T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF BIOMOLECULAR SCIENCES

SYNTHESIS OF ISOTHIOCYANATE DERIVATIVES AND BIOLOGICAL ACTIVITIES OF H_2S RELEASING COMPOUNDS

MASTER OF SCIENCE THESIS

YAKUP BERKAY YILMAZ

Thesis Supervisor

PROF. DR. TUĞBA TÜMER

Second Thesis Supervisor

PROF. DR. MEHMET AY

ÇANAKKALE – 2022

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PLAGIARISM DECLARATION PAGE

I declare that all the information and results offered in visual, audio and, written form are obtained by myself observing the academic and ethical rules. Moreover, all other results and information referred to in the thesis but not specific to this study are cited.

Yakup Berkay YILMAZ

24/01/2022

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Çanakkale, January 2022

ÖZET

İZOTİYOSİYANAT TÜREVLERİNİN SENTEZLENMELERİ VE H_2S SALAN BİLEŞİKLERİN BİYOLOJİK AKTİVİTELERİ

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İnflamasyon, çeşitli patojenler ve hücre hasarı dahil olmak üzere zararlı uyaranları ortadan kaldırmak için çeşitli yolaklar ve uyarı işlemleri içeren bir süreçtir. Uzun süreli inflamasyon, kronik inflamasyon olarak adlandırılır ve kanser dahil çok sayıda hastalıkla ilişkilidir. Steroid olmayan anti-inflamatuar ilaçlar (NSAID'ler), çeşitli anti-inflamatuar etkinlikleri ile, çoğunlukla siklooksijenaz (COX) yolakları üzerindeki etkileri nedeniyle inflamasyona karşı iyi tanınan ve yaygın olarak kullanılan ilaç sınıfıdır. Son birkaç yıldır, NSAID'ler antikanser etkinlikleri ile de dikkat çekmiştir. Yaygın kullanımlarının yanı sıra, NSAID'lerin uzun süreli kullanımı, kardiyovasküler bozukluklar ve gastrointestinal hasar dahil olmak üzere birçok rahatsızlığa yol açmaktadır. Bu istenmeyen etkilerin üstesinden gelmek için yüksek yeterliliğe ve daha az yan etkiye sahip yeni bileşiklere ihtiyaç vardır.

Hidrojen sülfür (H_2S), nitrik oksit (NO) ve karbon monoksitten (CO) sonra gelen üçüncü endojen gaz aktarıcı (gazotransmitter) moleküldür. Memeli hücrelerinde homeostazın korunması ve sitoproteksiyon dahil olmak üzere çeşitli fizyolojik özelliklere sahiptir. H_2S, fizyolojik özelliklerinin yanı sıra kanser hücresinin hayatta kalmasına karşı da etkilidir. H_2S salan NSAID'lerin, anti-inflamatuar ve antikanser etkinliklerinden dolayı kanserin önlenmesi için güçlü ajanlar olduğu düşünülmektedir. Öte yandan, H_2S salmalarıyla da bilinen izotiyosiyanatlar (ITC'ler), doğal olarak turpgillerde bulunur ve antikanser etkinlikleri ile bilinir. Bu çalışmada, H_2S salan bileşikler (**S1**-**S19**) ve ITC türevleri (**I1**-**I3**), antikanser etkinlikleri için denenmiştir. Buna göre, tüm H_2S salan bileşikler arasında, **S6** ve **S8**, 100 µM'den düşük IC_{50} değerleri ile MCF-7 meme ve PC-3 prostat kanseri hücre

hatlarında umut vadeden antiproliferatif etkinlik göstermiştir. Bu süreçte, ITC türevleri sentezlenmiştir ve yapıları çeşitli spektrofotometrik tekniklerle doğrulanmıştır. Daha sonra, bu bileşikler de antiproliferatif etkinlikleri açısından denenmişler ve **I1** ve **I2** bileşikleri, 100 µM'da MCF-7, PC-3 ve HT-29 hücre hatlarında umut vadeden antiproliferatif etkinlik göstermiştir. **I1** ve **I2** bileşikleri için IC_{50} değerleri sırasıyla, PC-3'te 70,7 µM ve 39,9 µM, MCF-7'de sırasıyla 52,2 µM ve 48,1 µM olarak bulunmuştur. HT-29 hücrelerinde sadece I2 bileşiği etken bulunmuştur ve IC_{50} değeri 39 µM olarak hesaplanmıştır. **I1** bileşiği apoptoz süreciyle ilişkili Bax/Bcl-2 gen ekspresyonu oranını MCF-7 hücrelerinde 1,58 kat artırmıştır. Ayrıca, üç izotiyosiyanat türevinin tümü, 4 saatlik süreç boyunca tercih edilen salınım olan yavaş H_2S salınımı göstermiştir. Bu sonuçlar bilinen izotiyosiyanatlar (ITCler) olan ve kontrol grubu olarak kullanılan sülforafan ve MIC-1 ile uyumlu sonuçlar göstermiştir. Sonuç olarak, **S6**, **S8**, **I1** ve **I2** bileşikleri kanser tedavisi ve önlenmesi için umut vadeden antikanser ajanları olabilir.

Anahtar Kelimeler: İzotiyosiyanatlar, Hidrojen sülfür salan bileşikler, NSAIDler, Antikanser

ABSTRACT

SYNTHESIS OF ISOTHIOCYANATE DERIVATIVES AND BIOLOGICAL ACTIVITIES OF H_2S RELEASING COMPOUNDS

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Inflammation involves various pathways and signaling processes to eliminate detrimental stimuli including various pathogens and cell damage. Prolonged inflammation is referred to as chronic inflammation and is related to numerous diseases including cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) are well known and widely used drug class against inflammation due to their anti-inflammatory activities, mostly for their action on cyclooxygenase (COX) pathways. For the past few decades, NSAIDs have also drawn attention for their anticancer activities. Besides their widespread use, long-term use of NSAIDs leads to several disorders including cardiovascular disruptions and gastrointestinal damage. To overcome these undesired effects, novel compounds with high proficiency and fewer side effects are needed.

Hydrogen sulfide (H_2S) is considered as the third endogenous gasotransmitter molecule following nitric oxide (NO) and carbon monoxide (CO). It has various physiological properties in mammalian cells including maintenance of homeostasis and cytoprotection. Besides its physiological properties, H_2S is also effective against cancer cell survival. H_2S releasing NSAIDs are thought to be potent agents for the prevention of cancer due to their anti-inflammatory and anticancer activities. On the other hand, isothiocyanates (ITCs), which happen to release H_2S, are naturally found in cruciferous vegetables and are known for their anticancer activities. In this study, H_2S releasing compounds (**S1**-**S19**) and ITC derivatives (**I1**-**I3**) were tested for their anticancer activities. Accordingly, among all H_2S releasing compounds, **S6** and **S8** showed promising antiproliferative activity in MCF-7

breast and PC-3 prostate cancer cell lines with IC_{50} values lower than 100 µM. ITC derivatives were synthesized and their structures were confirmed with various spectrophotometric techniques. Then, they were also tested for their antiproliferative activities and compounds **I1** and **I2** showed promising growth inhibition in MCF-7, PC-3 and, HT-29 cell lines at 100 µM. IC_{50} values were found in PC-3 as 70.7 µM and 39.9 µM, in MCF-7 as 52.2 µM and 48.1 µM for **I1** and **I2**, respectively. In HT-29 cells, only compound **I2** was found effective and the IC_{50} value was 39 µM. Compound **I1** was able to increase Bax/Bcl-2 gene expression levels related to the apoptosis process by 1.58-fold in MCF-7 cells. Also, all three isothiocyanate derivatives showed preferable slow H_2S release as control ITC compounds sulforaphane and MIC-1 over 4 hours time period. Overall, compounds **S6**, **S8**, **I1** and, **I2** could be promising anticancer agents for the treatment or prevention of cancer.

Keywords: Isothiocyanates, Hydrogen sulfide releasing compounds, NSAIDs, Anticancer

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ABBREVIATIONS

NSAID	Non-steroidal Anti-inflammatory Drug
COX	Cyclooxygenase
H_2S	Hydrogen Sulfide
ITC	Isothiocyanate
SFN	Sulforaphane
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
PBS	Phosphate-Buffered Saline
SRB	Sulforhodamine B
TP	Thiophosgene
NBS	*N*-bromosuccinimide
DMF	Dimethylformamide
Et_3N	Triethylamine
THF	Tetrahydrofuran
NMP	1-Methyl-2-pyrrolidinone
$Ni(CN)_2$	Nickel(II) Cyanide
$Zn(OAc)_2$	Zinc Acetate
Na_2S	Sodium Sulfide
$FeCl_3$	Iron(III) Chloride
APE	2-(4-Aminophenyl)ethan-1-ol
APE-Br	2-(4-Amino-3-bromophenyl)ethan-1-ol
APE-CN	2-Amino-5-(2-hydroxyethyl)benzonitrile
Na_2SO_4	Sodium Sulfate
MCF-7	Human Breast Cancer Cell Line
PC-3	Human Prostate Cancer Cell Line
HT-29	Human Colon Cancer Cell Line
HUVEC	Human Healthy Endothelial Cell Line
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2

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CHAPTER I
INTRODUCTION

One of the most central mechanisms for the defense of animal cells against microbial infections, stressful conditions, or certain injuries is called inflammation. Inflammation consists of a series of dynamic, organized responses involving both vascular and cellular events with certain humoral secretions. In these events, plasma, fluids, and white blood cells including neutrophils, monocytes, basophils, and eosinophils change location towards to inflamed site. Also, various mediators and other signaling molecules including prostaglandins, oxygen and, nitrogen-derived free radicals are secreted into the inflamed site by immune defense cells with different mechanisms to contribute to a series of events (Anwikar & Bhitre, 2010). Factors that mediate inflammatory responses can be categorized into four groups: 1) a potent endogenous vasodilator nitric oxide (NO), which is inducible nitric oxide synthase (iNOS)-derived under inflammatory conditions and can produce peroxynitrite ($ONOO^-$/ONOOH) by reacting with superoxide (O^{2-}) (Förstermann & Sessa, 2012); 2) lipid metabolites and various derivatives of arachidonic acid such as prostaglandins; 3) three cascades of soluble proteases and/or substrates such as clotting; 4) cytokines which are a group of cell-derived polypeptides. Most of the cytokines are multifunctional and they exert their effects in a paracrine or autocrine manner locally or systemically. They can also work in many pathways involving antagonistic and synergistic interactions to have both positive or negative regulatory effects on different target cells. These responses normally occur in a few minutes rapidly when triggered by inflammatory stimuli such as microbial infection (Nguyen, 2012).

Short-term inflammation is referred to as acute inflammation and it is the regulated form of inflammation. After the acute inflammation process is over, the body returns to a state of homeostasis. However, if stimuli persistent in the environment or inflammation response is prolonged due to various factors, it is referred to as chronic inflammation and it is the dysregulated form of inflammation (Figure 1). Chronic inflammation is harmful to the organism itself and it is related to many negative health outcomes such as Alzheimer's disease, congestive heart failure and, even cancer. Due to the role of chronic inflammation in mentioned diseases and more, many studies are undergoing for several decades based on the molecular mechanisms of inflammation including the use of NSAIDs.

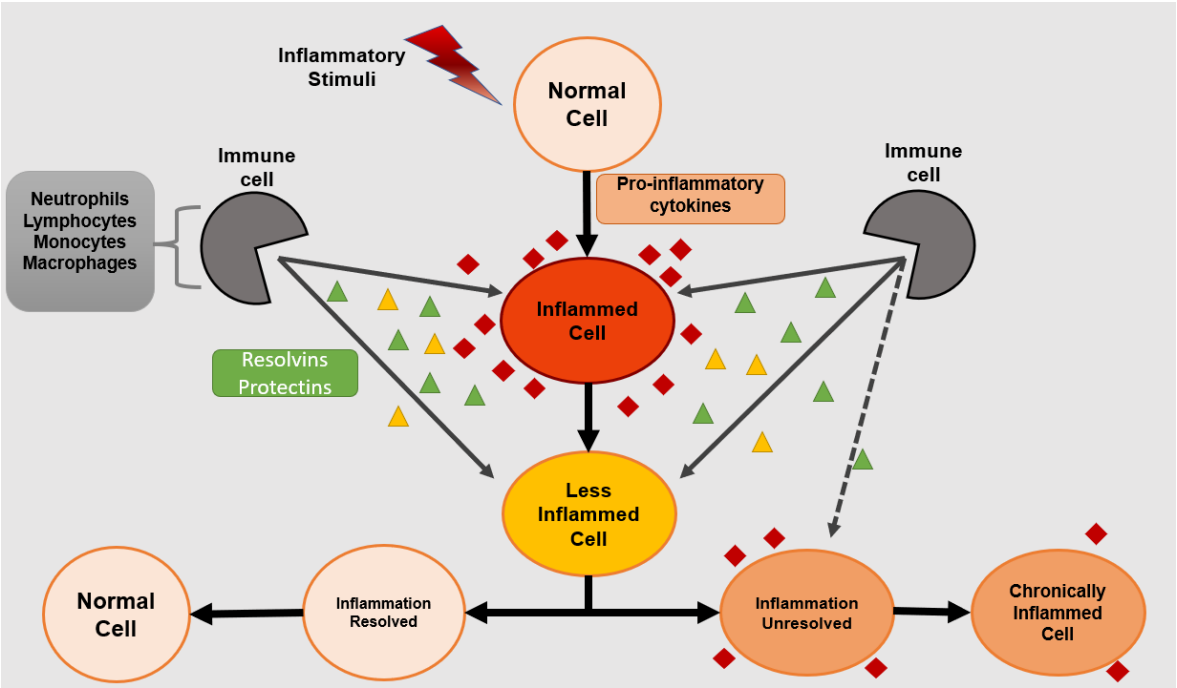


Figure 1. Simple presentation of chronic inflammation.

1.1. Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most commonly and widely used drug family for their easy accessibility and low prices, resulting in accounting for nearly 10 % of all prescribed medications (Abdulla et al., 2013). They are a heterogeneous and broad group of drugs containing various chemical classes and with a chemical perspective these drugs can be divided into three groups; 1) phenazones (oxicams, pyrazolones), 2) carboxylic acids (salicylic acid and its esters, fenamic acids, acetic acids, propionic acids), 3) non-acidic compounds (Vitale et al., 2015). First NSAIDs, including aspirin, were found more than 100 years ago. Nowadays, more than 50 NSAIDs are being consumed in health services for symptomatic treatment of acute and chronic inflammation, fever, and pain without a medical prescription. In the United States alone, 30 billion NSAID doses are consumed and over 70 million prescriptions are written in a year (Kumar & Swee, 2015).

1.1.1. NSAIDs And Their Relation With COX-2

Prostanoids are a bioactive lipid family consisting of thromboxane A2 (TXA_2), prostacyclin (PGI_2), and prostaglandins. Biosynthesis of prostanoids is a rate-limiting step catalyzed by cyclooxygenases (COXs). There are two major isoforms of COXs; COX-1 which is expressed continuously in most tissues, and COX-2 which is expressed by certain inflammatory stimuli. Both COX-1 and COX-2 mediate the conversion of arachidonic acid into thromboxanes (TXs), prostaglandins (PGs), and other eicosanoids, and, both virtually have the same enzymatic activity. However, each isoform shows discrete functional coupling to prostanoid synthases and expression patterns depending on cell types. COX-1 is responsible for maintaining reparative and protective mechanisms including renal homeostasis, production of mucus on gastrointestinal mucosal surfaces, and coagulation (Warner & Mitchell, 2002). It is commonly expressed in low levels in most cell types; however, high levels of expression for COX-1 are found in gastric mucosa and platelets. On the other hand, COX-2 is mitogen inducible in response to growth factors and pro-inflammatory events, and, via the arachidonic pathway, it is the source of the inflammatory mediator prostacyclin (PGI_2) and prostaglandin E2 (PGE_2). COX-2 is also constitutively expressed in certain regions such as the brain, gut, and kidney but is not induced by inflammatory factors. Prostaglandins, especially PGE_2, are important mediators that play a crucial role in the occurrence of inflammation, growth of several solid tumors, and angiogenesis. Prostaglandins show their effects through G-coupled proteins on cell surfaces in paracrine and/or autocrine manner (Hirata & Narumiya, 2011).

NSAIDs inhibit COXs by binding to their active site of them and stop the conversion of arachidonic acid (AA) to prostaglandins by preventing AA binding. NSAIDs can be divided into three subgroups; 1) rapid, reversible inhibitors followed by irreversible, covalent modification of the COXs (e.g. acetylation by aspirin); 2) lower affinity, rapid, reversible inhibitors followed by higher affinity, time-dependent, slowly reversible binding (e.g. indomethacin); 3) reversible and rapid competitive inhibitors (e.g. naproxen) (Perrone et al., 2010). Most of the NSAIDs are non-selective inhibitors of COXs, whereas, there are many COX-2 selective inhibitors such as celecoxib. Hence, different NSAIDs inhibit both isoforms to various degrees. Inhibition of COX-2 is responsible for the analgesic and

antipyretic effects of NSAIDs with a decrease in pro-inflammatory prostaglandins E2 and I2 (prostacyclin). In long-term use and/or chronic administration of NSAIDs, several side effects can be observed in patients due to inhibition of COX-1 and preventing protective properties of this isoform including gastrointestinal (GI) ulceration and renal injury (Monteiro-Steagall et al., 2013). The carboxylic acid functional group of most NSAIDs may lead to local irritation and is partly responsible for GI toxicity (Figure 2). Co-administration of gastro-protective drugs and prescription of the possible lowest dose for a limited amount of time are commonly used methods to prevent these adverse effects of NSAIDs. Along these lines, selective COX-2 inhibitor NSAIDs gained a slope for research to eliminate the effects of COX-1 inhibition. However, since COX-2 inhibitors are unable to recognize inflammation-related COX-2 from constitutively expressed COX-2, increased cardiovascular events occur in prolonged use of drugs (MacDonald et al., 2017). Nowadays, researchers are trying to find alternative drugs with different functional groups and chemical structures to overcome and diminish the side effects of NSAIDs.

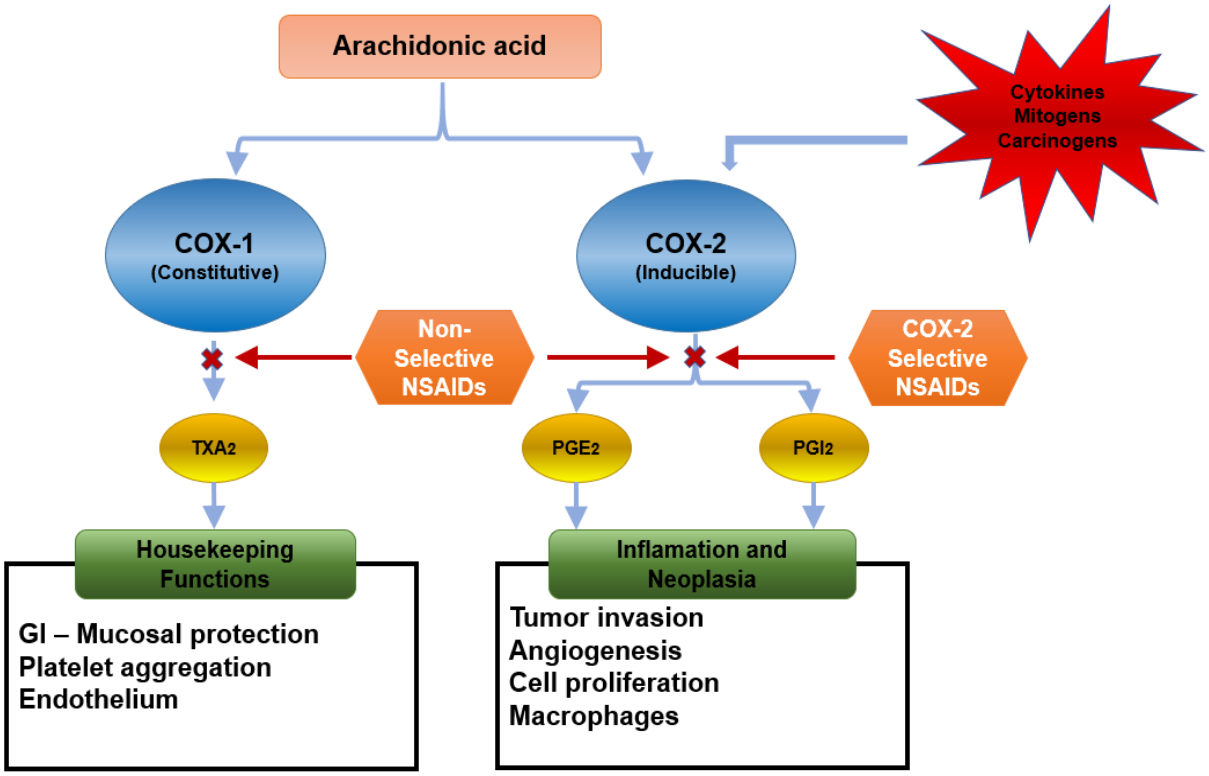


Figure 2. Mechanism of modulation of COXs.

1.1.2. NSAIDs As A Biological Target For Cancer

Chronic inflammation may lead to cancer involving various physical, chemical, and biological determinants along with immune-mediated damage, prolonged exposure to irritants, and infections. Epigenetic and genetic alterations play role in the carcinogenesis process and unfortunately modify tissue homeostasis that leads to induction of chronic inflammatory response. Additionally, inflammatory mediators and cells contribute to the occurrence of the tumor-promoting microenvironment. The relationship between inflammation and cancer is a promising way to prevention of cancer via anti-inflammatory treatment. COX-2 involves in angiogenesis and is over-expressed in all metastatic cancer processes. Furthermore, it plays role in proliferation, apoptosis, invasion, and host immune response in the tumorigenesis process (Toomey et al., 2009). In several cancer types, increased levels of PGE_2 are present and it is documented that they act through multiple cell signaling pathways involved in tumor malignant phenotype maintenance and induction (Nakanishi & Rosenberg, 2013). Prostaglandins are released from tumor cells, stromal cells within the microenvironment of the tumor, and physiological systems due to stress and coping with cancer, pain, tissue damage and, various surgical treatment. Removal of tumor is the common therapeutic strategy for treatment, although surgical processes accelerate reappearance and occurrence rate of the tumor (Liu et al., 2017). All circumstances mentioned above make COX-2 an attractive target to prevent increased levels of PGE_2 and prevent or diminish the promotion of cancer.

NSAIDs are considered to have positive effects on both tumor therapy and prevention of cancer and several epidemiological studies proved this consideration. Reduced incidences were shown in several cancer types including gliomas, breast and, lung with regular use of NSAIDs (Vallée et al., 2018). Also, several hypotheses suggested that NSAIDs could act on both oxidative stress and chronic inflammation to decrease tumor growth. Several molecular mechanisms exploit the link between cancer prevention and NSAIDs such as COX inhibition, decreased glycolytic signaling, and inflammatory response. NSAIDs also can induce apoptosis through mitochondria with intrinsic apoptotic pathways (Jana, 2008). Other signaling pathways including interleukins, TNF-α, and iNOS are also documented to be acted on by NSAIDs (Vallée et al., 2018). In clinical trials, it is reported that some of the

NSAIDs have protective effects such as aspirin may have protective effects against prostate cancer in long-term use and the overall survival rate is increased in patients with ovarian cancer (Ma & Brusselaers, 2018).

1.2. H_2S-donor Compounds

Hydrogen sulfide (H_2S) is a smelly, colorless water-soluble gas and is firstly mentioned in the 17th century. After the production of endogenous H_2S and its relation with physiology discovered by Abe and Kimura, H_2S was recognized as the third most important endogenous gas molecule in organisms following carbon monoxide (CO) and nitric oxide (NO). Recently, it has emerged as an important signaling molecule and H_2S is commonly known as a gasotransmitter. It is produced in mammalians and it is endogenously produced by enzymatic reactions of homocysteine, cysteine, and cystathionine mediated by cystathionine γ-lyase (CSE) and, cystathionine β-synthase (CBS). Furthermore, it has been identified that a third enzyme mainly specific to mitochondria called 3-mercaptopyruvate sulfurtransferase (MPST) produces H_2S (Abe & Kimura, 1996; Altaany et al., 2013). CBS, mainly present in the brain, liver, and nervous system, can convert homocysteine and cysteine into cystathionine and release H_2S. CSE, mainly active in the portal vein, aorta, and other vascular tissues, is responsible for the conversion of L-cystathionine and cystine to pyruvate and L-cysteine. On the other hand, the cooperation of MPST with cysteine aminotransferase (CAT) results in the production of H_2S in the presence of α-ketoglutarate. Although all of the enzymes are specifically active in different tissues, they convert cysteine and cysteine derivatives to H_2S and, precisely contribute to the regulation of H_2S (Figure 3). H_2S can traverse the cell membrane, play role in diverse systems and can be synthesized by mammalian tissues thus, can activate specific pathways or react directly with biological targets as a gasotransmitter (Wallace & Wang, 2015). It relaxes blood vessels, modulates neuronal excitability, regulates cell growth, and hyperpolarizes the cell membrane. Moreover, it is documented in both *in vivo* and *in vitro*, H_2S showed notable effects against inflammation and oxidative stress by behaving as an antioxidant molecule and activating the machinery mediated by Nrf-2 against the reactive oxygen species (Gerő et al., 2016). H_2S is a powerful reducing agent and it can be consumed by superoxide ($O^{2.-}$), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) (Filipovic et al., 2012). Therefore, the situations

mentioned above make H_2S is an important molecule in cardiovascular disease, endocrine regulation, neurophysiology, and other pathological and physiological processes.

Due to interesting biological activities, compounds that are able to generate exogenous H_2S are considered promising agents. Various classes of H_2S-donors have been reported in the literature, such as arylthioamides, mercaptopyruvate, thiadiazolidin-3,5-diones, and dithioates (Ercolano et al., 2019). Additionally, natural and synthetic isothiocyanates (ITC) are described as H_2S generating compounds in an L-Cysteine-dependent manner (Rapposelli et al., 2017). Sulforaphane (SFN) is a slow H_2S releasing compound, it belongs to the ITC family and is one of the most studied ITC. The natural ITCs derive from *Brassicaceae* plants, mostly studied in broccoli and broccoli sprouts. ITCs, produced by endogenous myrosinase through enzymatic hydrolysis of glucosinolates, inhibit cancer activity in both animal models and cell culture. It is known that SFN has growth inhibitory effects on several cultured cancer types including prostate, breast, and colorectal cancer (Lucarini et al., 2018). Furthermore, the generation of H_2S-donor derivates of NSAIDs is suggested by many authors to reduce or overcome the side effects of NSAIDs. In contrast, a study has shown that gastric damage was radically better tolerated with the use of H_2S-donor derivative of diclofenac than diclofenac itself. Compared to H_2S-donor moiety, diclofenac decreased haematocrit by 50% with widespread bleeding in the GI tract in rats, and, overall increased anti-inflammatory potency of diclofenac (Wallace et al., 2007). Similarly, in a rat model of visceral pain, an H_2S-donor moiety of mesalamine showed superior anti-inflammatory actions in experimental colitis and improved analgesic effects (Fiorucci et al., 2007).

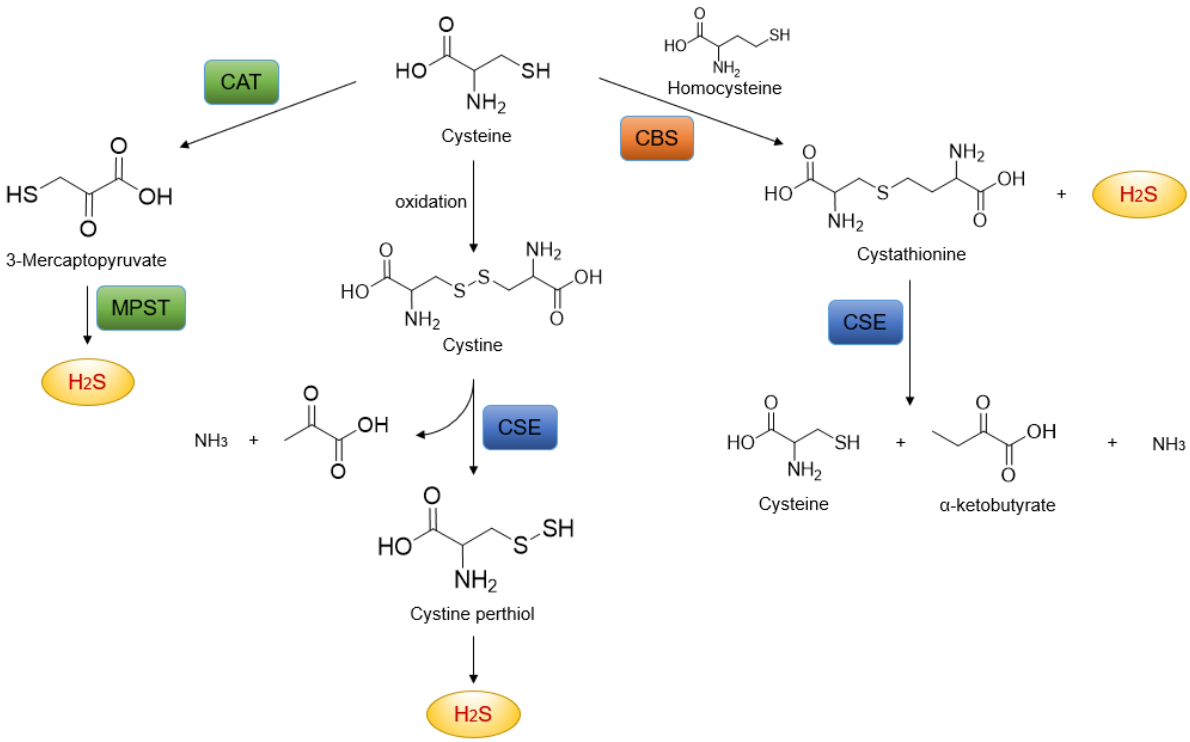


Figure 3. Enzymatic synthesis of H_2S.

1.3. The Aim Of The Study

This study aims to investigate the biological activity of eighteen novel H_2S-donor NSAIDs against cancer. All of the H_2S-donor compounds (**S1-S19**) were synthesized by Semra Altunsoy in her master thesis under the supervision of Prof. Dr. Mehmet Ay in association with the TUBİTAK project (117Z398)(Altunsoy, 2021). Also, three isothiocyanate derivatives (**I1-I3**) were synthesized in the scope of the thesis and these compounds were also a part of the same TUBİTAK project. The biological activities of the mentioned compounds were not known in the literature. The study includes the screening of the compounds for their anti-cancer, proliferation inhibition properties. Breast, prostate, colon cancer cells were used, healthy endothelial cells were used for comparison and determination of drug selectivity.

Overall, in the scope of this thesis;

- Three isothiocyanate derivatives were synthesized in the Natural Products and Drug Research Laboratory.
- H_2S releasing capacities of I compounds were evaluated.
- Antiproliferative effects of the H_2S-donor compounds (S series) and naturally H_2S releasing ITC derivatives (I series) were investigated on the above-mentioned cancer cells using SRB assay.
- For promising compounds with antiproliferative activities, mechanisms of action were partially investigated by qPCR assay.

CHAPTER 2
PREVIOUS STUDIES

The compounds mentioned in the thesis (compounds **S1**-**S19**) were not studied until the beginning of this thesis except for their H_2S releasing capacities and their activity against SIM-A9 microglia cell line for cell viability and NO release inhibition in the thesis of (Altunsoy, 2021). Other than that, there was no evidence related to the biological activities of these novel H_2S-releasing compounds. ITC derivatives were also synthesized (compounds **I1**-**I3**) which happen to release H_2S, in the scope of this thesis and there was no evidence related to their synthesis and biological activity in the literature. All of the investigations taken part in the thesis were part of the TUBİTAK 117Z398 project. Due to the cytoprotective effects of H_2S, the side effects of NSAIDs have been trying to be solved by synthesizing their H_2S releasing derivatives. Various efforts have been put throughout the years to evaluate the effects of H_2S-releasing NSAIDs. To summarize them;

In the study of Chattopadhyay et al. (2016) effects of AVT-219, NO, and H_2S-releasing derivative of naproxen, against induced GI damage in rats were evaluated. AVT-219 did not induce significant gastric damage compared to naproxen and was able to reduce PGE_2 levels. Furthermore, AVT-219 selectively inhibited the enzymatic activity in a dose-dependent manner of pure ovine COX-1 and COX-2. Plasma levels of TNF-α were reduced three-fold compared to naproxen-treated rats. Cell growth inhibition rates of AV-219 were also investigated by this group on different cancer cell types including HT-29 and MCF-7. AVT-219 has effectively inhibited the growth of these cell lines compared to naproxen with IC_{50} values which were at least 18,000-fold more potent. Moreover, colon cancer injected male athymic nude (NU/NU) mice treated with naproxen died due to GI bleeding and damage in two weeks, whereas, when compared to the control group, AVT-219-treated mice showed no overt signs of toxicity and showed a reduction in tumor growth due to enhanced apoptosis and reduced cell proliferation. These findings suggested that AVT-219 could be a more GI-safe agent compared to its native form naproxen and may be useful as a chemotherapeutic agent (Chattopadhyay et al., 2016).

Effects of ATB-346, an H_2S-releasing derivative of naproxen, on gastric lesion, gastric blood flow, and its influence on systemic inflammation in water immersion and restraint stress-induced (WRS) rats compared to naproxen were investigated by Magierowski et al. (2017). ATB-346 reduced dot-like lesions compared to naproxen and protein expression levels significantly decreased in gastric mucosa compared to naproxen. Heme Oxygenase-1 (HO-1) and nuclear factor erythroid 2-related factor 2 (Nrf-2) protein expression levels were drastically increased but Hypoxia-inducible factor (HIF-1α) did not change in ATB-346 treated group, however, naproxen increased protein expression of Nrf-2 and HIF-1α, which upregulation of HIF-1α protein involve in stress ulcerogenesis. Also, ATB-346 was able to reduce COX-2 protein levels in gastric mucosa Also, ATB-346 reduced cytokine concentrations in plasma except for Interleukin-1β (IL-1β), Interleukin 2 (IL-2), and Granulocyte-macrophage colony-stimulating factor (GM-CSF). These findings suggested that H_2S release from ATB-436 is more GI-safe than the naproxen (Magierowski et al., 2017).

Another study was conducted by Dingenen et al., (2019) that showed effects of ATB-346 against gastrointestinal injury in murine postoperative ileus (POI). Il-1β and Monocyte Chemoattractant Protein-1 (MCP-1) expression levels caused by intestinal manipulation reduced significantly in ATB-346 treated group compared to the naproxen and control groups. COX-2 activity reduced significantly in both treatment groups however, they were unable to reduce iNOS activity significantly. Less intestinal damage was detected in the ATB-346 treated group than in the naproxen group. Results suggested that the H_2S-releasing derivative of naproxen is more effective with increased protective activities against POI (Van Dingenen et al., 2019).

Similarly, gastric injury was reduced in aged high fructose diet rats with the use of ATB-340, an H_2S-releasing derivative of aspirin, in the study of Pavlovskiy et al. (2020) as well reduced gastric mucosa damage index compared to the aspirin and control group. ATB-340 was also able to reduce oxidative stress index but the reduction was similar to the aspirin group in terms of CBS, CSE, and Thiosulfate-dithiol sulfurtransferase (TST) activity. This study was suggested that gastroprotection properties of ATB-340 may be related to increased activity in H_2S signaling in terms of attenuated oxidative stress (Pavlovskiy et al., 2020).

Gyöngyösi et al. (2021) synthesized a new H_2S-releasing derivative of ibuprofen called EV-34 and evaluated its oxidative stability, H_2S releasing ability, and cytotoxicity *in vitro*. Model of phase I biotransformation was used to test the stability of EV-32 in phase I metabolic reactions and EV-32 was found to be stable under experimental conditions. Since ibuprofen is metabolized via Cytochrome P450 (CYP) enzymes, synthetic porphyrin was used to mimic CYP450 oxidation. It was found that CYP enzymes may also play a role in EV-32 metabolism. EV-32 was cleaved in the biological system and H_2S release was shown in rat blood and, liver. Moreover, the cell cytotoxicity assay confirmed that EV-32 was non-toxic to rat myoblast cells. COX-1 and COX-2 inhibition rate of EV-32 was found similar to ibuprofen in carrageenan-induced inflammation tests. Thus, it is suggested that EV-32 could be used in patients with higher cardiovascular risk in prolonged ibuprofen treatment as it can release H_2S as a cardioprotective agent (Gyöngyösi et al., 2021).

Anticancer effects of two H_2S-releasing derivatives of diclofenac called ACS15 and ACS32 showed significant effects on human MDA-MB-23, mouse MCF-7 and 4T1 breast cancer lines on inhibition of osteoclast formation which increased levels is supportive for breast cancer, in the study of Frantzias et al. (2012). Cancer cells were viable in the process. Additionally, conditioned media from human cell line enhanced IκB phosphorylation and osteoclast formation. ACS15 and ACS32 were able to significantly inhibit these processes, however, diclofenac itself did not show any effect. ACS15 and ACS32 were able to inhibit the receptor activator of NF-κB ligand (RANKL)-induced IκB phosphorylation and Nuclear Factor Kappa B (NFκB) DNA binding strongly (Frantzias et al., 2012).

In the study of Kodela et al. (2013) anticancer effects of NO and H_2S-releasing derivatives of naproxen and sulindac (NOSH-NAP and NOSH-SUL) were evaluated on the human colon, pancreas, breast, and leukemia cell lines. Both of the compounds inhibited cell growth on all cell lines better than parent compounds naproxen and sulindac in a dose-dependent manner. Their inhibitory effects on normal human lung fibroblast, primary pancreatic epithelial and, human mammary epithelial cell lines. It is reported that both of the compounds inhibited cell growth preferentially in cancer cells. Furthermore, both of the compounds inhibited the growth of colon cancer by a combination of induction of G_0/G_1 or G_2/M arrest and apoptosis (Kodela et al., 2013).

Due to higher anti-inflammatory and higher anticarcinogenic effects with lower side effects of H_2S-releasing compounds, novel H_2S-releasing derivatives are thought as promising therapeutic agents against cancer development. On the other hand, isothiocyanates are effective in inhibiting pro-inflammatory factors in low doses. ITC derivatives were synthesized according to the most known and effective ITC sulforaphane with *in silico* analysis showing higher biocompatibility, binding efficiency.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and Essential Items

All of the chemicals and essential items used for experiments and analysis were listed in Table 1.

Table 1

Chemicals and essential items used for experiments and analysis

Sigma-Aldrich Chemical	Sodium sulfate (238597)
Methanol (24229)	Iron(III) chloride (451649)
Acetic acid (1.00056.2500)	Zinc acetate (383317)
Hydrochloric acid (320331)	*N*,*N*-dimethyl-*p*-1,4-phenylenediamine sulfate salt (186384)
Trypan blue solution (T6146)	L-Cysteine (C7352)
Sodium pyruvate (P5280)	Sodium sulfide (407410)
Dimethyl sulfoxide (DMSO; D2650)	**Alfa Aesar**
Sulforhodamine B sodium salt (S1402)	1-Methyl-2-pyrrolidinone (A12260)
Doxorubicin hydrochloride (D2975000)	*N*-Bromosuccinimide (A15922)
Trisma base (T1503)	Nickel(II) cyanide tetrahydrate (39480)
Chloroform (102431)	2-(4-Aminophenyl)ethan-1-ol (L15223)
Silica gel (89943)	**Life Technologies, Thermo Fisher Scientific**
Silica gel on TLC plates (99569)	Dulbecco's Modified Eagle's Medium (DMEM) (12800017)
Dimethylformamide (PHR1553)	Trypsin-EDTA (25200056)
Ethyl acetate (109623)	Fetal bovine serum (FBS, 10500064)
Hexane (HX0302)	Penicillin-streptomycin (15140122)
Diethyl ether (309966)	**Santa Cruz Biotechnology**
Thiophosgene (330752)	Dimethyl sulfoxide (DMSO; sc-358801)
Triethylamine (471283)	**Caisson laboratories**
Tetrahydrofuran (401757)	Dulbecco's PBS (PBP01)

3.1.2. Equipment

All of the equipment used for experiments and analysis were given in Table 2.

Table 2

List of equipment used for experiments and analysis

Brand	Model	Equipment
Electrothermal	IA9100	Melting point apparatus
CAMAG	022.9120	UV cabinet
IKA	RV 10 control	Rotary evaporator
BUCHI	R-300	Rotary evaporator
BUCHI	V-300	Vacuum pump
Perkin Elmer	Spectrum 100	FT-IR spectrophotometer
Jeol	400 MHz	NMR spectrophotometer
Agilent	600 MHz	NMR spectrometer
Shimadzu	8040 LCMS	LC/MS/MS spectrometer
CEM	Discover SP-909155	Microwave
Acculab	-	Weighing balance
Esco	CCL-170T-8	Cell culture CO_2 incubator
Esco	Class II BSC	Biological safety cabinet
Hettich	Universal 320R	Standard centrifuge
TECAN	Infinite 200 PRO	Microplate reader
Scientific Industries	Genie 2	Vortex
HANNA Instruments	HI-2211	pH meter

3.1.3 Cell Lines

Following cell lines given in Table 3 were purchased from American Type Culture Collection (ATCC). Cells were optimized in laboratory conditions and incubated at 37 ºC in humidified air containing 5% CO_2, then stored in liquid nitrogen for later experiments. Growth medium choice and supplementation materials were given in Table 3.

Table 3

Growth medium and supplementation materials for cell lines

Cell Lines	HT-29, MCF-7, HUVEC	PC-3
Growth Medium	DMEM	DMEM
Supplementation	10% Fetal Bovine Serum, 100 µg/mL streptomycin, 100 U/mL penicillin	20% Fetal Bovine Serum, 100 µg/mL streptomycin, 100 U/mL penicillin

3.2. Methods

After all equipment and materials were gathered, synthesis of the isothiocyanate derivatives was started. As compounds synthesized, biological activities of the synthesized compounds were tested alongside H_2S releasing compounds.

3.2.1. Synthesis of Isothiocyanate Derivatives (I Series)

In the synthesis part, aromatic-ring containing three isothiocyanate derivatives were synthesized that have potential anti-inflammatory and anticarcinogenic properties. These compounds were selected based on the drug sulforaphane and natural compounds containing the isothiocyanate functional group. Structural properties of the compounds were characterized with FT-IR, ^{1}H-NMR, ^{13}C-NMR and, MS analysis.

I1 Compound: Reaction of 2-(4-aminophenyl)ethan-1-ol (**APE**) and thiophosgene (**TP**) in the presence of triethylamine expected to yield 2-(4-isothiocyanatophenyl)ethan-1-ol (**I1**) (figure 4).

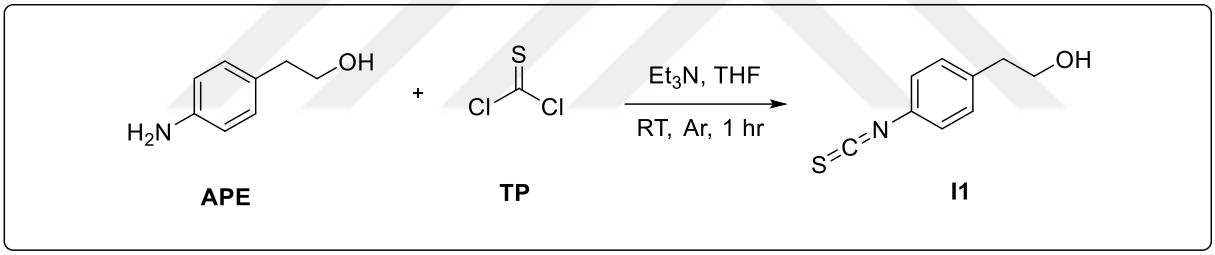


Figure 4. Synthesis of compound **I1**.

I2 Compound: Bromination reaction of **APE** with *N*-bromosuccinimide (**NBS**) expected to yield 2-(4-amino-3-bromophenyl)ethan-1-ol (**APE-Br**). Then the reaction of **APE-Br** and **TP** in the presence of triethylamine is expected to yield 2-(3-Bromo-4-isothiocyanatophenyl)ethan-1-ol (**I2**) (figure 5).

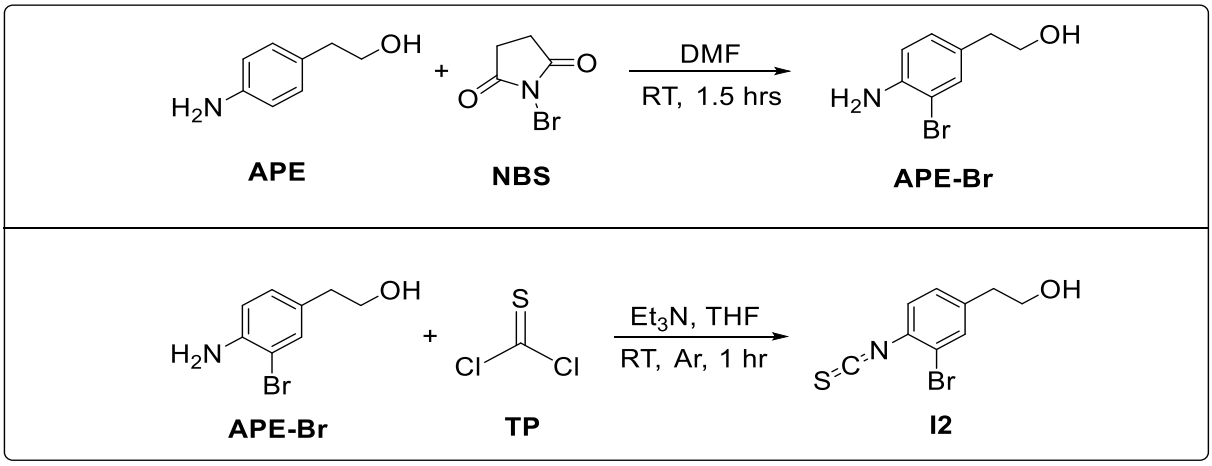


Figure 5. Synthesis of compound **I2**.

I3 Compound: Reaction of **APE-Br** and nickel(II) cyanide expected to yield 2-amino-5-(2-hydroxyethyl)benzonitrile (**APE-CN**). Then, the reaction of **APE-CN** and **TP** in the presence of triethylamine is expected to yield 5-(2-hydroxyethyl)-2-isothiocyanatobenzonitrile (**I3**) (figure 6).

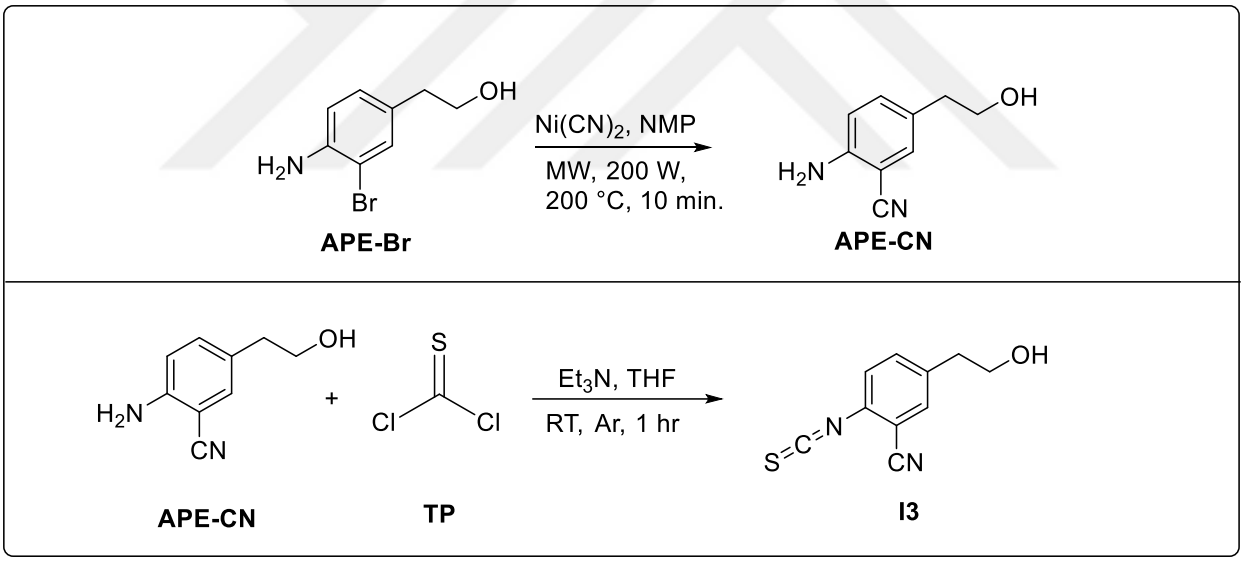


Figure 6. Synthesis of compound **I3**.

3.2.2 Structures of H_2S Releasing Compounds (S Series)

All H_2S Releasing compounds (**S1**-**S19**) were synthesized in the same TUBİTAK 117Z398 project by Semra ALTUNSOY (Altunsoy, 2021). Structures of compounds were given in figure 7.

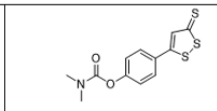
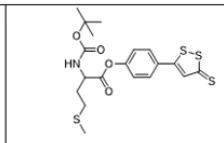
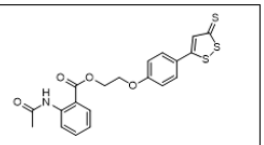
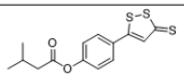
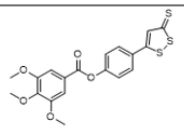
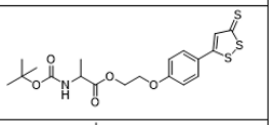
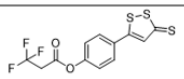
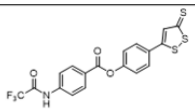
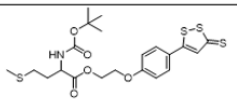
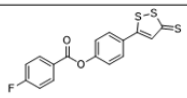
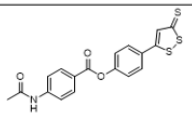
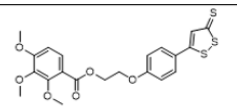
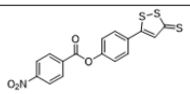
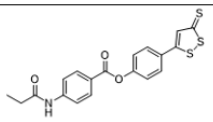
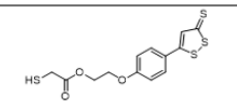
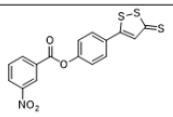
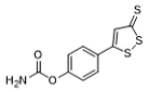
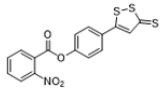
S1		S8		S15	
S2		S9		S16	
S3		S10		S17	
S4		S11		S18	
S5		S12		S19	
S6		S13			
S7					

Figure 7. Structures of S Series.

3.2.3. H_2S Release Measurement

The H_2S release capacities of I compounds were tested according to the methylene blue method by (Li et al., 2017). Briefly, *N*,*N*-Dimethyl-*p*-1,4-phenylenediamine sulfate salt, and $Zn(OAc)_2$ were reacted with H_2S in the presence of Fe^{+3} ions and in strong acidic conditions (Figure 8). Firstly, a standard curve was obtained with the use of Na_2S (0-150 µM) to generate H_2S in increasing concentrations after mixing increasing concentrations of Na_2S with (500 µL) methylene blue solution. Then, (100 µM in final) each compound was mixed with (1mM) L-cysteine containing (20 mM, pH: 7.4) phosphate buffer. The mixture of L-cysteine and compounds were taken into a 24-well plate (1 mL). Methylene blue solution was added to the compound mix (500 µL). The plate was read immediately, then every 5 minutes with a microplate reader at 670 nm until a meaningful increase was not observed.

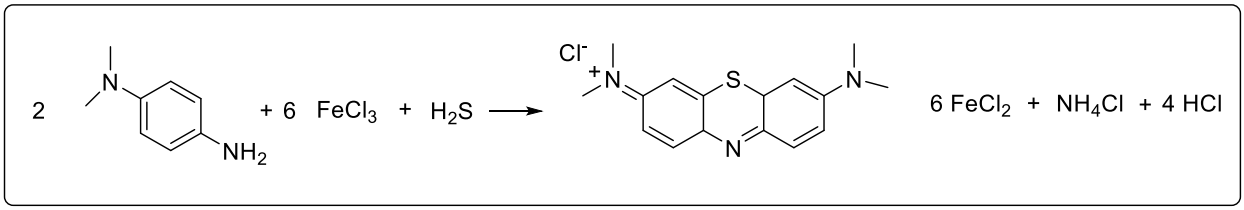


Figure 8. General reaction scheme of methylene blue method.

Components of methylene blue for each reaction are given below (final 500 µL);

- 30 mM $FeCl_3$ in 1.2 M HCl solution (200 µL)
- 20 mM *N*,*N*-dimethyl-*p*-1,4-phenylenediamine sulfate salt in 7.2 M HCl solution (200 µL)
- 1% $Zn(OAc)_2$ solution in dH_2O (100 µL).

3.2.4. Gene Expression Analysis From Total RNA

RNA isolation from MCF-7 cells was executed by using PureLink® RNA mini kit with TRIzol®. RNA isolation and cDNA synthesis were carried out as previously summarized (Tumer et al., 2018). The expression of apoptotic genes was investigated by the Quantitative Polymerase Chain Reaction (qPCR) technique. In the end, the effects of compounds **I1** and **I2** on Bax (Bcl-2-associated X protein) and Bcl-2 (B-cell lymphoma 2) expression levels were investigated using specific TaqMan ® probes with the use of β-actin as endogeneous control. The treatment doses were chosen as IC_{50} and IC_{50}x2 values. Steps in the total process were given in figure 9.

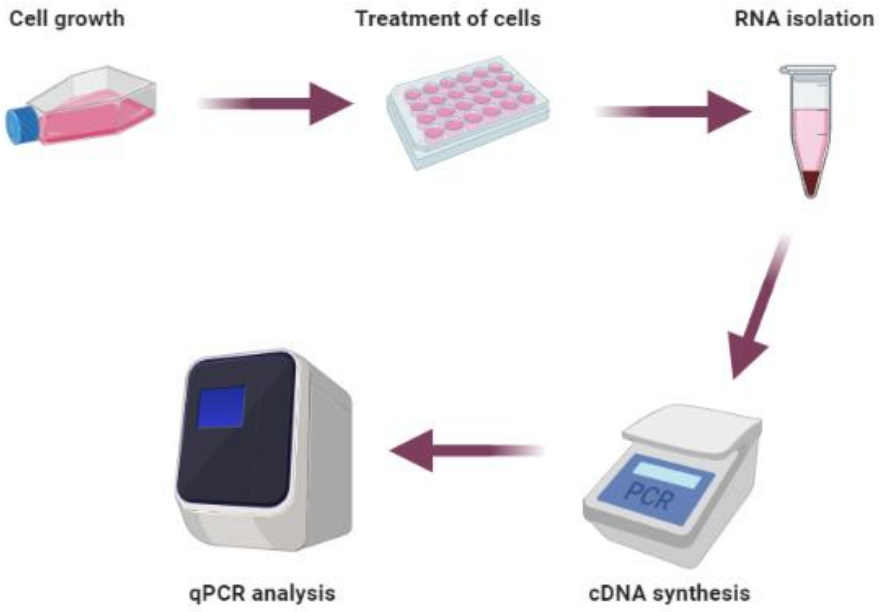


Figure 9. Steps for gene expression analysis.

3.2.5. Cytotoxicity Assay and IC_{50} Determination

The potential antiproliferative effects of S and I compounds were tested by using Sulforhodamine B (SRB) assay on human-derived cancer cell lines namely PC-3, HT-29, and MCF-7 along with healthy cell line, HUVEC. Cells were seeded as $5x10^3$ concentration in each well of 96-well plate. After adherence and 24 hours, cells were treated with compounds and reference compound in an increasing dose-dependent manner (1-200 µM) for 48 hours. After incubation time is finished, media was aspirated from wells, and cells were fixated with 10% (m/V) trichloroacetic acid (TCA) solution for an hour at 4 °C. Cells were washed with dH_2O and air-dried after incubation was finished. Then, 100 µL of SRB dye (0.04% m/V) was poured onto cells and incubated for 30 min at RT. After 30 min, the unbound SRB dye was discarded, and the remaining SRB residue on the cells was washed with 1% (V/V) acetic acid solution. Finally, 10 mM of Tris base solution (pH: 10.5) (200 µL) was used to dissolve the protein-bound dye, and absorbance was recorded by using a microplate reader at 565 nm. IC_{50} values of the compounds were determined by GraphPad Prism 6 software.

3.2.6. Statistical Analysis

Graphpad Prism 6 software was used to perform statistical analyses to determine significant differences. Data were obtained through technical and biological replicated experiments. Results were displayed as the standard error of mean ± (SEM). For comparison between control and treatment groups, One-way analysis of variance (ANOVA) was used.

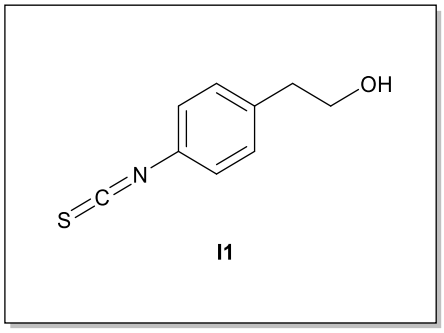
CHAPTER 4
RESULTS AND DISCUSSION

4.1. Results

At the beginning of the studies, optimization steps for the synthesis of compounds were performed to find the optimal time and heat conditions for reactions to increase yield. After optimization steps, all three isothiocyanate derivatives were synthesized starting with **I1**, then, **I2**, and finally, **I3**. After synthesis of the isothiocyanate derivatives was finished, all compounds, including H_2S releasing compounds (S compounds from **S1** to **S19**) and isothiocyanate derivatives (I compounds from **I1** to **I3**) were examined for their antiproliferative activities. Human-derived cell lines were used for antiproliferative activities and before evaluation, several optimizations including cell seeding concentration were performed. Finally, the H_2S releasing capacities of synthesized isothiocyanate derivatives were examined.

4.1.1. Synthesis of Compound I1

2-(4-Isothiocyanatophenyl)ethan-1-ol (I1)



I1

In a 10 mL two-neck round bottom flask, 2-(4-aminophenyl)ethan-1-ol (**APE**) (1.82 mmol; 0.25 g) was dissolved in 1 mL of THF and placed in an ice bath, then, triethylamine (4.36 mmol; 0.60 mL; 2.4 equiv.) was added and stirred under Argon gas for 15 minutes. A solution of thiophosgene (2.18 mmol; 0.21 mL; 1.2 equiv.) in 1 mL of THF was added dropwise into the flask for 30 minutes and stirred in an ice bath for one hour. When it was understood that the starting material was finished with TLC (hexane:ethyl acetate / 2:1) and the solvent was evaporated with a rotary evaporator under reduced pressure. The resulting yellow oily product was extracted with EtOAc (3x30 mL), dried over anhydrous Na_2SO_4.

After removing the solvent under reduced pressure with a rotary evaporator, column chromatography (silica gel) was used to purify the oily product with hexane:ethyl acetate (3:1) solvent system. When the light yellow viscous liquid obtained was kept in the refrigerator at +4 °C. Cream-colored solid, 97 mg, yield 30%, m.p. 44-45 °C (lit. m.p. 42-44 °C) (Park et al., 2003).

IR (υ/cm^{-1}): 3236 (O-H stretching), 3073, 3049, 3037 (aromatic C-H stretchings), 2918, 2872 (aliphatic C-H stretches), 2172, 2073 (-N=C=S stretches), 1500 (aromatic C=C stretching), 1051 (alcohol C-O stretching) (Appendix - figure 1).

^{1}H NMR (600 MHz, DMSO-d$_6$, ppm): δ 7,31 (s,2H), 7,27 (s, 2H), 4,65 (s, 1H), 3,58 (d, J= 6,16 Hz, 2H), 2,70 (d, J= 5,98 Hz, 2H) (Appendix - figure 2).

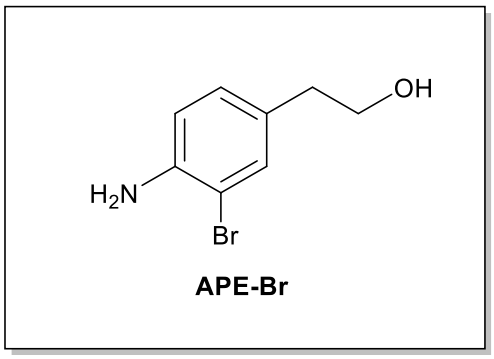
^{13}C-NMR (150 MHz, DMSO-d$_6$, ppm): δ 140.47, 133.13, 130.77, 128.03, 126.08, 62.12, 38.86 (Appendix - figure 3).

LC/MS (ESI) m/z: 180 ([M+H]$^+$, 60), 162 ([M-OH]$^+$, 100), 124 ([M-NCS]$^+$, 40) (Appendix - figure 4).

4.1.2. Synthesis of Compound I2

Compound **I2** was synthesized in 2 steps. In the first step bromination reaction of **APE** was reacted with *N*-bromosuccinimide (**NBS**) in DMF at room temperature for 3.5 hours to yield 2-(4-amino-3-bromophenyl)ethan-1-ol (**APE-Br**). Then, the **APE-Br** compound was reacted with thiophosgene in THF for 1 hour to convert amino group (-NH_2) to isothiocyanate group (N=C=S).

2-(4-Amino-3-bromophenyl)ethan-1-ol (APE-Br)



APE-Br

In a 250 mL one-neck round bottom flask, **APE** (14.28 mmol; 1.96 g) was dissolved in 71 mL of DMF and **NBS** (14.28 mmol; 2.5 g) was added to the ice bath-cooled flask and

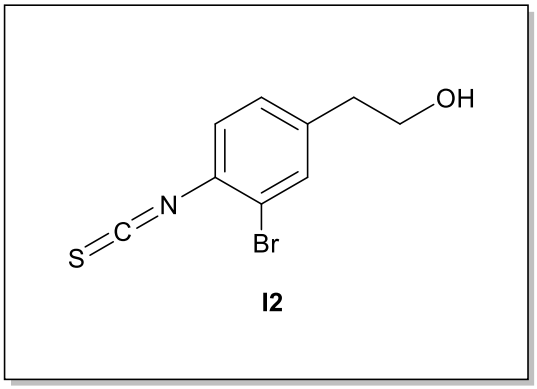
stirred in an ice bath for 5 minutes. Then, the flask was taken to room temperature and stirred for 1.5 hours. When it was understood that the starting material was finished with TLC (hexane:ethyl acetate), DMF was evaporated and 10 mL of dH_2O was added to the medium, and the product was extracted with ethyl acetate (3x30 mL). The organic phase was washed with dH_2O (50 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated with a rotary evaporator under reduced pressure. Column chromatography (silica gel) was used to purify the crude product in hexane:ethyl acetate (1:3) solvent system (Choi et al., 2007). Light yellow solid, 2.44 g, yield: 80%, m.p. 66-68 °C.

IR (υ/cm^{-1}): 3386 and 3275 (N-H and O-H stretchings), 3056, 3031 (aromatic C-H stretchings), 2951, 2923, 2861 (aliphatic C-H stretchings), 1630, 1583 (N-H stretching and, aromatic C=C stretching), 1498, 1363 (aliphatic C-H stretchings), 1047 (alcohol C-O stretch), 766 (C-Br stretching (Appendix - figure 5).

^{1}H-NMR (600 MHz, DMSO-d_6, ppm): 7,16 (s, 1H), 6,88 (dd, j= 8,15, 1,17, 1H), 6,68 (d, j= 8,14 Hz, 1H), 5,06 (s, 2H), 4,56 (t, j= 5,02 Hz, 1H), 3,48 (m, 2H), 2,52 (t, j= 7,03 Hz, 2H) (Appendix - figure 6).

^{13}C-NMR (150 MHz, DMSO-d_6, ppm): 144.13, 132.53, 129.31, 129.10, 115.73, 107.77, 62.78, 38.07 (Appendix - figure 7).

2-(3-Bromo-4-isothiocyanatophenyl)etan-1-ol (I2)



I2

APE-Br (1 mmol; 0.22 g; 1 equiv.) was taken into a 25 mL two-neck round bottom flask permeated with argon gas and dissolved in 3 mL of THF. Thiophosgene (1.2 mmol; 92 µL; 1.2 equiv.) and triethylamine (2.4 mmol; 0.33 mL; 2.4 equiv.) were added to the ice-bath-cooled medium, respectively. After stirring in an ice bath for 10 minutes, the reaction mixture was stirred for one hour at room temperature. When it was understood that the

starting material was finished with TLC (hexane:ethyl acetate), 20 mL of dH_2O was added to the medium and extracted with ethyl acetate (3x20 mL). The organic phase was washed with dH_2O (20 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated with a rotary evaporator under reduced pressure. The crude product was purified by column chromatography (silica gel) in a hexane:ethyl acetate (1:4) solvent system. Orange oily liquid, 77 mg, yield: 30%.

IR (υ/cm^{-1}): 3334 (O-H stretch), 3063, 3033 (aromatic C-H stretch), 2945-2875 (aliphatic C-H stretch), 2042 (N=C=S stretch), 1596 (C=C stretch), 1485, 1399 (aliphatic C-H stretch), 1037 (alcohol C-O stretch), 520 (C-Br stretch) (Appendix - figure 8).

^{1}H-NMR (400 MHz, DMSO-d_6, ppm): δ 7,60 (d, j= 1,8 Hz, 1H), 7,43 (d, j= 8,2 Hz, 1H), 7,27 (dd, j= 8,2 ve 1,8 Hz, 1H), 4,64 (s, broad, 1H), 3,57 (t, j= 6,6 Hz, 2H), 2,69 (t, j= 6,6 Hz, 2H) (Appendix - figure 9).

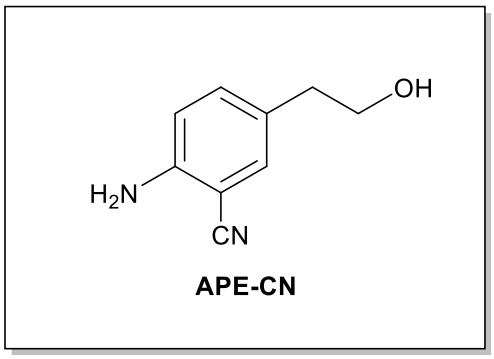
^{13}C-NMR (100 MHz, DMSO-d_6, ppm): δ 142.64, 136.02, 133.97, 130.18, 128.08, 127.60, 120.06, 61.84, 38.42 (Appendix - figure 10).

LC/MS (ESI) m/z: 257 ([M-H]$^-$, 44), 241 ([M-OH]$^-$, 84), 227 ([M-OH-CH_2]$^-$, 76), 223 (100), 201 ([M-NCS]$^-$, 78). (Appendix - figure 11).

4.1.3. Synthesis of Compound I3

Compound **I3** was synthesized in 2 steps. In the first step **APE-Br** was reacted with nickel(II)cyanide in *N*-methyl-2- pyrrolidinone (**NMP**) for 10 minutes with microwave energy (200 °C, 200 Watt) to yield 2-amino-5-(2-hydroxyethyl)benzonitrile (**APE-CN**). Then, **APE-CN** was reacted with thiophosgene in THF for 1 hour to convert amino group (-NH_2) to isothiocyanate group (N=C=S).

2-Amino-5-(2-hydroxyethyl)benzonitrile (APE-CN)



APE-CN

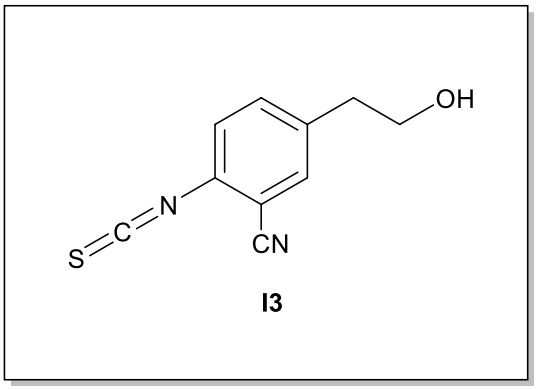
In a 35 mL microwave reaction vessel, **APE-Br** (3.47 mmol; 0.75 g; 1 equiv.) was dissolved in 1 mL of **NMP**. Nickel(II) cyanide tetrahydrate (2.09 mmol; 0.38 g; 0.6 equiv.) was added and stirred for 2 minutes at room temperature. 200 watts of microwave energy was applied at 200 °C for 10 minutes. When it was understood that the starting material was finished with TLC (hexane:ethyl acetate), 30 mL of water was added to the medium and extracted with ethyl acetate (3x30 mL). The organic phase was washed with dH_2O (30 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated with a rotary evaporator under reduced pressure. The crude product was purified by column chromatography (silica gel) in hexane:ethyl acetate (1:2) solvent system (Choi et al., 2007). Cream solid, 0.31 g, yield: 56%, m.p. 68-70 °C.

IR (υ/cm^{-1}): 3336, 3180 (N-H and O-H stretchings), 3027 (aromatic C-H stretching), 2964, 2931, 2878 (aliphatic C-H stretchings), 2217 (C≡N stretching), 1615, 1575 (N-H and, aromatic C=C stretchings), 1024 (alcohol C-O stretch) (Appendix - figure 12).

^{1}H-NMR (600 MHz, DMSO-d_6, ppm): 7,18 (s, 1H), 7,14 (d, j= 8,54 Hz, 1H), 6,69 (d, j= 8,49 Hz, 1H), 5,81 (s, 2H), 4,57 (t, j= 5,17 Hz, 1H), 3,48 (m, 2H), 2,52 (t, j= 6,9 Hz, 2H) (Appendix - figure 13).

^{13}C-NMR (150 MHz, DMSO-d_6, ppm): 150.33, 135.43, 132.32, 127.54, 118.76, 115.68, 93.68, 62.43, 37.82 (Appendix - figure 14).

5-(2-Hydroxyethyl)-2-isothiocyanatobenzonitrile (I3)



I3

APE-CN (1 mmol; 0.16 g; 1 equiv.) was dissolved in 3 mL of THF in a 25 mL two-neck round bottom flask and permeated with argon gas. Thiophosgene (1.2 mmol; 92 µL; 1.2 equiv.) and triethylamine (2.4 mmol; 0.33 mL; 2.4 equiv.) were added to the ice-cooled flask, respectively. After stirring in an ice bath for 10 minutes, then it was stirred for 1 hour at room temperature. When it was understood that the starting material was finished with TLC (hexane:ethyl acetate), 20 mL of dH_2O was added to the medium and extracted with ethyl acetate (3x10 mL). The organic phase was washed in 20 mL of dH_2O and dried over anhydrous Na_2SO_4. A rotary evaporator was used to evaporate the solvent under reduced pressure. In a hexane:ethyl acetate (1:3) solvent system, the crude product was purified using column chromatography (silica gel). Orange solid, 72 mg, yield: 36%.

IR (υ/cm^{-1}): 3356 (O-H stretch), 3073, 3037 (aromatic C-H stretch), 2948, 2878 (aliphatic C-H stretches), 2229 (CN stretch), 2022 (-N=C=S stretch), 1603, 1568 (C=C stretch), 1490, 1386 (aliphatic C-H stretches), 1035 (alcohol C-O stretch) (Appendix - figure 15).

^{1}H-NMR (400 MHz, DMSO-d_6, ppm): δ 7,77 (d, j=2,0 Hz, 1H), 7,60 (dd, j= 8,3 ve 2,0 Hz, 1H), 7,54 (d, j= 8,3 Hz, 1H), 4,67 (s, 1H), 3,58 (t, j= 6,5 Hz, 2H), 2,74 (t, j= 6,5 Hz, 2H) (Appendix - figure 16).

^{13}C-NMR (100 MHz, DMSO-d_6, ppm): δ 139.33, 138.30, 138.13, 135.08, 134.42, 133.83, 127.47, 117.03, 61.78, 38.20 (Appendix - figure 17).

LC/MS (ESI) m/z: 204 ([M+H]$^+$, 100), 180 ([M-CN]$^+$, 70), 163 ([M-CN-OH]$^+$, 96), 149 ([M-NCS]$^+$, 52) (Appendix - figure 18).

4.1.4. H_2S Releasing Capacities of I Compounds

H_2S releasing capacities of I compounds were tested by using the methylene blue method as mentioned above. Briefly, compounds were incubated in L-cysteine containing phosphate buffer and then reacted with methylene blue solution. As a result, the color change was measured with a microplate reader at 670 nm and H_2S concentration was found according to the standard curve. The released H_2S concentration for each compound were given in figure 10.

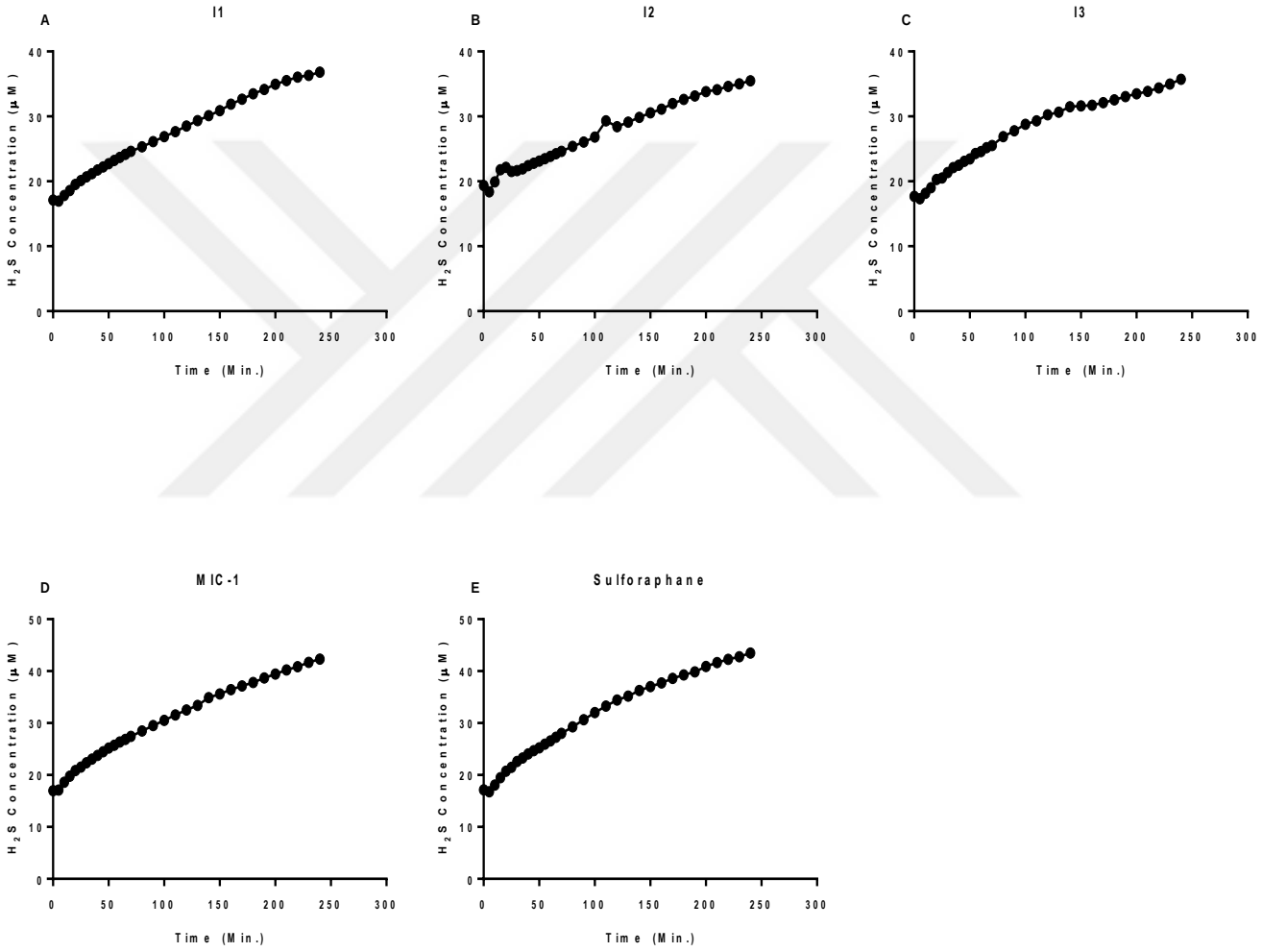


Figure 10. H_2S releasing capacities of (A) compound **I1**, (B) compound **I2**, (C) compound **I3**, (D) Moringin (MIC-1) and, (E) Sulforaphane as µM.

According to the results, compound **I1** was able to release 36.80 µM H_2S, compound **I2** was able to release 35.46 µM H_2S, and, compound **I3** was able to release 35.70 µM H_2S after a

total of 4 hours time period. Reference ITCs, moringin, and sulforaphane were able to release 42.29 µM and, 43.45 µM H_2S, relatively, in the same time period.

4.1.5. Bax/Bcl-2 mRNA Expression Levels of I Series

After cells were treated, RNAc isolation was finished and, cDNA synthesis was completed, the Bax/Bcl-2 mRNA expression levels were assessed by qPCR analysis. Bax (apoptotic) and, Bcl-2 (anti-apoptotic) are apoptosis-related genes. The ratio of Bax/Bcl-2 determines the cell susceptibility to undergo apoptosis, therefore, a higher ratio suggests cells may go through apoptotic pathways. As shown in figure 11, **I2** did not significantly change the ratio of Bax/Bcl-2, whereas, **I1** was able to increase the ratio of Bax/Bcl-2 by 1.58-fold at 104 µM (IC_{50}x2 value) dose.

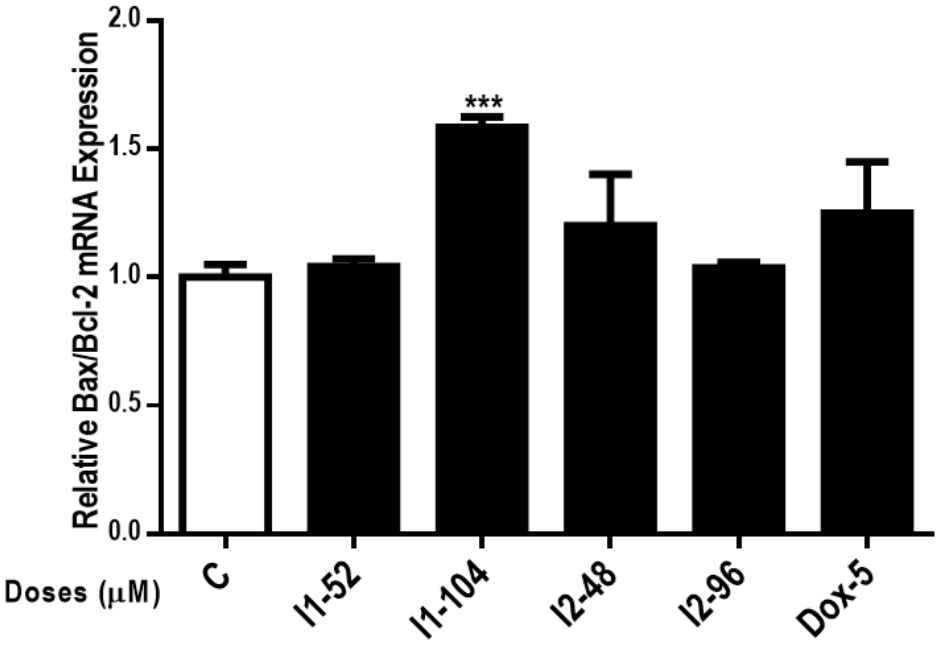


Figure 11. Effects of **I1** and **I2** on Bax/Bcl-2 mRNA expression levels in MCF-7 breast cancer cells. C: Control, Dox: Doxorubicin. ***p < 0.001.

4.1.6. Evaluation of Anticancer Effects of S Series and Determination of IC_{50} Values

The antiproliferative effects of S compounds were evaluated by using human-derived cell lines including colon, prostate, and breast cancer cell lines along with healthy endothelial cell line by SRB assay. Cells were treated with increasing doses of the

compounds (1-200 µM) for 48 hours. The cell viability was assessed, Graphpad Prism 6 Software was used to determine IC_{50} values and given in table 4. According to the results, the IC_{50} values of most of the compounds were higher than 100 µM. Only compound **S6** showed IC_{50} values under 100 µM for HUVEC as 32.77 µM, for MCF-7 as 41.61 µM, for PC-3 as 62.34 µM, and for HT-29 as 68.84 µM, respectively. Moreover, IC_{50} values were under 100 µM for **S8** and **S2** except for the HT-29 cell line. Compounds **S1**, **S4**, **S5**, **S7**, **S10**, **S12**, **S15**, **S16**, **S17** did not show toxic effects against any of the cell lines. Also, any of the compounds found selective against cancer cells.

Table 4

The cytotoxic effect of S compounds on HUVEC endothelial healthy and, cancer cell lines. Doxorubicin was used as a reference drug.

Compounds	IC_{50} (μM)			
	HUVEC	MCF-7	PC-3	HT-29
S1	>100	>100	>100	>100
S2	59.19	51.66	94.38	>100
S3	>100	84.53	97.44	>100
S4	>100	>100	>100	>100
S5	>100	>100	>100	>100
S6	32.77	41.61	62.34	68.84
S7	>100	>100	>100	>100
S8	47.86	38.87	76.75	>100
S9	61.29	>100	>100	>100
S10	>100	>100	>100	>100
S11	>100	>100	>100	73.13
S12	>100	>100	>100	>100
S13	92.72	>100	>100	>100
S15	>100	>100	>100	>100
S16	>100	>100	>100	>100
S17	>100	>100	>100	>100
S18	81.87	>100	>100	>100
S19	>100	86.26	>100	84.64
Doxorubicin	11.4	13.8	15.6	3.16

4.1.7. Evaluation of Anticancer Effects of I Series and Determination of IC_{50} Value

To evaluate the antiproliferative effects of I compounds, the same cell lines mentioned above were used. Firstly, cell lines were treated with 100 µM of I compounds. As shown in figure 12, compounds **I1** and **I2** effectively inhibited the proliferation of each cell line. **I1** inhibited the proliferation of PC-3, MCF-7, HT-29 and, HUVEC by 78%, 88%, 81.5% and, 82%, respectively. **I2** inhibited the proliferation of cell lines by 91%, 92%, 82.5% and, 93% respectively. However, **I3** was unable to inhibit the proliferation of cancer cells even at a 100 µM dose.

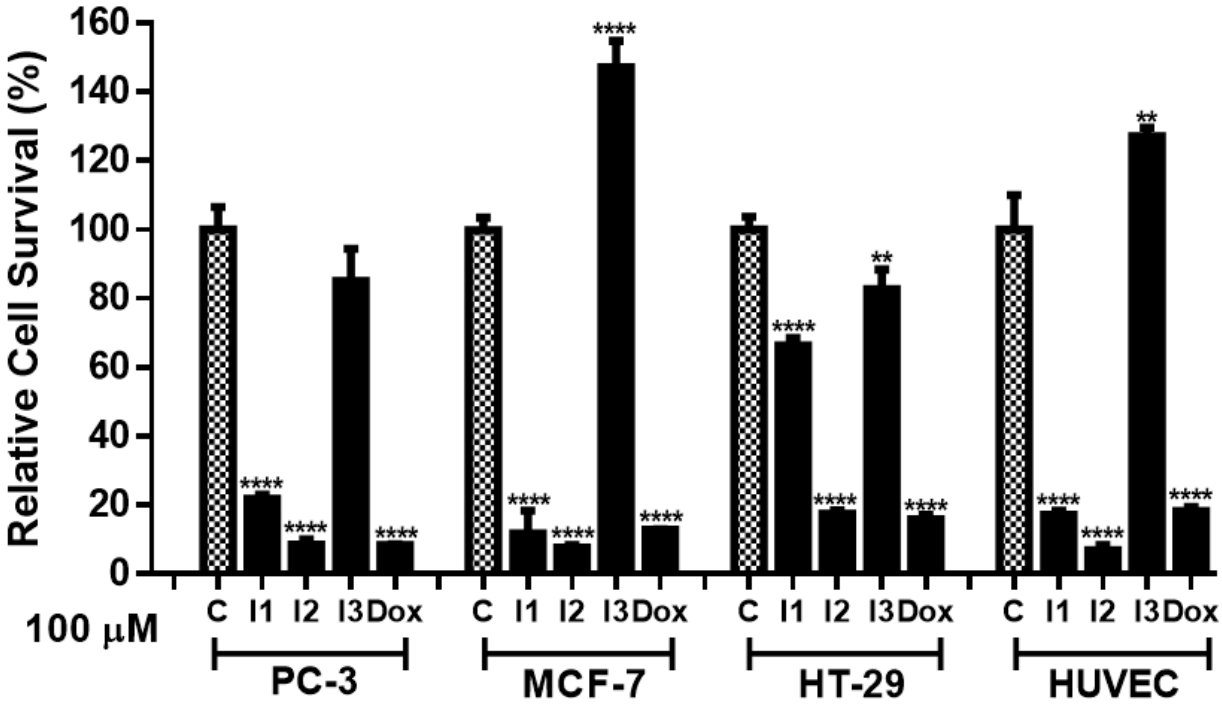


Figure 12. The cytotoxic effect of I compounds on cancer cell lines and, HUVEC healthy cell line at 100 µM dose. C: Control, Dox: Doxorubicin. **p < 0.01, ****p < 0.0001.

After the screening of I compounds against proliferation, cells were treated with increasing doses of the compounds (1-200 µM) to evaluate the IC_{50} values of **I1** and **I2**. Then, IC_{50} values were determined by using GraphPad Prism 6 Software and given in figure 13 for PC-3 cells, in figure 14 for MCF-7 cells and, in figure 15 for HT-29 cells.

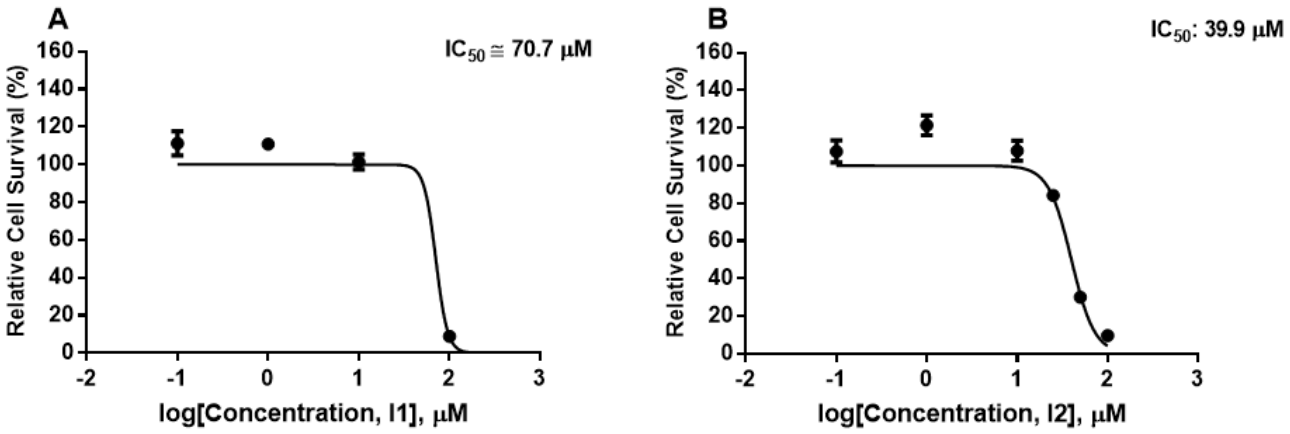


Figure 13. The IC_{50} concentrations of (A) compound **I1** and (B) compound **I2** in the PC-3 cancer cell line.

The IC_{50} concentrations of compounds **I1** and **I2** in the PC-3 cancer cell lines were found as 70.7 µM and 39.9 µM, respectively.

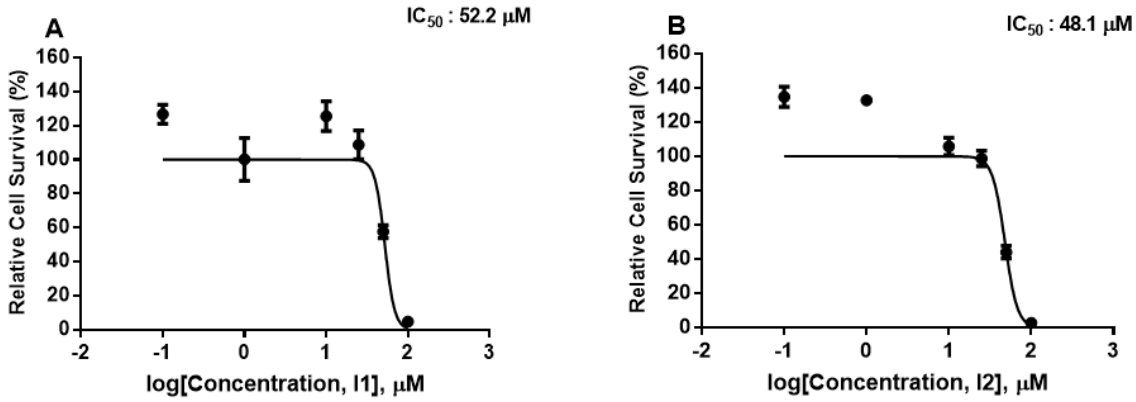


Figure 14. The IC_{50} concentrations of (A) compound **I1** and (B) compound **I2** in the MCF-7 cancer cell line.

The IC_{50} concentrations of compounds **I1** and **I2** in the MCF-7 cancer cell line were found as 52.2 µM and 48.1 µM, respectively.

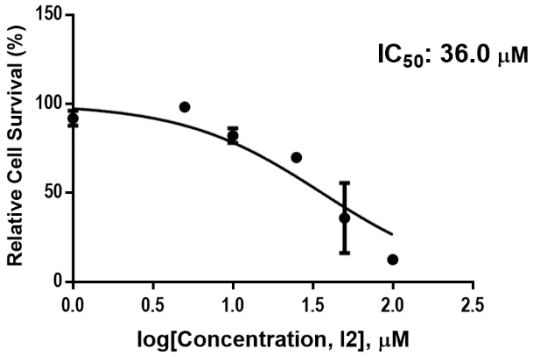


Figure 15. The IC_{50} concentration of compound **I2** in HT-29 cancer cell line.

The IC_{50} concentration of compound **I2** in the HT-29 cancer cell line was found as 36 μM.

4.2. Discussion

Cancer is still one of the diseases with high mortality rates in everyday life. Cancer is known for its unregulated cellular functions, increased pro-inflammatory tumor microenvironment, continuos activation of pro-survival transcription factors, having replicative immortality, promoting angiogenesis, and sustained cell proliferation. Many of the above-mentioned properties of cancer have been studied broadly *in vitro*, *in vivo*, pre-clinical and, clinical drug development. Among all cancer types, prostate, colorectal, breast, and lung are the most diagnosed types of cancer in Europe. Cancer treatment paradigms significantly changed with increasing knowledge about molecular biology during the past 15 years (Ferlay et al., 2013). These changes mostly occur in terms of novel drug development with multi-targeted effects and display fewer side effects, while efficiently inhibiting cancer cell growth.

NSAIDs are a drug class used for their anti-inflammatory purposes, treatment of pain and fever worldwide for a long time. Their common effect mechanism was mentioned in section 1.1.1. Due to the anti-inflammatory purposes of NSAIDs, their anticarcinogenic properties were investigated past few decades, since chronic inflammation is closely related to cancer development and progression. Plentiful studies are revealing cancer-preventive

effects of NSAIDs including breast, prostate, and colon cancer. Additionally, it was shown that NSAIDs can inhibit cancer progression by inducing apoptosis, enhancing cellular immune response and, inhibiting angiogenesis. However, NSAIDs are also known for their adverse effects such as GI-tract damage and cardiovascular disease development. To reduce adverse effects of NSAIDs, design, and synthesis of novel compounds that are more effective against inflammation and have fewer side effects needed to be found. In this thesis, two classes of NSAIDs were investigated for their anticancer activities; H_2S releasing compounds and isothiocyanate derivatives.

H_2S is known as the third gasotransmitter signaling molecule after NO and CO. It has many physiological properties mentioned in section 1.2. It even leads to apoptosis under various pathological conditions. In cancer, it was shown that H_2S can play a vital role in terms of cancer cell survival. H_2S inhibits the growth of PC-3 prostate cancer cells in a dose-dependent manner. H_2S production by CSE is also responsible for inhibition of growth of PC-3 cells when compared with CSE deficient mice (Pei et al., 2011). Since, cancer cells accumulate glucose at higher rates and convert glucose to lactate by H_2S induction, H^+ ions inside the cell increase as a result. If the activity of regulators of intracellular pH is disrupted by an agent, intracellular acidification leads cancer cells to death eventually. A candidate example for this process was the use of GYY4137. GYY4137 is a slow H_2S-donor, it induces glucose uptake, disrupts the export of H^+ ions accumulate inside the cell, and leads to cell death compared to its control compound which cannot release H_2S and had no effect (Lee et al., 2014). Additionally, GYY4137 was able to suppress signal transducer and activator of transcription 3 (STAT3) and its related downstream proteins and led to apoptosis in hepatocellular carcinoma cells. These findings suggest that H_2S and its related pathways can be manipulated to gain an advantage against cancer cells. In the current study, all of the S compounds which happen to release H_2S were tested against cell survival on MCF-7 breast, PC-3 prostate, and HT-29 colon cancer cell lines along with HUVEC healthy endothelial cells. All of the S compounds were firstly designed and synthesized after *in silico* analysis for their anti-inflammatory effects. All experiments carried out in the current study were for their secondary anticancer effects. Among all compounds, **S6** had IC_{50} values in every cell line under 100 µM dose for inhibition of cell proliferation. It is followed by **S2** and **S8** which had IC_{50} values under 100 µM dose against all cell lines except for HT-29. This suppression

may be related to inhibitory activities of H_2S mentioned above. However, as seen in table 4, not all the compounds were found cytotoxic. This can be due to the incapacity of the compounds to interfere with signaling pathways. More detailed studies are needed to enlighten the working mechanism of S compounds.

In the second part of the study, isothiocyanate derivatives were synthesized and tested for their anticancer effects. ITCs are sulfur-containing substances mainly found in cruciferous vegetables. They generally exert their anticancer effects firstly by modulating phase I and phase II enzymes; while downregulating phase I enzymes to prevent carcinogen activation, they upregulate phase II enzymes which are related to cytoprotective properties such as detoxification and prevention of reactive oxygen species induced damage. It is also known that ITCs can induce cell cycle arrest and eventually cause cell death. They effectively inhibit the occurrence of metastasis and angiogenesis. Another common feature of ITCs is they can release H_2S that can enhance their effectiveness against cancer cells. SFN is well known ITC for its anticancer activities and is a slow H_2S-donor. Interestingly, it was shown that H_2S release from SFN greatly inhibited PC-3 cancer cell growth, however, it lose its effectiveness when SFN was used with H_2S scavengers methemoglobin and oxidized glutathione (Pei et al., 2011). This report suggested that H_2S release partly modulates and enhances anticancer activities of ITCs. It is important to mention that the slow release of H_2S is favorable for drug compounds to have such preferable effects. In the current study, all three isothiocyanate derivatives were synthesized successfully. Their structures were confirmed with FT-IR, ^{1}H NMR, ^{13}C NMR and, MS spectroscopic methods. As mentioned above, I compounds except compound I1 also were firstly designed and synthesized after *in silico* analysis for their anti-inflammatory effects. All experiments carried out in the current study were for their secondary anticancer effects. Among all I compounds, at 100 µM dose, both the compounds **I1** and **I2** effectively inhibited proliferation of PC-3 prostate cancer cells by 78% and 91%, inhibited proliferation of MCF-7 cancer cells by 88.2% and 92.4%, and, inhibited the proliferation of HT-29 colon cancer cells by 81.5% and 82.5%, respectively. This inhibition may be related to ITCs anticancer effects as mentioned above and may be related to their H_2S-releasing capacities. Also, **I1** and **I2** effectively inhibited the proliferation of HUVEC healthy endothelial cells which showed that **I1** and **I2** were not selective against cancer cells. However, the **I3** compound was not able to inhibit the growth

of any cell lines. All of the I compounds released H_2S as shown in figure 11. According to the results, I compounds showed slow H_2S release as sulforaphane over 4 hours period. **I1** compound released 36.80 µM, **I2** compound released 35.46 µM and, **I3** compound released 35.70 µM, while, SFN released 43.45 µM H_2S. I compounds showed a similar release pattern as sulforaphane in terms of released H_2S concentration. Moringin (MIC-1) from *Moringa oleifera* is another well-known ITC that has many anti-inflammatory and anticancer activities was also tested for its H_2S releasing capacity. Its H_2S release pattern was similar to SFN and I compounds. Overall, all tested compounds and known H_2S-donors showed similar slow H_2S-releasing capacities over the same period of time, which is preferable for the drug compounds.

Bax is an apoptotic gene and Bcl-2 is an anti-apoptotic gene. The ratio of Bax/Bcl-2 determines the cell susceptibility to apoptosis. The higher the ratio, the more likely cells go through apoptosis. Effects of compound **I1** and **I2** on Bax/Bcl-2 mRNA expression levels in MCF-7 breast cancer cells were investigated and only compound **I1** was found effective with 1.58-fold at 104 µM. This finding suggests that compound **I1** may lead breast cancer cells to apoptosis by Bax/Bcl-2-mediated gene expression.

Originally all of the compounds including the S and I series were synthesized to evaluate their anti-inflammatory activities. However, due to the dissolve problem of the S series and giving orange-yellowish color to the assays related to anti-inflammatory activity. The occurrence of such problems led biological activities towards anticarcinogenic activity, which is the secondary part of the same TUBİTAK project. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay is used for cell viability assays and, normally, at the end of MTT assay, wells should be pink in order to get accurate results after absorbance reading at 570 nm. However, as seen in figure 16, the S series made the wells darker even at the end of the experiment, which, interferes with the absorbance reading.

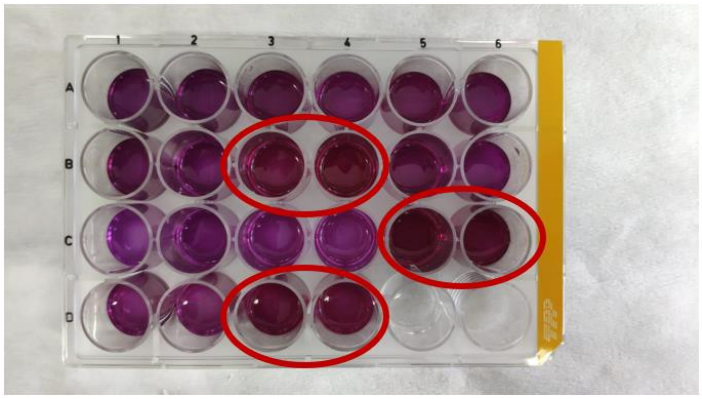


Figure 16. Color occurrence in MTT assay after S series treatment.

Even cells were washed with phosphate buffer saline (PBS), S series were still giving colors to the wells as seen in figure 17, which suggested S series could be sticky and present at the bottom of the well and, with the addition of the media, PBS, etc., compounds partially dissolve in the environment.

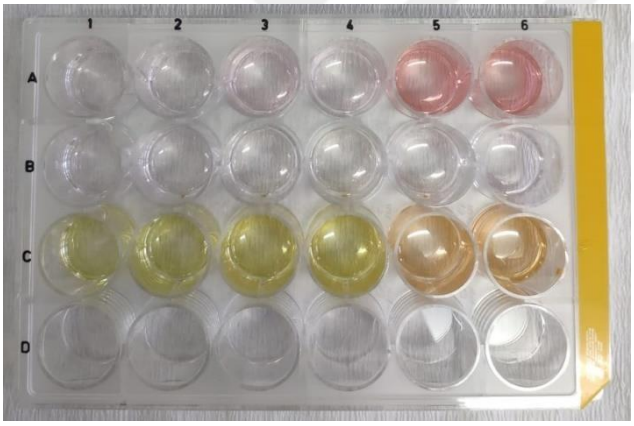


Figure 17. Color occurrence in the wells of S series after PBS wash.

The same problem was happened in the NO and ELISA assays due to color change in the medium, which prevented measurement of components from media with interference with absorbance reading as seen in figure 18.

Figure 18. Color occurrence in the wells of S series in medium collected from cells.

In the light of the situations mentioned above, anticarcinogenic activities of the S series were carried out along with I compounds which the results of the studies were mentioned in this thesis.

CHAPTER 5
CONCLUSION

In this study, novel H_2S releasing compounds (S series) and naturally H_2S releasing isothiocyanate derivatives (I Series) were tested against cancer cell lines for their anticancer activities. Among all H_2S releasing compounds, the compounds **S6** and **S8** showed promising antiproliferative activity against MCF-7 breast and PC-3 prostate cancer cell lines. In the second part of the study, three ITC derivatives were synthesized and their structures were confirmed with FT-IR, ^{1}H NMR, ^{13}C NMR and, MS analysis. Then, they were also tested for their anticancer activity, and among all three ITC derivatives, compounds **I1** and **I2** were found effective against MCF-7 and PC-3 cancer cells, only **I2** was found effective against HT-29 cancer cells. **I1** was able to increase Bax/Bcl-2 mRNA expression levels which suggest that cells may be going through apoptosis. Additionally, the H_2S releasing capacities of synthesized ITC derivatives were measured. The release of H_2S in the cancer environment is associated with inhibition of growth of cancer cells and, slow release of H_2S is superior over immediate release for drug compounds. All ITC derivatives showed preferable slow H_2S release as sulforaphane and MIC-1 which are well-known ITCs over 4 hours time period. Collectively, all these findings suggested that **S6**, **S8**, **I1** and, **I2** may present promising scaffolds as new anticancer agents which can be further developed by *in silico* design and be tested by more detailed *in vitro*/*in vivo* studies.

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APPENDIX

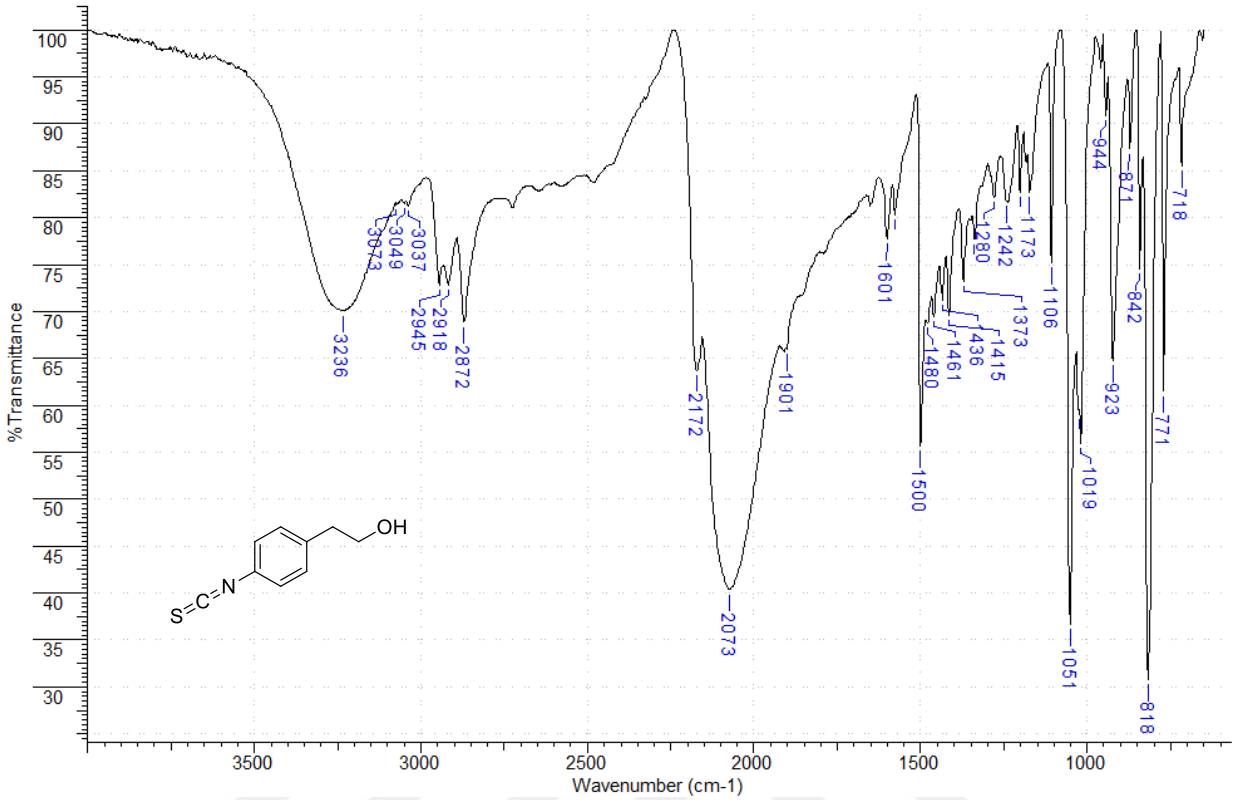

Figure 1. FT-IR spectrum of 2-(4-Isothiocyanatophenyl)ethan-1-ol (**I1**).

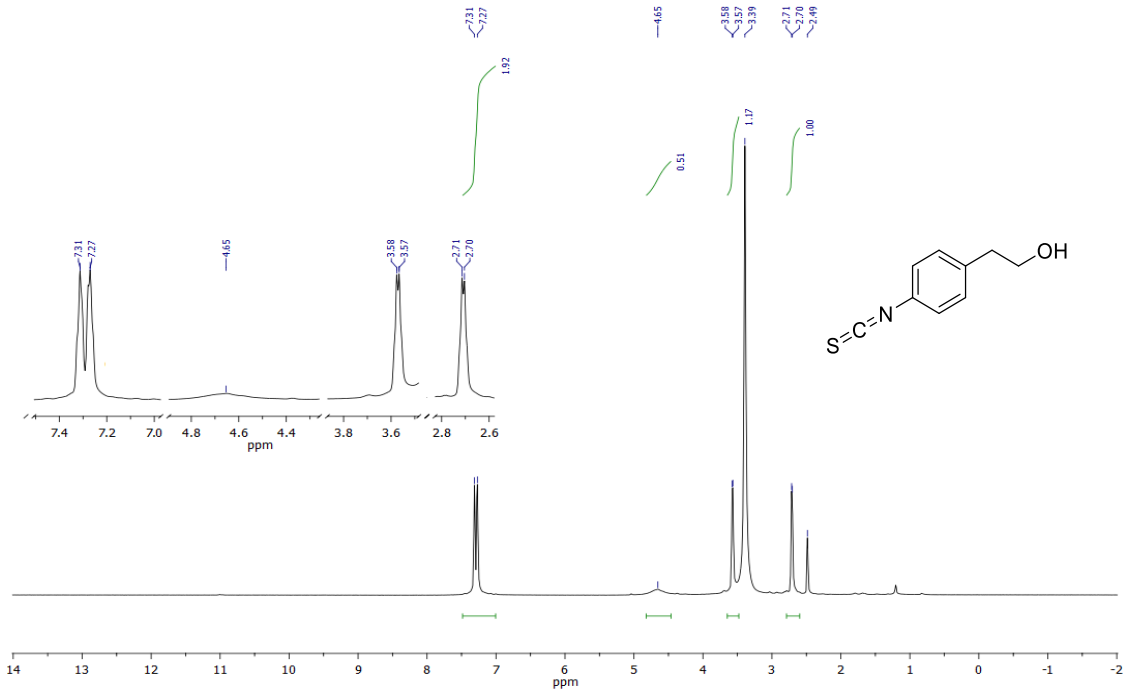

Figure 2. ^{1}H NMR spectrum of 2-(4-Isothiocyanatophenyl)ethan-1-ol (**I1**).

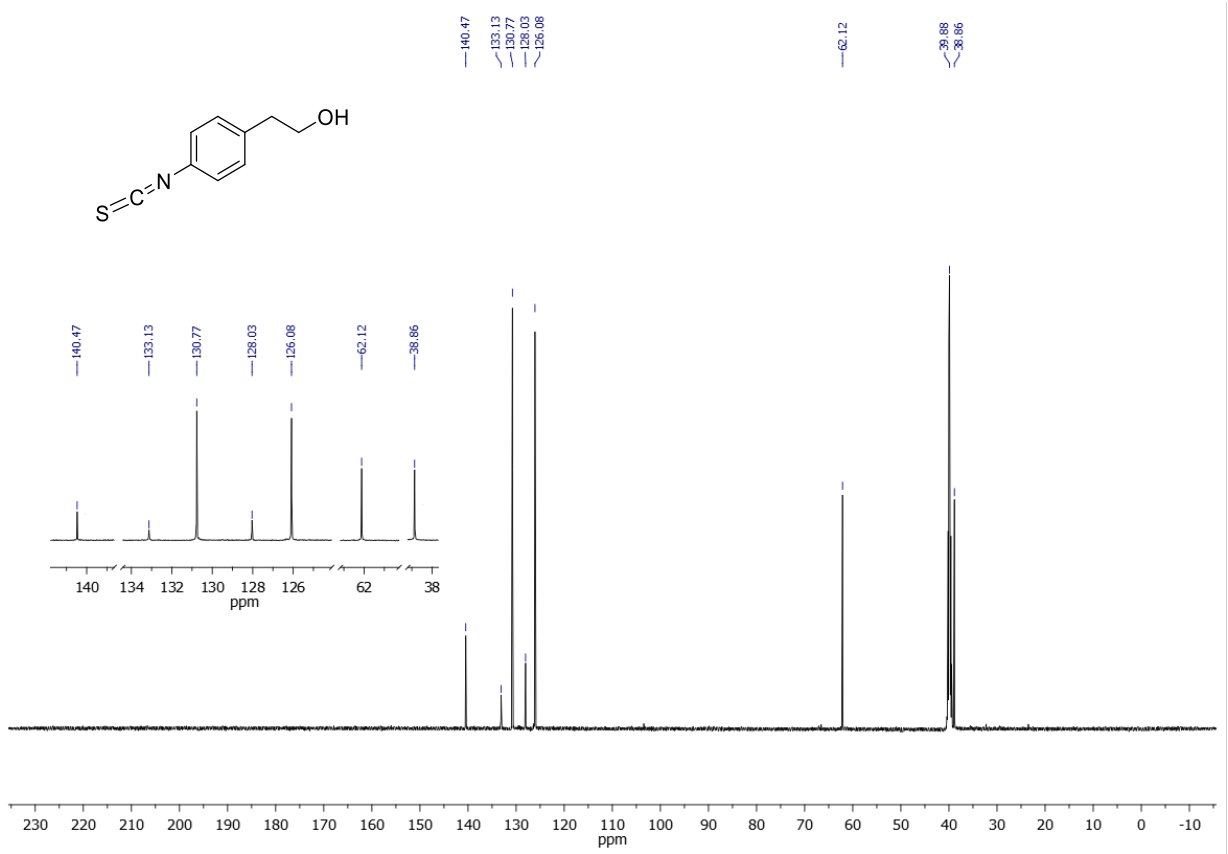

Figure 3. ^{13}C NMR spectrum of 2-(4-Isothiocyanatophenyl)ethan-1-ol (**I1**).

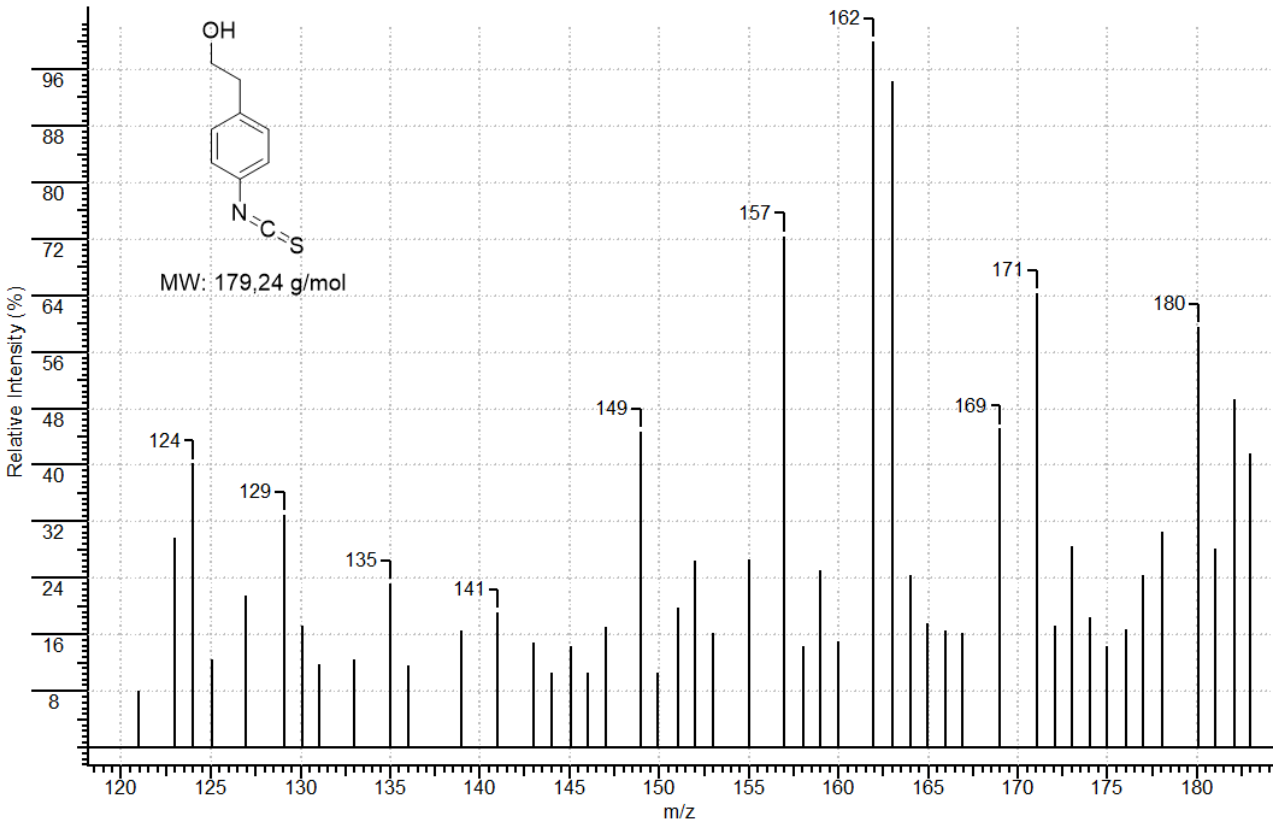

Figure 4. LC/MS spectrum of 2-(4-Isothiocyanatophenyl)ethan-1-ol (**I1**).

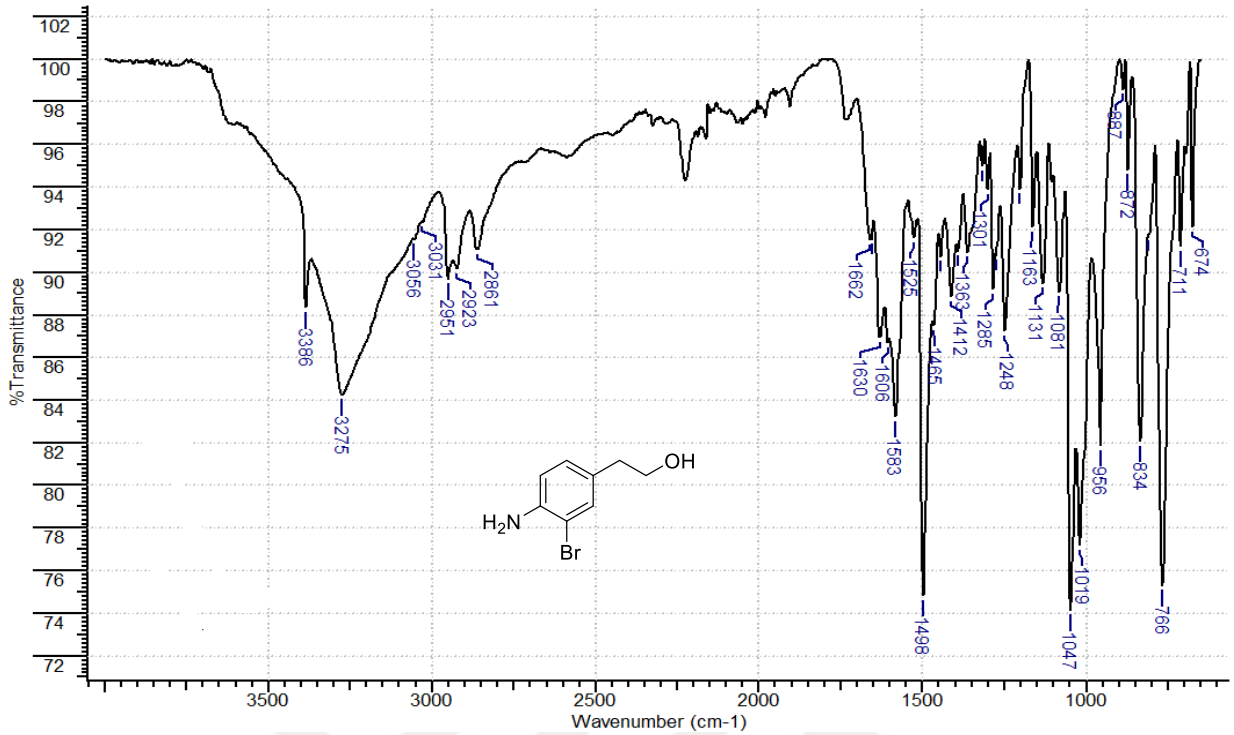

Figure 5. FT-IR spectrum of 2-(4-Amino-3-bromophenyl)ethan-1-ol (**APE-Br**).

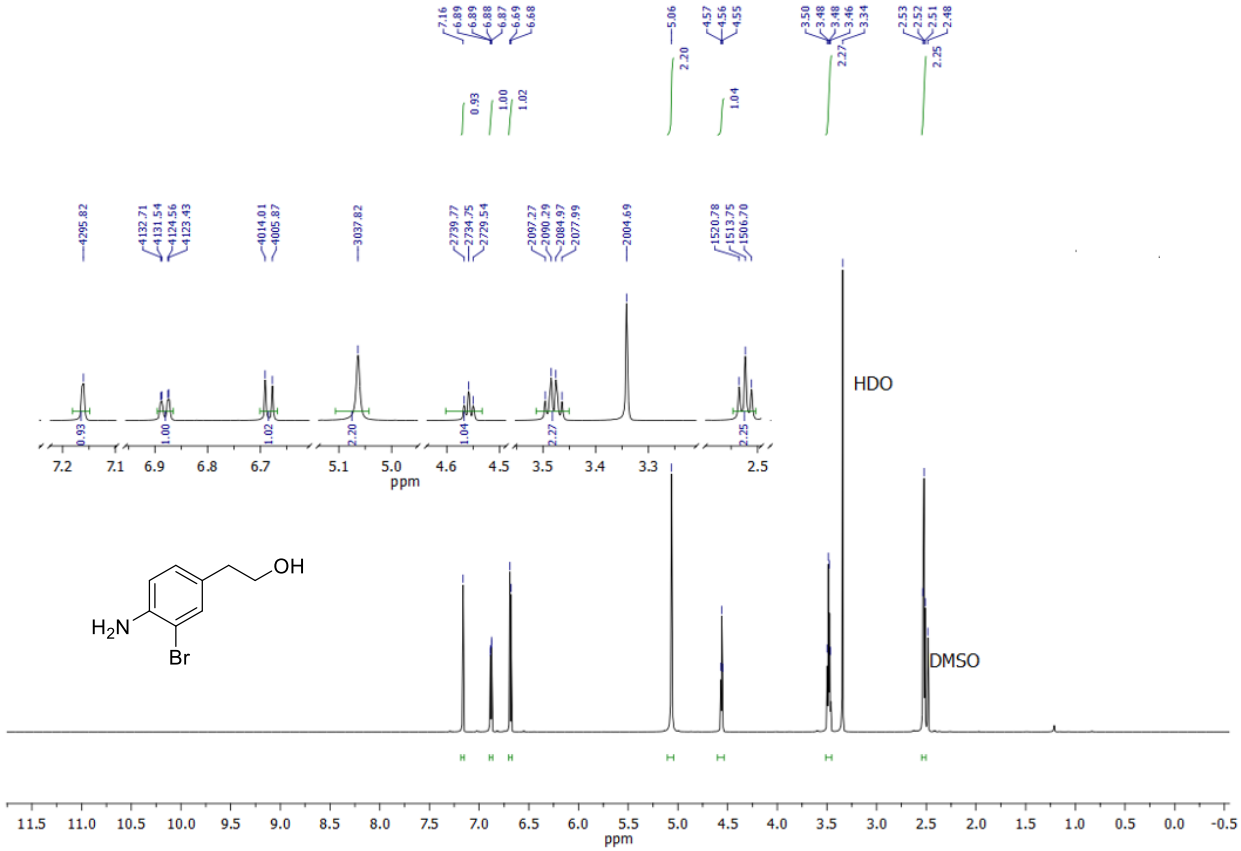

Figure 6. ^{1}H NMR spectrum of 2-(4-Amino-3-bromophenyl)ethan-1-ol (**APE-Br**).

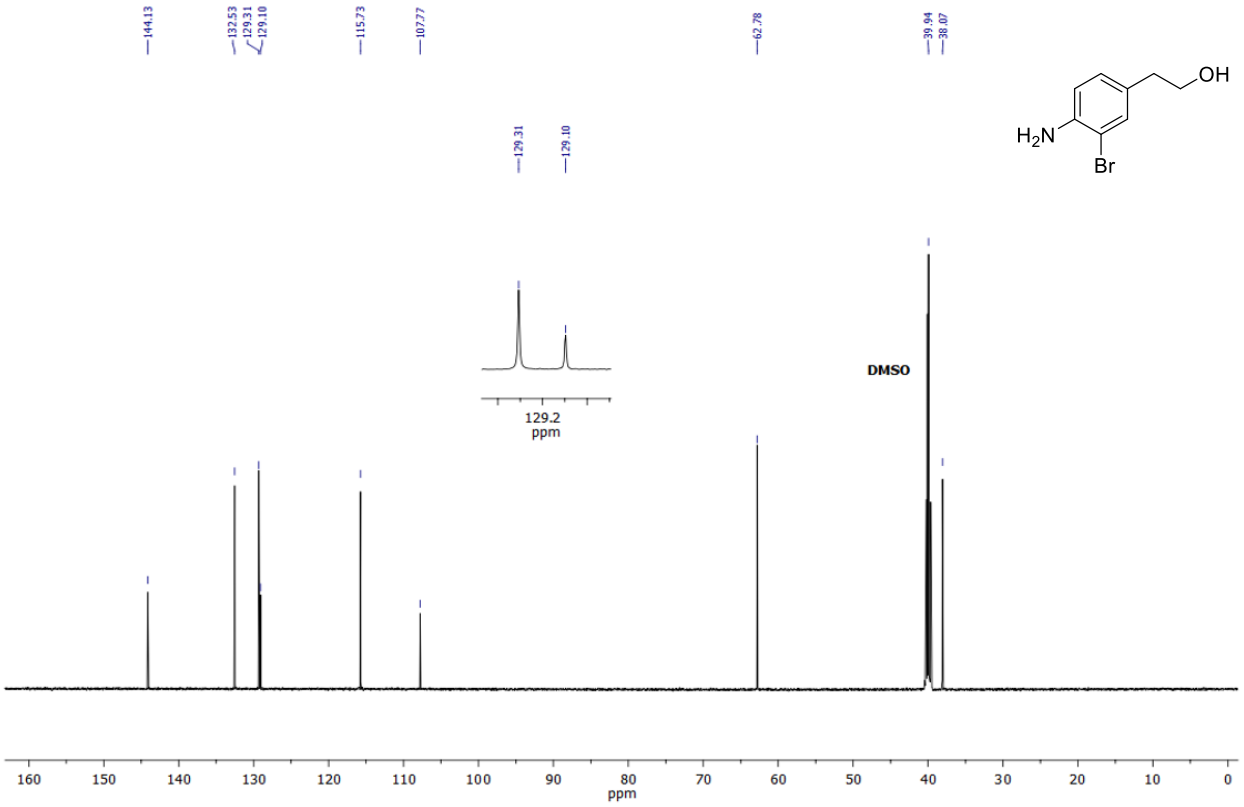

Figure 7. ^{13}C NMR spectrum of 2-(4-Amino-3-bromophenyl)ethan-1-ol (**APE-Br**).

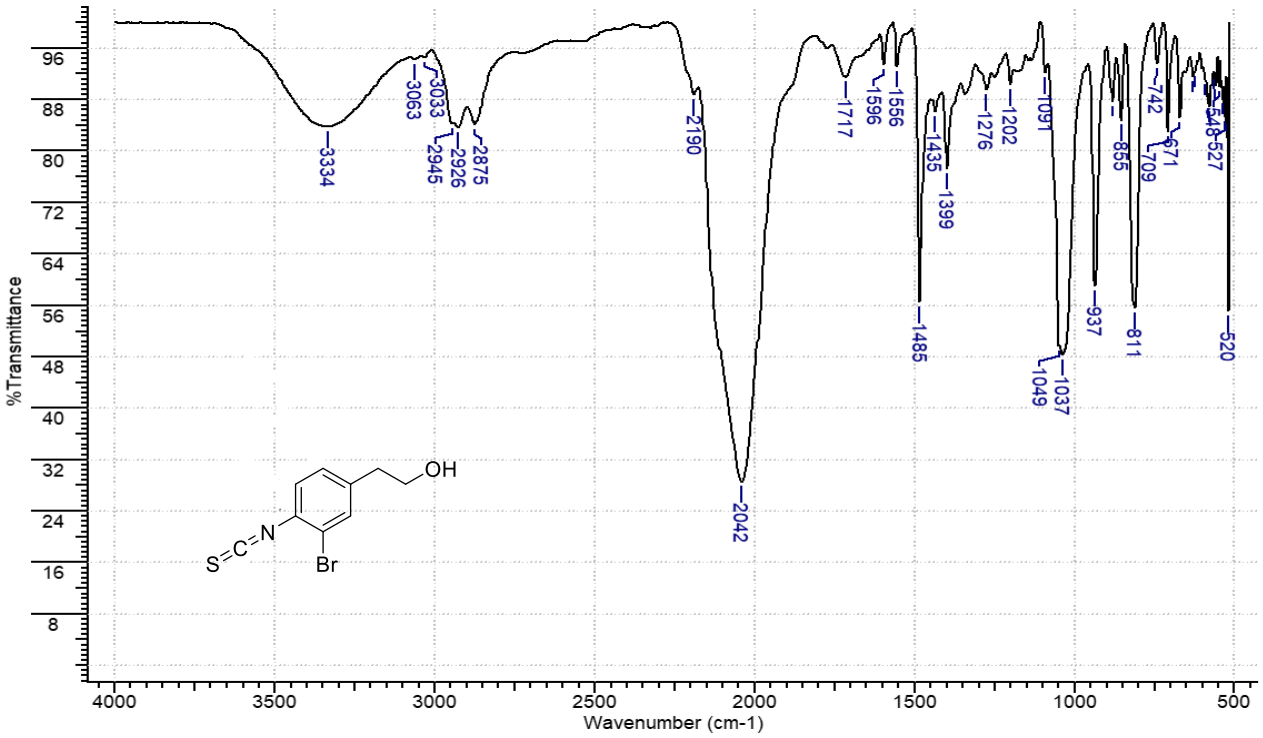

Figure 8. FT-IR spectrum of 2-(3-Bromo-4-isothiocyanatophenyl)etan-1-ol (**I2**).

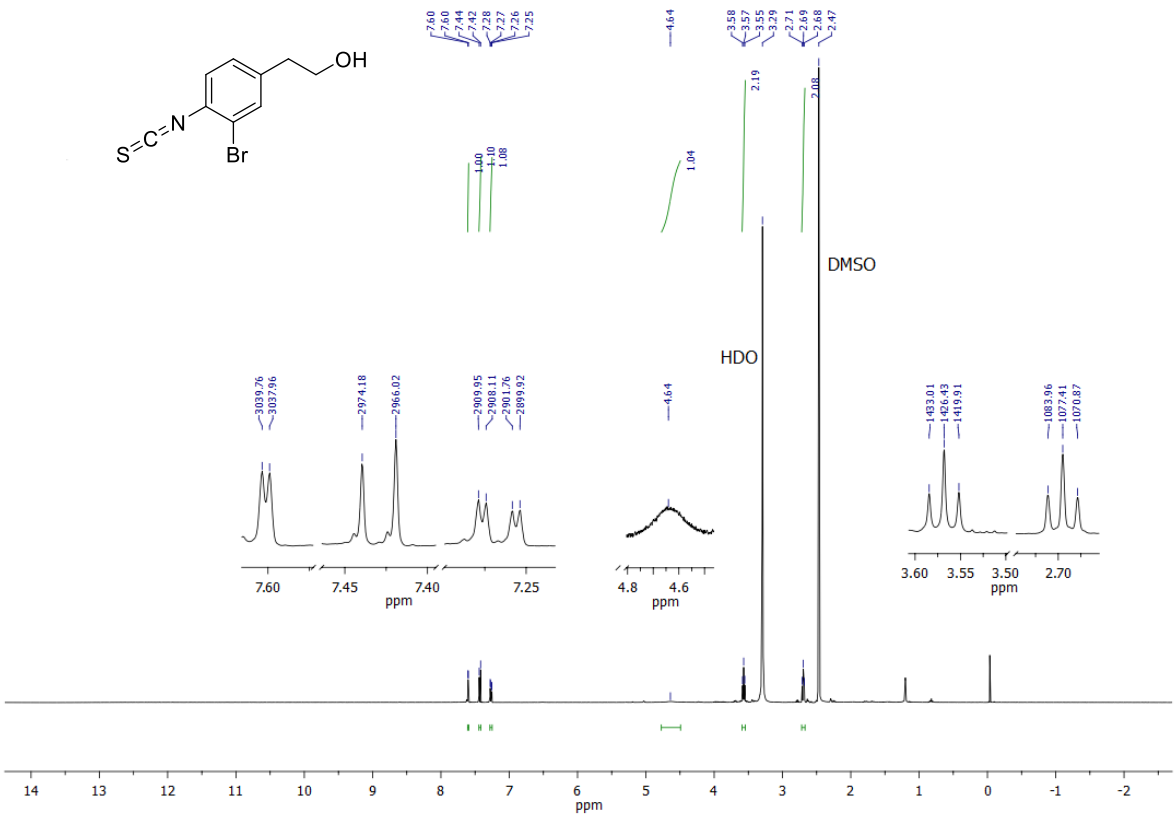

Figure 9. ^{1}H NMR spectrum of 2-(3-Bromo-4-isothiocyanatophenyl)etan-1-ol (**I2**).

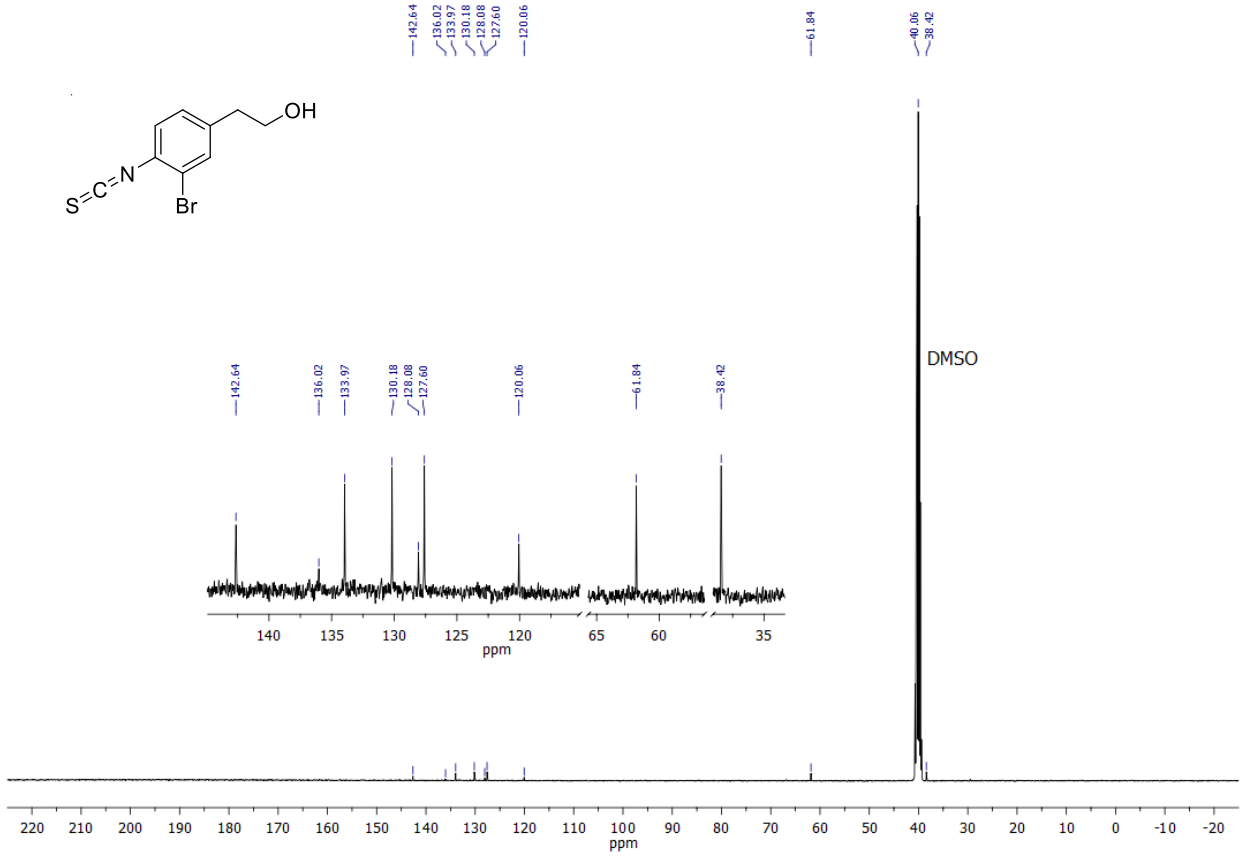

Figure 10. ^{13}C NMR spectrum of 2-(3-Bromo-4-isothiocyanatophenyl)etan-1-ol (**I2**).

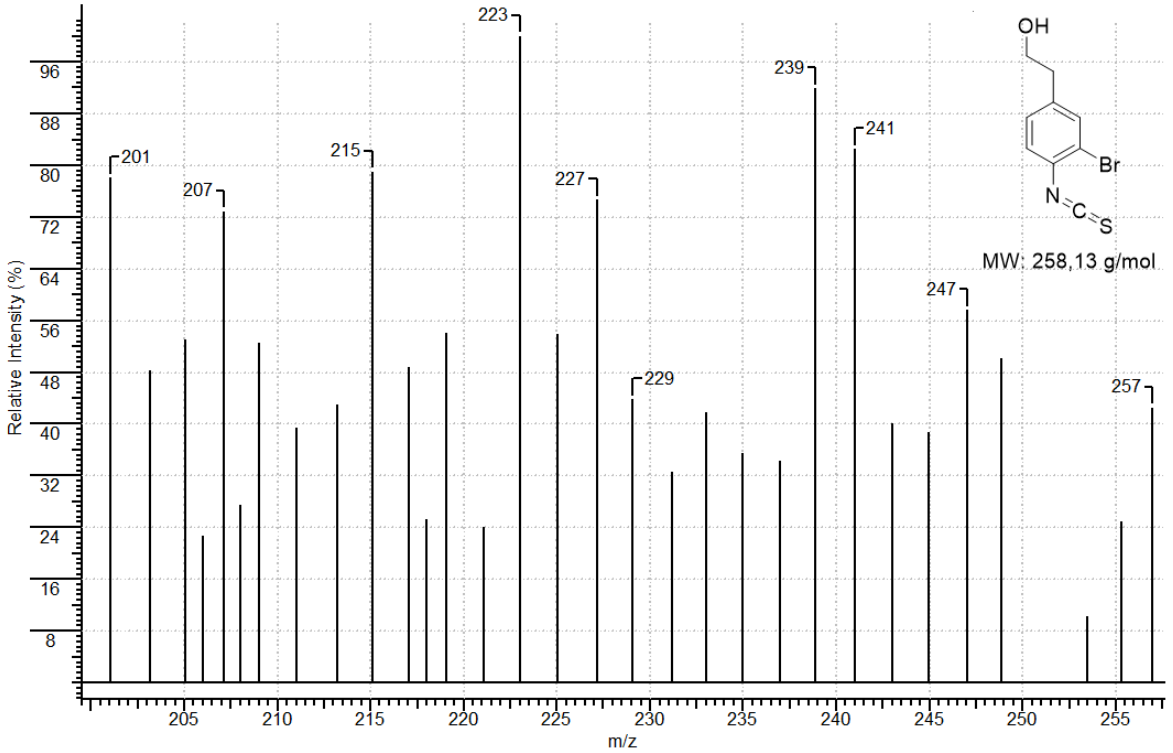

Figure 11. LC/MS spectrum of 2-(3-Bromo-4-isothiocyanatophenyl)etan-1-ol (**I2)**.

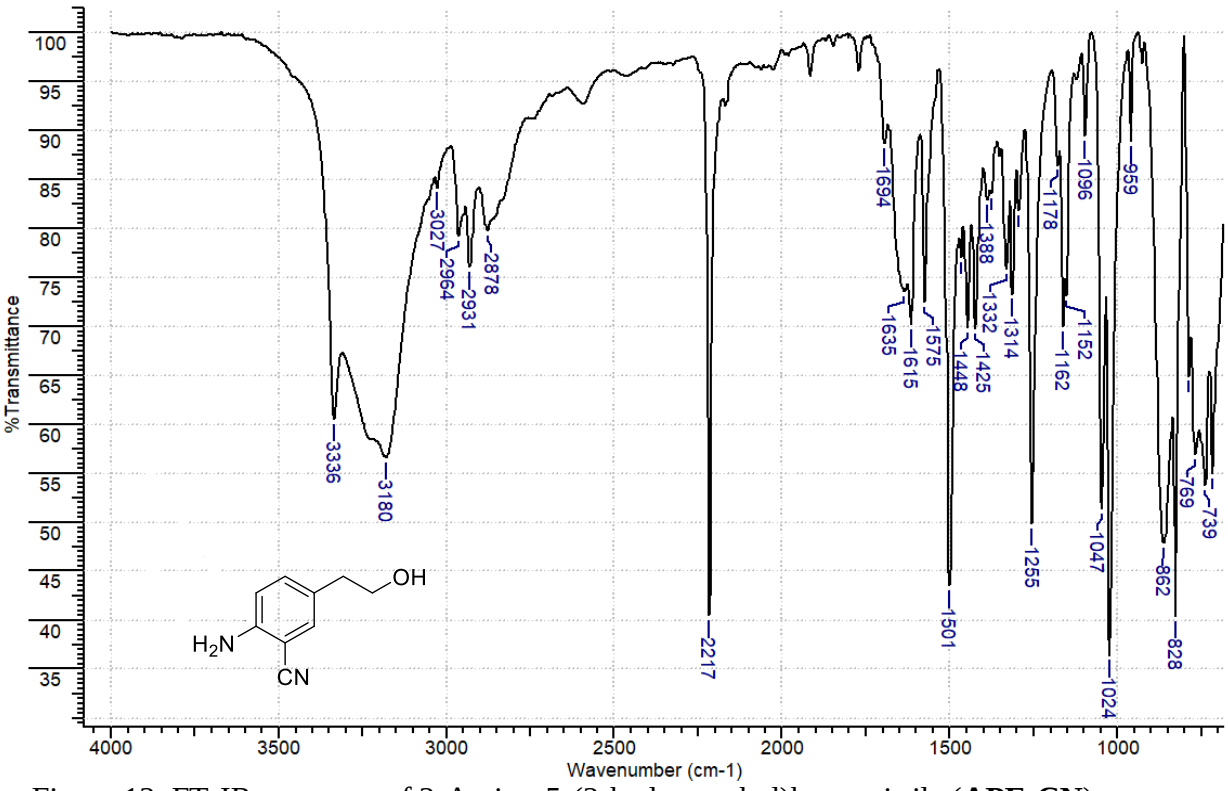

Figure 12. FT-IR spectrum of 2-Amino-5-(2-hydroxyethyl)benzonitrile (**APE-CN**).

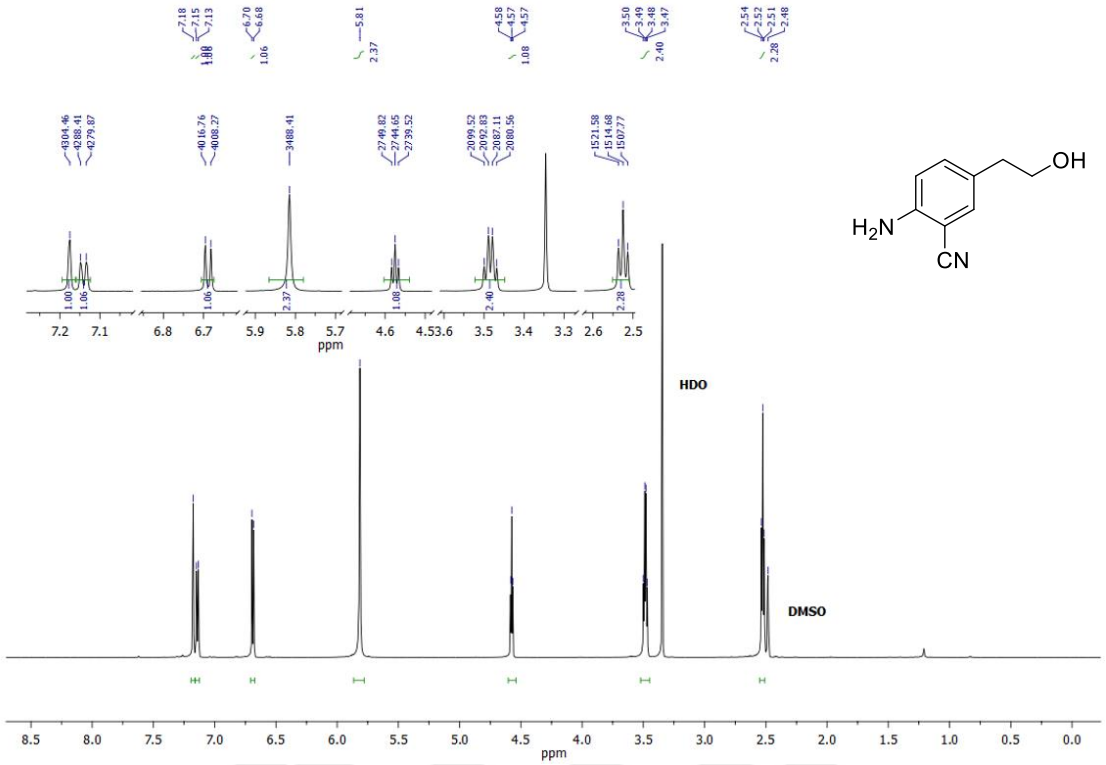

Figure 13. ^{1}H NMR spectrum of 2-Amino-5-(2-hydroxyethyl)benzonitrile (**APE-CN**).

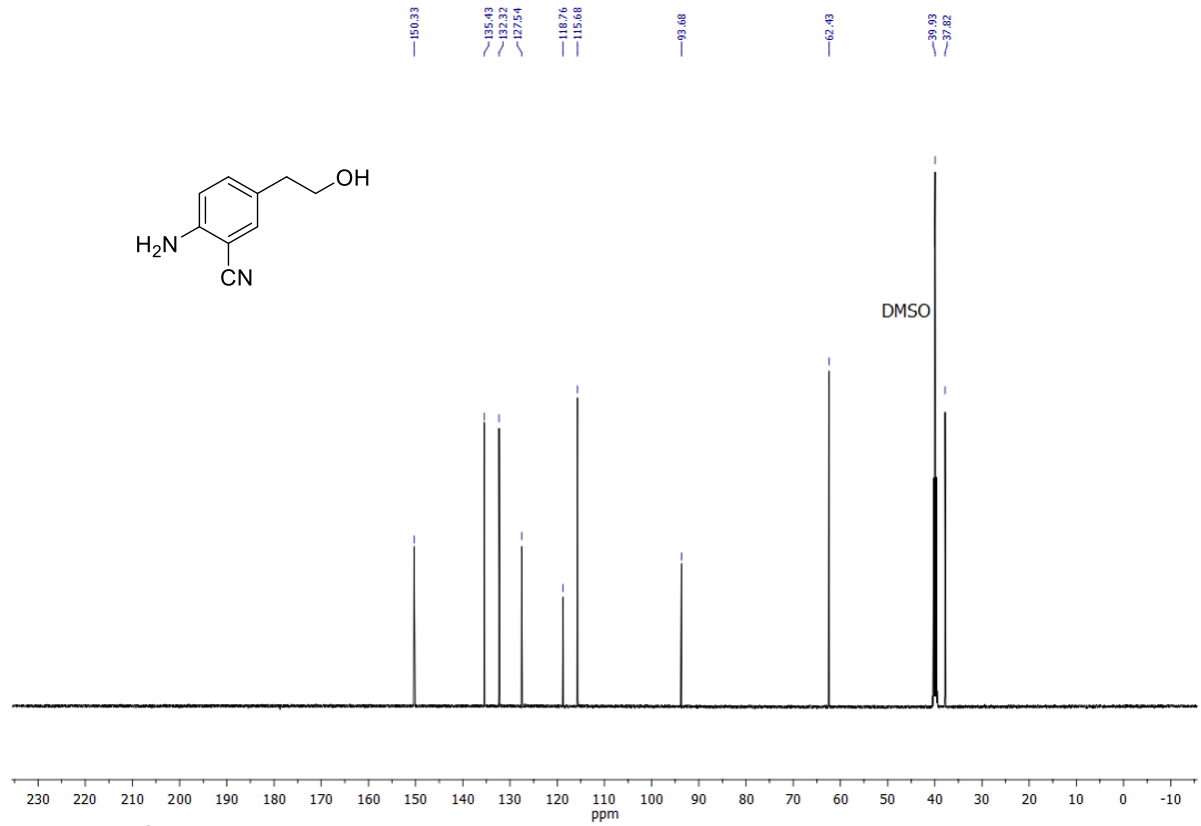

Figure 14. ^{13}C NMR spectrum of 2-Amino-5-(2-hydroxyethyl)benzonitrile (**APE-CN**).

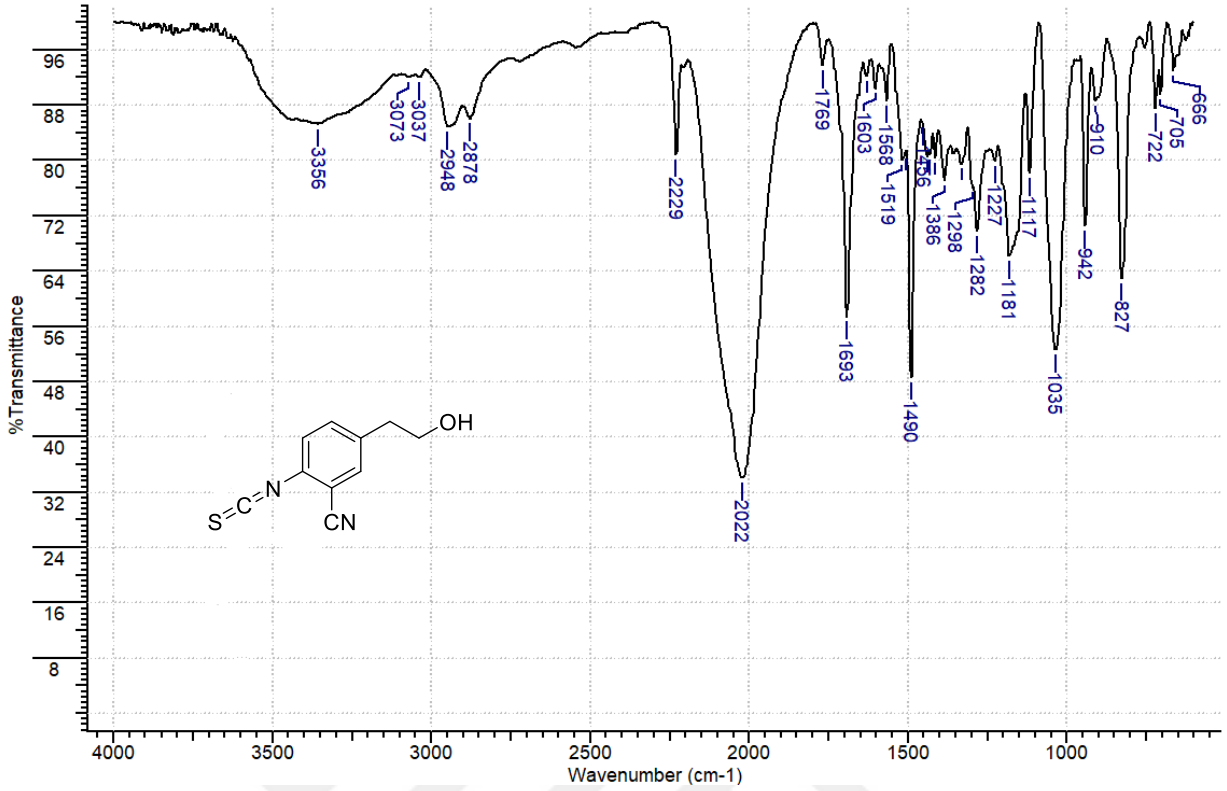

Figure 15. FT-IR spectrum of 5-(2-Hydroxyethyl)-2-isothiocyanatobenzonitrile (**I3**).

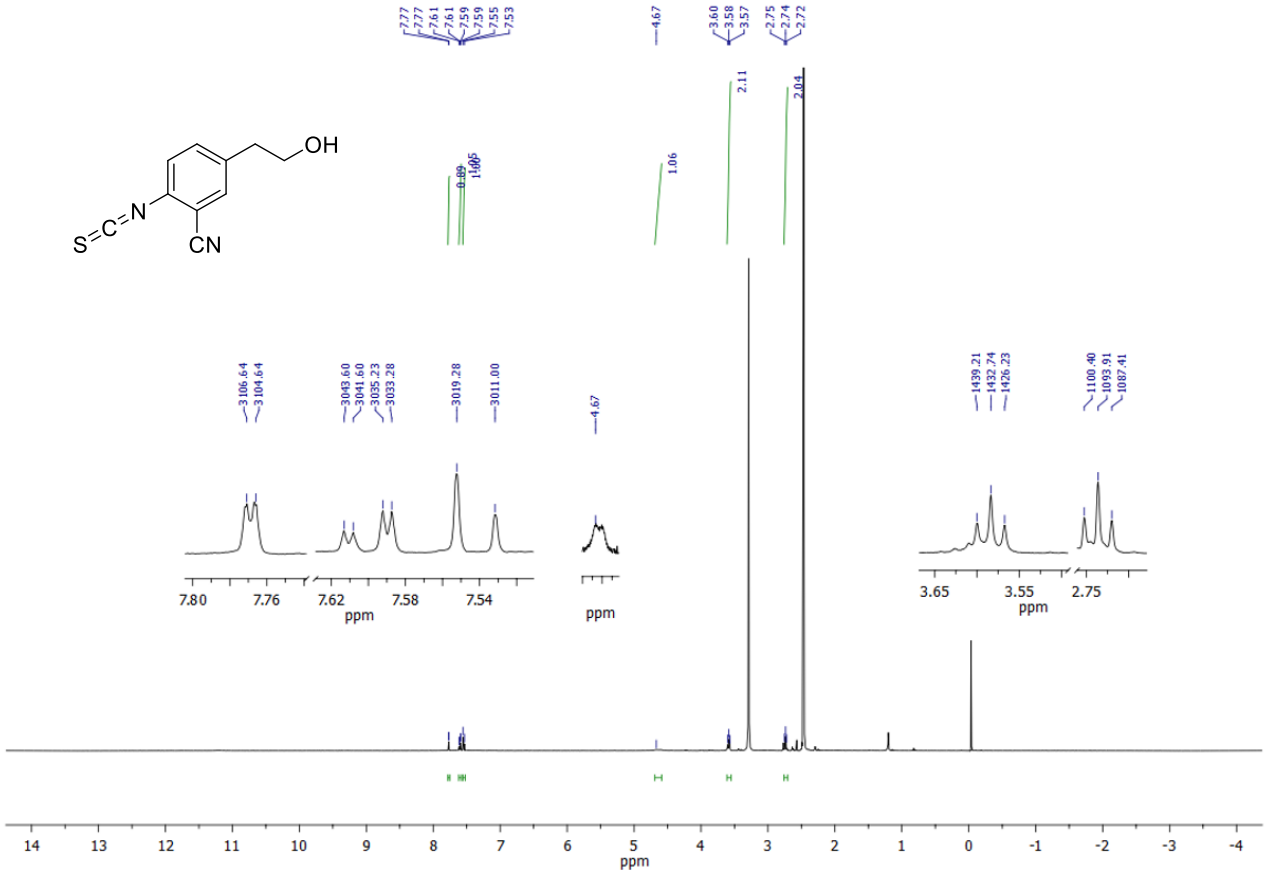

Figure 16. ^{1}H NMR spectrum of 5-(2-Hydroxyethyl)-2-isothiocyanatobenzonitrile (**I3**).

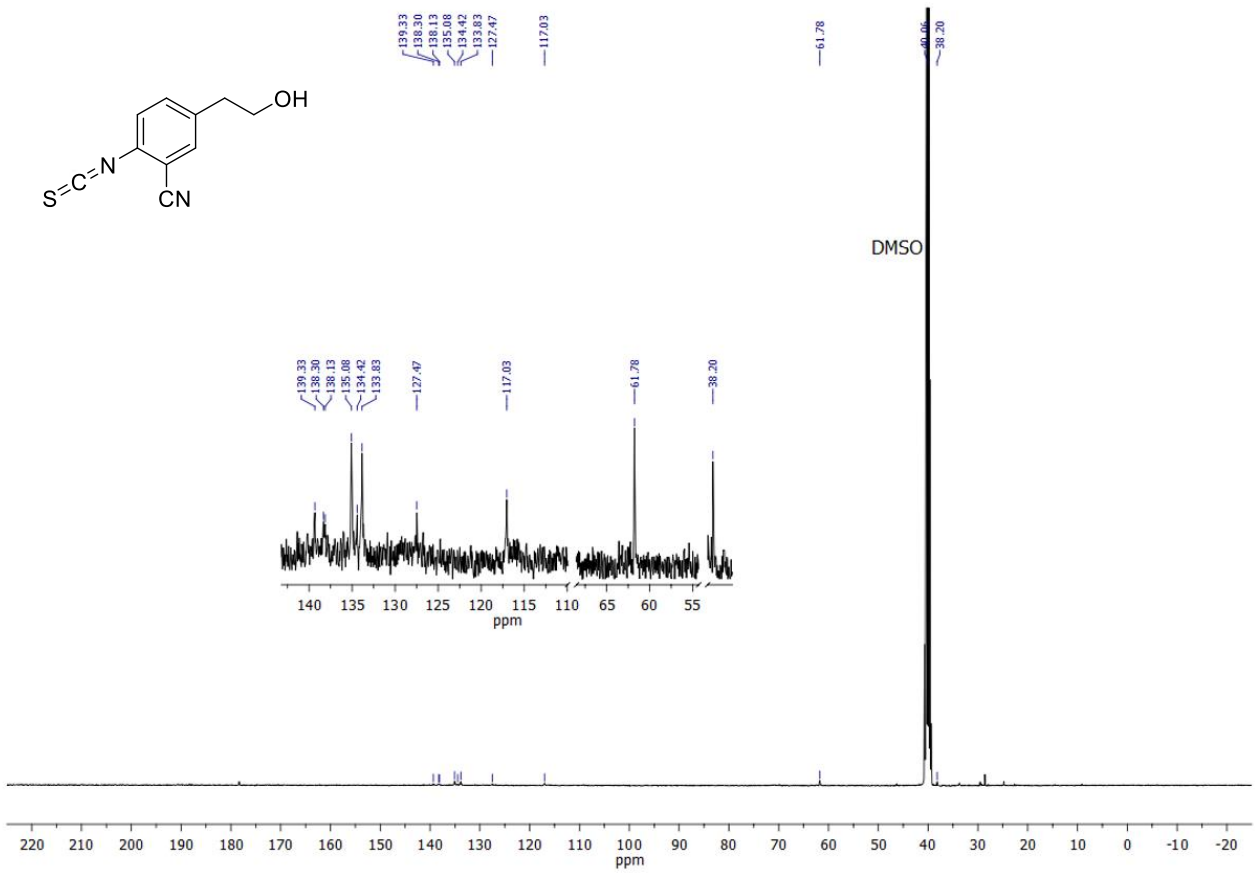

Figure 17. ^{13}C NMR spectrum of 5-(2-Hydroxyethyl)-2-isothiocyanatobenzonitrile (**I3**).

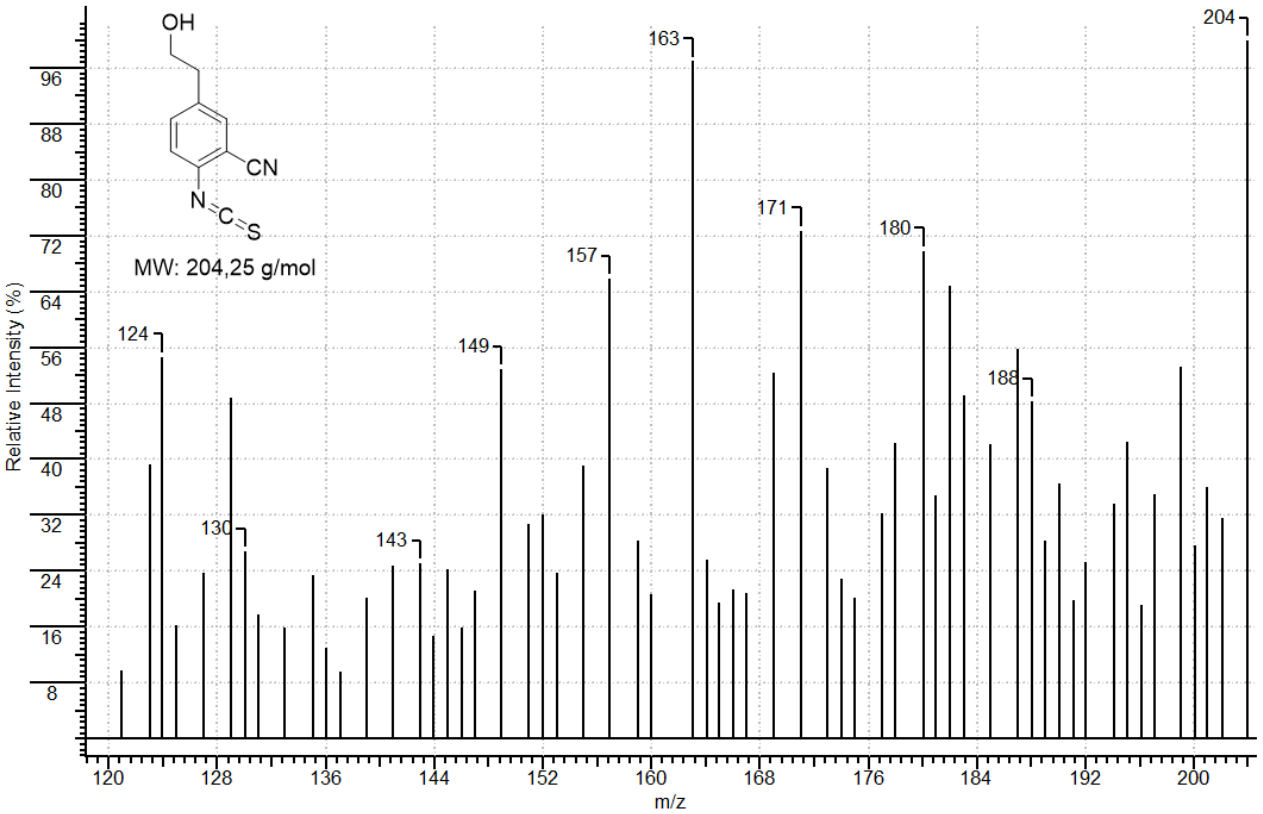

Figure 18. LC/MS spectrum of 5-(2-Hydroxyethyl)-2-isothiocyanatobenzonitrile (**I3**).