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Design and Synthesis of Novel Flavonoids

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ABSTRACT

Flavonoids encompass a family of organic, naturally occurring polyphenolic compounds, with a general structure consisting of a 15-carbon skeleton, 2 phenyl rings, and a heterocyclic ring. Flavonoids include various subcategories – chalcones, flavones, flavanones, flavanols, isoflavones, anthocyanins – all of which have demonstrated differential health benefits such as antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Panche et al., 2016). Compounds extracted from natural sources – such as flowering plants, wine, cocoa – or synthesized in lab, flavonoids have been extensively studied in their natural state as well as their synthetically modified versions, demonstrating the rising interest in flavonoid applications.

To further explore the medicinal potential of this group of compounds, a variety of flavonoids were synthesized with a focus on modification of the carbonyl moiety and the carbon alpha to the carbonyl. Various experimental designs were tested – bromination, coupling, hydrazone synthesis, indole and thiazolidinone trials, and introduction of double bonds – the most promising route being flavonoid hydrazone synthesis. The potential biological applications of these synthesized flavonoids for treating COVID-19 were studied using computational analysis in collaboration with Neela Yennawar at the Huck Institute at University Park. Additional biological studies will be performed by collaborators to examine the anti-aging and anti-parasitic applications of these compounds in yeast and *T. brucei* respectively.

Of the various synthetic experimental protocols attempted, flavonoid hydrazone synthesis resulted in both sufficient yields and manageable protocols that could be further implemented for the modulation of novel flavonoids. Additionally, further bromination via copper bromide demonstrated some indication of dimerization via mass spectrometry analysis, indicating additional synthetic products that could be created from the novel flavonoid hydrazones.

Further research should focus not only on the synthesis of novel flavonoid hydrazones, but also on their biological implications, such as potential extensions of existing anti-aging or anti-inflammatory properties or novel emergent properties.

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GLOSSARY

TLC – Thin layer chromatography. A method of analysis that differentiates multiple compounds in a mixture based on polarity. When using a TLC plate composed of polar silica gel, the more nonpolar compounds will travel with the nonpolar solvent system and will thus be farther up the TLC plate, while the more polar compounds will "stick" to the polar silica gel and travel a shorter distance or not at all.

NMR Spectroscopy – Nuclear magnetic resonance. A method of analysis in which a sample, frequently a solid dissolved in a deuterated solvent, is exposed to a magnetic field in which the atomic radii of the atoms in the compound are excited. The resulting resonance that is produced is then detected by the NMR machine and depicted in frequency peaks. Differential forms of NMR were utilized, specifically ^{1}H and ^{13}C NMR, that detect unique protons and carbons in a sample respectively. This method of analysis can assist in identifying the nature of a compound based on characteristic protons or carbons present in its structure. For example, if a goal of a certain reaction is to replace a hydrogen atom with a halogen, a proton NMR can help differentiate whether that reaction successfully took place through the NMR comparisons of both the reagent and the product and the disappearance of a characteristic peak.

Deuterated solvent – a solvent in which the hydrogens are replaced with deuterium atoms – an isotope of hydrogen.

Mass Spectrometry – A method of analysis in which a sample is analyzed and then passed through a mass analyzer. The detector is then able to measure the mass-to-charge ratio of the ions in the sample, providing a data spectrum of distinguishable peaks. This method allows for the mass of various compounds in the sample to be identified, providing some insight into the

nature of the compound produced. The mass can then be compared to the mass of the expected compound, and the relative abundance of the compound with that mass relative to potential other compounds in the sample mixture.

Flavonoid – plant-derived polyphenolic organic compounds composed of a general structure of a heterocyclic ring and two phenyl rings (Figure 1).

Hydrazine – family of compounds with a general structure of two nitrogen atoms bound together, further bound to additional hydrogen atoms. The simplest form is the hydrazine, composed of two nitrogen atoms bound together, each further bound to two additional hydrogen atoms (Table 1a).

Thiazolidinone – Family of compounds composed of a thiazole ring and a ketone bound to any of the three carbon atoms (Table 1b).

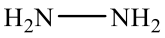
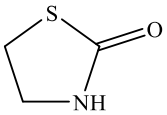
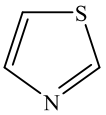
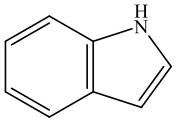
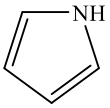
Thiazole – Family of heterocyclic compounds with a general structure of a five-membered ring containing a nitrogen and sulfur atom (Table 1c).

Indole – Family of heterocyclic compounds composed of a benzene ring fused to a pyrrole ring (Table 1d).

Pyrrole – Family of heterocyclic compounds composed of a five-membered nitrogen-containing ring (Table 1e).

α-Bromination – A reaction scheme in which an alpha hydrogen is substituted by a bromine atom. This process is more broadly called alpha-halogenation but substituting with a specific halogen is accounted for in the differential name.

Table 1. General structures for a) hydrazines, b) thiazolidinones, c) thiazoles, d) indoles, and e) pyrroles.

 Hydrazine	 Thiazolidinone	 Thiazole	 Indole	 Pyrrole
a)	b)	c)	d)	e)

ACKNOWLEDGEMENTS

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Chapter 1: Flavonoids – Plant-based compounds with a tremendous diversity of biological properties

When considering flavonoids by their general chemical definition, flavonoids are described as polyphenolic, plant-derived compounds with a general structure including a 15-carbon skeleton, 2 phenyl rings, and a heterocyclic ring. Flavonoids, while being definitive in their general structure, are characterized as a large-spanning family, encompassing various types of naturally occurring compounds.

Characterized as an over-arching family of distinct compounds, flavonoids include subgroups such as chalcones, flavones, flavanones, flavanols, isoflavones, and anthocyanins (Figure 1). These compounds are found in substances such as cocoa, wine, tea, flowering plants, and a variety of other natural sources, with their extractions or chemical syntheses being the general method for their access in synthetic chemistry. Experimentation and observation of flavonoid effects in vivo is not a novel craft, with their implications including antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties (Panche at al., 2016). Additionally, flavonoids have been identified to modulate various key enzymatic processes, such as the inhibition of xanthine oxidase, cyclo-oxidase, lipoxygenase, and phosphoinositide 3-kinase (Panche at al., 2016). In nature, flavonoids have also been characterized as pigments in some angiosperm families, while some vegetables utilize flavonoids for defense against plaques (Panche at al., 2016).

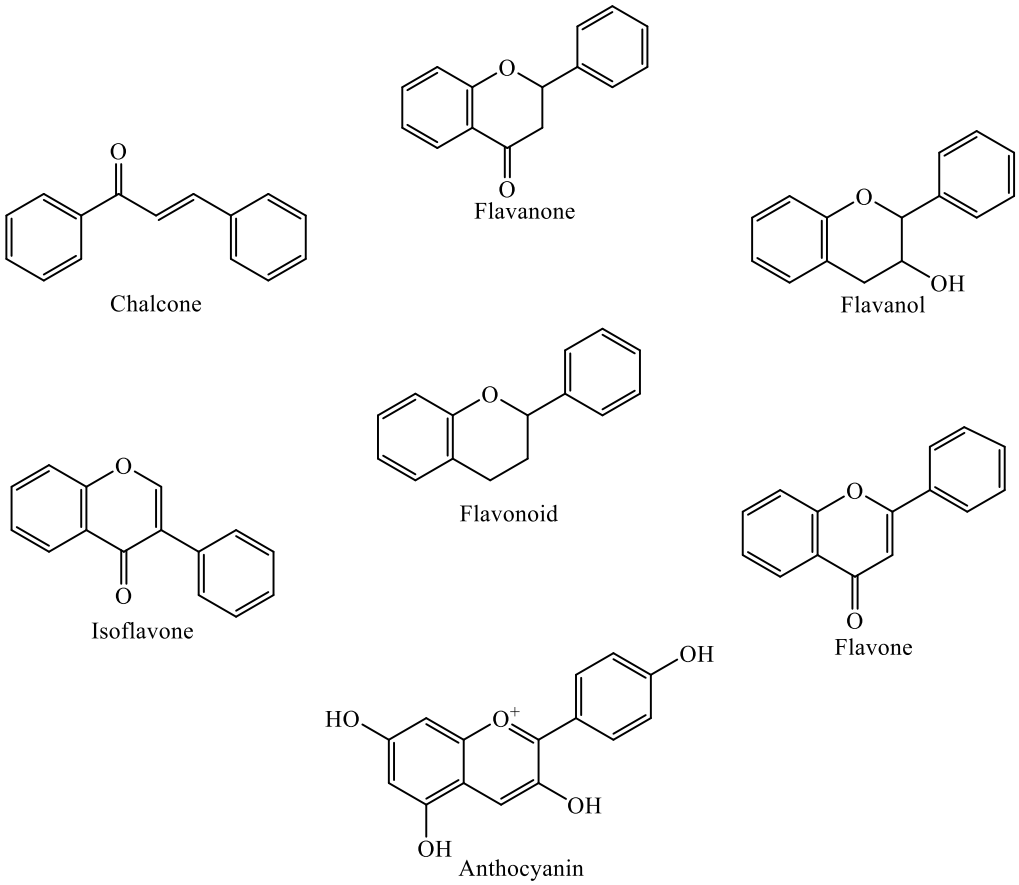


Figure 1. Types of flavonoids

As these compounds have demonstrated variable promising implications for anti-aging and anti-inflammatory applications, additional benefits associated with diabetes and neuroprotection have also been noted (Panche at al., 2016). With respect to anti-aging applications, flavonoids quercetin and fisetin have demonstrated promising anti-aging properties in mice through senolytic-like activity. These flavonoids functioned to clear senescent cells and reduce oxidative stress (Yousefzadeh et al., 2018). Additionally, another flavonoid – epicatechin – demonstrated additional anti-aging benefits in skeletal muscle of healthy mice (Si et al., 2019). Anthocyanins – flavonoids found in blueberries, strawberries, and red wine – were found to reduce age-associated lung function decline when consumed in high amounts (Morris, D., 2018).

Yet another example includes a flavonoid extracted from a flowering plant *Saussurea involucrate* – Rutin – that functions via antioxidative mechanisms to elicit anti-aging processes (Shoyama et al., 2012). Specifically, rutin was found to protect against free radical induced oxidative stress in D-galactose treated mice (Shoyama et al., 2012). Anti-aging properties were further identified in catechins, flavonoids derived from green tea in addition to improving special cognition learning in age-accelerated rats (Haque et al., 2006). Therefore, while the natural properties of flavonoids have been extensively studied, such findings elicit questions as to whether structural modifications could further enhance existing properties.

Chapter 2: Rationales for Synthesizing Novel Flavonoid Derivatives

Two main rationales were prioritized throughout the conduction of experimental research. First, as discussed above, flavonoids are known to have a variety of important biological properties. Through the utility of a biologically privileged scaffold, there are heightened chances for developing compounds with enhanced biological implications than if the starting reagent did not initially possess such properties. Flavonoids have been extensively studied and referenced in their multitude of biological functions, while specific flavonoid derivatives have demonstrated pharmacological implications from cytotoxic to cardioprotective effects (Murti et al., 2021). For example, an ethyl carbamate naringenin derivative was cited by Murti et al. to elicit cytotoxic effects (2021). Such extensive studies and general promising results support the primary rationale for flavonoid derivative synthesis. While personal research focused on the synthesis portion of this approach, collaborations were made with biologists capable of testing such novel molecules for anti-aging, anti-parasitic and anti-COVID applications.

The second rationale for exploring flavonoid syntheses is that it is a sustainable method to access biologically valuable compounds. Synthetic organic chemistry relies almost exclusively on petrochemical feedstocks such as natural gas, coal and petroleum. This reliance is contributing to climate change, and chemists must find other sustainable sources for chemicals. One exciting area that is emerging to address this problem is xylochemistry, wherein wood or plant-based biomass is used as a source of raw materials for synthesis instead of fossil fuel sources (Groß et al., 2020). The flavanones used as starting materials are derived from plants. For example, hesperetin is a flavonone found in citrus fruits, while naringenin can be extracted from grapes (Panche at al., 2016). Novel methods are currently being developed to extract these

compounds from their plant sources in energy efficient ways. Alternatively, they can be genetically engineered in environmentally benign methods. Therefore, by using flavanones as starting materials, more sustainable approaches to the synthesis of biologically relevant products are employed.

Accessing Flavonoid Derivatives via Modification of C3 and C4 of the Flavonoid Scaffold

Synthesis primarily focused on the modification of C3 and C4 of the flavonoid skeleton as there is ample chemistry known for modifying the carbonyl functional group or the carbon alpha to that carbonyl group (Figure 2). The intent for the modification of such target carbons was for the transformation of the carbonyl into imine or hydrazone functional groups, which could be varied by simply using different hydrazines or amines. Hydrazones could be further transformed into indole or thiazolidinone rings. It was envisioned that modifying the alpha carbon and subsequently performing coupling reactions would introduce aromatic rings at that position.

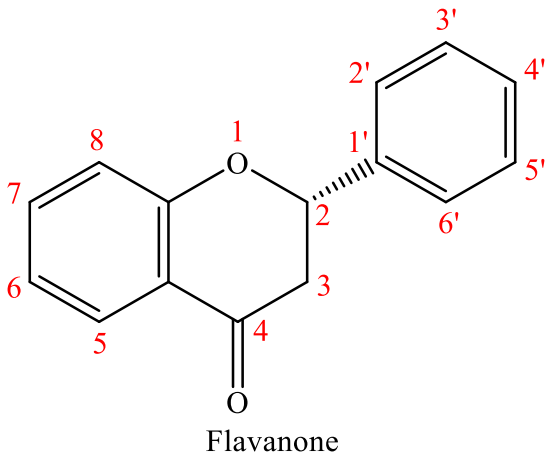


Figure 2. Flavonoid with designated carbons

Chapter 3: Modifications at the Alpha Carbon

Flavone Bromination and Coupling Trials

Flavone was the starting flavonoid of choice, a relatively simple flavonoid compound lacking any hydroxyl groups that could potentially interfere with reaction processes. After consulting literature, methods and protocol employed by Quintin et al. (2006) allowed for successful synthesis of bromo-flavone (Figure 3), a compound that would then function as a reagent in subsequent coupling trials. While TLC analysis was employed as a measure of reaction completion, NMR spectroscopy was utilized as a primary measure of analysis for product formation, specifically looking for the disappearance of the characteristic C3 proton peak. The synthetic procedure involved a 3:1 equivalent of NBS to flavone dissolved in methylene chloride and pyridine (0.09M). NMR analysis was conducted on the dried product, indicating a disappearance of a characteristic proton peak located at 6.8 ppm. This disappearance provided insight into the potential success of the reaction – the characteristic hydrogen was likely replaced with a bromine atom. Flavone was not the only flavonoid utilized for halogenation trials, but it did appear to be the most successful in the preliminary steps of subsequent coupling.

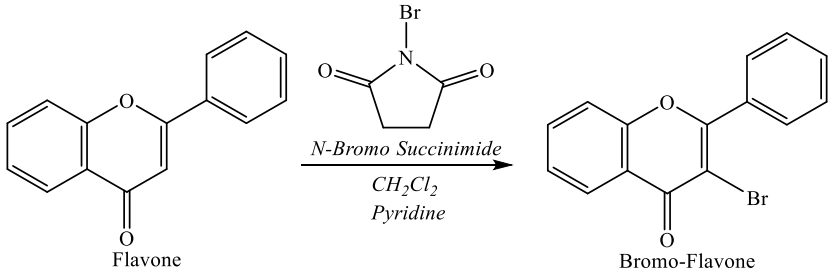


Figure 3. Bromination of starting flavone

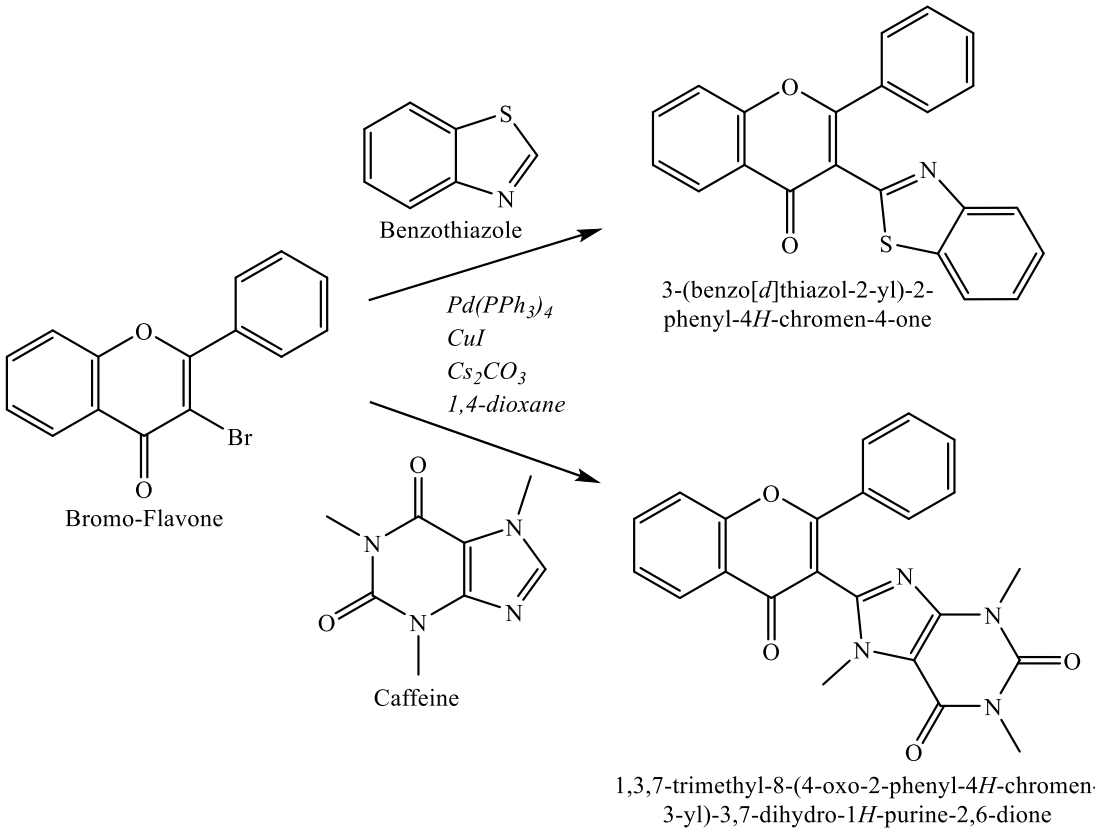


Figure 4. Proposed syntheses utilizing the synthesized bromo-flavone as a reagent

Following successful bromination, additional compounds were chosen for coupling trials. Available and testable compounds included caffeine and benzothiazole (Figure 4). A 2:1 ratio of caffeine/benzothiazole to bromo-flavone was employed in a metal-catalyzed coupling reaction scheme, utilizing palladium triphenyl phosphine, copper iodide, and cesium carbonate in 1,4-dioxane corresponding to protocol illustrated by Min et al. (2012). The reaction mixture was heated to reflux and stirred. Coupling reaction trials were performed twice, each yielding minimal product that could encourage further attempts. Thus, while bromination was successful, coupling trials did not demonstrate promising results for further experimentation.

Chapter 4: Transformation of the Carbonyl Group

Synthesis of Naringenin Phenylhydrazone

With the results from metal-catalyzed coupling reactions not being promising, experimental focus shifted towards hydrazone synthesis. Hydrazone synthesis posed an intriguing field of flavonoid modification as the introduction of the hydrazine moiety would allow for further manipulation either through thiazolidinone or indole ring formation. Hydrazone synthesis was also viewed as an interesting field on its own, as novel flavonoid hydrazones could also be tested in their biological significance. Naringenin phenylhydrazone was successfully synthesized following the protocol presented by Bak et al. (2011), involving a 1.2:1 ratio of phenylhydrazine to starting naringenin dissolved in ethanol and glacial acetic acid. The resulting yellow precipitate was then dried and analyzed via NMR and mass spectrometry. Such analyses confirmed the successful synthesis of naringenin phenylhydrazone (Figure 5), and more stock was made for subsequent reaction schemes.

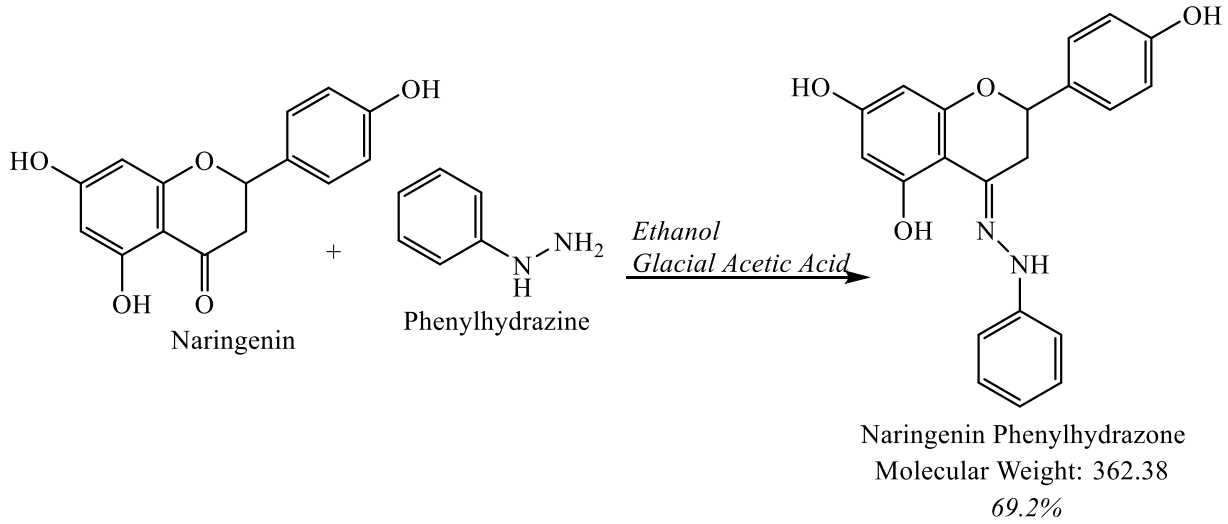


Figure 5. Reaction scheme for naringenin phenylhydrazone synthesis

Potential Implication for Indole and Thiazolidinone Products

Thiazolidinones

Thiazolidinones are characterized as either solitary compounds or as functional groups composed of five-membered thiazole rings with an additional ketone moiety (Table 1b). The carbonyl group can be present on any of the remaining carbons composing the ring, therefore there is variability in derivative structures. Thiazoles and thiazolidinones have been synthesized and studied for potential health implications, with results providing notable and promising anti-inflammatory, antiviral, analgesic, anti-carcinogenic, and anti-bacterial properties (Cascioferro, et al., 2020). For example, specific synthesized 4-thiazolidinone derivatives tested against *S. aureus* demonstrated even more potent effects than reference treatment vancomycin (Cascioferro, et al., 2020). With the biological implications of synthetic thiazolidinones being a prevalent course of modern pharmaceutical synthesis, modifications of health-enhancing flavonoids to include thiazolidinone scaffolds were considered.

Indoles

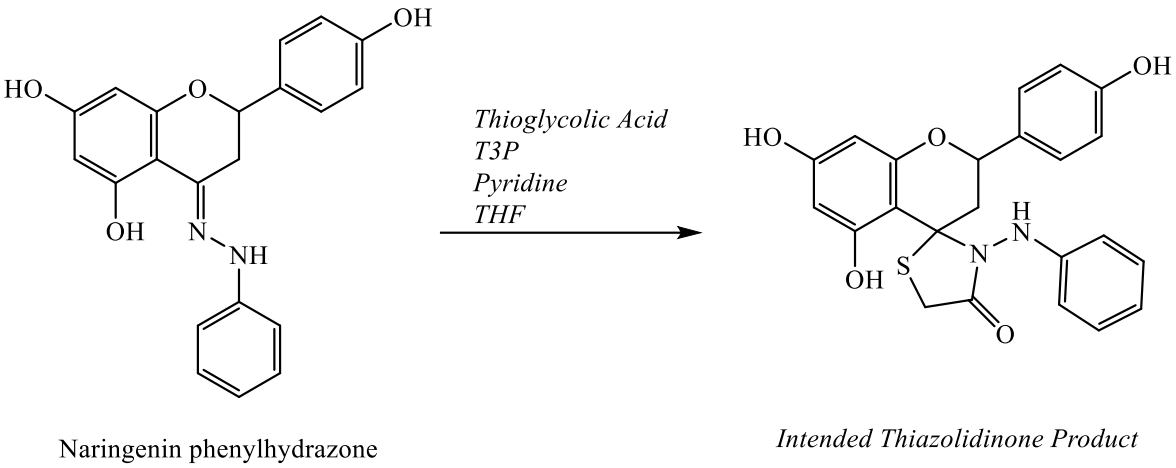
Characterized as aromatic, heterocyclic compounds or functional groups composed of a benzenoid nucleus and nitrogen-containing pyrrole ring (Table 1d), indoles are found in many naturally occurring compounds, tryptophan being an especially prominent one (Kumar & Ritika, 2020). Due to their prevalence, pharmaceutical intentions have been sought and tested, the biosynthesis of novel indole derivatives proposing antiviral, anti-inflammatory, antimicrobial, anticarcinogenic, and other health enhancing capabilities (Kumar & Ritika, 2020). For example, 6-amino-4-isobutoxy-1H-indole-2-carboxylate, a prepared indole derivative, demonstrated

inhibitory activity against influenza A (Xue et al. qtd. in Kumar & Ritika, 2020). With such pharmaceutical applications considered, intentions for experimentation shifted to include the synthesis of indole-containing flavonoid derivatives from starting flavonoid hydrazones.

Flavonoid Hydrazone Modifications

Thiazolidinone Trials

Literature scarcity was a common theme throughout thiazolidinone synthesis investigations, and even with available sources, reported yields did not demonstrate much promise. Even still, reputable articles were referenced when devising thiazolidinone synthesis reaction schemes, one being by Silverberg et al. (2020). This protocol involved a T3P-assisted procedure with a 1:1 naringenin phenylhydrazone to thioglycolic acid, 2 equivalents T3P, and 3 equivalents of pyridine dissolved in THF (2.6 M). Reactions were modified to include or exclude pyridine, testing to see whether its presence was required for the reaction to proceed (Figure 6). Both reactions produced viscous products that, even upon purification, did not yield promising results when analyzed via NMR and mass spectrometry. While there was some indication of product, the yields compared to mass of reagents used did not encourage further experimentation in this field.



Naringenin phenylhydrazone

Intended Thiazolidinone Product

Figure 6. Reaction scheme for thiazolidinone product formation from starting naringenin phenylhydrazone

Indole Trials

After the consultation of literature, a patent presented by Marzabadi et al. (2004) provided a basis for fischer indole synthesis utilizing the naringenin phenylhydrazone previously formed. The reaction mixture included a 1:1 ratio of naringenin phenylhydrazone to zinc chloride to which acetic acid was added. In parallel, a similar procedure was conducted with hydrochloric acid instead of zinc chloride (Figure 7). These reaction trials were of small volume and were thus conducted in small capped glass vials heated and stirred in hot water baths. TLC comparisons between starting naringenin phenylhydrazone and the reaction mixture assisted in referencing the progress of the reactions. After theoretical completion, the reactions were then worked-up via separatory funnel to extract any residual acid into the aqueous layer, the hypothetical product being extracted into chloroform. Multiple trials for indole synthesis were attempted, however, analytical methods such as TLC, NMR, and mass spectrometry did not demonstrate promising results to continue attempts in indole synthesis.

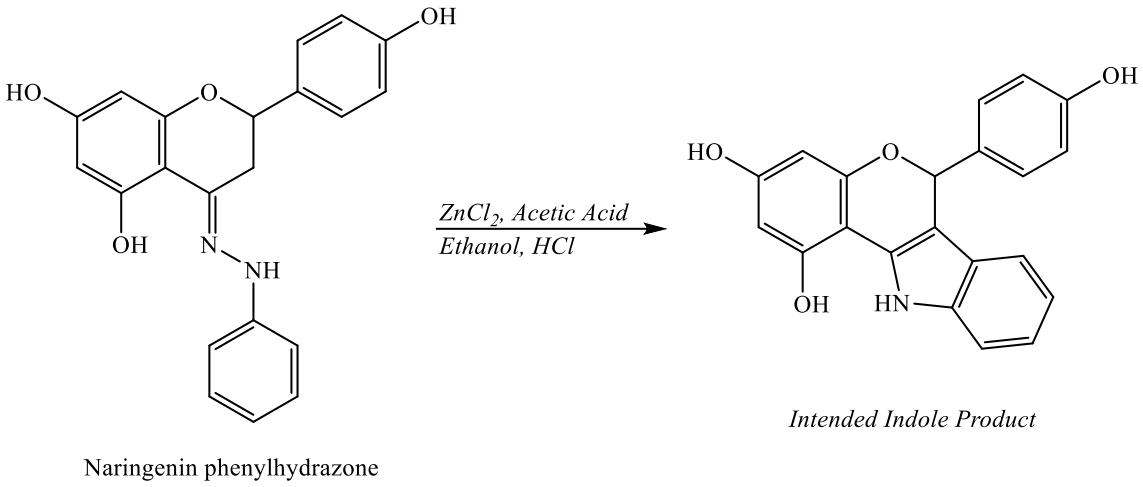


Figure 7. Reaction scheme for indole product formation from starting naringenin phenylhydrazone

α-Bromination of Flavonoid Hydrazones

Contrary to the previous two approaches centered on modifying the added phenylhydrazone through either indole or thiazolidinone ring formation, α-bromination of the previously synthesized naringenin phenylhydrazone was also considered for further coupling schemes. This process aimed to brominate C3 of naringenin phenylhydrazone, resulting in the formation of a subsequent reagent that could then undergo further reactions to introduce a double bond at that location (Figure 8a). A 2:1 ratio of copper bromide and naringenin phenylhydrazone were dissolved in chloroform and ethyl acetate according to the procedure outlined by Anker et al. (2010). The reaction mixture was stirred at reflux for multiple hours. TLC analysis demonstrated an appearance of a potential product spot different from the starting naringenin phenylhydrazone. Following a flash column chromatography work-up, the three resulting fraction flasks were evaporated and submitted for mass spectrometry analysis. Interestingly enough, while some compounds in the sample indicated correspondence to the desired brominated hydrazone, there was also indication of dimerization (Figure 8b). As dimerization is

a relatively difficult synthetic process, the prevalence of some dimer formation was a promising and interesting procedural discovery that could be explored in further research.

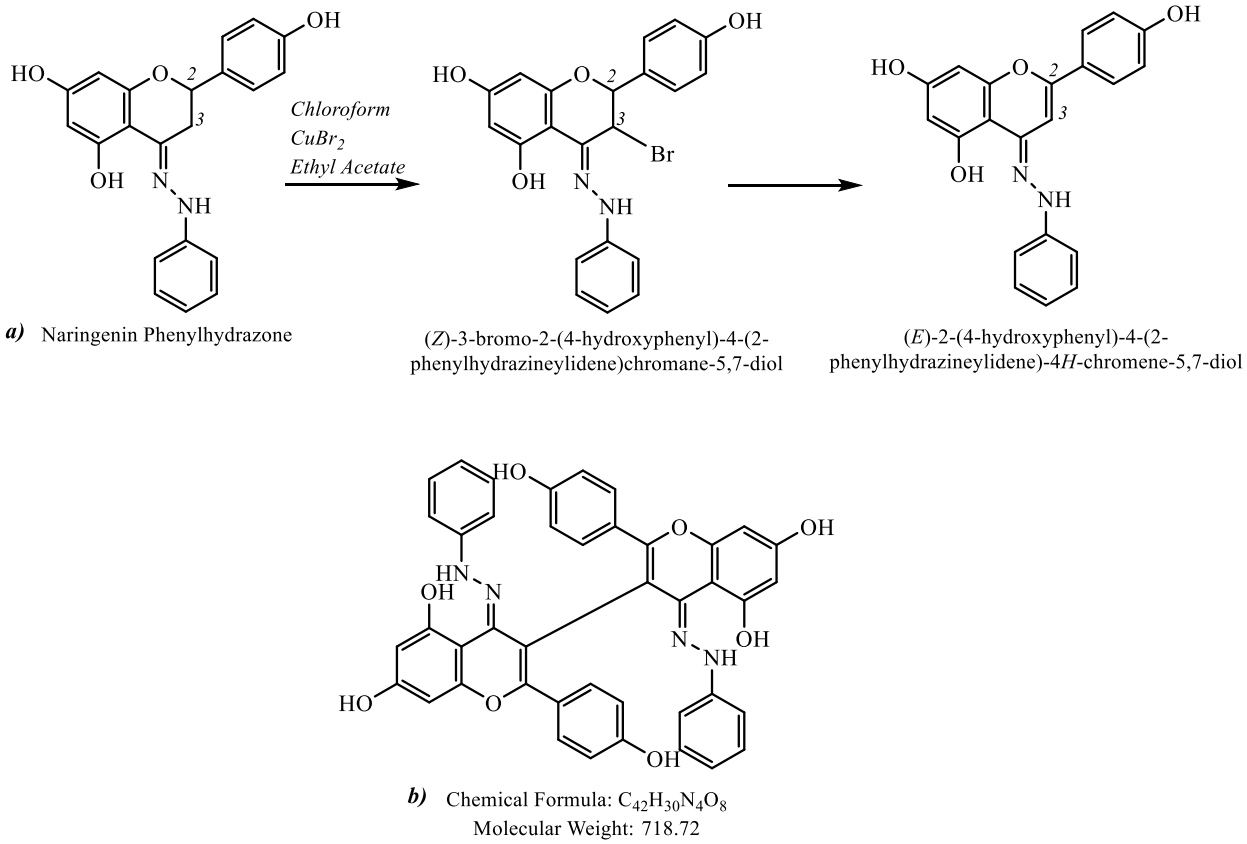


Figure 8. Demonstrates a) intended bromination and subsequent introduction of double bond between C2 and C3, and b) hypothetical dimer indicated by mass spectrometry analysis.

Chapter 5: Return to Flavonoid Hydrazone Synthetic Reactions

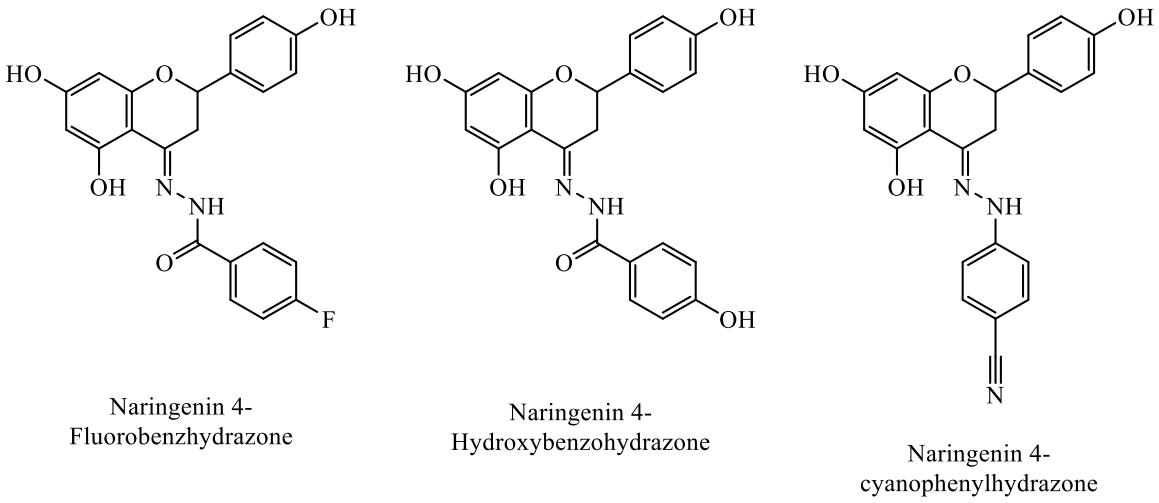
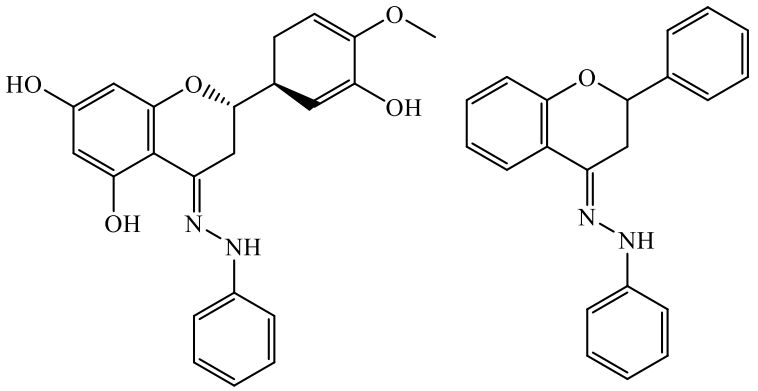


Figure 9. Structures of novel flavonoid derivatives successfully synthesized in lab.

Flavonoid hydrazone derivatives have been previously tested in their pharmacological efficacy, naringenin phenylhydrazone itself being cited by Bak et al. (2011) and Kim et al. (2012) for lung and cervical cancer applications respectively. Specifically, both references demonstrated that this flavonoid derivative induces apoptosis in carcinogenic cells, illustrating an enhanced anti-carcinogenic functionality of a starting flavonoid with an added hydrazine moiety. With extensive experimentation and analysis of flavonoid synthetic modifications, the most promising results were those obtained from flavonoid hydrazone synthetic trials, providing both sufficient yields and well-adapted procedures. The previous successes in naringenin phenylhydrazone synthesis and the potential pharmacological implications encouraged further experimentation in flavonoid hydrazone synthesis.

As naringenin demonstrated the best results in hydrazone synthesis – when compared to flavanone and hesperetin (Figure 10) – this compound was chosen as the reagent flavonoid for modification. Hydrazines were selected from those already available and ones that would yield

novel products; additional hydrazines were also purchased for utility. Of those available, 4-fluorobenzhydrazide, 4-hydroxybenzohydrazide, and 4-cyanophenylhydrazine hydrochloride were utilized in experimental procedures similar to that employed in naringenin phenylhydrazone synthesis.



Hesperetin Phenylhydrazone Flavanone Phenylhydrazone

Figure 10. Proposed structures for hesperetin phenylhydrazone and flavanone phenylhydrazone.

For naringenin 4-fluorobenzhydrazone synthesis and naringenin 4-hydroxybenzo-hydrazone synthesis a 1:1.2 mmol ratio of naringenin to hydrazine was employed, combined with glacial acetic acid and ethanol (0.551M). As 4-cyanophenylhydrazine was coupled to hydrochloric acid in the form that was available in lab, the experimental protocol was similar to those outlined above, but did not include glacial acetic acid (Figure 11).

All reactions were monitored via TLC, and upon completion, the precipitate was filtered through a funnel lined with filter paper. The precipitate was then left to dry and was followed with NMR and mass spectrometry analysis techniques. Preliminary TLC observations demonstrated promising results, as additional spots appearing at lower R_f values were not indicative of either starting naringenin or hydrazine. Mass spectrometry data additionally

confirmed product formation for naringenin 4-fluorobenzhydrazone and naringenin 4-hydroxybenzohydrazone.

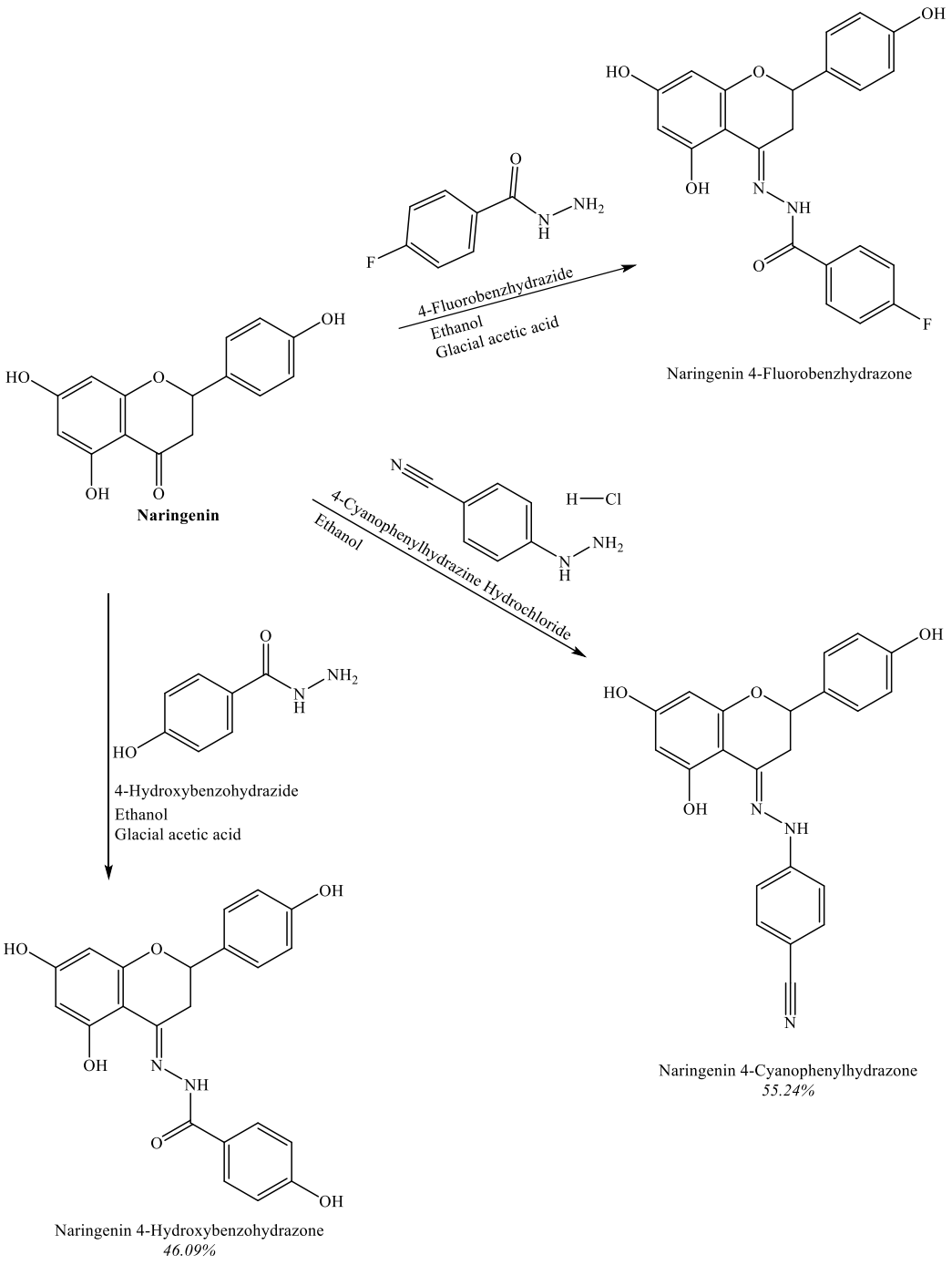


Figure 11. Reaction scheme for the formation of novel naringenin derivatives: Naringenin 4- Fluorobenzhydrazone, Naringenin 4-Cyanophenylhydrazone, and Naringenin 4-Hydroxybenzohydrazone

Chapter 6: Biological Studies

While synthetic organic chemistry was the focus of thesis-centered research, biological applications of the synthetic products was a significant rationale for such reactions. Such biological applications included computational analysis centered on ligand binding affinity against the papain-like protease of COVID-19, anti-aging benefits in yeast, as well as anti-parasitic properties in *T. brucei*.

Through a collaboration with Dr. Neela Yennawar at University Park, various docking servers were utilized to obtain estimated binding affinities of the hypothetical novel compounds and their corresponding COVID-19 targets, particularly the papain-like protease (PL-protease) and Main protease (MPro) of COVID-19. Specific docking servers included an open-access COVID-19 Docking Server (https://ncov.schanglab.org) which estimated the hypothetical compounds' binding affinity towards the PL-protease. Additionally, PyMOL – a downloadable program – was used for protease and compound visualization. HADDOCK ProDIGY served as another docking server for the identification of hypothetical binding affinity. Two different docking servers were used to provide an average binding affinity estimate, illuminating whether a compound could effectively inhibit protease functionality.

A total of eight flavonoid derivatives were identified to have the highest binding affinities for the papain-like protease of COVID-19 (Figure 12). Some of these compounds have been successfully synthesized and will further be tested in biological assays with a local company 'Reaction Biology.'

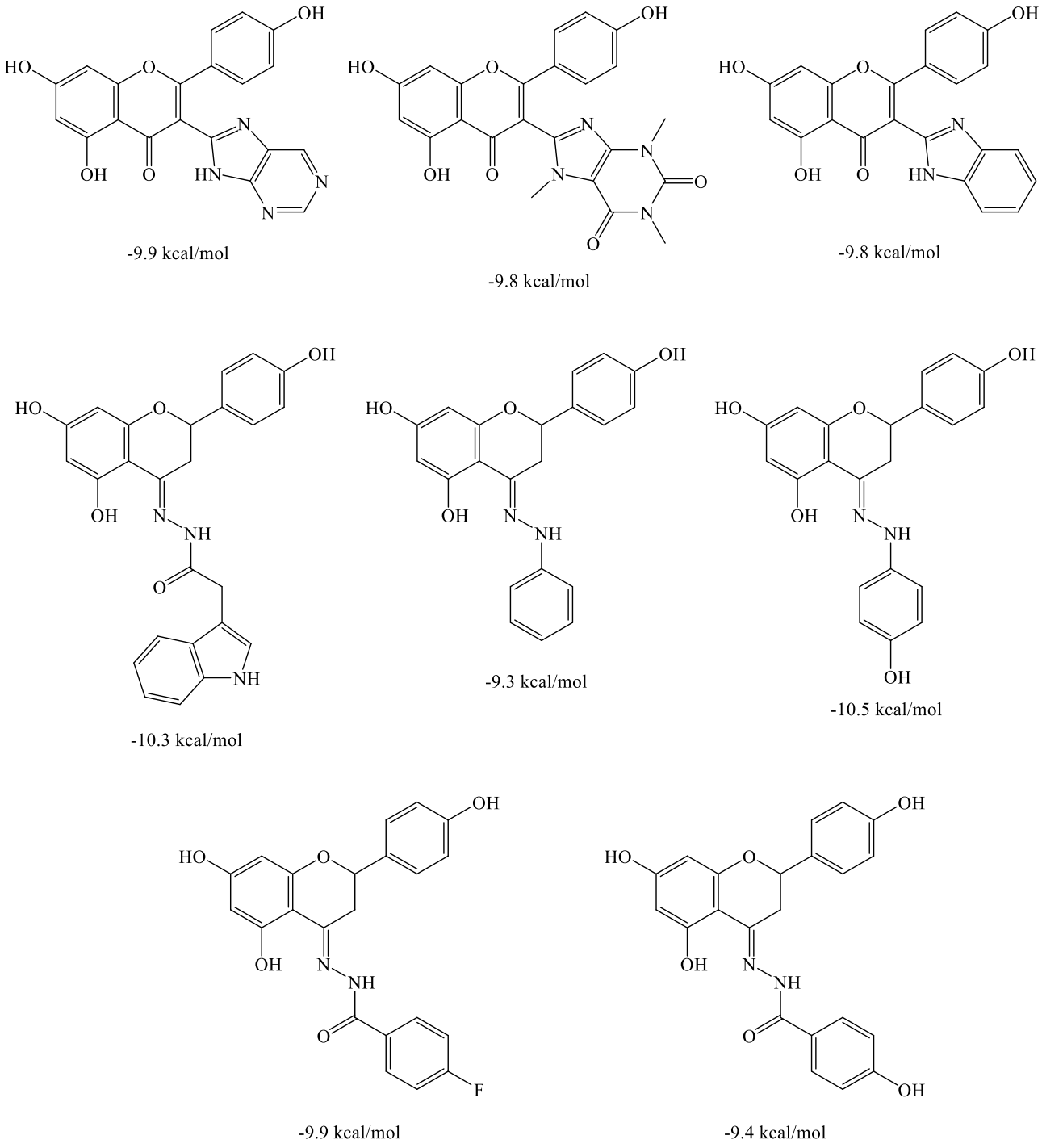


Figure 12. Proposed Covid-19 ligands with corresponding hypothetical binding energies derived from docking servers.

Separate from COVID-19 implications, additional biological assays have been performed through a collaboration with Dr. Megan Povelones at Villanova University. Initial biological assay exposure was conducted in the summer of 2022, in which pilot experimentation warranted some experience in how such assays are conducted. With such exposure, the successfully synthesized flavonoid hydrazones – such as naringenin phenylhydrazone, naringenin 4-fluorobenzhydrazone, naringenin 4-hydroxybenzohydrazone, and naringenin 4-cyanophenylhydrazone – will be tested against *T. brucei* at Villanova University in summer 2023.

Chapter 7: Conclusion and Discussion

The purpose and course of this research experience embodied a general observation of flavonoids, with the intention of synthesizing novel compounds for further biological testing. While intentions of application shifted throughout means of synthesis – whether novel compounds would be tested for COVID-19, anti-bacterial, anti-aging, anti-inflammatory implications – the general objective revolved around exploring synthetic organic chemistry in detail, highlighting characteristics associated with one broad group of organic compounds – flavonoids.

Initial observations highlighted computational modeling programs – PyMOL, Autodoc Vina, PRODIGY and HADDOCK – for hypothetical binding affinity estimates of intended compounds. As such, this portion was predominantly virtual and did not require laboratory practices, a type of research rather fitting during the peak prevalence of COVID-19. While some team members pursued COVID-19 applications in their synthetic procedures, personal research aimed to expand existing anti-aging and anti-inflammatory properties of flavonoids.

Various synthetic procedures were tested, all with distinct mechanisms. Initial trials focused primarily on C3 bromination and coupling, the former being successful while the latter posing difficulties in the presented laboratory conditions. Such procedures frequently require sensitive laboratory conditions, while the literature itself (Min et al., 2012) did not demonstrate outstanding yields. Subsequent trials intended to construct indole or thiazolidinone moieties from flavonoid hydrazones; the flavonoid hydrazone synthesis was successful in both yield and efficiency, while indole and thiazolidinone yields were not significant enough for further experimentation. Additional experimentation on the synthesized flavonoid hydrazone – naringenin phenylhydrazone – included C3 bromination for subsequent elimination and

introduction of a double bond between C2 and C3. While bromination was indeed successful, according to mass spectrometry data, additional unexpected results included potential dimerization of the naringenin phenylhydrazone. As dimer formation is frequently viewed as a difficult procedure, this discovery uncovered a potential new path for future experimentation in this scheme of synthetic organic chemistry.

As the most successful synthetic procedure was that of flavonoid hydrazone synthesis, these synthetic products became the main course of experimental study. Similar protocols were utilized as those outlined by Bak et al. (2015), with some modifications. Acid was not added to one reaction as the starting hydrazine included hydrochloride, indicating some required modifications and adaptations throughout the course of literary reference and application.

In conclusion, current research and laboratory experimentation demonstrated the most effective methods for minimally sensitive laboratory methods of novel flavonoid synthesis, specifically in synthesizing those with hydrazone moieties. Further research and experimentation is required to 1) validate the true nature of the compounds formed, 2) generate higher yields, as well as 3) identify any biological significance of such compounds in their anti-aging and anti-inflammatory applications. Mass spectrometry data of the synthesized novel flavonoid hydrazones – naringenin 4-fluorobenzhydrazone and naringenin 4-hydroxybenzohydrazone – confirmed the successful synthesis of these compounds, allowing for subsequent steps to be taken to both synthesize more compounds by similar means and to biologically test existing ones.

Appendix

Methods for Literary Consultation, Protocol, and Analysis

Literary Consultation

As synthetic chemistry – similar to any other fields of research – requires consultation of previously conducted experimental techniques, various databases were consulted to devise reproducible protocols. Such databases included SciFinder, Google Scholar, and MedLine all for the purposes of referencing synthetic procedures and methods. SciFinder specifically allowed for advanced searches of compounds similar in structure to desirable products, allowing to understand which methods have provided viable results and whether the intended products were novel. Google Scholar was predominantly used for the purposes of elucidating any questions concerning experimental protocols, identifying which methods may have worked effectively in the past and what work-up procedures may be required to extract and purify products. MedLine was utilized in understanding any biological significance that the products could provide.

Protocol Methods

With reaction mechanisms predominantly requiring the utility of condensers, heating mantles, and stir plates, procedural methodology for setting up experiments was relatively similar. These methods included combining the starting reagents into round-bottom flasks with a stir bar, placing the flask into a heating mantle if the experiment required heating, and resting both on a stir plate. If the experiment required that the reaction mixture was heated to reflux, a condenser would be inserted into the flask and held in place via clamps and an iron stand.

Working up the reaction mixture or extracting and purifying the product, while being similar between experiments, was more variable in approach. Methods for extraction included utilizing a simple funnel, a separatory funnel, a column, or recrystallization. Some reaction mixtures in which precipitate formed did not require additional purification for preliminary analysis, therefore, a standard funnel allowed for the extraction of precipitate from filtrate. If acid or another aqueous-soluble compound was used, a separatory funnel was utilized to remove the acid or water-soluble compound and extract the desired product into the organic layer. A column was predominantly used for the purpose of product purification through flash column chromatography. This method allowed for separation of different compounds based on their different polarities. Recrystallization involved the dissolution of a solid product into a slowly evaporating solvent – commonly ethanol or ethyl acetate – for the purpose of crystallizing the product for x-ray crystallography. This method was not frequently used as the primary analytical methods included NMR and mass spectrometry, both of which did not require crystalline forms of the product.

Another important protocol method to note when considering reaction methodology is one that does not directly fall into the two aforementioned categories. The rotary evaporator (Rotovap) was a device utilized for the purpose of evaporating residual solvent from the reaction mixture, leaving a dry product for further analysis.

Analytical Methods

Whether for the purpose of tracking reaction progression or for interpreting the effectiveness of product formation, various analytic methods were employed, specifically thin layer chromatography (TLC), nuclear magnetic resonance (NMR), and mass spectrometry. TLC

was a primary method for determining reaction progress and functions by separating compounds in a sample by polarity. The more polar compounds "stick" to the polar silica on the plate, while the more nonpolar compounds tend to travel up the plate with the solvent system. As such, reaction progression was identified by comparing the reaction mixture with starting reagents, identifying whether the starting compound was still present in the reaction mixture. NMR served as a form of product visualization, identifying whether the sample was different from the starting reagents based on the presence of unique protons in the sample and their corresponding shifts. This method of analysis was predominantly employed after the reaction was halted and the product was thoroughly dried. The product was then redissolved in deuterated solvent and placed into the on-campus NMR machine for visualization. Mass spectrometry was an off-campus method of analysis, with the product samples being thoroughly dried and sent to either the University of Delaware or to Penn State University Park. The mass spectrometry data provided insight into the exact masses of compounds present in the sample, illuminating whether desired product formed and in what proportion of the overall submitted sample.

Methodology

Various methodologies were employed throughout the course of synthetic research, differing in experimental protocol and work-up methods. The main reaction types outlined – bromination, coupling, hydrazone, thiazolidinone, and indole synthesis – all involved variability in procedures, and require some explanation and elaboration. Reagents and corresponding proportions are outlined in Table 1.

Table 2. Outline of the general ratio, reagents, solvents, and work-up procedures for the discussed experimental protocols.

<table>
<tr><td colspan="4">Bromination (Quintin et al., 2006)</td></tr>
<tr><td colspan="4">General Ratio:

1:3 flavonoid to NBS; 0.09M reaction concentration</td></tr>
<tr><td>Flavonoid</td><td colspan="2">Reagent</td><td>Solvent</td></tr>
<tr><td>Flavone</td><td colspan="2">NBS</td><td>CH_2Cl_2

Pyridine</td></tr>
<tr><td>Chrysin</td><td colspan="2">NBS</td><td>Acetone or Ethanol

Pyridine</td></tr>
<tr><td>Apigenin</td><td colspan="2">NBS</td><td>DMF

Pyridine</td></tr>
<tr><td colspan="4">Work-up:

Solid redissolved in CH_2Cl_2 and washed with 0.1M sodium thiosulfate

Purification via flash column chromatography</td></tr>
<tr><td colspan="4">Coupling Trials (Min et al., 2012)</td></tr>
<tr><td colspan="4">General Ratio:

1:2:3:2 bromo-flavonoid to coupling compound to $Pd(PPh_3)_4$ to CuI to Cs_2CO_3; 0.1M reaction concentration</td></tr>
<tr><td>Flavonoid</td><td>Coupling Compounds</td><td>Reagent</td><td>Solvent</td></tr>
<tr><td>Bromo-flavone</td><td>Caffeine

Benzothiazole</td><td>$Pd(PPh_3)_4$

CuI

Cs_2CO_3</td><td>1,4-dioxane</td></tr>
</table>

Work-up:

Methylene chloride wash through celite

Purification via flash column chromatography

Hydrazone Synthesis (Bak et al., 2011)

General Ratio:

1.2:1 hydrazine to flavonoid; 0.52M reaction concentration

Flavonoids	*Hydrazines*	*Solvents*	*Acids*
Naringenin Flavanone Hesperetin	Phenylhydrazine	Ethanol	Glacial acetic acid
Naringenin	4-Fluorobenzhydrazide 4-Hydroxybenzohydrazide	Ethanol	Glacial acetic acid
Naringenin	4-Cyanophenylhydrazine Hydrochloride	Ethanol	--

Work-up:

Filtration through filter paper

On occasion required purification via flash column chromatography

Thiazolidinone Trials (Silverberg et al., 2020)

General Ratio:

1:1:2:3 flavonoid hydrazone to thioglycolic acid to T3P to Pyridine; 2.6M reaction concentration

Flavonoid	*Reagent*	*Solvents*	*Acid*
Naringenin phenylhydrazone	T3P	Pyridine THF	Thioglycolic acid
Work-up: Water and ethanol wash Purified via flash column chromatography			
Indole Trials (Marzabadi et al., 2004)			
General Ratio: 1:1 flavonoid hydrazone to $ZnCl_2$ or HCl; 0.87M reaction concentration			
Flavonoid	*Reagent/Solvent*	*Acid*	
Naringenin phenylhydrazone	$ZnCl_2$	Acetic acid	
	Ethanol	HCl	
Work-up: Extraction into chloroform via separatory funnel; acid neutralized with sodium bicarbonate			
Naringenin Phenylhydrazone α-Bromination (Anker et al., 2010)			
General Ratio: 1:2 flavonoid to $CuBr_2$; 0.8M reaction concentration			
Flavonoid	*Reagent*	*Solvents*	
Naringenin phenylhydrazone	$CuBr_2$	Chloroform Ethyl acetate	
Work-up: Purification via flash column chromatography with chloroform: acetone as solvent			

Flavone Bromination and Coupling Trials

The bromination protocol employed a 3:1 equivalent of NBS to flavone dissolved in methylene chloride and pyridine (0.09M). The reaction mixture was stirred at room temperature and was monitored via TLC. After completion, the reaction mixture was dissolved in methylene chloride, washed with water, and evaporated to dryness. The product work-up protocol included dissolution in methylene chloride and an HCl and sodium thiosulfate wash, the HCl functioning to extract residual pyridine and the sodium thiosulfate to extract residual NBS. NMR analysis was then conducted, indicating the disappearance of a characteristic proton peak located at 6.8 ppm. The disappearance of this peak indicated the replacement of that unique proton with a bromine atom. Flavone was not the only flavonoid utilized for halogenation trials, but it was the most successful in the halogenation process.

Coupling trials included a 1:2:3:2 bromo-flavonoid to caffeine/benzothiazole to $Pd(PPh_3)_4$ to CuI to Cs_2CO_3, all dissolved in 1,4-dioxane for a 0.1M reaction mixture. The reaction mixture was heated to reflux, stirred, and monitored via TLC. Following perceived reaction completion, the reaction mixture was dissolved in methylene chloride and filtered through celite. Flash column chromatography was employed to purify the resulting sample.

Synthesis of Flavonoid Hydrazones

Following the ratios presented in Table 1 – 1:1.2 flavonoid to hydrazine dissolved in ethanol and glacial acetic acid – the reaction mixture was heated to reflux and stirred over the course of two days with 1:1 hexane/ethyl acetate TLC serving as a primary method for monitoring reaction progress. After the perceived completion of the reaction, the product was dissolved in more ethanol and filtered through filter paper. In some cases, some purification via

flash column chromatography was required, however, the precipitate product was frequently pure. An additional point to note is that one hydrazone synthesis reaction did not require the use of glacial acetic acid as its dry reagent state contained a hydrochloride – 4-cyanophenylhydrazine hydrochloride. Thus, that particular hydrazone synthesis reaction only involved the dissolution of naringenin and 4-cyanophenylhydrazine hydrochloride in ethanol.

Thiazolidinone Trials

In thiazolidinone synthesis trials, all compounds were added to a round-bottomed flask via ratios presented in Table 1. This protocol involved a 1:1:2:3 ratio of naringenin phenylhydrazone to thioglycolic acid to T3P to pyridine dissolved in THF (2.6 M). The reaction mixture was heated to reflux and stirred until perceived completion. The viscous reaction mixture was then extracted into methylene chloride via separatory funnel, with the organic layer then being analyzed via TLC. The separated reaction mixture was evaporated, and the dry product was submitted for mass spectrometry analysis.

Indole Trials

For indole synthesis, all starting reagents were dissolved in the corresponding solvents with acid (Table 1). It is important to note that two different procedures were attempted in indole synthesis: one reaction involved naringenin phenylhydrazone (1 equiv.), $ZnCl_2$ (1 equiv.), and acetic acid and another employed naringenin phenylhydrazone (1 equiv.), HCl (1 equiv.) and ethanol. As these reactions were initially tested in small proportions, compounds were placed into small glass vials, capped, and placed into a hot water bath on a hot plate. Small stir bars were added into the vials and the reactions were left to heat and stir. The products were extracted

into chloroform via a separatory funnel, while the acid was neutralized and extracted with sodium bicarbonate. The final reaction mixture was then evaporated, and the solid was sent for mass spectrometry analysis.

Naringenin Phenylhydrazone α-Bromination

All starting reagents (1:2 flavonoid hydrazone to $CuBr_2$) were dissolved in the designated solvents, heated to reflux, and stirred for two hours. The reaction mixture was then cooled and purified via flash column chromatography with a 1:1 chloroform/acetone solvent system. This purification yielded three different fraction flasks: one flask contained an upper spot, a second contained a combination of an upper and lower spot, and a final flask contained only the lower spot. These samples were evaporated, and the dry solids were submitted for mass spectrometry analysis.

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