Sara Kärppä

Synthesis and Characterization of Novel Tilorone Analogues

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Abstract

Author:	Sara Kärppä
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Supervisors:	Paula Kiuru, Docent Manuela Vorácová, Doctoral Researcher Kari Raatikainen, Senior Lecturer

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive lung disease characterized with abnormal scarring of lung tissue, for which current treatment options are limited. This thesis study contributes to the ongoing drug discovery efforts within the University of Helsinki's medicinal chemistry research group, in collaboration with Helsinki University Hospital (HUS), by focusing on the synthesis and characterization of novel tilorone-based analogues.

Alkylation reactions, nucleophilic addition reactions, and acetylation reactions were the three sets of reactions that made up the thesis study. Various synthetic techniques, including conventional and microwave-assisted heating, were used and optimized. The nucleophilic addition reactions were proved to be the most effective ones, producing four new compounds with excellent purity and yields, while the alkylation reactions had difficulties with product isolation and poor yields. Although, there was difficulties, one compound was isolated with good purity. These analogues were characterized using nuclear magnetic resonance (NMR) spectroscopy, ensuring their structural identity and quality.

A total of five novel compounds and one known standard were successfully selected and submitted for biological testing at HUS, where they will be evaluated using three different cell lines. These results support the continued exploration of tilorone-based structures in the search for new drug candidates for IPF.

Keywords: Idiopathic pulmonary fibrosis, medicinal chemistry, organic synthesis, tilorone analogues, thin-layer chromatography, drug development

Tiivistelmä

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Idiopaattinen keuhkofibroosi (IPF) on krooninen sairaus, joka johtaa keuhkojen etenevään arpeutumiseen. Tällä hetkellä käytössä olevat lääkehoidot, kuten nintedanibi ja pirfenidoni, hidastavat taudin etenemistä mutta eivät paranna sitä, ja niiden käyttöön saattaa liittyä haittavaikutuksia. Tämän opinnäytetyön tavoitteena oli edistää uusien lääkeaineiden kehittämistä idiopaattisen keuhkofibroosin hoitoon osana Helsingin yliopiston lääkekemiallisen tutkimusryhmän ja Helsingin yliopistollisen sairaalan (HUS) välistä yhteistyötä. Työssä keskityttiin tiloroniin pohjautuvien uusien molekyylien synteesiin ja karakterisointiin.

Työ jakautui kolmeen reaktiosarjaan: alkylointi-, nukleofiiliset additio- ja asetylointireaktiot. Synteesissä käytettiin sekä mikroaaltoreaktioita että perinteisiä refluksireaktioita, ja menetelmiä optimoitiin reaktio-olosuhteita säätämällä. Alkylointireaktioiden tuotteiden puhdistaminen osoittautui haastavaksi, minkä vuoksi saannot jäivät alhaisiksi. Tästä huolimatta yksi yhdiste saatiin eristettyä hyvällä puhtaudella. Sen sijaan nukleofiiliset additioreaktiot tuottivat neljä erittäin puhdasta yhdistettä, joiden saannot nousivat jopa 98 %:iin. Yhdisteiden rakenne ja puhtaus varmistettiin yhdinmagneettisella resonassispektroskopialla (NMR).

Kaikki viisi uutta yhdistettä sekä yksi vertailustandardi lähetettiin biologisiin testeihin, jotka tullaan suorittamaan kolmella eri solulinjalla HUS:ssa. Työn tulokset tukevat tiloroniin perustuvien yhdisteiden jatkokehittämistä keuhkofibroosin mahdollisina lääkeaineina.

Avainsanat:	idiopaattinen keuhkofibroosi, lääkeainekemia, orgaaninen synteesi, tiloroni analogit, ohutkerroskromatografia, lääkekehitys

Confidential Information and Use of Artificial Intelligence

Due to intellectual property considerations, the chemical structures and synthetic routes are not presented in the public version of this thesis. However, they are included in a confidential appendix available only to the thesis supervisor and the evaluation committee.

OpenAI’s ChatGPT (version GPT-4o mini) was used to assist with the structuring, language refinement and formatting the written part of this thesis. I have not substituted my own writing with AI-generated content but have followed the revision suggestions provided by the AI. No confidential information has been shared with ChatGPT, and as the author of this thesis, I take full responsibility for all its content.

Contents

List of Abbreviations

α-SMA	Alpha smooth muscle actin
APCI	Atmospheric pressure chemical ionization
DCM	Dichloromethane
ECM	Extracellular matrix
EtOAc	Ethyl acetate
HUS	Helsinki university hospital
IPF	Idiopathic pulmonary fibrosis
MeOH:	Methanol
MERS-Cov:	Middle East respiratory syndrome coronavirus
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
TEA	Triethylamine
TGF-β	Transforming growth factor beta
TLC	Thin-layer chromatography
UV	Ultraviolet

1 Introduction

The thesis study was implemented as a part of the medicinal chemistry research group at the Faculty of Pharmacy, Division of pharmaceutical chemistry and technology, at the University of Helsinki. The study was carried out in a collaboration with Prof. Marjukka Myllärniemi group, Helsinki University Hospital (HUS), contributing to ongoing efforts to develop new drug candidates for idiopathic pulmonary fibrosis (IPF). This thesis study focuses on the synthesis and characterization of novel tilorone analogues to explore their structural modifications and potential pharmaceutical applications. The study involved optimizing synthetic methods through both conventional and microwave-assisted synthesis, followed by the characterization of the compounds using nuclear magnetic resonance (NMR), and mass spectrometry (MS). The thesis lab work was conducted in the research group's well-established laboratory, in Viikki campus in Helsinki.

Idiopathic pulmonary fibrosis is a chronic and progressive lung disease characterized by excessive scarring of lung tissue, leading to a decline in lung function. Despite advances in understanding its pathogenesis, effective treatment options remain limited. Current drugs, such as nintedanib and pirfenidone slow the progression of the disease but do not cure the condition, and their use is often associated with adverse effects. Because of this, there is an urgent need for new therapeutic agents to target molecular mechanisms of fibrosis. Tilorone (figure 6), a small-molecule interferon inducer, has known immunomodulatory properties, and its derivatives have demonstrated anti-inflammatory and antifibrotic effects, making them potential candidates for further study in medicinal chemistry [1.]

The goal of this work was to develop and refine synthetic strategies for tilorone analogues while assessing their structural properties. By modifying these molecules and evaluating their chemical characteristics, this study aimed to contribute to the field of medicinal chemistry and support the search for

potential IPF drug candidates. The findings may serve as a foundation for future research and preclinical studies.

2 Theoretical Background

2.1 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis is a specific type of chronic, progressive lung disease characterized by fibrosis of the lung tissue [1]. The term “idiopathic” reflects the unknown cause of disease, which primarily affects adults over 50 years old and is more common in men and smokers. IPF leads to decreasing lung function, resulting breathlessness, dry cough and reduced quality of life. In IPF, the healing process of the lungs is being disturbed and instead of repairing damaged tissue, the lungs develop more scar tissue, which makes them stiff and not able to function properly. This reduces the ability of the lungs to take oxygen in, leading to breathing difficulties that worsen over time. Many studies have shown that the median of survival time is somewhere between two to three years after diagnosis and the diagnosis is often delayed because of nonspecific symptoms. [1; 2.]

Currently, there is no cure for IPF, however, treatments such as pirfenidone and nintedanib are available and have been utilized to slow the progression of the disease [3]. These therapies help reduce the formation of scar tissue and improve the quality of life for the patient [2]. However, research to find better treatments is necessary, because the disease remains difficult to manage. [4; 5.]

Idiopathic pulmonary fibrosis is a rare but serious disease, with an estimated global prevalence varying between 2–29 cases per 100 000 persons. The incidence rate is about 5–10 new cases per 100 000 persons annually, although this can vary depending on the population and region. At the age of 50, the incidence tends to rise. [1.]

Several factors have been identified as potential risk contributors for IPF, even though the exact reason behind the disease is still unknown. These factors include:

- smoking
- environmental exposures
- microbial agents
- gastroesophageal reflux
- genetics
- viral infections.

Among these factors, smoking has emerged as the most strongly associated risk factor. [3; 4.]

2.1.1 Mechanism of IPF

The pathogenesis of IPF can be understood within the framework of the body's natural wound-healing process, which includes three distinct phases: injury, inflammation and repair [6]. In response to lung injury, the alveolar epithelial cells release inflammatory mediators, triggering platelet activation. Under physiological conditions, this would lead to a wound-healing process and formation of a wound clot with subsequent tissue repair. However, in IPF patients, these cells become dysfunctional. Instead of proper repair, the damaged cells release a cascade of various bioactive molecules, such as cytokines, chemokines and extracellular matrix (ECM) proteins. These substances drive the formation of scar tissue (fibrosis) in the lungs, disrupting normal lung healing and architecture, lung function and leading to respiratory decline. [6.]

One of the key elements of IPF is excessive activity of fibroblasts and myofibroblasts, which are the contributors to the scarring process. Myofibroblasts are specific cells that produce a large amount of ECM proteins, including collagen. This causes damage and scarring the lung structure.

Myofibroblasts are mediate wound contraction, they express proteins like α-smooth muscle actin (α-SMA) and fibroblast activating protein. α-SMA gives properties to myofibroblasts that allow them to contract and stiffen surrounding tissue, which contributes to the thickening and reduce flexibility of the lungs in IPF. Fibroblast activating protein is an enzyme that remodels the extracellular matrix and increases fibroblasts activity, contributing to tissue scarring. [4.]

There are three main sources of myofibroblasts in the lungs:

1. Fibroblasts that already exist in lung tissue and become overactive.
2. Alveolar epithelial cells that transform into myofibroblasts through a process called epithelial-to-mesenchymal transition, driven by a protein called transforming growth factor beta (TGF-β).
3. Circulating fibroblasts from the bone marrow that can travel to the lungs and become myofibroblasts. [4.]

The protein transforming growth factor beta (TGF-β) is a significant driver in this process. It stimulates fibroblast proliferation and leads to epithelial-to-mesenchymal transition, in which epithelial cells transform into myofibroblasts. This transformation leads to increased collagen production and scarring in the lungs. The interactions between epithelial cells, fibroblasts, immune cells and signaling pathways like TGF-β play key roles in driving the disease. Understanding these mechanisms may lead to better treatments and improved therapies in the future. [5; 6; 7.]

2.1.2 Treatment Options and Research

IPF has no known cure, and now, only two medications have been approved to treat its symptoms. These are small-molecule compounds called pirfenidone (figure 1) and nintedanib (figure 2). However, ongoing research for many other compounds and antibodies has entered the clinical evaluation of numerous compounds, many of which demonstrated promising anti-fibrotic activities. [6.]

Pirfenidone, approved in Japan 2005 and in EU 2011 [8], has been used as a treatment for IPF for its anti-inflammatory, antioxidant and anti-fibrotic effects. Even though the exact mechanism of action remains unknown, it is known that pirfenidone inhibits the expression of the TGF-β and cytokines with fibrotic and inflammatory properties, as well as acts as an antioxidant by scavenging hydroxyl radicals and superoxide anions. That is beneficial to reduce the production of fibroblasts and collagen synthesis. Pirfenidone has a specific structure that contains oxidized pyridine ring and has phenyl and methyl functional groups (figure 1). It is classified as a pyridone derivative. [8.]

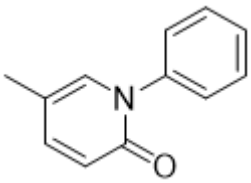

Figure 1 Structure of pirfenidone.

Another available treatment for IPF is nintedanib, a tyrosine kinase inhibitor originally developed for cancer therapy. It targets multiple growth factors involved in fibrosis, including platelet-derived growth factors, fibroblast growth factors, and vascular endothelial growth factors, by inhibiting their respective tyrosine kinase receptors. Several studies have shown that nintedanib is safe to use and effective in IPF, although it is not recommended in patients with low lung capacity. The chemical structure of nintedanib contains a 2-indolone skeleton (figure 2). [9.]

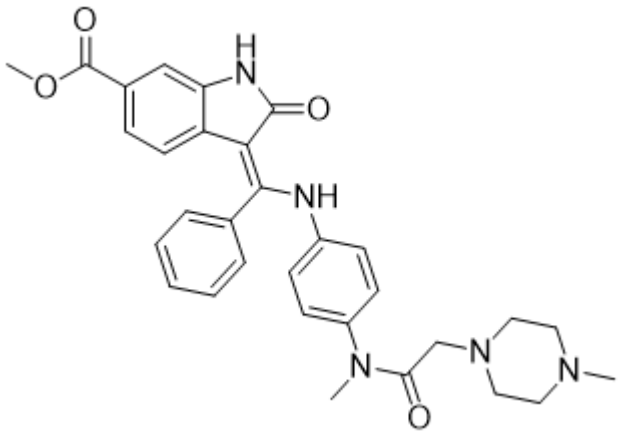

Figure 2 Structure of nintedanib.

Currently, research efforts are aimed at researching potential TGF-β inhibitors, as these are significant signaling factors in fibrosis. TGF-β plays a crucial role in changing fibroblasts to myofibroblasts and weakens the activity of the enzymes that break down the ECM, as well as stimulates cell proliferation. As an example, there exists an anti-allergic drug, that has been used in skin fibrosis in Japan called Tranilast® and it has been proved to inhibit the expression of TGF-β. [6.]

Pulmonary fibrosis is a complex disease, and while understanding its pathogenesis deepens, the effective treatments remain limited. While drugs like nintedanib and pirfenidone have shown promising results, they cannot fully reverse fibrosis. Additionally, both medications are associated with difficult adverse effects. In a clinical trial, over 90% of the patients had reported at least one side effect. Nintedanib is primarily linked to gastrointestinal issues (e.g. nausea and diarrhea), while pirfenidone has also been associated with skin related adverse effects. When it comes to drug treatment, research needs to be done even though the process might be lengthy. [6.]

2.2 Coumarins

Coumarins are plant-based aromatic organic molecules, that have been first described in 1822 and first synthesized in 1868 [10]. Known for their pleasant

aroma, coumarins have historically been used in perfumes and flavoring. However, more than 200 years of studies have proven that derivates of coumarins have pharmacological and biological effects, and because of those, they have been used in medicine for example as an anti-inflammatory, antihypertensive, antioxidant, anticoagulant and neuroprotective agents. [10; 11.]

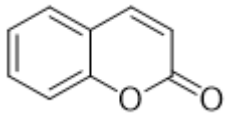

Figure 3 Coumarin structure.

The basic form of coumarin (2*H*-1-benzopyran-2-one) has a characteristic benzopyrone structure, consisting of a benzene ring fused to lactone ring (figure 3). Their core structure plays a role for numerous naturally occurring and synthetic derivatives. The basic molecular framework can be modified by various functional groups, resulting in diverse chemical and biological properties. [11.]

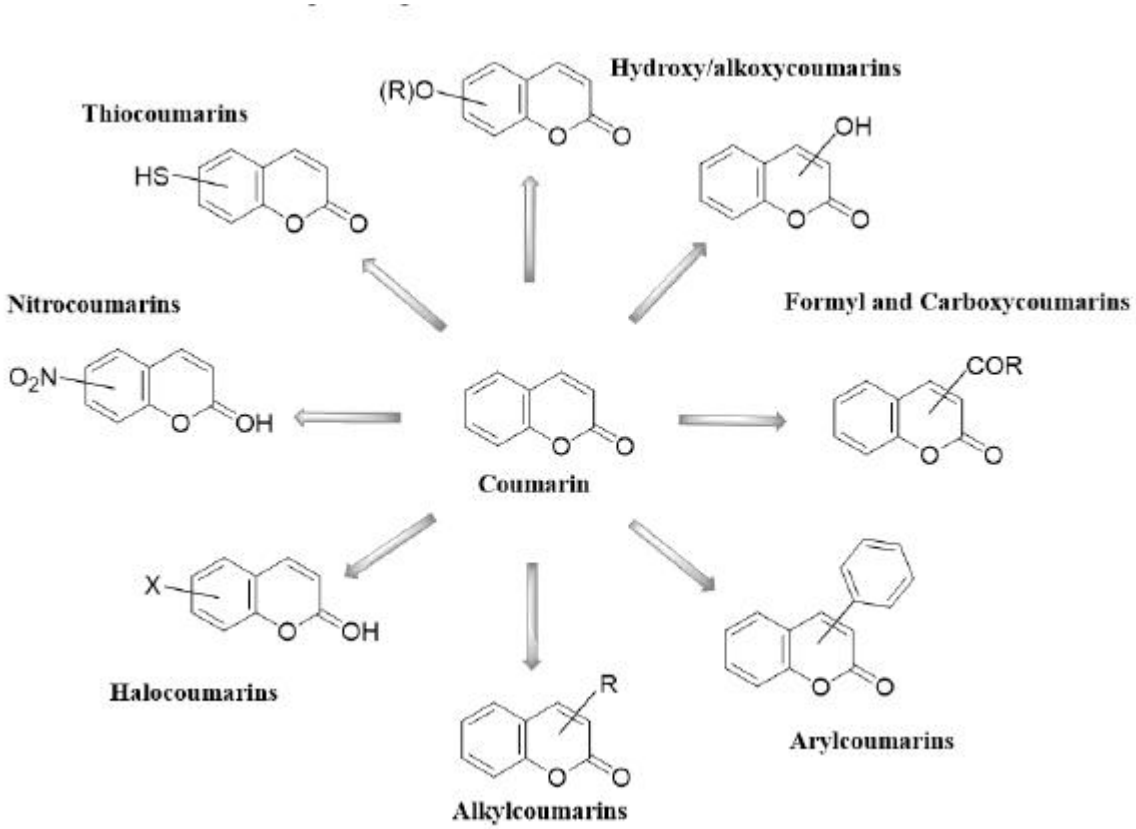

Figure 4 Example of simple coumarins. [11.]

The conjucated benzopyrone structure of coumarins gives them a versatile reactivity profile, with various examples seen in figure 4. The benzene ring is prone to electrophilic substitution reactions, allowing the introduction of functional groups that can modify their properties. The lactone ring is reactive under basic conditions, where nucleophilic attack can open the ring and form coumaric acid derivates. Coumarins can also be hydroxylated, which improves their solubility and changes their activity. The lactone ring can be reduced to dihydrocoumarins or then they can be oxidized. These reactions make coumarins useful in chemical synthesis, and drug development. [11.]

Coumarins are typically crystalline solids with sweet odor. They are moderately soluble in organic solvents (e.g. ethanol, acetone) but less soluble in water due to their hydrophobic aromatic ring. Coumarins exhibit strong ultraviolet (UV) absorption due to their conjugated structure, making many of them useful as fluorescent markers. [12.]

2.2.1 Pharmacological Activities of Coumarins

Coumarins have a wide range of biological and pharmacological activities, making them valuable in medicinal chemistry and therapeutic applications [13]. Their diverse activities arise from their ability to interact with various biological targets and influence cellular processes. One of the most well-known pharmacological properties of coumarins is their anticoagulant effect. Compounds like warfarin and acenocoumarol are synthetic derivatives that prevent blood clot formation by inhibiting vitamin K epoxide reductase, an enzyme essential for blood clotting. Their structures are presented in figure 5. [13; 14.]

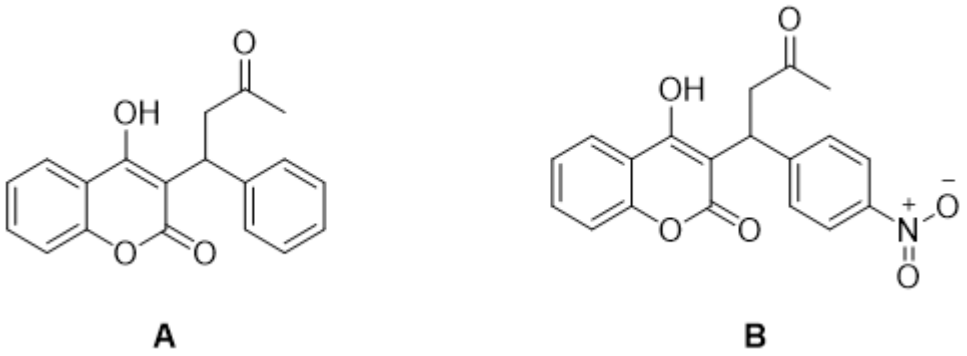

Figure 5 Compound A warfarin and compound B acenocoumarol.

Coumarins have shown promising anticancer properties by inducing apoptosis in cancer cells and inhibiting tumor growth. Some coumarins act as angiogenesis inhibitors, blocking the blood supply to tumors, while others inhibit specific signaling pathways critical for cancer progression. Coumarins also possess antimicrobial effects against bacteria, fungi and viruses. [13; 14.]

Of particular relevance in this context is the potential of coumarins to exhibit anti-fibrotic effects, which makes them candidates for treating fibrotic diseases. They inhibit the activation of myofibroblasts, which are the primary drivers of collagen production. Additionally, coumarins can interfere with transforming growth factor-beta signaling, a critical pathway that triggers fibrotic responses. By reducing TGF-β activity or blocking its downstream signaling, coumarins

prevent the excessive production and deposition of collagen and other extracellular matrix proteins [14].

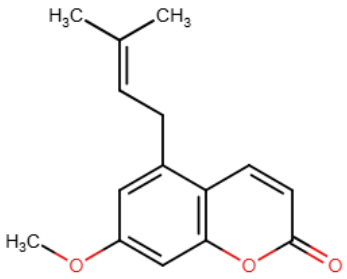

Figure 6 Structure of osthole.

One of the compounds that has shown anti-fibrotic effects with suppressing collagen expression and inhibiting TGF- β-induced migration is osthole (figure 6). [15.]

2.3 Tilorone

Tilorone (figure 7) is a synthetic drug that helps the immune system fight viruses by stimulating the production of interferons; protein that plays a key role in protecting cells from infection. Interferons activate the body's antiviral defenses, blocking viruses from multiplying and spreading. Tilorone has been shown to work against many types of viruses, including influenza, herpes, West Nile virus and even coronaviruses like MERS-CoV [16]. This drug is mainly used in Russia and a few neighboring countries, where it is sold under the names Amixin® and Lavomax®, with over 20 years of clinical experience, though it remains less studied globally. [16.]

Because of tilorone's immunomodulatory properties, it may also have potential for treating IPF [16]. It has been shown to inhibit the pro-fibrotic TGF-β pathway

and activate anti-fibrotic bone morphogenetic protein signaling in mouse models [17]. These mechanisms suggest it could reduce inflammation and fibrosis. [17.]

Tilorone is an aromatic compound that has an aliphatic chain that gives it a symmetrical structure which is seen in figure 7.

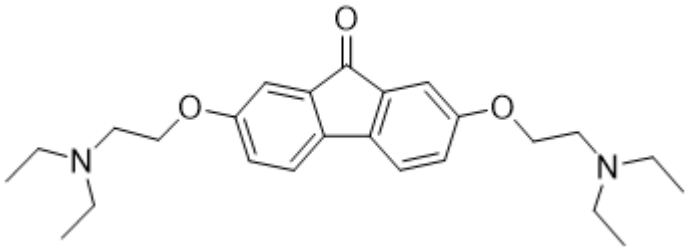

Figure 7 Structure of tilorone.

Tilorone's structure consists of a planar fluorenone core with a central ketone functional group, along with aminoalkoxy chains extending from the aromatic system. These aminoalkoxy side chains contribute to its solubility and potential interactions with biological targets, while the fluorenone backbone plays an important role in its structural stability. [18.]

2.3.1 Tilorone's Pharmacological Activities

As mentioned earlier, tilorone's main mechanism of action circles around inducing the production of interferons, which are critical proteins in the body's defense against pathogen. These interferons, such as interferon-alpha and interferon-beta have a central role in antiviral immunity by activating cellular pathways that inhibit viral replication and spread. [18.]
Along to its antiviral properties, tilorone exhibits significant immunomodulatory effects. It enhances the activity of natural killer (NK) cells and T-cells, both of which are vital for identifying and destroying virus-infected cells. Moreover, tilorone promotes the release of cytokines and other immune mediators, amplifying the immune system's ability to respond to infections. [18.]

Tilorone's anti-inflammatory activities are another important aspect of its pharmacological profile. By regulating the production of interferons and cytokines, tilorone can reduce excessive inflammatory conditions, although it has not been extensively studied in such contexts. [19; 20.]

One area of interest for future research is tilorone's potential effects on fibrosis, or tissue scarring, which is a central feature in IPF. Interferons, which are stimulated by tilorone, have been shown in some studies to reduce the proliferation of fibroblasts and decrease the deposition of extracellular matrix proteins, both key processes in fibrosis. While this antifibrotic potential has not been primary focus of tilorone's clinical use, it suggests that the drug might offer benefits in treating conditions where fibrosis plays a significant role. [19.]

While tilorone's primary use has been as an antiviral agent, its broader impact on immune regulation and inflammation suggests it may have untapped potential in treating conditions like IPF and other inflammatory diseases. Further studies are necessary to fully understand how tilorone could be utilized for the purpose. [20.]

2.4 Characterization Techniques

2.4.1 Thin layer chromatography coupled with mass spectrometry (TLC-MS)

Thin layer chromatography as itself is a simple, easy to perform and inexpensive technique, and needs minimal amount of solvents and for that reason it is widely used in laboratory to separate compounds from reaction mixtures. With TLC only, it is impossible to directly identify or characterize the analytes from the TLC plates. TLC is often combined with mass spectrometry, which is one of the most efficient analytical techniques to identify structures. [21.]

2.4.2 Fundamentals of TLC

TLC works like any other chromatographic analysis, where the compounds interact with the stationary and mobile phase based on their chemical properties. In TLC, compounds separate based on their polarity. Unlike other chromatographic analyses, with TLC there is no fear of the so called “memory effect”, because with every analysis there is a new plate (stationary phase) used for each separation. The memory effect refers to a residual compound from previous analyses interfering with new samples, which can happen with techniques like gas-chromatography, where some high-boiling-point compounds can adsorb strongly to the silica and not elute completely, leading to contamination of subsequent separation. In addition to these advantages, TLC is conducted under ambient conditions, it is an ideal separation method for high-throughput analysis. [21.]

In TLC analysis the stationary phase is typically organic or inorganic materials, such as silica, alkyl-silica, cellulose and monolithic polymer, which are coated onto metal, plastic, or glass sheets. The mobile phase consists of organic solvents with varying hydrophobicities, selected based on the analyte’s properties. During the separation process, the edge of the TLC plate is placed in the mobile phase, which moves upward through the stationary phase by capillary action. The placement and the plate on a chamber is seen below on a figure 8. The interaction between the analyte, mobile phase and stationary phase determines the movement of each compound, resulting in different migration rates. The separation is quantified using the retention factor (R_f), calculated as the ratio of the distance travelled by the analyte to the distance travelled by the mobile phase. [22.]

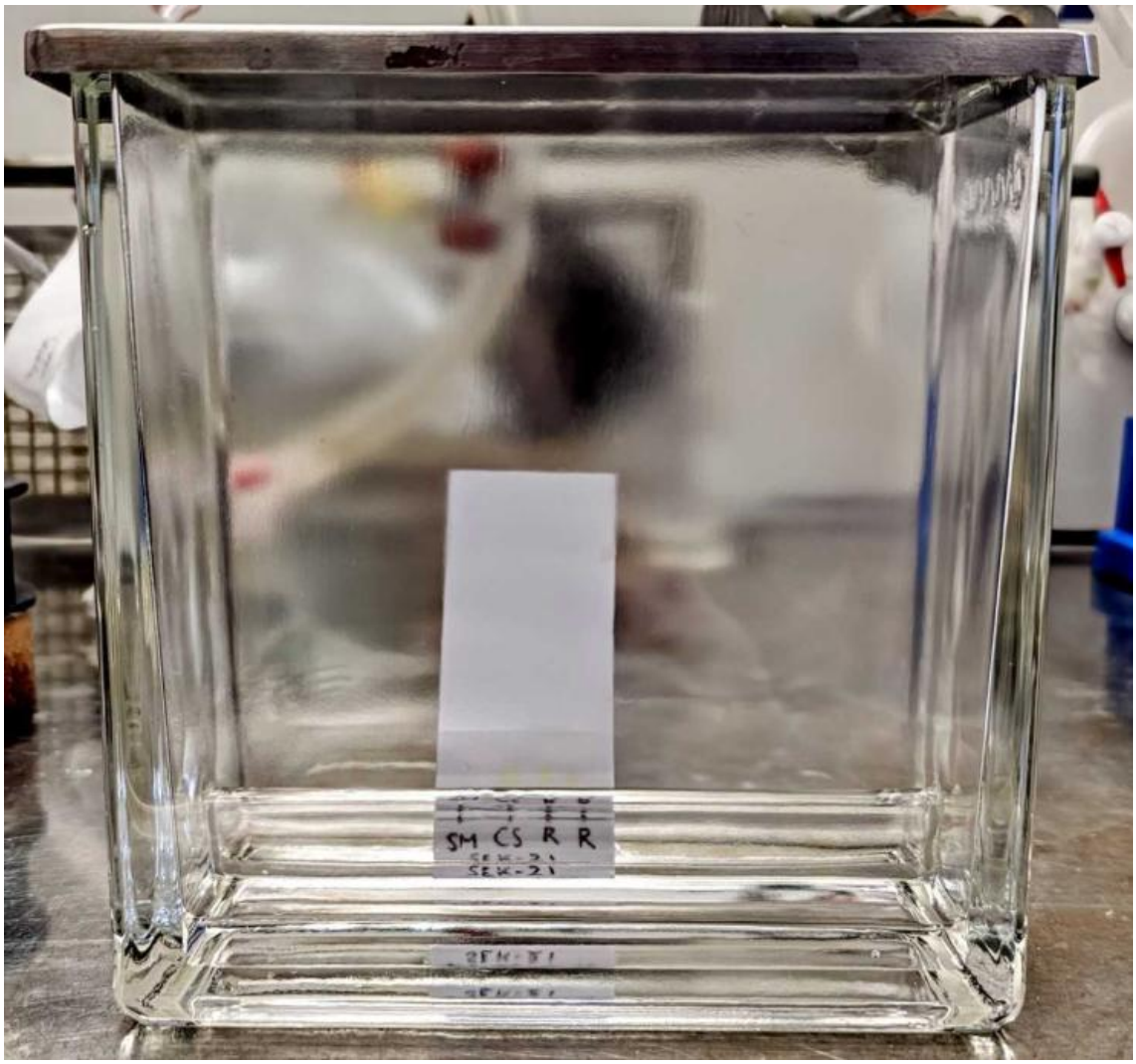

Figure 8 TLC chamber, with TLC plate in it.

The most difficult part with TLC analysis is usually to find a suitable mobile phase, so called solvent system. The solvent system needs to be homogeneous, and selecting the right solvents requires careful consideration of several factors, such as solubility, which guarantees that the sample dissolves well in the solvent, affinity, which balances sample's attraction to the solvent and the stationary phase, and resolution, which refers to the degree of separation between closely eluting compounds. [22.]

Typically, the mobile phase is a mixture of a polar solvent and less polar solvent (e.g. heptane – ethyl acetate -mixture). The simplest way to find a perfect solvent system is to start with a less polar combination and observe the

separation. If the components do not move very far, then the polarity should be increased and compare the results from this more polar solvent system to the less polar one. [22.]

After running the sample on a TLC plate, the separated compounds can be visualized using UV light or by staining the plate. In figure 9, the TLC plate is exposed to UV light. [22.]

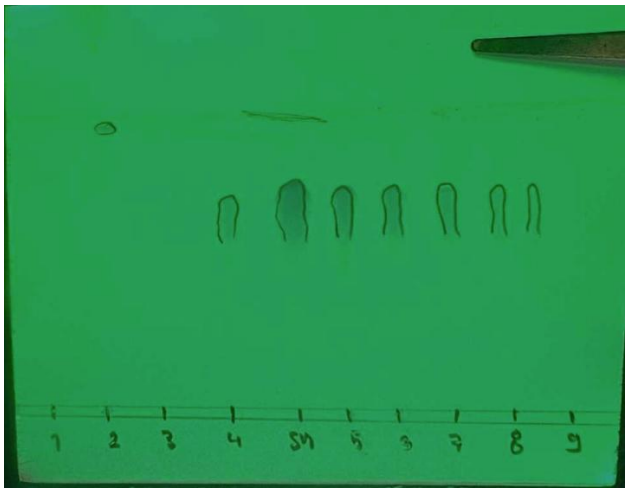

Figure 9 TLC plate under UV-light.

Especially the staining might be necessary because most of the organic compounds are colourless, as seen on figure 10, where the highest purple compound was not visible under UV-light. There are several types of staining reagents that are commonly used for visualizing compounds on TLC plates [22], depending on their chemical structure:

- Iodine: Temporary and general stain.
- Vanillin: Another good general stain.
- Potassium permanganate: Good for alkenes, alkynes and aromatics.
- Bromocresol green: For acidic compounds
- Ninhydrin: Excellent for amino acids and amines. [22.]

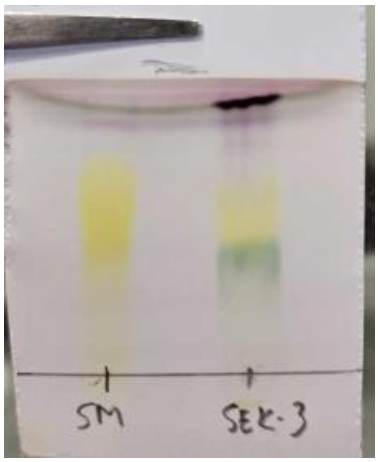

Figure 10 TLC plate stained with vanillin. First spot is the starting material and the second one the reaction mixture. From the mixture can be seen, that there is still some starting material left, but two other compounds are also seen (green and purple).

Staining the TLC plate with a chemical dye is a destructive visualization method, meaning that once stained, the original compounds can no longer be detected under UV light, as they undergo a chemical transformation during the staining process. In contrast, UV light visualization is a non-destructive method, allowing compounds to remain unchanged after detection. TLC plates are typically coated with a fluorescent indicator, which causes the background to emit a green glow under UV light at 254 nm, making non-fluorescent compounds appear as dark spots. Additionally, UV light detection at 365 nm can be used, as some compounds fluoresce and become visible under this longer wavelength. [22.]

2.4.3 Fundamentals of TLC-MS

One of the most popular and useful methods for describing chemical and biological substances is mass spectrometry (MS). By ionizing molecules and analysing their mass-to-charge ratio (*m/z*), an analytical method can be used to determine the molecular weight and structural composition of the substance. The process consists of three main stages: ionization, mass analysis and detection [23]. In the ionization stage, the sample is converted into charged particles using techniques like electrospray ionization (ESI) or atmospheric

pressure chemical ionization (APCI). The resulting ions are then separated in the mass analyser, which can be based on principles like time-of-flight (TOF), quadrupole or ion trap analysis, depending on the instrument. Finally, the ions are detected, and their intensities are recorded to generate a mass spectrum, which provides information about the molecular structure, fragmentation patterns and chemical composition of the sample. The MS can be coupled with liquid chromatography or gas chromatography, or as in this study, with TLC. [21.]

Thin-layer chromatography coupled with mass spectrometry (TLC-MS) is widely used technique for the direct analysis of separated compounds. The system typically consists of a compact single-quadrupole mass spectrometer (covering a broad mass range, e.g. 10-2000 m/z) [23] equipped with ionization sources to accommodate a variety of analytes. One commonly used ionization method for drug-like molecules in these systems is atmospheric pressure chemical ionization (APCI). [21; 23.]

In APCI-based TLC-MS setups, an atmospheric solids analysis probe enables direct analysis of solid and liquid samples with minimal preparation. In this method the sample is introduced via a glass capillary into a heated nitrogen gas stream inside the ionization source, where it vaporizes before undergoing ionization. This setup, integrated into the main part of the mass spectrometer, allows for rapid analysis and is particularly effective for reaction monitoring and characterization of reaction mixtures. For TLC-MS coupling, an additional device can be added, the TLC plate reader, that can be used to directly extract and transfer compounds from TLC plates into the mass spectrometer. [23.]

In addition to TLC-MS, these instruments often support flow injection analysis, where liquid samples are directly introduced into the system without chromatographic separation. Flow injection analysis provides a rapid method for monitoring reaction progress and assessing purity by delivering the sample in a continuous solvent flow to the mass spectrometer. [23.]

TLC-MS integration is achieved through a TLC reader, which extracts compounds with methanol directly from a developed TLC plate. A specialized elution head creates a seal over the targeted TLC spot, delivering a solvent flow that dissolves and transfers the sample into the mass spectrometer. This technique eliminates the need for additional sample processing, allowing for efficient identification of chromatographically separated compounds. [24.]

As a low-resolution mass spectrometry technique, TLC-MS provides qualitative information with sufficient accuracy for structural confirmation and compound identification. This makes it a valuable tool in synthetic chemistry applications, where quick and reliable characterization of reaction product is essential. [24.]

2.4.4 Nuclear Magnetic Resonance (NMR)

When examining the structure of organic substances, nuclear magnetic resonance (NMR) spectroscopy is a versatile analytical method. It analyses the magnetic properties of certain atomic nuclei, like hydrogen (^{1}H) and carbon (^{13}C), to give detailed information about molecule's structure, and behaviour [25]. Unlike other spectroscopic methods such as infrared or ultraviolet/visible spectroscopy, NMR requires an artificial magnetic field to work. [25.]

The functionality of NMR is based on certain nuclei and their behaviour in a magnetic field. These nuclei absorb electromagnetic radiation at specific frequencies based on their magnetic spin states. The NMR spectrum is formed by detecting these absorption signals. When placed in a strong external magnetic field, certain nuclei behave like small magnets and align with or against the field, creating distinct energy levels. Upon exposure to a radiofrequency pulse, some nuclei are promoted to a higher energy state. As they relax back to the lower energy state, they emit radiofrequency signals, which are detected and Fourier transformed into an NMR [25.]

One of the most important features of NMR spectrum is the chemical shift, which gives the information about the electronic surroundings of a nucleus.

Chemical shifts occur because the electron density near a nucleus affects the magnetic field it experiences. A chemical shift shows what kind of "neighbourhood" an atom is in. If there are more electrons around the atom, they act as a "shield", weakening the magnetic field the atom experiences and slightly changing its signals. Measured in parts per million (ppm), these shifts help identify different types of nuclei and their locations within a molecule. For instance, in proton NMR, hydrogen atoms in varying environments resonate at different frequencies, enabling the identification of specific hydrogen and offering valuable information of the molecular structure. Typical chemical shift values in NMR spectroscopy are illustrated in figure 11. [25.]

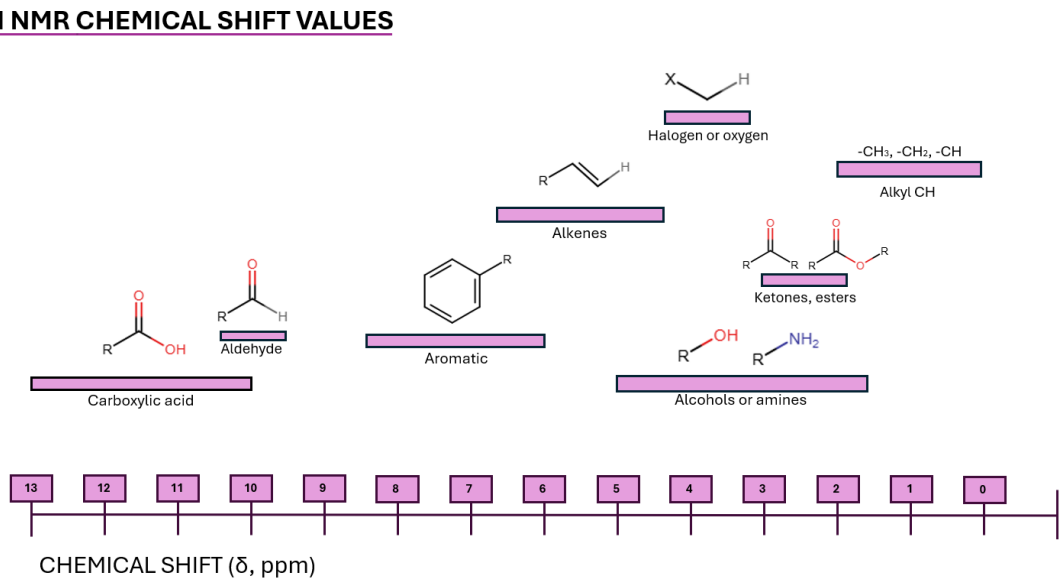

Figure 11 Chemical shift values of ^{1}H NMR. [25.]

Another important feature of NMR spectra is spin-spin coupling, or *J*-coupling, which occurs when two or more nuclei interact with each other through chemical bonds. This interaction causes the NMR signals to split into multiple peaks, known as splitting. The pattern of these splits gives clues about the number of nearby nuclei, their surroundings and their distances from each other. *J*-coupling is measured in hertz (Hz) and helps to understand how atoms in a molecule are connected and arranged [25]. An example of this is shown in a figure 12, where the ^{1}H-NMR spectrum of 1,1,2-trichloroethane exhibits characteristic splitting due to interactions between neighbouring hydrogen

atoms. From the figure 9 and be seen a triplet approximately 5.5 ppm corresponding to the proton on the carbon bearing two chlorine atoms ($CHCl_2$), while the doublet at around 3.9 ppm is assigned to the methylene protons (CH_2) adjacent to it.

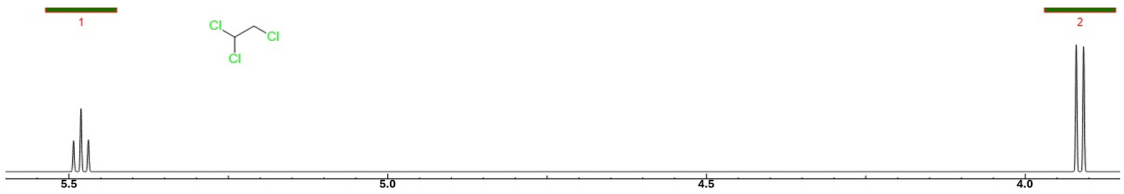

Figure 12 Example of spin-spin coupling of a 1,1,2-trichloroethane. The peaks split into patterns due to neighbouring hydrogen atoms affecting each other.

Two of the most common types of NMR spectroscopy are ^{1}H-NMR and ^{13}C-NMR. Proton NMR detects hydrogen atoms (protons) in a molecule and gives details about how many hydrogens are present, and where they are located. In ^{13}C-NMR the focus is on carbon atoms, providing insights into the arrangement of the molecule's carbon framework. These two techniques complement each other ^{13}C-NMR providing information on the carbon backbone of the molecule and ^{1}H-NMR offering more detailed data on the hydrogen atoms and their environments. [25.]

NMR spectroscopy is widely used in organic chemistry for structural elucidation of compounds and determining the purity of a sample. It is also valuable in identifying functional groups, confirming the presence of specific chemical bonds, and assessing molecular conformations. NMR spectroscopy is essential in the pharmaceutical industry for drug development, as it enables the determination of the structure of small molecules and their interactions with biological targets. Additionally, NMR is employed in a variety of other fields, including biochemistry, materials science, and food chemistry. [25.]

3 Aims of the Thesis

This thesis aims to support ongoing medicinal chemistry research focused on the development of new drug candidates for IPF, in collaboration with the University of Helsinki and HUS. The primary objective was to synthesize and characterize novel analogues based on two scaffolds: coumarin and tilorone. These scaffolds were selected due to their previously reported activities, tilorone for its antiviral and immunomodulatory properties [17] and coumarin for its known anti-inflammatory and antifibrotic effects [10]. The goal was to introduce structural modifications to these molecules and characterize these with spectroscopic methods like NMR, and MS.

The work involved optimizing synthetic methods using both conventional heating and microwave-assisted synthesis to improve reaction efficiency and product yields. After purification, the structures and purity of the resulting compounds were verified through NMR and MS analysis.

Throught the successful preparations and analysis of novel analogues, this research contributed valuable knowledge to the University of Helsinki's medicinal chemistry research group and supported its efforts to discover potential therapeutic agents for treatment of the IPF.

4 Results and Discussion

TLC-MS analysis was originally planned to support compound identification, but the instrument was under maintenance during the laboratory work period, and therefore no TLC-MS results are included in this thesis. The purity and structure of compounds were confirmed with NMR.

4.1 Alkylated Derivatives

The synthesis of novel tilorone analogues began with a series of alkylation reactions carried out under an argon atmosphere. These reactions (A1-A9)

were performed under various conditions, utilizing different alkylating agents, solvents and both microwave-assisted and conventional reflux set-ups. The general reaction scheme is shown in figure 13. Chloride and bromide alkylating agents were tested, with bromides demonstrating higher reactivity. Among these, reaction A3, which was prepared with microwave-assisted heating, gave a higher yield compared to its conventional counterpart A2, suggesting that microwave-assisted irradiation may enhance reaction efficiency under certain conditions. Microwave-assisted synthesis gives rapid and consistent heating, which often results shorter reaction times and higher yields, where conventional reflux depends on slower heat transfer and longer reaction times [26]. However, apart from the A2-A3 comparison, no significant differences were observed between two heating methods in the other experiments. Despite these variations the isolation of the product proved to be challenging, as several experiments resulted in only the starting material being recovered after purification.

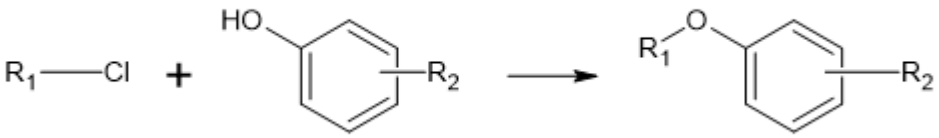

Figure 13 General scheme of the alkylation reactions.

Table 1 presents a summary of experiments carried out using the same synthetic method. In experiments A6-A8, only starting material was recovered after extraction, while in experiment A9, TLC analysis indicated that no reaction had occurred, and the experiment was subsequently discarded.

To improve yields, several bases were tested, including potassium carbonate, cesium carbonate and sodium hydride, both with and without catalytic potassium iodide. And still, these adjustments did not improve the product formation. Poor solubility of the starting material in some solvents and formation of heterogeneous suspensions likely contributed to low reactivity and incomplete conversions.

Table 1 Summary of experiments performed using the same synthetic methodology.

EXPERIMENT	BASE	REACTION AGENT	CATALYST	SOLVENT
A1	K_2CO_3	Aminoalkyl chloride	KI	Acetone
A2	K_2CO_3	Bromoalkane	-	Acetone
A3	K_2CO_3	Bromoalkane	-	Acetone
A4	K_2CO_3	Aminoalkyl chloride	KI	Acetone
A5	K_2CO_3	Aminoalkyl chloride	KI	Acetone
A6	KOH	Aminoalkyl chloride	-	Ethanol
A7	Cs_2CO_3	Aminoalkyl chloride	-	DMF
A8	NaH 60 %	Aminoalkyl chloride	-	DMSO
A9	K_2CO_3	Aminoalkyl chloride	-	Acetone

NMR analysis of crude reaction products supported these findings (A1-A5, appendix 1). Among these alkylation reactions only one compound was successfully isolated and purified (with a yield of 38%) and this product was produced using an alkylating agent without amine functionality (A3, appendix 1). The successful formation and isolation of this compound suggest that the absence of polar or reactive functional groups, such as amine, may contribute to better chemical stability and cleaner reaction outcomes under tested conditions. One possible explanation why this experience was more successful than the others with the amine group on an alkylating agents could be that the presence of nucleophilic amines could either promote side reactions or increase the polarity and wated solubility, making the products more difficult to extract and purify using standard workup procedures.

These findings indicate that the nature of alkylating agent has a significant influence on the outcome of the reaction and must be carefully considered when designing the future synthetic strategies.

4.2 Nucleophilic Addition Derivatives

The second series of reactions focused on modifying the parent compound through nucleophilic addition with alkoxyamines derivatives (B1-B5, appendix 1)

as illustrated in the general reaction scheme in figure 14. The preparation of this series of reactions were started with a reaction found from literature [27], and it acted as a foundation for synthesizing a set of structurally related analogues. This approach proved to be more successful than the earlier alkylation series, yielding significantly higher product recoveries and improved purity.

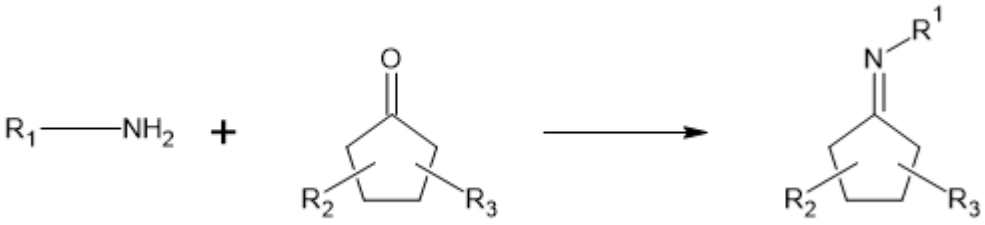

Figure 14 General scheme of the nucleophilic addition reaction.

The first compound using this method yielded 87% (B1, appendix 1), and the same reaction conditions were then applied to four other derivatives. These reactions produced excellent yields of 64%, 89%, 98% and 95%. The slightly lower 64% yield can be explained with a product loss during multiple purification steps, which were part of the method development process (B2, appendix 1).

The reaction set-up worked smoothly from the beginning, but purification required more optimization. The initial TLC monitoring reaction involved testing various solvent systems:

- Dichloromethane – Methanol (15%)
- Dichloromethane – Methanol (30%)
- Heptane – Ethyl acetate (30%)

These systems showed limited movement of the product. Reverse-phase TLC using H_2O – methanol (50%) was also tested but compound did not move under these conditions either. Eventually, using dichloromethane (DCM) - methanol (MeOH) (20%) with 1% addition of triethylamine (TEA) enabled the product to move clearly on normal phase TLC plate. This improvement of mobility was likely due to triethylamine suppressing interactions between the basic compound and the acidic silanol groups on the silica plate, which often cause tailing or immobilization of basic analytes, which is demonstrated in a figure 15.

Figure 15 Tailing on a TLC plate by the reaction mixture. First from the right starting material, second cross spot with starting material and reaction mixture, and the two left on the left from reaction mixture and it's duplicate.

Further experiments with amine-functionalized TLC plates and various solvent mixtures were conducted, but the best TLC results were obtained using a 50% ethyl acetate - heptane solvent system, which provided clear separation and consistent R_f-values, as seen in a figure 16.

Figure 16 TLC plate from a reaction mixture where starting material has a Rf-value of 0.48 and the reactions value is 0.6.

The preliminary purification was performed using a silica column with DCM-MeOH-TEA solvent system. While this allowed for initial separation of the target compound, the results were not completely optimal. Some remaining impurities could be seen in NMR spectra. TLC experiments during method development

provided insights that guided the purification strategy. The product showed limited mobility on standard silica plates when using various solvent systems, but improved with the presence of triethylamine, indicating that basic compound-surface interaction was affecting to mobility. These observations prompted a shift to amine functionalized stationary phase.

Subsequent purifications using amino columns yielded markedly better separation and higher purity. The amino stationary phase, being more compatible with basic or polar compounds, reduced undesired interactions and the purification with the amino column produced the cleanest product with the highest efficiency and selectivity.

4.3 Acetylated Derivatives

A final series of reactions was performed to synthesize acetylated derivatives (C1-C2) from the B1 compound, with varying reaction conditions (appendix 1). In the first approach, the reaction was carried out under basic conditions using a dry solvent and a base catalyst. While the reaction appeared to proceed based on visual observations, analysis by TLC indicated that the R_f-values of the products were nearly identical with the starting material, making it difficult to assess the extent of product formation. This overlap limited the ability to confirm successful transformation solely through TLC monitoring, and because of this one of the products end up discarded only based on the TLC.

In parallel, reaction was also performed under acidic conditions. However, this attempt was unsuccessful, as the crude product partitioned into the aqueous layer during extraction (TLC of organic and aqueous layer seen in figure 17), complicating recovery and suggesting that the product was in a salt form.

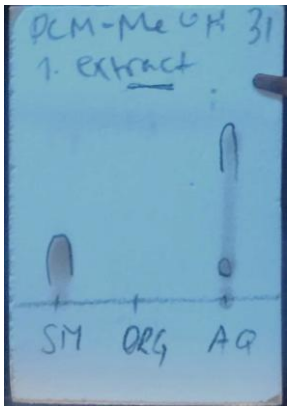

Figure 17 Result of the TLC after extraction, where the product can be seen on an aqueous layer, not in an organic layer.

NMR of the crude product done in basic conditions indicated the formation of desired product, but the product end up decomposed during purification with flash-column chromatography, indicating that the product was not that stable or that the purification method needs more optimization in the future.

5 Conclusion

The aim of this thesis study was to contribute to the development of novel therapeutic candidates for idiopathic pulmonary fibrosis by synthesizing and characterizing new tilorone analogues. Three series of reactions were explored: alkylation, nucleophilic addition and acetylation, each offering insights into the synthetic behaviour and analytical characteristics of the compounds under investigation.

The alkylation reactions proved challenging, with poor yields and difficulties in isolating pure products. These issues were likely due to solubility problems, side reactions involving amine-functionalized alkylating agents, and incomplete reactions. Despite extensive optimization attempts involving various bases and solvents, only one compound was successfully purified from this series.

In contrast, the nucleophilic addition series demonstrated promising outcomes. With well-established reaction conditions and work-up, these reactions consistently produced compounds with high purity and excellent yields. Minor

purification challenges were overcome by optimizing chromatographic conditions, especially by switching to amino-functionalized stationary phase.

The third set of reactions focused on acetylated derivatives yielded mixed results. While the crude product from one reaction showed good formation, decomposition occurred during purification, and acidic reaction conditions failed to produce the desired compound at all. These findings highlight the sensitivity of such derivatives to specific conditions and suggest a need for further method development.

In total, five novel compound and one known standard were successfully prepared and are being sent for biological testing at HUS. These compounds will be tested using three different cell lines to evaluate their potential antifibrotic activity and relevance for future drug development targeting IPF.

Overall, this study successfully synthesized and characterized several novel tilorone-based analogues, particularly from the nucleophilic addition pathway. These results contribute valuable knowledge to the University of Helsinki's ongoing medicinal chemistry efforts in collaboration with HUS. The compounds prepared in this work provide a foundation for further biological evaluation and future research into the structure-activity relationships of tilorone-based drug candidates. Continued optimization of synthetic methods and purification strategies will be essential for expanding this chemical series and improving its pharmacological potential.

6 Experimental Section

6.1 Reagents and Devices

Unless otherwise specified, all reagents and solvents were obtained from commercial suppliers and used without purification. Microwave reactions were performed with a fixed hold time in capped microwave vials using a Biotage Initiator+. Completion of reactions and purifications were monitored with TLC,

which was performed on silica gel 60 F_{254} -plates or silica gel 60 NH_2 F_{254} -plates, using UV light (254 and 366 nm). Flash chromatography was performed using Biotage Isolera One with Sfär cartridges as well as Sfär amino-catridges. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 101 MHz, respectively, using an Ascend 400 (Bruker). $CDCl_3$ was used as the NMR solvent unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) with solvent residual peaks as reference. Multiplicities are indicated by s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), dt (doublet of triplets), q (quartet), p (pentet), and m (multiplet). The coupling constants J were quoted in hertz (Hz). Melting point for solid compounds were confirmed using Stuart SMP 40 -melting point apparatus. Melting points are given in Celsius (ºC). Detailed experimental and characterization data is presented in a confidential appendix.

6.2 General Procedure of Alkylated Derivatives

A series of alkylation reactions were carried out under an inert argon atmosphere using various base and solvent systems. In a typical reaction, to the starting material (0.2 g, 1.12 mmol) the selected base (1.0–8.0 equiv.) was added with an appropriate solvent. In some cases, catalytic potassium iodide (KI) was added to enhance reactivity. The alkylating agent (2.0–4.0 equiv.) was then added dropwise, and the reaction mixture was either heated under reflux or the reaction was completed with microwave, depending on the setup. Reaction temperatures ranged from room temperature to 80 ºC, with reaction times varying from 5 to 48 hours. The solvent was evaporated under reduced pressure, and the crude residue was extracted with different solvent systems (e.g. diethyl ether and sodium hydroxide, or ethyl acetate and water) and with a separation funnel. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. Crude products were analysed by TLC and NMR, and when possible purified by automated flash chromatography. Isolated yields were generally low to moderate, and in several cases, no product was obtained after purification.

6.3 General Procedure of Nucleophilic Addition Derivatives

The general procedure for the second series of reactions involved dissolving the starting material (0.25 g, 0.517 mmol) and the alkoxyamine reagent (1.1 equiv.) in a solvent mixture, followed by refluxing at 85 ºC for 2.5 hours. After solvent evaporation, the residue was dissolved in water and the product was extracted with ethyl acetate and water using a separation funnel. The organic phase was dried over Na_2SO_4, filtered and concentrated, and the crude product was purified by automated flash chromatography. The products were obtained in good to excellent yields, ranging from 64% to 98%.

6.4 General Procedure of Acetylated Derivatives

General procedure for the third series of reactions involved the functionalization of a premade intermediate under an inert argon atmosphere. The starting material (0.05–0.1 g, 0.117–0.235 mmol) was dissolved in dry or acidic solvent system, and the reaction mixture was treated with a base or acidic reagent depending on the specific setup. In some cases, the solution was first cooled in an ice bath before the dropwise addition of the electrophilic reagent, while other reactions were performed entirely at room temperature. Reaction times varied from few hours to 48 hours. Upon completion, the reactions were quenched with water or aqueous base, and the crude product was extracted with ethyl acetate and water using a separation funnel. When necessary, the aqueous layer was basified prior to extraction to ensure product recovery. The combined organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude products were purified. Reaction outcomes varied depending on the conditions employed. One reaction, initiated under cold conditions, resulted in a high crude yield, but the product decomposed during purification by automated flash chromatography, resulting only recovery of the starting material.

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