CYCLOKETONE AND COUMARIN-BASED CHALCONE AND PYRAZOLINE DERIVATIVES: SYNTHESIS, IN SILICO STUDIES, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES AGAINST MCF-7 CELL LINE

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by

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LIST OF SYMBOLS

Å	Angstrom
α	Alpha
β	Beta
π	Pi
ρ	Density
µ	Micro
g	Gram
T	Temperature
δ	Chemical shift
℃	Degree celsius
m	Mili
M	Molar
n	Nano
k	Kilo
ns	Nanosecond
cal	Calori
mol	mol
ΔG	Binding energy
IC_{50}	Inhibition constant
mm	Milimol
µg	Microgram
µL	Microliter
%	Percent
µM	Micromolar

LIST OF ABBREVIATIONS

^{13}C NMR	Carbon Nuclear Magnetic Resonance
^{1}H NMR	Hydrogen Nuclear Magnetic Resonance
DEPT	Distortionless Enhancement by PolarizationTransfer
COSY	Correlated spectroscopy
HSQC	Heteroatom single quantum correlation
HMBC	Heteroatom multiple bond correlation
DMSO-d6	Deuterated dimethyl sulfoxide
$CDCl_3$	Deuterated chloroform
FTIR	Fourier Transform Infra-Red
MeOH	Methanol
EtOH	Ethanol
MP	Melting point
BBB	blood-brain barrier
PPB	Plasma protein binding
Pgp	P-glycoprotein
HIA	Human intestinal absorption
LogP	Distribution coefficient P
LogS	solubility
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donour
TPSA	Topographical polar surface area
Mw	Molecular weight
T1/2	Half-life time
ALA	Alanine
ARG	Arginine
ASN	Asparagine
ASP	Aspartate
CYS	Cysteine
GLN	Glutamine
GLY	Glycine
HIS	Histamine

LEU	Leucine
LYS	Lysine
MET	Methionine
PHE	Phenylalanine
SER	Serine
THR	Threonine
TYR	Tyrosine
VAL	Valine

LIST OF APPENDICES

TERBITAN KALKON DAN PIRAZOLINA BERASASKAN SIKLOKETON DAN KUMARIN: SINTESIS, KAJIAN *IN SILICO*, AKTIVITI ANTIOKSIDAN DAN SITOTOKSIK TERHADAP TITISAN SEL MCF-7

ABSTRAK

Kajian ini memfokuskan kepada sintesis, pencirian, kajian *in silico* dan penilaian biologi sebatian sikloketon dan sebatian kalkon dan pirazolina berasaskan kumarin sebagai agen antioksidan dan antikanser yang berpotensi. Kesemua sebatian ini telah berjaya disintesis melalui kondensasi Claisen-Schmidt diikuti dengan tindak balas siklo kondensasi. Kesemua struktur sebatian ini telah disahkan dengan menggunakan pelbagai teknik spektroskopi termasuk FT-IR dan NMR. Kajian pengedokan molekul telah dilakukan bagi meramal pengikatan sebatian yang disintesis terhadap protein sasaran reseptor estrogen alfa, ERα (3ERT) yang terlibat dalam laluan kanser payudara. Interaksi pengikatan yang berpotensi ini menunjukkan pengikatan efektif sebatian kepada asid amino utama ERα. Aktiviti antioksidan sebatian ini telah ditentukan menggunakan ujian radikal hapus sisa DPPH (2,2-difenil-1-pikrilhidrazil), yang berfungsi sebagai kaedah saringan awal untuk mengenal pasti perencat radikal bebas yang berpotensi. Sebatian **2c** (IC_{50} = 20.49 µM) dan **3c** (IC_{50} = 24.49 µM) menunjukkan aktiviti yang lebih baik sedikit daripada Trolox (IC_{50} = 30.83 µM), mencadangkan potensi mereka sebagai pemusnah radikal yang berkesan. Sebatian pirazolina **1civ** dan **1div** dalam siri 1 menunjukkan aktiviti antioksidan yang baik, masing-masing dengan IC_{50} 29.70 dan 20.74 µM manakala sebatian pirazolina **2di, 2dii** dan **2div** dalam siri 2 menunjukkan IC_{50} masing-masing sebagai 26.93, 27.49 dan 24.68 µM. Begitu juga, sebatian pirazolina **3ciii** dan **3dii** dalam siri 3 mempamerkan aktiviti antioksidan yang baik dengan nilai IC_{50} masing-masing sebagai 25.24 dan

17.01 µM. Aktiviti sitotoksik sebatian telah dinilai secara *in vitro* terhadap garisan sel kanser payudara MCF-7 dan sel manusia normal menggunakan ujian MTT. Keputusan menunjukkan bahawa sebatian tertentu menunjukkan ketoksikan terpilih terhadap garis sel kanser, dengan ketoksikan minimum terhadap sel normal. Sebatian **1b** dan **1d**, masing-masing dengan nilai IC_{50} 5.41 dan 1.89 µM, mempamerkan ketoksikan yang ketara, yang mana sebatian **1d** didapati lebih efektif daripada Tamoxifen (10.81 µM). Selain itu, sebatian **2c** menunjukkan ketoksikan tertinggi antara semua sebatian yang diuji, dengan nilai IC_{50} 0.65 µM dan indeks selektiviti 46.83. Sebatian lain dengan aktiviti ketoksikan yang baik termasuk sebatian **2d, 3a** dan **3c**, masing-masing dengan nilai IC_{50} 1.71. 8.26 dan 3.38 µM dan indeks selektiviti 8.06, 11.77 dan 9.04. Indeks selektiviti mencadangkan bahawa sebatian ini menawarkan kelebihan terapeutik dengan menyasarkan sel kanser tanpa menganggu tisu yang sihat. Kajian lanjut, termasuk penyiasatan mekanistik dan penilaian *in vivo*, adalah wajar untuk memahami sepenuhnya potensi terapeutik sebatian ini dalam pembangunan sebatian baharu untuk rawatan kanser.

CYCLOKETONE AND COUMARIN-BASED CHALCONE AND PYRAZOLINE DERIVATIVES: SYNTHESIS, IN SILICO STUDIES, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES AGAINST MCF-7 CELL LINE

ABSTRACT

This study focuses on the synthesis, characterisation, *in silico* study and biological evaluation of cycloketone and coumarin-based chalcone and pyrazoline compounds, as potential antioxidant and anticancer agents. All these compounds were successfully synthesised through Claisen-Schmidt condensation and subsequent cyclo-condensation reaction. The structure of the compounds was confirmed using various spectroscopic techniques, including FTIR and NMR. The molecular docking study was performed to predict the binding affinity of the synthesised compounds towards the target estrogen receptor alpha ERα (3ERT) protein involved in breast cancer pathways. The potential interactions suggest the effective binding of these compounds to the key amino acids associated with ERα. The antioxidant activity of the compounds was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, which serves as a preliminary screening method to identify potential free radical inhibitors. Compounds **2c** (IC_{50} = 20.49 µM) and **3c** (IC_{50} = 24.49 µM) showed a slightly better activity than Trolox (IC_{50} = 30.83 µM), suggesting their potential as effective radical scavengers. Pyrazoline compounds in series 1 such as **1civ** and **1div** showed good antioxidant activity of IC_{50} 29.70 and 20.74 µM, respectively, while pyrazoline compounds in series 2 namely **2di**, **2dii** and **2div** showed the IC_{50} of 26.93, 27.49 and 24.68 µM, respectively. Likewise, pyrazoline compounds **3ciii** and **3dii** in series 3 exhibited good antioxidant activity with IC_{50}

values of 25.24 and 17.01 μM, respectively. The cytotoxicity activity of the compounds was assessed *in vitro* against the MCF-7 breast cancer cell line and normal human cells using the MTT assay. The results revealed that certain compounds exhibited selective cytotoxicity towards the cancer cell line, with minimal toxicity to normal cells. Compounds **1b** and **1d** with IC_{50} values of 5.41 and 1.89 μM, respectively, exhibited significant cytotoxicity, with **1d** being particularly more effective than Tamoxifen (10.81 μM). Moreover, compound **2c** showed the highest cytotoxicity among all tested compounds, with an IC_{50} value of 0.65 μM and high selectivity index (SI) of 46.83. Other compounds with good cytotoxicity activity include compounds **2d**, **3a** and **3c** with IC_{50} of 1.71, 8.26 and 3.38 μM, respectively, with good selectivity index (SI) values of 8.06, 11.77 and 9.04, respectively. The selectivity index suggested that these compounds may offer a therapeutic advantage by targeting cancer cells while sparing healthy tissues. Further studies, including mechanistic investigations and *in vivo* evaluations to fully understand their therapeutic potential in the development of lead compounds for cancer treatment.

CHAPTER 1

INTRODUCTION

1.1 Background study

Cancer is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells in the body (Miller *et al.*, 2022). Failure to control this condition could ultimately lead to death (Key *et al.*, 2020). Generally, cancer is caused by genetic mutations of DNA cells which can be inherited or acquired by environmental factors (Brennan & Davey-Smith, 2022). Other causes are environmental exposure to carcinogens such as tobacco smoke, chemicals, and radiation. Lifestyle, infections, and hormonal imbalances can also contribute to cancer (Di Lonardo *et al.*, 2015). There are over 100 types of cancer, each classified by the nature of the cell initially affected. The common types of cancer are carcinomas, which originate in the skin or tissues that line internal organs like the breast, lung, prostate, and colorectal (Huang *et al.*, 2017). Others are sarcomas cancers that arise from connective tissues such as bone, cartilage, fat, muscle or blood vessels and central nervous system cancers that affect the brain and spinal cord (Razek & Huang, 2011). Cancer treatment depends on the type, location, cancer stage and the patient's overall health. Common cancer treatments include surgery, radiation therapy, chemotherapy, hormone therapy, immunotherapy, targeted hormone therapy and stem cell transplant (Saad & Fizazi, 2015; Rachner *et al*., 2018; Mitra *et al*., 2022; Wang *et al*., 2018). Cancer research is continually advancing with ongoing studies in genetics, molecular biology, and immunotherapy, leading to more effective treatments and potential cures (Wang *et al.*, 2021). Personalised medicine that adapts treatment to an individual genetic makeup, is an emerging and promising area (Goetz & Schork, 2018).

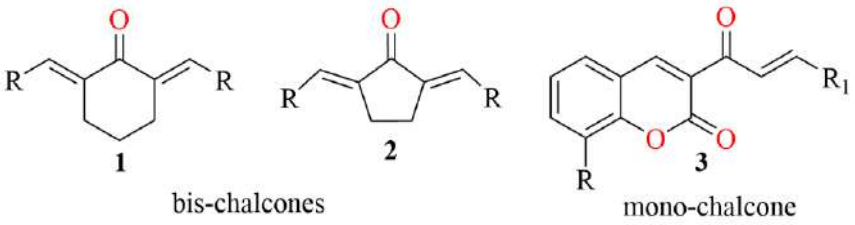
Meanwhile, breast cancer as one of the cancer types is a prevalent and life-threatening disease that affects a significant number of individuals worldwide (Akram *et al.*, 2017). Breast cancer comprises 25% of newly diagnosed cancer cases among women on a global scale, with an approximated total of over 2 million incidents of female breast cancer manifesting across the world (Houghton & Hankinson, 2021). Surgery treatment could be considered best at the initial stage of tumour growth where the breast cancer cells have not spread out to other parts of the organ or body (Tohme *et al.*, 2017). However, chemotherapy is the common type of treatment when cancer reaches an advanced state, where it starts to spread to other parts of the breast or body (Anand *et al.*, 2023). It is a type of cancer treatment that uses drugs to kill cancer cells. Chemotherapy drugs target rapidly dividing cells, a common characteristic of cancer cells (Aggarwal *et al.*, 2021). These drugs can work in various ways including interfering with DNA replication, damaging cell structures and inhibiting cell division (Maqbool *et al.*, 2023). Effectiveness in chemotherapy depends on the type and stage of cancer, the specific drugs used, and individual patient factors. However, these chemotherapeutic drugs have negative side effects that lower the quality of life for cancer patients by harming healthy body cells and causing organ toxicity (Chang, 2012). Therefore, it is important to continually discover new drugs that are highly selective toward cancer cells. To combat this disease, extensive research is being conducted to develop novel compounds with anti-breast cancer properties.

The development of drugs derived from medicinal plants has greatly advanced cancer therapy. New compounds with natural product scaffolds have attracted considerable interest. Numerous secondary metabolite constituents, particularly chalcone and pyrazoline scaffolds have been utilised. To develop new molecules with improved therapeutic potential, researchers modify the chemical structures of

compounds to increase the compounds' potency, selectivity for cancer cells, and metabolic stability, while minimising toxicity to healthy cells. (Guo, 2017). Researchers often aim to design small molecules with good pharmacokinetic properties, such as improved solubility, permeability, and bioavailability, which make them more suitable for oral administration and easier to absorb in the body since large-size molecules usually have solubility and absorption limitations (Liew *et al.*, 2020). Hybrid compounds, which combine two or more pharmacologically active scaffolds into a single molecule through covalent linkage, have garnered significant interest in drug discovery and development. These hybrid molecules can exhibit enhanced pharmacological potential due to the synergistic effects of their scaffolds, leading to improved efficacy, selectivity, and reduced side effects. Such compounds have improved pharmacological profiles because they can simultaneously target more than one mechanism enhancing the activity or selectivity and reducing the adverse drug effects. In this study, hybrid compounds with secondary metabolite scaffolds such as chalcone and pyrazoline attached to the coumarin ring have been of great interest.

Compounds with chalcone scaffolds have been exploited for their wide application in the pharmacological area due to their potential therapeutic benefits and have become a centre of attention in anticancer drug discovery (Rocha *et al.*, 2021; Nematollahi *et al.*, 2023). The unique chemical structure and naturally occurring characteristics of these compounds give them diverse pharmacological activities, including anti-inflammatory (Bensalah *et al.*, 2023; Emam *et al.*, 2021), antioxidant (Onar *et al.*, 2023; Xue *et al.*, 2018), and anticancer (Wang *et al.*, 2020) properties. Chalcones are found in nature and predominantly originate from medicinal or dietary plants (Gaonkar & Vignesh, 2017). These compounds are postulated to serve as the antecedents of flavonoid biosynthesis (Nematollahi *et al.*, 2023). Chalcone is a

phenolic compound belonging to the flavonoid family, which is widely recognised as an open-chain flavonoid (Rudrapal *et al.*, 2021). The general structure of a chalcone typically constitutes two aromatic rings connected by a three-carbon α,β-unsaturated carbonyl system C=C-C=O (Mahapatra *et al.*, 2015a; Pinto, 2016; Rosa *et al.*, 2019). In this study, we focused on two classes of chalcones viz; bis and mono chalcones from two cycloketone (cyclopentanone and cyclohexanone) and mono chalcones derived from coumarin scaffolds. Bis-chalcones are also of significant interest due to their diverse biological activities (Pereira *et al.*, 2023). Their synthesis and structural diversity allow the exploration of new derivatives with enhanced properties and therapeutic benefits (Ouyang *et al.*, 2021).



Coumarin-based chalcones are a fascinating class of organic compounds that combine the structural features of coumarins and chalcones, two groups known for their diverse biological activities and potential therapeutic applications (Zhuang *et al.*, 2017; Raad *et al.*, 2021). Coumarins are benzopyrone derivatives, consisting of a benzene ring fused to a lactone ring (Garg *et al.*, 2020). Coumarin-based chalcones integrate the coumarin moiety with the chalcone structure, typically by attaching the chalcone side chain to the coumarin ring system (Cao *et al.*, 2019). These compounds exhibit a range of biological activities and have attracted interest for their potential applications in medicinal chemistry and pharmacology (Barot *et al.*, 2015; Pasricha & Gahlot, 2020). Biological activities such as anticancer, antidiabetic, anti-inflammatory, antimicrobial and antioxidant have been reported (Xue *et al.*, 2018;

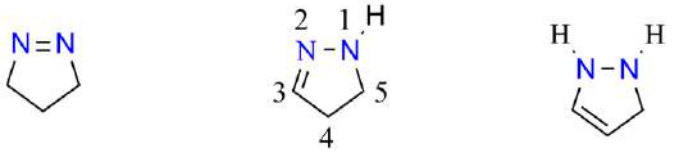
Onar *et al.*, 2023; Al-Warhi *et al.*, 2020; Wang *et al.*, 2020; Emam *et al.*, 2021). Coumarin-based chalcones represent a promising area of research due to their structural versatility and wide range of biological activities. Continued exploration of their synthesis and therapeutic potential could lead to the developing of new drugs for various diseases (Rohman *et al.*, 2024).

Nitrogen-based heterocyclic ring present in a wide variety of drugs has a broad range of pharmacological applications and has become important in the development of new effective drugs for the treatment of cancer (Hosseinzadeh *et al*., 2018). Molecular modelling and pharmacokinetic studies have shown that the incorporation of the nitrogen atom in the ring of a molecule can change its flexibility, metabolic profile, polarity, and capacity to form hydrogen bonding (Ooms, 2000; Van De Waterbeemd & Gifford, 2003). Most medicinal compounds contain a lot of these nitrogen heterocycles, particularly pyrazoline, which have become important in the creation of novel, efficient cancer drugs (Kerru *et al*., 2020).

Pyrazoline occurs naturally as alkaloids, vitamins and pigments of plant and animal cells (Varghese *et al.*, 2017). Pyrazolines are a class of aromatic five-membered heterocyclic compounds containing three carbon atoms, two adjacent nitrogen atoms at positions 1 and 2, and one terminal β-hydrogen with one endocyclic double bond. The position of the endocyclic double bond differs between the three types of pyrazoline which are 1-pyrazoline, 2-pyrazoline, and 3-pyrazoline. Among its various derivatives, the 2-pyrazoline scaffold is the most frequently studied because it has shown a wide range of possible pharmacological effects (Bhutani *et al*., 2015; Praceka *et al*., 2021).

They are being studied for their unique properties and several applications. They have been found to display anticancer, anti-inflammatory, and antimicrobial activities, among others (Karrouchi *et al.*, 2018). Pyrazolines are being extensively investigated as potential agents for combating cancer due to their cyclic structure and the various possible variations of the amine and R-groups (Ahmed *et al.*, 2019). The favourable antibacterial, antifungal, and even anticancer effects observed in other azomethine compounds have led researchers to hold a positive outlook on exploration of new pyrazoline compounds (Bhat *et al.*, 2023). In addition to these potential biological applications, they are also used as intermediates for many heterocyclic compounds (Varghese *et al.*, 2017). How these compounds work and precipitate numerous reactions has been the paramount issue, and many studies have been conducted to unravel their reaction mechanisms. Studies of these pyrazoline compounds have been pursued to investigate the organic synthesis methods, reaction mechanisms, and understanding of structure-activity relationships (Vahedpour *et al.*, 2021). The interest in pyrazolines has grown due to their presence in several pharmaceutical agents. Because of the medicinal relevance factors, many synthetic pathways have been examined for producing pyrazoline compounds. In light of these facts, the reactions of pyrazolines are of paramount importance in the field of organic chemistry. Pyrazoline compounds possess potential steroid hormone antagonist activity. So, many methods have been proposed for the syntheses of pyrazolines, and they have been subjected to many pharmacological evaluations (Matiadis & Sagnou, 2020). A variety of condensation reactions for the synthesis of pyrazolines have been reported. Aldol condensations, Michael addition, and Claisen-Schmidt condensations are some of these condensation reactions (Yadav & Wagh, 2020; Mandal *et al.*, 2016). Pyrazoline compounds are of interest due to their potential biological activities besides

their use as intermediates. Pyrazolines exist in several isomeric forms and research has shown that these isomers exhibit different reactions and yield different products (Şenol *et al.*, 2020). Pyrazolines have found increasing interest in medicinal chemistry because they are part of some commercial drugs. For the reason pyrazolines have the potential for biological applications, the development of new synthetic and systematic methods for their preparation has been growing (Mantzanidou *et al.*, 2021). The schematic representation below shows the various types of pyrazolines.



4 1-Pyrazoline **5** 2-Pyrazoline **6** 3-Pyrazoline

1.2 Problem statement

Breast cancer remains a major global health concern, with current treatments such as chemotherapy, radiation, and targeted therapies often leading to severe side effects and drug resistance. The search for novel, effective, and less toxic therapeutic alternatives is crucial. Chalcone and pyrazoline derivatives, particularly those based on cycloketone and coumarin scaffolds, have demonstrated promising anticancer properties, including apoptosis induction, cell cycle arrest, and inhibition of metastasis Ouyang *et al.*, 2021, Haider, *et al.*, 2022). However, despite their potential, their efficacy, selectivity, and mechanisms of action are not fully understood, limiting their development as alternative or complementary cancer therapies. This study aims to explore the synthesis, biological evaluation, and therapeutic potential of cycloketone and coumarin-based chalcone and pyrazoline derivatives, assessing their potential role in breast cancer treatment. Address toxicity and improve selectivity that target cancer

cells more specifically while sparing healthy cells, thereby improving patient outcomes.

1.3 Objective of the research

This research work focuses on five main objectives:

a. To synthesise and characterise a new series of mono and bis chalcones, coumarin-based chalcones, and their pyrazoline derivatives.
b. To evaluate the binding interactions of the synthesised compounds with estrogen receptor alpha (3ERT) using molecular docking and molecular dynamics simulations.
c. To analyse the in silico ADME properties of the synthesised compounds using the SwissADME tool to assess their therapeutic viability
d. To screen the synthesised compounds for antioxidant activity via DPPH radical scavenging assay and assess their cytotoxicity against breast cancer cells (MCF-7) through cell viability assays.

1.4 Scope of the study

Through a combination of experimental and computational approaches, new compounds against breast cancer can be developed. In this work, fifteen (15) coumarin-chalcone compounds from three categories; non-substituted, methoxy and ethoxy-substituted coumarins with different heterocyclic scaffolds have been synthesised and characterised using available spectroscopic instrument such as FTIR and NMR. Ten (10) new compounds were confirmed by Reaxys and ChemSpider chemicals databanks. In addition, twenty-four (24) cycloketone-based chalcones were produced. Similarly, forty-five (45) coumarin based and twenty (20) fused pyrazolines were produced and characterised as above. These novel compounds were also

subjected to antioxidant and cytotoxic techniques vis; Radical scavenging using DPPH assay and cytotoxicity using MCF-7 cell lines. The exploration of antioxidant activity shows the potential role in combating oxidative stress-related diseases as a therapeutic strategy. Furthermore, the use of MCF-7 cell line subtypes of breast cancer is to investigate potential therapeutic targets and their application in the study of estrogen receptor-positive breast cancer. The compounds were tested on MCF-7 breast cancer and L-929, a normal fibroblast cell line only. Not testing on a broader range of breast cancer subtypes (e.g., triple-negative breast cancer - MDA-MB-231). The study does not include *in vivo* testing of the compounds in animal models. In parallel, computational profiling techniques were employed to assess some properties. Computational tools such as molecular docking simulations was used to predict the binding affinities of these compounds to the estrogen receptor alpha (3ERT). Molecular dynamics (MD) simulations provide a more dynamic view of the interaction between ligands and receptors, to examine how the compounds behave in a biological environment and how stable these interactions are over time. *in silico* ADME profiling was done using the SwissADME web tool to evaluate the physicochemical properties, drug-likeness, and pharmacokinetic properties of the compounds, to predict their potential for oral bioavailability and drug development. The rationale behind the design of new anticancer compounds; such as chalcone, coumarin-chalcone and coumarin-pyrazoline compounds and their derivatives lies in their ability to target the key molecular pathways involved in breast cancer progression such as cell proliferation, apoptosis, angiogenesis, invasion, and metastasis. By modulating these pathways, these compounds can potentially inhibit tumour growth, induce cancer cell death, prevent angiogenesis and hinder the spread of cancerous cells (Mahapatra *et al.*, 2015b; Shrihastini *et al.*, 2021). Furthermore, the structure-activity relationships of

these derivatives are being explored to identify the key chemical features responsible for their anticancer activity. This information can guide the synthesis of analogues with improved potency, selectivity, and safety profiles (Rani *et al.*, 2019; Luo *et al.*, 2021; Satija *et al.*, 2022).

CHAPTER 2

LITERATURE REVIEW

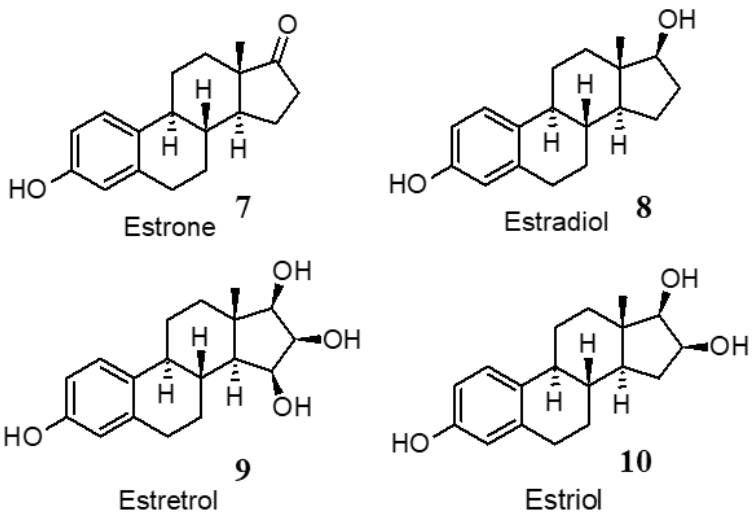
2.1 Breast cancer

Many cases of breast cancer, although not all, are impacted by variations in estrogen hormone levels (Azim and Partridge, 2014). The predominant viewpoint regarding estrogen function in breast cancer is that it serves as a trigger for the spread of cancer by promoting the division and proliferation of breast tissue, a mechanism that entails the potential for the occurrence of cancer-inducing genetic alterations (Hilton *et al.*, 2018). The human body is composed of numerous quadrillions of cells. The majority of these cells are continually undergoing division and duplication, a mechanism that maintains the operation of organs daily, throughout man's lifetime (Hammarlund *et al.*, 2020). A cell undergoes division to replicate its chromosomes, which are clusters of densely packed DNA, into a newly formed cell (Reyes-Lamothe and Sherratt, 2019). However, occasionally, this process does not occur as intended, resulting in DNA breakages. In the majority of instances, these breaks in DNA are promptly repaired by the molecular machinery responsible for safeguarding the integrity of the genome. Even though, on rare occasions, the repair of broken DNA is unsuccessful, leading to the misplacement or scrambling of chromosomes within a cell (Kleine *et al.*, 2009).

During cellular division, human cancers can develop through the rearrangement of chromosomes, leading to the activation of dormant cancer genes that initiate the growth of tumours (Duijf and Benezra, 2013). This chromosomal recombination event transpires when a chromosome breaks and generates a duplicate copy before the repair of the damage. Subsequently, in an unfortunate attempt at mending the damage, the broken end of one chromosome becomes fused with the broken end of its sister copy instead of its original counterpart. Consequently, the resulting structure is an aberrant

and dysfunctional chromosome (Burssed *et al.*, 2022). In the subsequent cellular division, the deformed chromosome undergoes elongation as it spans the two nascent progeny cells (Hanscom and McVey, 2020). Subsequently, the aforementioned chromosome "bridge" fractures, resulting in fragmented remnants harbouring cancerous genes that proliferate and become activated (Gusa and Jinks-Robertson, 2019). Certain types of malignancies in humans, such as specific forms of breast tumours, develop due to chromosomal rearrangements within the affected cells (Burssed *et al.*, 2022).

Estrogen, in conjunction with its corresponding receptors, combines to constitute a complex that penetrates the cellular nucleus. The estrogen receptors ERα and ERβ, which exhibit differential affinities for estrogen, serve as significant prognostic markers for the identification of breast carcinoma (Crowe, 2018). Estrogen is a collection of female hormones, comprising of estrone **7**, estradiol **8**(Jameh-Bozorghi *et al.*, 2015), estretrol **9** and estriol **10** (Malainou *et al.*, 2023). All four estrogens possess 18 carbons ($C_{18}H_{24}O_2$) and are collectively identified as C_{18} steroids (Nairne, 2009), consisting of one benzene ring, a phenolic hydroxyl group, and a ketone group (estrone), or one (17β-estradiol), two (estriol), or three (estretrol) hydroxyl groups (Mazurek *et al.*, 2020).



Estrone **7** Estradiol **8**

9 **10**

Estretrol Estriol

Estrogen and estrogen receptors combine to form a unit that enters the cell nucleus. The estrogen receptors ERα and ERβ, which have variable estrogen attraction are notable prognostic indicators to identify breast cancer tumours (Pagano *et al*., 2020). ERα (Estrogen receptor alpha) and ERβ (Estrogen receptor beta) are two main types of estrogen receptors, which are proteins that mediate actions of estrogen, but they play distinct roles in different tissues (Venkatesan *et al.*, 2010). ERα is responsible for cell proliferation (Fox *et al*., 2008; Liu *et al*., 2020; Saha *et al.*, 2021) and is the most common trigger of breast cancer (Omoto & Iwase, 2015; Akram *et al*., 2017) while ERβ contributes to tumour suppression. ERα which is associated with breast cancer in humans (Menendez & Lupu, 2017) consists of six domains with a total of 595 amino acids while ERβ comprises of six domains with 530 amino acids (Arnal *et al.*, 2017; Yaşar *et al.*, 2017). Several drugs and small molecules have been investigated for their ability to bind ERα which is important in the genesis and evolution of hormone-dependent breast cancer (Bafna *et al*., 2020).

Selective estrogen receptor modulators (SERMs) are a diverse group of nonsteroidal compounds that function as ligands for estrogen receptors, ERα and ERβ. When SERMs bind to these receptors, they induce a conformational change that can activate or inhibit the receptor's ability to regulate gene expression (Herfindo et al., 2020). Tamoxifen is an example of a SERM and is the most readily used adjuvant hormonal therapy for breast cancer, which can minimise the risk of breast cancer reappearance and death (Yao *et al*., 2020). Depending on the tissue in which it acts, it can function as an estrogen receptor antagonist (blocker) and an agonist (activator). In breast tissue, Tamoxifen acts as an antagonist and competes with estrogen for binding to ERα, thereby blocking estrogen proliferative action and thus inhibiting the growth of breast cancer cells. Tamoxifen has cytotoxic activity against MCF-7 breast cancer cells

(Huang *et al.*, 2015). Since tamoxifen also has adverse effects (Fisher *et al.*, 2005), alternative treatment is needed and research in the area has been continuously developed. The possibility of resistance also restricts the efficacy of Tamoxifen. Understanding the diverse mechanisms behind this resistance is essential for developing new therapeutic strategies and optimising treatment outcomes (Yao *et al.*, 2020). Ongoing research continues to explore ways to predict, prevent, and overcome resistance, thereby improving the prognosis for patients with hormone-dependent breast cancer.

2.2 Computational approach in drug design

Modern drug design involves a multidisciplinary strategy that utilises advanced technologies and scientific knowledge to explore and create novel drugs for a diverse array of diseases (Prieto-Martínez *et al.*, 2019; Mitra *et al.*, 2022), as illustrated in Figure 2.1. The objective is to create drugs with enhanced safety, greater efficacy, and precision in targeting particular diseases while causing minimal adverse effects (Manzari *et al.*, 2021). There are some key aspects of modern drug design which include;

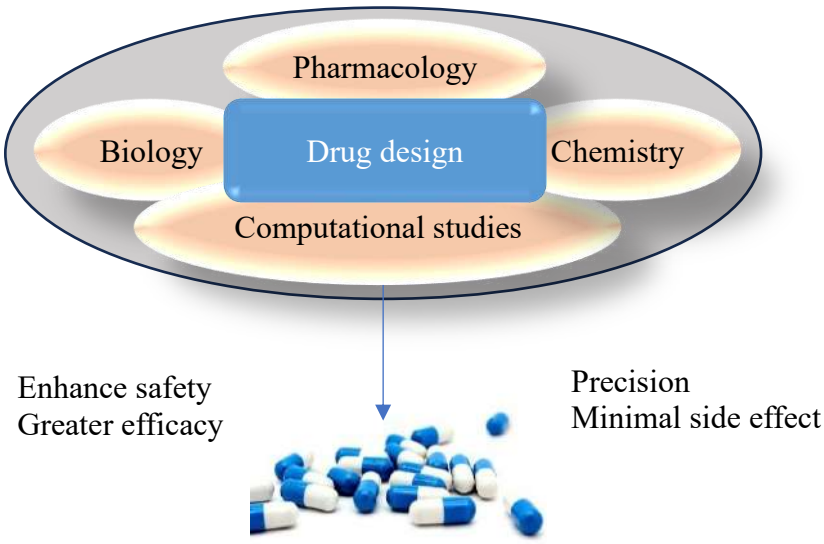


Figure 2.1 Modern drug design technology

2.2.1 Computational modelling in drug design

Advances in computational chemistry and molecular modelling have facilitated this study area, allowing researchers to simulate the complex interactions between drugs and their respective target molecules (Haldar *et al.*, 2018). The methodology and predictions regarding potential drug candidates' binding affinity and effectiveness are made to expedite the drug discovery process (Leelananda and Lindert, 2016). With the increased accessibility of extensive omics data and computational resources, scholars are exploring established drugs for novel therapeutic purposes. The drug repurposing strategy offers a cost-efficient approach to discovering innovative applications for approved or trial compounds potentially accelerating their transition to clinical application (Talevi and Bellera, 2020). Molecular modelling technologies have developed an automated platform for computer-aided drug design (CADD) which is time and cost-effective, a fast track to some limiting factors of drug discovery. CADD provides *in silico* study of a structure and the prediction of its biological activity (Huang *et al.*, 2015; Meng *et al.*, 2011). In drug discovery, two techniques of CADD are structure-based drug design (SBDD) and ligand-based drug design (LBDD), as illustrated in Fig 2.2.

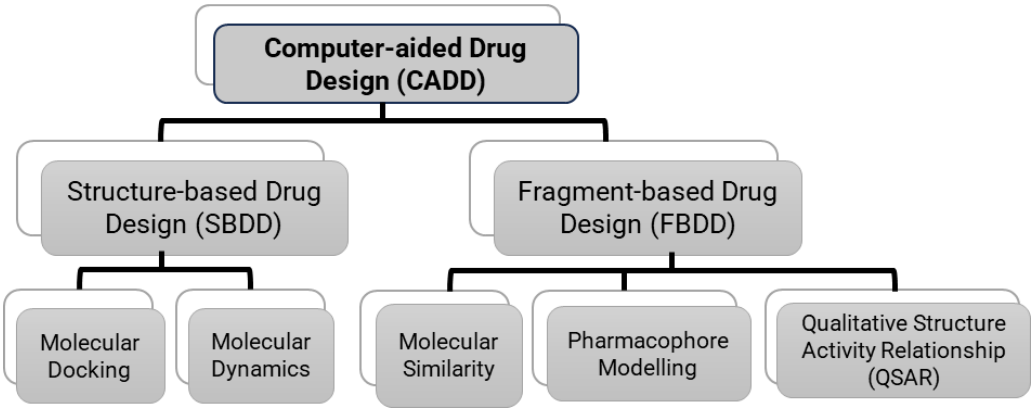


Figure 2.2 Computer-aided modelling in drug design

2.2.1(a) Structure-based drug design (SBDD)

Structure-based drug design (SBDD) is another method in medicinal chemistry that uses the three-dimensional structures of biological targets to design effective drugs (Wang *et al.*, 2018). By understanding the precise shape and chemical properties of the target molecule, usually a protein or enzyme, researchers can design compounds that bind specifically and tightly to the target, modulating its activity. Estrogen receptor alpha, ERα (3ERT-protein) was the protein identified and used in this research. Structure-based drug design continues to be a cornerstone of modern medicinal chemistry, offering a rational and efficient approach to developing new therapeutics that can effectively target complex diseases (Van der Greef *et al.*, 2007, Van Montfort and Workman, 2017).

2.2.1(b) Fragment-based drug design (FBDD)

Fragment-based drug design (FBDD) is a strategy for discovering lead compounds in drug development. Unlike traditional high-throughput screening, which uses large molecules, FBDD begins with smaller chemical fragments. These fragments are simpler, often with a molecular weight of less than 300 Daltons, and they tend to bind with lower affinity but higher efficiency (Bon *et al.*, 2022). This was among the reason of selecting chalcones and pyrazolines used in this research. Fragment-based drug design represents a powerful approach in modern medicinal chemistry, offering the potential to discover new drugs with high efficiency and precision. By focusing on the interactions of smaller fragments, FBDD leverages detailed structural insights to guide the development of effective therapeutics (Erlanson *et al.*, 2016).

For this research, we only concentrate on computational modelling, synthesis and in vitro assays of chalcone and pyrazoline compounds. Computational modelling

consists of molecular docking, molecular dynamics (MD) simulation and *in silico* ADME screening of the compounds as mentioned earlier.

2.2.2 Molecular docking

Molecular docking is a computational tool employed within molecular biology and bioinformatics to predict a molecule-favoured orientation (binding mode) towards a designated target receptor (Maden *et al.*, 2022). This approach is prominently palpable in drug design and discovery, facilitating a comprehensive understanding for researchers of the interplay between small molecules, like potential drug candidates, and protein targets within the biological system (Honarparvar *et al.*, 2014). Molecular docking is the most common SBDD approach in drug development because it can predict the conformation of small molecules (ligands) within the receptor/protein/molecular target (Ferreira *et al.*, 2015; Yuriev et al., 2015). The interaction forms a protein-ligand complex which is important to predict the biochemical phenomenon. A protein can be downloaded from a protein data bank (PDB), and docking is conducted using software such as AutoDock 4.2, one of the most used software programs to design protein-ligand interactions. The ligand can be docked to best fit on the active site, indicating the most favourable energetic conformation within ligand-protein complexes and the biological effects of the interactions (Udatha *et al.*, 2012; Meenambiga *et al.*, 2015). Molecular docking is important in virtual screening, lead optimisation, and understanding structure-activity correlations in drug discovery (Rudrapal and Chetia, 2020). Despite the valuable insights and guidance offered by molecular docking to experimental activities, it is constrained by limitations it should be employed in cycles with alternative experimental and computational techniques in the field of drug discovery (Temml and Kutil, 2021).

2.2.3 Molecular dynamics (MD) simulation

This is another computational technique used to explore the behaviour of a molecule over time (Adcock and McCammon, 2006). Molecular dynamics (MD) simulations are commonly employed in multiple scientific disciplines such as chemistry, biophysics, materials science, and pharmaceutical research (Salo-Ahen *et al.*, 2021). It involves the numerical integration of the locations and velocities of atoms in a molecular system by applying Newton's laws of motion (Smith, 2014). Researchers can use this technique to model the motions and interactions of molecules in many scenarios, including solutions, varying temperatures, or when subjected to external forces (Boldon *et al.*, 2015). These insights offer essential information about the dynamics and behaviour of molecular systems at the atomic level. They work alongside experimental techniques to aid researchers in comprehending complex biological systems and creating innovative materials. Nevertheless, molecular dynamics (MD) simulations can be expensive in terms of computational power, necessitating substantial computational resources and knowledge for efficient setup and analysis (de Vivo *et al.*, 2016).

2.2.4 *in silico* ADME properties

in silico ADME (Absorption, Distribution, Metabolism, and Excretion) focuses on understanding how a drug behaves pharmacokinetically (Chandrasekaran *et al.*, 2018; Kar & Leszczynski, 2020; Wu *et al.*, 2020). These are the important properties used in drug development to determine drug safety and efficacy. Both methods rely on computational models to predict these properties without conducting experiments. *in silico* ADME analysis predicts potential compounds to be synthesised (Dar *et al.*, 2019) so that molecules with a greater probability of success can be identified (Daina *et al.*,

2017). Nevertheless, these *in silico* predictions should be employed with experimental data and other computational techniques (Gleeson *et al.*, 2012).

2.3 *In vitro* analysis

In vitro refers to experiments or research conducted in an artificial setting outside a living organism, usually within a regulated laboratory setting (Knudsen *et al.*, 2015). I*n vitro* means ‘in glass’ in Latin, which signifies "in glass," denoting experiments performed in glass containers such as test tubes, Petri dishes or multi-well plates (Kurhekar *et al.*, 2019). *In vitro* studies are fundamental in numerous scientific fields, such as biology, chemistry, pharmacology, and medicine. They enable researchers to examine biological processes, validate hypotheses, and assess the impact of drugs or other substances in controlled settings before advancing to more complex and expensive *in vivo* experiments conducted within a living organism (Wikswo, 2014). It involves isolated cells, tissues, or biomolecules, providing a streamlined model system for investigating distinct facets of biological processes or evaluating compound pharmacological or toxicological characteristics (Sorger *et al.*, 2011). The *in vitro* assay involves standard methods which comprise cell cultivation, enzyme analyses, protein-protein interaction examinations, and drug screening assays (Nierode *et al.*, 2016). *In vitro* assays control experimental conditions such as pH, temperature and concentration of substances to isolate and study specific factors. It also reduces the need for animal testing, which is quicker and less expensive than *in vivo* studies, allowing large experiments to be conducted. Furthermore, it provides detailed mechanistic insight into the biological process or the mode of action of drugs or compounds (Blomme and Will, 2016). However, *in vitro* assay might not entirely emulate the details of biological processes in living organisms, and data obtained from *in vitro* experiments function as

an initial phase in scientific research, guiding the preparation and analysis of subsequent *in vivo* assays (Eisenbrand *et al.*, 2002).

2.3.1 Radical scavenging assay

A radical scavenging assay is an *in vitro* laboratory technique employed to assess the antioxidant potential of substances by determining their efficacy to counteract or neutralise free radicals (Amorati and Valgimigli, 2015). Free radicals are extremely reactive molecules containing unpaired electrons, capable of inducing harm to cellular structures and tissues via oxidative stress (Ifeanyi, 2018). Conversely, antioxidants are substances that can donate electrons to stabilise free radicals thereby reducing their damaging effects (Sisein, 2014). Radical scavenging is extensively used in both academic research and industry for screening and assessing the antioxidant capabilities of organic substances, food additives and medicinal agents (Gupta, 2015). There are various techniques for conducting radical scavenging studies, however, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is the most commonly used (Melendez *et al.*, 2014). The radical scavenging efficacy of the compounds is quantified in terms of the fraction of DPPH radicals neutralised by the antioxidant agents. A higher percentage of scavenging activity correlates with the strong antioxidant ability exhibited by the examined compounds (Gulcin and Alwasel, 2023).

2.3.2 Cytotoxicity assay

Cytotoxicity assay is also an *in vitro* laboratory method applied to evaluate the cytotoxic impacts of substances on cultured cells, assessing their capability to induce cell death or inhibit cell proliferation (Niles *et al.*, 2008). This technique offers significant insights into the potential toxicity of a compound. In pharmacology, toxicology, and drug discovery, cytotoxicity assays are extensively employed to examine the safety profiles of various substances like chemicals, pharmaceuticals, and

environmental agents. This assay is fundamental in drug development and safety assessment, aiding in the early detection of possible adverse effects of substances and facilitating decisions on further development or optimisation (Liu *et al.*, 2013).

The IC_{50} values of these compounds were compared with the standard references used. Cytotoxic activity is the ability of a test sample to cause toxic effects in the cancer cells which can be measured by assessing the proliferation of the cells. The grade of the activity is based on the IC_{50} values: IC_{50} of < 1 µM (strong potency), IC_{50} of 1–10 µM (strong potency), IC_{50} of 10–20 µM (moderate potency), IC_{50} of > 20 µM (low potency) and IC_{50} of > 100 µM (inactive) (Doganay et al., 2016). IC_{50} is the compound concentration required to inhibit a specific biological function or activity by 50%. It measures how effectively a compound can inhibit a particular target. GI_{50} is the compound concentration needed to inhibit cell population growth by 50% compared to an untreated control. It measures the compound's effect on cellular proliferation or growth. IC_{50} (Inhibitory Concentration 50) and GI_{50} (Growth Inhibition 50) are used to assess the efficacy of compounds, but they measure different aspects of biological activity. Both values provide a more comprehensive view of a compound's biological activity and potential therapeutic effects (Fiorentino *et al*., 2018).

2.4 Coumarins

Coumarin is a fragrant organic compound found naturally in many plants, such as sweet clover and cinnamon, and is known for its distinctive sweet, hay-like aroma. It has been extensively studied for its diverse biological activities, including anticoagulant, anti-inflammatory, antioxidant, and anticancer properties. Coumarin derivatives, such as warfarin, have been widely used in medicine as anticoagulants to prevent blood clots. Additionally, coumarins have applications in perfumery, cosmetics,

and as flavouring agents, though their use in food is regulated due to potential hepatotoxicity at high doses. Recent research continues to explore novel coumarin-based compounds for their therapeutic potential, particularly in targeting oxidative stress-related diseases and cancer. The versatility of coumarin and its derivatives makes it a significant focus in both chemical and pharmacological studies.

2.5 Cyclo ketones

Cycloketones, such as cyclopentanone and cyclohexanone, are cyclic organic compounds characterised by a carbonyl group (C=O) within a five- or six-membered ring structure, respectively. These compounds are widely utilized as intermediates in organic synthesis and industrial applications, particularly in the production of pharmaceuticals, agrochemicals, and polymers. Both cyclopentanone and cyclohexanone exhibit unique reactivity due to their ring strain and carbonyl functionality, making them valuable in various cyclization, oxidation, and condensation reactions. Recent studies have also explored their potential in green chemistry, such as their use as solvents or starting materials for sustainable chemical processes. Additionally, their structural motifs are found in numerous natural products and bioactive molecules, driving ongoing research into their synthetic applications and biological significance.

2.6 Chalcones

Chalcone is a secondary metabolite from plants flavonoids and isoflavonoids that can act widely on pharmacological targets (Rammohan *et al.*, 2020). Chalcone comes from the Greek word "chalcos" means bronze which refers to the colour of the natural chalcone (Zhuang *et al.*, 2017). Chalcone scaffolds are commonly used as

medicinal chemical templates and can target important chemical processes contributing to cancer treatment (Mahapatra *et al.*, 2015b).

2.6.1 Synthesis of chalcones

Chalcone is synthesised via a straightforward chemical process that allows for numerous alternatives. Several contemporary methods and techniques have been developed in chalcone synthesis (Elkanzi *et al.*, 2022). The most important procedure is condensing two aromatic systems (with electrophilic and nucleophilic groups) under base or acid catalysis, which results in the chalcone scaffold. Chalcones are employed as intermediates in synthesising useful pharmaceutical compounds due to their simple structures and diverse pharmacological actions (Bukhari *et al.*, 2012). Despite the wide range of substitutions, some common methods for chalcone preparation are discussed.

2.6.1(a) Claisen-Schmidt condensation

This reaction named after Rainer Ludwig Claisen and J. G. Schmidt, two pioneering chemists, is the most common method to synthesise chalcone from benzaldehyde and acetophenone (Zhuang *et al*., 2017). Generally, chalcones are produced through the reaction of aromatic aldehydes and ketones in the presence of acid or base (Anam *et al*., 2017) at either room or high temperature. Usually, an alcohol medium is used as the solvent for this reaction (Yadav & Wagh, 2020; Alidmat *et al.*, 2021). A base-catalysed condensation of 4-bromoacetophenone and 4-iodobenzaldehyde in methanol was stirred at room temperature, followed by a dropwise addition of NaOH as shown in Figure 2.3. The reaction gave a pure chalcone compound after recrystallisation (Zainuri et al., 2017).

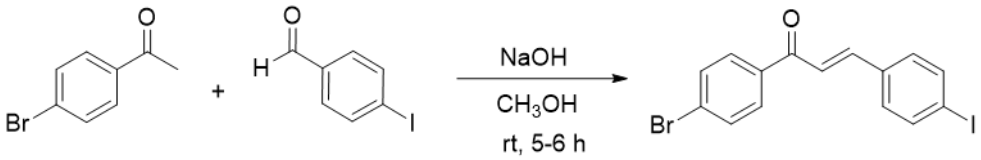


Figure 2.3 A base-catalysed Claisen-Schmidt condensation

An acid-catalysed Claisen-Schmidt condensation between substituted acetophenone and benzaldehyde using BF_3-Et_2O as an acid catalyst as illustrated in Figure 2.4. The reaction mixture was washed with water to remove BF_3 and recrystallisation formed a pure chalcone compound (Narender & Papi Reddy, 2007).

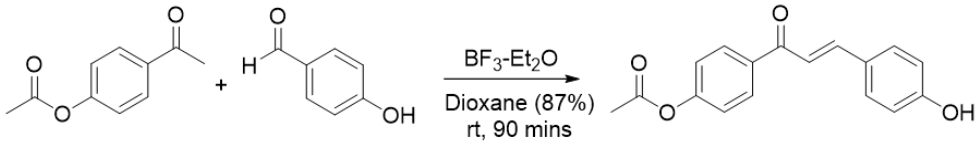


Figure 2.4 An acid-catalysed Claisen-Schmidt condensation

2.6.1(b) Suzuki-Miyaura coupling

Suzuki reaction is a widely used cross-coupling reaction of aryl or vinyl halides with boronic acids or boronate esters to form the carbon-carbon bonds (Goyal *et al.*, 2021). Suzuki reaction was introduced by Akira Suzuki in 1979, and later the synthesis of chalcone using this reaction was reported (Eddarir *et al.*, 2003). Suzuki-Miyaura coupling is another interesting metal-catalysed cross-coupling reaction. Figure 2.5 shows a preparation of chalcones via the Suzuki-Miyaura coupling of styryl boronic acid and various benzoyl chlorides which reported a very high yield of 80% (Haddach & McCarthy, 1999).

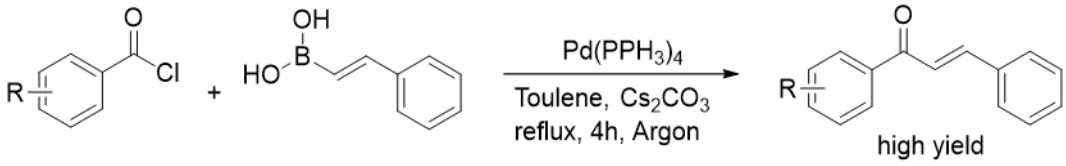


Figure 2.5 A Suzuki-Miyaura coupling of chalcones