# OPTIMIZING ARGININE FOR ENANTIOSELECTIVITY IN WARFARIN SYNTHESIS

by

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**Abstract**

Warfarin is an anticoagulant medication that is important for the treatment and prevention of blood clots and clotting-related disorders. While it is typically prescribed as a racemic mixture, the R- and S- enantiomers of warfarin have large differences in their anti-coagulant activities. In previous work, it was demonstrated that L-arginine is an effective catalyst in an enantioselective synthesis of Warfarin. The aim of this study is to determine whether various derivatives of L-arginine can promote a more enantioselective synthesis of Warfarin. Using nine L-arginine derivatives, separate warfarin reactions were created and allowed to run for a week. Thin Layer Chromatography (TLC), Flash Chromatography, and High-Performance Liquid Chromatography (HPLC) techniques were applied to determine, isolate, and assess the production of warfarin in each reaction. In cases where low enantioselectivities were observed, an acetic acid-assisted strategy was investigated for its potential to increase enantioselectivities. Data collected from the research suggested no substantial increase in percent yield and no significant assistance to enantioselectivity. Although the research revealed that the L-arginine derivatives investigated in this study did not present any enhancements in selectivity for the R-enantiomer, a comparison of L-arginine and L-citrulline indicated the importance of the guanidinium amine groups in L-arginine for driving its organocatalytic activity.

*Keywords:* Warfarin, enantioselective, synthesis, catalyst

## Optimizing Arginine for Enantioselectivity in Warfarin Synthesis

### Introduction

This project explores the potential of L-arginine as a biocatalyst for the enantioselective synthesis of R-warfarin as a greener and more sustainable approach aligning with the principles of green chemistry (Pleissner, et. al., 2020). Research on this topic has been conducted previously and demonstrated the feasibility of utilizing L-arginine for warfarin synthesis, achieving modest enantioselectivity (Wurz, et. al., 2025). The purpose of this project focuses on optimizing this process by investigating modification to the L-arginine molecule, through L-arginine derivatives, that could enhance its selectivity for the R-enantiomer. Through this scope, performing the same applications with D-arginine variants would presumably favor S-warfarin. Additionally, the efficiency of the reaction will be assessed by observing the percent yield of the newly tested biocatalyst, which will help determine the cost-effectiveness of optimizing warfarin production.

Building upon the success of the 2023-24 Organic Chemistry Course-based Undergraduate Research Experience (CURE) course, which demonstrated the effectiveness of L-arginine as a biocatalyst in enantioselective warfarin production, we aim to determine if different L-arginine derivatives are able to provide higher percent yields (Wurz, et. al., 2025). By following similar reaction schemes that yielded good percent yields and enantioselectivity in the CURE course, this research project aims to enhance L-arginine's ability to selectively produce the R-enantiomer of warfarin through structural modifications to the molecule. Success in this project may ultimately lead to an optimized biocatalyst for production of enantiomer-specific warfarin products.

**Warfarin**

Warfarin is an important pharmaceutical compound that is characterized for its anticoagulant properties. Although previously known for its initial commercial use as a rat poison, the current use of the medication assists in the prevention of blood clot formation. The production of warfarin is thus vital to mitigate the symptoms and conditions individuals who suffer from heart attacks, strokes, tachycardia, and other disorders experienced as a result of blood coagulation (Cleveland Clinic Medical, 2025). The molecule, known as Warfarin, has R and S enantiomer configuration, as illustrated in Figure 1. Each of the enantiomers have different potencies, metabolisms, and interactions with other drugs (Pleissner, et. al., 2020). The S enantiomer of Warfarin is three to five times more potent than the R configurations, where the anticoagulant desired effects are primarily attributed to the S-enantiomer form of Warfarin. The anticoagulant warfarin drug is metabolized through various cytochrome P450 (CYP) enzymes. Although CYP2C9 is the primary enzyme responsible for S-warfarin metabolism, R-Warfarin is metabolized by a larger scale of CYP isoforms which include: CYP1A2, CYP2C19, and CYP3A4. Current research has focused significantly on the impacts of CYP2C9 polymorphisms, particularly the CYP2C91 wild-type allele and the functionally significant CYP2C92 and CYP2C93 variants, on S-warfarin response. However the influence of these polymorphisms and the role of other CYP enzymes on R-warfarin metabolism remain less explored (Lane et al., 2011).

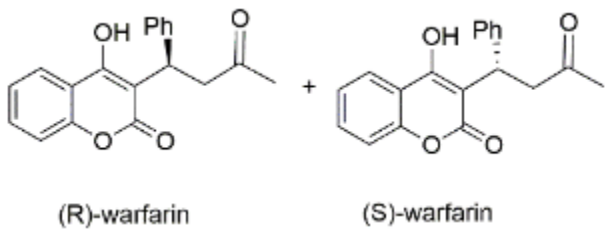



**Figure 1.** R- and S- Enantiomers of Warfarin

**Green Chemistry**

Current production of pharmaceuticals have impacted the world adversely through soil contamination, carbon footprints, but primarily through wastewater discharges from manufactures which damage aquatic ecosystems (Kayode-Afolayan et al., 2022). To make warfarin in a more effective manner, ideology from green chemistry was applied to the studies conducted. Green Chemistry aims to develop new chemical reactions and conditions that improve resource efficiency, energy efficiency, product selectivity, operational simplicity, as well as health and environmental safety (Terence et al., 2010). Following this "green" method to facilitate the reduction of waste, conserve energy, and develop new eco-friendly reactions, the synthesis of S-warfarin through such an approach will assist in reducing the pollution current manufacturers introduce into the environment.

**Previous Work: 2023-24 CURE**

Previously working on this study under the 2023-2024 Organic Chemistry Course-based Undergraduate Research Experience (CURE) course, the laboratory supported warfarin synthesis and provided a learning opportunity for students to practice green chemistry principles (Wong, T.C. et al., 2010). The success of natural L-arginine as a biocatalyst in enantioselective warfarin production, compared to other amino acids used in the CURE lab, revealed contrasting enantioselectivity results between L-arginine and D-arginine. Although selectivity is initially modest, recrystallization techniques can be utilized to obtain 97% purity of R- and S- enantiomer excess although it results in loss of product (Wurz, et. al., 2025).

This paper discusses the data collected in this research study that expanded on the work from CURE. These experiments concentrated on nine commercially available arginine derivatives which studied the potential improvements it could implicate on the enantioselective

synthesis of R-warfarin. Figure 2 illustrates the reaction scheme that was predominantly used within the CURE course. As established, the reaction scheme provided will be the same one utilized for purposes of this project to analyze the optimization of Arginine derivatives for the enantioselectivity in Warfarin synthesis.

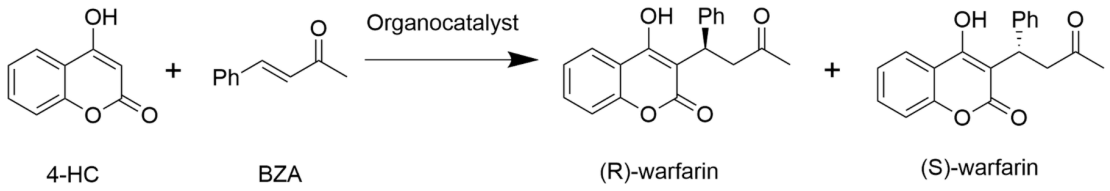
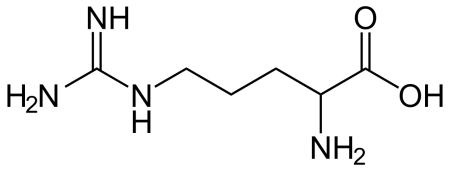
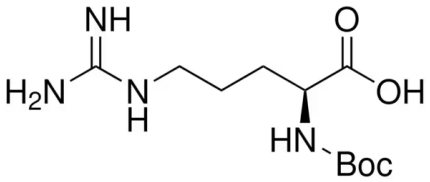
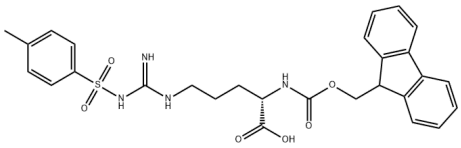
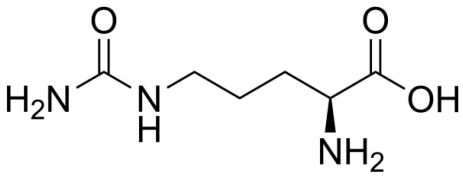
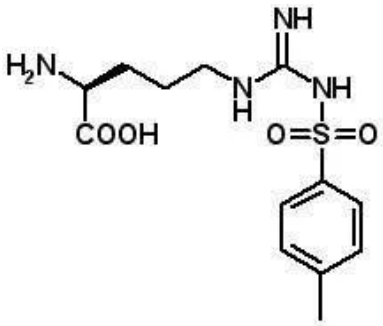


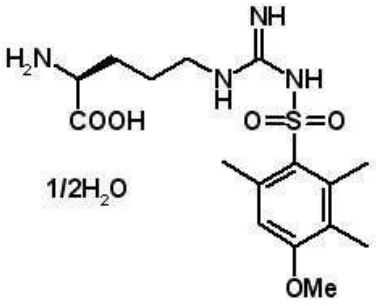
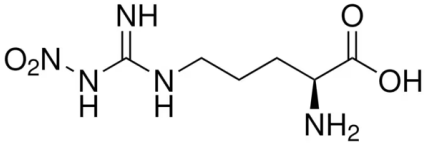
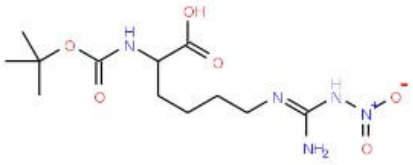
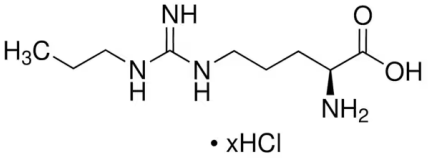

**Figure 2.** Warfarin Reaction Scheme for this study. Starting from the left to right, the reactants for the reaction include: 4-hydroxycoumarin (81 mg), benzylideneacetone (88 mg), its respective organocatalyst listed in Table 1 as labeled above the arrow, (R)-warfarin and (S)-warfarin end products. All reactions obtained 900 µ$L$ of DMSO and 100 µ$L$ of distilled water and incubated in an end-over-end rotator at room temperature for 7 days.

## Methods

The procedure was initiated following a warfarin synthesis reaction with a respective amino acid biocatalyst experiment to drive the detection of warfarin in each reaction. Nine biocatalysts were commercially purchased, eight of which were arginine derivatives and one (L-citrulline) of which had a similar molecular structure as L-arginine. In a screw top reaction vial all reactants where combined: 4-hydroxycoumarin (0.0810 g), benzylideneacetone (0.0880 g), DMSO (900 µ$L$), $ddH_2O$ (100 µ$L$), and the catalyst (0.0300 g). The same procedure was executed for the following reagents as listed in Table 1 with their respective weights.

**Table 1.** Warfarin Synthesis with a Amino Acid Biocatalyst Reagents

| Rxn # | Catalyst | Source | Structure |
|---|---|---|---|
| R1 | L-Arginine | Thermo Scientific |  |
| R2 | Boc-Arg-OH | Sigma Aldrich |  |
| R3 | N-Fmoc-N'-tosyl-L-Arginine | Fisher Scientific |  |
| R4 | L-Citrulline | Sigma Aldrich |  |
| R5 | H-ARG(Tos)-OH | aaptech |  |

| | | | |
|---|---|---|---|
| R6 | H-ARG(MTR)-OH | aaptech |  |
| R7 | Nω-Nitro-L-Arginine | Sigma Aldrich |  |
| R8 | Boc-N'-Nitro-L-homoarginine | Santa Cruz |  |
| R9 | Nω-Propyl-L-Arginine hydrochloride | Sigma Aldrich |  |
| R10 | No catalyst | N/A | N/A |

All reaction vials were placed on an end-over-end rotator and left to mix for a week at room temperature. Thin Layer Chromatography (TLC) techniques were followed for all the reactions to detect if warfarin production was present. TLCs were completed using 1$\mu L$ of a sample from the reaction on a silica gel sheet and placed in the solvent (2:1 hexane:ethyl acetate). Results for the TLC silica gel sheet staining were identified using a UltraViolet light to detect warfarin production, depicted through purple coloration. Upon identifying warfarin on the TLC Silica Gel sheets, Hitachi High-Performance Liquid Chromatography (HPLC) techniques were followed for all reactions with warfarin production. HPLC techniques were executed to

separate, identify, and quantify warfarin products to determine the concentrations produced by each organocatalytic warfarin reaction. $100\ \mu L$ of each sample was measured and placed into a filter tube and placed into a centrifuge for two minutes. In a separate glass tube $1,000\ \mu L$ of ethanol was measured and integrated into the tube. From the filter tubes, $5\ \mu L$ of each solution was placed into the $1,000\ \mu L$ ethanol tube and tested on the Hitachi HPLC machine. The Hitachi HPLC system (Hitachi High-Tech, Schaumburg, IL, USA) was equipped with a Zorbax-SB-C18 (Agilent, Santa Clara, CA, USA), 3.5 $\mu m$, $4.6 * 150$ mm column; 40% 0.1% formic acid and 60% methanol mobile phase; 1 mL/min flow rate; 280 nm wavelength UV detector (Hitachi High-Tech, Schaumburg, IL, USA); 20 min run time; and PC/Chrom software (H&A Scientific, Greenville, NC USA). The apparatus was connected to a computer at East Carolina University where standard curves were used to determine reaction yields.

Flash Chromatography was conducted for the L-arginine, BOC-ARG-OH, L-citrulline, H-ARG(Tos)-OH, and Nω-Nitro-L-arginine catalyst reactions, as a result of high percent yields determined by HPLC results. Flash Chromatography was initiated by washing the column with 4:1 Hexane:ethyl acetate to clean the instrument from potential contaminants. The entire L-arginine reaction solution was pipetted into the flash column and pushed down the silica gel utilizing the gas inlet to reduce the run time through added pressure. Upon the reaction having passed the sand, 8 mL of 4:1 Hexane:ethyl acetate was added into the column and flushed through until all the solvent passed down and was collected into a waste beaker located under the instrument. To collect the catalyst reactions, the column was washed with three separate buffer solutions and collected in nine separate vials. 24 mL of 4:1 Hexane:ethyl acetate was entered into the flash column and collected in three separate vials with 8 mL of the catalyst solution in each. 24 mL of 2:1 Hexane:ethyl acetate followed and was added into the flash column and collected

in three separate vials with 8 mL of the solution in each. The column was finally washed with 24 mL of Ethyl acetate and distributed into three separate vials to collect the remaining catalyst solution. Fractions 7-9 were tested through TLC, using the same procedure mentioned previously, to detect which fraction contained warfarin. The same Flash Chromatography and TLC procedures were followed for the BOC-ARG-OH, L-citrulline, H-ARG(Tos)-OH, and Nω-Nitro-L-arginine catalyst reactions.

Chiral chromatography tests were performed for the L-arginine, BOC-ARG-OH, L-citrulline, H-ARG(Tos)-OH, and Nω-Nitro-L-arginine catalyst reactions. This test was performed utilizing the fractions collected from the Flash Chromatography that contained warfarin. Each purified sample was analyzed by integrating it into the Shimadzu HPLC (Scimadzi Corp, Kyoto, Japan) which was equipped with a Chiralcel OD-H (Daicel Corp, Tokyo, Japan) chiral column: 50% IPA, 0.1% acetic acid mobile phase. Warfarin enantiomer and its percentages were monitored through samples by UV/VIS detection at 220 and 280 nm.

After assessing results from HPLC and Chiral Chromatography, an L-arginine acetic acid study was completed to determine if alterations to the reaction's pH environment could lead to the obtainment of better enantioselectivity. The procedure for this study was initiated following a screw top reaction vial (2 mL), 4-hydroxycoumarin (0.081 g), benzylideneacetone (0.088 g), DMSO (900 µ*L*), and H-ARG(Tos)-OH (0.0300 g) for all four reactions. Ratios of water to glacial acetic acid was used in the reaction series for this part of the study. Glacial acetic acid to $ddH_2O$ ratios for reactions 1, 2, 3, and 4, respectively, are as follows: 0 µ*L* to 100µ*L*, 20 µ*L* to 80 µ*L*, 30 µ*L* to 70 µ*L*, and 40 µ*L* to 60 µ*L*. HPLC and chiral chromatography were completed, using the same procedure described above.

## Results

The Arginine derivatives tested in this study did not improve upon the enantioselectivity observed with L-arginine. After running the warfarin synthesis reactions with all the arginine derivatives, as listed in Table 1, results from the TLC Silica Gel experiment indicated that all samples were successful at warfarin production and needed further testing through the Hitachi HPLC machine. The Hitachi HPLC apparatus results showed that arginine derivatives used in reactions 5 and 6 had the highest percent yields; although, the percent yields are significantly lower when compared to using L-arginine as a biocatalyst, as seen in Table 2.

**Table 2.** Presents Percent Yields after HPLCs were tested on all the Organocatalytic Reactions

| Organocatalytic Reactions | Percent Yield from HPLC |
|---|---|
| R1 | 97.94% |
| R2 | 8.71% |
| R3 | 1.93% |
| R4 | 12.56% |
| R5 | 56.45% |
| R6 | 41.14% |
| R7 | 15.60% |
| R8 | 1.82% |
| R9 | 1.73% |
| R10 | 1.44% |

These results determined that reactions 1, 2, 4, 5, 6, and 7 should be used for further flash chromatography to isolate warfarin products for chiral chromatography testing. After performing flash chromatography, TLCs were completed to indicate which fractions included warfarin

product for chiral chromatography. Chiral chromatography was used to test the enantiomer excess of the warfarin that was produced in the six reactions, this is reported in Table 3.

**Table 3.** Percent Enantioselectivity for all Organocatalytic Reactions Upon Completing Chiral Chromatography

| **Organocatalytic Reactions** | **Percent ee (R) from Chiral Chromatography** |
|---|---|
| R1 | 44 |
| R2 | 22 |
| R4 | 20 |
| R5 | 38 |
| R6 | 32 |
| R7 | 28 |

These results prompted further experimentation by changing the environmental conditions, such as pH, to hopefully lead to improved enantioselectivity of the biocatalyst. Therefore, using the organocatalyst in reaction 5, the arginine derivative with the highest percent yield, was used in the acetic acid study. Reactions were conducted using four different ratios of acetic acid to water to compare enantioselectivity in different pH levels. Executing the same procedures on these reactions to those applied for the original biocatalytic reactions, HPLCs and Chiral chromatography displayed no significant improvements as the highest percent yield was 55.51% and the highest enantioselectivity (R) was 47.9 for reactions with 0% and 30% of acetic acid in its reaction vial, respectively, as seen in Table 4.

**Table 4.** Percent Yields and Percent ee's for all Four Acetic Acid Reactions after HPLCs and Chiral Chromatography were Completed, Respectively.

| %Acetic Acid in Reaction | Percent Yield from HPLC | Percent ee (R) from Chiral Chromatography |
|---|---|---|
| 0% | 55.51% | 47.7 |
| 20% | 51.46% | 47.7 |
| 40% | 47.52% | 47.9 |
| 60% | 45.85% | 47.1 |

## Discussion

The results of this study indicate that among the L-arginine derivatives tests, none enhanced their effectiveness as organocatalysts for warfarin synthesis. Despite initial expectations, none of the nine L-arginine derivatives tested produced higher percent yields or greater enantiomeric excess (ee) compared to unmodified L-arginine. In fact, L-arginine consistently outperformed its derivatives in both categories, with a percent yield of 97.94% and a percent enantiomeric excess of 44% (R). The derivative reactions that did show relatively high yields, such as H-ARG(Tos)-OH (56.45%) and H-ARG(MTR)-OH (41.14%), still failed to surpass L-arginine's performance and did not lead to higher enantioselectivity.

Importantly, the study also explored environmental modifications to the reaction, specifically the introduction of acetic acid to alter the pH. However, this adjustment showed no significant impact on either the yield or the enantioselectivity of the biocatalytic reactions. Even at varying concentrations of acetic acid, the percent yields remained under L-arginine's baseline, and the enantiomeric excess values plateaued at approximately 48% (R). These findings suggest

that within the tested conditions, pH adjustment alone is not a viable strategy for optimizing this synthesis.

The comparative performance of L-citrulline, a structurally similar compound to L-arginine, provided further insight. L-citrulline lacks the guanidino group present in L-arginine and produced a significantly reduced percent yield of 12.56%, representing approximately a 53% decrease. This pronounced difference highlights the importance of the guanidino group in L-arginie's ability as an effective biocatalyst, indicating that its unique electronic or structural properties play a key role in the synthesis of warfarin.

Taken together, these findings reinforce the notion that L-arginine remains the most effective biocatalyst tested for the enantioselective synthesis of warfarin. Its consistent performance in both percent yield and ee supports its continued use in green chemistry approaches to pharmaceutical synthesis. Future research should focus on further exploring the mechanistic role of the guanidino group and L-arginine; as well as systematically varying other reaction conditions such as temperature, pH, and water concentration. These efforts may help establish optimal parameters that enhance enantioselectivity without the need for derivative modification or further purification methods.

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