



ALLEGHENY COLLEGE

**Tolcapone's potentially DEAD-ly influence: A stepwise synthesis and characterization of a
Rok1p modulator**

Walker Cunningham

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Allegheny College, Department of Biochemistry

**Tolcapone's potentially DEAD-ly influence: A stepwise synthesis and characterization of a
Rok1p modulator**

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**A senior comprehensive project in partial fulfillment of the requirements for a Bachelor of
Science degree from Allegheny College.**

*I hereby recognize and pledge to fulfill my responsibilities, as defined in the Honor Code, and to
maintain the integrity of both myself and the college community as a whole.*

Senior Thesis Committee:

Dr. Matthew Betush

Dr. Mahita Kadmiel

Dr. P.J. Persichini III

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Abstract

Colorectal cancer (CRC) is a major contributor to global cancer mortality, with increasing emphasis on identifying molecular targets for therapeutic intervention. DEAD-box proteins, a family of ATP-dependent RNA helicases characterized by the conserved Asp-Glu-Ala-Asp (D-E-A-D) motif, are integral to RNA metabolism, including ribosome biogenesis, splicing, and translation. Because DEAD-box helicases like DDX3 and DDX52 aid in cellular proliferation, their overexpression has been connected to the development of tumors. The yeast homolog of DDX52, Rok1p, provides a tractable model for researching the inhibition of RNA helicases. Tolcapone has been discovered as a possible Rok1p inhibitor by prior computational screening. To facilitate the subsequent assessment of Tolcapone's inhibitory action, this study offers a synthetic pathway toward the drug as an affordable substitute for commercial purchase. Key intermediates were successfully synthesized and described; however, the synthesis was not completed because of purification issues that arose in the concluding step. These findings lay the groundwork for further optimization and biological testing relevant to CRC research, as well as the creation of chemical factors targeted at DEAD-box helicases.

Background and significance

Colorectal cancer (CRC) is one of the leading cancers worldwide in terms of morbidity and mortality.¹ According to the World Health Association, it ranks third in incidence and second in cancer deaths globally. In the United States alone, it is estimated that 150,000 individuals will be diagnosed and 50,000 will die because of CRC in 2025.² Despite declines in the number of CRC-affected patients over time, high volumes of CRC have overwhelmed the CDC, making this form of cancer an area of focus and research in terms of understanding its molecular mechanisms to improve future therapeutic interventions (Figure 1). CRC in younger individuals is also on the rise. Younger people have a difficult time discerning their vague symptoms, falling victim to CRC.³ As such, attention has been brought to understanding the molecular mechanism of CRC to model different targets for intervention.^{3,4}

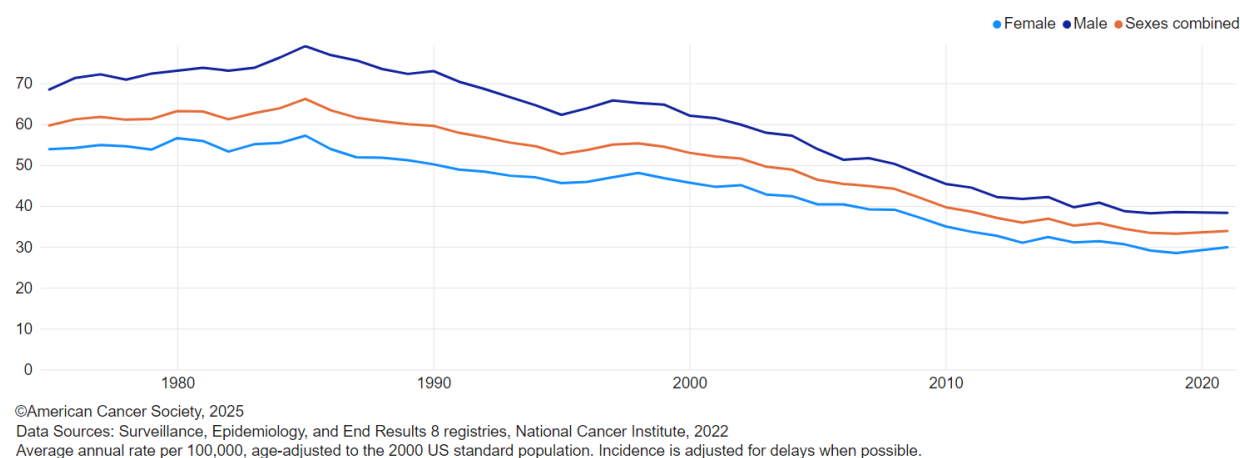


Figure 1: Death rates of colorectal cancer over time. Retrieved from American Cancer Society.²

One class of molecules gaining more attention in cancer research at the molecular level is the DEAD-box family of RNA helicases.⁵ In general, helicases are organized into several superfamilies (SF1, SF2, SF3, etc.) that are characterized by different motifs and mechanisms of action specific to each family.⁶ A majority of RNA helicases belong to SF2, which is a group that

includes five subfamilies, one of such subfamilies being the DEAD-box.^{7,8} This family is characterized by the presence of a conserved motif that includes the amino acid sequence, Asp-Glu-Ala-Asp (D-E-A-D). DEAD-box proteins share a condensed helicase core that shares two identical domains resembling the bacterial protein recombinase A (RecA).^{9,10} Within these two domains, there are motifs that create active sites for ATP binding and hydrolysis, as well as RNA binding (Figure 1). Some of these motifs are found throughout the entirety of SF2, whereas others are only specific to DEAD-box proteins. Motif II contains the “D-E-A-D” sequence, creating the DEAD-box name.⁹ The N- and C- terminal extensions on either side of the two domains play roles in regulation and ligand binding, which are included in protein function and where DEAD-box proteins differ.^{7,9,10}

As represented in Figure 2, these proteins contain 12 conserved motifs in each of the two domains. Domain 1 is equipped with motifs Q, I, Ia, Ib, Ic, II and III, whereas Domain 2 is equipped with IV, Iva, V, Va and VI. Initially, ATP binding happens in Domain 1 with Motifs Q, I and II. RNA binding happens in Domain 2 and binds to the most positive part of the molecule. RNA's net negative charge is due to its phosphate backbone, leading it to seek out motifs that tend to be more positive. The combination of ATP and RNA binding results in a structural rearrangement, as an ATP pocket is formed when Domain 1 moves closer to Domain 2.⁹ This conformational change is essential for the helicase's activity, as it allows the protein to effectively unwind the RNA helix.⁹ As helicase undergoes this transformation, the ATP-binding pocket becomes more defined, allowing for efficient ATP hydrolysis and the release of energy necessary for the unwinding process.⁹ Ribosome biogenesis feeds off the unwinding process, as helicases unwind rRNA to facilitate the proper folding and assembly of ribosomal components, essential for ribosome function.¹¹

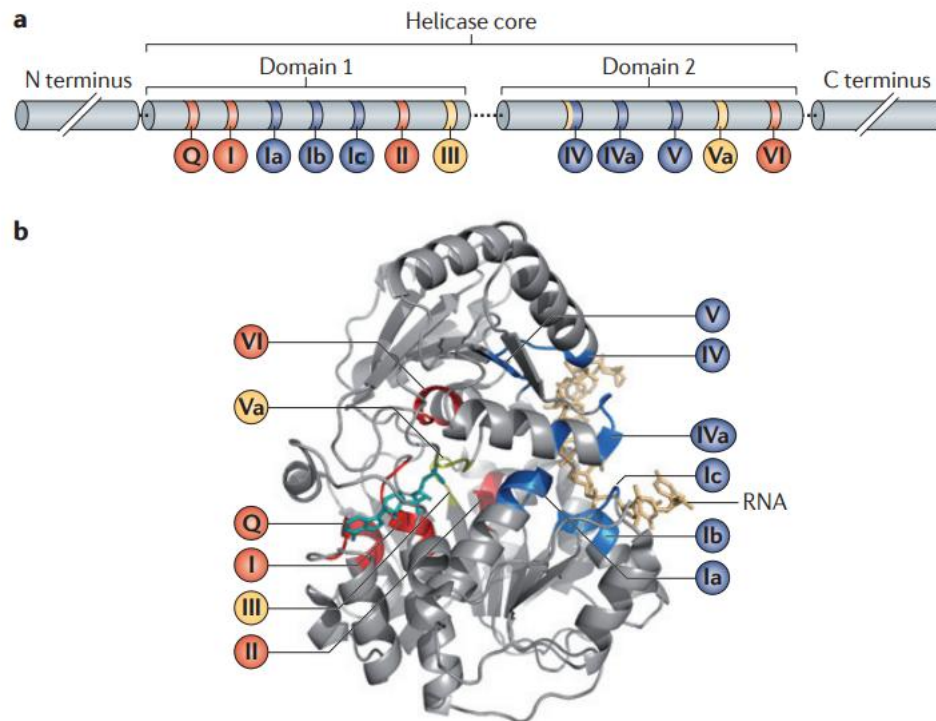


Figure 2: The structure and key features of the condensed form of DEAD-box protein Vasa from *Drosophila melanogaster*, the common fruit fly. Red motifs aid in ATP binding and hydrolysis while the blue motifs aid in RNA binding. Retrieved from Linder and Jankowsky.⁹

RNA helicases are increasingly recognized as important platforms facilitating interactions such as gene expression, cell cycle regulation, and drug design.^{7,12} Notably, dysregulation of DEAD-box helicases has been observed in various cancers, including CRC, where they may contribute to tumorigenesis, metastasis, and drug resistance.¹³

One notable player in the DEAD-box family is DEAD-box helicase 3 (DDX3), as it has been extensively studied for its roles in cancer. This protein has been shown to influence multiple

cancer-related pathways, including Wnt/ β -catenin signaling,¹⁴ cell cycle control, and apoptosis.¹⁵ In CRC, DDX3 overexpression correlates with increased proliferation and poor prognosis, and its inhibition has demonstrated anti-tumor effects in preclinical studies.^{15,16} Importantly, DDX3's dual role as both a tumor suppressor and oncogene, depending on cellular context, emphasizes the complexity of targeting RNA helicases for cancer therapy.

Building on the understanding gained from DDX3, other DEAD-box proteins such as DEAD-box helicase 52 (DDX52), have emerged as potentially significant in cancer biology. Although less thoroughly characterized, recent studies have reported that DDX52 is involved in ribosome biogenesis and RNA processing, both of which are essential for supporting the high metabolic demands of proliferating tumor cells and essential for protein synthesis and cellular function.¹¹ Increased expression of DDX52 has been noted in several tumor types, and early functional studies suggest it may support cancer cell growth and survival.¹⁶ While its role in CRC has not been definitively established, the growing body of evidence supports further investigation into DDX52 as a relevant player in tumorigenesis.¹⁷

To clarify the molecular functions of DDX52 and explore strategies for its inhibition, researchers often rely on model organisms. In the budding yeast *Saccharomyces cerevisiae*, the protein Rok1p serves as a homolog of DDX52, sharing both sequential and functional similarities.¹⁸ Rok1p is a specific DEAD-box protein found in yeast that also contains the characteristic DEAD-box motif. As a DEAD-box protein, Rok1p utilizes the energy from ATP binding and hydrolysis to unwind RNA duplexes or remodel RNA-protein complexes.¹⁹ Rok1p is one of many DEAD-box proteins found in eukaryotic cells that specialize in different aspects of RNA metabolism, such as translation, splicing, and RNA transport.^{18,19} It is also involved in the biogenesis and maturation of a small ribosomal subunit (40S) in the nucleolus.²⁰ It is necessary

in various steps, such as the removal of the U3 small nucleolar RNA (snoRNA) from the pre-ribosomal particle and the restructuring of pre-rRNA. The DEAD-box activity and RNA-remodeling functions of Rok1p are essential for its role in 40S ribosome biogenesis. Inhibition of Rok1p can lead to defects in small subunit formation and impair cellular growth.²⁰

As detailed in Miss Emma Cartney's comprehensive project, work was done with virtual screening and fragment libraries to produce new inhibitors for Rok1p. EasyVS, a web-based program, produced resultant compounds, and those results were confirmed in SwissDock. The top six compounds with acceptable toxicities were ordered and trialed for binding affinities against Rok1p (Figure 3).

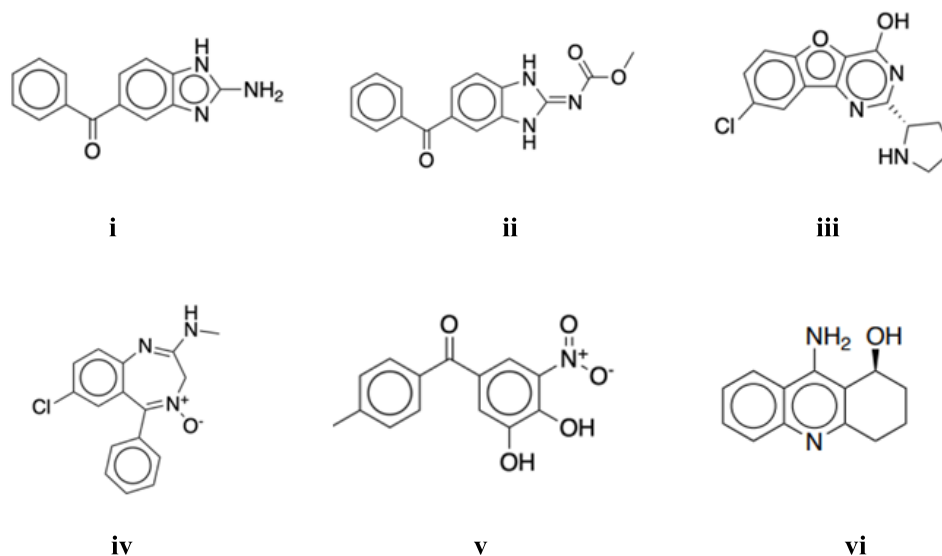


Figure 3: Purchased compounds based on EasyVS analysis. Binding affinities ranged from -6 to -6.8 kcal/mol when docked to Rok1p.

Compound **v** from the virtual screening is known as Tolcapone and is used in treatment for Parkinson's disease.²¹ Cartney discovered that Tolcapone would function as either an activator or an inhibitor depending on the concentration, a notable activity for this compound.

An additional study looked at other DEAD-box inhibitors that had been developed for DDX3. Recalling that DDX3 and DDX52 had similar motifs, the inhibitors that were identified for DDX3 can be connected to Rok1p. Radi et al. designed inhibitors for DDX3 to target HIV-1 after the same lab designed ATP competitive inhibitors for DDX3.²² (figure 3).

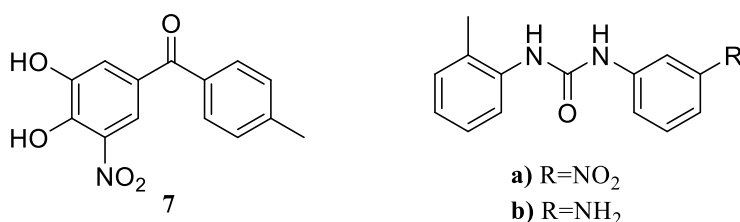


Figure 3: Comparison of Rok1p inhibitor Tolcapone (left) to the compound identified to be DEAD-box protein DDX3 inhibitor by Radi et al.²² (right).

Based on the results from that study, Tolcapone posed the most promise for molecular modifications because of its similarities in structure. Even though it showed the most consistent inhibition across all conformations and at low concentrations, the fact that activation is a possibility cannot be ignored. Modifications to Tolcapone proposed by Radi et al. should increase the binding affinity, which is a good indicator that inhibition will also increase.²³

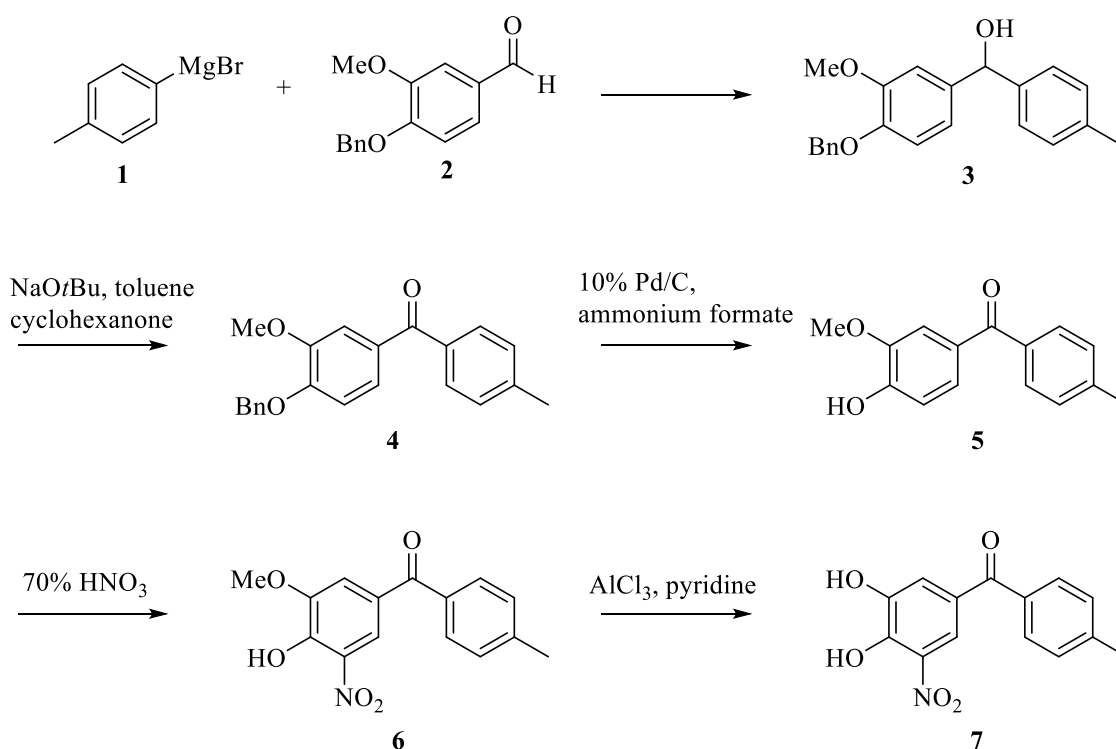
This study outlines the synthesis of Tolcapone as a cost-effective alternative to purchasing the commercially available drug. Once Tolcapone has been synthesized, confirmed, and shown to align with Cartney's reported inhibitory trends against Rok1p, structurally-

modified analogs can be developed. These chemical modifications, inspired by the proposals of Radi et al., aim to enhance the binding affinity between Tolcapone and Rok1p, thereby potentially improving its inhibitory effectiveness.

Results and Discussion

The synthesis of Tolcapone was conducted as outlined in Scheme 1 and described as a detailed synthesis by Manikumar et al.²⁴

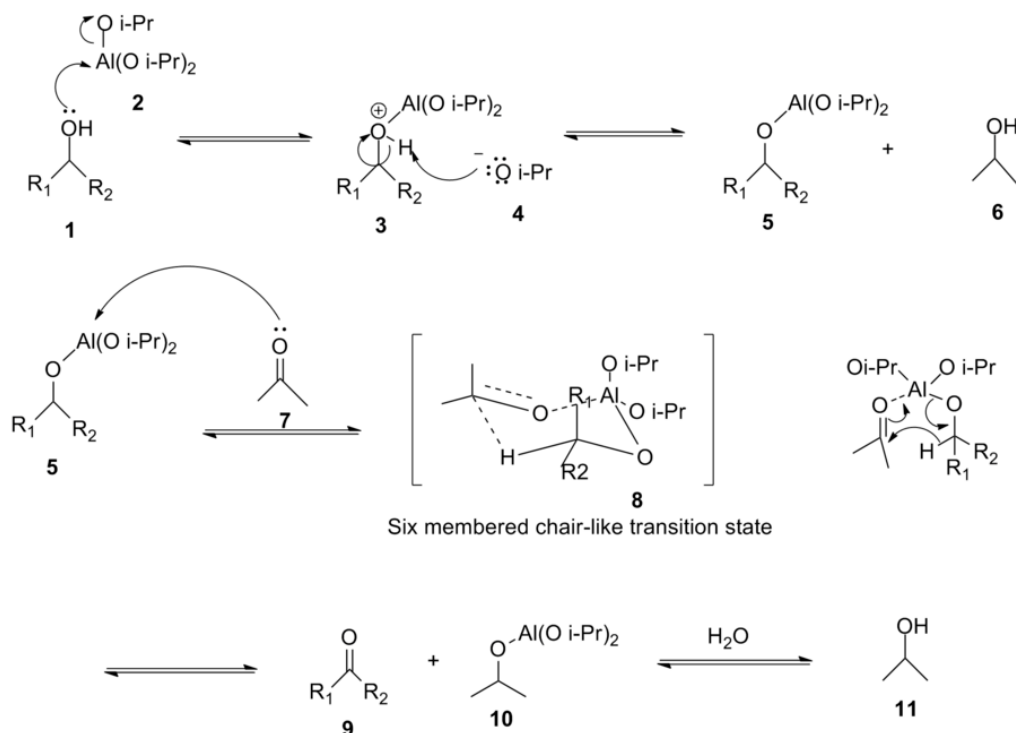
Scheme 1: Synthesis of Tolcapone (**7**)



Treatment of magnesium turnings in a solution of tetrahydrofuran (THF) with 4-bromotoluene under N₂ afforded *p*-tolylmagnesium bromide (**1**)^{25,26}. Challenges arose during synthesis when the Grignard reagent needed to be stored. Prolonged delays between its

preparation and use increased the risk of exposure to air and moisture, which can compromise the reagent's reactivity. The best results were achieved when the Grignard reagent was prepared and immediately used in the next step.

Next, treatment of commercially purchased 4-benzyloxy-3-methoxybenzaldehyde (**2**) with (**1**) in THF at 0°C afforded 1-(4-Benzyloxy-3-methoxyphenyl)-1-(4-methylphenyl)methanol (**3**) at % to complete the formation of the secondary alcohol. An Oppenauer-Woodward oxidation of the recently made alcohol was needed to afford (**4**) (Scheme 2). One of the key benefits of the Oppenauer oxidation lies in its use of affordable and non-toxic reagents. The reaction proceeds under mild conditions, typically involving gentle heating in a mixture of acetone and benzene. What sets this method apart from other common oxidation techniques, such as pyridinium chlorochromate (PCC) or Dess–Martin periodinane, is its pronounced chemoselectivity. Specifically, secondary alcohols are preferentially oxidized over primary alcohols, allowing for these chemoselective transformations. Additionally, unlike methods such as the Jones oxidation, the Oppenauer oxidation avoids over-oxidation of aldehydes to carboxylic acids, making it especially valuable in complex syntheses.²⁷

Scheme 2: Oppenauer oxidation²⁷

The Woodward modification of this oxidation comes from the substitution of the aluminum alkoxide to potassium tert-butoxide. In the tolcapone synthesis, NaOtBu is used as the base, and cyclohexanone is the hydrogen acceptor. This modification is used when certain alcohols do not prefer to oxidize under normal Oppenauer conditions.

Palladium-catalyzed deprotection of the -BnO protecting group was then combined with **(4)** using ammonium formate as the hydrogen donor to afford **(5)**. Following that, treatment of **(5)** with 70% HNO_3 at $0^\circ C$ afforded a crude mixture of **(6)**.

One challenge encountered during the purification process of **(6)** was the removal of excess acetic acid from the reaction mixture. An initial strategy to remove residual acetic acid

from (6) involved a basic aqueous extraction. A small volume of 1 M sodium hydroxide was added to the reaction mixture with the goal of deprotonating the acetic acid, thereby facilitating its partition into the aqueous layer. However, this approach proved ineffective, as the basic conditions also deprotonated (6) itself, resulting in the unintended transfer of the desired product into the aqueous phase. Subsequent analysis by ^1H NMR confirmed the absence of (6) in the organic layer, indicating significant product loss.

To recover (6), the aqueous phase was carefully re-acidified to a neutral pH using dilute acid, as confirmed by litmus paper. Extraction with ethyl acetate was then performed, followed by multiple washes with brine to enhance separation and encourage removal of any remaining acetic acid. Despite these adjustments and purification attempts, residual traces of acetic acid were still observed in the final reaction mixture, highlighting the persistent difficulty of fully removing polar, water-miscible impurities from small-scale organic syntheses.

In considering other potential solutions, an azeotropic distillation using toluene was evaluated as a viable strategy. Acetic acid and toluene form a low-boiling azeotrope, which could have been used to co-distill and effectively remove residual acetic acid under reduced pressure. This approach is commonly employed in organic synthesis to streamline solvent removal and minimize aqueous workup.²⁸ However, this method was not implemented due to the technical limitations of the available rotary evaporator, which lacked the vacuum strength necessary to efficiently evaporate the azeotropic mixture. Attempting this distillation risked incomplete solvent removal and potential degradation of sensitive intermediates due to prolonged heating. As a result, more conventional purification techniques were employed, although they proved less effective in fully eliminating acetic acid from the system. This limitation may have contributed to reduced purity of the intermediate and hindered progress in the ultimate step of the synthesis. In

future work, access to a higher-capacity vacuum system or alternative azeotropic solvents with lower boiling points could improve solvent removal efficiency and overall reaction purity.

Conclusions and Future Work

While the synthesis of Tolcapone could not be fully completed, the progress laid a foundation for future work. The difficulties encountered during purification underscore the importance of optimizing both reaction conditions and workup protocols in organic synthesis. Moving forward, improved isolation techniques and reaction monitoring will be key to successfully completing the synthesis and evaluating Tolcapone's potential as a DEAD-box helicase inhibitor.

To proceed with the last step of the Tolcapone synthesis, it is necessary to obtain a purer sample of **(6)** to minimize the formation of side products in the subsequent reaction. Since each step in the synthesis did not quantitatively convert all material from the previous step, additional material must be prepared to ensure sufficient yield for downstream processing. Analysis of the crude ^1H NMR spectrum indicates that **(6)** was successfully synthesized, though it was obtained in an impure form.

Although the reaction protocol was precisely followed, it is possible that the nitration reaction did not reach completion. One potential contributing factor was the physical behavior observed during the addition of the nitric acid–acetic acid mixture. While the round-bottom flask was maintained at 0 °C, the reaction mixture that contained compound **(5)** and acetic acid began to freeze during the slow addition of the nitrating reagent. This partial freezing may have interfered with proper mixing, limiting the conversion of compound **(5)** to compound **(6)**.

Following the formation of **(6)**, tolcapone can be assembled in its entirety with the deprotection of the last methoxy group, using aluminum chloride and pyridine in ethyl acetate at a reflux. Once Tolcapone is synthesized, trials to assess its effect on Rok1p inhibition can be run in Dr. Ivelitza Garcia's lab, where research has already begun to look at other areas of Rok1p activity. Based on Cartney's findings, it is expected that at lower concentrations (0–150 μM), Tolcapone should activate Rok1p, displaying a negative correlation between inhibitor concentration and Rok1p activity. At higher concentrations (0–800 μM), Tolcapone is expected to function as an inhibitor, displaying a positive correlation between concentration and activity (Figure 4).



Figure 4: Inhibition activity of tolcapone at lower micromolar amounts (left) versus higher micromolar amounts (right). Figures taken from Cartney.

As for the structural changes to Tolcapone, an earlier claim made by Radi et al., a compound for DDX3 was identified with certain modifications that can be mapped onto Tolcapone (Figure 5). These structural changes offer a promising direction for rational drug design, suggesting that similar modifications to Tolcapone could enhance its binding affinity and specificity toward DEAD-box helicases like DDX52.

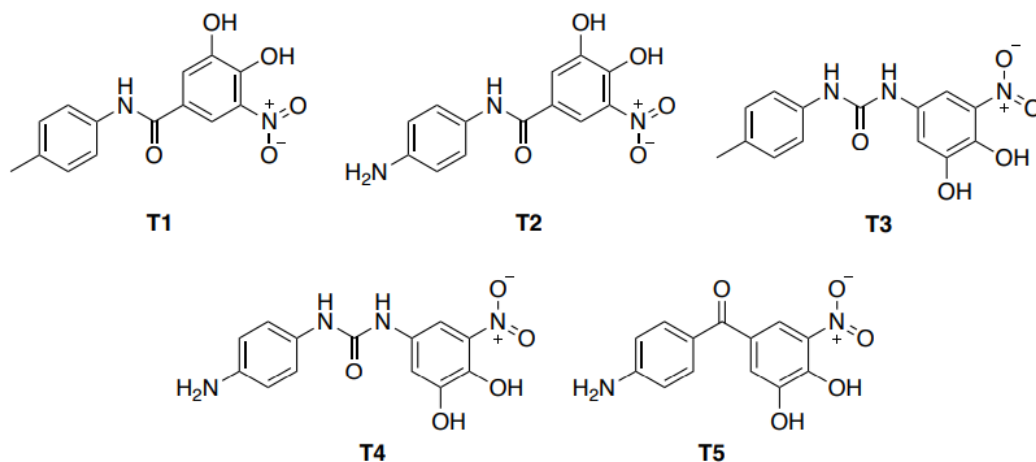


Figure 5: Proposed modifications to Tolcapone based on identifications of Radi et al. Binding affinities are **T1** = 9.70 kcal/mol, **T2** = -10.46 kcal/mol, **T3** = -10.19 kcal/mol, **T4** = -9.61 kcal/mol, and **T5** = -10.05 kcal/mol. Retrieved from Cartney's docking simulations.

Experimental

All compounds were characterized by ^1H NMR and spectra were obtained using a 400 MHz NMR spectrometer. Chemical shifts were reported in parts per million (ppm) and NMR spectra are provided in the appendix. Synthesis was followed based on Manikumar et al.²⁴

p-tolylmagnesium bromide (1)

A 100 mL round-bottomed flask, equipped with a reflux condenser, was charged with magnesium turnings (0.627g, 25.7mmol) and a magnetic stir bar and was purged with N_2 . Anhydrous THF (15mL) was added followed by the initial addition of 4-bromotoluene (ca.

0.25mL). The mixture was stirred and heated with a heat gun to initiate the reaction. Once the initial vigorous reaction subsided, an additional quantity of 4-bromotoluene (totaling 3.055mL, 25mmol) was added dropwise at a rate such that the reaction gently refluxed. The addition took 30 minutes and was completed after much of the magnesium had dissolved. Stirring continued for another 1 hour.

1-(4-Benzyloxy-3-methoxyphenyl)-1-(4-methylphenyl)methanol (3)

In a clean and dry round-bottomed flask, a solution of 4-benzyloxy-3-methoxybenzaldehyde (**2**) (2.907g, 12mmol) in THF (12mL) was slowly added to the same stirred mixture of *p*-tolylmagnesium bromide from above (15mL, 25mmol) at 0°C. The reaction mixture was then stirred at room temperature for 1 hour and concentrated in vacuo. The dark oily residue was dissolved in Et₂O (45mL) and cooled to 0°C, and 2M HCl (5mL) was carefully added. The phases were separated, and the product was extracted with Et₂O (50mL), washed with brine (100mL), dried (Na₂SO₄), and concentrated in vacuo. The crude mixture was recrystallized from DCM–hexanes to give (**3**) (2.538g) as a white solid; ¹H NMR (CDCl₃) δ 2.19 (1H, d), 2.32 (3H, s), 3.84 (3H, s), 5.11 (2H, s), 5.72 (1H, d), 6.79–7.42 (12H, m).

1-(4-Benzyloxy-3-methoxyphenyl)-1-(4-methylphenyl)methanone (4)

Sodium *tert*-butoxide (0.53 g, 5.50 mmol) was added to a stirred solution of (**3**) (1.25 g, 3.73 mmol) in toluene (20 mL) at room temperature under N₂ followed by the addition of cyclohexanone (2.53 mL). After the solution was refluxed for 1 hour, the mixture was cooled to 50°C, and H₂O (6mL) was slowly added while stirring continued. The phases were separated

with the aqueous phase being extracted with EtOAc (6mL). The resultant organic extract was washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The resultant crude mixture was recrystallized from EtOH to give **(4)** (0.70 g) as a yellow tinted solid. ^1H NMR (CDCl_3) δ 2.42 (3H, s), 3.93 (3H, s), 5.23 (2H, s), 6.87– 7.67 (12H, m)

1-(4-Hydroxy-3-methoxyphenyl)-1-(4-methylphenyl)methanone (5)

(4) (0.34 g, 1.00 mmol), a pinch of 10% Pd-C, and ammonium formate (0.293 g, 4.62 mmol) were mixed in a solution of MeOH (10 mL) and refluxed under N_2 for 30 min. After being cooled to 0°C , H_2O (4 mL) was slowly added followed by CH_2Cl_2 (15 mL). The mixture was filtered through a short pad of Celite[®], and the filter cake was washed with CH_2Cl_2 . The organic phase of the filtrate was separated, washed with brine, dried (Na_2SO_4), and concentrated in vacuo. The resultant crude mixture was recrystallized from CH_2Cl_2 -Hexanes to give **(5)** (0.10 g) as a yellow tinted solid. ^1H NMR (CDCl_3) δ 2.43 (3H, d), 3.95 (3H, s), 6.04 (1H, s), 6.93 (1H, d), 7.25– 7.67 (6H, m)

1-(4-Hydroxy-3-methoxy-5-nitrophenyl)-1-(4-methylphenyl)methanone (6) (crude)

A mixture of 70% HNO_3 (0.4 mL) and acetic acid (0.5 mL) was slowly added to a stirred solution of **(5)** (0.060 g, 0.025 mmol) in acetic acid (5 mL) at 0°C under N_2 . After being stirred for 30 min, the reaction mixture was poured into ice water. After chilling in the fridge overnight, the mixture was neutralized with 1M NaOH, extracted with ethyl acetate, washed with a series of water, sat. Na_2CO_3 , and brine. The extract was then dried (Na_2SO_4) and concentrated in vacuo to afford an acetic acid-smelling oily residue.

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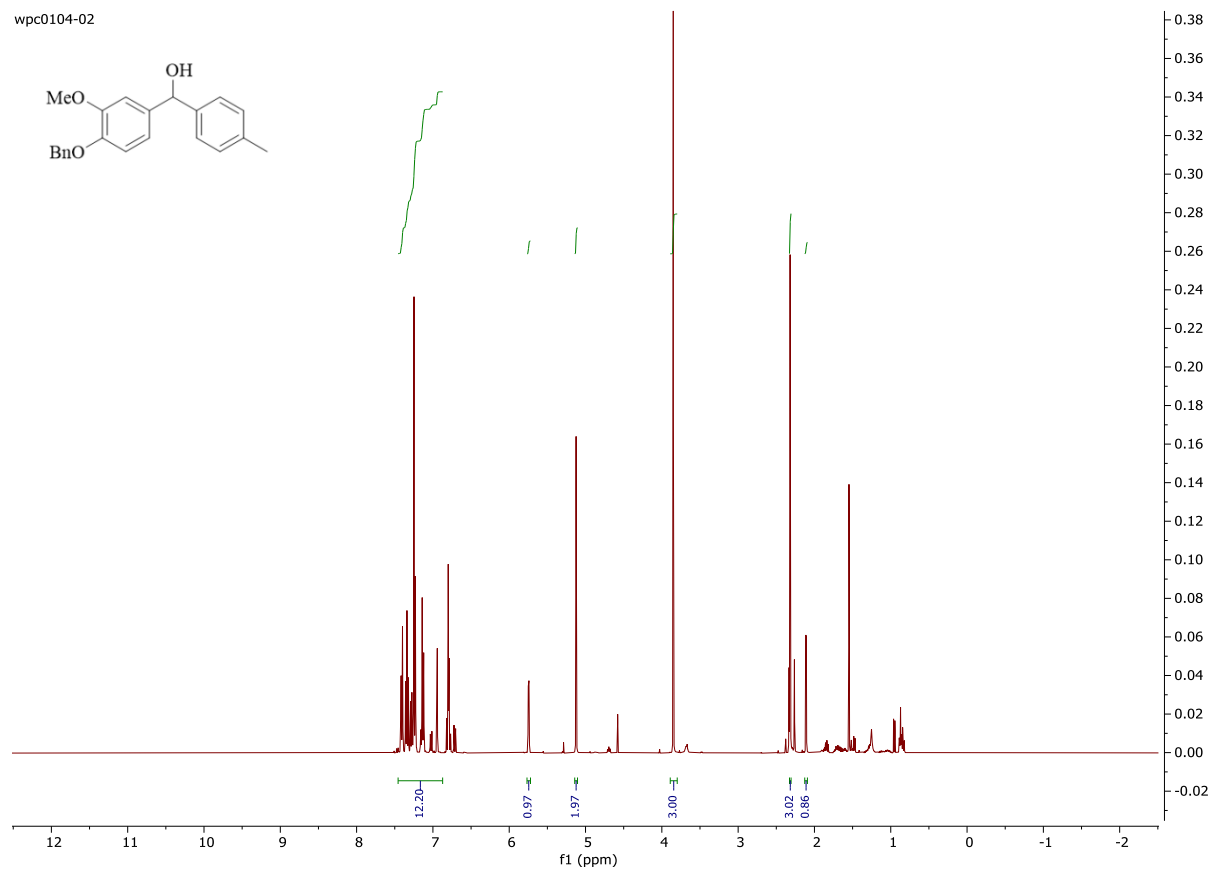
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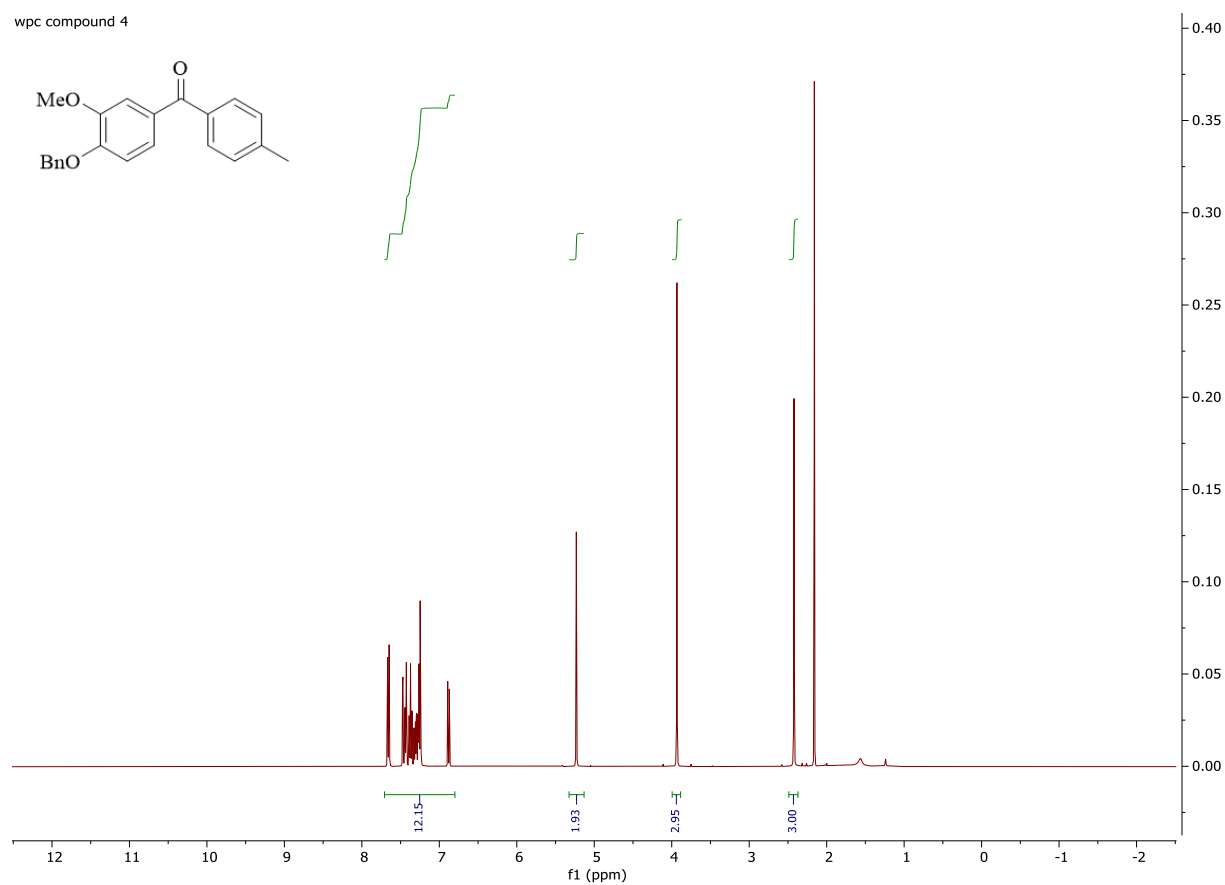
Appendix

NMR Traces

 ^1H NMR of 1-(4-Benzyloxy-3-methoxyphenyl)-1-(4-methylphenyl)methanol (**3**)

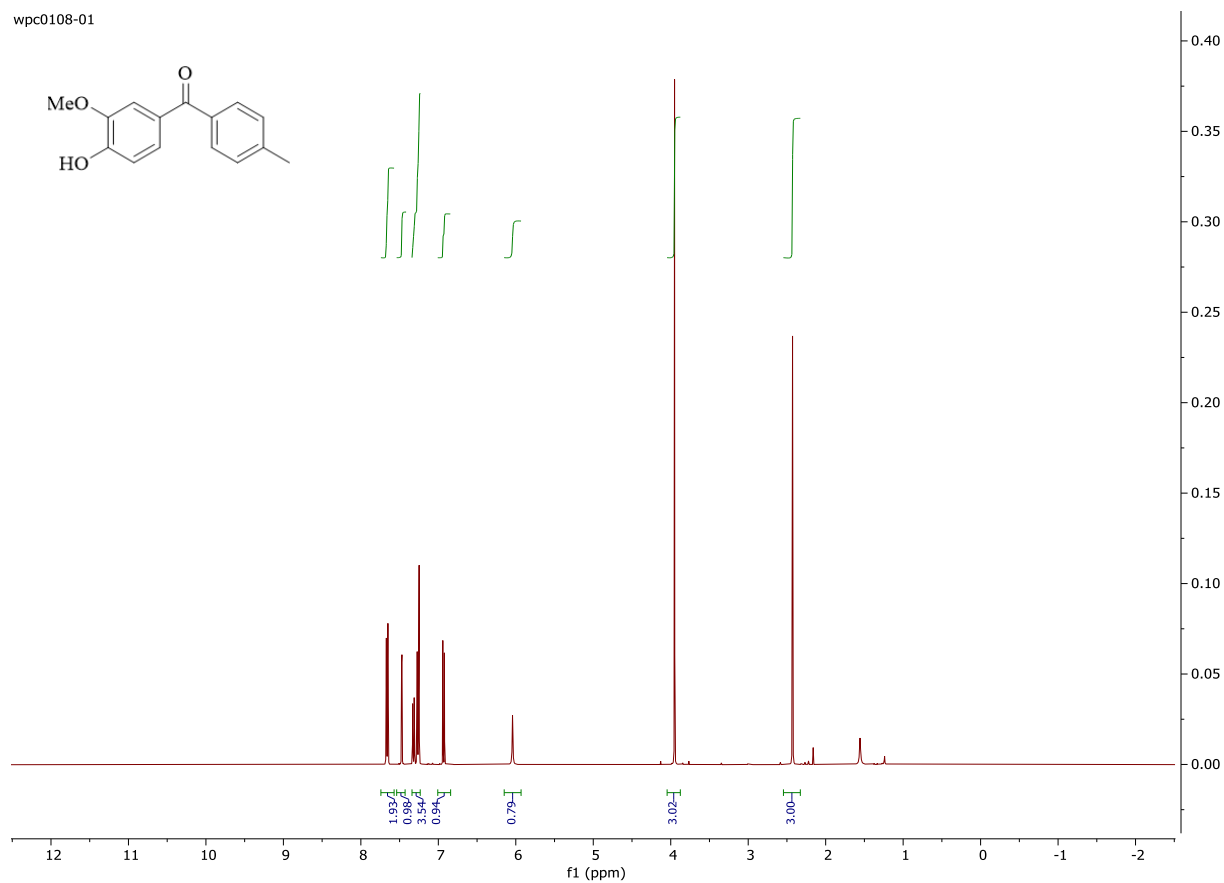
^1H NMR of 1-(4-Benzyloxy-3-methoxyphenyl)-1-(4-methylphenyl)ethanone (**4**)

wpc compound 4



^1H NMR of 1-(4-Hydroxy-3-methoxyphenyl)-1-(4-methylphenyl)methanone (**5**)

wpc0108-01



Crude ^1H NMR of 1-(4-Hydroxy-3-methoxy-5-nitrophenyl)-1-(4-methylphenyl)methanone (**6**)