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SYNTHESIS AND BIOLOGICAL ACTIVITY STUDIES ON 4-NITROPHENYLPIPERAZINE SUBSTITUTED 2(3H)-BENZOXAZOLONE DERIVATIVES

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APPROVAL PAGE

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ABSTRACT

The main objective of the pharmaceutical chemistry is to synthesize the compounds that show promising activity as therapeutic agents with lower toxicity. 2-(3H)-Benzoxazolone and its derivatives are compounds having diverse pharmacological activities. Recent studies show that these molecules could also show promising cytotoxic activity. Consequently, they can be used in the development of new candidates for anticancer drugs.

In this research study, three different 2-(3H)-Benzoxazolone derivatives were synthesized. Compound **1**, 6-[4-(4-nitrophenyl) piperazin-1-yl]acyl-2-Benzoxazolone was made by substitution reaction at the sixth position of benzoxazolone derivative at room temperature. Compound **2**, 3[4-(4-nitrophenyl) piperazin-1-yl[methyl-2-Benzoxazolone and compound **3**, 5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl[methyl-2-benzoxazolone were synthesized by substitution in the third position of benzoxazolone core structure via Mannich reaction, using reflux method. The identification and characterization of the synthesized compounds were carried out by melting point determination, thin layer chromatography, FT-IR and ^{1}H NMR.

The synthesized compounds were tested for cytotoxicity activity using MTT-assay. Our results show that compounds **2** and **3** have promising cytotoxic activity at low concentrations (50 μM) at 48h incubation time, as they were effective in inhibiting cell viability and growth of MCF-7 cell line.

Keywords: 2(3H)-Benzoxazolone, Mannich Reaction, Cytotoxicity, MTT-assay

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LIST OF ABBREVIATIONS

NSAIDs	Non-Steroidal Anti-inflammatory Drugs
FT-IR	Fourier Transform Infra-red
H NMR	Proton Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ADME	Absorption, Distribution, Metabolism and Excretion
PPA	Polyphosphoric Acid
CA-4	Combretastatin A-4
MES	Maximal electroshock
MIC	Minimum inhibitory concentration
NC	Nucleocapsid protein
HIV	Human immunodeficiency virus
LDL	Low-density lipoprotein
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
ET_3N	Triethylamine
s	Singlet
m	Multiplet
t	Triplet
Pip	Piperazine
Arom	Aromatic

1. INTRODUCTION

2(3H)-Benzoxazolone and its derivatives are important compounds in the medicinal chemistry. Since they can be used as starting materials for synthesis of various compounds with diverse biological activities. These activities include anticancer, anti-inflammatory, hypnotic, analgesic, anticonvulsant, antimicrobial. Also they are in demand because they can be easily modified and not expensive to obtain. (1-3)

The nitrogen atom that present at the third position of the 2(3H)-Benzoxazolone moiety play important role in its biological activity due to the electronic characteristics of the atom (4). Potentially useful drug has been developed in recent years based on these pharmacophores for example: chlorzoxazone (sedative analgesic). (5,6)

Cancer is a group of complex diseases characterized by uncontrolled cell division. In recent years, the morbidity and mortality of cancer has reached a high level and is a major public health problem worldwide. (7) nowadays, there are several classes of drugs that have therapeutic effects against tumor cells. The major problem is that they lack selectivity toward cancer cells which lead to severe adverse effect. Finding and synthesis of novel small molecules that have ability to selectively block cancer cell consider now as one of major medicinal chemist goals and point of view of modern medicinal chemistry. Therefore, new molecule needs to be found urgently that could be effective candidate for anticancer activity (6,7)

The aim of this research study, is the synthesis of 2(3H)-Benzoxazolone derivatives by substitution reaction in two different positions, namely third and sixth positions. Third position substitution was done with 2(3H)-Benzoxazolone and 5-chloro-2(3H)-Benzoxazolone via Mannich reaction by using reflux method. Sixth position substitution was done with acylbenzoxazolone at room temperature. The compounds were characterized by Proton Nuclear Magnetic Resonance (^{1}H-NMR) and Fourier Transform Infra-Red (FT-IR) spectroscopy. The purity of the compounds was determined by melting point and thin layer chromatography (TLC). The cytotoxicity of compounds was tested by using MTT assay toward MCF-7 cell line.

2. LITERATURE REVIEW

2.1 Anticancer

Cancer is a large group of complex diseases that have different type and can be seen in different locations but have one thing in common that is the abnormal cells growing out of control. Is consider as one of the most serious and dangerous diseases in the world. (8,9)

Anticancer drugs, also named antineoplastic drugs, agents that demonstrate activity against cancerous disease. They are classified to several classes for example: alkylating agents, antimetabolites, natural products, and hormones.

The selection of anticancer drug depends on several factors, which include the type and location of the disease, severity of cancer, whether radiation therapy or surgery have to be used or not, and also the adverse effects related to the drug.

Chemotherapy is mostly used for treating cancer. Cross resistance associated with the specificity and selectivity of drugs that is used now has restricted the application of chemotherapy. Because of the failure of available chemotherapeutics to fully treat cancer there is an urgent need for developing new chemical molecule as anticancer candidate. (7,10)

One of the most important tests for evaluation of anticancer agent is cytotoxicity tests and can be consider as one of the first steps in discovery new anticancer drug. (10)

2.2 Cytotoxicity

Cytotoxicity is a description of the extent of the killing or destructive capacity of an agent. Most often used to describe toxicity of certain drugs or biological active compounds that limit the development of cancer cells. It is an in vitro test and consider as one of the most essential indicators in research studies for biological evaluation. It is a useful first step in

assessing a test substance's potential toxicity which include biologically active compounds. (11)

Because minimal to no toxicity is important for the successful developing a new drug candidate, cytotoxicity tests are widely performed. Alternatively, chemicals can be intended to act as anticancer drugs, where selective cytotoxicity to cancer cells is vitally important and cell toxicity studies play a key role in this regard. (12)

It is in vitro cell culture systems, if a tested chemical compound interferes with attachment of cells is considered cytotoxic. This interfering includes changing morphology or when the rate of cell growth is adversely affected or inducing cell death. A dependable, not expensive and reproducible short-term cytotoxicity and cell viability assays are needed to detect the cell death caused by these pathways

In either the fields of pharmacology or medicinal chemistry, variable types of cytotoxicity assays are currently being used. These assays have different classifications:1) Dye exclusion: Trypan blue, eosin, Congo red, erythrosine B assays. 2) Colorimetric assays: MTT assay, MTS assay, XTT assay, WST-1 assay, WST-8 assay, LDH assay, SRB assay, NRU assay and Crystal Violet assay. 3) Fluorometric assays: alamarBlue assay and CFDA-AM assay. 4) Luminometric assays: ATP assay and real-time viability assay

It's important to choose the appropriate method among these assays to ensure precise, reliable results. There are several factors to consider when choosing the cytotoxicity and cell viability tests to be used in the study. Such as the availability in the laboratory where the study is to be conducted, test compounds, detection mechanism, sensitivity and specificity. (11,12)

Cellular testing is less humanely ambiguous, easy to control and reproduce, and less expensive in comparison to animal studies. Because of their sensitivity to change in environment there are factors important to consider in cytotoxicity experiments to get accurate results these include the concentration of the potentially toxic agent being tested, variations in temperature, pH and concentrations of nutrients and waste may affect the results. Therefore, to ensure the measured cell death corresponds to the toxicity of the added compounds, it is important to control the experimental conditions. (14)

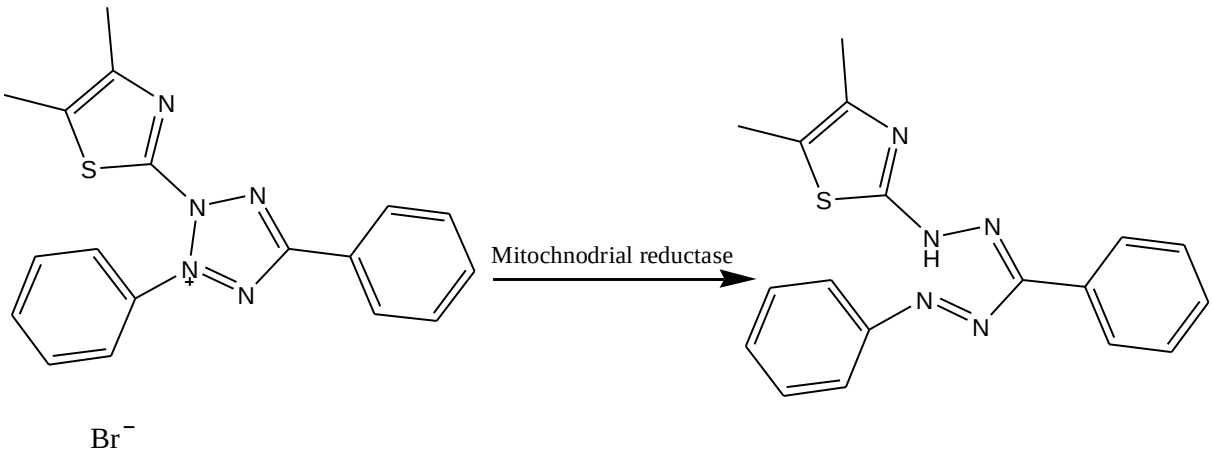
2.2.1 Colorimetric assays

The colorimetric assay principle is the evaluation of a biochemical marker to assess the cells ' metabolic activity. Reagents utilized in colorimetric assays generate a color in response to cell viability, enabling colorimetric estimation of cell viability through a spectrophotometer. For adherent or suspended cell lines, colorimetric assays are applied, simple to perform and are comparatively economical. Commercial colorimetric assay kits are accessible from numerous companies and experimental procedures of these assays are usually available in kit packages. (15)

These colorimetric assays can be further classified into tests measuring mitochondrial activity and integrity of the plasma membrane.

2.2.2 MTT assays

The MTT assay is a colorimetric reaction, a sensitive and reliable indicator of the cellular metabolic activity. It depends on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to reduce the yellow water-soluble tetrazolium dye -[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to an insoluble MTT-formazan product which has a purple color. In the mitochondria of living cells this enzymatic conversion of the tetrazolium compound into water-insoluble formazan crystals by dehydrogenases is take place. This test therefore evaluates the viability of cells in terms of reduction activity, as well as reducing agents and enzymes existed in other organelles, such as the endoplasmic reticulum are also included. (16,17) A solubilization solution (acidified ethanol or dimethyl sulfoxide) is used in the MTT assay to break down the insoluble formazan product into a colored solution. by a spectrophotometer the colored solution absorbance can then be quantified measuring at a specific wavelength (mostly between 500 and 600 nm).

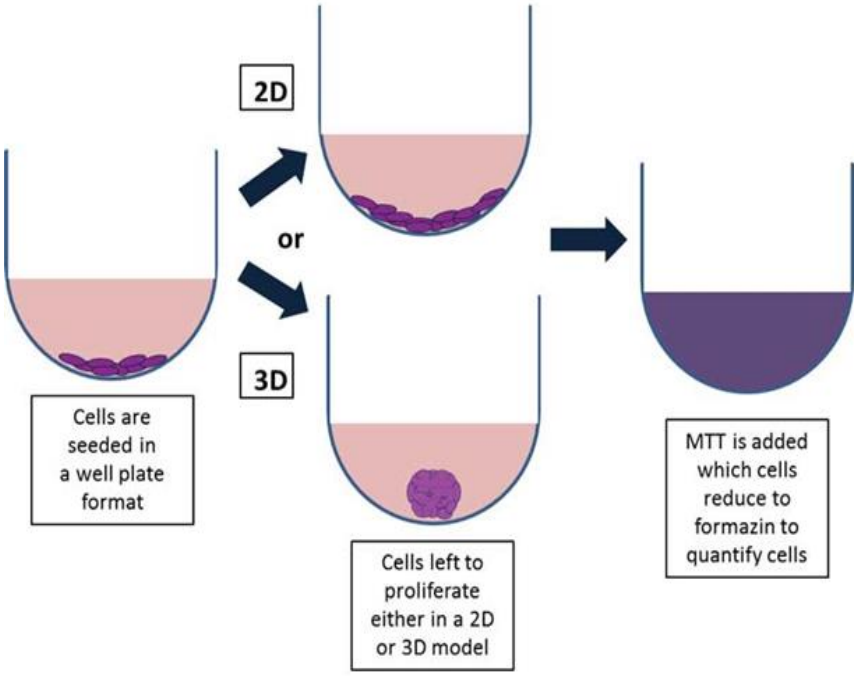


3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl -2H-tetrazolium bromide (MTT)

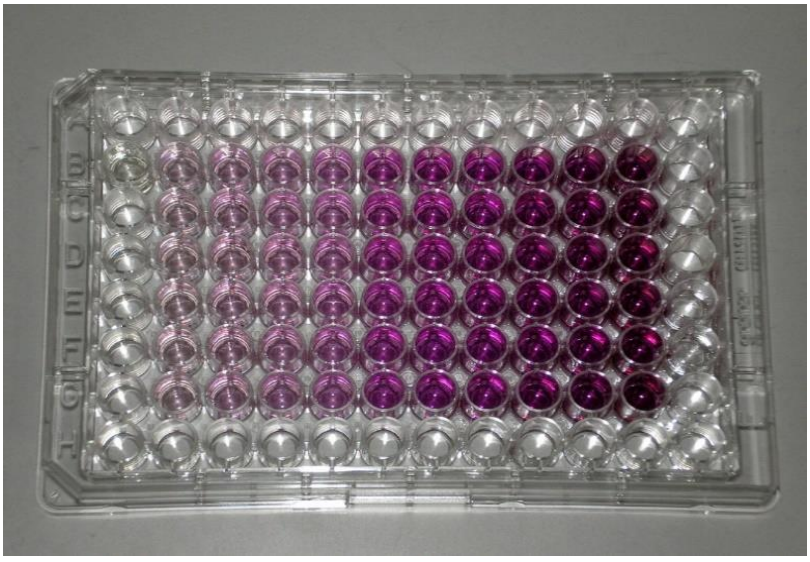
(E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl-formazan (formazan)

Figure 2.1 Enzymatic reduction of MTT to formazan

MTT is a standout among the most broadly utilized methods for analyzing the proliferation of cell viability. (18) MTT is absorbed by endocytosis and reduced by mitochondrial enzymes and endosomal / lysosomal compartments after that it form needle-like MTT formazans in the surface of the cell. MTT may activate apoptosis-related factors such as caspase-3, caspase-8 or cell contents spillage triggered after MTT formazan crystals appear. MTT method should therefore be carefully selected, otherwise the viability of cells might be underestimated and incomparable. MTT assay is a dependable method for cytotoxicity testing and this is the main advantage of it. Although the disadvantage is that the formation of formazan crystals depends on the number of mitochondria and the metabolic rate that results in several known obstructions. (19) Figure 2.2 pictures of MTT plated and procedure



a)



b)

Figure 2.2. (a) image of MTT procedure and (b) MTT plate

2.3. 2-(3H)-Benzoxazolone (Benzoxazolinone)

Benzoxazolinone is a heterocycle with bicyclic ring system containing a phenyl ring fused to carbamate. Which is IUPAC Name: 3*H*-1,3-benzoxazol-2-one. It is a powdered material with light brown color, having 138 ºC melting point and pKa = 8.7

The hydrophilicity and lipophylicity possessed give valuable properties that have several important consequences. Hydrophilicity is due to presence of oxygen and nitrogen atom which is important for hydrogen bonding, two hydrogen bonding accepting sites and one hydrogen bonding donating site present in the molecule. Lipophilicity is due to the bulkiness factor of the compound. Its lipophilicity play a vital role for structure binding to hydrophobic protein receptor. This dichotomy is reflected by a rather a discrete partition coefficient (log P = 0.97) and high dipole moment (4.47 Debye). These properties of Benzoxazolinone have important role in its absorption, distribution, metabolism, and excretion (ADME) in the body (20,21)

The structure of Benzoxazolinone is shown in figure 2.3. In research studies, several derivatives of benzoxazolinone were shown to have a variety of therapeutic activities. In fact, this template has wide range of clinical applications, that ranging from anti-inflammatory-analgesic compounds to anti-HIV and antipsychotic compounds. (22)

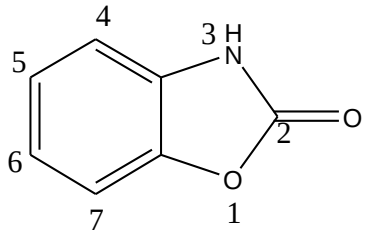


Figure 2.3 Structure and numbering of 2(3H)-Benzoxazolone

2.4. Chemical Reactivity of 2(3H)-Benzoxazolone

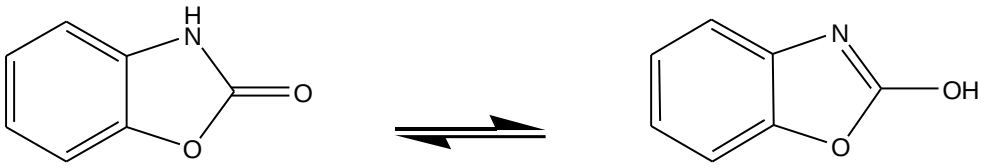
Many studies reported in the literature reveal the reactivity of Benzoxazolinones as three major types of reactions;

1)N-substitution reaction (either alkylation or acylation)

2)Aromatic ring electrophilic substitution reaction

3)Ring opening or expansion reactions

Several useful transformations at the level of the N (3) position of the heterocycle can be done because of enolizable character of the amide moiety. (21) Keto and enol form structure shown in figure (2.4).



keto form

enol form

Figure 2.4 Tautomers of 2(3H)-Benzoxazolone

2.4.1 N- substitution reaction (alkylation and acylation)

N-alkylation of 2(3H)-Benzoxazolone proceeds under base-catalyzed conditions to produce N alkyl derivatives, (23) as shown in figure 2.5.

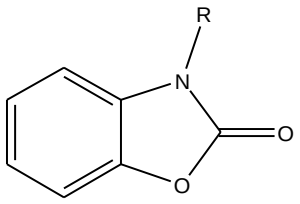


Figure 2.5. N-alkyl derivatives of 2(3H)-benzoxazolone

N-acylation is submitted to generalized acid-base catalysis to give N- acyl derivatives, shown in figure 2.6. (24)

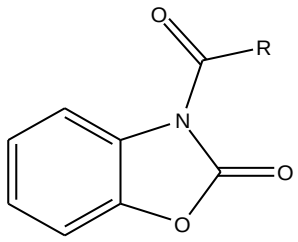


Figure 2.6. N-acyl derivatives of 2(3H)-benzoxazolone
R=CH_3

Base catalyzed Michael addition of acrylonitrile leads to N-cyanoethyl derivative, as shown in figure 2.7. (25)

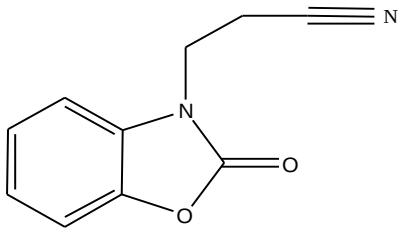


Figure 2.7. N-cyanoethyl derivative of 2(3H)-benzoxazolone

Mannich reaction with 2(3H)-Benzoxazolone produces immediate access to N-aminomethyl derivatives, as shown in figure 2.8. (26)

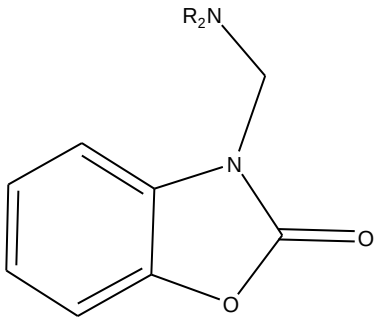


Figure 2.8. N-aminomethyl derivative of 2(3H)-benzoxazolone

Another example of N- substitution reaction of 2(3H)-Benzoxazolone with hydroxaminosulfuric acid which gives the cyclic hydrazide structure as shown in figure 2.9. (27)

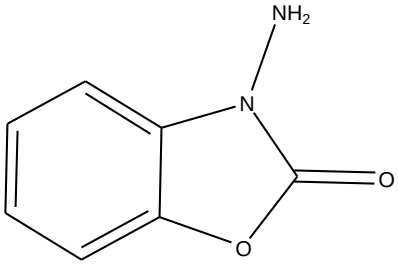


Figure 2.9. Cyclic hydrazide derivative of 2(3H)-benzoxazolone

2.4.2 Aromatic ring electrophilic substitution reaction

Aromatic electrophilic substitution is preferred to be done at sixth position. which can be detected not only for the sulfonation, halogenation, nitration, and chlorosulfonation reactions, but also for the Friedel-Crafts acylation.

In Friedel-Crafts reaction due to electron-rich character of Benzoxazolinone that make it extremely protonated by Lewis acid ($AlCl_3$) presented in reaction medium and become consequently extremely deactivated in this electrophilic aromatic substitution process.

to solve that problem, the reaction can take place by using either a less reactive electrophilic species for example (polyphosphoric acid, PPA) or the $AlCl_3$.DMF complex which more preferred to produce 6-acyl derivatives, shown in figure (2.10). (28)

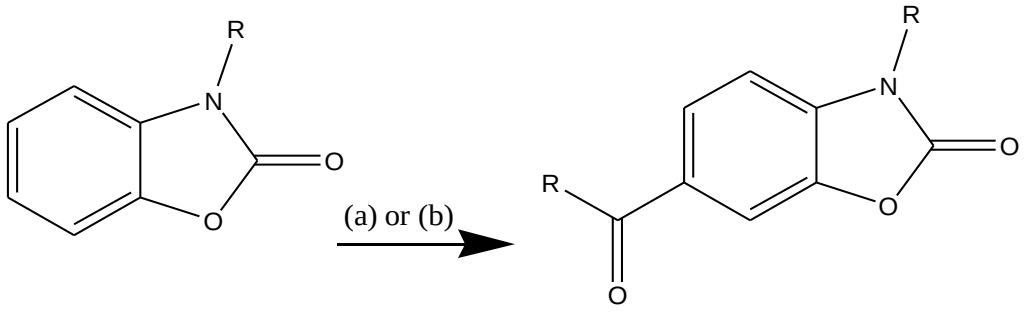


Figure 2.10.. Access to 6-acyl-2(3H)-benzoxazolone derivatives.
Methods: (a) RCOOH, PPA, (b) RCOCl, $AlCl_3$, DMF

As a most productive alternative, N-acyl derivatives can be rearranged at high temperature (160°C) in a Fries-like reaction promoted by $AlCl_3$, to 6-acyl derivatives as in figure 2.11(28)

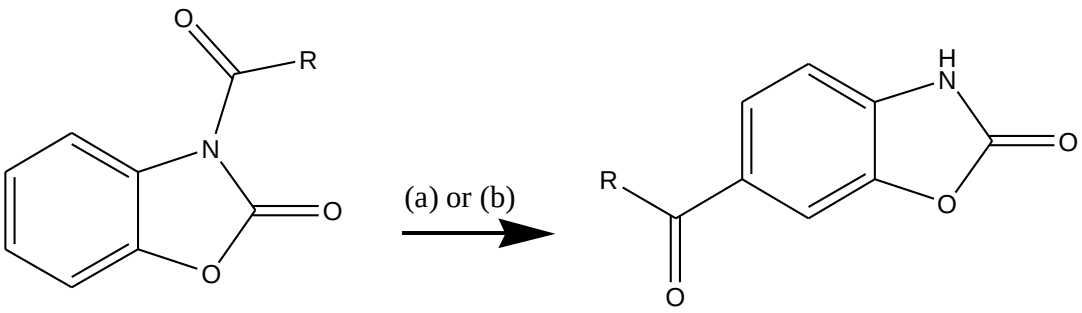


Figure 2.11. General synthesis of 6-acyl-2(3H)-benzoxazolone derivatives. Methods: (a) RCOOH, PPA, (b) RCOCl, AlCl3, DMF

2.4.3 Ring opening or expansion reactions

While 2(3H)-Benzoxazolone derivatives in acid medium are fairly stable, they are rapidly hydrolyzed in the basic medium, resulting in ring opening products such as 2-aminophenols, Figure 2.12(a) These 2-aminophenols can also be acylated in 4th position, (b), subsequent ring closure leads to 5-acyl-2(3H)-Benzoxazolone derivatives which are otherwise inaccessible, (c).Ring expansion of 2(3H)-Benzoxazolone derivatives to Benzoxazinones can be affected through a similar 2-aminophenols, (d). General synthesis of (3H)-Benzoxazolone derivatives is given in Figure 2.12. (29)

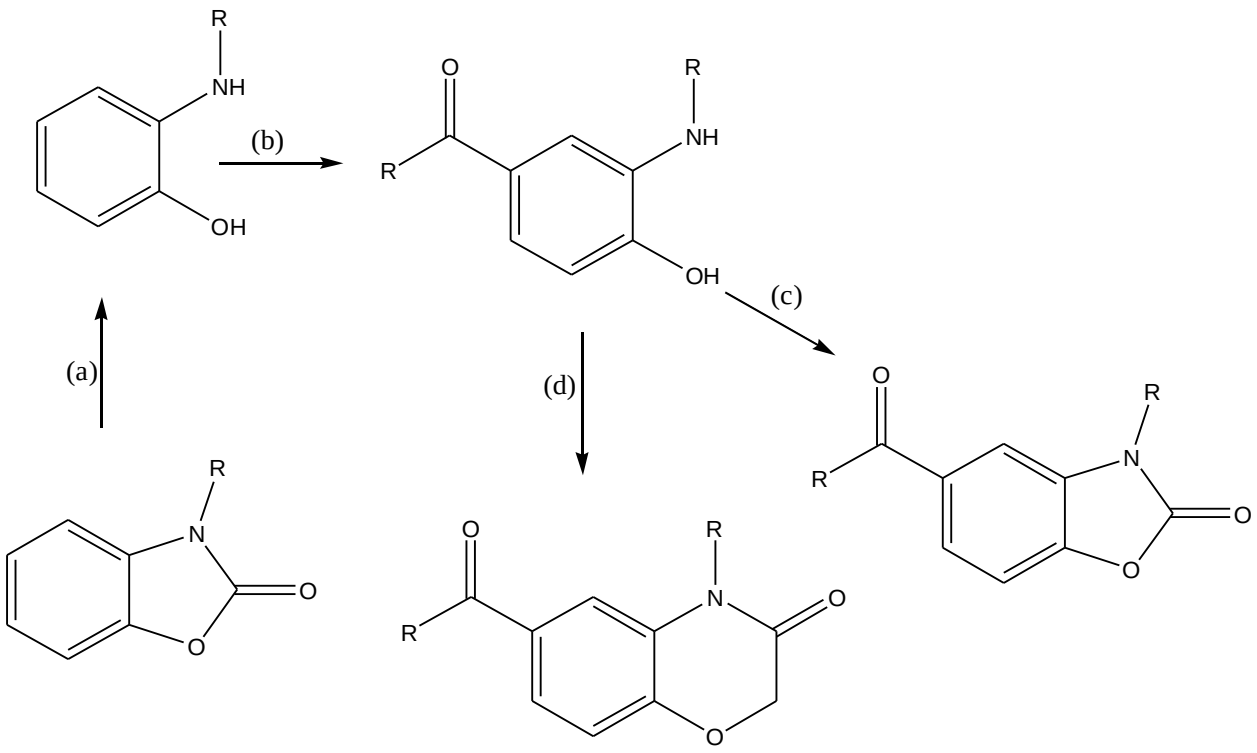


Figure:2.12. General Synthesis of 5-acyl-2(3H)-benzoxazolone and benzoxazinone derivatives. Methods: (a) aq. NaOH, (b) RCOCl, $AlCl_3$.DMF; (c) ClCOOEt, TEA; (d) $BrCH_2COOEt$, TEA

2.5 Biological Activity of Benzoxazolinone

Benzoxazolinone derivatives have been described by research studies that have several biological activities such as anticancer, analgesics, anti-bacterial, anti-fungal, antiviral and anti-inflammatory activities. (30,31)

In this section several examples of Benzoxazolinone derivatives reported in literatures are described in term of their structure and biological activities.

Ivanova et al reported the cytotoxic effect of Mannich bases of 6-(3-aryl-2-propenoyl)-2(3H)-Benzoxazolones, figure 2.13. Through N-aminomethylation of two parents 6-(3-aryl-2-propenoyl)-2(3H)-Benzoxazolones, Mannich bases with chalcone core structure were produced as potential anticancer agents. Using the MTT-dye reduction assay, the newly synthesized compounds were evaluated for cytotoxicity in the human pre-B-cell leukemia cell line BV-173. The cytotoxic effects of tested compounds showed concentration-dependent at low micromolar concentrations. At a concentration of 2,5 mM, Mannich bases induced programmed cell death in BV-173. (32)

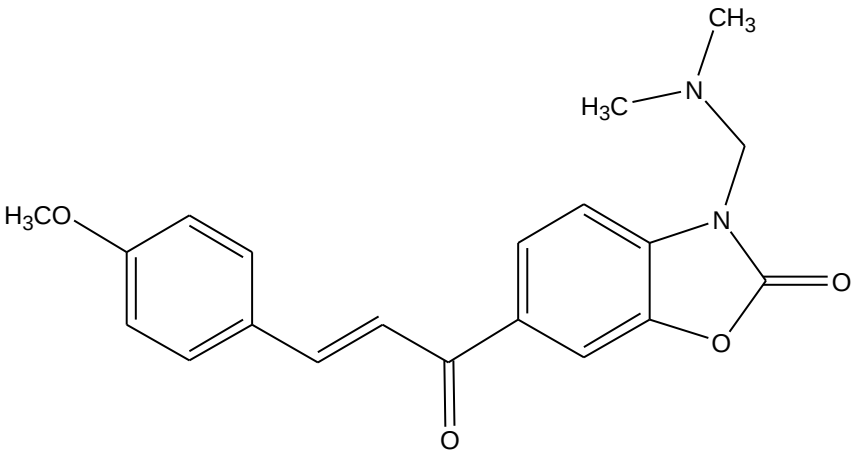


Figure 2.13. Example of benzoxazolinone structure that is shown cytotoxic activity [6-(3-methoxyphenyl-2-propenoyl)-3-(N,N,N trimethylamino)-2(3H)-benzoxazolones].

Fadda et al reported synthesis of new Benzoxazole derivatives and evaluate their cytotoxic effect to human cancer cell lines. Series of Benzoxazole derivatives were synthesized by the reaction of 2-mercaptobenzoxazole as a core compound with some chloroacetamide derivatives and hydrazine. Compounds (a) and (b) given in figure 2.14 and 2.15, displayed the stronger cytotoxic effects against hepatocellular carcinoma, HepG-2 (IC_{50} 5.5±0.22µg/ml) and breast cancer MCF-7. (IC50 5.6±0.32 µg/ml) cell lines. (33)

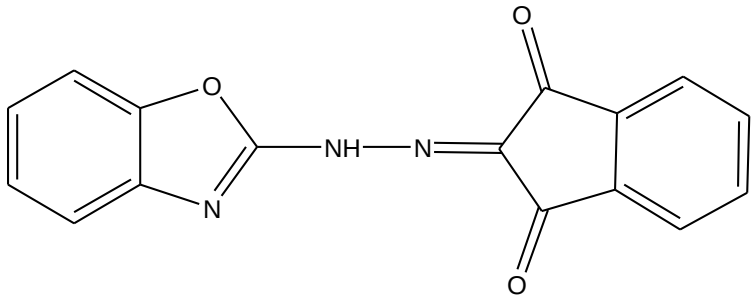


Figure 2.14 .(a) Example of benzoxazolinone derivative with cytotoxic activity [2-(2-(benzoxazol-2-yl)hydrazono)-1H-indene-1,3(2H)–dione]

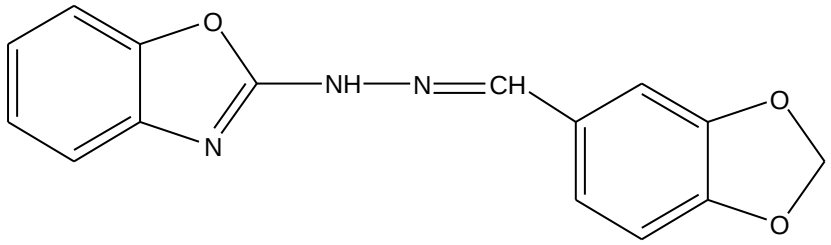


Figure 2.15.(b**)** Example of benzoxazolinone derivative with cytotoxic activity 2-(2-(benzo[d][1,3]dioxol-5-ylmethylene)hydrazinyl)benzoxazole

In order to design a new class of heterocyclic stilbenes as novel structural analogues of natural combretastatin (A-4), the Benzoxazolone ring was selected by Gerovas et al. 28 cis- and trans-styrylbenzoxazolones was made by a modified Wittig reaction under Boden's conditions. The in vitro cytotoxic effect of styrylbenzoxazolones against different cell lines was tested. Stilbene derivative 16Z, (Z)-3-methyl-6-(3,4,5-trimethoxystyryl)-2(3H)-Benzoxazolone, (figure 2.16). exhibited potent anti-proliferative and proapoptotic effects in liver cancer cells, suggested to be similar or better compared to CA-4. The inhibition of cellular proliferation is due to induction of mitotic arrest. (34)

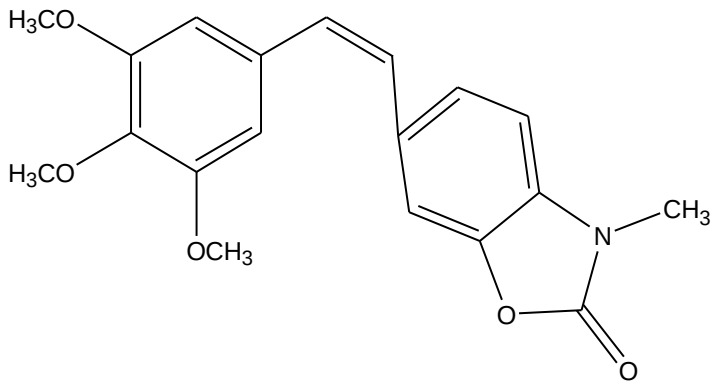


Figure 2.16 Example of benzoxazolinone derivative with cytotoxic activity (Z)-3-methyl-6-(3,4,5-trimethoxystyryl)-2(3H)-benzoxazolone

Ortega et al. synthesized a series of Benzimidazole derivative as potent acid ceramidase inhibitor in malignant melanoma cells (35), examples of synthesized compounds are shown in figure 2.17.

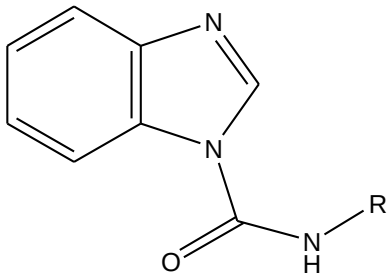


Figure 2.17 2-Benzimidazole derivative with cytotoxic activity
R= 4-phenylbutyl

Benzoxazolinones with arylpiperazine substituented were of interest as they could affect the central nervous system. In Mulazim et al research study microwave assisted heating method was utilized for the preparation of piperazine substituted 5-Chloro-2(3H)-Benzoxazolone derivatives. Since research studies have found that these types of compounds have anti-inflammatory and analgesic activities, their biological activities have also been investigated. 5-chloro-2(3H)-Benzoxazolone was reacted with piperazine derivatives through Mannich reaction to produce 3-substituted-5- chloro-2(3H)-Benzoxazolone compounds as shown in figure 2.18. Compound 1 with 2-fluorophenyl piperazine substituent had the longest anti-inflammatory activity (100 mg / kg dose). Hot-plate and tail-fick tests were used for the analgesic activities. Compound 3 was found to have the highest activity in the hot plate test while compounds 1 and 2 showed increased anti-nociceptive activity in the tail-fick test.. (1)

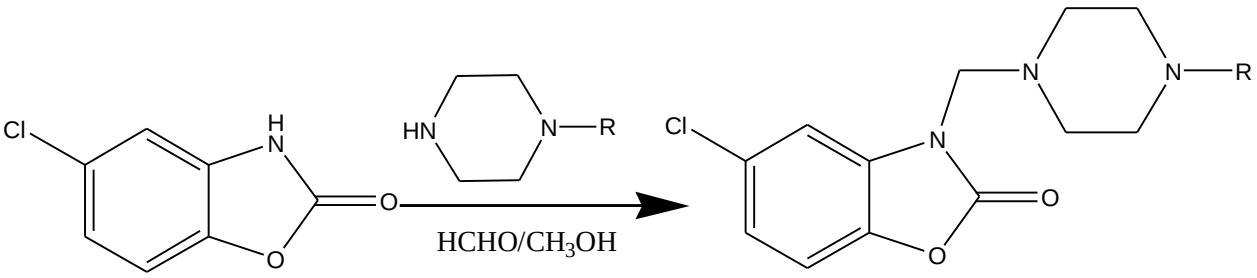


Figure 2.18. Synthesis of piperazine substituted 5-chloro-2(3H)-benzoxazolone derivatives R=2-flurophenyl (1) ,2-methoxyphenyl (2) ,2-pyrimidyl (3)

A series of 2(3H)-Benzoxazolone and 2(3H)-Benzothiazolone derivatives were prepared and investigated for anticonvulsant activity by Ucar et al. The compounds were synthesized to see the relation between the 2(3H)-Benzoxazolone and 2(3H)-Benzothiazolone derivatives' structures and anticonvulsant activity. Significant anticonvulsant activity showed by several of these compounds. The most active of the series against seizures induced by maximal electroshock (MES) were compounds (a) and (b) shown in figure 2.19 with ED_{50} values of 8.7 and 7.6 mg/kg, respectively. (36)

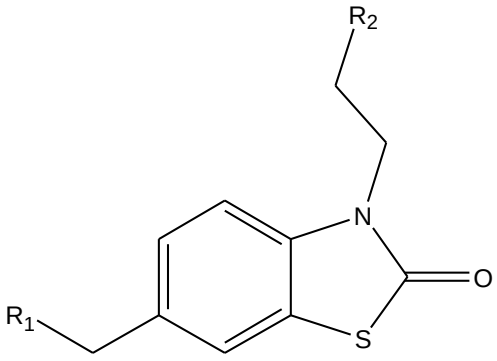


Figure 2.19. structure of 2(3H)-benzothiazolone derivatives
(a) $R_1=C_2H_5CO$ $R_2=C_5H_{10}N$ (b) $R_1=C_3H_7$ $R_2=C_5H_{10}N$

Evaluation of antimicrobial activities of 2(3H)-Benzoxazolone derivatives was done by Soyer et al. The potential antimicrobial activity of a group of 2-(2-oxo-2- benzoxazoline-3-yl)-N-phenylacetamide and propionamide derivatives bearing substituents with different lipophilic and electronic nature on N-phenyl ring have been investigated. Minimal effective concentrations of the compounds were determined against Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis and Candida albicans by using microdilution method. In terms of Gram (-) and Gram (+) antibacterial activity in the series studied compound 6, namely 3-(2-oxo-2- benzoxazoline-3-yl)-N-(m-tolyl) propionamide, figure 2.20, was the most active derivative. (37)

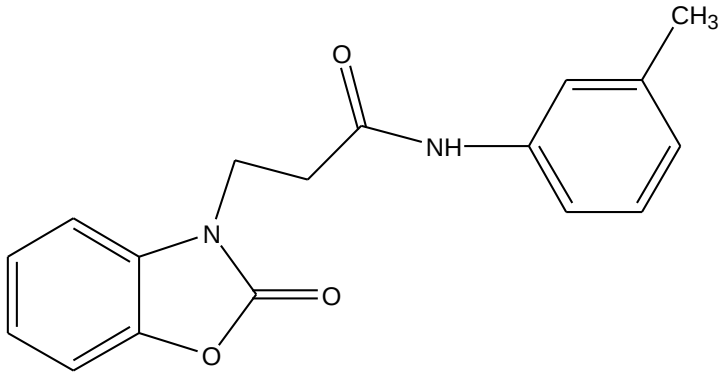


Figure 2.20 Example of benzoxazolinon derivative with antimicrobial activity 3-(2-oxo-2- benzoxazoline-3-yl)-N-(m-tolyl) propionamide

Erol et al synthesize and investigate the antibacterial and antifungal activity of thiazolinoethyl 2(3H))-Benzoxazolone derivatives. Cyano derivatives of 6-acyl 2(3H)-Benzoxazolones were reacted with cysteamine HCI in ethanol to produce the corresponding 6-acyl-3-thiazolinoethyl2(3H)-Benzoxazolones and then the antibacterial and antifungal activities of synthesized compound were evaluated. The antifungal studies against C albicans (a and b, MIC = 67.5 yg/mL), C stellaatoidea (c, MIC = 67.5 pg/mL) and C parapsilosis (d, MIC = 67.5 pg/mL) and were more successful in comparison. Chemical structures of them shown in figure 2.21. (38)

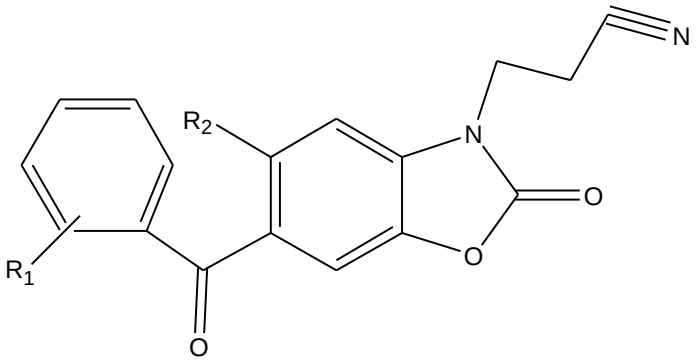


Figure 2.21 . Thiazolinoethyl-2(3H)-benzoxazolone derivatives
(a) R_1=H ,R_2=Cl (b) R_1=4-CH_3O , R_2=H (c) R_1=H, R_2=H (d) R_1=4-NO_2 R_2=H

Gamba et al presented a new Benzoxazole derivative that have inhibitory activity against the HIV-1 nucleocapsid protein (NC). Their data manifested that 2-benzoxazolinones have ability to bind to the hydrophobic pocket of NC and decrease its nucleic acid chaperone activity. The research study showed that all compounds in the series were able to develop non-covalent interactions with protein, however with different affinities. The results highlighted compound that shown in figure 2.22 as the most active analogue in the series, with low μM IC50 values for antiviral activity in HIV-infected cells. (39)

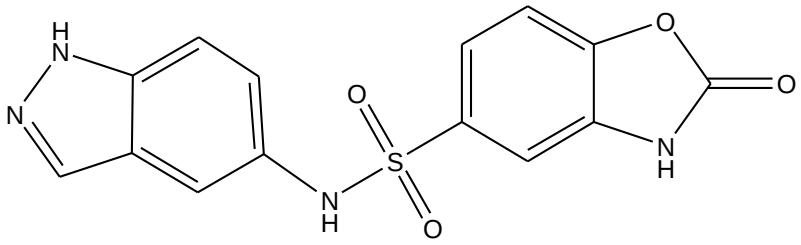


Figure 2.22 Example of benzoxazole derivative with anti-HIV activity
N-(1H-indazol-5-yl)-2-oxo-2,3-dihydro-1,3-benzoxazole-5-sulfonamide

Hadizadeh et al described the design and synthesis of four bupropion analogues, 3-Methyl-6- (substituted amino) propionyl]-2-benzoxazolinone as antidepressant. Using forced swimming test in mice. All analogues were found to be effective at the doses 2.5-20 mg/kg. (40)

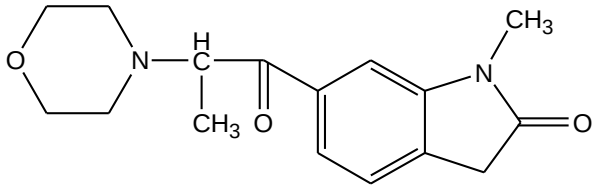


Figure 2.23 2-Benzoxazolinone derivative with antidepressant activity

Aichaoui et al. synthesized chalcone derivatives of 2(3H)Benzoxazolone and investigate them for antioxidant activity performed in vitro studies. Biological results highlight the compound produced, shown in figure 2.24, as the most effective agent as it has the ability to inhibit copper-mediated human LDL oxidation with an activity ten times more potent than that of Probucol, a reference antioxidant drug. (41)

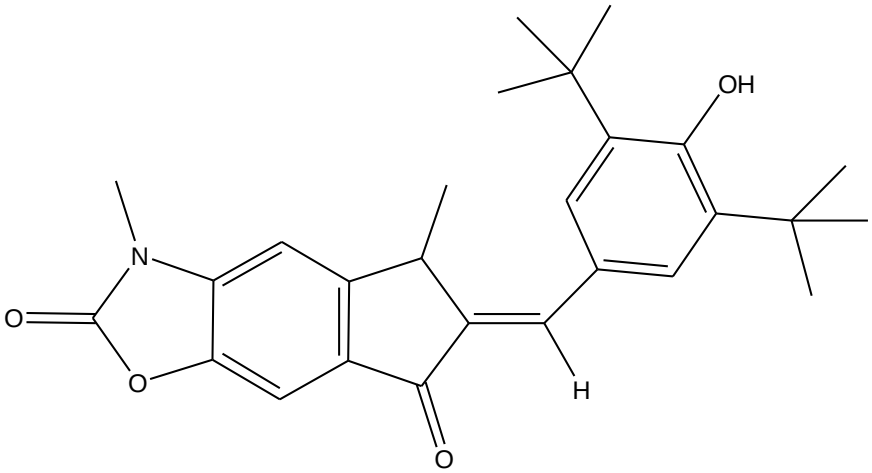


Figure 2.24 2(3H)Benzoxazolone derivative with antioxidant activity

Novel 1,2,3-triazole-based benzoxazolinone derivatives have been synthesized by Panda et al. to have antinociceptive property. The antinociceptive activity was investigated by using writhing test and tail immersion methods. As the result of this research study, compound that presented in figure 2.25, show the most potent antinociceptive activity with 41.83% compared to indomethacin, the reference drug. (44.69%) inhibition. (42)

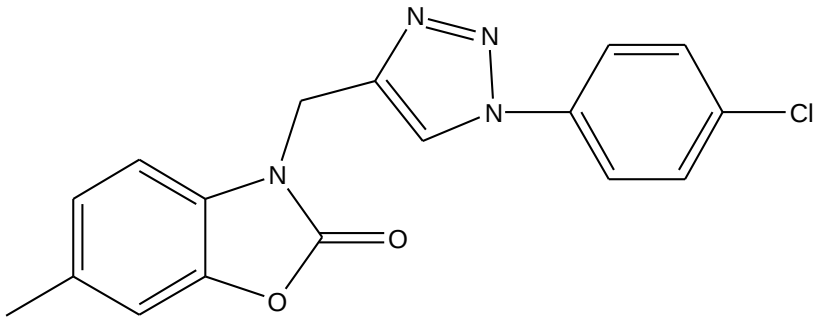
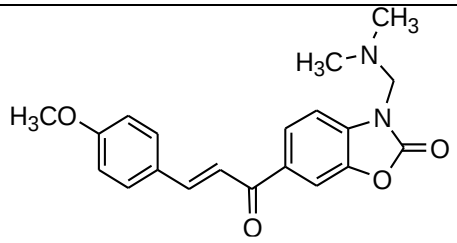
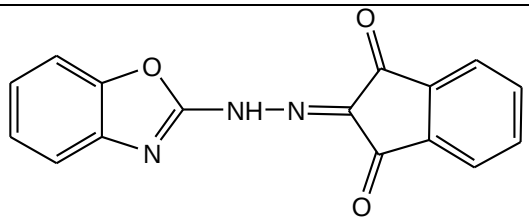
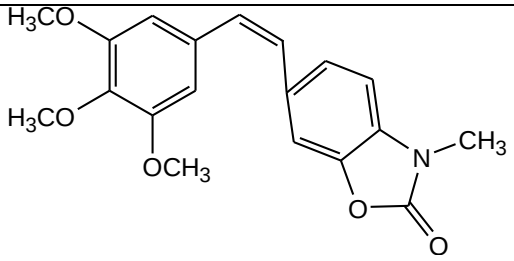
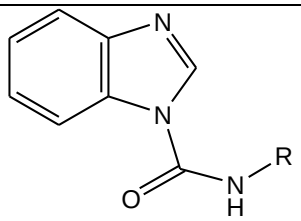
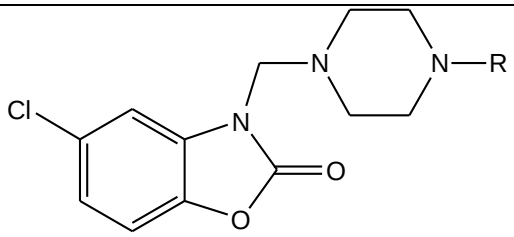
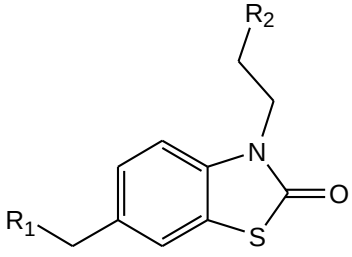
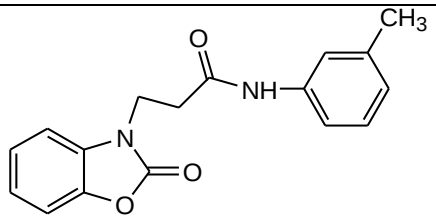
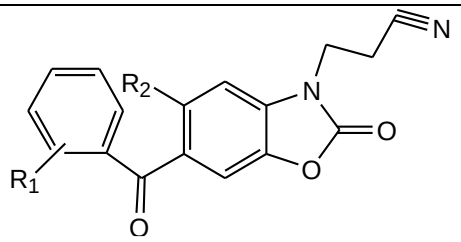
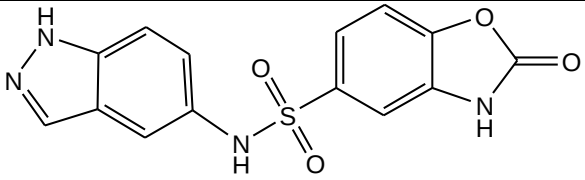
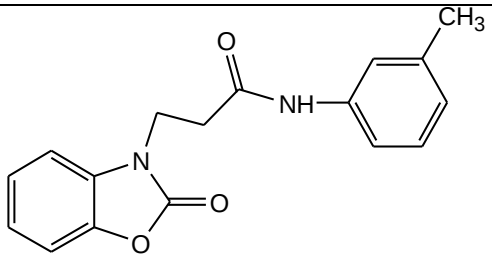
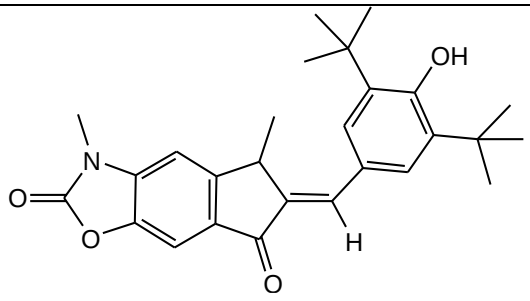
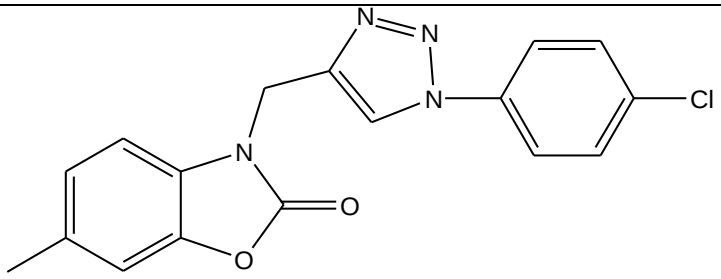


Figure 2.25 Benzoxazolinone derivative with antinociceptive activity

Table 1; Some examples of Benzoxazole derivatives and their activity types

Biological activity	Example of 2(3H)Benzoxazolone derivatives	Reference no.
Cytotoxicity ,BV-173 cell line		**32**
Cytotoxicity , HepG-2 and MCF-7cell line		**33**
Cytotoxicity , HepG2 cell line		**34**
Cytotoxicity , melanoma cells		**35**
Anti-inflammatory-analgesic		**1**
Anticonvulsant		**36**

Antibacterial		**37**
Antifungal		**38**
HIV-1 nucleocapsid protein		**39**
Antidepressant activity		**40**
Anti-oxidant		**41**
Antinociceptive		**42**

2.6 Mannich Reaction

Many authors defined the aminoalkylation of CH-acid compounds as early as the nineteenth century. However, Carl Mannich was the first one who identify the massive importance of this type of reaction and was he who broadened the chemistry through systematic research into a broad synthetic methodology. This reaction, which now bears his name, has become one of the most significant C-C bond-forming reactions in organic chemistry. (43)

The Mannich reaction is a traditional method for β-amino carbonyl compound production (Mannich bases). is extremely useful for the construction of nitrogenous molecules and, because of its diverse application in various pharmaceutical production is standout amongst the most significant basic reaction types in organic and medicinal chemistry. (44)

The Mannich reaction is a nucleophilic addition reaction that included condensation of an aldehyde, usually formaldehyde, with ammonia or primary or secondary amine, and an activated hydrogen compound. The active hydrogen compound is most commonly ketone, acid, or ester, although nitro alkane has recently been used. (45)

The schematic representation of general Mannich reaction is given in figure 2.26

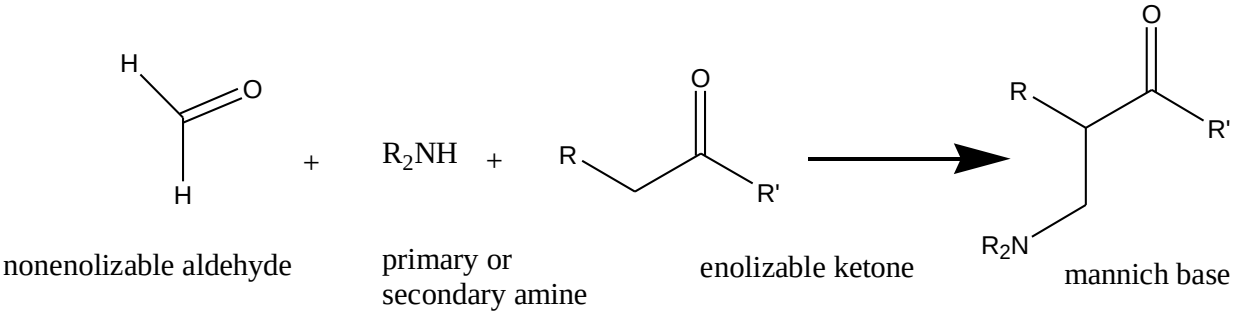
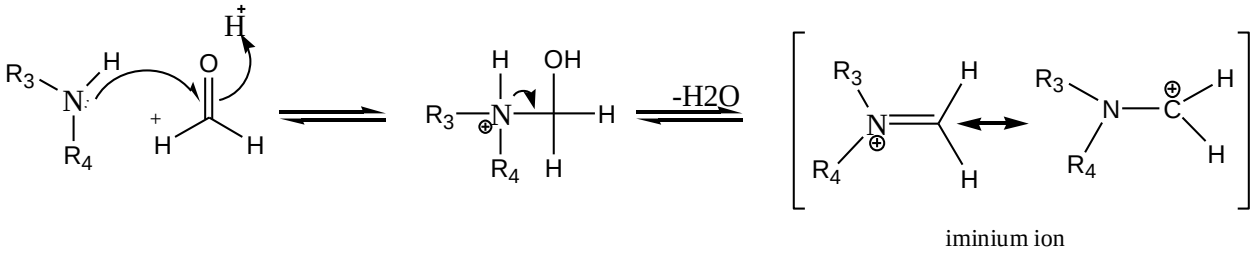


Figure 2.26 General scheme of mannich reaction

Mechanism of reaction shown in figure 2.27



The reaction is take place in acidic conditions, the enolizable carbonyl compound is converted to enol form, which attacks the iminium ion at positively charged carbon adjacent to nitrogen to produce a ß-aminocarbonyl compound

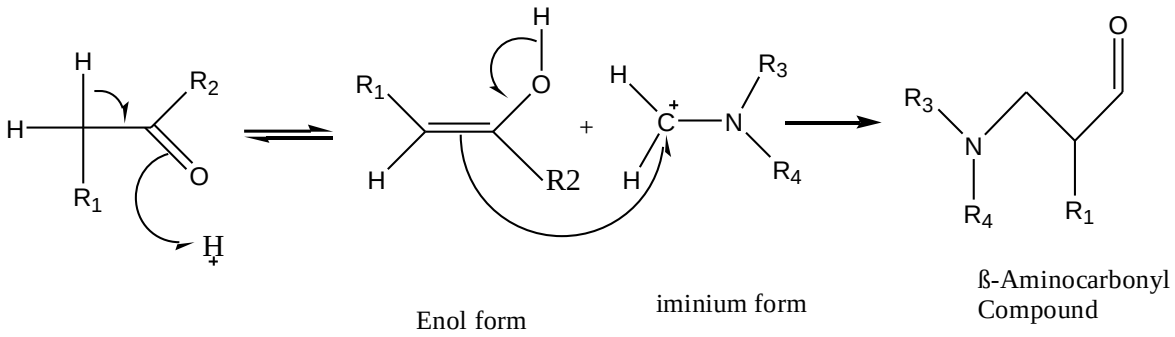


Figure 2.27 Mechanism of Mannich reaction

This type of reaction is important and has a broad range of uses. In particular, this means that it starts from inexpensive starting materials and produces key building blocks in an cheap and effective manner for pharmaceuticals and natural products. In terms of yields and resources, it is efficient.

The increasing popularity of the Mannich reaction has been endorsed by the pervasive nature of nitrogen in medications and natural products along with the possibility of this multi-component reaction to produce diversity. The utilization of Mannich bases in cancer treatment is one of several research areas that currently in progress. (46)

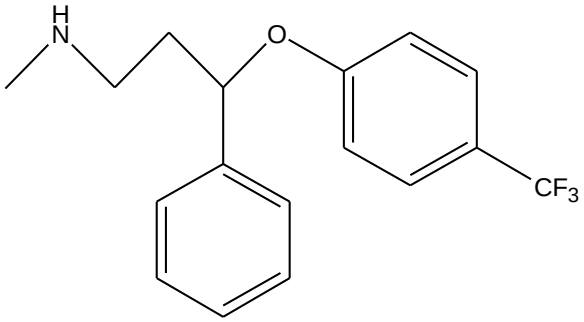
2.6.1 Mannich Bases

Mannich bases are the final products of Mannich reaction and identified as beta-amino ketone bearing compounds which possess amino alkyl chain (NCH2X moiety)

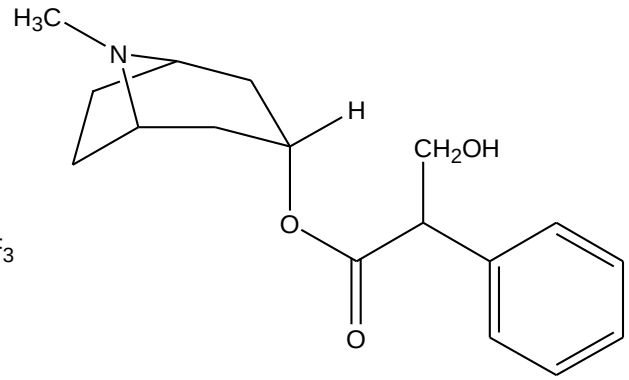
Mannich bases are highly reactive and recognized as having a large variety of activities. Such as: anti-inflammatory-analgesic, antibacterial, anticancer, antifungal, antiviral, anticonvulsant, anti-HIV, antimalarial, antipsychotic, antitubercular, activities and so on. Mannich bases ' biological activity is fundamentally attributed to α, β-unsaturated ketone that can be produced by deamination of the hydrogen atom of the amine group. (47)

The versatility of Mannich bases is demonstrated by the large major type of reactions these compounds can be subjected to. They are useful intermediates in synthetic chemistry for the preparation of a various new compounds. Examples of therapeutically effective Mannich bases which have amino alkyl chain in their structure are e ranitidine (histamine H2- receptor antagonist), atropine (competitive antagonist of the muscarinic receptor), cocaine(stimulant), fluoxetine (antidepressant), procyclidine (anticholinergic drug), biperiden (antiparkinsonian agent) and so forth. (48)

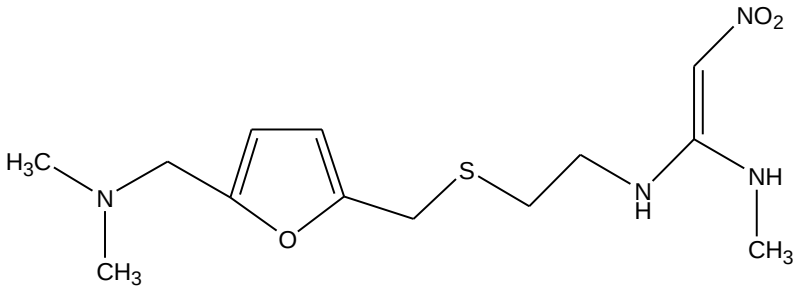
Mannich bases are established to play an important role in synthetic medicinal chemistry development. The research studies manifested that Mannich bases are highly reactive and can readily be transformed to other compounds, such as physiologically active amino alcohol that is produced from reduction of mannich base. Mannich bases are also recognized for their use in detergent additives, polymers, resins, surface active agents. Mannich bases prodrugs of various active compounds were prepared to eliminate the restrictions. (49)



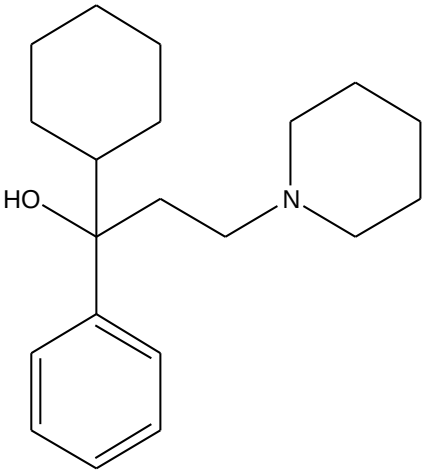
Flouxetine



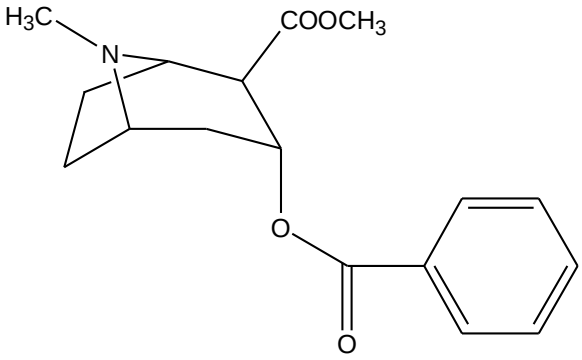
Atropine



Ranitidine



Trihexyphenidyl



Cocaine (benzoylmethylecgonine)

Figure 2.28 Examples of clinically useful Mannich bases (45)

3. MATERIALS AND METHODS

3.1 Materials

All reactions were carried out using standard laboratory equipment and standard laboratory glassware. The starting materials, 5-chloro-2 (3H)-benzoxazolone,4-(4-nitrophenyl) piperazine, methanol, triethylamine and formaldehyde, acylbenzoxazolone used in this study were obtained from Sigma Aldrich Chemical Company and were used without further purification.

3.2. Thin Layer Chromatographic Method

3.2.1. Material

Thin layer chromatography (TLC) was used to monitor the progression of the reaction carried out on Silica gel/TLC-plates (DC-AlufplienKieselgel, Germany) and solvents used were benzene, acetonitrile, hexane and methanol. Silica gel plate was detected under UV-light (254 nm).

Three different mobile phases were prepared and used, with different ratios as follows;

M-1/ Acetonitrile: Methanol (2:1).

M-2/ Hexane: Methanol (3:1)

M-3/ Benzene: Methanol (5:1)

3.2.2 Method

The mobile phase (solvents) was poured into the TLC chamber to a depth of about 0.5 cm. The chambers were covered with watch glass, gently swirled and allowed to stand while assembling the plates**.** TLC plates were cut horizontally into plates of about 5 cm tall by

different widths and pencil is used to draw a line across the plat at 0.5 cm from the bottom of the plate. 6-(bromoacyl)Benzoxazolinone the starting material of compound 1 dissolved in Dimethylformamide (DMF) while the product (compound 1) dissolved in acetonitrile. For compound 2&3 the starting materials (Benzoxazolinone and 6-chlorobenzoxazoline) and their products dissolved in chloroform. Spots were made on the plate with the aid of a microcapillary and gently placed to the TLC chamber, covered with watch glass and left undisturbed. The plate was allowed to develop, once the solvent front was about half a centimeter just under the top of the plate the plate was removed, and the solvent front was marked by pencil and left to dry. The spots were viewed under UV light at 254 nm and Rf values calculated.

3.3. Melting Point

The melting point determination of the synthesized compounds was carried out with Mettler Toledo (FP90 central processor) melting point apparatus.

3.4. Spectroscopy

Fourier Transform Infra-Red (FT-IR) The FT-IR spectra of the products were recorded on Perkin Elmer 630 Spectrometer at Eastern Mediterranean University (EMU), Faculty of Pharmacy.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) The ^{1}H-NMR spectra of the products were recorded on a Mercury Varian 400 MHz spectrometer where deuterated chloroform was ($CDCl_3$) used as a solvent. The test was conducted at Ankara University, Central Instrumental Analysis Laboratory, Faculty of Pharmacy.

3.5 Cell viability and growth assay

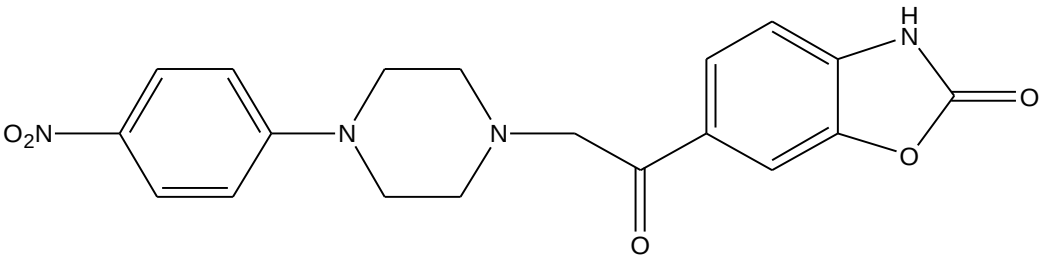
The MTT assay (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to measure the cytotoxicity . MTT is depend on colorimetric measurement of reduction of 3-(4, 5-dimethylthialzol-2-yl) -2, 5-diphenyltetrazoliumbromide which is reduced by living cells to produce purple formazan . Compound **2**, (3[4-(4-nitrophenyl) piperazin-1-yl**]**methyl-2-benzoxazolone) and compound **3**, (5-chloro**-**3[4-(4-nitrophenyl) piperazin-1-yl**]**methyl-2-benzoxazolone) were prepared in dimethyl sulfoxide (DMSO), 100 mg/ml. and diluted in culture medium with five different concentrations (5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and 100 µg/ml). MCF-7 cells were collected, suspended in medium and seeded in 96-well culture dishes at a density of 5 × 104/ml cells in each well with 100 µl medium. Negative control row contained neither cells nor extracts and only cells were seeded in positive control row. Extract dilutions were triplicated and cell lines were incubated for 24 and 48 h. After incubation MTT solution was heated to 37 °C and then 10 µl were added to the each well. After 24 h incubation at 37 °C in 5% CO_2, 200 µl DMSO was added to dissolve the formazan salts. The absorbance was measured at 570 nm with spectrophotometer (Versa Max, Molecular Device, Sunnyvale). All experiments were performed in triplicate for each compound.

3.6. Experimental

Experimental procedures were taken from the literatures (1,50)

3.6.1. Synthesis of Compound 1

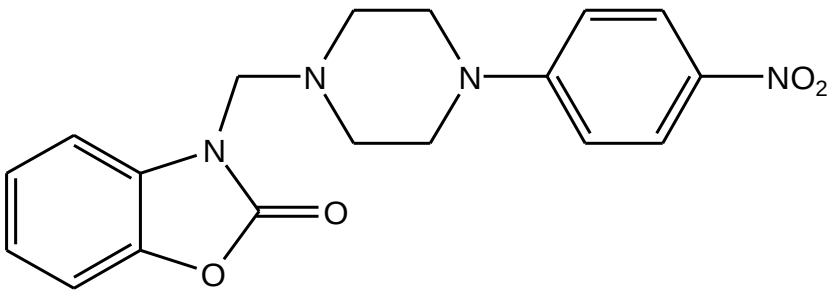
6-[4-(4-nitrophenyl) piperazin-1-yl]acyl-2-Benzoxazolone



0.781 mmol (200mg) 6-(bromoacyl)Benzoxazolone solution in 5ml of DMF was added dropwise to 0.781(160mg) mmol 4-(4-nitrophenyl)piperazine and 1.562 mmol (0.2 ml) triethylamine solutions in 5 ml of DMF. The mixture was stirred at room temperature for 26 h. after that crushed ice was added into the reaction mixture. The obtained solid was filtered using vacuum filtration method to yield a crude product which was subsequently washed with water and leaved to dry at room temperature. The reactions were monitored by TLC and resulting precipitate was purified by recrystallization using acetonitrile as solvent.

3.6.2. Synthesis of compound 2

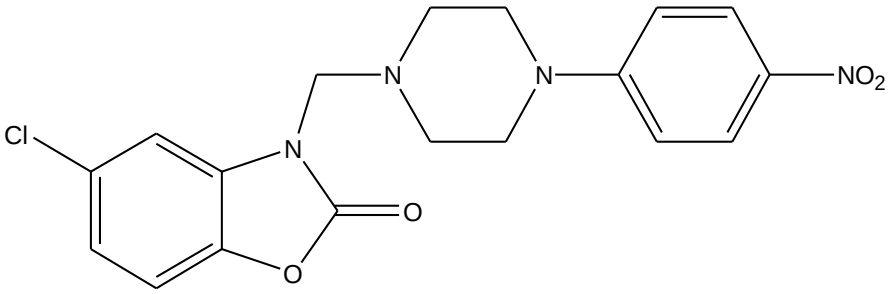
3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-Benzoxazolone



(200mg) 1.480 mmol of 2(3H)-benzoxazolone and 1.480 mmol (308mg) of 4-(4-nitrophenyl) piperazine were dissolved in 8 ml of methanol. 0.2 ml of formalin and 2 ml of methanol solution are then added. After that mixture was placed in water bath to be refluxed for one hour. then crushed ice was added to the mixture and the obtained precipitate was filtered off, washed with ethanol, and by recrystallization the product purified and ethanol used as a solvent.

3.6.3 Synthesis of compound 3

5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-benzoxazolone

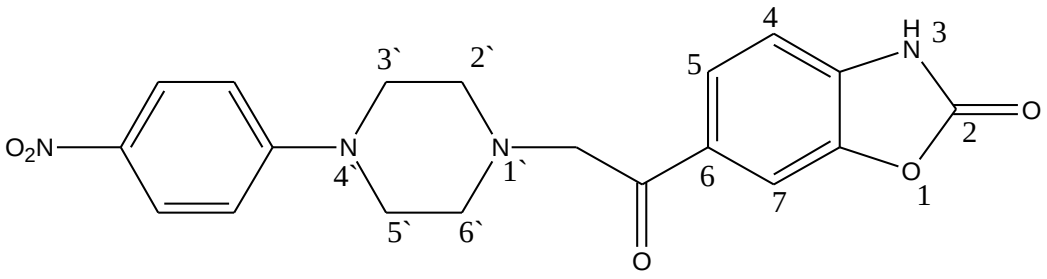


1.179 mmol (200mg) of 5-chloro-2-benzoxazolone and 1.179 mmol (mg) of 4-(4-nitrophenyl) piperazine were dissolved in 8 ml of methanol. 0.2 ml of formalin and 2 ml of methanol solution are then added. After that mixture was placed in water bath to be refluxed for one hour. then crushed ice was added to the mixture and the obtained precipitate was filtered off, washed with water leaved to dry and purified by recrystallization and using cyclohexane as solvent.

4. RESULTS AND DISCUSSION

4.1. Results

Compound 1



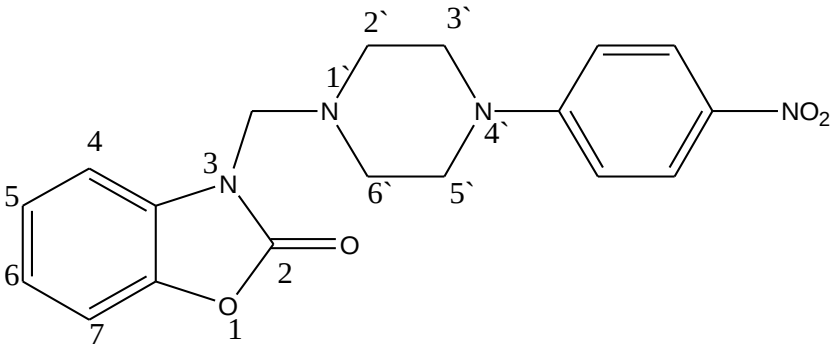
− yellow crystalline solid was obtained with a yield of 50.6% (151 mg) and a melting point of 134.1 °C

− TLC in the M1 mobile phase gave Rf values of 0.76

− **Fourier Transforms Infra-Red (FT-IR) Spectroscopy (IR v_{max}):** FT-IR showed stretches around 3200-3500 cm^{-1} (N-H) and around 2700-3300 cm^{-1}(C-H) and around 1787.05 cm^{-1} (C=O of carbonyl)

-**Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR, $CDCl_3$; ppm) δ:** 7-8.1 (m; 7H; Aromatic-H); 4.7 (2H; s; CH_2); 3.2-3.7 (t; 4H; pip H3`-H5`);2.4- 2.8 (t; 4H; pip H2` -H6`) ppm

Compound 2



-Brownish-yellow crystalline solid was obtained with a yield of: 45.3% (0.237 g) and a melting point of 170.8 °C.

− TLC in the M3 mobile phase gave Rf values of 0.21

_ **Fourier Transforms Infra-Red (FT-IR) Spectroscopy (IR v_{max}):** FT-IR showed stretches at around 2700-3300 cm^{-1}(C-H) and 1760.33 cm-1 (C=O of carbonyl)

_**Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR, $CDCl_3$; ppm) δ:** 6.8-8.1 (m; 8H; Aromatic-H); 4.7 (2H; s; CH_2);3.4 (t; 4H; pip H3`-H5`); 2.8 (t; 4H; pip H2` -H6`) ppm

_**Cytotoxicity results:** shown in figure below

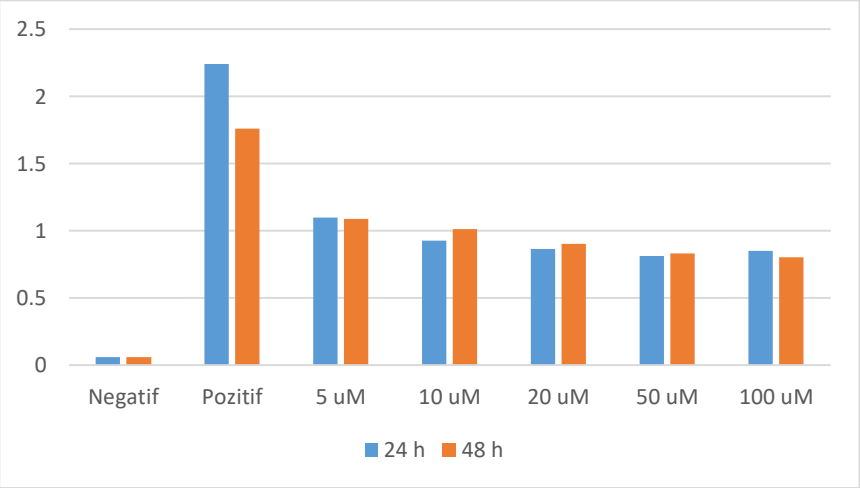
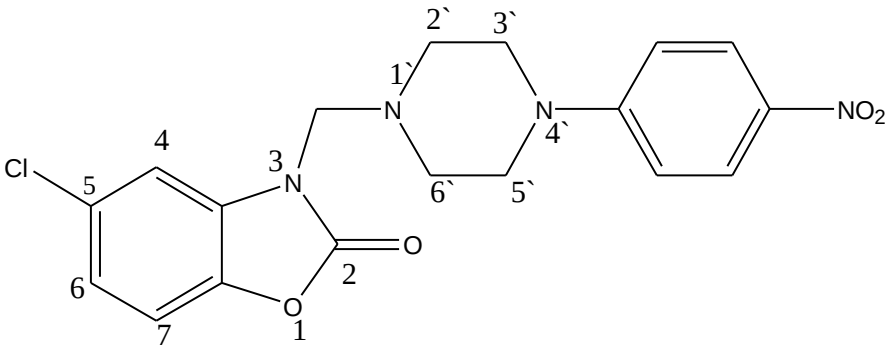


Figure 4.1. Effect of compound **2**, (3[4-(4-nitrophenyl) piperazin-1-yl[methyl-2-Benzoxazolone), on cell viability of MCF-7 cells**.**

Compound 3



−yellow crystalline solid was obtained with a yield of 44.6% (0.203 g) and a melting point of 170°C.

− TLC in the M3 mobile phase gave Rf values of 0.17

− **Fourier Transforms Infra-Red (FT-IR) Spectroscopy (IR v_{max}):** FT-IR showed stretches at around 2700-3300 cm^{-1}(C-H) and around 1781.15 cm^{-1} (C=O of carbonyl)

__ **Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR, $CDCl_3$; ppm) δ:** 6.8-8.1 (m; 7H; Aromatic-H); 4.7 (2H; s; CH_2); 3.4 (t; 4H; pip H3`-H5`); 2.8 (t; 4H; pip H2` -H6`) ppm

_**Cytotoxicity results:** shown in figure below

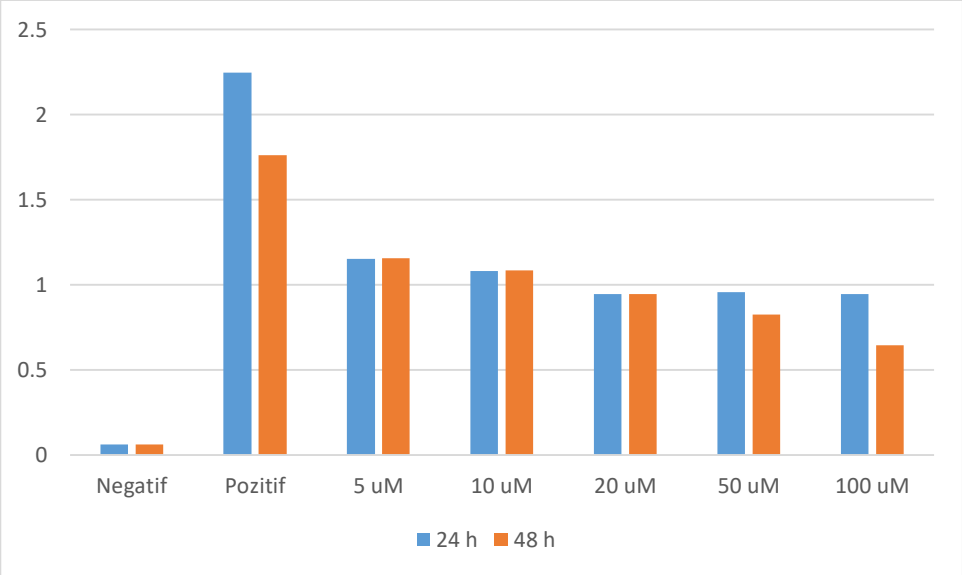


Figure 4.2. Effect of compound **3**, (5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-Benzoxazolone) on cell viability of MCF-7 cells

4.2. Discussion

In this research study three derivatives of benzoxazolinone were synthesized following literature procedures. (1, 50) 4-(4-nitrophenyl) piperazine was employed as the amine substituent in all three molecules. First compound was synthesized at room temperature by substitution reaction at the sixth position of acylbenzoxazolinone. The product was evaluated for the cytotoxicity activity against MCF-7cell line. But due to insolubility of compound **1** in DMSO, the solvent used for analysis, the cytotoxicity test could not be performed.

Compounds **2** and **3** were synthesized via Mannich reaction by third position substitution at benzoxazolinone and 5-chloro-2-benzoxazolinone, respectively. These compounds were also evaluated for their cytotoxicity against MCF-7 cell line

1) Reaction scheme of 6-substituted 2-(3H)-Benzoxazolone derivative given in figure 4.3

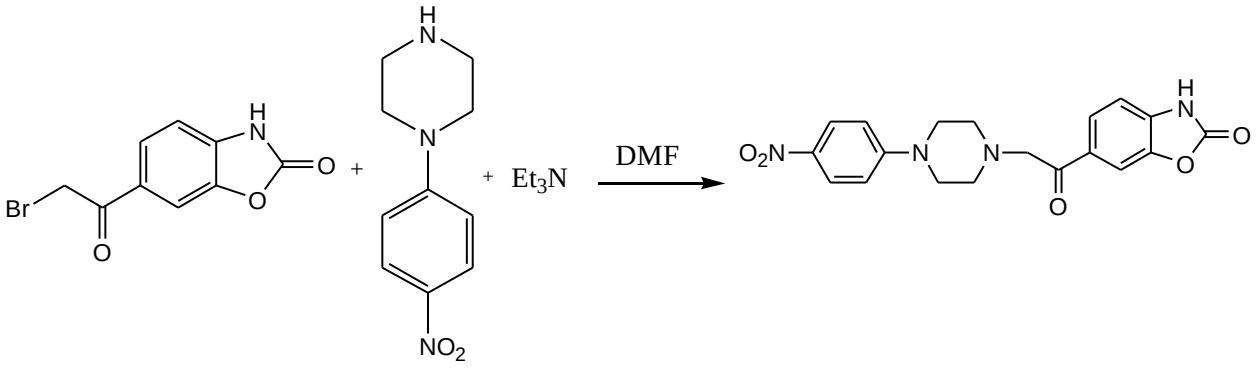


Figure 4.3. Synthesis of 6-bromoacyl-3-substituted benzoxazolone molecules

2) Mannich reaction of 3-substituted Benzoxazolone derivatives given in figure 4.4

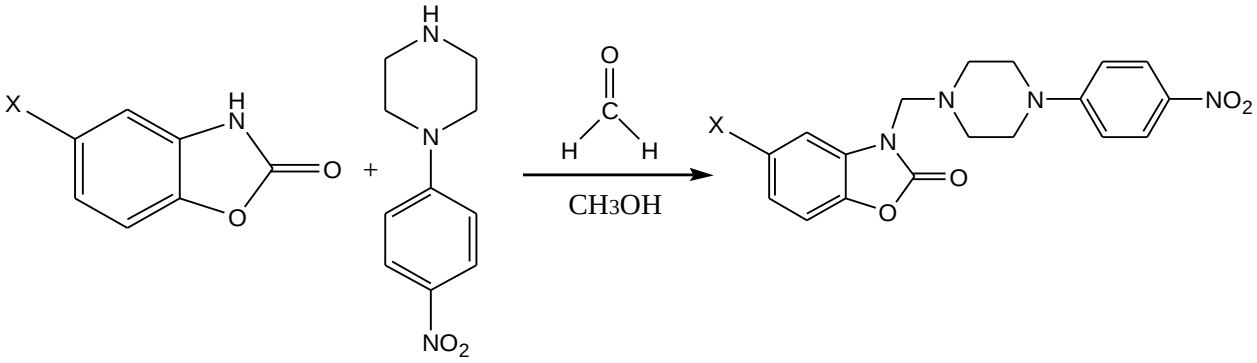
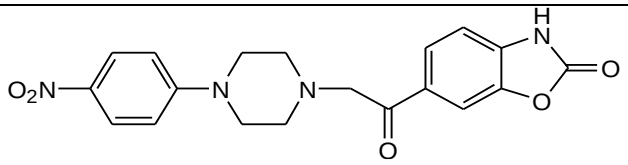
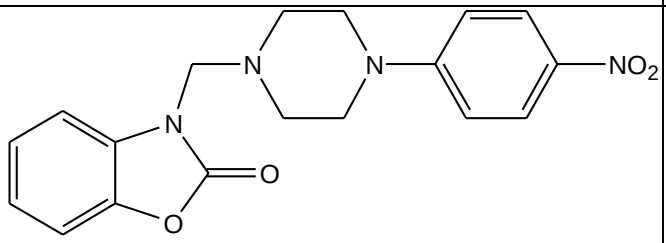
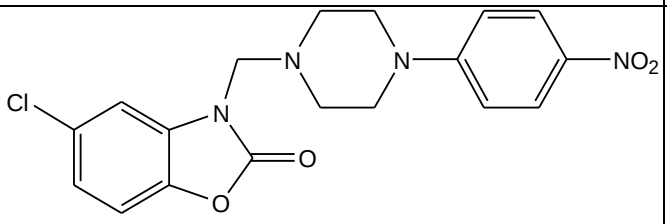


Figure 4.4. General synthesis of 3-substituted benzoxazolone molecules
compound 2 X=H
compound 3 X=Cl

Summary of the yields and melting points of three compounds are shown in table 4.1.

Table 4.1: Structure, name, melting point and yield of compound 1- 3

Compound Number	Structure& Name	Melting point	Yield %
1	 6-[4-(4-nitrophenyl)piperazin-1-yl]acyl-2-Benzoxazolone	134.1 ℃	50.6%
2	 3[4-(4-nitrophenyl) piperazin-1yl]methyl-Benzoxazolone	170.8 ℃	45.3%
3	 5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-benzoxazolone	170 ℃	44.6%

The synthesized compounds were characterized by Fourier Transform Infra-Red (FT-IR) and Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR). Thin layer chromatography and melting point were used to check the purity and also to cross reference to the previously synthesized compounds.

The FT-IR spectra of compounds **2** and **3** show the absence of N-H stretch, which indicates that the reaction has actually taken place at position 3 of 5-chloro-2(3H)-Benzoxazolone and benzoxazolone. While for compound **1**, a weak peak present around 3200-3500cm^{-1} indicates the presence of N-H stretch as expected. Around 1760-1790 cm^{-1} carbonyl (C=O) stretch was observed in FT-IR spectra of all three compounds. Also C-H stretches are observed around 2700-3300 cm^{-1} as expected.

The FT-IR spectra of the compounds synthesized are shown in Fig. 4.5, Fig. 4.6 and Fig. 4.7 below;

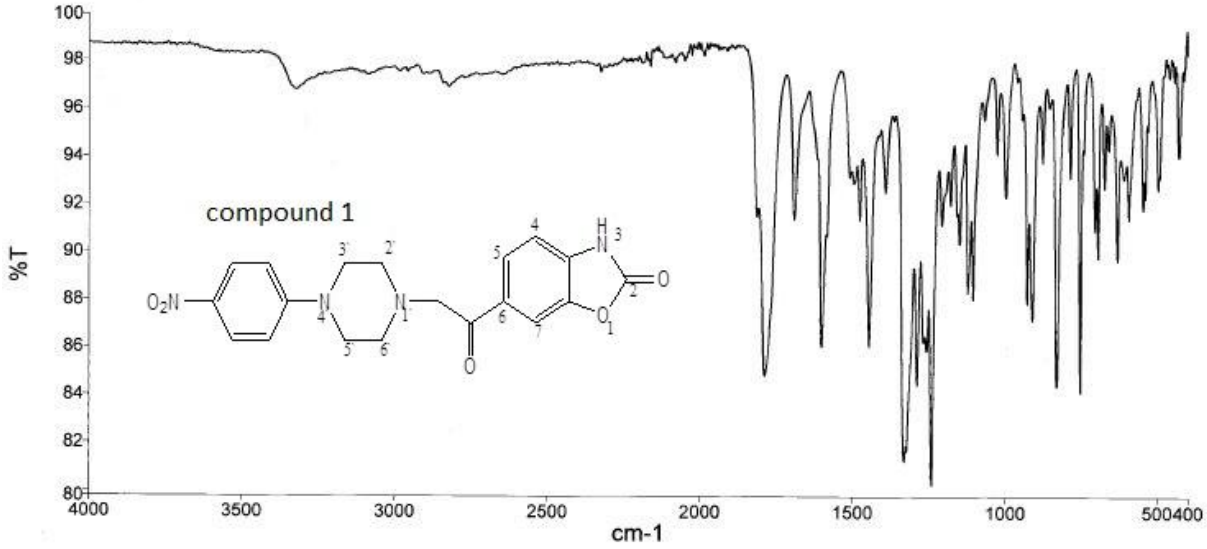


Figure 4.5: FT-IR Spectrum of 6-[4-(4-nitrophenyl) piperazin-1-yl]acyl-2-Benzoxazolone

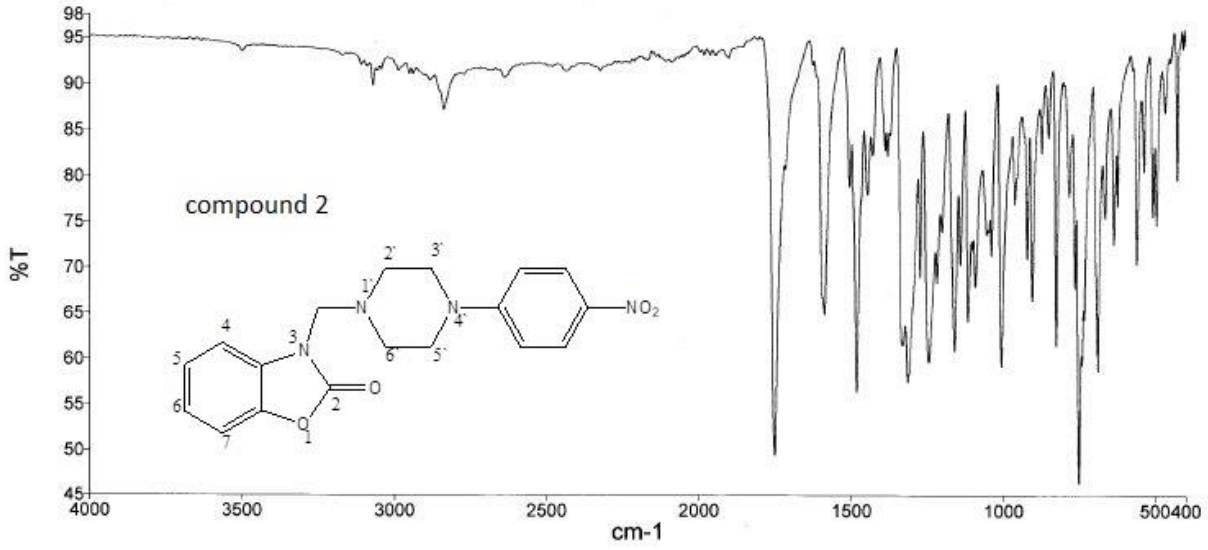


Figure 4.6 FT-IR Spectrum of 3[4-(4-nitrophenyl) piperazin-1yl]methyl-Benzoxazolone

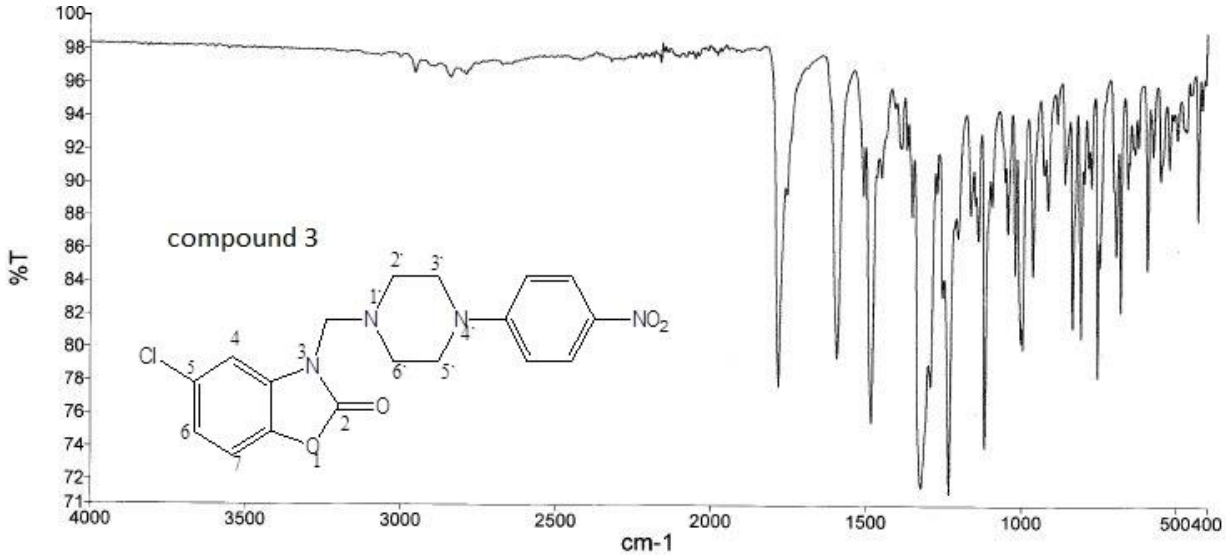


Figure 4.7 FT-IR Spectrum of 5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-Benzoxazolone

^{1}H-NMR spectra of compounds **1**, **2** and **3** in $CDCl_3$ show peaks at expected chemical shifts values. In all spectra, relative to the starting materials, there is an additional CH_2 (methylene) peak as a singlet observed at 4.7 ppm for three compounds. This also proves that the reaction has taken place at third and sixth position of benzoxazolone molecules via a CH_2 bridge. Investigations of ^{1}H-NMR spectra reveal the presence of aromatic peaks as multiples between 6.8 to 8.1 ppm for compound **2** and **3** and 7-8.1 ppm for compound **1** as also observed for similar compounds reported in the literature. The piperazine protons (H3`-H5`) and (H2` -H6`) were seen as triplets at 3.4 and 2.8 ppm respectively for both compounds **2** and **3**. This indicated that less shielded protons (H3`-H5`) are closer to the piperazine nitrogen next to the electron withdrawing group, benzene, while more shielded protons (H2` -H6) are closer to the piperazine nitrogen next to the electron releasing group methylene. By same principle the piperazine proton of compound **1** seen as triplet at 3.2-3.7 (H3`-H5`) and 2.4- 2.8 (H2` -H6`) ppm.^{1}H NMR spectra of compounds **1**, **2** and **3** in $CDCl_3$ are given in Fig. 4.8, Fig. 4.9and Fig. 4.10.

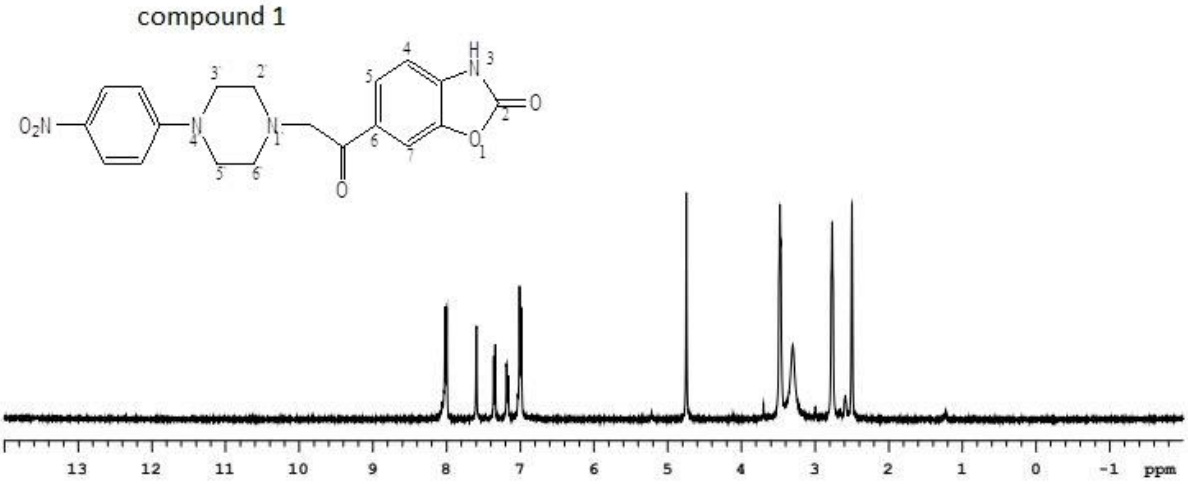


Figure 4.8:^{1}H NMR Spectrum of 6-[4-(4-nitrophenyl) piperazin-1-yl]acyl-2-Benzoxazolone

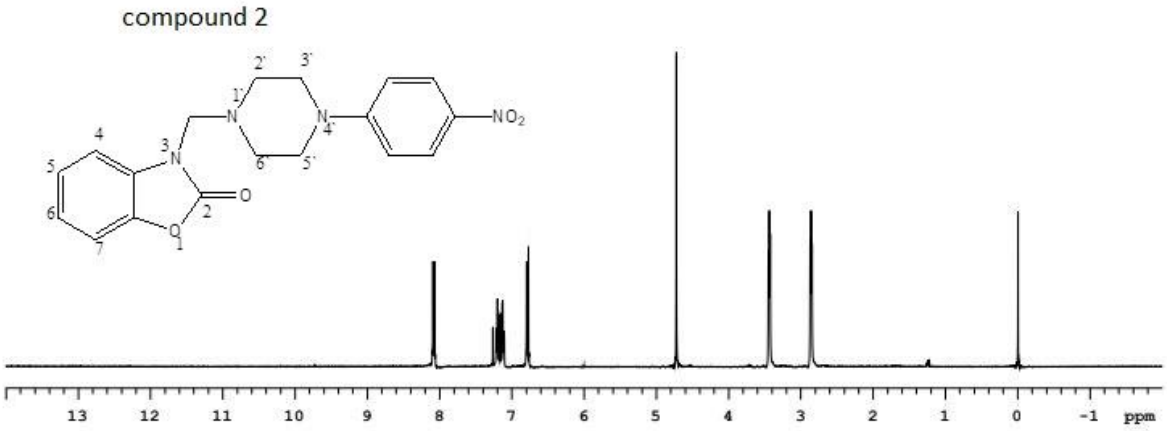


Figure 4.9:[1]H NMR Spectrum of 3[4-(4-nitrophenyl) piperazin-1yl]methyl-Benzoxazolone

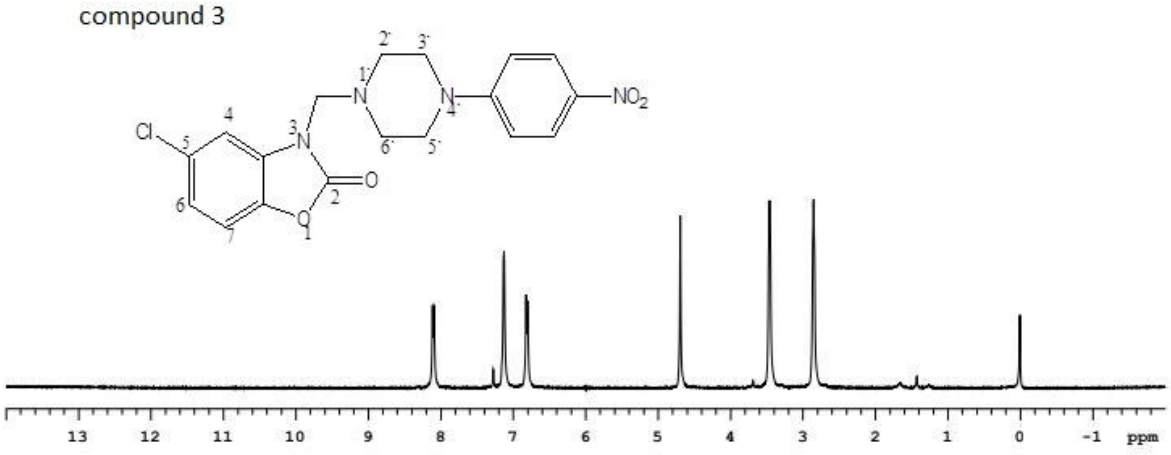


Figure 4.10:[1]H NMR spectrum of 5-chloro-3[4-(4-nitrophenyl) piperazin-1-yl]methyl-2-Benzoxazolone

In the cytotoxicity test five different concentrations of 2(3H)-Benzoxazolone derivatives were used (5, 10, 20, 50 and 100 μg/ml) in 24 hours and 48 hours of incubation period. The 50 μM was the most effective one in inhibiting MCF-7 cells viability and growth at 48 hours incubation period.

5. Conclusion

This research study focus on the synthesis and cytotoxic evaluation of three different derivatives of Benzoxazolone. We have chosen benzoxazolone core structure because it possible to do different substitutions which give us molecules that could have potentially biological activity.

The cytotoxicity of these three compounds was examined by using MTT-assay in 24 h and 48 h incubation period. The results were promising because even at low concertation (50 μM) it show growth inhibition toward MCF-7 cells line.

Depending on these results synthesis of similar compounds is planned to continue investigating more of the biological activities of these compounds.

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