure A 35: ¹H spectrum (DMSO-d₆, 400MHz) of 4.4
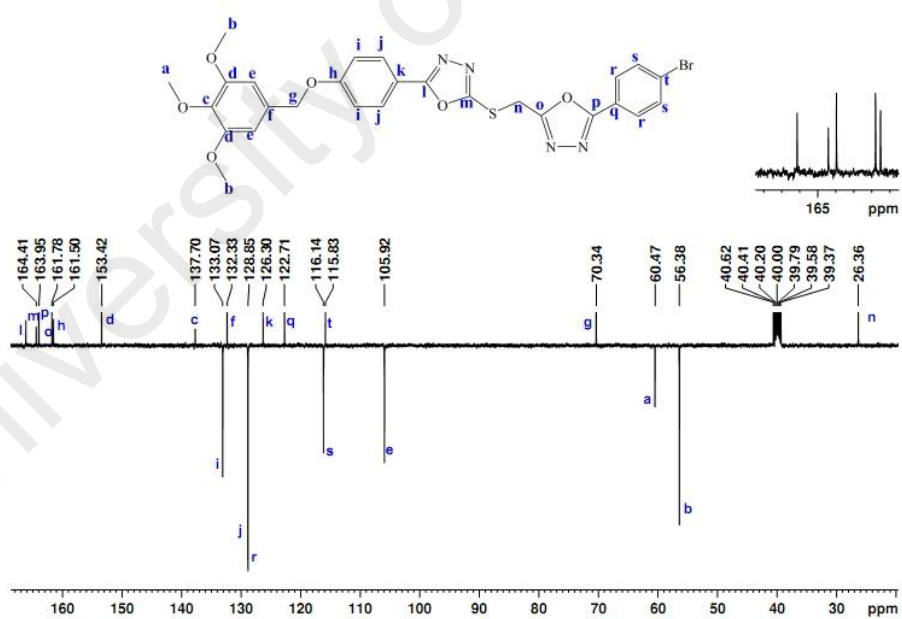
Figure A 36: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.4
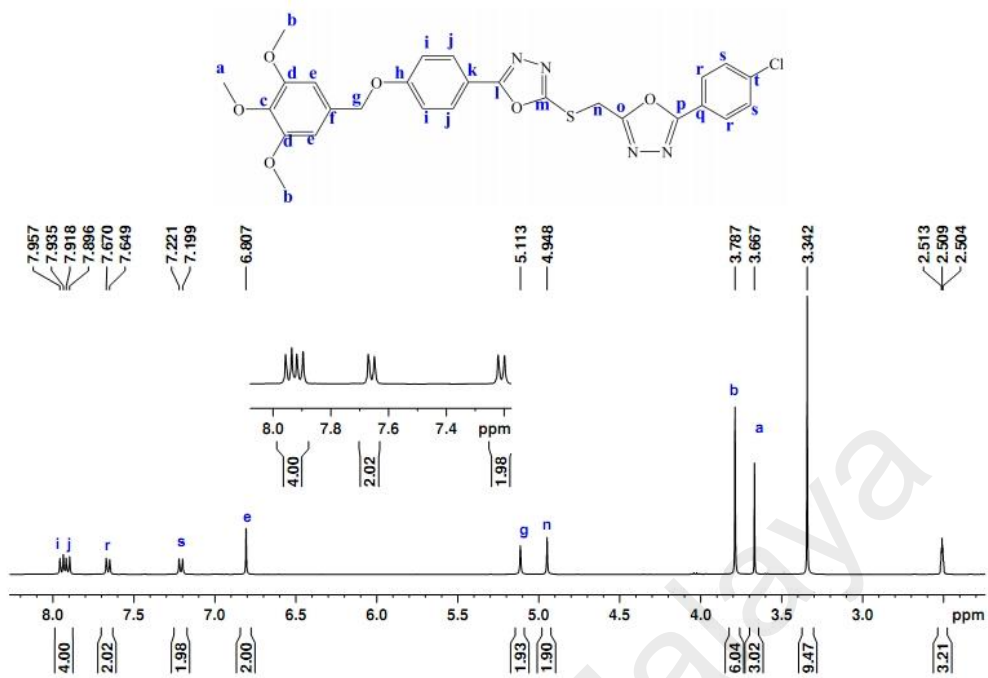
Figure A 37: ¹H spectrum (DMSO-d₆, 400MHz) of 4.5
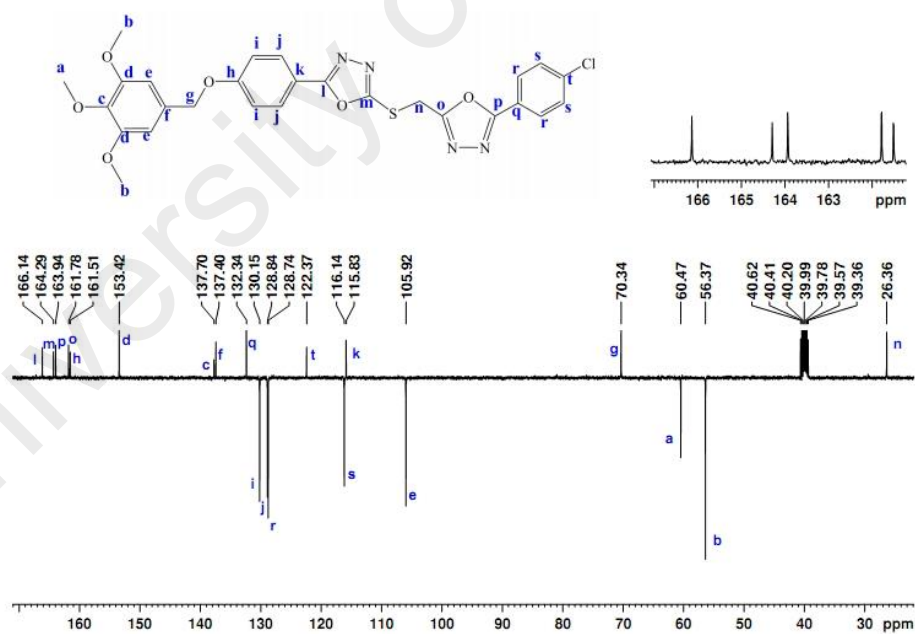
Figure A 38: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.5

171
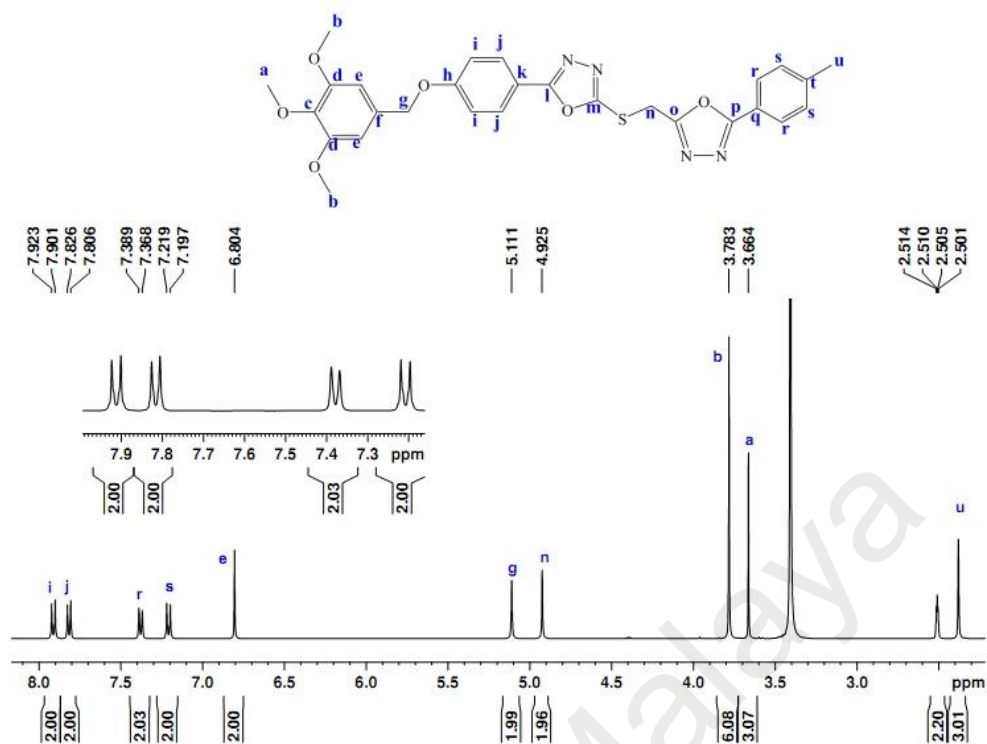
Figure A 39: ¹H spectrum (DMSO-d₆, 400MHz) of 4.6
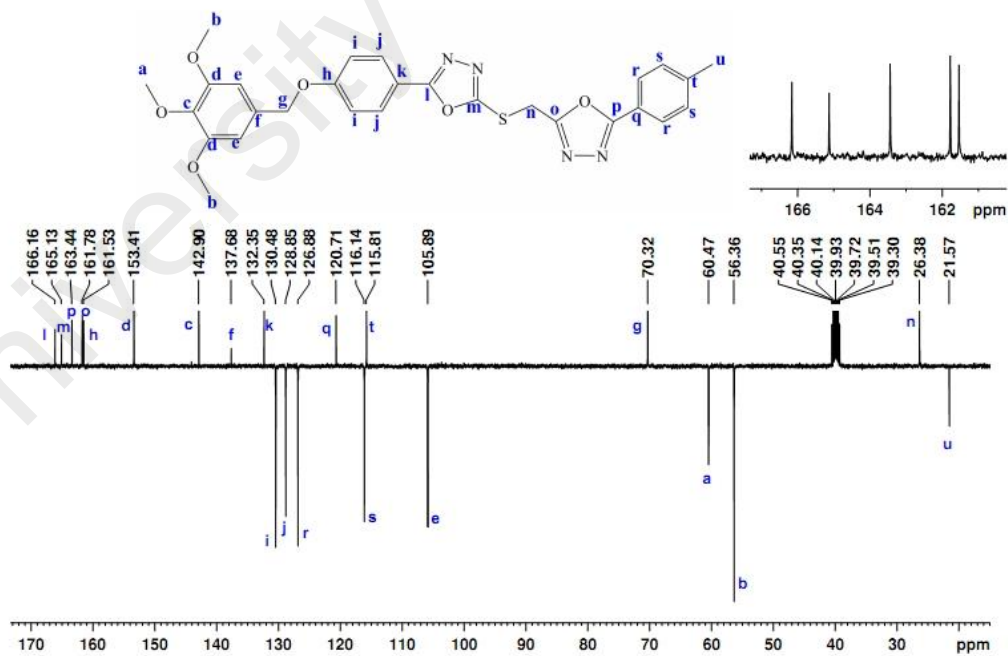
Figure A 40: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.6

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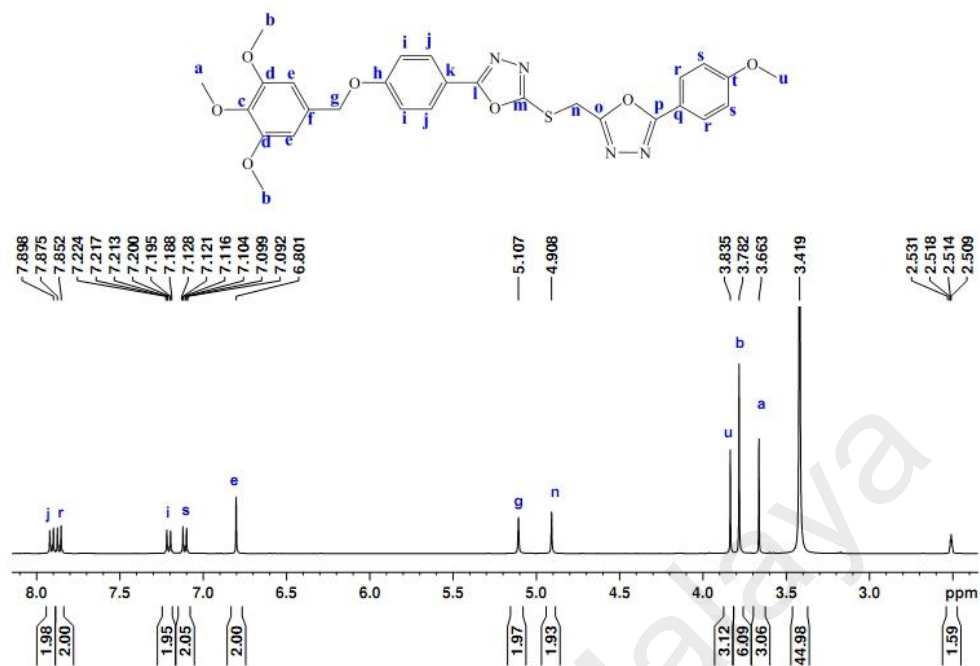
Figure A 41: ¹H spectrum (DMSO-d₆, 400MHz) of 4.7
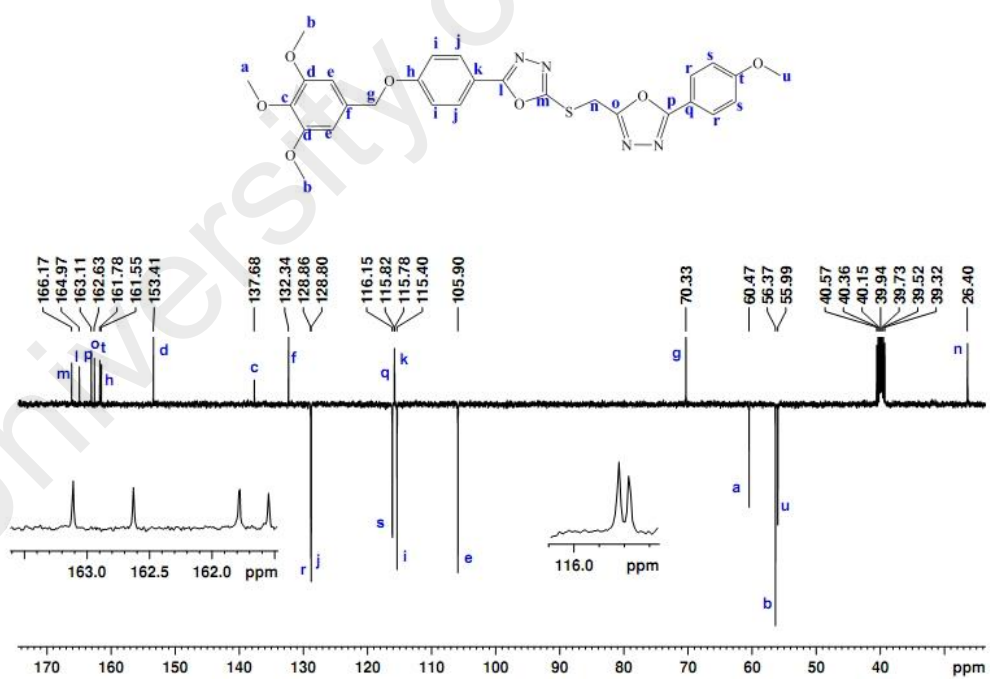
Figure A 42: ¹³C spectrum (DMSO-d₆, 100MHz) of 4.7
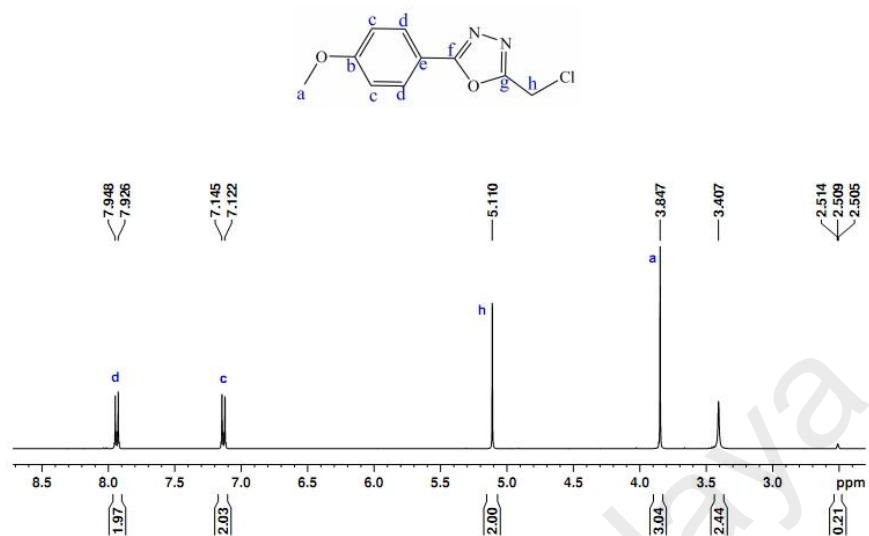
Figure A 43: ¹H spectrum (DMSO-d₆, 400MHz) of 4f
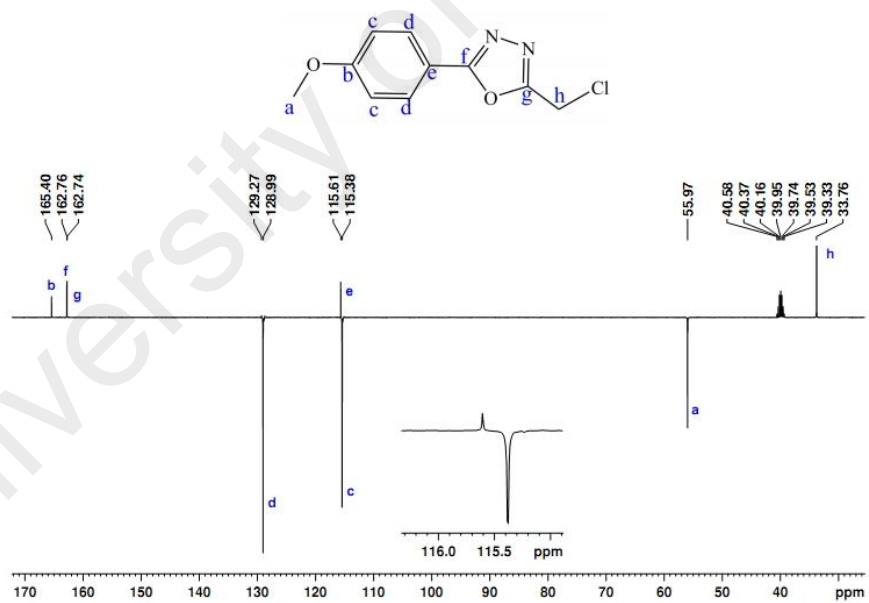
Figure A 44: ¹³C spectrum (DMSO-d₆, 100MHz) of 4f
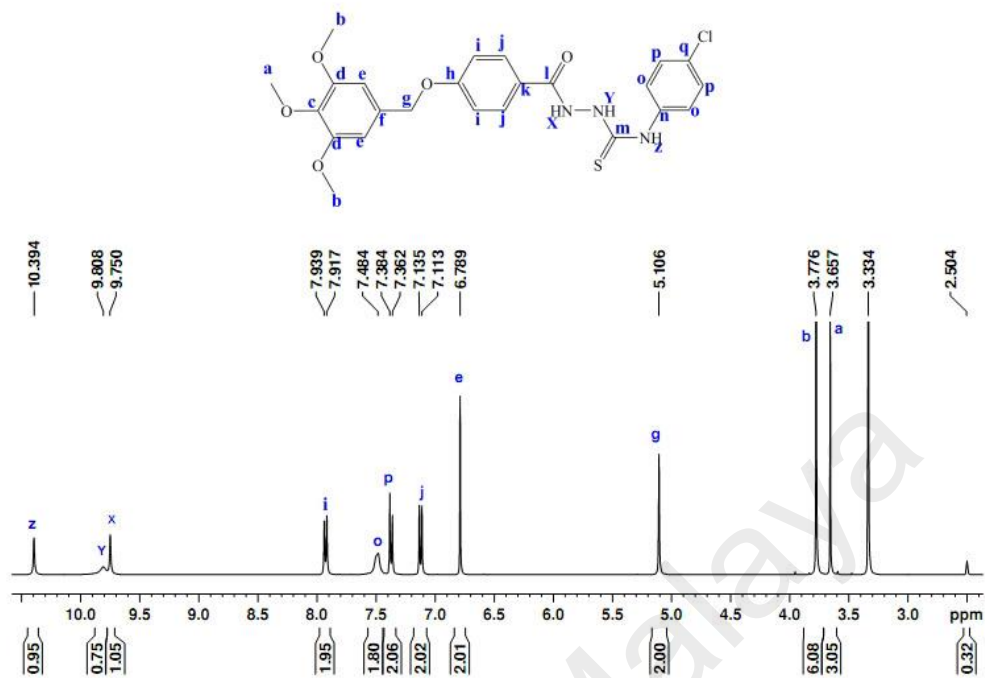
Figure A 45: ¹H spectrum (DMSO-d₆, 400MHz) of 5.1
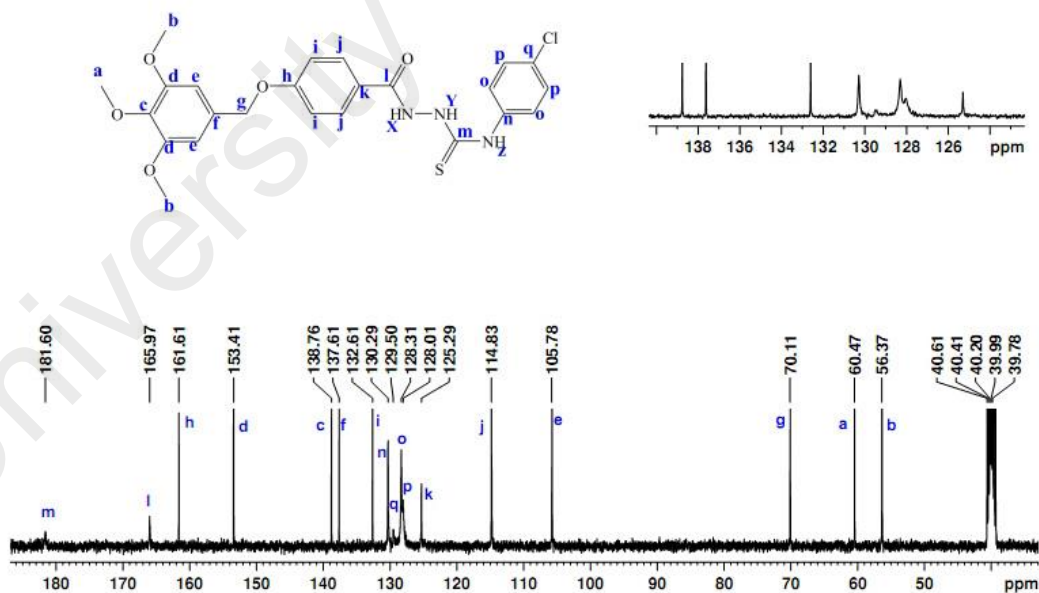
Figure A 46: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.1

175
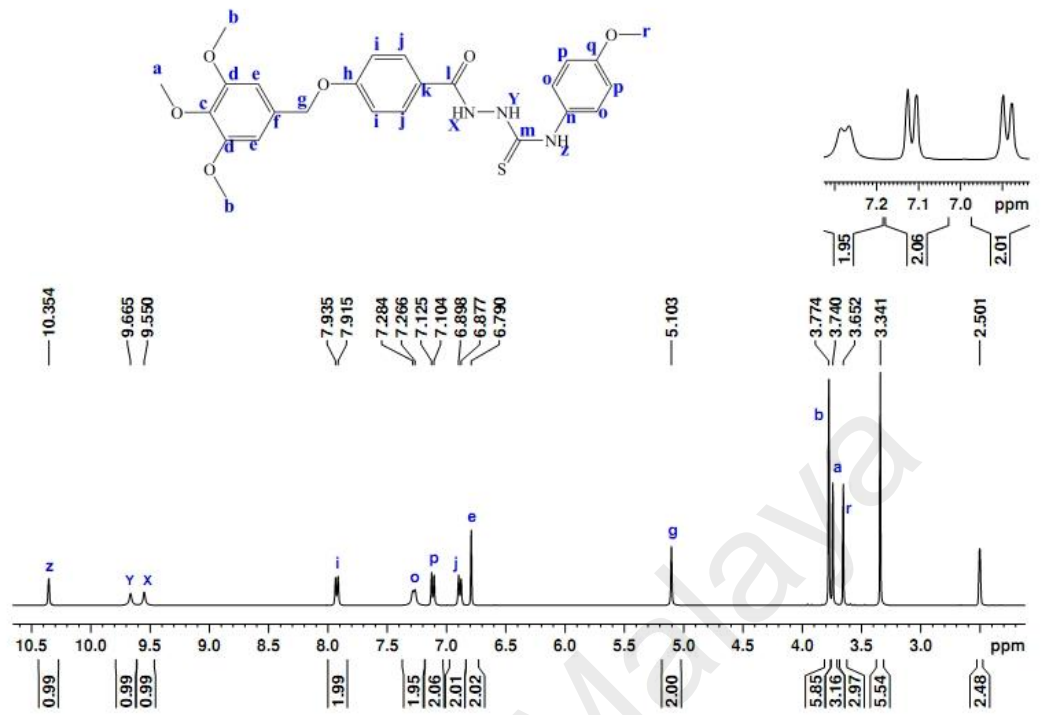
Figure A 47: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 5.2
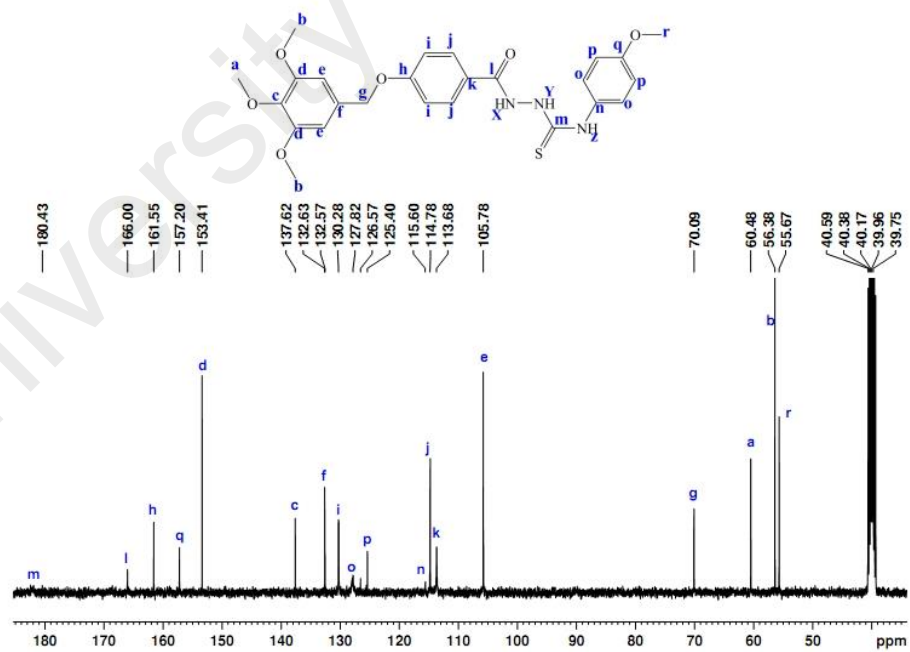
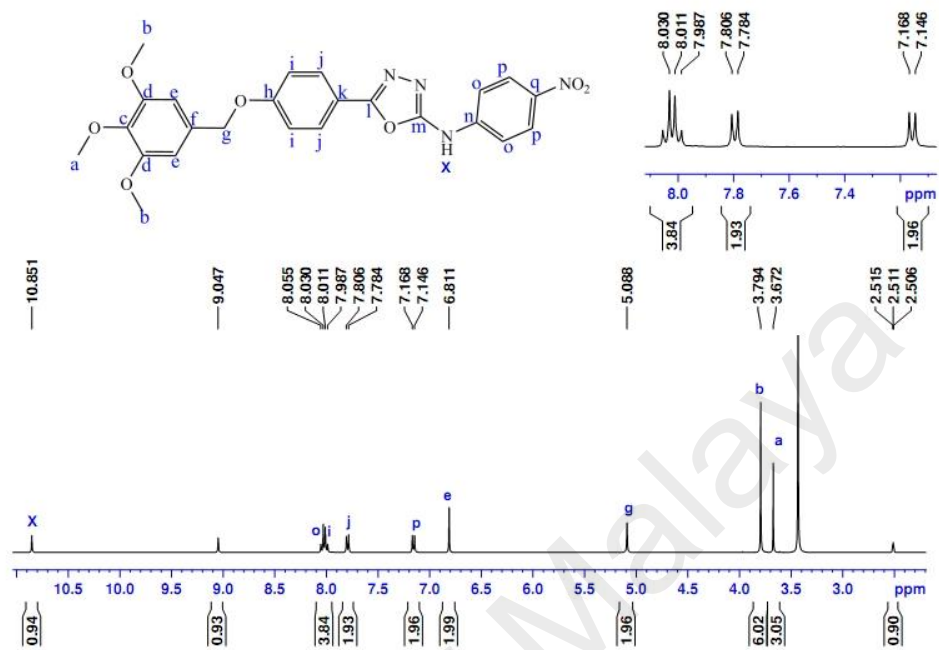**Figure A 48: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.2**
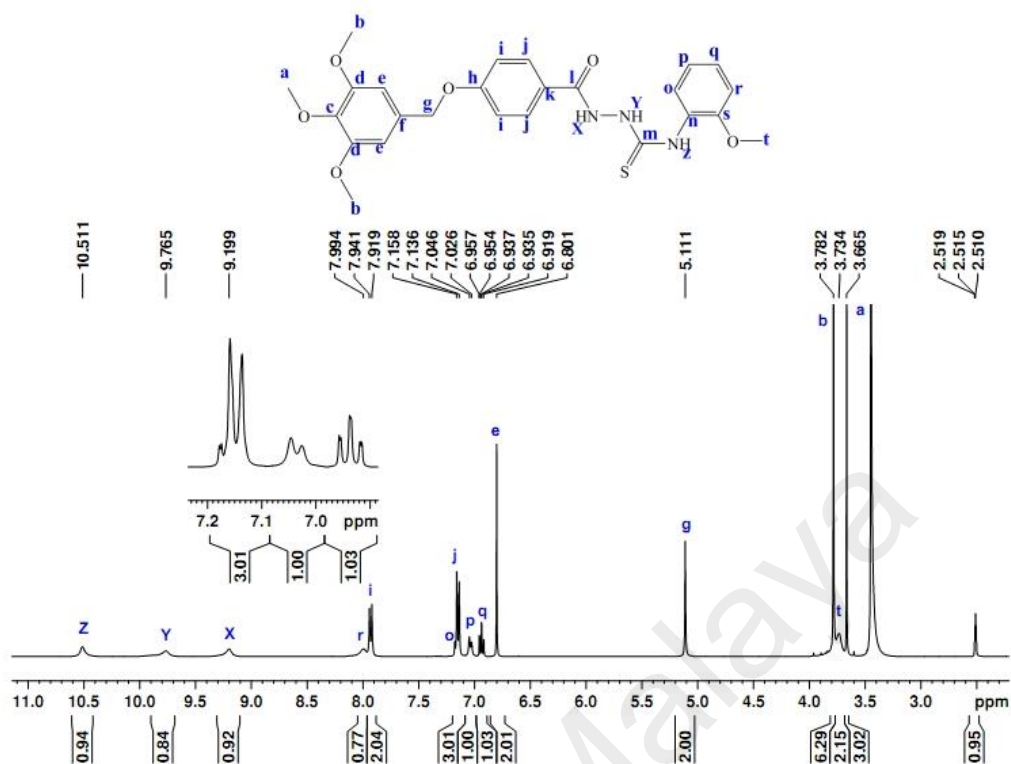
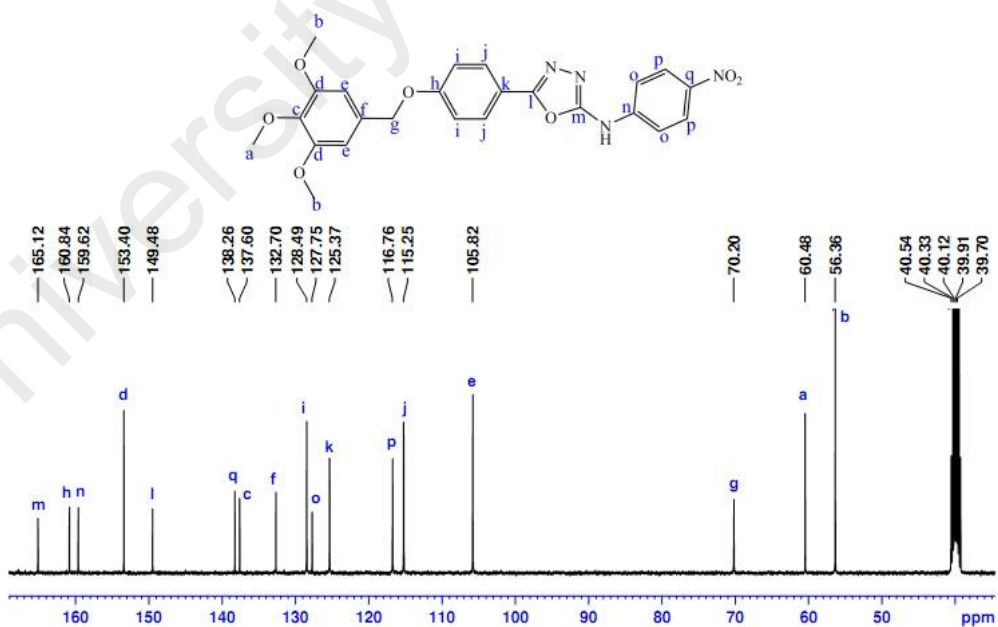**Figure A 49: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 5.3**
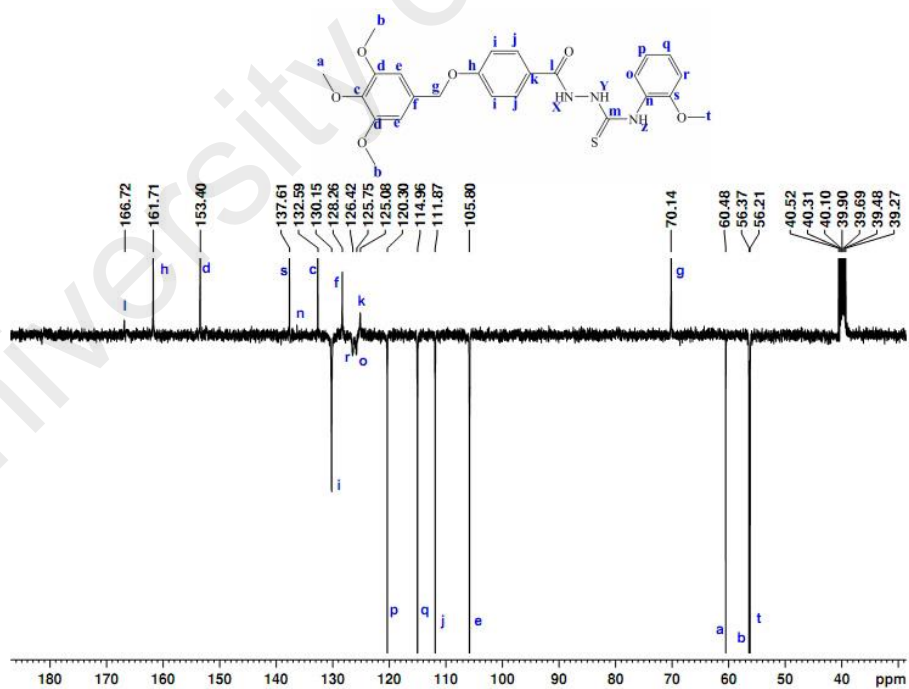
Figure A 50: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.3

177
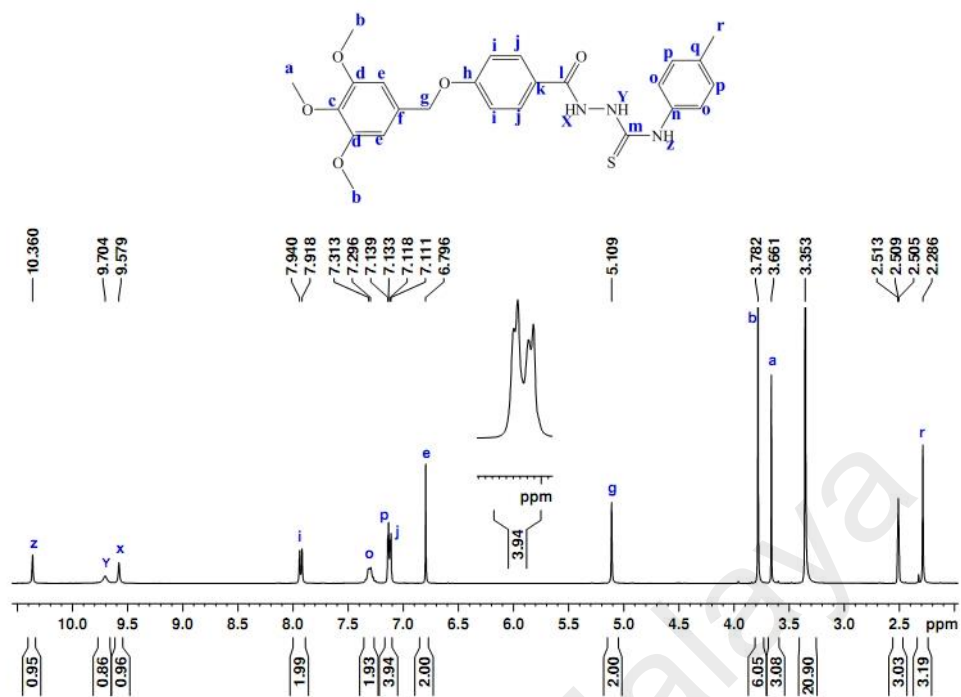
Figure A 51: ¹H spectrum (DMSO-d₆, 400MHz) of 5.4

Figure A 52: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.4

Figure A 53: ¹H spectrum (DMSO-d₆, 400MHz) of 5.5

Figure A 54: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.5

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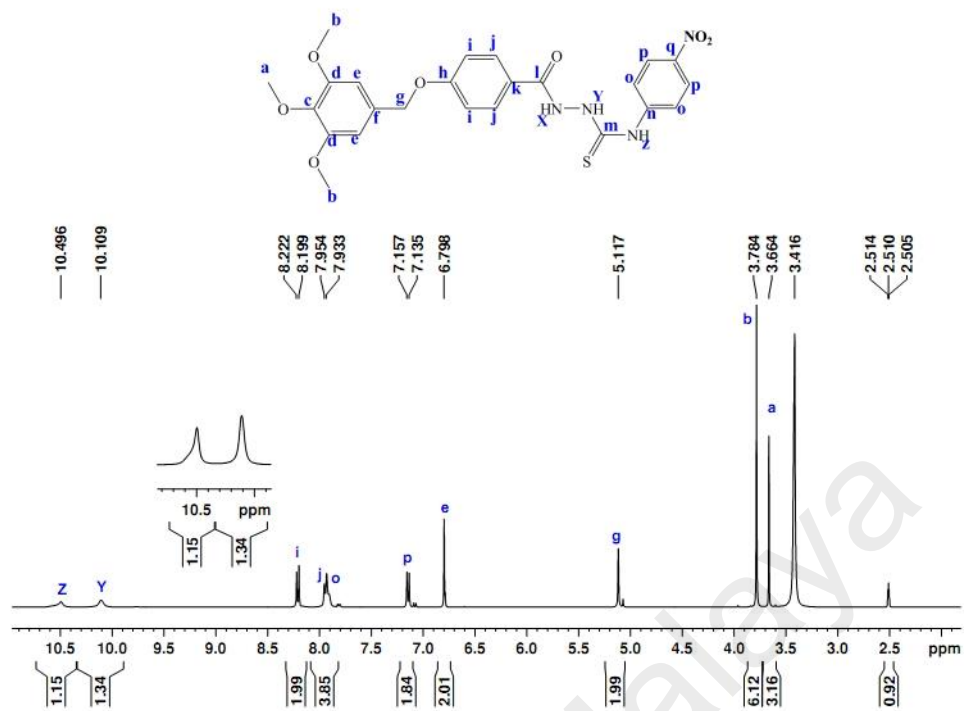
Figure A 55: ^1H spectrum (DMSO-d$_6$, 400MHz) of 5.6

Figure A 56: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.6
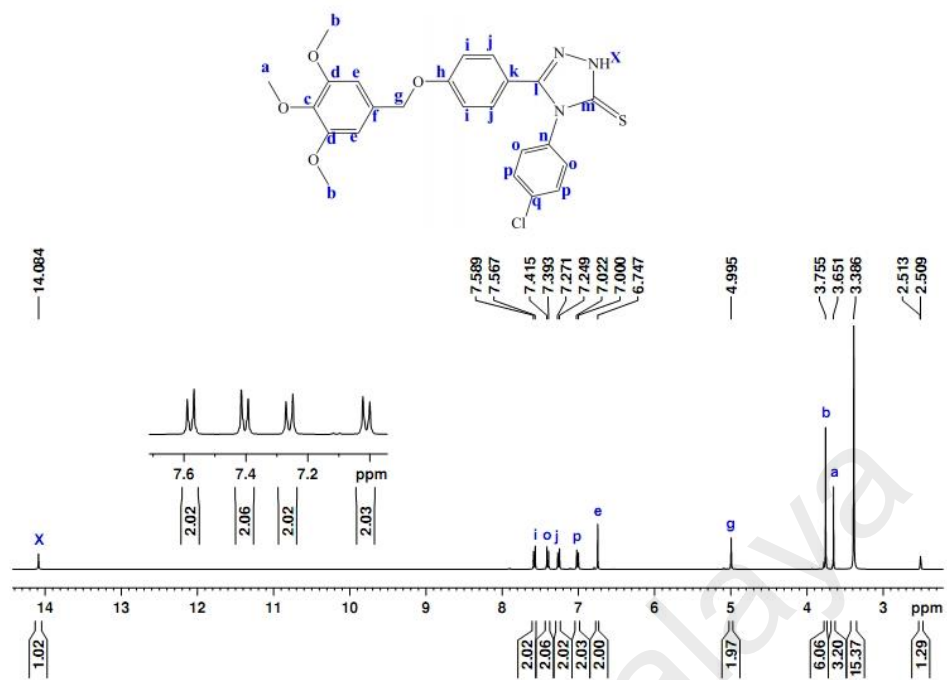
Figure A 57: ¹H spectrum (DMSO-d₆, 400MHz) of 5.7

Figure A 58: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.7
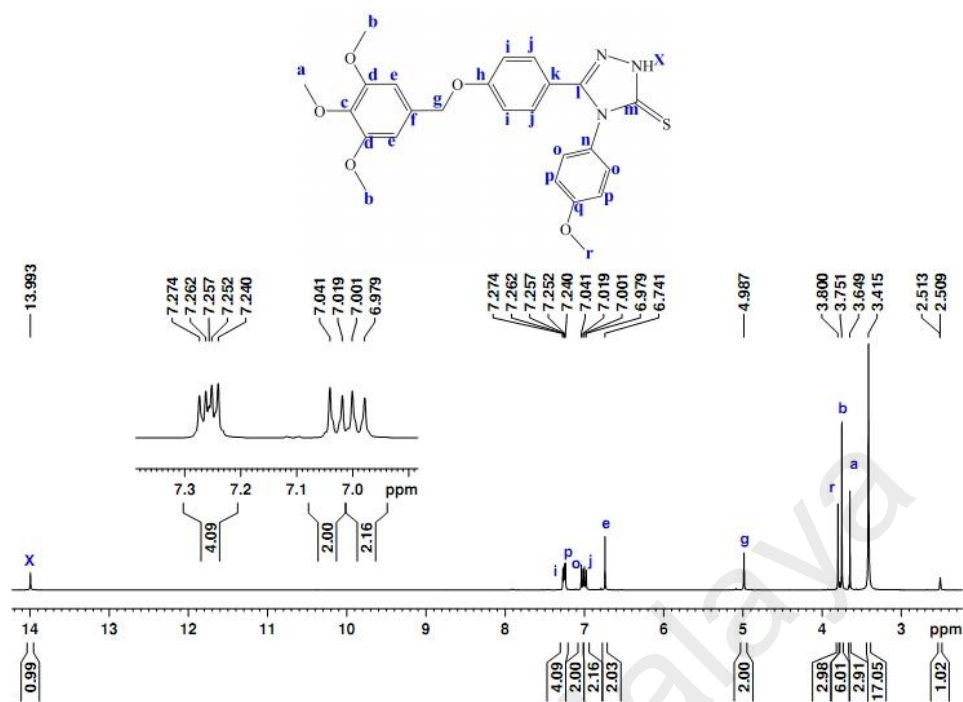
Figure A 59: ¹H spectrum (DMSO-d₆, 400MHz) of 5.8
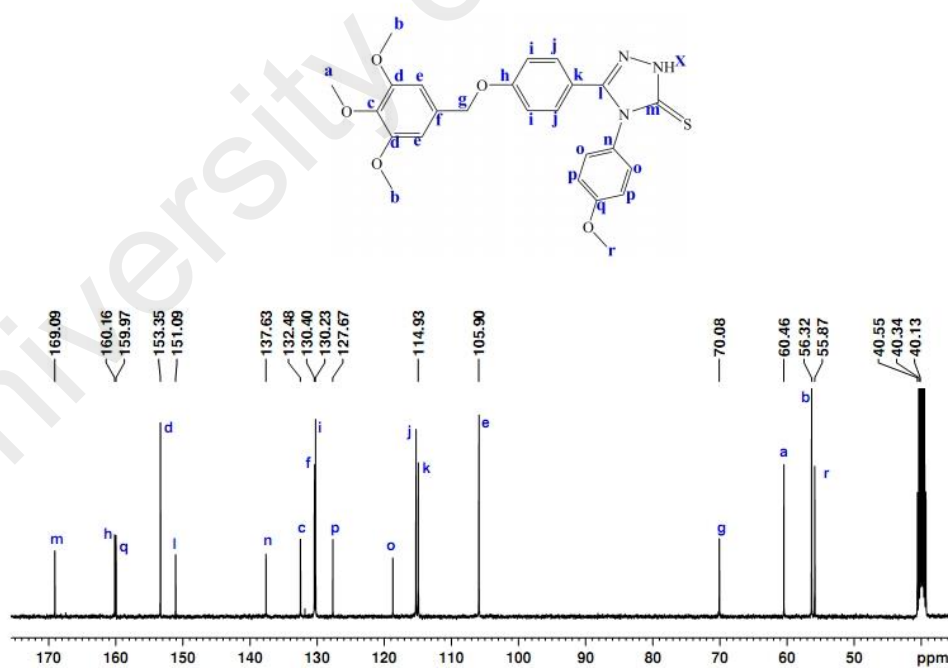
Figure A 60: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.8
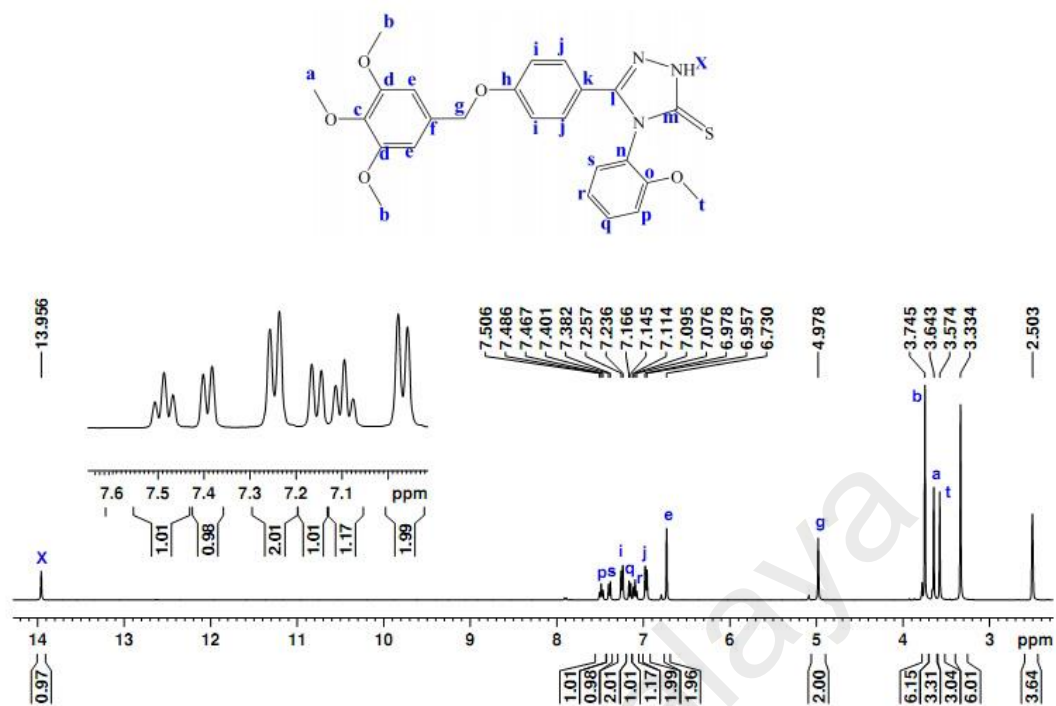
Figure A 61: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 5.9
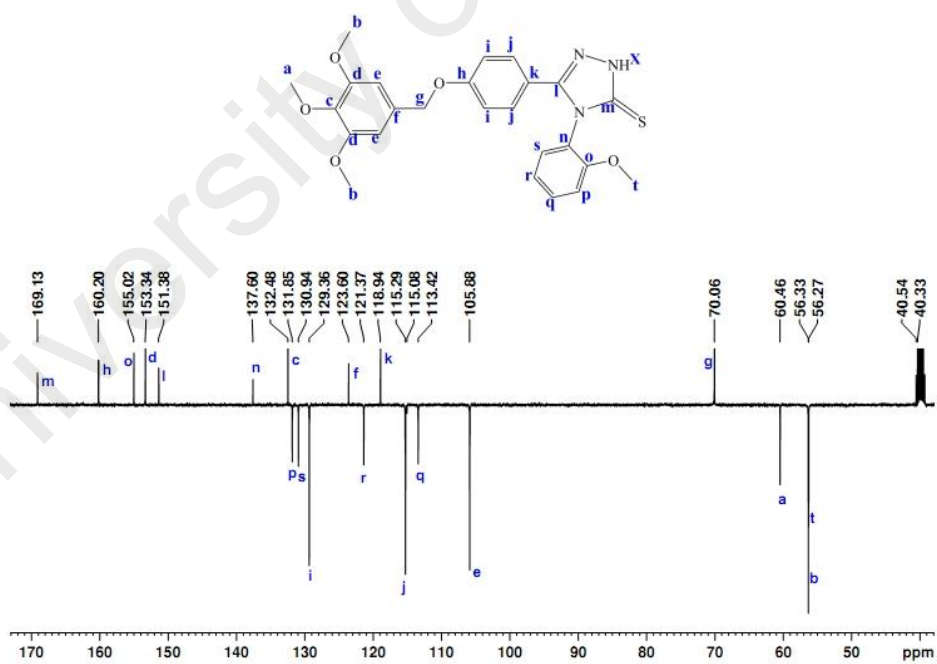
Figure A 62: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.9
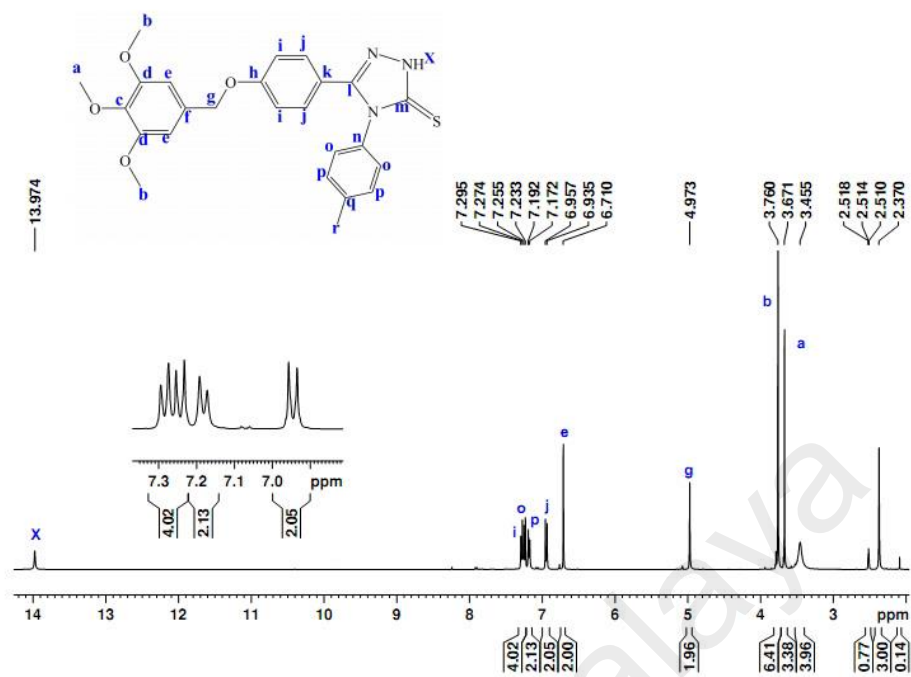
Figure A 63: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 5.10
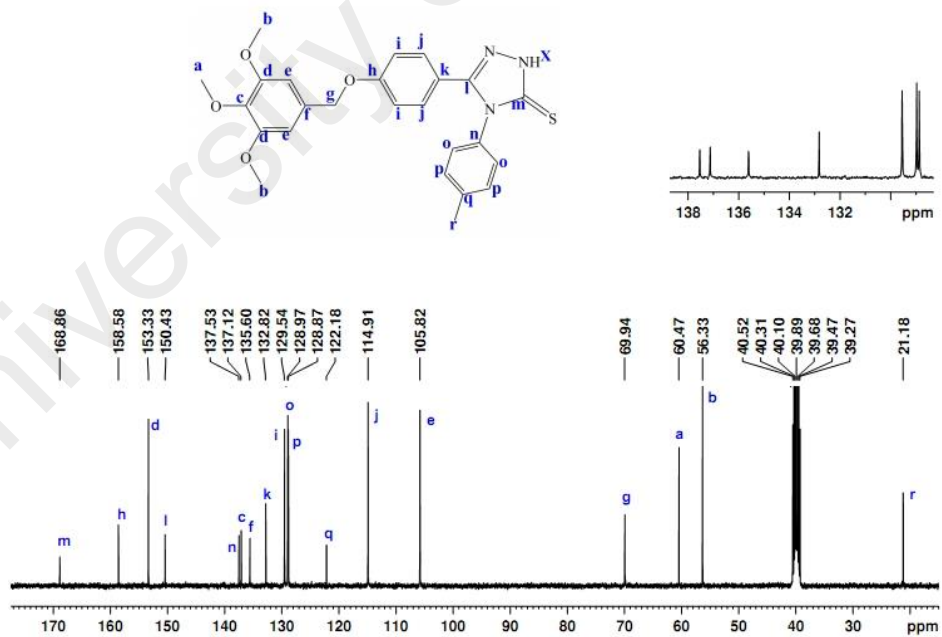
Figure A 64: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.10

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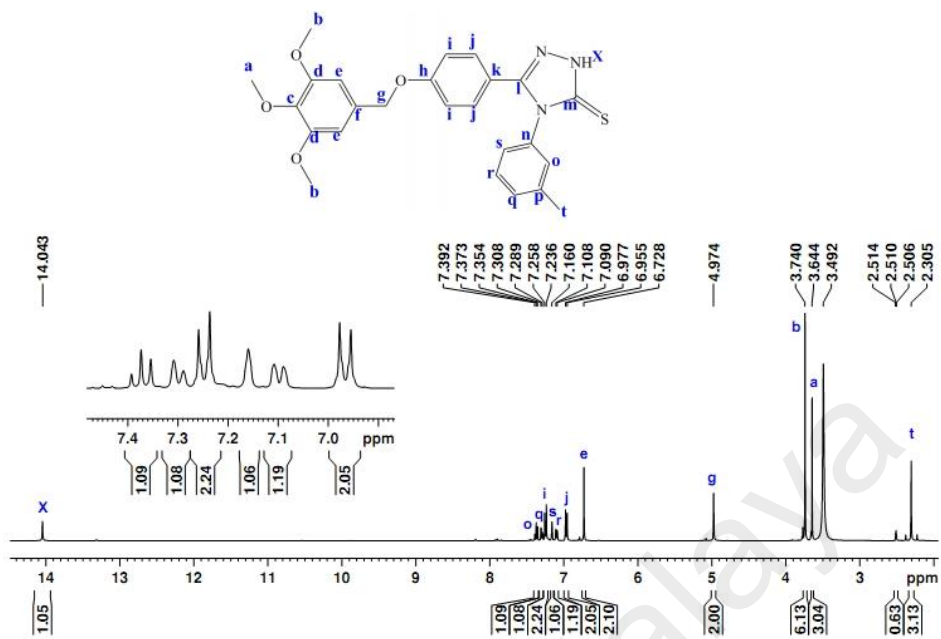
Figure A 65: ¹H spectrum (DMSO-d₆, 400MHz) of 5.11
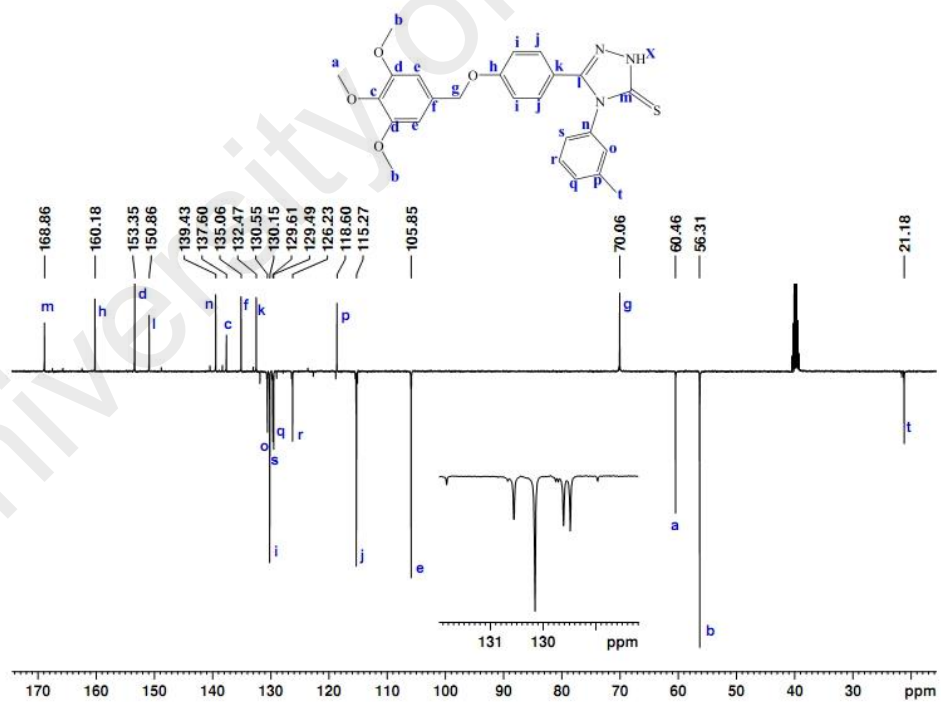
Figure A 66: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.11

185
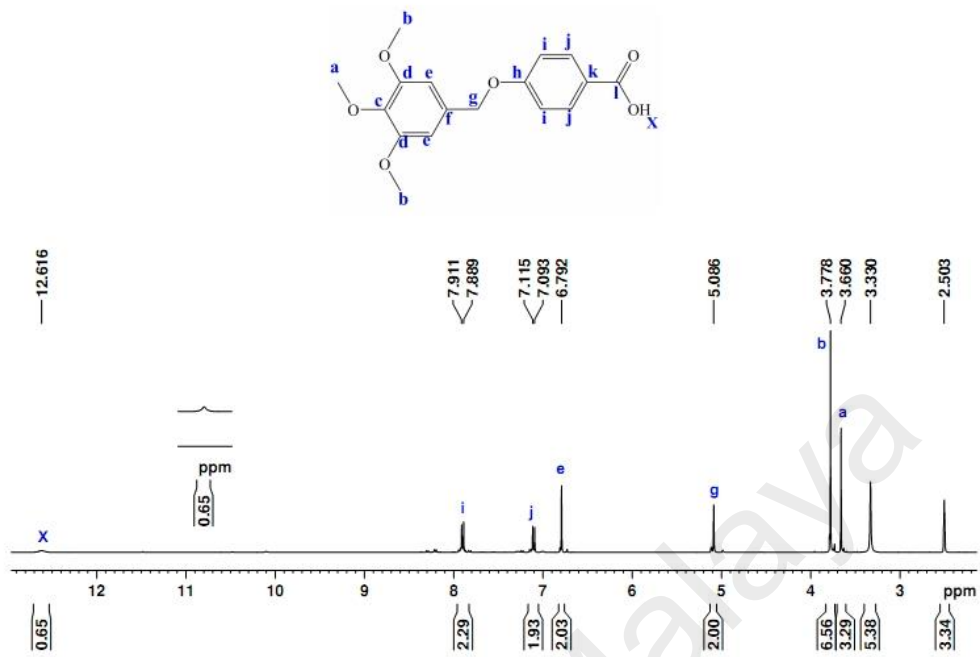
Figure A 67: ^{1}H spectrum (DMSO-d$_6$, 400MHz) of 5.12
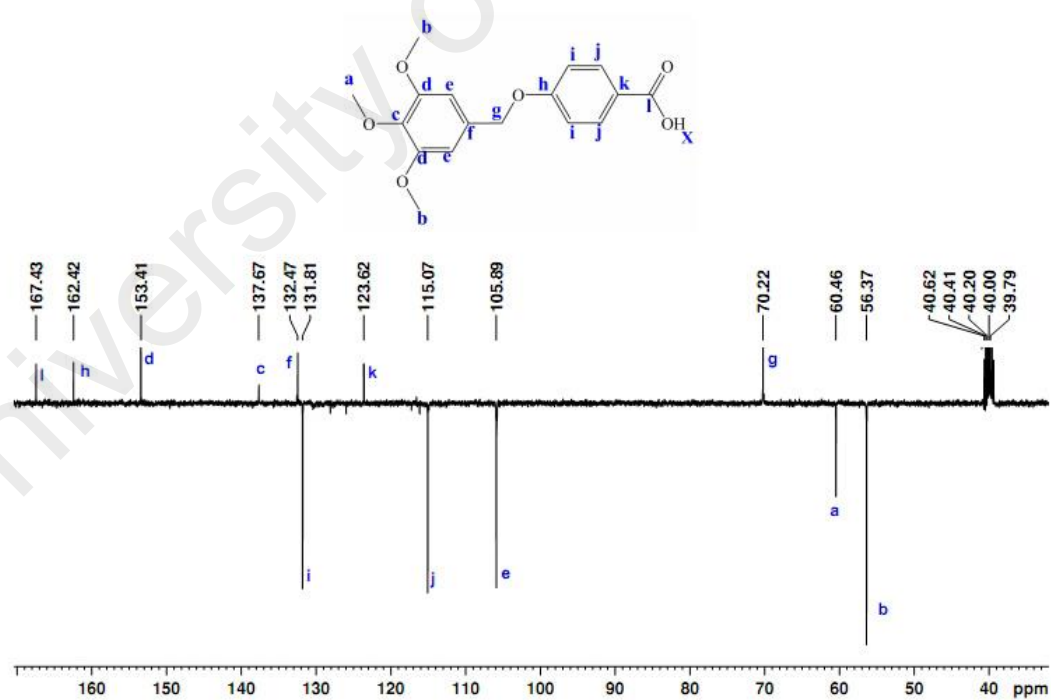
Figure A 68: ^{13}C spectrum (DMSO-d$_6$, 100MHz) of 5.12

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Figure A 69: ¹H spectrum (DMSO-d₆, 400MHz) of 5.12b

Figure A 70: ¹³C spectrum (DMSO-d₆, 100MHz) of 5.12b
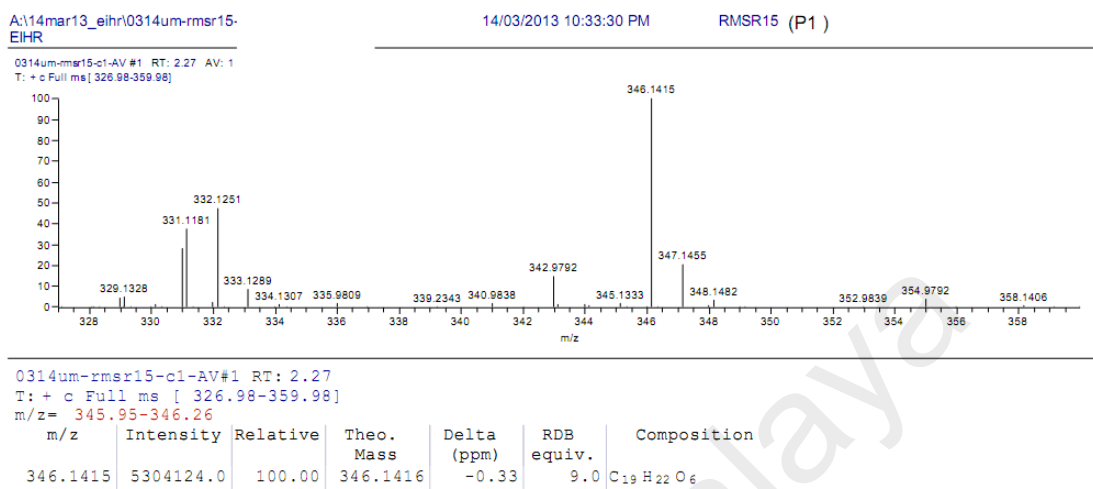
Figure B 1. HREIMS spectrum of 2.5
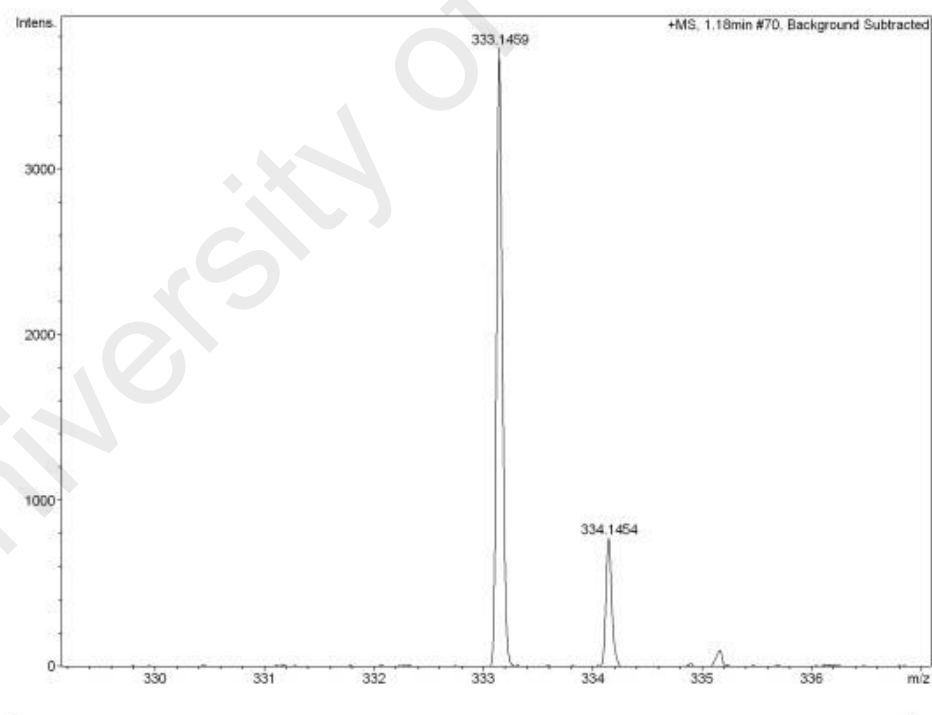
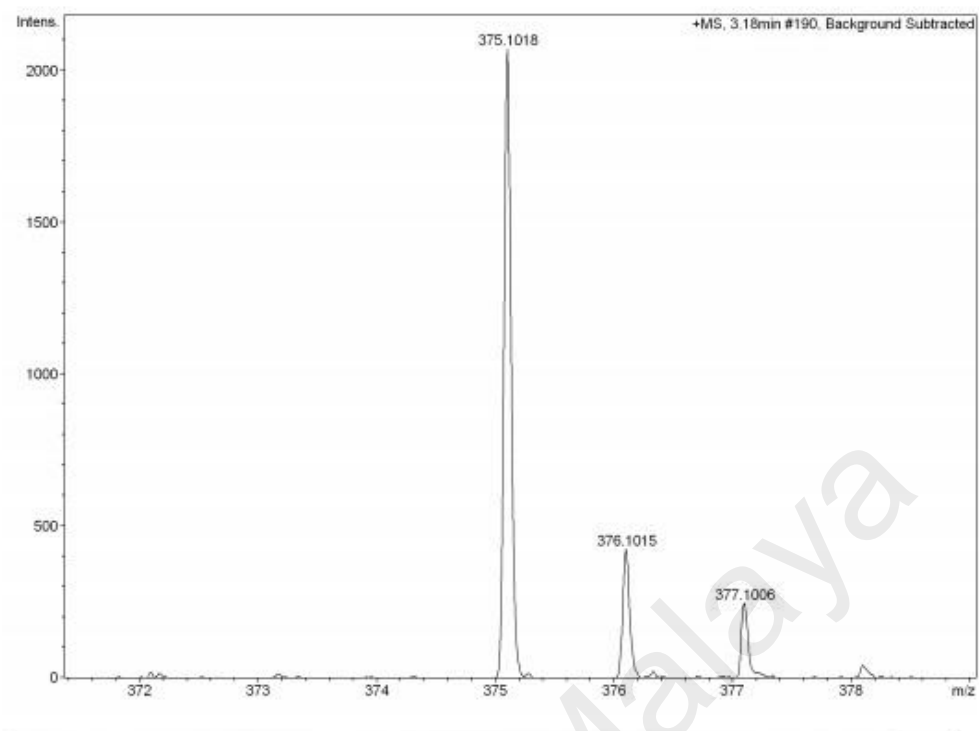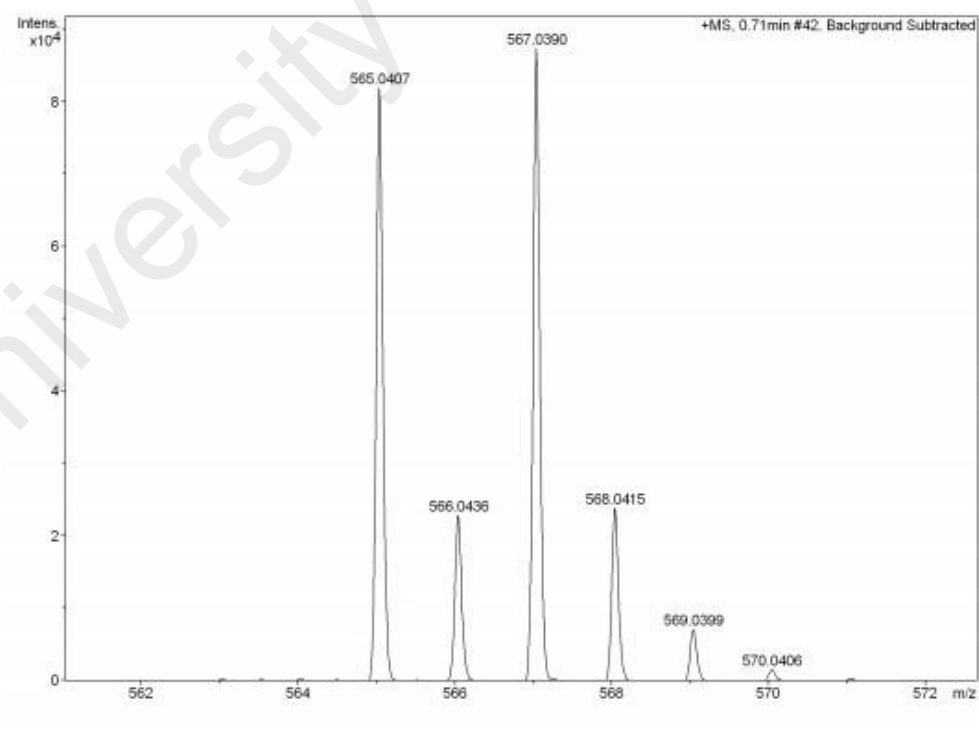

Figure B 2. HREIMS spectrum of 2.6
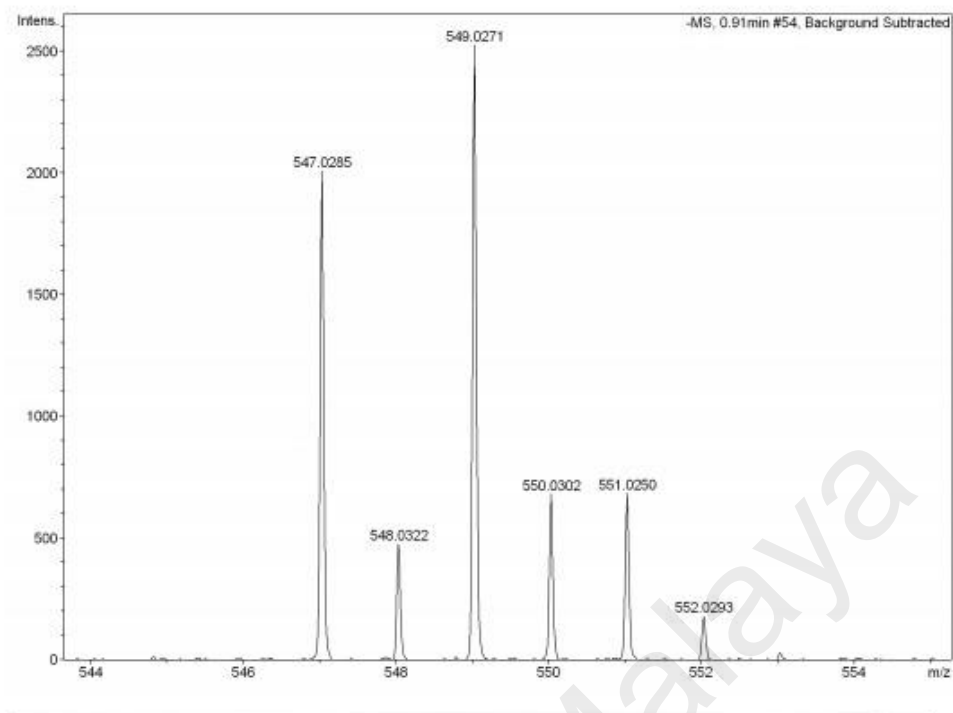
Figure B 3. HREIMS spectrum of 3.1
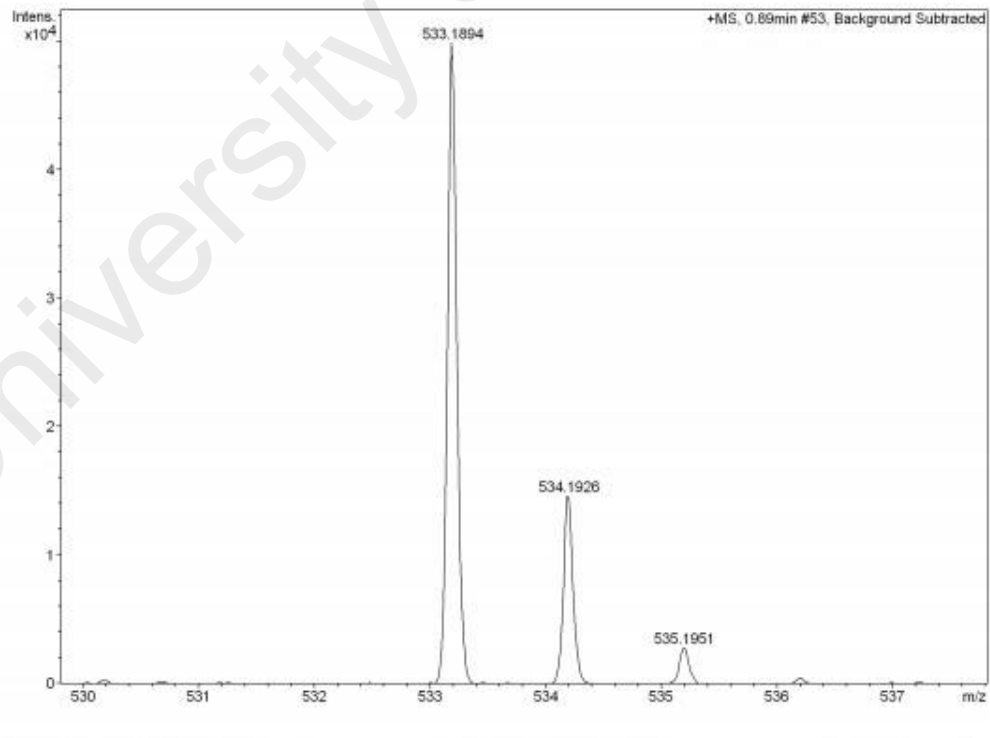
Figure B 4. HREIMS spectrum of 3.6
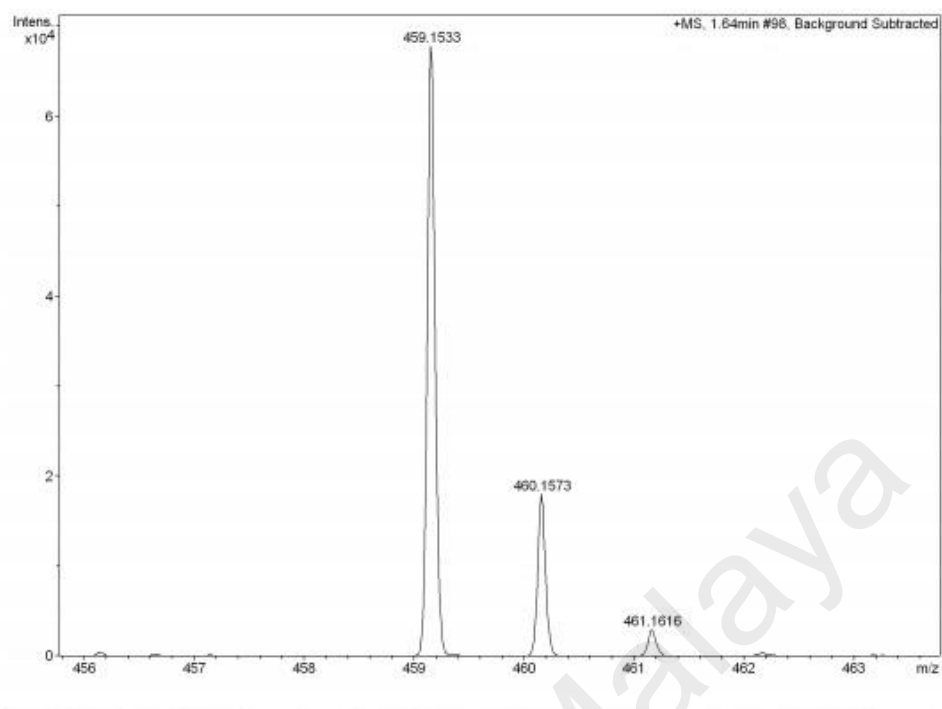
Figure B 5. HREIMS spectrum of 3.7
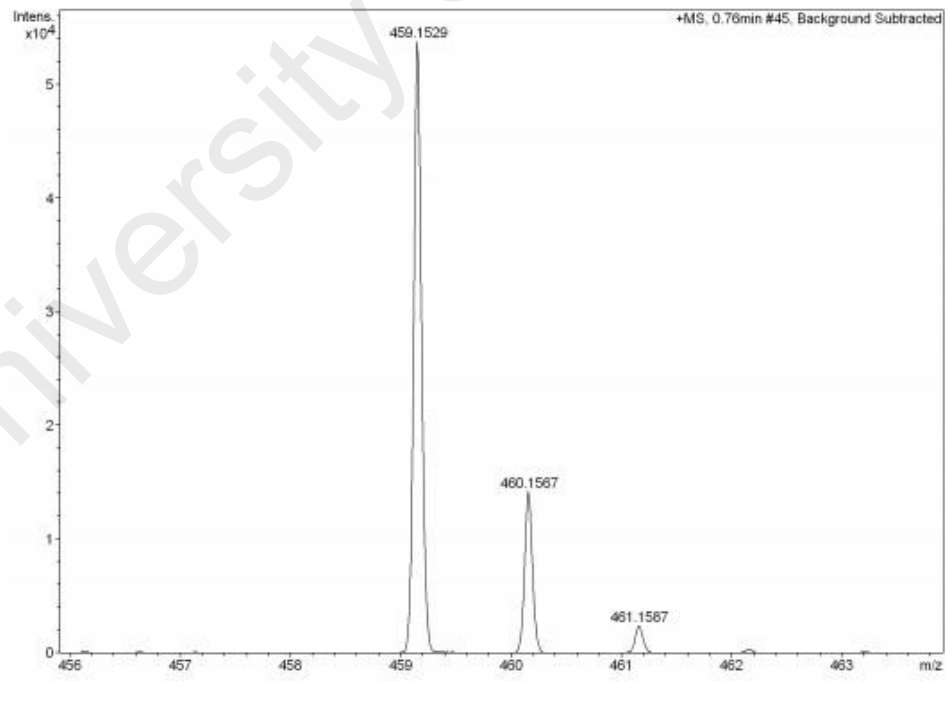
Figure B 6. HREIMS spectrum of 3.9

Figure B 7. HREIMS spectrum of 4.1

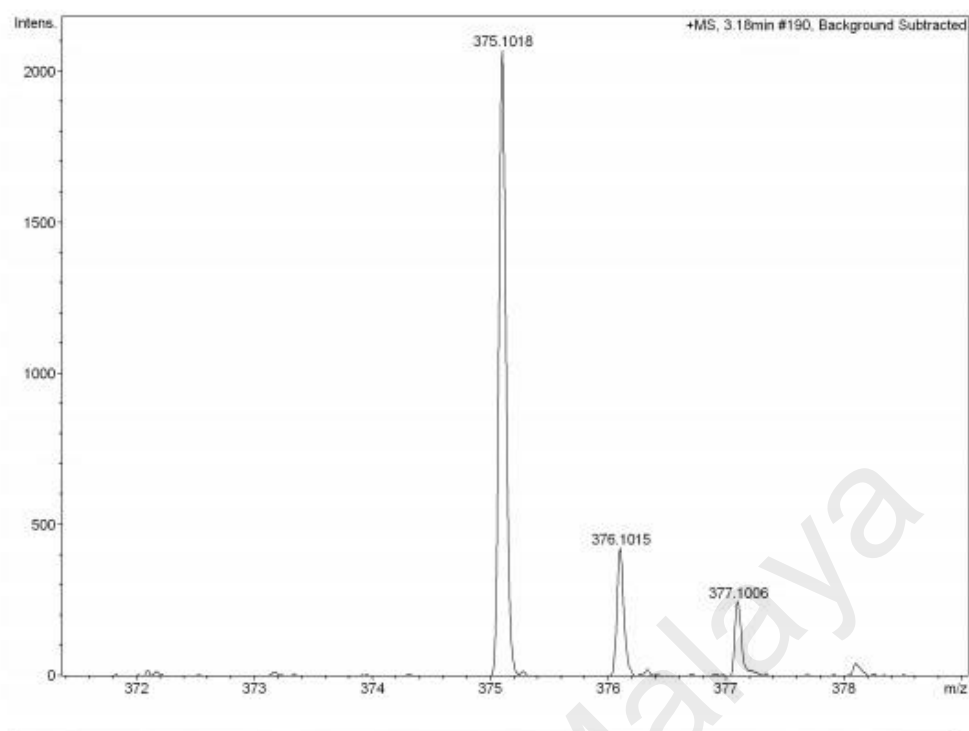**Figure B 8. HREIMS spectrum of 4.2**

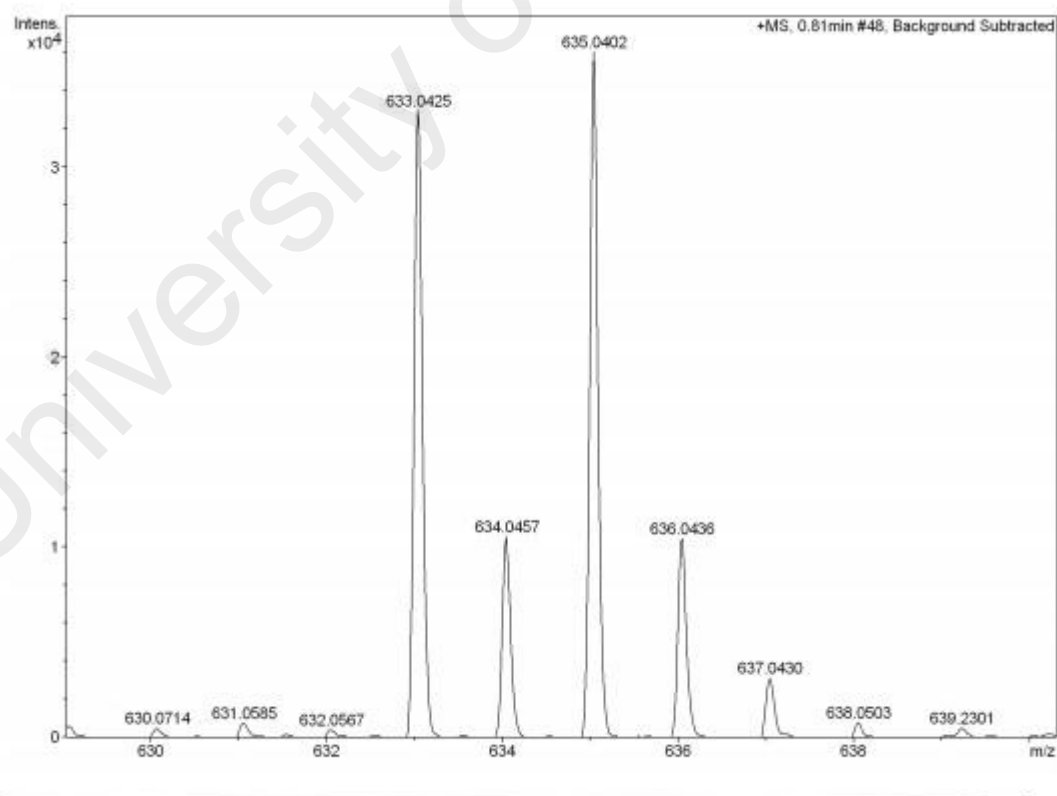**Figure B 9. HREIMS spectrum of 4.3**

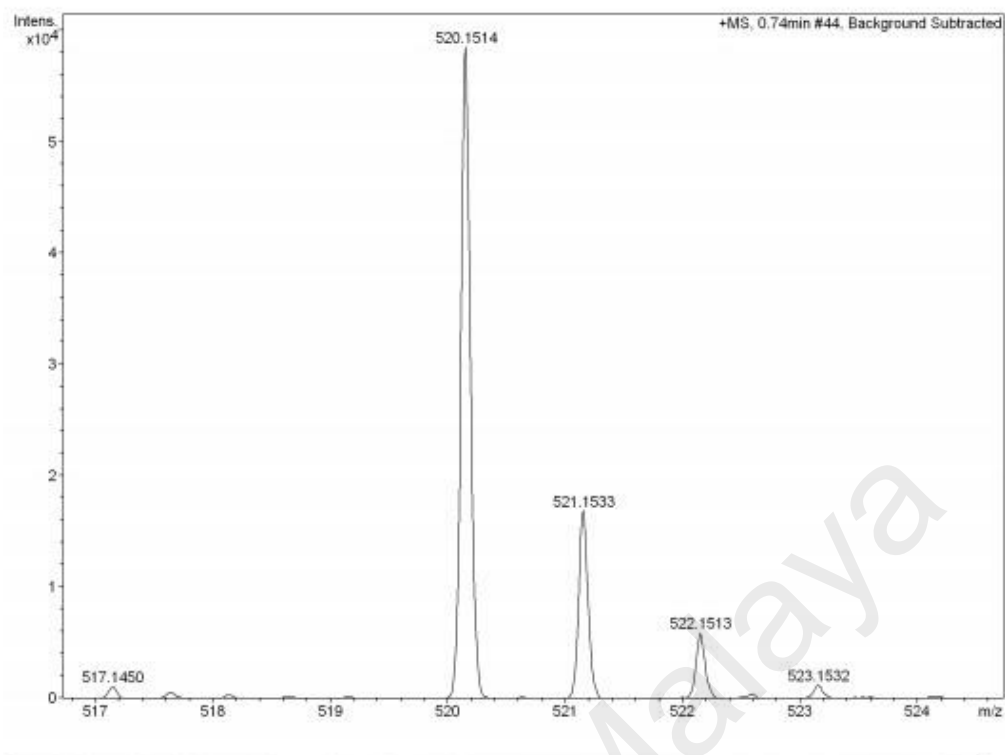**Figure B 10. HREIMS spectrum of 4.4**

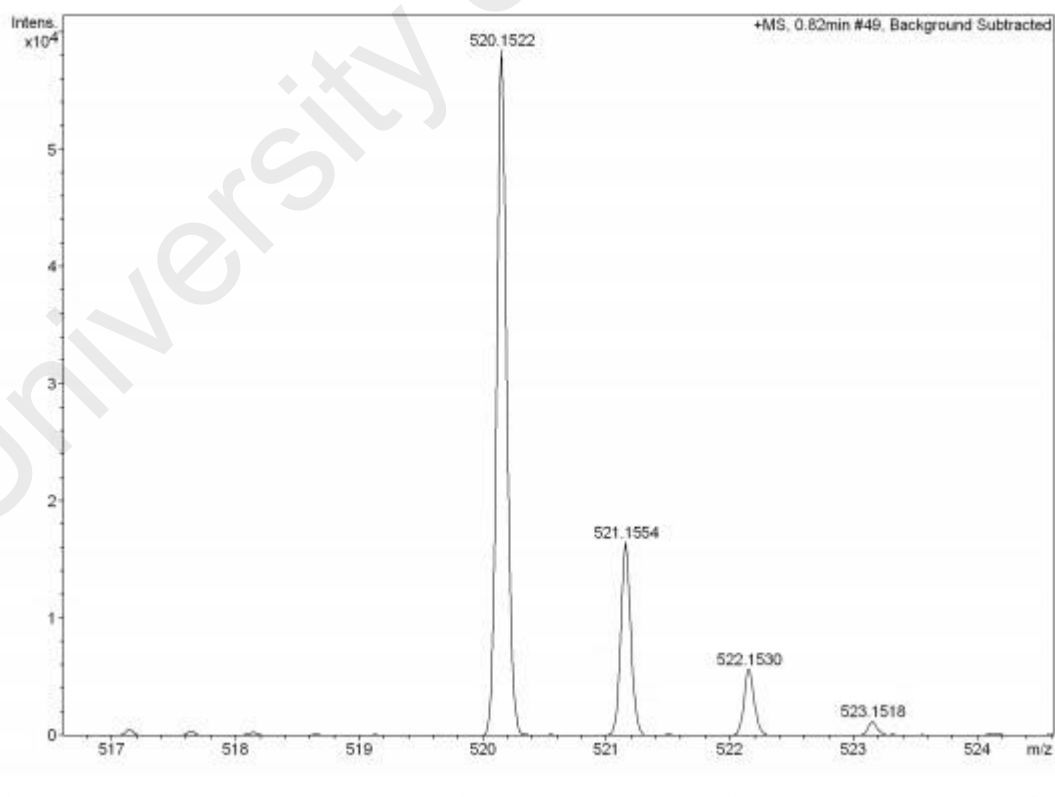**Figure B 11. HREIMS spectrum of 5.2**
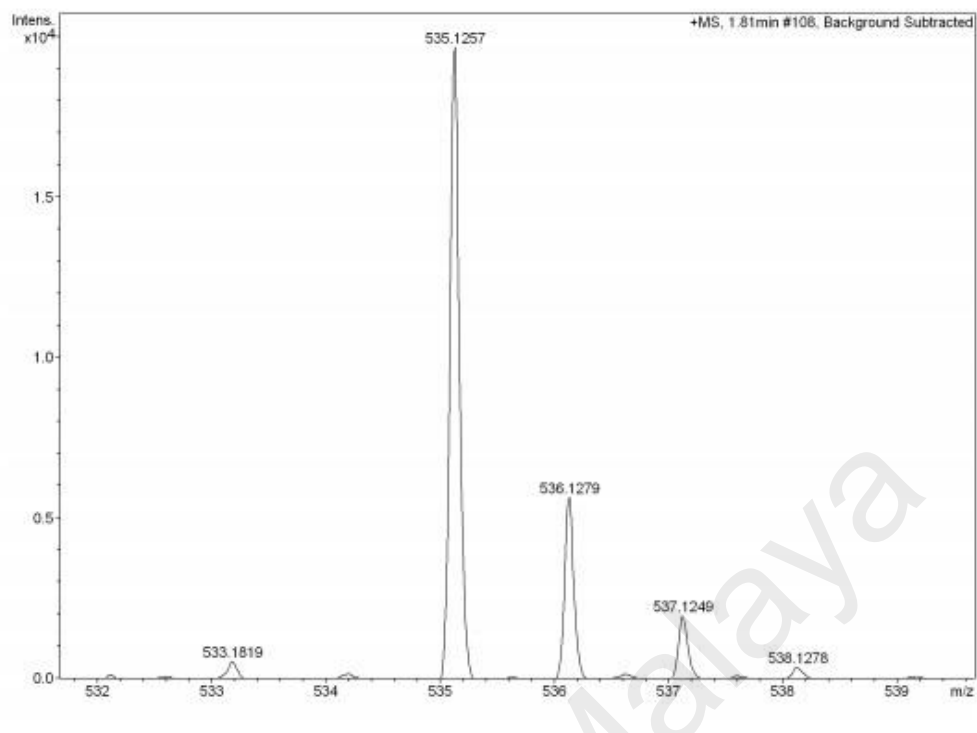

Figure B 12. HREIMS spectrum of 5.3
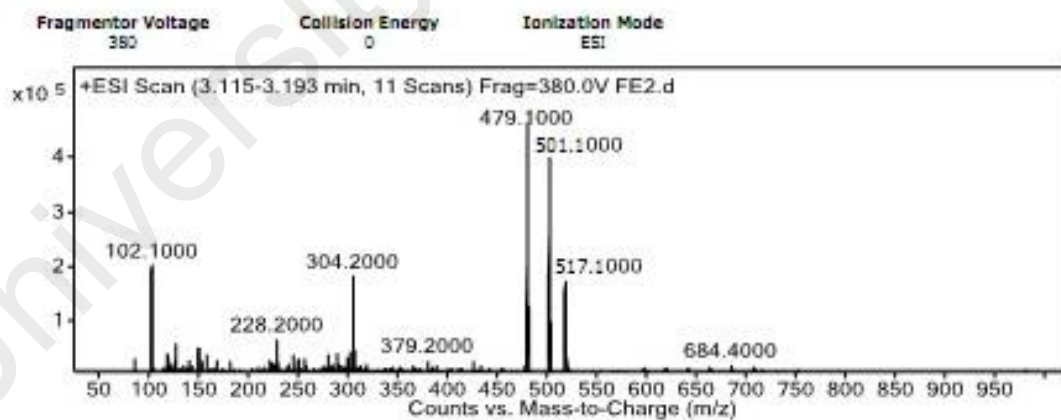
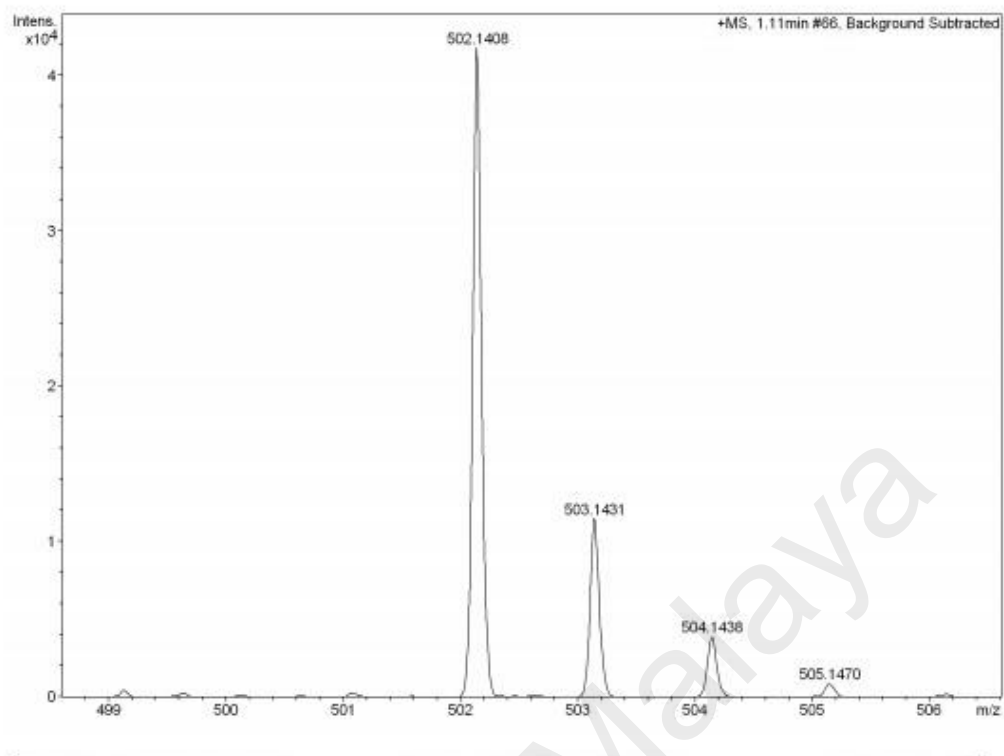**Figure B 13. HREIMS spectrum of 5.6**

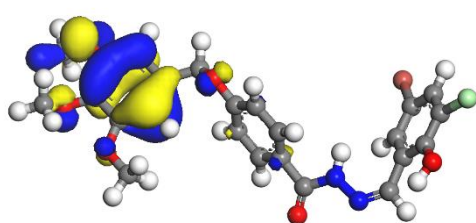**Figure B 14. MS spectrum of 5.12b**

Figure B 15. HREIMS spectrum of 5.8
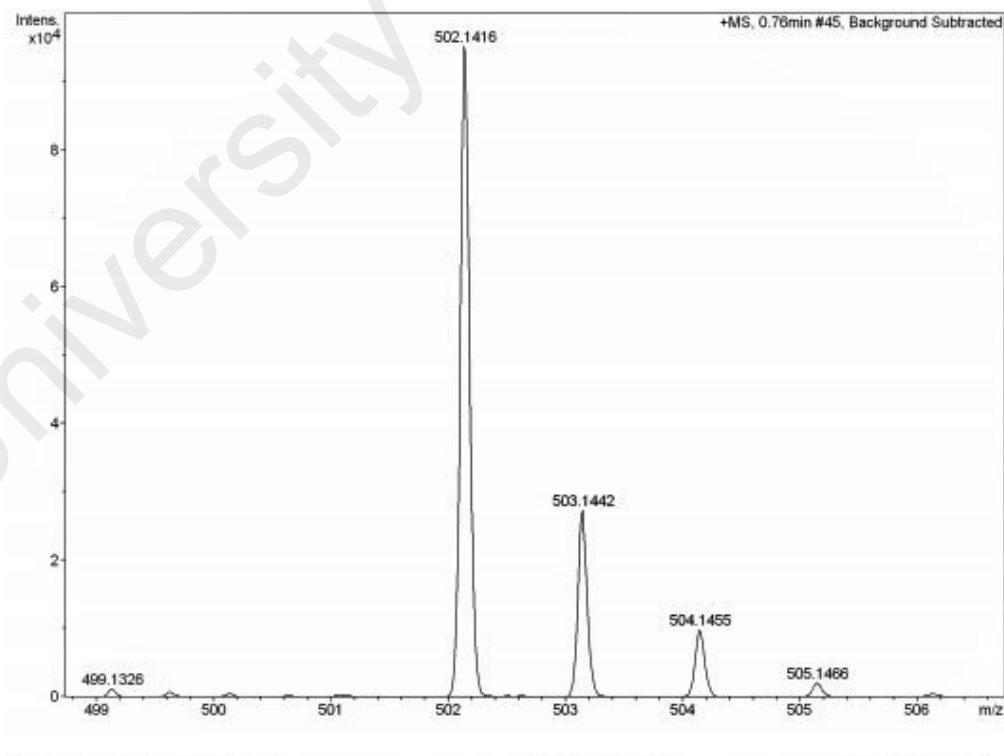
Figure B 16. HREIMS spectrum of 5.9

Figure C 1: Compound 3.1
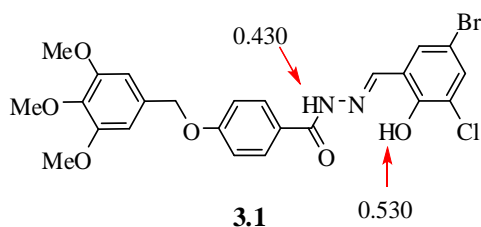
HOMO   LUMO
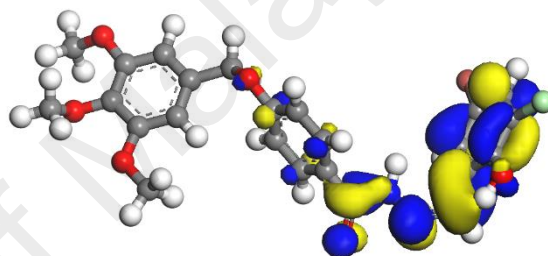
```
Energy of Highest Occupied Molecular Orbital: -0.19427Ha   -5.286eV

Energy of Lowest Unoccupied Molecular Orbital: -0.10021Ha   -2.727eV

Energy components:

    Sum of atomic energies =    -4510.8388518Ha

               Kinetic =     -14.5517906Ha

          Electrostatic =      -2.5990702Ha

    Exchange-correlation =       4.0472479Ha

       Spin polarization =       2.7760162Ha

 Ef       -4521.166448Ha     -10.3275966Ha     5.21E-06      3.0m
15
```

```
df binding energy  -10.3275966Ha      -281.02832eV   -6480.794kcal/mol

Energy of Highest Occupied Molecular Orbital: -0.19711Ha   -5.364eV

Energy of Lowest Unoccupied Molecular Orbital: -0.10206Ha   -2.777eV
```

Figure C 2: Compound 3.2
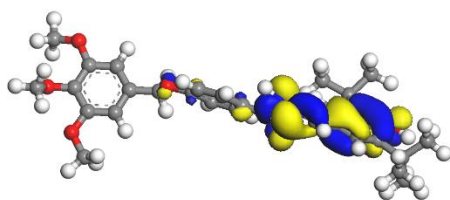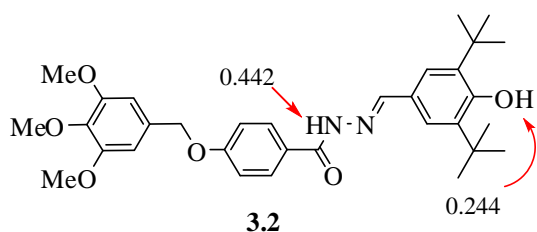
HOMO   LUMO

```
Energy of Highest Occupied Molecular Orbital:  -0.17295Ha    -4.706eV

Energy of Lowest Unoccupied Molecular Orbital: -0.06395Ha    -1.740eV

Energy components:

   Sum of atomic energies =    -1788.3255244Ha

               Kinetic =        -17.0112602Ha

           Electrostatic =       -6.7550403Ha

      Exchange-correlation =      5.3056159Ha

         Spin polarization =      4.2220584Ha

   Ef        -1802.564151Ha      -14.2386262Ha     3.98E-06     3.8m
15
```

```
df binding energy -14.2386262Ha      -387.45290eV    -8935.051kcal/mol

Energy of Highest Occupied Molecular Orbital:  -0.17180Ha    -4.675eV

Energy of Lowest Unoccupied Molecular Orbital: -0.05956Ha    -1.621eV
```

Figure C 3: Compound 3.3
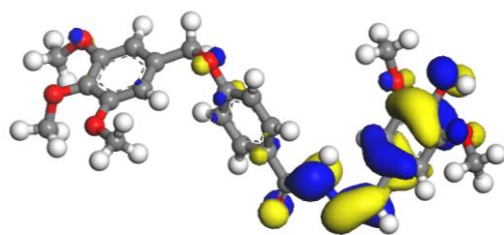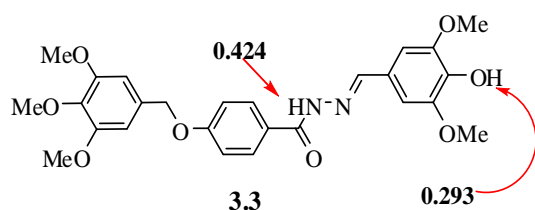
HOMO   LUMO

```
Energy of Highest Occupied Molecular Orbital: -0.18312Ha    -4.983eV

Energy of Lowest Unoccupied Molecular Orbital: -0.07937Ha    -2.160eV

Energy components:

    Sum of atomic energies =     -1705.5942483Ha

                  Kinetic =        -16.3489301Ha

             Electrostatic =         -3.2982634Ha

      Exchange-correlation =          4.4585263Ha

         Spin polarization =          3.4592295Ha
```

```
   Ef    -1717.323686Ha      -11.7294377Ha      4.34E-06      2.6m
15
```
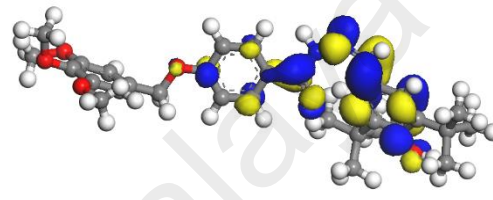
```
  Energy of Highest Occupied Molecular Orbital: -0.18302Ha    -4.983eV

  Energy of Lowest Unoccupied Molecular Orbital: -0.07422Ha    -2.020eV
```

Figure C 4: Compound 3.6
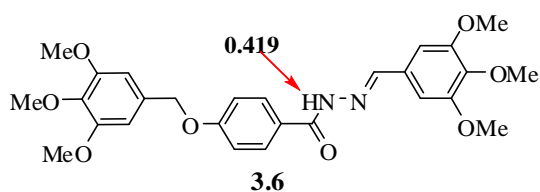
HOMO   LUMO
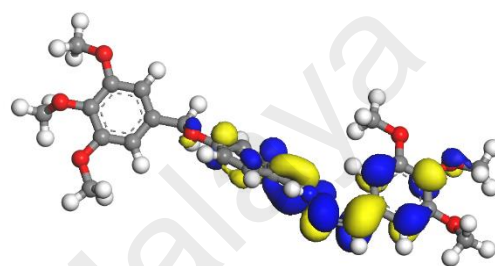
```
Energy of Highest Occupied Molecular Orbital:  -0.16996Ha    -4.623eV

Energy of Lowest Unoccupied Molecular Orbital: -0.06561Ha    -1.780eV

Energy components:

    Sum of atomic energies =     -1744.3828292Ha

                 Kinetic =         -15.8927548Ha

             Electrostatic =         -4.5085454Ha

      Exchange-correlation =          4.6196857Ha

        Spin polarization =          3.6061237Ha


       Total Energy            Binding E                  Time    Iter

Ef   -1756.558320Ha         -12.1754907Ha               51.4m     10
```

Figure C 5: Compound 3.7

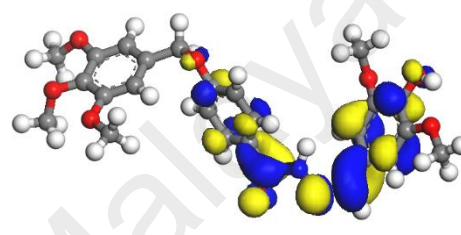HOMO   LUMO
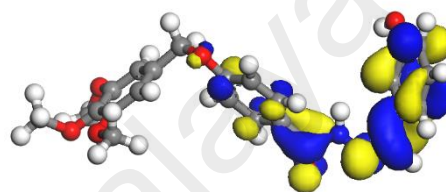
```
Energy of Highest Occupied Molecular Orbital:  -0.21186Ha    -5.765eV

Energy of Lowest Unoccupied Molecular Orbital: -0.07923Ha    -2.156eV


Energy components:

  Sum of atomic energies   =    -1478.0168769Ha

                Kinetic =       -14.4491047Ha

            Electrostatic =      -2.9756409Ha

      Exchange-correlation =     3.9387806Ha

        Spin polarization =      3.0469047Ha

  Ef      -1488.455937Ha      -10.4390603Ha      4.21E-06      2.1m
15
```
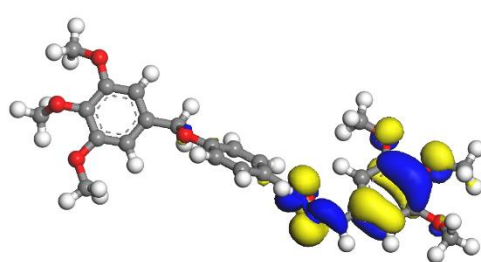
```
Energy of Highest Occupied Molecular Orbital:  -0.19237Ha    -5.235eV

Energy of Lowest Unoccupied Molecular Orbital: -0.07919Ha    -2.155eV
```

Figure C 6: Compound 3.8
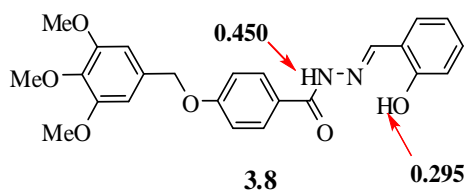
HOMO   LUMO
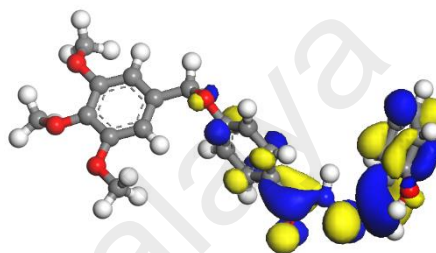
Energy of Highest Occupied Molecular Orbital: -0.219945Ha   -5.985eV

Energy of Lowest Unoccupied Molecular Orbital: -0.085148Ha   -2.317eV

Energy components:

    Sum of atomic energies =   -1478.0168769Ha

                Kinetic =   -14.8586565Ha

   Electrostatic =   -2.5643173Ha

    Exchange-correlation =   3.9414217Ha

        Spin polarization =   3.0469047Ha

        Total Energy   Binding E   Time
Iter

  Ef   -1488.451524Ha   -10.4346475Ha   34.2m   10

Energy of Highest Occupied Molecular Orbital: -0.17894Ha   -5.075eV

Energy of Lowest Unoccupied Molecular Orbital: -0.08325Ha   -2.267eV

Figure C 7: Compound 3.9
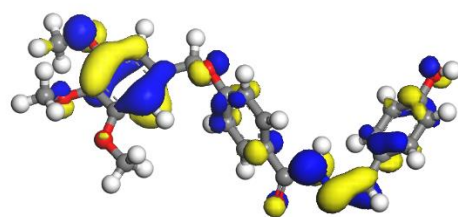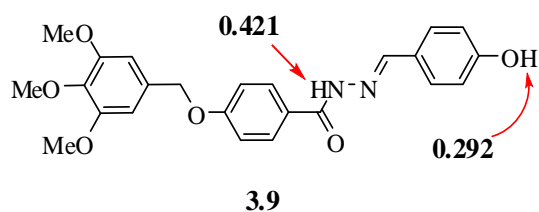
HOMO   LUMO

Energy of Highest Occupied Molecular Orbital: -0.18652Ha   -5.075eV

Energy of Lowest Unoccupied Molecular Orbital: -0.07613Ha   -2.072eV

Energy components:

    Sum of atomic energies =   -1478.0168769Ha
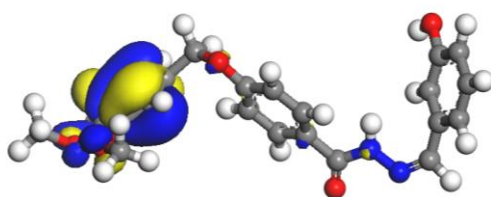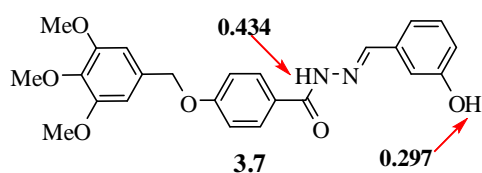
              Kinetic =   -14.3137854Ha

         Electrostatic =   -3.1083113Ha
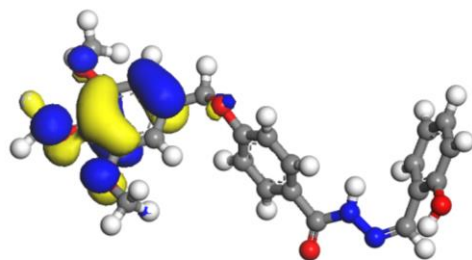
   Exchange-correlation =   3.9388519Ha

     Spin polarization =   3.0469047Ha

Ef   -1488.453217Ha   -10.4363402Ha   4.24E-06   2.3m
15

Energy of Highest Occupied Molecular Orbital: -0.17911Ha   -4.874eV

Energy of Lowest Unoccupied Molecular Orbital: -0.076256Ha   -2.072eV

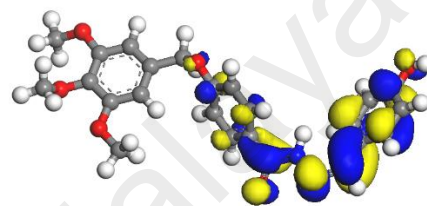**Figure C 8: Compound 4.2**
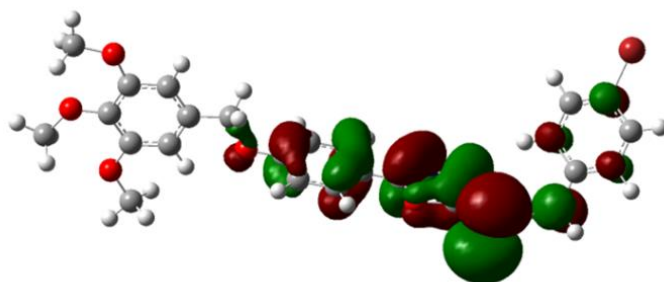
4.2

```
Zero-point correction=                              0.451221
(Hartree/Particle)
 Thermal correction to Energy=                      0.568231
 Thermal correction to Enthalpy=                    0.569124
 Thermal correction to Gibbs Free Energy=           0.562234
 Sum of electronic and zero-point Energies=        -4135.975609
 Sum of electronic and thermal Energies=           -4135.978015
 Sum of electronic and thermal Enthalpies=         -4135.954114
 Sum of electronic and thermal Free Energies=      -4135.976213

          E (Thermal)             CV                S
           KCal/Mol        Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      298.413            118.120           225.451
```

Figure C 9: Compound 4.2-r1

4.2-r1

```
Zero-point correction=                              0.435813
(Hartree/Particle)
 Thermal correction to Energy=                      0.468444
 Thermal correction to Enthalpy=                    0.469388
 Thermal correction to Gibbs Free Energy=           0.362148
 Sum of electronic and zero-point Energies=        -4135.421677
 Sum of electronic and thermal Energies=           -4135.389047
 Sum of electronic and thermal Enthalpies=         -4135.388103
 Sum of electronic and thermal Free Energies=      -4135.495343

          E (Thermal)             CV                S
           KCal/Mol        Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      293.953            119.790           225.706
```

Figure C 10: Compound 4.2-r

4.2-r2

```
Zero-point correction=                                0.435309
(Hartree/Particle)
 Thermal correction to Energy=                        0.467942
 Thermal correction to Enthalpy=                      0.468887
 Thermal correction to Gibbs Free Energy=             0.363327
 Sum of electronic and zero-point Energies=          -4135.420798
 Sum of electronic and thermal Energies=             -4135.388164
 Sum of electronic and thermal Enthalpies=           -4135.387220
 Sum of electronic and thermal Free Energies=        -4135.492779

            E (Thermal)             CV                  S
            KCal/Mol          Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      293.638               120.466           222.169
```
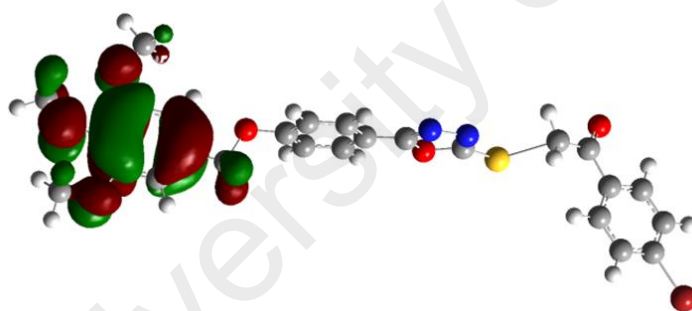
Figure C 11: Compound 4.3

4.3

```
Zero-point correction=                                0.482112
(Hartree/Particle)
 Thermal correction to Energy=                        0.501234
 Thermal correction to Enthalpy=                      0.503026
 Thermal correction to Gibbs Free Energy=             0.403978
 Sum of electronic and zero-point Energies=          -4420.897388
 Sum of electronic and thermal Energies=             -4420.876376
 Sum of electronic and thermal Enthalpies=           -4420.827331
 Sum of electronic and thermal Free Energies=        -4420.873271

            E (Thermal)             CV                  S
            KCal/Mol          Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      289.137               119.411           224.432
```

Figure C 12: Compound 4.3-r1

4.3-r1

```
Zero-point correction=                              0.426512
(Hartree/Particle)
 Thermal correction to Energy=                      0.459224
 Thermal correction to Enthalpy=                    0.460168
 Thermal correction to Gibbs Free Energy=           0.353528
 Sum of electronic and zero-point Energies=        -4420.282688
 Sum of electronic and thermal Energies=           -4420.249976
 Sum of electronic and thermal Enthalpies=         -4420.249031
 Sum of electronic and thermal Free Energies=      -4420.355671

                    E (Thermal)            CV               S
                     KCal/Mol        Cal/Mol-Kelvin   Cal/Mol-Kelvin
 Total               288.167            119.456           224.443
```
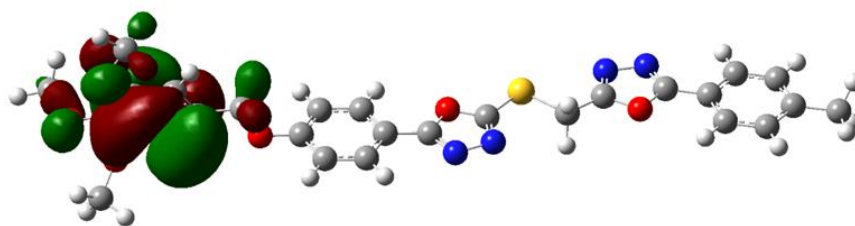
Figure C 13: Compound 4.3-r2

4.3-r2

```
Zero-point correction=                              0.426439
(Hartree/Particle)
 Thermal correction to Energy=                      0.459121
 Thermal correction to Enthalpy=                    0.460065
 Thermal correction to Gibbs Free Energy=           0.353179
 Sum of electronic and zero-point Energies=        -4420.291536
 Sum of electronic and thermal Energies=           -4420.258855
 Sum of electronic and thermal Enthalpies=         -4420.257910
 Sum of electronic and thermal Free Energies=      -4420.364797

                    E (Thermal)            CV               S
                     KCal/Mol        Cal/Mol-Kelvin   Cal/Mol-Kelvin
 Total               288.103            119.679           224.961
```

Figure C 14: Compound 4.6

4.6

```
Zero-point correction=                            0.502455
(Hartree/Particle)
 Thermal correction to Energy=                    0.539401
 Thermal correction to Enthalpy=                  0.540345
 Thermal correction to Gibbs Free Energy=         0.422612
 Sum of electronic and zero-point Energies=       -2150.614196
 Sum of electronic and thermal Energies=          -2150.577251
 Sum of electronic and thermal Enthalpies=        -2150.576306
 Sum of electronic and thermal Free Energies=     -2150.694039

            E (Thermal)             CV                  S
             KCal/Mol         Cal/Mol-Kelvin     Cal/Mol-Kelvin
 Total         338.479             134.573            247.789
```
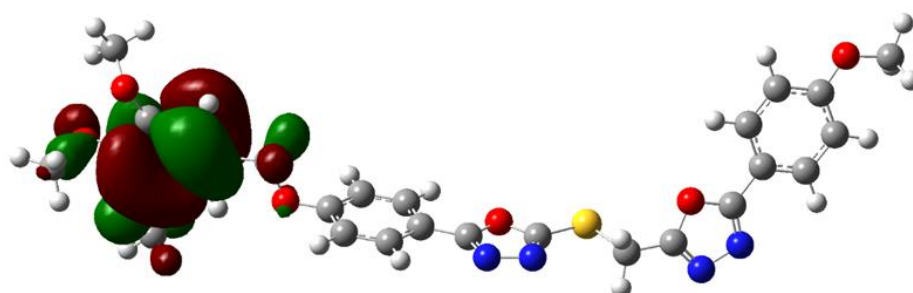
Figure C 15: Compound 4.6-r1

4.6-r1

```
Zero-point correction=                            0.488769
(Hartree/Particle)
 Thermal correction to Energy=                    0.525582
 Thermal correction to Enthalpy=                  0.526526
 Thermal correction to Gibbs Free Energy=         0.409200
 Sum of electronic and zero-point Energies=       -2149.986588
 Sum of electronic and thermal Energies=          -2149.949775
 Sum of electronic and thermal Enthalpies=        -2149.948831
 Sum of electronic and thermal Free Energies=     -2150.066157

            E (Thermal)             CV                  S
             KCal/Mol         Cal/Mol-Kelvin     Cal/Mol-Kelvin
 Total         329.807             134.908            246.934
```

Figure C 16: Compound 4.7

4.7

```
Zero-point correction=                               0.508304
(Hartree/Particle)
 Thermal correction to Energy=                       0.545626
 Thermal correction to Enthalpy=                     0.546571
 Thermal correction to Gibbs Free Energy=            0.429944
 Sum of electronic and zero-point Energies=         -2225.816610
 Sum of electronic and thermal Energies=            -2225.779288
 Sum of electronic and thermal Enthalpies=          -2225.778343
 Sum of electronic and thermal Free Energies=       -2225.894971

          E (Thermal)            CV                S
           KCal/Mol         Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      342.386             137.567           245.462
```

Figure C 17: Compound 4.7-r1

4.7-r1

```
Zero-point correction=                               0.454447
(Hartree/Particle)
 Thermal correction to Energy=                       0.531747
 Thermal correction to Enthalpy=                     0.532692
 Thermal correction to Gibbs Free Energy=            0.415957
 Sum of electronic and zero-point Energies=         -2225.188388
 Sum of electronic and thermal Energies=            -2225.151088
 Sum of electronic and thermal Enthalpies=          -2225.150144
 Sum of electronic and thermal Free Energies=       -2225.266879

          E (Thermal)            CV                S
           KCal/Mol         Cal/Mol-Kelvin    Cal/Mol-Kelvin
 Total      333.677             137.929           245.689
```

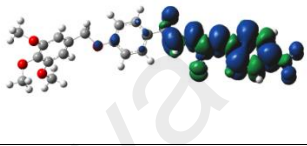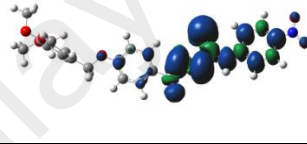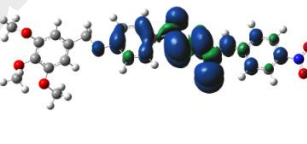**Figure C 18: Calculation properties for compound 5.6 and its radicals**

Entry	Et (hartree)	ZPE (hartree)	BDE (kcal/mol)	Spin Density	Density distribution of the SOMO for radicals of compound **5.6**
5.6	-2060.70073	0.50257	-	-	-
5.6-r1	-2071.52418	0.48896	277.99	0.3946	
5.6-r2	-2071.96206	0.44853	279.79	0.4444	
5.6-r3	-2071.53218	0.45097	280.06	0.7894	

Figure D 1. Comparison DPPH radical scavenging activity plots in μg/mL and μM concentration of the hydrazone compounds 3.1-3.9
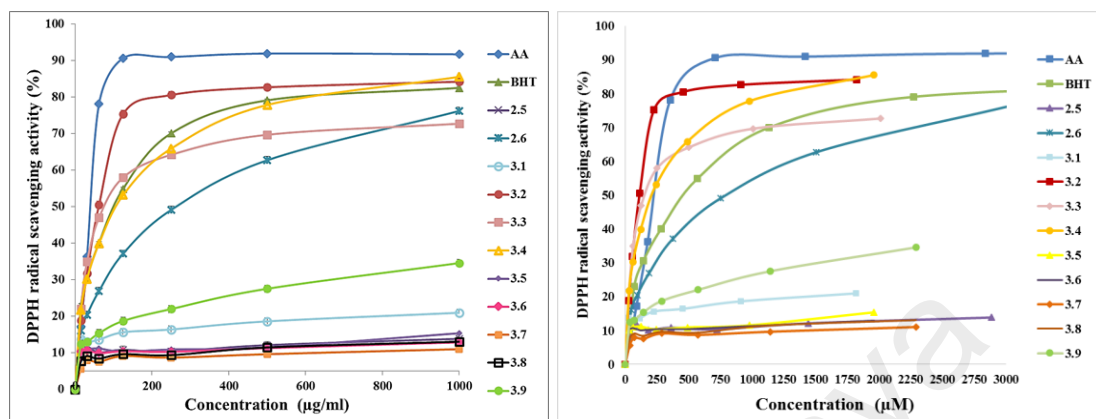

Figure D 2. Comparison DPPH radical scavenging activity plots in μg/mL and μM concentration of 1,3,4-oxadiazole compounds 4.1-4.7
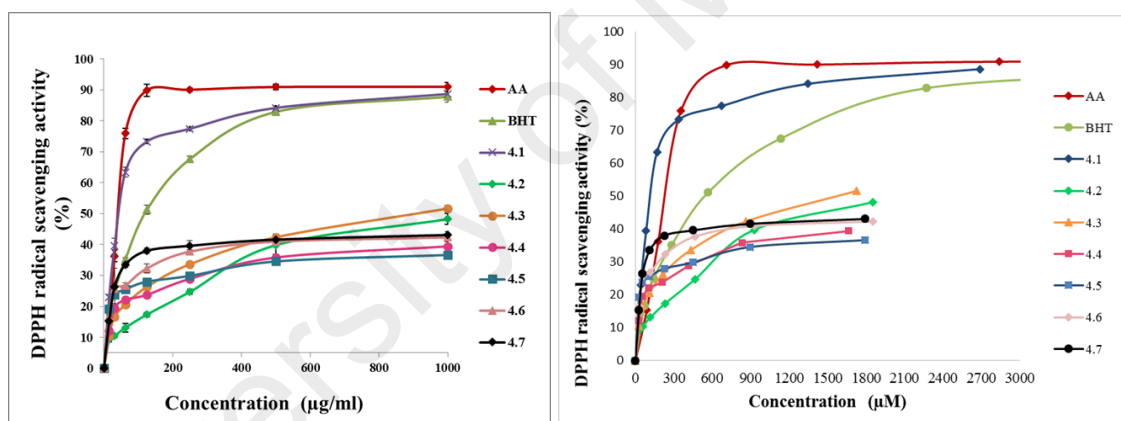

Figure D 3. Comparison DPPH radical scavenging activity plots in μg/mL and μM concentration of thiosemicarbazides 5.1-5.6 and 1,2,4-triazoles 5.7-5.11

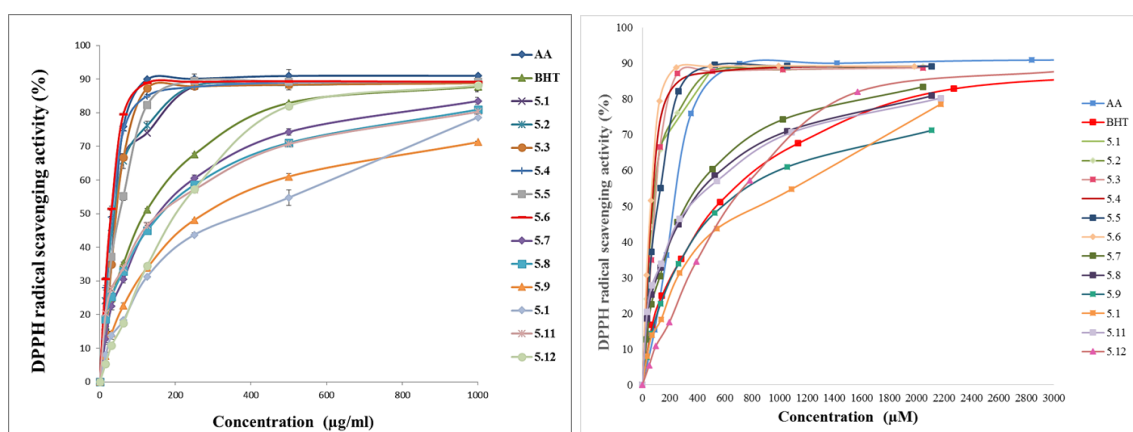**Figure D 4. Comparison IC$_{50}$ value of the active compounds in concentration µg/mL and µM**